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DISSERTATION

DEVELOPMENT OF THE TECHNOLOGY OF AN ANTIBACTERIAL CREAM BASED ON THE EXTRACT OF *SPERANSKIA TUBERCULATA* (BUNGE) BAILL.

226 – Pharmacy, industrial pharmacy 22 – Healthcare

Applying for the degree of Doctor of Philosophy

The dissertation contains the results of my research. The use of ideas, results and texts of other authors is linked to the corresponding sources.

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Abstract

Sun Z. Development of the technology of an antibacterial cream based on the extract of *Speranskia tuberculata* (Bunge) Baill. – Qualifying scientific work on manuscript rights.

Dissertation for the Doctor of Philosophy degree in the field of knowledge 22 – Healthcare by speciality 226 Pharmacy, Industrial Pharmacy. – Kyiv National University of Technologies and Design, Kyiv, 2026.

The dissertation aims to solve a relevant scientific and applied problem: developing the composition and technology of an antibacterial cream for topical use. The active pharmaceutical ingredient (API) of the developed cream is a dry ethyl acetate extract of the herb *Speranskia tuberculata* (Bunge) Baill. The feasibility of using the specified extract as an API is substantiated theoretically and experimentally; in particular, the antioxidant, antibacterial and cytotoxic activity of the extracts has been established, the main groups of biologically active substances have been identified, and approaches to the standardisation of the dry ethyl acetate extract have been proposed; the composition and technology of the cream have been developed, its quality indicators have been determined, the irritant potential and antibacterial activity of the cream *in vitro* have been investigated, stability studies have been conducted, and its shelf life has been substantiated. The results justify further research into the developed antibacterial cream based on the extract of the herb *Speranskia tuberculata* (Bunge) Baill., for topical use in the complex therapy of acne.

The object of the study is the development process of an antibacterial cream based on an extract of the herb *Speranskia tuberculata* (Bunge) Baill.

The study focuses on the composition, technology, and approaches to standardising an antibacterial cream based on a dry ethyl acetate extract of the herb *Speranskia tuberculata* (Bunge) Baill.

The aim of the work is to develop the composition, technology, and control methods for an antibacterial emulsion cream of the “oil in water” type (o/w) with a dry ethyl acetate extract of the herb *Speranskia tuberculata* (Bunge) Baill. for topical use.

Scientific novelty of the obtained results. In the work, for the first time:

- a comprehensive study of extracts of the herb *Speranskia tuberculata*, obtained using solvents of different polarity, was carried out, and the advantages of the ethyl acetate extract in terms of antibacterial, antioxidant and antitumor activities and *in vitro* cytotoxic activity in the investigated human tumour-cell models were established; favourable *in vitro* cytocompatibility of TGC-EA was additionally demonstrated in HaCaT keratinocyte and HFF-1 fibroblast models;

- the relationship between the biological activity of the extracts and the content of the main classes of biologically active substances was established, and the minimum necessary concentrations of phenols, flavonoids, terpenoids and alkaloids were determined, which provide a pronounced antibacterial effect of the ethyl acetate extract;

- the composition and technology for obtaining an antibacterial emulsion cream of the "oil in water" type (o/w) with dry ethyl acetate extract of the herb *Speranskia tuberculata* (Bunge) Baill. as an active pharmaceutical ingredient were developed and scientifically substantiated; quality indicators were determined, and methods for their control were proposed.

- the main technological equipment was selected, and a quality risk analysis was conducted based on the proposed Ishikawa diagram;

- the critical parameters of the technological process were substantiated.

The approaches to standardisation of extracts of medicinal plant raw materials were improved through comprehensive control of the content of biologically active substances and toxicologically significant impurities in accordance with the requirements of ICH Q3C and ICH Q3D.

The following were further developed:

- scientific ideas about the pharmacological potential of plants of the genus *Speranskia*;

- methodology for a comprehensive combination of phytochemical, pharmacological and technological research in the development of herbal medicines.

The practical significance of the results lies in developing the composition, technology, and draft specification for quality control of an antibacterial cream based on dry ethyl acetate extract of *Speranskia tuberculata*, which can be used for further implementation in production.

Specifications for the active pharmaceutical ingredient and the antibacterial cream were proposed. A technological scheme for producing an oil/water antibacterial emulsion cream was developed, including the determination of critical process parameters and control points, as well as key equipment units, creating the prerequisites for industrial implementation of the developed product.

The results indicate the potential of the developed antibacterial cream, based on the extract of the herb *Speranskia tuberculata*, for topical use in acne and other inflammatory skin diseases and provide the basis for further research and implementation in pharmaceutical production.

Chapter 1 systematically analyses and summarises the scientific basis for using the medicinal plant *Speranskia tuberculata* (Bunge) Baill. in traditional medicine and modern pharmaceutical research. This chapter clarifies the relationship between *Speranskia tuberculata* and the traditional Chinese medicinal name Tou Gu Cao (TGC) and justifies selecting this plant as the research subject. Literature analysis shows that *Speranskia tuberculata* has various pharmacological properties, including anti-inflammatory, analgesic, antitumor, antioxidant, and antibacterial activities. Among these effects, anti-inflammatory and analgesic activities align with the plant's traditional use for rheumatic pain, traumatic injuries, and local inflammatory disorders. At the same time, available studies indicate that its extracts can inhibit certain tumour cell lines and pathogenic and opportunistic skin microorganisms, which provides a scientific basis for considering this plant as a potential source for the production of modern active pharmaceutical ingredients.

The section also supports the feasibility of using *Speranskia tuberculata* herb to develop soft dosage forms for topical application. Compared with individual synthetic antibacterial agents, plant extracts usually contain several classes of biologically active compounds and can exert a combined effect through antibacterial, antioxidant and anti-

inflammatory mechanisms, as well as by modulating the local skin microenvironment. Given that infectious and inflammatory skin diseases are often associated with impaired barrier function, microbial colonisation and local inflammatory reactions, the development of a modern soft dosage form for topical application based on *Speranskia tuberculata* herb extract meets both the traditional experience of its topical use and the modern requirements for safe, stable, standardised and industrially applicable pharmaceutical products. Therefore, creams and gels for external administration were identified as priority dosage forms for modern pharmaceutical transformation of this medicinal plant.

Chapter 2 describes the materials, experimental design and methodological basis of the dissertation. The whole herb *Speranskia tuberculata* was used as the plant raw material. The extracts were obtained using solvents of different polarity, namely ethyl acetate, petroleum ether, n-butanol and purified water, and the corresponding dry extracts were prepared by drying. By comparing extracts obtained with different solvents, the effect of solvent polarity on extract yield, chemical composition, and biological activity was systematically investigated, which served as the experimental basis for selecting the active pharmaceutical ingredient.

The *in vitro* cytotoxic, antioxidant, and antibacterial activities of the obtained extracts were evaluated. Cytotoxicity was assessed using the MTT assay in four human tumour cell lines (A549, Hep G2, A-375, and HeLa). At the same time, supplementary cytocompatibility studies were performed using HaCaT (human immortalised keratinocytes) and HFF-1 (human fibroblasts) following the selection of TGC-EA for topical formulation development. The DPPH free radical scavenging assay and the superoxide anion radical scavenging assay were used to determine antioxidant activity. Antibacterial activity was studied against *Staphylococcus aureus*, *Escherichia coli* and *Cutibacterium acnes* as test microorganisms relevant to the direction of the study; *C. acnes* was included because of its recognised involvement in the multifactorial pathogenesis of acne. For the final cream formulation, the *in vitro* antibacterial effect was evaluated using a liquid co-incubation method combined with colony counting,

which more realistically reflects the release, diffusion, and biological activity of the active ingredient in a semisolid cream matrix.

Chapter 2 also provides physicochemical, pharmacotechnological and microbiological methods necessary for assessing the quality of extracts and cream formulations. High-performance liquid chromatography combined with mass spectrometry was used to identify the main organic compounds in the extracts. Spectrophotometric methods were used to determine the total content of polyphenols, flavonoids, terpenoids, and alkaloids; these analytical methods were validated for specificity, linearity, precision, accuracy, range, robustness, and sensitivity. ICP-AES, AAS and gas chromatography were used to monitor heavy metals and residual solvents. A comprehensive evaluation system was developed for the cream formulation, including appearance, pH, dynamic viscosity, centrifugal stability, cold stability, heat stability, microbiological purity, irritant potential, and antibacterial activity. Experimental design and statistical methods were used to optimise the formulation and analyse the data.

To carry out all the planned research during the course of the dissertation, the experimental facilities of the following organisations were utilised: the laboratories of the School of Chemistry and Chemical Engineering at Qilu University of Technology, the laboratory of the School of Pharmacy and Pharmaceutical Sciences at Shandong First Medical University & Shandong Academy of Medical Sciences, and the Shandong Analysis and Test Centre.

Chapter 3 systematically studied the chemical composition and biological activity of different *Speranskia tuberculata* extracts and identified the dry ethyl acetate extract (TGC-EA) as an active pharmaceutical ingredient for further formulation development. Experimental results showed that the extracts obtained with solvents of different polarities differed significantly in biological activity and chemical composition. The ethyl acetate extract showed the strongest antibacterial activity against *Staphylococcus aureus* and *Cutibacterium acnes*, with IC_{50} values of approximately 112–135 μg/mL. In contrast, the other extracts did not exhibit comparable antibacterial activity in the same experimental range. These results indicate

that medium-polarity compounds enriched in TGC-EA may be the basis of its antibacterial activity.

HPLC-MS analysis revealed a complex chemical composition in *Speranskia tuberculata* extracts. The petroleum ether extract showed about 300 peaks of organic compounds, while the ethyl acetate extract showed 75 unique peaks selected for further analysis based on the intensity criterion (amplitude $\geq 10^4$ a.u.). Further analysis of the chemical composition of the ethyl acetate extract showed that more than 75% of the identified compounds selected for detailed analysis belonged to four main classes: phenolic compounds, flavonoids, terpenoids and alkaloids. Comparing the chemical composition with *in vitro* antibacterial activity confirmed that the extract's antibacterial activity is closely related to the levels of these main classes of biologically active compounds.

On this basis, we propose key parameters for standardising the dry extract of TGC-EA. To ensure consistent *in vitro* antibacterial activity, the ethyl acetate dry extract should meet the following minimum requirements for the content of the main classes of active compounds: total polyphenol content — not less than 255 mg GE/g, total flavonoid content — not less than 154 mg PE/g, total terpenoid content — not less than 192 mg UAE/g and total alkaloids content — not less than 62 mg TE/g. At the same time, the levels of heavy metals and residual solvents met pharmacopoeial and relevant international requirements, indicating a favourable safety profile and the controllability of TGC-EA as a herbal active pharmaceutical ingredient. Therefore, TGC-EA was selected as the optimal active pharmaceutical ingredient for developing an antibacterial cream.

Chapter 4 is devoted to the composition of the formulation, technological process and technological scheme of the antibacterial cream TGC-EA. In accordance with the principles of pharmaceutical development described in ICH Q8, the research was conducted within the framework of the “Quality by Design” concept. The stability of the active pharmaceutical ingredient, the organoleptic, rheological, and functional characteristics of the developed cream, and the technological parameters of its production process were comprehensively investigated. A soft dosage form was chosen

for the development of the medicinal product – an emulsion cream of the “oil-in-water” type. Based on the results, the introduction of dry TGC-EA extract into the cream's oil phase was justified, ensuring proper dispersion and stability in the emulsion system. Excipients were selected based on their functional roles in emulsification, thickening, structure formation, moisturising, antioxidant protection, preservation, and chelation and stabilisation.

In optimising the cream base, an orthogonal L9 experimental design was used to study three key formulation factors: the ratio of gum arabic to tragacanth, the total amount of two gum-based rheology modifiers, and the mass ratio of the water phase to the oil phase. We developed a comprehensive evaluation system based on indicators of appearance, colour, light transmission, texture, rub-in ability, centrifugal stability, cold resistance, heat resistance, pH, and dynamic viscosity. As a result, we identified a cream base with good stability, appropriate rheological properties, and favourable consumer characteristics. The study showed that the combined use of the two natural gums contributed to the formation of a stable three-dimensional network, providing effective structural support for oil droplets, increasing the cream’s resistance to phase separation, and improving its properties during rubbing.

The concentration of TGC-EA in the cream composition was justified by the results of a study of its antibacterial activity *in vitro*. Compared with the extract in solution, the cream matrix may restrict the release, diffusion and contact of the active ingredient with microorganisms; therefore, a higher loading level is required in the formulation to maintain the desired *in vitro* antibacterial activity. The experimental results showed that incorporation of TGC-EA at 0.10% (w/w), corresponding to 0.10 g per 100 g of cream, provided pronounced *in vitro* antibacterial activity against *Staphylococcus aureus* and *Cutibacterium acnes*, while maintaining satisfactory homogeneity and stability of the cream formulation. These results indicate that the developed cream base can not only carry the plant extract but also preserve its biological activity.

Further, a technological process for producing the antibacterial cream TGC-EA was developed. The proposed process includes the preparation of raw materials and

excipients, preparation of the oil phase, dispersion of the active ingredient in the oil phase, preparation of the aqueous and colloidal phases, emulsification, homogenization with high shear forces, cooling, deaeration, storage of the intermediate product, filling into tubes and packaging. For industrial production, the main equipment units suitable for a production scale of 300 kg per batch with packaging in 30 g tubes were determined. Critical process parameters, including temperature, homogenization rate, mixing duration, order of component introduction, vacuum conditions, and cooling rate, were identified. Their correlation with critical quality indicators was established to ensure process reproducibility and stable product quality.

To assess intermediate product quality, FTIR spectroscopy was used to evaluate the chemical compatibility of the formulation components. No characteristic changes indicating obvious chemical incompatibility or anomalous changes in the main functional groups were detected. Visual inspection, microscopic examination, a scraping test, and stability testing confirmed the homogeneity of the active ingredient in the formulation. The semi-finished cream product appeared homogeneous, finely dispersed, and soft, with no visible particles, oil-and-water stratification, or graininess. The pH and dynamic viscosity values were within the ranges compatible with the skin and acceptable for soft dosage forms for topical application. The results of the study of microbiological purity, irritation and antibacterial activity *in vitro* confirmed the feasibility of the selected composition and technology of the developed cream.

Chapter 5 is devoted to the study of tube packaging, quality control, stability, standardisation and risk management of TGC-EA antibacterial cream. Considering the characteristics of soft dosage forms for topical use, appropriate tube packaging was selected to ensure tightness, stability, and ease of administration during storage and use. The quality assessment of the finished product showed that the cream packaged in tubes is a homogeneous, milky-white, finely dispersed soft preparation without visible particles or phase separation, with easy extrusion and application. These properties meet the basic requirements for the appearance, texture, and ease of use of topical creams. The pH of the finished cream was 5.68 ± 0.18, which falls within the weakly acidic physiological pH range of the skin and may help reduce the risk of local irritation

while maintaining formulation stability. The dynamic viscosity was 28.54±3.65 Pa·s, indicating that the cream has a structure consistent with a soft dosage form for cutaneous use. This viscosity ensured convenient extrusion from the tube and application to the skin and preserved the shape during storage. Microbiological purity testing confirmed the absence of specified microorganisms, including *Staphylococcus aureus* and *Escherichia coli*, indicating that the technological process and packaging system meet microbiological control requirements for non-sterile topical drugs. Additionally, the absence of *Cutibacterium acnes* was confirmed, which is important given the intended purpose of the developed antibacterial cream.

The irritation potential of the finished cream was assessed using the HET-CAM method. During the 300-s observation period, no haemorrhage, vascular lysis or coagulation was observed. These findings indicate a low irritation potential of the tested cream under the conditions of the applied HET-CAM model. Antibacterial activity testing showed that five randomly selected tube-filled samples maintained an inhibition rate of 96.87%–98.85% against *Cutibacterium acnes* and 99.49%–99.75% against *Staphylococcus aureus*. These findings demonstrate that the cream formulation had not only clear antibacterial activity but also good within-batch uniformity and functional reproducibility.

The analysis of elemental impurities showed that the contents of Cd, Pb, As, and Hg in the finished cream were 0.762±0.138 ppm, 1.279±0.313 ppm, 0.417±0.091 ppm and 0.068±0.024 ppm, respectively, all of which were below the relevant limit values. These results indicate that although the formulation contains a plant extract and natural polymeric excipients, the risk of heavy metal contamination can be effectively managed through raw material control, process control and finished product testing. Together, the microbiological purity, elemental impurity analysis and HET-CAM results provide complementary information on the quality and selected risk-related characteristics of the developed cream.

Stability studies demonstrated that the TGC-EA antibacterial cream maintained good overall stability during the 12-month observation period. During storage, no adverse changes were observed in appearance, colour, odour, homogeneity or particle

size distribution, and there was no oil phase separation, water exudation, disruption of the emulsion system or obvious particle aggregation. Variations in pH and dynamic viscosity remained within acceptable limits, and the *in vitro* antibacterial activity was preserved. The total polyphenol, flavonoid, and terpenoid contents demonstrated a high degree of retention, which may indicate the effectiveness of the combined stabilising factors of the formulation, including the antioxidant system, chelating agent system, and structural components of the cream matrix.

Based on these results, a standardised quality control system for TGC-EA antibacterial cream was established. It covers the appearance, pH, dynamic viscosity, microbiological purity, antibacterial activity, heavy metal content, total content of major classes of biologically active compounds, packaging integrity and stability.

In addition, we applied quality risk management to the process analysis. An Ishikawa causal diagram was constructed based on factors related to personnel, equipment, materials, methods, measurement, and environment, and the main sources of risk affecting product quality were identified, along with corresponding control strategies. The results show that the developed antibacterial cream TGC-EA has a clearly defined formulation, reproducible technological process, stable quality indicators, and prospects for further scaling up the technology and implementation in pharmaceutical production. The developed TGC-EA antibacterial cream can be considered a promising topical formulation for further investigation in the context of acne and other inflammatory skin conditions, providing an experimental basis for subsequent preclinical studies, clinical evaluation, and further technological development and scale-up.

Keywords: medicinal plant raw materials; *Speranskia tuberculata* (Bunge) Baill.; Tou Gu Cao; ethyl acetate dry extract; active pharmaceutical ingredient; O/W cream; soft dosage form; antibacterial activity; cream manufacturing technology; quality control; standardisation; stability; HPLC-MS; spectrophotometry; FTIR.

Annotation (in Ukrainian)

Сунь Ц. Розроблення складу та технології антибактеріального крему на основі екстракту *Speranskia tuberculata* (Bunge) Baill. – Кваліфікаційна наукова робота на правах рукопису.

Дисертація на здобуття наукового ступеня доктора філософії в галузі знань 22 – Охорона здоров'я за спеціальністю 226 Фармація, промислова фармація. – Київський національний університет технологій та дизайну, Київ, 2026.

Дисертаційна робота спрямована на вирішення актуального науково-прикладного завдання — розроблення складу та технології антибактеріального крему для місцевого застосування. Активним фармацевтичним інгредієнтом (АФІ) розробленого крему є сухий етилацетатний екстракт трави *Speranskia tuberculata* (Bunge) Baill. Доцільність використання зазначеного екстракту як АФІ обґрунтовано теоретично та експериментально; зокрема встановлено антиоксидантну, антибактеріальну та цитотоксичну активність екстрактів, визначено основні групи біологічно активних речовин та запропоновано підходи до стандартизації сухого етилацетатного екстракту; розроблено склад і технологію крему, визначено показники його якості, досліджено подразнювальний потенціал та антибактеріальну активність крему *in vitro*, проведено дослідження стабільності та обґрунтовано термін його придатності. Отримані результати обґрунтовують перспективність подальшого дослідження розробленого антибактеріального крему на основі екстракту трави *Speranskia tuberculata* (Bunge) Baill. для місцевого застосування в комплексній терапії акне.

Об’єкт дослідження – процеси розроблення антибактеріального крему на основі екстракту трави *Speranskia tuberculata* (Bunge) Baill.

Предметом дослідження є склад, технологія та підходи до стандартизації антибактеріального крему на основі сухого етилацетатного екстракту трави *Speranskia tuberculata* (Bunge) Baill.

Метою роботи є розроблення складу, технології та підходів до методів контролю антибактеріального емульсійного крему типу «олія у воді» (о/в) із

сухим етилацетатним екстрактом трави *Speranskia tuberculata* (Bunge) Baill. для місцевого застосування.

Наукова новизна отриманих результатів. У роботі вперше:

- проведено комплексне дослідження екстрактів трави *Speranskia tuberculata*, отриманих з використанням розчинників різної полярності, та встановлено переваги етилацетатного екстракту з точки зору антибактеріальної, антиоксидантної та протипухлинної активності, а також цитотоксичної активності *in vitro* на досліджуваних моделях пухлинних клітин людини; додатково продемонстровано сприятливу цитосумісність TGC-EA *in vitro* на моделях кератиноцитів HaCaT та фібробластів HFF-1;

- встановлено взаємозв'язок між біологічною активністю екстрактів та вмістом основних класів біологічно активних речовин, визначено мінімально необхідні концентрації фенолів, флавоноїдів, терпеноїдів та алкалоїдів, які забезпечують виражену антибактеріальну дію етилацетатного екстракту;

- розроблено та науково обґрунтовано склад і технологію одержання антибактеріального емульсійного крему типу «олія у воді» (о/в) із сухим етилацетатним екстрактом трави *Speranskia tuberculata* (Bunge) Baill. як активним фармацевтичним інгредієнтом; визначено показники якості та запропоновано методики їх контролю.

- підібрано основне технологічне обладнання та проведено аналіз ризиків для якості на основі запропонованої діаграми Ішікави;

- обґрунтовано критичні параметри технологічного процесу.

Удосконалено підходи до стандартизації екстрактів лікарської рослинної сировини шляхом комплексного контролю вмісту біологічно активних речовин і токсикологічно значущих домішок відповідно до вимог ICH Q3C та ICH Q3D.

Дістали подальшого розвитку:

- наукові уявлення про фармакологічний потенціал рослин роду *Speranskia*;

- методологія комплексного поєднання фітохімічних, фармакологічних та технологічних досліджень при розробленні фітопрепаратів.

Практичне значення отриманих результатів полягає у розробленні

складу, технології та проєкту специфікації контролю якості антибактеріального крему на основі сухого етилацетатного екстракту *Speranskia tuberculata*, що може бути використано при подальшому впровадженні у виробництво.

Запропоновано специфікації для активного фармацевтичного інгредієнта та антибактеріального крему. Розроблено технологічну схему виробництва антибактеріального емульсійного крему типу олія/вода з визначенням критичних параметрів процесу та точок контролю, а також ключових одиниць обладнання, що створює передумови для промислового впровадження розробленого лікарського засобу.

Отримані результати свідчать про перспективність розробленого антибактеріального крему на основі екстракту трави *Speranskia tuberculata* для місцевого застосування при акне та інших запальних захворюваннях шкіри та створюють передумови для його подальшого дослідження і впровадження у фармацевтичне виробництво.

Розділ 1 системно аналізує та узагальнює наукові основи застосування лікарської рослини *Speranskia tuberculata* (Bunge) Baill. у традиційній медицині та сучасних фармацевтичних дослідженнях. Уточнено зв'язок між *Speranskia tuberculata* та традиційною китайською лікарською назвою Тоу Гу Цао (TGC), обґрунтовано доцільність вибору цієї рослини як об'єкта дослідження. Аналіз літератури показав, що *Speranskia tuberculata* має різноманітні фармакологічні властивості, зокрема протизапальну, знеболювальну, протипухлинну, антиоксидантну та антибактеріальну активність. Серед цих ефектів протизапальна та знеболювальна активність узгоджуються з традиційним застосуванням рослини при ревматичних болях, травматичних ушкодженнях та місцевих запальних розладах. Водночас наявні дослідження свідчать про те, що її екстракти можуть пригнічувати певні лінії пухлинних клітин (які) та патогенні та умовно-патогенні мікроорганізми шкіри, що надає наукове підґрунтя для розгляду цієї рослини як потенційного джерела для виробництва сучасних активних фармацевтичних інгредієнтів.

У розділі також обґрунтовано доцільність використання трави *Speranskia*

tuberculata для розроблення м’яких лікарських форм для місцевого застосування. Порівняно з окремими синтетичними антибактеріальними засобами, рослинні екстракти зазвичай містять кілька класів біологічно активних сполук і можуть чинити комбіновану дію через антибактеріальні, антиоксидантні та протизапальні механізми, а також шляхом модуляції місцевого мікросередовища шкіри. З огляду на те, що інфекційні та запальні захворювання шкіри часто пов'язані з порушенням бар'єрної функції, мікробною колонізацією та місцевими запальними реакціями, розроблення сучасної м'якої лікарської форми для місцевого застосування на основі екстракту трави *Speranskia tuberculata* відповідає як традиційному досвіду його місцевого використання, так і сучасним вимогам до безпечних, стабільних, стандартизованих та промислово придатних фармацевтичних продуктів. Тому креми та гелі для зовнішнього ~~введення~~ застосування були визначені як пріоритетні лікарські форми для сучасної фармацевтичної трансформації цієї лікарської рослини.

Розділ 2 описує матеріали, план експерименту та методологічну основу дисертації. Як рослинна сировина використовували цілу траву *Speranskia tuberculata*. Екстракти отримували з використанням розчинників різної полярності, а саме етилацетату, петролейного ефіру, н-бутанолу та очищеної води, і готували відповідні сухі екстракти шляхом висушування. Шляхом порівняння екстрактів, отриманих різними розчинниками, системно досліджено вплив полярності розчинника на вихід екстракту, хімічний склад та біологічну активність, що стало експериментальною основою для вибору активного фармацевтичного інгредієнта.

Фармакологічну активність отриманих екстрактів оцінювали за трьома основними напрямами: протипухлинна, антиоксидантна та антибактеріальна. Протипухлинну активність оцінювали методом MTT на клітинних моделях A549, HepG2, A375, HeLa та RAW264.7. Для визначення антиоксидантної активності використовували метод поглинання вільних радикалів DPPH та метод поглинання аніон-радикалів супероксиду. Антибактеріальну активність вивчали щодо *Staphylococcus aureus, Escherichia coli ma Cutibacterium acnes* (раніше —

Propionibacterium acnes), обраних як тест-мікроорганізми для оцінки антибактеріальних властивостей досліджуваного екстракту. Для остаточної рецептури крему антибактеріальну активність *in vitro* оцінювали методом рідкофазного спільного інкубування з подальшим підрахунком колоній, що дозволило оцінити антибактеріальну дію активного інгредієнта у складі готової фармацевтичної композиції.

У розділі 2 також наведено фізико-хімічні, фармако-технологічні та мікробіологічні методи, необхідні для оцінки якості екстрактів і рецептур кремів. Для ідентифікації основних органічних сполук в екстрактах використовували високоефективну рідинну хроматографію у поєднанні з мас-спектрометрією. Спектрофотометричні методи застосовували для визначення загального вмісту поліфенолів, флавоноїдів, терпеноїдів та алкалоїдів; ці аналітичні методи валідували за специфічністю, лінійністю, прецизійністю, правильністю, діапазоном, робасністю та чутливістю. Для контролю важких металів та залишкових розчинників використовували ІЗП-АЕС, ААС і газову хроматографію. Для рецептури крему було розроблено комплексну систему оцінювання, що включала зовнішній вигляд, pH, динамічну в'язкість, центрифужну стабільність, холодостійкість, термостійкість, мікробіологічну чистоту, подразнювальний потенціал та антибактеріальну активність. Для оптимізації рецептури та аналізу даних застосовували методи планування експерименту та статистичні методи.

Для виконання всіх запланованих у межах дисертаційного дослідження робіт було використано експериментальну базу таких установ: лабораторії Школи хімії та хімічної інженерії Технологічного університету Цілу, лабораторію Школи фармації та фармацевтичних наук Шаньдунського першого медичного університету й Шаньдунської академії медичних наук, а також Шаньдунського центру аналізу та тестування.

Розділ 3 містить систематичне дослідження хімічного складу та біологічної активності різних екстрактів *Speranskia tuberculata* та визначає сухий етилацетатний екстракт (TGC-EA) як активний фармацевтичний інгредієнт для

подальшого розроблення рецептури. Експериментальні результати показали, що екстракти, отримані з розчинниками різної полярності, суттєво відрізнялися за біологічною активністю та хімічним складом. Етилацетатний екстракт продемонстрував найбільш виражену антибактеріальну активність відносно *Staphylococcus aureus* та *Cutibacterium acnes* зі значеннями IC_{50} приблизно 112–135 мкг/мл. Натомість інші екстракти не проявляли порівнянної антибактеріальної активності в тому ж експериментальному діапазоні. Ці результати свідчать про те, що сполуки середньої полярності, збагачені в TGC-EA, можуть являти собою матеріальну основу його антибактеріальної дії.

Аналіз методом ВЕРХ-МС виявив складний хімічний склад екстрактів *Speranskia tuberculata*. В екстракті петролейного ефіру виявлено близько 300 піків органічних сполук, тоді як в етилацетатному екстракті — 75 унікальних піків, відібраних для подальшого аналізу за критерієм інтенсивності (амплітуда $\geq 10^4$ a.u.). Подальший аналіз хімічного складу етилацетатного екстракту показав, що понад 75% ідентифікованих сполук, відібраних для детального аналізу, належали до чотирьох основних класів: фенольних сполук, флавоноїдів, терпеноїдів та алкалоїдів. Зіставляючи хімічний склад з антибактеріальною активністю *in vitro*, було підтверджено, що антибактеріальна активність екстракту тісно пов'язана з рівнем вмісту цих основних класів біологічно активних сполук.

На цій основі запропоновано ключові параметри стандартизації сухого екстракту TGC-EA. Для забезпечення антибактеріальної ефективності сухий етилацетатний екстракт повинен відповідати таким мінімальним вимогам щодо вмісту основних класів активних сполук: загальний вміст поліфенолів — не менше 255 мг ГЕ/г, загальний вміст флавоноїдів — не менше 154 мг РЕ/г, загальний вміст терпеноїдів — не менше 192 мг UAE/г та загальний вміст алкалоїдів — не менше 62 мг ТЕ/г. Водночас рівні важких металів та залишкових розчинників відповідали фармакопейним та відповідним міжнародним вимогам, що свідчить про сприятливий профіль безпеки та контрольованість TGC-EA як рослинного активного фармацевтичного інгредієнта. Тому TGC-EA було обрано

як оптимальний активний фармацевтичний інгредієнт для розроблення антибактеріального крему.

Розділ 4 присвячено складу рецептури, технологічному процесу та технологічній схемі антибактеріального крему TGC-EA. Відповідно до принципів фармацевтичного розроблення, описаних у ICH Q8, дослідження проводили в рамках концепції «Якість, закладена при розробленні» (Quality by Design). Комплексно досліджено стабільність активного фармацевтичного інгредієнта, органолептичні, реологічні та функціональні характеристики розробленого крему, а також технологічні параметри процесу його одержання. Для розроблення лікарського засобу обрано м’яку лікарську форму — емульсійний крем типу «олія у воді». За результатами проведених досліджень обґрунтовано введення сухого екстракту TGC-EA до олійної фази крему, що забезпечувало його належне диспергування та стабільність у складі емульсійної системи. Допоміжні речовини підбирали з урахуванням їх функціональної ролі в емульгуванні, загущенні, структуроутворенні, зволоженні, антиоксидантному захисті, консервуванні, а також хелатуванні та стабілізації.

При оптимізації основи крему застосовували ортогональний план експерименту L9 для вивчення трьох ключових факторів рецептури: співвідношення гуміарабіку до трагаканту, загальної кількості двох реологічних модифікаторів на основі камедей та масового співвідношення водної фази до масляної. Комплексна система оцінювання була розроблена на основі показників зовнішнього вигляду, кольору, пропускання світла, текстури, здатності до розтирання, центрифужної стабільності, холодостійкості, термостійкості, pH та динамічної в'язкості. В результаті було визначено основу крему з хорошою стабільністю, відповідними реологічними властивостями та сприятливими споживчими характеристиками. Дослідження показало, що синергетичний ефект двох природних камедей здатний формувати стабільну тривимірну сітку, забезпечувати ефективну структурну підтримку крапель олії та підвищувати стійкість крему до розшарування фаз і його властивості при розтиранні.

Концентрацію TGC-EA у складі крему обґрунтовано за результатами

дослідження його антибактеріальної активності *in vitro.* Встановлено, що введення TGC-EA у концентрації 1,0 % (мас./мас.) забезпечувало антибактеріальну активність крему щодо *Staphylococcus aureus* та *Cutibacterium acnes* при збереженні однорідності та стабільності емульсійної системи. На підставі отриманих результатів концентрацію TGC-EA 1,0 % (мас./мас.) обрано для подальшого розроблення складу крему. Отримані результати свідчать, що розроблена кремова основа забезпечує можливість введення рослинного екстракту до складу лікарської форми зі збереженням його біологічної активності.

Надалі розроблено технологічний процес виробництва антибактеріального крему TGC-EA. Запропонований процес включає підготовку сировини та допоміжних речовин, приготування масляної фази, диспергування активного інгредієнта в масляній фазі, приготування водної та колоїдної фаз, емульгування, гомогенізацію з високими зусиллями зсуву, охолодження, деаерацію, зберігання напівпродукту, фасування в туби та пакування. Для промислового виробництва визначено основні одиниці обладнання, придатні для виробничого масштабу 300 кг на серію з фасуванням у туби по 30 г. Визначено критичні параметри процесу, включаючи температуру, швидкість гомогенізації, тривалість перемішування, порядок введення компонентів, умови вакуумування та швидкість охолодження. Встановлено їх кореляцію з критичними показниками якості, що забезпечує відтворюваність процесу та стабільну якість продукту.

При оцінюванні якості напівпродукту методом FTIR-спектроскопії досліджували хімічну сумісність компонентів рецептури. Характерних змін, що свідчать про очевидну хімічну несумісність або аномальні зміни основних функціональних груп, не виявлено. Однорідність активного інгредієнта в рецептурі підтверджено методами візуального огляду, мікроскопічного дослідження, скребкового тесту та випробування на стабільність. Напівпродукт крему мав вигляд однорідної, дрібнодисперсної, м’якої системи без видимих частинок, розшарування олії та води або зернистості. Значення pH та динамічної в'язкості знаходились в діапазонах, сумісних зі шкірою та прийнятних для м'яких

лікарських форм для місцевого застосування. Результати дослідження мікробіологічної чистоти, подразнювальної дії та антибактеріальної активності *in vitro* підтвердили доцільність обраного складу та технології розробленого крему.

Розділ 5 присвячено дослідженню пакування у туби, контролю якості, стабільності, стандартизації та управління ризиками антибактеріального крему TGC-EA. З урахуванням характеристик м’яких лікарських форм для місцевого застосування було підібрано відповідне пакування у туби для забезпечення герметичності, стабільності та зручності введення під час зберігання та використання. Оцінювання якості готового продукту показало, що крем, розфасований у туби, є однорідним, молочно-білим, дрібнодисперсним м’яким препаратом без видимих частинок або розшарування фаз, з легким видавлюванням та нанесенням. Ці властивості відповідають основним вимогам до зовнішнього вигляду, текстури та зручності використання кремів для місцевого застосування.

Значення pH готового крему становило 5,68±0,18, що відповідає слабокислому фізіологічному діапазону шкіри та сприяє зниженню ризику місцевого подразнення при збереженні стабільності рецептури. Динамічна в'язкість склала 28,54±3,65 Па·с, що свідчить про наявність у крему структури., відповідної м’якій лікарській формі для нашкірного застосування. Ця в'язкість забезпечувала як зручне видавлювання з туби та нанесення на шкіру, так і збереження форми під час зберігання. Випробування на мікробіологічну чистоту підтвердило відсутність специфікованих мікроорганізмів, включаючи *Staphylococcus aureus* та *Escherichia coli*, що свідчить про відповідність технологічного процесу та системи пакування вимогам мікробіологічного контролю для нестерильних препаратів для місцевого застосування. Додатково підтверджено відсутність *Cutibacterium acnes*, що є важливим з огляду на цільове призначення розробленого лікарського засобу.

Подразнювальний потенціал зразка крему оцінювали методом HET-CAM. Протягом 300 с спостереження зразок крему не спричиняв геморагії, лізису

судин або коагуляції, що свідчить про хорошу місцеву біологічну сумісність та низький ризик гострого подразнення. Випробування на антибактеріальну активність показало, що п'ять випадково відібраних зразків крему у тубах зберігали ступінь інгібування 96,87–98,85% відносно *Cutibacterium acnes* та 99,49–99,75% проти *Staphylococcus aureus*. Ці результати свідчать про те, що рецептура крему відрізнялась не лише вираженою антибактеріальною активністю, а й хорошою функціональною відтворюваністю.

Аналіз елементних домішок показав, що вміст Cd, Pb, As та Hg у готовому кремі становив 0,762±0,138 ppm, 1,279±0,313 ppm, 0,417±0,091 ppm та 0,068±0,024 ppm відповідно, що є нижчим за відповідні граничні значення. Ці результати свідчать про те, що незважаючи на наявність у рецептурі рослинного екстракту та природних полімерних допоміжних речовин, ризик забруднення важкими металами може ефективно контролюватися за допомогою контролю сировини, контролю процесу та випробування готового продукту. У сукупності з результатами випробувань на мікробіологічну чистоту та подразнювальний потенціал ці дані підтверджують сприятливий профіль безпеки розробленого крему.

Дослідження стабільності показало, що антибактеріальний крем TGC-EA зберігав стабільність досліджуваних показників протягом 12 місяців спостереження. Під час зберігання не спостерігалося небажаних змін зовнішнього вигляду, кольору, запаху, однорідності або розподілу частинок за розміром, не відмічалося розшарування масляної фази, виділення води, порушення емульсійної системи або очевидної агрегації частинок. Коливання pH та динамічної в'язкості залишалися в прийнятних межах, а антибактеріальна активність *in vitro* зберігалась. Основні сполуки, пов'язані з ефективністю, включаючи загальний вміст поліфенолів, флавоноїдів та терпеноїдів, показали високий ступінь збереження, що свідчить про те, що антиоксидантна система, система хелатуючих агентів, структура включення в олійну фазу та колоїдна мережа спільно підвищували хімічну стабільність активних інгредієнтів.

На підставі вищезазначених результатів було розроблено стандартизовану

систему контролю якості антибактеріального крему TGC-EA. Вона охоплює зовнішній вигляд, pH, динамічну в'язкість, мікробіологічну чистоту, антибактеріальну активність, вміст важких металів, загальний вміст основних класів біологічно активних сполук, цілісність пакування та стабільність. Крім того, до аналізу технологічного процесу було застосовано управління ризиками для якості. Побудовано причинно-наслідкову діаграму Ішікави на основі факторів, пов'язаних з персоналом, обладнанням, матеріалами, методами, вимірюванням та навколишнім середовищем, та ідентифіковано основні джерела ризику, що впливають на якість продукту, а також відповідні стратегії контролю. Результати свідчать, що розроблений антибактеріальний крем TGC-EA має чітко визначену рецептуру, відтворюваний технологічний процес, стабільні показники якості перспективи для подальшого масштабування технології та впровадження у фармацевтичне виробництво. Він може розглядатися як перспективний рослинний препарат для місцевого застосування у комплексній терапії акне та інших запальних захворювань шкіри, що забезпечує експериментальне підґрунтя для подальших доклінічних досліджень, клінічної оцінки та промислового виробництва.

Ключові слова: лікарська рослинна сировина; *Speranskia tuberculata* (Bunge) Baill.; Тоу Гу Цао; сухий етилацетатний екстракт; активний фармацевтичний інгредієнт; крем типу O/B; м’яка лікарська форма; антибактеріальна активність; технологія виробництва крему; контроль якості; стандартизація; стабільність; ВЕРХ-МС; спектрофотометрія; FTIR-спектроскопія.

List of published works

Scientific works containing the main scientific results of the dissertation:

1. Zeyuan Sun, T. M. Derkach. (2024). Biologically active compounds in the extracts from the *Speranskia Tuberculata* (Bunge) Baill. herb and their effect on the viability of cancer cells of five different lines. *Pharmaceutical Review,* No.1(69), 23—34. https://doi.org/10.11603/2312-0967.2024.1.14441

2. Zeyuan Sun, T. M. Derkach. (2024). Anti-Atherosclerosis Effect of the Herb *Speranskia Tuberculata* (Bunge) Baill. *Health & Education,* No. 2, 142–148. https://doi.org/10.32782/health-2024.2.18

3. Zeyuan Sun, T. M. Derkach. (2024). Bioactive Compounds Extracted from the Herb *Speranskia Tuberculata* (Bunge) Baill. *Journal of Chemistry and Technologies,* 32, No. 3, 580–590. https://doi.org/10.15421/jchemtech.v32i3.305125 (indexed in the SCOPUS database)

4. Zeyuan Sun, T. M. Derkach. (2024). Antioxidant Activities of Petroleum Ether, Ethyl Acetate, N-butanol and Aqueous Extracts from *Speranskia Tuberculata* (Bunge) Baill. herb. *Modern Medicine, Pharmacy and Psychological Health*. No 2 (16), 101–105. https://doi.org/10.32689/2663-0672-2024-2-16

5. Zeyuan Sun, T. M. Derkach. (2024). Antibacterial Properties of Extracts of the Herb *Speranskia Tuberculata* (Bunge) Baill. *Farmatsevtychnyi zhurnal.* No4, 72–82. https://doi.org/10.32352/0367-3057.4.24.07

6. Zeyuan Sun, T. M. Derkach. (2026). Chemical Characterisation, Biological Activity and Emulsion Dosage Form Development of *Speranskia tuberculata* (Bunge) Baill. (2026). *Journal of Chemistry and Technologies,* 34(2), 499–511. https://doi.org/10.15421/jchemtech.v34i2.352308 (indexed in the SCOPUS database)

7. Zeyuan Sun, T. M. Derkach. (2026). Development of a Technology for Producing an Antibacterial Cream Based on the Herb *Speranskia Tuberculata* (Bunge) Baill. *Farmatsevtychnyi zhurnal,* No3, 84–96. https://doi.org/10.32352/0367-3057.3.26.07

Publications certifying the approval of the dissertation materials

8. Zeyuan Sun, Tao Wu, Shu Xing, Zhou Mingyang, Tetiana M Derkach. (2021). Anti-hepatocellular carcinoma: effect of bioactive components of *Speranskia Tuberculata* (Bunge) Baill. In: *Innovation in Education, Science and Business: Challenges and Opportunities*. *Materials of the II All-Ukrainian Conference of Higher Education Students and Young Scientists*, Kyiv, November 18, 2021, vol. 1. Kyiv: Kyiv National University of Technologies and Design, pp 189–193.

9. Zeyuan Sun, Tetiana Derkach. (2023). Study on Evaluation of Vitro Coagulation Effect of Extracts of Mugwort. In: *KyivLvivPharma-2023. Pharmaceutical Technology and Pharmacology in Ensuring Active Longevity*. Kyiv-Lviv, Ukraine, November 16-18, 2023.

10. Sun Z., Derkach T. (2024). Ternopil Anti-Atherosclerosis Effect Of The Herb *Speranskia Tuberculata* (Bunge) Baill. In: *Scientific and Technical Progress and Optimization of Technological Processes for the Creation of Medicinal Products. Materials of the Xth Scientific and Practical Conference*. Eds.: M.M. Korda et al. I.Ya. Gorbachevsky Ternopil National Medical University, Ternopil, October 17-18, 2024, pp. 148–149.

11. Zeyuan Sun, T. Derkach. (2024). The study of the free radical scavenging rate of one kind of microbial fermentation product based on Chinese traditional medicine. In: *Collection of Materials of the XIIth Scientific and Practical Conference "Science, Innovation and Quality in Modern Pharmaceutical Production*". Kyiv: JSC "Farmak", pp. 8–10.

12. Sun Z., Derkach T. (2025). Bioactive Compounds and Standardisation Extracted from The Herb *Speranskia Tuberculata* (Bunge) Baill. In: *Zaporizhzhia Pharmaceutical Forum – 2025: Pharmacy of the Future – From the Current State to Global Challenges*, 20-21 November 2025, Zaporizhzhia, ZDMFU, pp. 167.

13. Sun Z., Derkach T. (2025). Research on the antibacterial efficacy and stability of a kind of medical cream based on the extracts of the herb *Speranskia Tuberculata* (Bunge) Baill. In: *Chemical and Biopharmaceutical Technologies in 2025. The collection of abstracts of VII International Scientific and Practical*

Conference "KyivLvivPharma-2025. Pharmaceutical Technology and Pharmacology in Ensuring Active Longevity", November 20-21, 2025 (Kyiv-Lviv)/ Tallinn: Nordic Sci Publisher, 2026, C. 181-183.

14. Sun Z., Derkach T. (2025). Study of Antibacterial Properties of Extracts of the Herb *Speranskia Tuberculata* (Bunge) Baill. In: *Advanced Materials and Technologies: Biotechnology, Applied Chemistry and Ecology*. Kyiv, Kyiv National University of Technologies and Design, Faculty of Chemical and Biopharmaceutical Technologies. October 30-31, pp. 26.

15. Sun Z., Derkach T. (2026). Phytochemical Profile and Antibacterial Activity of Secondary Metabolites from *Speranskia Tuberculata* (Bunge) Baill. In: *BioChemSecurity 2026 - International Conference on Biosecurity, Chemical Safety, and Global Cooperation*. Kryvyi Rih, Ukraine. March 20 2026. https://www.google.com/url?q=https://drive.google.com/file/d/1inBrKspdJhAOTBKpyLMZalFS5r1PP4ab/view?usp%3Ddrive_link&sa=D&source=editors&ust=1774069059662465&usg=AOvVaw3xI1ylJqtvLHOfvhGgT2x4

Articles that additionally reflect the scientific results of the dissertation.

16. T. Wu, X. Liu, Z. Sun, S. Xing, L. Han, X. Li, X. Pan, J. Chen, M. Zhou, T. Derkach, & J. K. Bielicki. (2022). Fruit of *Phyllanthus emblica* L. suppresses macrophage foam-cell genesis and vascular lipid deposition using in vivo and *in vitro* models of early atherosclerosis development. *Food Science and Technology Research*, 28(4), 317–328. https://doi.org/10.3136/fstr.FSTR-D-22-00002 (indexed in the SCOPUS database)

17. Y. Ren, Z. Sun, Y. Huang, X. An, X. Bian, Z. Cao, Y. Liu, K. Javed, T. Derkach, X. Li. (2024). Preparation of fluorinated chitosan-based multifunctional materials with rapid hemostasis, anti-infection, and non-adhesive properties for wounds. *Journal of Polymer Science,* 62(12), 2762–2779. https://doi.org/10.1002/pol.20240038 (indexed in the SCOPUS database)

18. Y. Ren, Z. Sun, Y. Huang, X. An, X. Bian, Z. Cao, Y. Liu, K. Javed, T. Derkach, X. Li. (2024). Preparation of TiO2 Nanorods Composites Doped with Silver Nanoparticles and Their Bactericidal Properties under Visible Light Irradiation.

Particles & Particle Systems Characterisation. 2300191. https://doi.org/10.1002/ppsc.202300191 (indexed in the SCOPUS database)

19. Xu, X., Xu, J., Sun, Z., & Derkach, T. (2024). Cyclodextrin-grafted redox-responsive hydrogel mediated by disulfide bridges for regulated drug delivery. *Designed Monomers and Polymers*, 27(1), 21–34. https://doi.org/10.1080/15685551.2024.2358581 (indexed in the SCOPUS database)

20. Chen, Z., Ge, C., Zhu, X., Sun, P., Sun, Z., Derkach, T., Zhou, M., Wang, Y., & Luan, M. (2024). A novel nanoprobe for visually investigating the controversial role of miRNA-34a as an oncogene or tumor suppressor in cancer cells. *Analytical Methods*, 16(5), 667–675. https://doi.org/10.1039/D3AY02270F (indexed in the SCOPUS database)

21. Wang, Y., Tong, M., Cao, Z., Sun, Z., Derkach, T., & Liu, B. (2024). Decoding the Molecular Mechanism of Sweet Taste of Amino Acids by Their Intrinsic Properties and Interactions with Human Sweet Taste Receptor. *Chemistry Select*, 9(37), e202403468. https://doi.org/10.1002/slct.202403468 (indexed in the SCOPUS database)

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Abbreviation

A375 – malignant melanoma

A549 – lung adenocarcinoma

AAS – atomic absorption spectroscopy

API – Active pharmaceutical ingredient

APT – Aluminium-plastic laminated tubes

ATR – Attenuated Total Reflectance

CAM – Chick chorioallantoic membrane

CIP/COP – cleaning in place/cleaning out of place

CCP– Critical control points

CC_{50} – half-maximal cytotoxic concentration

CFU/g – Colony Forming Units per gram

CPP – Critical process parameter

CQA – Critical quality attribute

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethylsulfoxide

DoE – Design of experiments

DPPH – 2,2-diphenyl-1-picrylhydrazyl

EGCG – epigallocatechin-3-gallate

EMA – European Medicines Agency

FBS – Fetal bovine serum

FTIR – Fourier Transform Infrared Spectroscopy

GE/g – Gallic Acid Equivalents per gram

GMP – Good manufacturing practice

HaCaT – human immortalised keratinocyte cell line

HDPE – High-density polyethene

HeLa – cervical carcinoma

HepG2 – hepatocellular carcinoma of the liver

HFF-1 – human foreskin fibroblast cell line

HPLC/MS – High-performance liquid chromatography- mass spectrometry

IC_{50} – half-maximal inhibitory concentration

ICH — International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

ICH Q8 – Pharmaceutical Development

ICH Q9 – Quality Risk Management

ICH Q10 – Pharmaceutical Quality System

ICP-AES – Inductively coupled plasma atomic emission spectrometry

IPC – in-process control (process parameters and in-process tests)

JP XVII – The Japanese Pharmacopoeia Seventeenth Edition (2016)

LOX – Lipoxygenases

MIC – Minimum Inhibitory Concentration

MVTR – Extremely Low Moisture Vapour Transmission Rate

PBS – Phosphate-buffered saline

PDI – polydispersity index

Ph.Eur. — European Pharmacopoeia

PTFE – Polytetrafluoroethylene

PVDF – Polyvinylidene fluoride

QbD – Quality by Design

QCM – Quality Control Methods

QRM – Quality Risk Management

RE/g – Rutin / Quercetin Equivalents per gram

SPhU – State Pharmacopoeia of Ukraine

SARS – superoxide anion radical scavenging

SOP – Standard Operating Procedure

TAMC – Total Aerobic Microbial Count

TE/g – Tannic / Thymol Equivalents per gram

TYMC – Total Yeasts and Moulds Count

UAE/g – Ursolic Acid Equivalents per gram

USP – American Pharmacopoeia

USP-NF – U.S. Pharmacopoeia National Formulary

Introduction

Justification of the choice of research topic. The modern development of pharmaceutical science and technology creates a demand for new active pharmaceutical ingredients (APIs) to ensure the production of effective and safe medicines. Plant raw materials are a valuable source of biologically active substances that meet these requirements to the greatest extent. Of particular interest are plants used in traditional Chinese medicine (TCM), many of which have a centuries-old history of use but remain insufficiently studied from the perspectives of modern pharmaceutical chemistry, drug technology, and evidence-based medicine. Such plants include *Speranskia tuberculata* (Bunge) Bail. — an endemic Chinese medicinal plant of the *Euphorbiaceae* family, known in TCM under the name Tou Gu Cao (TGC). Despite the long-term use of *Speranskia tuberculata* in TCM, its chemical composition, the relationships among individual groups of biologically active substances and their therapeutic effects, as well as optimal approaches to obtaining standardised extracts and creating modern dosage forms, remain insufficiently studied today. An additional problem is the terminological ambiguity between the botanical species *Speranskia tuberculata* (Bunge) Baill. and the generalised name Tou Gu Cao to designate a group of plants with similar medicinal effects in TCM. It complicates the standardisation of raw materials, the reproducibility of pharmacological effects, and the introduction of drugs into pharmaceutical practice.

The relevance of the work is determined by the need for scientific justification of the use of extracts of *Speranskia tuberculata* (Bunge) Baill. in industrial pharmacy, which includes determining the field of application, optimal dosage form, standardisation of medicinal plant raw materials, development of the composition and production technology of a medicinal agent (MA) that will meet modern pharmacopoeial and international requirements for quality, safety and efficacy.

Connection of work with scientific programs, plans, topics, and grants. The work was carried out in accordance with the scientific direction KNUTD No. 21/24 "Fundamental technologies of active longevity", Research issue No. 21-1/26 “Use of methods of in silico modelling, complex chemoinformatics, computational biochemistry,

pharmacology, pharmaceutical chemistry and expertise in the early stages of projects for the search for new drugs".

The research purpose and tasks. The purpose of the work is to develop a scientifically based composition and technology for an oil/water type antibacterial cream with an extract of the medicinal plant *Speranskia tuberculata* (Bunge) Baill. as an API, and to study its technological, physicochemical, and microbiological properties.

The following tasks were solved to achieve the goal:

- to analyse modern literature on the pharmacological use of individual species of plants of the genus *Speranskia* and to clarify the features of the use of the term TGC;

- to systematise and summarise data from literary sources on existing approaches to pharmacotherapy of skin infections with the use of herbal preparations;

- to develop methods for obtaining dry extracts of *Speranskia tuberculata* using solvents of different polarity;

- to investigate the *in vitro* cytotoxic, antioxidant and antibacterial activities of the obtained extracts, and, following selection of TGC-EA for topical formulation development, to evaluate its cytocompatibility in human skin-related keratinocyte and fibroblast models;

- to establish the relationship between the biological activity of the extracts and their chemical composition;

- to identify and quantify the main classes of biologically active substances in extracts;

- to optimise sample preparation conditions and validate spectrophotometric methods for determining the total content of phenols, flavonoids, terpenoids and alkaloids in extracts and cream;

- to substantiate the specification requirements for the active pharmaceutical ingredient;

- to develop the composition and technology of an antibacterial cream of the oil/water type with an extract of the medicinal plant *Speranskia tuberculata* (Bunge) Baill. as an API, with the selection of the main technological equipment;

- to investigate the organoleptic, physicochemical and technological characteristics of the developed cream, its microbiological purity and *in vitro* antibacterial activity, and its irritation potential using the HET-CAM model;

- to develop a draft quality control method (QCM), investigate stability indicators over time, and substantiate the storage conditions and shelf life of the developed medicinal product.

Object of research: technologies for manufacturing soft dosage forms with antibacterial action based on plant raw materials.

Subject of research: theoretical and experimental substantiation of the composition, technology, and quality control methods of extracts of the medicinal plant *Speranskia tuberculata (Bunge) Bail.* and an antibacterial cream based on it; study of its technological, physicochemical, and microbiological properties; justification of its shelf life.

Research methods. To obtain extracts of *Speranskia tuberculata* (Bunge) Baill. sequential Soxhlet extraction with solvents of different polarity, ethyl acetate (EA), petroleum ether (PE), n-butanol (n-but), and purified water was used. The *in vitro* cytotoxic activity of the extracts was evaluated using the MTT assay in four human tumour cell lines: A549 (human lung adenocarcinoma), Hep G2 (human liver tumour cell line), A-375 (human malignant melanoma), and HeLa (human cervical carcinoma). Following the selection of TGC-EA for topical formulation development, supplementary MTT experiments were performed using HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts to assess *in vitro* cytocompatibility in human skin-related cellular models. The DPPH radical-binding and pyrogallol autoxidation methods were used to determine the antioxidant activity of the extracts. Antibacterial properties against *Staphylococcus aureus*, *Escherichia coli*, and *Cutibacterium acnes* (formerly *Propionibacterium acnes*; both names are still used in the scientific literature) were investigated by the agar disk diffusion method for extracts, and by the liquid co-incubation method with cell counting (quantitative liquid co-incubation assay with CFU enumeration) for the cream. Identification of biologically active compounds was carried out using quadrupole time-of-flight tandem liquid chromatography/mass spectrometry methods. Spectrophotometric methods were used to quantitatively determine the total content of flavonoids, polyphenols, terpenoids and alkaloids. Organoleptic, physico-chemical, and microbiological indicators were used to evaluate the pharmaco-

technological properties of cream compositions. Technological stability indicators (hot, cold and colloidal stability) were studied. The FTIR method was used to determine the compatibility and stability of individual components. Microbiological studies were carried out in three directions: microbiological purity and *in vitro* antibacterial activity were assessed, and the irritation potential of the cream was assessed using the HET-CAM method, and the irritation potential of the cream was assessed using the HET-CAM method. Experimental planning and statistical analysis of results were carried out using Design of Experiments approaches and pharmacopoeial statistical methods.

The scientific novelty of the obtained results. In the work, for the first time:

- a comprehensive study of *Speranskia tuberculata* extracts obtained using solvents of different polarity was carried out, and the advantages of the ethyl acetate extract in terms of antibacterial and antioxidant activities and *in vitro* cytotoxic activity in the investigated human tumour-cell models were established; favourable *in vitro* cytocompatibility of TGC-EA was additionally demonstrated in HaCaT keratinocyte and HFF-1 fibroblast models over the investigated concentration range;

- the relationship between the biological activity of the extracts and the content of the main classes of biologically active substances was established, and the minimum necessary concentrations of phenols, flavonoids, terpenoids and alkaloids were determined, which provide a pronounced antibacterial effect of the ethyl acetate extract;

- the composition, technology and quality control system for the production of an antibacterial cream of the oil/water type with an extract of the medicinal plant *Speranskia tuberculata* (Bunge) Baill. as an active pharmaceutical ingredient were developed, the main technological equipment was selected, and a risk analysis for quality was performed based on the proposed Ishikawa diagram;

- the critical parameters of the technological process and the indicators of standardisation of the herbal active pharmaceutical ingredient were substantiated.

The approaches to the standardisation of extracts of medicinal plant raw materials were improved through comprehensive control of the content of biologically active substances and toxicologically significant impurities in accordance with the requirements of ICH Q3C and ICH Q3D.

The following were further developed:

- scientific ideas about the pharmacological potential of genus *Speranskia* plants;

- the methodology for a comprehensive combination of phytochemical, pharmacological and technological research in the development of herbal medicines.

The practical significance of the obtained results lies in the development of a scientifically based technology for an antibacterial cream based on a dry ethyl acetate extract of *Speranskia tuberculata*, with standardised quality characteristics and experimentally evaluated *in vitro* antibacterial activity and irritation potential. Specifications for the active pharmaceutical ingredient and the finished medicinal product are proposed in accordance with the applicable pharmaceutical requirements. A technological scheme for the production of an oil/water type cream has been developed, defining critical process parameters and control points, as well as key equipment units, thereby creating the prerequisites for further pharmaceutical development and potential industrial implementation of the developed product. The obtained results provide an experimental basis for further preclinical investigation and subsequent evaluation of the clinical efficacy and safety of the developed cream.

The personal contribution of the applicant consists of the following. The author directly conducted a literature review and analysis of the current state of research on the dissertation topic. The doctoral candidate, together with the scientific supervisor, determined the goal, objectives and methods of experimental research. The author independently obtained and studied dry extracts of *Speranskia tuberculata* using solvents of varying polarity, assessed their *in vitro* biological activities, determined the most promising field of application, and developed the composition and manufacturing technology for the cream, selecting the necessary key equipment. Together with the scientific supervisor, the technological scheme and a draft of the QCM were improved, and a risk assessment of the QCM was conducted. The author independently manufactured a trial batch of cream packaged in tubes and conducted all necessary studies. The author independently validated the applied spectrophotometric methods. He personally conducted statistical analysis of the results, systematised the data, and formulated the work's conclusions.

Personal contribution of the applicant to publications. 13 scientific articles were published based on the materials of the dissertation. Among them, 7 publications had only 2 co-authors, the applicant and the supervisor; the contribution of each of them is described above. The remaining six articles, in addition to the applicant and the supervisor, were co-authored by scientists with whom the research was conducted. In these works, the applicant's contribution consisted of developing and improving experimental methods and techniques, as well as collecting experimental data in the part directly related to the dissertation's materials.

Approbation of the results of the dissertation. The main provisions of the work were presented and discussed at international and all-Ukrainian scientific and practical conferences: II All-Ukrainian Conf. of Higher Education Students and Young Scientists “Innovation in Education, Science and Business: Challenges and Opportunities” (Kyiv, 2021); VI and VII International Scientific and Practical Conf. “KyivLvivPharma. Pharmaceutical Technology and Pharmacology in Ensuring Active Longevity” (Lviv, 2023 and Kyiv, 2025); Xth Scientific and Practical Conf. “Scientific and Technical Progress and Optimization of Technological Processes for the Creation of Medicinal Products” (Ternopil, 2024); XIIth Scientific and Practical Conf. "Science, Innovation and Quality in Modern Pharmaceutical Production" (Kyiv, 2024); “Zaporizhzhia Pharmaceutical Forum – 2025: Pharmacy of the Future – From the Current State to Global Challenges” (Zaporizhzhia, 2025); “Advanced Materials and Technologies: Biotechnology, Applied Chemistry and Ecology” (Kyiv, 2025); International C. on Biosecurity, Chemical Safety, and Global Cooperation (Kryvyi Rih, 2026).

Publications. The main study results were published in 7 scientific articles, including 5 papers in Ukrainian journals of Category B (speciality 226) and 2 papers in the Scopus database. Also, 8 research reports were presented at various conferences and published in conference proceedings or abstracts. Articles that additionally reflect the scientific results of the dissertation were published in 6 papers in the Scopus database.

Structure and size of the dissertation. The dissertation consists of an introduction, 5 chapters, general conclusions, a reference list (254 items on 29 pages), 68 tables and 35 figures. The main text involves 236 pages. The total volume of the work is 265 pages.

Chapter 1. Healing plant *Speranskia tuberculata* (Bunge) Baill. in medicine

1.1 Origin, growth area and botanical species of *Speranskia tuberculata*

1.1.1 Origin and growth areas

Today, natural herbal remedies represent perhaps the most essential source for creating new drugs, including new APIs. Several factors contribute to this. First, large areas with many plants, some of which remain unexplored. This situation is particularly characteristic, for example, of Asia, a large continent with a rich tradition of developing traditional means of medicine. For example, these include traditional Chinese medicine or Indian medical practices [1].

Pharmaceutical science has not kept pace with the development of traditional medicine, leaving many plants used for centuries absent from pharmacopoeias. The reason lies in the lack of research. Chinese plant *Speranskia tuberculata* (Bunge) Baill. is a typical example. It is a perennial herbaceous plant endemic to Northeast China that grows on grassy slopes, meadows, or thickets, usually in dry places, reaching 300-1900 m in height [2, 3]. *Speranskia tuberculata* is a drought-resistant plant. Hairy spherical buds along the stem produce tiny white flowers. The plant is well-known in TCM; however, it is still not listed in the Pharmacopoeia of the People's Republic of China, let alone in the Pharmacopoeia of Ukraine.

Although the plant is not listed on the International Union for Conservation of Nature's (IUCN) Red List of Endangered Plants, its range is declining. Due to the gradual intensification of agriculture, fewer and fewer areas of this plant's natural habitat are available. However, it is pretty widespread in many Chinese provinces, such as Anhui, Hebei, Henan, Jilin, Liaoning, Nei Mongol, Ningxia, Shaanxi, Shandong and Shanxi [3].

1.1.2 Botanical species

Macroscopic characteristics. *Speranskia tuberculata* (Bunge) Baill. plant (known also in China as Tou Gu cao (TGC)) does not correspond to a single botanical species, but rather to a group of phyto-materials derived from multiple plant sources recorded in different regions of China [4]. It has been reported that as many as 44 plant species have been used under this name. Nevertheless, only three major categories appear to be employed more commonly in traditional Chinese medicine. These include, firstly, the

dried whole herb of *Speranskia tuberculata,* conventionally known as “Zhenzhu Tou Gu Cao”; secondly, the dried stem of *Impatiens balsamina Linnaeus*, known as “Fengxian Tou Gu Cao”; and thirdly, the whole herbs of several *Ranunculaceae* species, including *Clematis intricata Bge*., *C. aethusaefolia Bge*., and *C. hexapetala*, which are generally classified as “Tiexian Tou Gu Cao”. The existence of multiple botanical origins under the same medicinal designation reflects the complexity of TGC's traditional use. It underscores the need to clarify its botanical basis for pharmacognostic identification, quality evaluation, and the standardisation of the medicinal raw material [5]. In the present study, *Speranskia tuberculata* (Bge.) Baill. (or Zhenzhu Tou Gu Cao) was selected as the medicinal material for the relevant academic research (Fig. 1.1).

The medicinal material of *Speranskia tuberculata* comprises both the rhizome and the aerial parts. The rhizome is cylindrical and may reach 20 cm or more in length, depending on the plant's age. Its external surface is yellowish-brown and marked by fine longitudinal wrinkles. The texture is firm and hard, and the rhizome is not readily broken. On fracture, the cortex appears reddish-purple to bluish-purple and can be easily separated, whereas the xylem is yellowish-white and the pith is relatively small.



Fig. 1.1 Wild *Speranskia tuberculata*

The aerial stem is profusely branched, measuring approximately 10–40 cm in length and 0.1–0.3 cm in diameter. The stem surface is pale green to greyish green and densely covered with greyish white, curled pubescence. The basal part is cylindrical and slightly ridged, whereas the upper part is flattened and somewhat triquetrous. The stem is brittle and prone to fracture. On a transverse fracture, the cortex is green, the xylem is pale, and the pith is usually hollow, loose, and fibrous. The leaves are greyish-green, alternate, and frequently curled, wrinkled, or fragmented. After moistening and flattening, intact leaves are lanceolate to elliptic-lanceolate,

measuring 1.5-7.0 cm in length and 0.5–2.0 cm in width. The leaf base is broadly cuneate to subrounded, and the apex is usually acuminate. The upper part of the leaf margin is entire, whereas the lower two-thirds bears sparse, blunt teeth. Both surfaces are covered with white pubescence, and the midrib is prominent on the abaxial surface. Racemes and fruits may be present. The capsule is green, triangular, and depressed-globose. The odour is slight, and the taste is initially bland, followed by slight bitterness [5, 6].

Microscopic characteristics. In transverse section, the leaf exhibits a typical dorsiventral structure. Both the upper and lower epidermis consist of a single layer of cells, and epidermal cells on both surfaces contain hesperidin crystals. Nonglandular trichomes are present on both surfaces, although they are more abundant on the lower epidermis, particularly in the region of the veins [6]. The mesophyll is differentiated into palisade tissue and spongy tissue. The palisade tissue forms a single layer immediately beneath the upper epidermis, whereas the spongy tissue is located beneath the lower epidermis. Calcium oxalate cluster crystals with sharply pointed angles are observed in both tissues, with diameters ranging from approximately 14 to 53 μm. The midrib projects on both the adaxial and abaxial surfaces, though it is more conspicuous on the lower surface. Collenchyma occurs beneath the epidermis on both sides of the midrib.

In epidermal peel preparations, the anticlinal walls of the upper epidermal cells are nearly straight, and nonglandular trichomes are rarely observed. The cells are polygonal and contain abundant hesperidin crystals. Stomata are sparse, predominantly paracytic and anomocytic, with occasional anisocytic stomata. By contrast, the anticlinal walls of the lower epidermal cells are sinuously curved, and nonglandular trichomes are more numerous. Stomata are abundant and are likewise predominantly paracytic and anomocytic, with anisocytic stomata occurring occasionally. On both epidermal surfaces, nonglandular trichomes are concentrated mainly near the veins [6]. These trichomes are usually unicellular and measure approximately 104–320 μm in length (Fig. 1.2a).

From the exterior to the centre, the stem consists of the epidermis, cortex, phloem, xylem, and pith. Epidermal cells are subquadrate or slightly tangentially elongated, and a cuticle and nonglandular trichomes cover the outer wall. Hesperidin crystals are present within the epidermal cells and generally measure 10–15 μm in diameter. The cortex

comprises 5–7 layers of cells and contains calcium oxalate cluster crystals measuring approximately 14–24 μm in diameter. Two to three layers of collenchyma are present in the outer cortex. In the pericyclic region, fibres are arranged in 2–4 interrupted rows, forming a discontinuous ring. These fibres possess thickened, slightly lignified walls and appear flattened and polygonal, with diameters of approximately 16–31 μm. The phloem is relatively narrow, whereas the xylem is well-developed. Vessels are approximately 10–45 μm in diameter and are arranged radially. The xylem rays are uniseriate, and numerous lignified fibres are present. The pith is broad, and a small number of pith cells contain calcium oxalate cluster crystals (Fig. 1.2b).

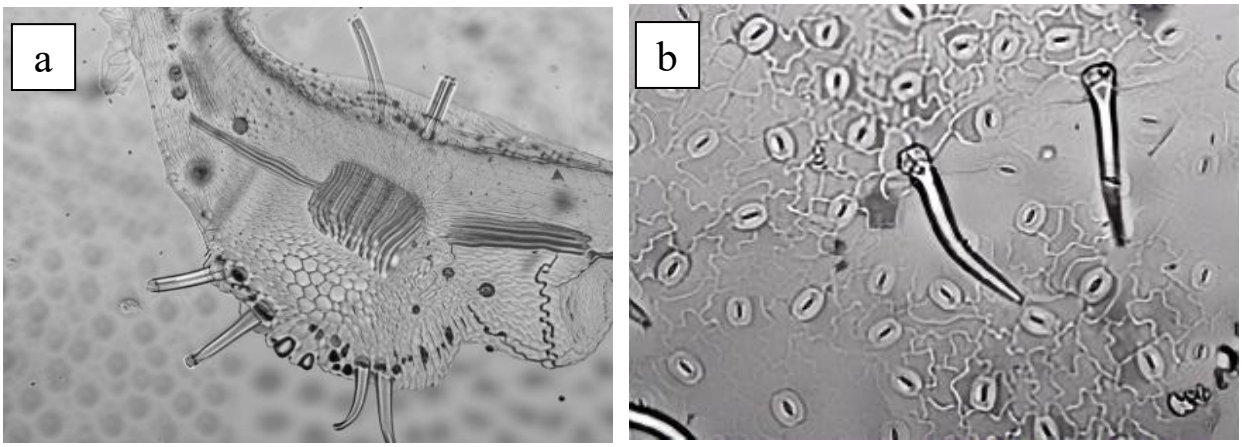


Fig. 1.2 Leaf (a) and stem (b) transections (10 × 20) of *Speranskia tuberculata*.

1.1.3 Availability of plant samples on the market

As mentioned, raw herbs *Speranskia tuberculata* (Bunge) Baill. were bought from Beijing Tongrentang Pharmacy Ltd. The seller provided all available certificates collected in Table 1.1. Two aspects seem particularly important for further consideration. First, the available sample is guaranteed to belong to the species *Speranskia tuberculata* in the form of dried aerial parts. Second, the collected plant was properly processed and stored. Post-harvest processing involved removing soil and extraneous impurities, sectioning, and subsequent natural drying. The processed material was stored in a cool, dry, and well-ventilated environment to mitigate moisture accumulation and insect infestation. The quality of certified herb samples on the market allows their use as raw materials for the manufacture of medicinal extracts. It serves as a guarantee of the stability of the results.

Table 1.1

Botanical, Ecological, and Pharmacognostic Specifications of *Speranskia tuberculata* (Bunge) Baill. provided by Beijing Tongrentang Pharmacy Ltd.

Characteristic / Parameter	**Detailed Specifications & Descriptions**
Category: Botanical Profiling	
Common / Trade Name	Zhenzhu Tougucao; formally recognised in Chinese botanical nomenclature as Digouye.
Category: Botanical Name	Speranskia *tuberculata* (Bunge) Baill.
Taxonomic Classification	Family: Euphorbiaceae; Genus: *Speranskia*.
Medicinal Part Used	Dried aerial parts (comprising stems, leaves, inflorescences, and fruits).
Category: Habitat and Edaphic Factors	
Geographical Site	Shandong Province, Zaozhuang City.
Planting Altitude	520 - 650 meters.
Temperature Requirements	The growth temperature range is 15°C to 28°C.
Moisture & Humidity	The soil relative humidity is maintained between 40% and 60%.
Photoperiodic Requirements	Heliophilous (sun-loving) with a moderate capacity for shade tolerance. The average daily sunshine duration is at least 8 hours.
Category: Phenology and Harvesting	
Life Cycle	Perennial herbaceous plant (allows for continuous annual harvesting post-establishment without the need for replanting).
Phenological Stages	Spring emergence and germination occur from March to April; anthesis (flowering phase) spans from May to August; fructification (fruiting phase) occurs from July to October.
Harvesting Time	2023.8.26
Category: Pharmacognosy and Storage	
Macroscopic Characteristics	Stems are cylindrical, greyish-green or purplish, and densely covered with grayish-white pubescence. Leaves are alternate with coarsely dentate margins. Capsules are densely covered with tuberculate (wart-like) protuberances.
Traditional Indications	Traditionally utilised to expel wind and dampness, relax sinews and activate collaterals, and disperse blood stasis to reduce swelling (Note: A qualified medical professional must direct clinical applications).
Processing and Storage	Post-harvest processing involves removing soil and extraneous impurities, sectioning, and subsequent natural sun-drying or shade-drying. The processed material must be stored in a cool, dry, and well-ventilated environment to mitigate moisture accumulation and insect infestation.
Category: Quality Control	
Moisture	≤ 8%
Acid-insoluble ash	≤ 3%
Total ash	≤ 15%
Extractives	≥ 10%

1.2 *Speranskia tuberculata* in traditional Chinese medicine

1.2.1 Known therapeutic properties

According to authoritative traditional sources, including Zhonghua Bencao, the National Compilation of Chinese Herbal Medicines, and the Dictionary of Chinese Materia Medica, the principal functions of *Speranskia tuberculata* are dispelling wind and removing dampness, relaxing the tendons and activating blood circulation, promoting blood circulation and relieving pain, dispersing blood stasis and reducing swelling, and detoxifying [7]. Its major indications include rheumatic arthralgia, contracture of the muscles and bones, traumatic injury, cold-damp beriberi, sores and carbuncles, and certain skin disorders [8]. These records suggest that the medicinal role of *Speranskia tuberculata* was established primarily through long-term empirical use. Its therapeutic scope was not confined to a single disease entity but extended to a range of conditions associated with pain, inflammation, local swelling, and musculoskeletal disorders.

From the perspective of traditional functional classification, the most important application lies in the treatment of rheumatic arthralgia, impaired joint mobility, and other painful disorders [9]. In traditional Chinese medicine, herbs of this type are not typically considered single-function agents equivalent to modern analgesics or anti-inflammatory drugs. Rather, their therapeutic significance is understood in terms of a broader regulatory effect on pathological processes such as invasion of wind-cold-dampness, blood stasis, and obstruction of the meridians and collaterals. In particular, the traditional use of *Speranskia tuberculata* for rheumatic pain, musculoskeletal contracture, and traumatic injury suggests that its principal historical application was concentrated in musculoskeletal disorders and localised pain syndromes. This therapeutic profile is consistent with traditional descriptions such as “relaxing the tendons and activating blood circulation” and “promoting blood circulation and relieving pain”, and it is also the aspect most readily supported by subsequent modern pharmacological studies [10].

Among the directions explored in modern research, anti-inflammatory and analgesic effects represent the most developed pharmacological field and the one most closely aligned with the traditional functions of TGC. Comparative studies of several medicinal materials classified as TGC have shown that *Speranskia tuberculata* exhibits

relatively strong anti-inflammatory and analgesic activity, with comparatively low overall toxicity [11]. Although different Tougucao-related medicinal materials appear to possess some degree of analgesic activity, their performance in inflammatory models is not fully consistent. In particular, *Speranskia tuberculata* demonstrated a more pronounced inhibitory effect on croton oil-induced mouse ear swelling, suggesting that it may be one of the more representative source species with respect to anti-inflammatory efficacy [12].

In addition to its anti-inflammatory and analgesic activity, TGC has been discussed regarding its antioxidant potential. Studies on the extraction of total flavonoids from *Speranskia tuberculata* (Zhenzhu Tou Gu Cao) and *Impatiens balsamina* (Fengxian Tou Gu Cao) have indicated measurable activity in *in vitro* free-radical scavenging and antioxidant assays [12, 13]. These findings may provide a partial chemical and pharmacological explanation for the traditional use of TGC in reducing swelling, alleviating inflammation, and promoting local recovery, since oxidative stress is closely associated with inflammatory responses, tissue injury, and chronic pathological states. However, the evidence in this area should still be interpreted cautiously. Many available studies focus on total flavonoid extraction, process optimisation, or *in vitro* evaluation and have not yet provided systematic evidence for a relationship between in vivo-active pharmacodynamic effects and the mechanisms of action of these constituents [13-15]. In fact, TGC shows research potential in antioxidant studies, rather than regarding antioxidant activity as a fully established pharmacological property.

A similar situation applies to antibacterial and antimicrobial research. Although some studies have reported that TGC extracts exhibit inhibitory effects against certain microorganisms, the overall body of evidence remains scattered and methodologically uneven [16, 17]. From the perspective of traditional applications, these findings are not unexpected, as TGC has long been used for sores, carbuncles, the washing of affected areas, and certain local skin disorders [16]. Such traditional patterns of external use imply that the herb may play an auxiliary role in conditions involving infection, irritation, or localised inflammatory reactions. Nevertheless, most current studies remain at the level of preliminary extract screening, and the available evidence remains insufficient to classify TGC as a natural antibacterial agent with robust pharmacological validation [17].

A more accurate interpretation is that TGC possesses exploratory value in antibacterial research, broadly consistent with its traditional background of external application. However, stronger support will require clearer evidence regarding active fractions, antimicrobial spectrum, and formulation dependence.

Overall, the available evidence supports three broad conclusions. First, at the level of traditional materia medica and long-term clinical experience, TGC is mainly considered an empirical medicinal material used for musculoskeletal contracture, rheumatic arthralgia, traumatic injury, and localised toxic swelling [7]. Second, among the currently investigated pharmacological directions, anti-inflammatory and analgesic effects are the most coherent and the most appropriate for focused academic discussion [9-11]. Third, antioxidant, antibacterial, immunomodulatory, and tumour-related activities have also been explored, but most results remain preliminary [13, 15-17]. Thus, rather than describing TGC as a multifunctional medicinal plant whose effects have already been comprehensively validated, it is more academically appropriate to characterise it as a medicinal plant with a relatively clear traditional therapeutic basis, moderate modern support in the anti-inflammatory and analgesic domain, and continuing research value in antioxidant- and external-use-related applications.

1.2.2 Dosage forms used

For medicinal plants, therapeutic properties and dosage forms cannot be regarded as entirely separate issues. The same plant, when administered as a decoction, powder, extract, external washing preparation, or semisolid formulation, may involve different patterns of constituent release, different local or systemic routes of action, and distinct quality-control requirements [18, 19]. This issue is particularly important for TGC, because its traditional use includes both internal and external administration. A systematic review of its dosage forms is valuable not only for understanding its historical application but also for establishing a basis for subsequent pharmaceutical development, including the development of the active pharmaceutical ingredient and topical cream formulations.

Among the dosage forms documented in the literature, the most fundamental and historically representative form of TGC is the decoction. In Zhonghua Bencao and related compilations, the internal use of Zhenzhu Tougucao is generally recorded as decocted in

water, usually with a customary dosage range. In contrast, external administration is often described as a decoction for fumigation and washing, or as an external wash [20]. It indicates that the medicinal use of TGC was originally established within the traditional mode of direct decoction of the crude herbal material. Decoctions are important not only because they are among the most common forms of administration in TMC, but also because they reflect the integral nature of crude herbal medicines. During water decoction, water-soluble constituents and relatively polar fractions enter the preparation simultaneously, thereby producing a complex multicomponent system rather than an isolated single-compound preparation. In traditional practice, this whole-extraction mode often more closely aligns with accumulated clinical experience. From the perspective of modern pharmaceutical research, however, it also introduces challenges such as compositional ambiguity, batch-to-batch variability, and difficulty in assigning specific pharmacological effects. Thus, the decoction represents both the most original form of TGC use and the starting point from which its modern standardisation must proceed.

In addition to simple decoction for oral administration, TGC has also been used in pills, powders, and other compound oral preparations. Traditional records indicate that it may be processed into pills or powders and used in combination with other medicinal materials that dispel wind-dampness, promote blood circulation, unblock the collaterals, disperse cold, or relieve pain [20]. This pattern suggests that TGC did not always function as an isolated single herb in traditional clinical practice, but was often incorporated into compound prescriptions together with herbs such as Fangfeng, Danggui, Haifengteng, and Shujincao [21]. This background of compatibility is highly significant because it shows that the traditional efficacy of TGC was embedded within a broader therapeutic framework focused on rheumatic arthralgia, musculoskeletal pain, and cold-damp obstruction of the channels, rather than being understood in isolation.

Another major dosage-form pathway is external application. Traditional sources indicate that TGC was used not only internally, but also in decocted washes, fumigation procedures, fresh-herb poultices, and other local applications [20]. This external-use profile is highly consistent with its range of traditional indications. For conditions such as sores and carbuncles, traumatic injury, and certain localised painful or inflammatory

disorders, local administration has a more direct therapeutic rationale than systemic oral administration alone [21]. In many traditional herbal medicines, washing, fumigation, and poultice application indicate that clinical value lies not only in systemic effects after absorption, but also in direct intervention in local inflammation, irritation, swelling, and pain. The traditional external use of TGC suggests that it was not a purely oral medicinal material but one capable of exerting local therapeutic effects. This point is especially important for modern formulation development, as it provides a traditional medical basis for advancing TGC into topical creams, gels, lotions, and other semisolid dosage forms. In this sense, the incorporation of TGC into modern topical formulations should not be regarded as an idea detached from tradition, but rather as a continuation, refinement, and standardisation of an established therapeutic logic [22, 23].

Overall, the dosage-form history of TGC does not indicate that it has already completed its transition into fully modern pharmaceutics. Rather, it reflects an intermediate stage characterised by clear traditional patterns of use, an emerging process of modern standardisation, and a relatively limited number of mature single-herb pharmaceutical products. TGC is therefore more appropriately regarded as a medicinal plant with a well-established traditional basis for decoction, washing, and external use, which has already entered research on decoction pieces, formula granules, and extracts, and which possesses realistic development potential for topical semisolid dosage forms.

1.3 Experience in disease treatment with herbal medicines

1.3.1 Herbal medicines as anticancer agents

Plant-derived compounds have made a major contribution to modern oncology. Among the different therapeutic areas associated with herbal medicine, cancer treatment provides one of the clearest examples of successful translation of botanical resources into modern, evidence-based pharmacotherapy [24]. Several compounds derived from medicinal plants or developed from plant-derived lead structures have advanced far beyond traditional use and experimental observation to become established components of standard anticancer therapy [25]. This degree of clinical translation highlights the continued value of medicinal plants not only as complementary health resources but also as sources of therapeutically valuable anticancer agents.

The significance of plant-derived anticancer agents lies not merely in their natural origin but in their chemical diversity and ability to interact with central molecular targets involved in malignant growth [26]. Some of these compounds disrupt the microtubule apparatus required for mitosis, whereas others interfere with DNA replication and repair by inhibiting topoisomerases. At the same time, many non-approved phytochemicals remain under active investigation because they modulate apoptosis, inflammation, oxidative stress, angiogenesis, invasion, and metastasis-related processes.

Clinically used and well-established plant-derived anticancer agents. Among clinically established plant-derived anticancer agents, the taxanes are among the best-established. Paclitaxel, originally isolated from Taxus species, and docetaxel, the most successful semisynthetic analogue of paclitaxel, are widely used taxanes in the treatment of breast cancer, ovarian cancer, non-small-cell lung cancer, and other malignancies [27, 28]. As a result, mitotic progression is disrupted, spindle function is impaired, and the proliferative capacity of rapidly dividing tumour cells is substantially reduced.

Beyond their mechanism of action, taxanes are notable for their evolution from natural products to routine oncology drugs. Their long-term clinical use depended not only on antitumor efficacy, but also on advances in semisynthetic production, formulation, and large-scale supply. In this sense, paclitaxel and docetaxel illustrate how a plant-derived scaffold can yield a clinically durable drug class [29].

Early development of paclitaxel was limited by low natural abundance, supply constraints, and formulation-related toxicity [30]. However, these problems were progressively addressed through semisynthetic production, improved formulation strategies, and the development of clinically useful derivatives such as docetaxel· [30]. This history is useful because it shows that the therapeutic value of a botanical resource does not depend solely on the direct extraction of the parent natural product. Clinical success also depends on whether the scaffold can be manufactured, modified, and formulated for sustained medical use. Paclitaxel activity depends on preservation of key structural features of the taxane scaffold, particularly the oxetane-containing core and the C13 side chain. It helps explain why taxane development relied heavily on semisynthetic

optimisation rather than simple extraction of the parent product. This dependence helps explain why taxane optimisation required both synthetic and semisynthetic work.

A second major clinically translated class is represented by camptothecin derivatives. Camptothecin, originally isolated from *Camptotheca acuminata*, introduced a pharmacological mechanism distinct from that of microtubule-targeting agents and thereby broadened the scope of plant-derived anticancer therapy. Instead of acting on the cytoskeleton, camptothecin derivatives inhibit topoisomerase I by stabilising the cleavage complex and converting transient DNA breaks into lethal replication-associated lesions. Because rapidly proliferating tumour cells rely heavily on efficient DNA replication and repair, this mechanism is central to anticancer treatment [31].

Camptothecin derivatives provide another major example of successful clinical translation from a plant-derived lead. Camptothecin itself exhibited a mechanism distinct from that of microtubule-targeting drugs by inhibiting topoisomerase I. However, the parent compound was limited by poor solubility and instability of its active lactone form. These limitations were partly overcome in derivatives such as irinotecan and topotecan, which retained the essential scaffold while improving pharmaceutical usability.

Additional clinically relevant examples include vincristine and etoposide. Vincristine, derived from *Catharanthus* roseus, inhibits tubulin polymerisation and disrupts spindle formation, thereby arresting mitosis and suppressing the proliferation of rapidly dividing cells [31]. Vincristine stabilises microtubules by a different mechanism than paclitaxel. It is a good example of how plant-derived compounds can target the same cellular system through opposite effects on microtubule dynamics [32].

Etoposide, by contrast, is a semisynthetic derivative of podophyllotoxin and primarily inhibits topoisomerase II, leading to DNA strand breaks, cell-cycle arrest, and apoptotic cell death [33]. It is used in the treatment of small-cell lung cancer, testicular cancer, malignant lymphoma, and leukaemia. Etoposide remains clinically relevant not only because it is still used in oncology. It also extends the range of DNA-targeting mechanisms represented among plant-derived anticancer drugs. Together, these agents show that medicinal plants have contributed clinically useful anticancer drugs through distinct mechanisms, including microtubule targeting and topoisomerase inhibition [34].

Promising herbal compounds with anticancer potential. Beyond clinically established plant-derived drugs, several herbal compounds have shown substantial anticancer potential but have not yet achieved comparable clinical translation. Curcumin, berberine, and epigallocatechin-3-gallate (EGCG) are among the most representative examples. Unlike paclitaxel or irinotecan, these compounds are not part of routine standard chemotherapy [35]. Their current value lies in their broad pharmacological activity, lead-compound potential, and possible future roles in adjunctive treatment, chemoprevention, or early development research.

These compounds are often discussed in the context of adjunctive treatment and chemoprevention because they influence multiple processes that contribute to tumour progression. Their possible value may lie in modulating oxidative stress, inflammatory signalling, survival pathways, and treatment-associated pathological responses rather than in replacing established cytotoxic drugs [35]. Another reason these compounds continue to attract attention is that they represent a different therapeutic profile from that of classic chemotherapeutic agents. Approved drugs such as paclitaxel and irinotecan achieved clinical significance through strong target-specific efficacy and extensive pharmaceutical development. By contrast, multifunctional phytochemicals such as curcumin, berberine, and EGCG derive much of their current interest from broad biological activity rather than clinically established efficacy. This distinction separates compounds with established chemotherapeutic status from phytochemicals whose current value is mainly mechanistic, adjunctive, or exploratory.

Major mechanisms underlying anticancer activity. Although plant-derived anticancer agents differ greatly in chemical structure, many converge on a small number of central pharmacological mechanisms. One of the central mechanisms is the disruption of microtubule dynamics. Paclitaxel and docetaxel stabilise polymerised microtubules and suppress dynamic instability, whereas vincristine inhibits tubulin polymerisation and interferes with spindle formation. Despite their opposite molecular effects, both strategies ultimately arrest mitosis and inhibit tumour-cell proliferation. This comparison clearly illustrates how plant-derived compounds can target the same essential cellular system through different pharmacological routes [32]. The inhibition of DNA topoisomerases is

a second major mechanism. Camptothecin derivatives act mainly through topoisomerase I inhibition, whereas etoposide targets topoisomerase II, promoting the accumulation of DNA strand breaks. Plant-derived compounds can interfere not only with the cytoskeletal machinery required for cell division, but also with the DNA-processing systems that support tumour growth and survival.

In addition to these established mechanisms, apoptosis induction and cell-cycle arrest are recurrent themes in the activity of many non-approved phytochemicals. Curcumin, berberine, and EGCG have all been associated with regulating apoptotic pathways, interfering with cell-cycle progression, and suppressing survival-related signalling in preclinical models [35]. Because tumour progression rarely depends on a single pathway, this broad activity profile helps explain continued interest in these compounds despite their limited clinical validation.

Herbal compounds have also been associated with inhibition of angiogenesis, invasion, and metastasis. Although much of this evidence remains preclinical, it broadens the anticancer scope of herbal compounds beyond simple direct cytotoxicity.

Translational limitations and future perspectives. Despite their clinical value, plant-derived anticancer agents still face substantial development challenges. Even for clinically successful drugs, sustainable production, formulation, and toxicity were major obstacles during development. As indicated by the taxane history, potent biological activity alone is not sufficient for long-term therapeutic success [30]. A natural product must be pharmaceutically tractable, manufacturable at scale, and suitable for repeated medical use [34]. It is a central lesson for herbal medicine-based drug discovery.

1.3.2 Herbal medicines as antioxidant agents

Oxidative stress contributes to the pathogenesis of many chronic diseases, including cardiovascular, metabolic, hepatic, and neurodegenerative disorders [36]. For this reason, herbal medicines with antioxidant activity have been widely investigated as supportive interventions that reduce oxidative injury, regulate endogenous defence systems, and attenuate inflammation-related tissue damage [36, 37]. Unlike plant-derived anticancer drugs, however, herbal antioxidants are used mainly in preventive or adjunctive settings rather than as universally accepted first-line therapeutics [38].

Well-established herbal antioxidants with validated efficacy. Among herbal antioxidants, silymarin is one of the better-validated examples. Derived from *Silybum marianum* (milk thistle), silymarin has long been used for liver-related disorders and is now widely discussed for its antioxidant and hepatoprotective properties [39]. Its therapeutic value is particularly relevant in chronic liver disease, where reductions in oxidative stress, attenuation of inflammation, and improvements in liver-related outcomes have been reported [40]. So, silymarin remains one of the clearest examples of an herbal antioxidant with meaningful clinical support. Silymarin also stands out because of the relative maturity of its clinical positioning. While many herbal antioxidant phytochemicals remain supported mainly by *in vitro* data or small heterogeneous clinical studies, silymarin has been repeatedly evaluated in disease-oriented settings, especially chronic liver disease [40]. It places silymarin on firmer clinical ground than many antioxidant phytochemicals, which are supported mainly by small, heterogeneous studies [39, 41].

Curcumin is another major example of a relatively well-supported herbal antioxidant. As the principal polyphenolic constituent of *Curcuma longa*, curcumin has been investigated extensively in both experimental and clinical settings.

TCM is a major part of antioxidant research on herbal medicines. Unlike the isolated-compound models from modern pharmacology, many Chinese medicinal herbs are used in disease settings in which oxidative stress is closely associated with chronic inflammation, metabolic dysfunction, vascular injury, or hepatic damage [42]. In recent years, TCM-derived antioxidants have increasingly been discussed in relation to drug-induced liver injury, cardiovascular disorders, chronic metabolic diseases, and other conditions in which redox imbalance contributes directly to tissue damage and disease progression [43]. Ginseng and ginsenosides can also be included in this higher-evidence group of plant-based antioxidant agents, although with more caution than silymarin or curcumin. Ginseng significantly reduced malondialdehyde levels and increased total antioxidant capacity, superoxide dismutase, glutathione, and glutathione reductase [44].

From the perspective of traditional Chinese medicine, several commonly used Chinese materia medica also fit well within the antioxidant framework of herbal therapy.

Salvia miltiorrhiza is often cited here because recent reviews describe it as a widely used East Asian medicinal herb with antioxidant, anti-inflammatory, vascular-protective, and hepatoprotective effects in cardiovascular and liver-related disorders. Astragalus membranaceus, especially its polysaccharide fraction, has increasingly been associated with antioxidative and mitochondria-protective actions in chronic degenerative and neurological conditions [45]. In parallel, Scutellaria baicalensis and its major flavonoids, including baicalin and baicalein, have been repeatedly reported to alleviate oxidative injury and enhance antioxidant defence, partly through Nrf2-related mechanisms [46, 47].

Promising herbal compounds with antioxidant potential. In addition to these comparatively well-supported agents, several herbs remain promising but less validated antioxidant candidates. Resveratrol is the most widely discussed example. Despite strong experimental support, meta-analyses of randomised clinical trials have shown limited and inconsistent overall effects on oxidative stress biomarkers, indicating that its antioxidant efficacy remains uncertain. Resveratrol remains important mainly in mechanistic and preclinical research, but current evidence supports describing it as a candidate antioxidant phytochemical rather than an established therapeutic antioxidant [48].

Quercetin is another important example in this category. It is a potent antioxidant phytochemical with protective effects against oxidative stress, inflammation, apoptosis, and tissue injury in multiple experimental settings [49]. However, much of the stronger evidence remains preclinical or disease-specific, and broader clinical validation is still needed before quercetin can be placed alongside the more established herbal antioxidants.

This distinction between well-supported and promising antioxidant phytochemicals is particularly important in the field of antioxidants because antioxidant claims are often easier to demonstrate *in vitro* than in clinical practice [37]. Many compounds exhibit strong radical-scavenging activity or beneficial redox effects in experimental systems, yet far fewer demonstrate consistent, clinically meaningful benefits in human trials [44].

Major mechanisms underlying antioxidant activity. The antioxidant activity of herbal medicines is not limited to direct free radical scavenging. Many phytochemicals exert antioxidant effects through a combination of direct and indirect mechanisms, including neutralising reactive oxygen species, inhibiting lipid peroxidation, preserving

mitochondrial function, and regulating endogenous antioxidant defence systems. This broader interpretation explains why herbal antioxidants are often associated not only with redox regulation but also with anti-inflammatory and cytoprotective effects.

One of the most important mechanisms involves modulation of the Nrf2/ARE signalling pathway. Natural products such as quercetin, curcumin, anthocyanins, and tea polyphenols have been reported to enhance antioxidant defence by activating Nrf2-dependent transcriptional programs, thereby increasing the expression of protective enzymes, including heme oxygenase-1, NAD(P)H quinone oxidoreductase 1, glutathione-related enzymes, and other cellular antioxidant systems [50]. In addition, maintenance of glutathione homeostasis, enhancement of superoxide dismutase and catalase activity, and preservation of mitochondrial redox balance are frequently described as part of the broader antioxidant action of plant-derived compounds [48].

Preservation of mitochondrial function is another important aspect of herbal antioxidant activity. Because mitochondria are both a major source and a major target of oxidative stress, compounds that reduce mitochondrial dysfunction may help limit downstream injury to lipids, proteins, and nucleic acids [51]. This mechanism is especially relevant in chronic metabolic and neurodegenerative diseases, where oxidative stress and mitochondrial impairment reinforce one another. From this perspective, antioxidant phytochemicals may be understood not simply as scavengers, but as modulators of broader cellular defence systems [48].

Antioxidant and anti-inflammatory actions are also closely interconnected. Oxidative stress and inflammation reinforce one another in many chronic disorders, and phytochemicals that reduce oxidative injury frequently also suppress inflammatory mediators and redox-sensitive signalling pathways [51]. As a result, the therapeutic relevance of herbal antioxidants lies not only in controlling oxidant burden but also in restoring cellular homeostasis under pathological conditions.

Translational limitations and future perspectives. Despite extensive interest in herbal antioxidants, several important limitations remain. Strong *in vitro* antioxidant activity does not necessarily translate into meaningful in vivo or clinical benefit, and many phytochemicals suffer from poor solubility, low bioavailability, rapid metabolism,

and inconsistent tissue exposure [37]. These factors are especially important for compounds such as curcumin and resveratrol, whose mechanistic promise is substantial, but whose clinical performance is often constrained by pharmacokinetic limitations [44].

Another major challenge is the heterogeneity of the available clinical evidence. Trials often differ in formulation, dosage, treatment duration, biomarker selection, and patient population, making direct comparison difficult and limiting the strength of conclusions. Some studies emphasise biochemical endpoints such as malondialdehyde, glutathione, superoxide dismutase, or total antioxidant capacity, whereas others focus more on inflammatory markers or broader disease-related outcomes. Consequently, even when a phytochemical shows encouraging biological activity, its therapeutic significance may remain difficult to define clearly [41, 44].

Future progress will therefore depend on better standardisation of herbal preparations, improved delivery systems, and more rigorous biomarker-guided clinical studies. The most valuable herbal antioxidants are likely to be those that can be linked not only to favourable changes in redox biomarkers, but also to clearly defined disease contexts and reproducible clinical outcomes [39]. Under these conditions, herbal antioxidants may become more useful not only as supportive agents, but also as more clearly defined therapeutic interventions in oxidative-stress-related diseases [40].

1.3.3 Herbal medicines as antimicrobial agents

Herbal medicines have long been used in the management of infectious diseases, but their translation into modern evidence-based anti-infective therapy has been less extensive than in oncology [52]. In contemporary clinical practice, the clearest and most firmly established examples of plant-derived anti-infective agents are concentrated in antimalarial therapy, particularly quinine from Cinchona bark and artemisinin derivatives from Artemisia annua [53]. Beyond these agents, a broad range of herbal extracts, essential oils, and phytochemicals exhibit antibacterial, antifungal, anti-biofilm, or anti-virulence activity. However, most remain topical, adjunctive, or investigational rather than universally accepted systemic anti-infective agents [54]. This distinction shows that the antimicrobial application of herbal medicines extends beyond direct drug substitution and often involves supportive or complementary strategies.

The growing interest in herbal antimicrobials is also closely related to the global problem of antimicrobial resistance. Conventional antibiotics remain indispensable, but increasing resistance, biofilm-associated persistence, and treatment-limiting toxicity have driven the search for alternative or complementary strategies [55]. This fact has resulted in renewed attention to plant-derived compounds, which often exhibit multitarget activity, including membrane disruption, antibiofilm effects, quorum-sensing inhibition, and antibiotic-potentiating properties [56, 57]. For this reason, the antimicrobial application of herbal medicines is best understood not only in terms of direct pathogen killing, but also in terms of modulation of virulence, persistence, and drug susceptibility [55].

Well-established herbal antimicrobials and clinically relevant examples. Among plant-derived anti-infective agents, quinine is among the earliest and most historically important. Derived from Cinchona bark, quinine became the first specific antimalarial medicine and remains clinically relevant today [53].

Artemisinin and its derivatives represent an even more important modern example of plant-derived antimicrobial success. Modern reviews continue to identify artemisinin-based combination therapy as central to current malaria management [58]. The artemisinin class demonstrates that a plant-derived scaffold can remain relevant not only historically, but also in modern globally important treatment strategies.

Outside malaria, the application of herbal antimicrobials is usually more limited and context-dependent. Tea tree oil from *Melaleuca alternif*olia is a representative example of a clinically used topical herbal antimicrobial. Reviews have long documented its antimicrobial properties and its use in dermatologic and oral health settings. At the same time, more recent systematic reviews indicate therapeutic promise in some human applications but also highlight variability and limitations in trial quality [54]. Tea tree oil is therefore best understood as a topical or external antimicrobial application of herbal medicine rather than as a systemically established anti-infective drug.

Berberine occupies a similarly intermediate position. It has a long history of use in intestinal and gastrointestinal disorders, and systematic review evidence suggests that it may improve clinical cure rates and shorten the duration of diarrhoea. However, the same literature also stresses the lack of consistently high-quality evidence, indicating that

berberine should be viewed as a clinically relevant herbal antimicrobial candidate rather than a fully established mainstream anti-infective medicine [59]. This distinction reflects the actual translational position of many herbal compounds: clinically meaningful in selected contexts, but not equivalent to universally accepted standard drugs [55].

Outside malaria, the application of herbal antimicrobials is usually more limited and context-dependent. Tea tree oil from *Melaleuca alternifolia* is a representative example of a clinically used topical herbal antimicrobial. Reviews have long documented its antimicrobial properties and its use in dermatologic and oral health settings. At the same time, more recent systematic reviews indicate therapeutic promise in some human applications but also highlight variability and limitations in trial quality [54]. Tea tree oil is therefore best understood as a topical or external antimicrobial application of herbal medicine rather than as a systemically established anti-infective drug.

Promising herbal compounds and TCM-oriented anti-infective applications. Beyond comparatively well-established topical or gastrointestinal applications, a large proportion of current research on herbal antibacterials is centred on compounds that have not yet become standard antibacterial drugs but show meaningful activity against resistant pathogens, biofilm-associated infections, or persistent bacterial colonisation. Among the most widely studied are allicin from garlic, EGCG from green tea, and essential-oil-derived compounds such as thymol, carvacrol, eugenol, and cinnamaldehyde [56, 60]. Research on these compounds focuses not only on their ability to directly inhibit bacterial growth but also on their capacity to alter bacterial adhesion, biofilm development, membrane integrity, and susceptibility to conventional antibiotics [60].

TCM-oriented antibacterial research is often framed less around isolated compounds and more around therapeutic contexts such as drug-resistant infection, intestinal dysbiosis, biofilm persistence, and antibiotic adjuvant therapy [61]. Such a broader framing reflects how Chinese materia medica is actually studied and applied: not only as a source of antibacterial molecules, but also as a system for modulating resistance, intervening in biofilms, and enabling combination treatment [62]. *Scutellaria baicalensis* is another useful example, especially because herbal flavonoids have been reviewed as a distinct class of antibacterial phytochemicals with activity against resistant bacteria. In

these discussions, compounds from S*cutellaria* are not presented merely as weak direct antibacterials, but as agents that may alter membrane function, interfere with nucleic-acid-related processes, suppress virulence, or potentiate conventional drugs [61].

Major mechanisms underlying antibacterial activity. The antibacterial mechanisms of herbal compounds are diverse, but several recurring patterns emerge from the current literature. One of the most consistent antibacterial mechanisms is membrane disruption. Essential oils and many lipophilic plant-derived compounds act at the bacterial membrane, altering permeability, destabilising membrane organisation, and promoting leakage of intracellular contents [63]. This mechanism has been repeatedly discussed for thymol, carvacrol, and related phenolics. It helps explain why these compounds exhibit broad-spectrum antibacterial activity. Their effects are not limited to a single canonical antibiotic target, and they may remain active even when target-specific antibiotics fail.

Herbal antibacterials can also interfere with intracellular homeostasis and bacterial metabolism. Allicin is highly relevant here because of its reactivity with thiol groups and its ability to disrupt bacterial proteins and essential cellular functions. Cinnamaldehyde has likewise been associated with changes in membrane-linked physiological processes and broader metabolic balances [57, 64]. Berberine, in turn, has been described as a multitarget antibacterial compound that can affect membrane function, transport systems, and resistance-related pathways [62, 55]. These observations are notable because many herbal antibacterials do not behave like narrowly targeted antibiotics; instead, they impose multisite physiological stress on bacterial cells. This mechanistic breadth helps explain continued developmental interest in them as antibacterial leads [57, 64].

Biofilms are central to chronic infection, antibiotic tolerance, and recurrence [65]. Bacteria in biofilms are protected by extracellular matrix, altered metabolic states, and coordinated regulatory behaviour, all of which reduce susceptibility to conventional drugs [60]. Against this background, phytochemicals that inhibit adhesion, disrupt matrix integrity, prevent maturation, or enhance antibiotic penetration become highly relevant even if their direct antibacterial potency is only moderate.

Some plant-derived compounds also reduce bacterial pathogenicity by interfering with quorum sensing and virulence-related behaviour. Reviews on Chinese medicinal

herbs and herbal flavonoids indicate that many plant-derived compounds may reduce bacterial pathogenicity by interfering with communication systems, suppressing virulence-associated phenotypes, and weakening coordinated pathogenic behaviour [63]. This line of work is conceptually important because it shifts the aim of antibacterial herbal therapy away from “killing the bacterium at all costs” toward reducing its ability to organise, persist, and damage host tissue [66]. In resistant infections, this may be particularly useful because anti-virulence strategies can complement conventional antibiotics rather than compete directly with them.

Antibiotic adjuvant activity may be one of the most clinically meaningful directions for future development. Recent work on berberine and on combinations of medicinal herbs with standard antibiotics suggests that some herbal compounds do not need to replace antibiotics to be therapeutically useful [67, 68]. Instead, they may restore susceptibility, reduce the effective antibiotic dose, or enhance activity against resistant strains such as MRSA or Gram-negative multidrug-resistant pathogens. This approach aligns more closely with the current translational state of the field than claims that most phytochemicals are ready to function as stand-alone systemic drugs [52, 67]. It also fits well with the historical logic of herbal medicine, in which combination-based therapeutic use has often been more important than single-compound substitution.

Translational limitations and future perspectives. Despite this breadth of antibacterial activity, several translational barriers remain. In particular, strong *in vitro* antibacterial activity does not automatically lead to useful clinical application. Many herbal compounds are unstable, poorly soluble, irritant at active concentrations, or difficult to formulate in ways that preserve activity while maintaining tolerability [69, 61]. The heterogeneity of the evidence also causes disorder. Some herbal antibacterials are supported mainly by planktonic growth assays, others by biofilm models, others by mechanistic cell studies, and only a minority by meaningful clinical trials [54, 59].

One more major issue is the scope of use. The current antibacterial relevance of herbal medicines is strongest in topical applications, gastrointestinal contexts, anti-biofilm research, resistance modulation, and antibiotic adjuvant strategies. It is much weaker in the form of universally accepted systemic antibacterial drugs. A clearer

definition of the antibacterial scope would make discussion of herbal antimicrobials more accurate and better align future research with the field's actual strengths [52, 67].

For TCM-derived antibacterials, future progress will likely depend on better standardisation, clearer separation between herb, extract, monomer, and formula-level evidence, and more rigorous evaluation of clinically relevant endpoints, such as synergy with antibiotics, biofilm suppression, virulence attenuation, and resistance reversal [70-72]. These endpoints are more closely aligned with the actual comparative advantages of herbal antibacterials than simple claims of broad-spectrum killing [73].

Overall, the antimicrobial field shows a pattern that differs from that of the anticancer field. In anticancer therapy, several plant-derived compounds have clearly become standard drugs. In antimicrobial therapy, the strongest translation is still seen in antimalarial agents such as quinine and artemisinin derivatives. At the same time, most antibacterial and antifungal herbal compounds remain in topical, adjunctive, or preclinical stages [61]. Even so, herbal medicines clearly contribute valuable anti-infective scaffolds, anti-biofilm mechanisms, resistance-modifying strategies, and integrated therapeutic approaches. From this perspective, the antimicrobial application of herbal medicines is best viewed not as a narrow search for antibiotic substitutes, but as a broader therapeutic field encompassing direct anti-infective agents, adjunctive phytochemicals, and TCM-based anti-virulence or supportive treatment strategies [74].

Conclusions to Chapter 1

1. The selection of *Speranskia tuberculata* (Bunge) Baill. as the primary object of study among the group of plants known as Tou Gu Cao has been justified. Despite its absence from official pharmacopoeias and its limited natural range in specific Chinese provinces, its unique botanical characteristics and therapeutic properties in TCM make it a promising candidate for integration into conventional medicine. A comprehensive morphological description of the raw material has been established, providing a necessary foundation for further pharmacognostic identification and quality evaluation.

2. The high therapeutic potential of *Speranskia tuberculata* has been demonstrated across several areas, most notably its anti-inflammatory and analgesic activities. These

properties exhibit the highest level of clinical evidence and directly correlate with the plant's traditional use in treating rheumatic conditions and musculoskeletal disorders.

3. Modern evidence-based medicine is actively moving towards revealing the potential of the *Speranskia tuberculata* herb in the following areas: analgesic and anti-inflammatory effects due to the ability of extracts to reduce swelling and relieve pain; antitumor potential as the herb can inhibit the growth of liver, cervical and melanoma cancer cells; and high antibacterial activity against some microbes due to the content of flavonoids and phenols. Such an observation confirms the feasibility of developing new, highly effective TGC-based agents to manage inflammatory processes.

4. The modes of application for the studied plant have been systematised, tracing the evolution of TGC use from traditional multi-component decoctions to localised applications (fumigation and washing). So, the development of modern topical dosage forms (creams and gels) is the most rational approach, as it maximises the plant's local therapeutic effect, as supported by centuries of clinical experience.

5. Global experience in the successful translation of herbal materials into official medicine (e.g., taxanes and artemisinin) has been summarised. The efficiency of this process depends not only on biological activity but also on the technological feasibility of producing active pharmaceutical ingredients and developing advanced delivery systems. Such a conclusion highlights the urgent need to identify optimal extraction and formulation methods specifically for the constituents of *Speranskia tuberculata*.

6. The advantages of TGC phyto-extracts over synthetic mono-preparations have been argued based on their multi-target mechanism of action. It justifies studying TGC as a holistic phytocomplex with significant potential for the therapy of chronic inflammatory and infectious states, particularly through its synergistic antioxidant, antibacterial, and immunomodulatory properties.

The research results in Chapter 1 are contained in the published article [75].

Chapter 2. Materials and methods

Like other pharmacy research, this work is primarily based on the current pharmacopoeia requirements. In this situation, the vast majority of references cite the 2nd edition of the State Pharmacopoeia of Ukraine (SPhU), comprising three main volumes and eight supplements [76-86]. As is well known, the SPhU is in the process of harmonisation with the EU Pharmacopoeia (Eur. Pharm.). Therefore, for monographs for which this process has not yet been completed, the corresponding articles of the Eur. Pharm., 11th edition, were used [87]. The pharmacopoeial volumes are listed only once to avoid repetition in the reference list. If it is necessary to refer to a particular monograph, issue, or page in the text, abbreviated references are provided, sufficient to identify the address in SPhU. These references are not duplicated, and they do not clutter the list.

2.1 Preparation of extracts

Extract preparations were based on the SPhU directions (SPhU, 2nd ed., v. 1, 5.23, p. 105-115). Dried and cleaned specimens of *Speranskia tuberculata* (Bunge) Baill. were purchased from Tongrentang Pharmacy Ltd. (Beijing, China). The samples were crushed to 20 mesh using a crusher to prepare crude extracts. Weighed 50 g of powder; 500 mL of analytically pure ethyl acetate (EA), petroleum ether (PE), n-butanol (n-but), produced by Tianjin Fuyu Fine Chemical Co., Ltd., and deionised water were added to a Soxhlet extraction flask in sequence. Extraction was performed at 72℃ for EA, 85℃ for PE, 80℃ for n-butanol, and 90℃ for water for 4 hours in each case.

After cooling to room temperature, the 4 crude extracts were centrifuged at 6000 rpm for 5 min. The resulting liquid crude extracts were dried, and the dried extracts were weighed. A stock solution at 50 mg/mL was prepared from these extracts (solvent: dimethyl sulfoxide). Later, this solution was used to prepare working solutions at concentrations of 10-1000 μg/mL for experimentation.

2.1.1 Preparation of plant raw materials

Materials. The dried whole herb of *Speranskia tuberculata (Bunge) Baill* was obtained from Tongrentang Pharmacy Ltd. (Beijing, China).

Equipment. Grinder/mill; 20-mesh sieve; analytical balance; clean, airtight containers.

Prior to extraction, the plant material was visually inspected, and foreign matter was removed to exclude samples with obvious mould, agglomeration, or deterioration. The dried herb was milled and passed through a 20-mesh sieve to obtain a powder of controlled particle size. The resulting powder was transferred into clean, dry, airtight containers and stored under conditions minimising exposure to moisture and light until use. For each extraction run, the plant powder was accurately weighed **(**50.0 g**)** using an analytical balance, and the mass was recorded. The use of a defined mesh size and a fixed starting mass served as a standardised starting point for subsequent fractionation and ensured traceability and reproducibility across extraction batches.

2.1.2 Extractants

Reagents. Petroleum ether (PE), ethyl acetate (EA), n-butanol (analytical grade; Tianjin Fuyu Fine Chemical Co., Ltd., Tianjin, China); purified water.

Equipment. Soxhlet apparatus with condenser; heating mantle or water/oil bath; volumetric glassware.

A polarity-graded solvent system was selected to obtain extract fractions spanning the low-to-high polarity range. Sequential Soxhlet fractionation was performed using petroleum ether → ethyl acetate → n-butanol → deionised water (aqueous solution or AS). For each solvent stage, a fixed solvent volume (500 mL) and a fixed plant mass (50.0 g) were used, thereby standardising the solid-to-solvent ratio. In the present project, the EA extract was designated as the API for the TGC cream. It was incorporated into the oil phase during cream manufacture. The remaining fractions were retained for comparative purposes during early-stage screening and method development, thereby maintaining a clear, traceable fractionation pathway.

2.1.3 Dissolution modes

Reagents. Petroleum ether, ethyl acetate, n-butanol, purified water; dimethyl sulfoxide (DMSO).

Equipment. Soxhlet extractor; heating mantle/bath; centrifuge; centrifuge tubes; vortex mixer; analytical balance.

Extraction was performed by sequential Soxhlet extraction. Briefly, the plant powder **(**50.0 g, 20-mesh) was loaded into a Soxhlet thimble and extracted under reflux using 500 mL of the respective solvent for 4 h per stage. The extraction sequence was petroleum ether → ethyl acetate → n-butanol → water**.** The heating temperature was adjusted according to the solvent system (PE: 85°C**;** EA: 72°C; n-Bu: 80°C; water: 90°C). After each stage, the system was cooled to room temperature and the corresponding crude extract solution was collected. To remove suspended particulate matter and improve downstream reproducibility, each crude extract solution was centrifuged at 6000 rpm for 5 min, and the supernatant was recovered for drying. Prior to the next solvent stage, the plant residue in the thimble was briefly air-conditioned under ventilation to reduce residual solvent and minimise cross-contamination; clean glassware and fresh solvent were then used for the subsequent extraction stage.

For downstream assays and analytical studies, dried extracts were reconstituted in DMSO to prepare stock solutions at 50 mg/mL. Working solutions were prepared by further dilution to concentrations in the range of 10–1000 μg/mL, as required by individual experiments. The above workflow (sequential Soxhlet extraction → centrifugation/clarification → drying → standardised stock/working solutions) ensured consistent preparation and traceability of extract inputs across the study.

2.1.4 Drying

Reagents. None beyond the clarified extract solutions obtained in Section 2.1.3.

Equipment. Rotary evaporator, freeze-drier, analytical balance.

Following centrifugation and recovery of the clarified supernatant, each solvent extract was concentrated to obtain the dried extract. This step removes residual solvents as completely as practicable, yielding a stable, weighable sample suitable for reproducible reconstitution, quantitative dosing, formulation development, and storage.

2.2 Testing the medicinal properties of extracts

2.2.1 In vitro cytotoxicity and cytocompatibility assessment

Six established human cell lines were used in the MTT studies. Four human tumour cell lines—A549 (lung adenocarcinoma-derived), A-375 (malignant

melanoma-derived), HeLa (cervical cancer-derived) and Hep G2 (human liver tumour-derived)—were included to characterise the *in vitro* cytotoxic activity of the investigated TGC extracts.

Following selection of the ethyl acetate extract (TGC-EA) as the active pharmaceutical ingredient for subsequent development of a topical antibacterial cream, two additional human skin-related cellular models were included: HaCaT human immortalised keratinocytes and HFF-1 human foreskin fibroblasts. These two cell lines were used to assess non-specific cytotoxicity and *in vitro* cytocompatibility of the extracts in cellular models more directly relevant to topical application. HaCaT and HFF-1 were not included as tumour-cell models. Detailed information on the origin, identification, authentication, traceability, and experimental roles of the human cell lines used in the MTT assays is provided in Table 2.1. All cell lines used in the MTT experiments were commercially obtained from Wuhan Pricella Biotechnology Co., Ltd. (Procell, Wuhan, China). The supplier name and catalogue number of each cell line were recorded, and the corresponding Cellosaurus accession numbers, Research Resource Identifiers (RRIDs), and recognised reference-collection cross-references were used to provide additional identity and traceability information. These identifiers allow the cell-line designation used in the present study to be linked to internationally recognised reference records.

Lot-specific Certificates of Analysis, STR authentication reports, and mycoplasma quality-control documents were not available for inclusion in the present dissertation. No independent STR authentication of the experimental cell stocks was performed by the authors. Therefore, the information presented in Table 2.1 should be interpreted as documentation of the commercial source, catalogue identity, and reference-database cross-references of the cell lines rather than as independent authentication of the specific cell stocks used in the experiments.

Table 2.1

Origin, identification, authentication, traceability and experimental role of the human cell lines used in the MTT assays *

Cell line	Experimental role	Biological origin	Supplier and catalogue No.	Authoritative identifier/reference cross-reference
A-549	Human tumour-cell model; used to evaluate *in vitro* cytotoxic activity	Human lung adenocarcinoma-derived cell line	Procell, Wuhan, China; Cat. No. CL-0016	Cellosaurus CVCL_0023; RRID:CVCL_0023; ATCC CCL-185
A-375	Human tumour-cell model; used to evaluate *in vitro* cytotoxic activity	Human malignant melanoma-derived cell line	Procell; Cat. No. CL-0014	Cellosaurus CVCL_0132; RRID:CVCL_0132; ATCC CRL-1619
HeLa	Human tumour-cell model; used to evaluate *in vitro* cytotoxic activity	Human cervical cancer-derived cell line	Procell; Cat. No. CL-0101	Cellosaurus CVCL_0030; RRID:CVCL_0030; ATCC CCL-2
HepG2	Human liver tumour-cell model; used to evaluate *in vitro* cytotoxic activity.	Human liver tumour-derived cell line	Procell; Cat. No. CL-0103	Cellosaurus CVCL_0027; RRID:CVCL_0027; ATCC HB-8065
HaCaT	Human skin-related keratinocyte model; used for *in vitro* cytocompatibility assessment	Spontaneously immortalised human keratinocyte cell line	Procell; Cat. No. CL-0090	Cellosaurus CVCL_0038; RRID:CVCL_0038
HFF-1	Human skin-related fibroblast model; used for *in vitro* cytocompatibility assessment	Human foreskin fibroblast cell line	Procell; Cat. No. CL-0352	Cellosaurus CVCL_3285; RRID:CVCL_3285; ATCC SCRC-1041

** For all cell lines listed in Table 2.1, lot-specific Certificates of Analysis and STR authentication records were not available for inclusion in the dissertation, and the authors performed no independent STR authentication of the experimental stocks. Supplier catalogue identifiers and recognised reference-database cross-references are provided for traceability.*

Cell culture media contain different concentrations of buffers, inorganic salts, glucose, amino acids, vitamins, and numerous bioactive compounds. All cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) + 10% FBS (Fetal Bovine Serum) medium. Cells were cultivated in dishes with a diameter of 10 cm. The cultivation box was kept at a constant temperature of 37°C and a CO_2 concentration of 5%; according to cell growth conditions, fluid exchange or passage was performed

every 2 days or every other day. Cultured cells were passaged in different ways depending on the cell type. Adherent cells were passaged by digestion. Part of the adherent growth, but not the firm cells, can also be passaged by direct blowing. Suspended cells can be separated and passaged by direct blowing, centrifugal precipitation, or natural sedimentation to remove the supernatant, then blown and passaged. The cell counting method was used to assess the growth state of cultured cells and, if necessary, to determine the biological effects of medium, serum, and drugs.

The extraction and concentration of 100 g *Speranskia tuberculata* (Bunge) Baill. yielded an EA-based crude extract weighing 1.53 g and a PE-based crude extract weighing 3.63 g. The concentrated extracts were dissolved in dimethyl sulfoxide to prepare a 50 mg/mL solution for the cell model experiment.

An MTT cytotoxicity assay was used to evaluate the concentration-dependent cytotoxic effects of the TGC extracts at concentrations of 12.5–75 μg/mL in four human tumour cell lines: A549, A-375, HeLa and Hep G2. Following the selection of TGC-EA for topical formulation development, supplementary MTT assays were performed in HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts over an extended concentration range of 12.5–1000 μg/mL to assess *in vitro* cytocompatibility. The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide, from Sigma-Aldrich) is a simple and reliable method for the preliminary evaluation of anticancer agents [88]. The experimental principle was as follows. MTT can react with succinate dehydrogenase in mitochondria of living cells to form a blue-purple crystalline formazan, an insoluble, water-soluble product. The blue-violet formazan exhibits a characteristic absorption peak at 492 nm upon dissolution in dimethyl sulfoxide (DMSO). When the number of collected cells was within a specific range, the number of living cells was proportional to the absorbance *A* after formazan dissolution. The following formula calculated cell viability:

$$Cell\ viability, \% = \frac{A_x}{A_0} \times 100\%, \quad (2.1)$$

where indexes *x* and *0* refer to the sample with concentration *x* and the blank sample.

The experimental procedure for the MTT assay was as follows. Each of the six human cell lines was seeded into 96-well plates at a density of approximately 0.5–1.0

× 10^4 cells/well in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were cultured for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 to allow adequate attachment. For comparative screening of extract-associated cytotoxic activity, the four human tumour cell lines A549, A-375, HeLa and Hep G2 were exposed to TGC extracts at final concentrations of 12.5, 25, 37.5, 50, 62.5 and 75 µg/mL. The final culture volume was 100 µL per well. Following selection of TGC-EA as the active pharmaceutical ingredient for subsequent topical formulation development, supplementary MTT experiments were performed using HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts. Both TGC-PE and TGC-EA extracts were evaluated at final concentrations of 12.5, 25, 37.5, 50, 62.5, 75, 100, 200, 500 and 1000 µg/mL. The extended concentration range was selected to characterise cytocompatibility over a broader exposure interval and to include concentrations overlapping with and exceeding those associated with the *in vitro* antibacterial activity of the extracts.

For each concentration, three replicate wells were prepared. Untreated cells cultured under the same conditions served as the control group. The outermost wells of the 96-well plates were filled with 100 µL PBS to minimise edge effects associated with evaporation. After exposure to the extracts for 24 h, the treatment medium was removed, and the cells were washed twice with PBS. Subsequently, 100 µL of MTT-containing medium was added to each well, and the plates were incubated for a further 3–4 h at 37°C. After incubation, the MTT-containing medium was carefully removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The plates were gently agitated for 10 min to ensure complete solubilisation, after which the absorbance of the resulting formazan solution was measured at 492 nm using a microplate reader. Cell viability was calculated relative to the untreated control using Equation (2.1). Each experiment was repeated independently three times. Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed as described in Section 2.6, with $p < 0.05$ considered statistically significant.

2.2.2 Antioxidant activity

Using 2,2-diphenyl-1-picrylhydrazyl (DPPH) is generally recognised as a rapid method for studying antioxidant activity [89]. It is based on the study of free radical behaviour and includes spectrophotometric measurements of the antioxidant capacity to scavenge DPPH radicals. This experiment consisted of a control group and 4 sample groups, with 96-well plates used for each group. Sample groups were prepared by dissolving 10 mg of the different extracts in 180 μL of deionised water, then adding 90 μL of 0.1 mM DPPH solution to the reaction system. The final concentrations of the extract samples were 5, 10, 20, 50 and 100 μg/mL. The control group used the same solvent volume to replace these 5 extracts, adding 90 μL of 0.1 mM DPPH solution to each well. After thoroughly mixing them, they were placed in a shaker and reacted in the dark for 15 min. The absorbance of the sample (OD_A) and control (OD_{A0}) groups was measured at 520 nm using a SpectraMax M5 spectrophotometer; the DPPH radical scavenging (DPPH) rate was calculated as

$$\text{DPPH rate} = \frac{OD_{A0} - OD_A}{OD_{A0}} 100\%. \quad (2.2)$$

Another well-known method of studying antioxidant properties is the Pyrogallol autoxidation method [90]. Pyrogallol rapidly self-oxidises in the presence of molecular oxygen in an aqueous or alkaline environment. The zero adjustment solution was prepared by placing 600 μL 0.1 M Tris-HCl buffer (pH=8.2) and 600 μL distilled water in a centrifuge tube. Mix well, then place in a 25°C water bath for 20 minutes. Solution I: Take 600 μ L 0.1 M Tris-HCl buffer solution (pH=8.2) and 580 μL distilled water. Mix them, place the samples, set a control group, and set the concentrations of the extracts to be tested in the sample groups to 5, 10, 20, 50, and 100 μg/mL. Solution II: 20 μL of 5 mM pyrogallol solution. Under water-bath conditions, solutions I and II were mixed at 37°C for 10 min, and the absorbance was measured at 325 nm. During the measurement process, the first recording began at 0 minutes, with a 1-minute interval between recordings. The results of 5 consecutive measurements were recorded. The superoxide anion radical scavenging (SARS) rate was calculated as

$$\text{SARS rate} = \frac{\Delta A_0 - \Delta A_x}{\Delta A_0} 100\%, \quad (2.3)$$

where ΔA_x and ΔA_0 refer to the self-oxidation rate of the sample and control groups.

The IC_{50} values, which characterise the inhibitor concentration required to inhibit a biological process by 50%, were calculated from graphs of SARS vs concentration.

2.2.3 Antibacterial activity

Agar disk diffusion method for extracts. Three bacteria were used to evaluate antibacterial activity: *Staphylococcus aureus (S. aureus or SA), Escherichia coli (E. coli or EC),* and *Cutibacterium acnes (C. acnes or CA).* Bacterial colonies for the studies were taken from a -80℃ refrigerator. After thawing at room temperature (25℃), 50 μL of bacterial liquid was inoculated into a test tube. Then, 5 mL of culture medium was added at a 100:1 ratio. The next step was incubation at 37℃ for 12 h. 100 μL of the bacterial solution was spread evenly on a plate containing solid culture medium. After standing for 12 min, the sterilised filter paper was attached to the surface of the culture medium.

The antibacterial capacity of 4 types of extracts (PE, EA, n-but and aqueous solution (AS)) was determined as follows. Experimental samples were divided into 2 groups: a control group (distilled water) and sample groups (4 types of extracts, each at concentrations of 10, 50, 100, 200, and 500 μg/mL). 5 μL of solutions at different concentrations were applied to filter paper for further solvent evaporation. The sample obtained this way was placed in a shaking incubator at a constant temperature to observe the results and measure the diameter of the antibacterial zone (D) after 12 h.

$$Antibacterial\ rate = \frac{D_{samples} - D_{control}}{D_{control}} \times 100\%, \quad (2.4)$$

$D_{samples}$ refers to the diameter of the antibacterial zone of the sample groups;

$D_{control}$ refers to the diameter of the antibacterial zone of the control groups.

Quantitative liquid co-incubation assay with CFU enumeration. The antibacterial experimental method and steps for studying cream samples were as follows. The reference strains *S. aureus* and *C. acnes* were utilised for the antibacterial assay. A single colony was inoculated into sterile Mueller-Hinton (MH) broth and incubated aerobically at 37°C with continuous shaking (150 rpm) for 24 h to reach the late logarithmic growth phase. The bacterial suspension was subsequently centrifuged

at 4000 rpm for 5 min. The supernatant was discarded, and the cell pellet was washed twice with sterile physiological saline (0.9% NaCl) to remove residuals. The bacterial concentration was adjusted using a spectrophotometer (OD600) and then diluted in sterile MH broth to achieve final inoculum concentrations of 10^9 CFU/mL for TGC extract and 10^6 CFU/mL for TGC cream. The *in vitro* antibacterial activity was evaluated using a liquid co-incubation model.

The experimental groups were established in sterile tubes (or 96-well deep-well plates) as follows: Treatment Group: 1 mL of the TGC extract (or TGC cream) working solution + 1 mL of the bacterial suspension. Negative Solvent Control: 1 mL of MH broth containing the equivalent concentration of DMSO + 1 mL of the bacterial suspension. Subsequently, 100 µL of the appropriately diluted suspensions were spread evenly onto MH agar plates using a sterile L-shaped spreader. The plates were inverted and incubated at 37°C for 24 h. All experiments were performed in triplicate.

Following incubation, plates exhibiting between 300 and 500 morphologically typical *S. aureus* and *C. acnes* colonies were selected for enumeration. The bactericidal rate was calculated using colony-forming units (CFU) from the vehicle control group as the baseline, accounting strictly for potential solvent interference. The calculation was performed according to the following equation:

$$Antibacterial\ rate = \frac{N_c - N_t}{N_c} \times 100\%, \quad (2.5)$$

where N_c and N_t are the average numbers of viable bacteria (CFU/mL) in the Negative Solvent Control and the treatment group, respectively.

2.3 Identification and study of bioactive organic compounds

2.3.1 High-performance liquid chromatography-mass spectrometry

The compounds in the obtained extracts were studied using a quadrupole time-of-flight tandem liquid chromatograph/mass spectrometer (QTOF-LC/MS, Agilent Technologies, USA). It is a high-resolution, accurate-mass instrument designed for the analysis of complex samples. Chromatographic separation was performed using an Agilent 1290 UPLC liquid chromatographic system coupled to an Agilent 6550 QTOF

mass spectrometer. The main specifications of the LC front-end and the MS platform are summarised in Tables 2.2 and 2.3, respectively.

Table 2.2

Main parameters of the Agilent 1290 UPLC liquid chromatographic system

Item	Specification / Resource	Description
System type	Analytical UHPLC/HPLC system	Liquid chromatographic front end for high-speed and high-pressure separations.
System pressure and flow range	Up to 1300 bar and 5 mL/min	Provides the operating range required for rapid and efficient UHPLC analysis.
Pump module	High-pressure binary solvent delivery	Used for accurate solvent delivery under high-pressure analytical conditions.
Autosampler/injector	Typically, 0–20 µL or 0–100 µL, depending on module configuration	Used for precise sample introduction; the injection range depends on the installed sampler.
Sample temperature control	4–40 °C	Maintains sample stability prior to injection.
Column temperature control	Typically, up to 80 °C	Provides controlled thermal conditions for chromatographic separation.

Table 2.3

Analytical performance, identification software, and database resources of the Agilent 6550 Q-TOF mass spectrometer

Item	Specification/Resource	Description
Mass resolution	Up to 40,000	High-resolution accurate-mass analysis for compound identification.
Mass accuracy	Sub-ppm	Enables reliable elemental composition assignment and compound confirmation.
Acquisition and control software	MassHunter Acquisition for LC/MS	Used for instrument control, method setup, tuning, calibration, and data acquisition.
Identification software	MassHunter Qualitative Analysis	Used for qualitative data processing, molecular formula generation, and compound identification.
Database/library management software	MassHunter PCDL Manager	Used for creating, editing, and managing personal compound databases and libraries.
Database/library type	PCD / PCDL	Used for accurate-mass screening and library-based identification.
Representative database resource	METLIN Metabolite PCD/PCDL	Commonly used for metabolite screening and tentative identification.
Other optional database resources	Application-specific PCDLs and MS/MS libraries	Applicable to different analytical purposes, such as extractables and leachables, mycotoxins, pesticides, and environmental contaminants.

Mass spectra were obtained in negative ESI mode (100–1500 m/z). The parameters were as follows: drying gas (nitrogen), 15 L/min; sheath gas temperature, 350°C; sheath gas flow rate, 12 L/min; capillary voltage, 3200 V.

Compounds were separated on a Waters C18 2.1×50 mm, 1.7 μm column in gradient mode. The mobile phase A (water with 0.1% formic acid) and mobile phase B (methanol) were set as follows: 70% A–30% B (0–7 min), 60% A–40% B (7–17 min), 20%A–80%B (17–26 min), 10%A–90%B (26–31 min), with a 4 min balance to 90%A–10% B. The injection volume was 20 μL, with a flow rate of 0.3 mL/min.

2.3.2 Classification of compounds by classes

As is known, the number and structure of organic compounds are extremely large and complex, respectively. Their classification based on certain characteristics is not easy. It should be noted that there are classifications based on chemical characteristics and on pharmaceutical characteristics. However, in all cases, some substances are difficult to classify within a single approach and require adjustments or exclusions. The classification proposed in [91] covers almost all substances. However, the result is a rather complex classification, which is difficult to generalise.

The classification used in the work is based on the division of substances into classes, taking into account their primary medical properties and the possibility of determination using identical chemical methods. Preliminary analysis showed that the proposed approach allows us to distinguish four classes of substances, namely polyphenolic substances, flavonoids, terpenes and terpenoids, and alkaloids. At the same time, such a division is not absolutely unambiguous, since, for example, flavonoids are a subgroup (variety) of a large family of polyphenols. At the same time, the proposed approach has a significant advantage: the substances considered in the vast majority have one or another therapeutic property (often close within the same class). They can therefore generally be considered biologically active.

Polyphenols are a general name for thousands of compounds that have more than one aromatic ring and more than one hydroxyl group (-OH) in their structure. They are found mainly in plants, function as powerful antioxidants that neutralise free radicals,

and exhibit anti-inflammatory effects. The main classes of polyphenols include flavonoids, phenolic acids, stilbenes, lignans, and others.

In turn, flavonoids are a large class of plant polyphenolic compounds that are secondary metabolites, play a key role in plant life, and have significant antioxidant, anti-inflammatory, and immunomodulatory effects on the human body. Flavonoids are distinguished by their specific structure: two benzene rings connected by three carbon atoms (C6-C3-C6 skeleton). Flavonoids are classified into subclasses according to the degree of oxidation of the central propane chain into flavones, flavonols, flavanones, isoflavones, anthocyanins, chalcones, and aurones.

Alkaloids are a large group of nitrogen-containing organic compounds with basic (alkaline) properties, mainly of plant origin but also of animal origin. A complex structure characterises them, often containing heterocyclic rings with nitrogen atoms, and they have a strong physiological effect on the human and animal body.

Terpenes are unsaturated hydrocarbons (isoprenoids) produced by many plants and some insects. Terpene molecules are built from five-carbon isoprene units (C_5H_8) that are connected "head to tail" and classified by the number of isoprene units - from hemiterpenes (1 unit), monoterpenes (2 units, $C_{10}H_{16}$) and to polyterpenes. Terpenes have a strong odour and biological activity (antibacterial and anti-inflammatory).

2.3.3 Pretreatment of TGC cream samples for spectrophotometric determinations

Because TGC cream is a typical O/W semi-solid system, and the active ingredient, TGC-EA Dry Extract, was incorporated into the oil phase during manufacture, the target finished product constituents are not simple aqueous solutions. They are distributed within a complex microstructure composed of lipid domains, interfacial regions, and the continuous phase. Also, the formulation contains stearic acid, cetearyl alcohol, petrolatum, apricot kernel oil PEG-6 esters, polysorbate 80, *Acacia Senegal Gum*, and *Astragalus Gummifer Gum*, all of which may interfere with direct spectrophotometric or colourimetric determination. That is why, prior to the determination of bioactive compounds, the cream samples were subjected to an appropriate pretreatment procedure involving demulsification, extraction, low-temperature lipid precipitation, centrifugation, and membrane filtration [92].

Since these four classes of constituents differ in extraction behaviour and polarity, a single extraction protocol was not appropriate for all analytes [93]. Accordingly, 1.00 g of TGC cream was weighed separately for each assay, and each analyte group was pretreated independently. A non-polar pre-defatting step with petroleum ether or n-hexane was deliberately avoided at the initial stage, since such treatment could result in the loss of analytes, particularly terpene-rich and medium-polarity constituents originally dissolved or dispersed in the oil phase. Instead, a release-oriented pretreatment strategy based on alcohol-assisted demulsification, analyte transfer into the extraction phase, low-temperature lipid precipitation, and clarification was employed.

2.3.4 Determination of the total flavonoids and polyphenol contents

Preparation of the Rutin Standard Curve. The aluminium nitrate–sodium nitrite colourimetric method was employed. Weigh 20.0 mg of rutin reference standard, dried to constant weight, and transfer to a 100 mL volumetric flask. Dissolve in methanol and dilute to volume to obtain the reference stock solution. Pipette 0.2, 0.8, 1.4, 2.0, 2.6, and 3.2 mL of the stock solution into separate 10 mL volumetric flasks. Add 4 mL methanol, then accurately add 0.4 mL of 5% (w/v) sodium nitrite solution, mix well, and leave for 6 min. Subsequently, add 0.4 mL of 10% (w/v) aluminium nitrate solution, mix well, and leave for 6 min. Add 4.0 mL of 4% (w/v) sodium hydroxide solution, add methanol to the mark, mix well, and leave for 15 min.

The use of $NaNO_2$ and NaOH as reagents in addition to $Al(NO_3)_3$ (or $AlCl_3$) results in a change in the resulting colour from light-yellow to intense red-pink. Accordingly, the absorption maximum shifts from 425 nm to 510 nm [247, 248]. It is believed that this avoids overlapping spectra of other pigments, which usually “fade out” after 500 nm. Finally, measure the absorbance at 510 nm and obtain the regression equation of the standard curve.

Preparation of the Gallic Acid Standard Curve. Accurately weigh 2.75 mg of gallic acid reference standard and transfer to a 25 mL volumetric flask. Dissolve in distilled water and dilute to volume; mix well to prepare a gallic acid reference stock solution at 0.11 mg/mL. Accurately pipette 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mL of the

stock solution into separate 10 mL volumetric flasks. Add 6 mL of distilled water and mix thoroughly, then add 0.5 mL of Folin–Ciocalteu reagent, mix well, and allow to stand for 5 min. Add 1.5 mL of 10% (w/v) sodium carbonate solution, dilute to the mark with water, and mix well. Keep in the dark for 1.5 h, measure the absorbance at 760 nm, and obtain the regression equation of the standard curve.

Rationale of the cream pretreatment route. Acidified 80% methanol was used because flavonoids and polyphenols are generally medium- to high-polarity constituents that are efficiently extracted in aqueous methanolic media. Mild acidification was applied to facilitate their release from the cream structure, especially from the O/W interfacial regions and the gum-based matrix. Low-temperature lipid precipitation followed by secondary centrifugation was used to minimise interference from petrolatum, fatty alcohols and acids during colourimetric assays.

Pretreatment for the TGC cream. A weighed portion of 1.00 g of TGC cream was transferred into a 50 mL centrifuge tube. 5.0 mL of 0.5% acidified 80% methanol was added, and the sample was vortex-mixed for 2 min to obtain a uniform dispersion. The mixture was then sonicated at 35–40°C for 30 min. After extraction, the sample was centrifuged at 9000 rpm for 10 min, and the supernatant was collected. The residue was re-extracted twice with 4.0 mL and 3.0 mL of the same solvent, respectively, by the same procedure. The three supernatants were combined. The combined extract was kept at 4°C for 30 min to promote lipid precipitation. The sample was subsequently centrifuged again at 10000 rpm for 10 min, and the clarified supernatant was recovered. When necessary, the extract was concentrated to approximately 2 mL at <40°C, then brought to a final volume of 5.00 mL with the same extraction solvent. The solution was filtered through a 0.45 μm PVDF membrane filter and subsequently placed in a lyophiliser for lyophilisation into a solid powder at -10°C. The acquired cream powders are used to determine total flavonoids and total polyphenols [94-96].

Determination of Total Flavonoid and Total Polyphenol Contents in TGC Extracts of different polarities and TGC cream. Weigh 0.2 g of each TGC dry extract or TGC cream powder accurately, transfer to a 50 mL volumetric flask, add 95% ethanol to volume, and mix well. Accurately pipette 3 mL of each of the four

solutions, and determine the contents as shown in the previous sections. Calculate the total flavonoid and polyphenol contents in the extracts and cream powders [97, 98].

2.3.5 Determination of the total content of terpenoids

Preparation of the Terpenoid Standard Curve. Prepare a 0.1 mg/mL ursolic acid reference standard solution for use. Aliquots of 0.0, 0.01, 0.02, 0.04, 0.1, 0.2, and 0.3 mL of the standard solution were heated to dryness. Subsequently, 0.1 mL of vanillin and 1 mL of sulfuric acid solution were added, mixed, and incubated in a 60°C water bath for 30 min. The mixture was removed, cooled immediately, kept in an ice-water bath for 15 min, and the absorbance was measured at 540 nm. With absorbance as the abscissa and ursolic acid mass as the ordinate, the standard curve was plotted.

Rationale for the cream pretreatment route. Compared with flavonoids and polyphenols, terpenes are relatively more lipophilic. Thus, a highly aqueous extraction solvent was considered less suitable. At the same time, non-polar pre-defatting at the beginning of sample preparation was avoided, because part of the terpene fraction could be lost together with the lipid phase. High-strength ethanol was selected as a compromise solvent capable of both demulsifying the cream and extracting terpene-rich constituents. Low-temperature lipid precipitation and secondary centrifugation were applied to reduce interference from residual oily excipients.

Pretreatment for the TGC cream. An accurately weighed portion of 1.00 g of TGC cream was placed in a 50 mL centrifuge tube, and 5.0 mL of 95% ethanol was added. After vortex mixing for 2 min, the sample was sonicated at 35–40°C for 30 min. The mixture was then centrifuged at 9000 rpm for 10 min, and the supernatant was collected. The residue was extracted twice with 4.0 mL and 3.0 mL of 95% ethanol, respectively. The extracts were combined and stored at 4 °C for 30 min to allow precipitation of residual lipids, followed by centrifugation at 10000 rpm for 10 min. The clarified supernatant was collected. If required, the extract was gently concentrated to approximately 2 mL at <35°C, then adjusted to 5.00 mL with 95% ethanol. The final extract solution was filtered through a 0.45 μm PTFE membrane filter, then placed in a lyophiliser and lyophilised to a solid powder at -10°C. The acquired TGC cream powders were utilised to determine total terpenes.

Determination of Total Terpenoid Contents in TGC Extracts of different polarities and TGC cream. Weigh 20 mg of the TGC dry extracts or TGC cream powders, add methanol, and make up to 10 mL. After ultrasonication for 10 min, centrifuge at 3,000 r/min for 10 min, and then use for analysis. Accurately transfer 200 μL of the prepared solution into an EP tube and evaporate the solvent to dryness. Add 0.1 mL of vanillin and 1.0 mL of sulfuric acid solution, mix well, and incubate in a 60°C water bath for 30 min. Measure the absorbance at 540 nm, and calculate the total terpene mass fraction of each extract by substituting into the regression equation.

2.3.6 Determination of the total content of alkaloids

Preparation of Reference Standard Solution. Weigh 8 mg of Tuberostemonine reference standard. Dissolve in chloroform, shake well, and make up to volume in a 50 mL volumetric flask. Accurately pipette 5 mL of this solution, add 5 mL of chloroform and 2 mL of 0.04% bromocresol green buffer solution, shake vigorously, and leave for 30 min. After phase separation, collect the lower chloroform layer and dilute to 10 mL to obtain a reference standard solution at 0.08 mg/mL.

Rationale for the cream pretreatment route. Alkaloids are generally more readily transferred into the extraction phase under acidic conditions, where they are present in protonated form. Acidified 70% ethanol was therefore used to improve the release of alkaloid components from the lipid domains and the cream matrix. If the subsequent total alkaloid assay required additional alkalinisation, phase transfer, or specific derivatisation, the filtered extract obtained in this pretreatment step was used as the starting solution for the corresponding analytical procedure.

Pretreatment for the TGC cream. A weighed portion of 1.00 g of TGC cream was transferred into a 50 mL centrifuge tube; 5.0 mL of 1% HCl in 70% ethanol was added. The sample was vortex-mixed for 2 min and sonicated at 35–40°C for 30 min. The extract was then centrifuged at 9000 rpm for 10 min, and the supernatant was collected. The residue was extracted twice more using 4.0 mL and 3.0 mL of the same acidified ethanol solution. The extracts were combined, stored at 4°C for 30 min to induce lipid precipitation, and centrifuged again at 10000 rpm for 10 min. The supernatant was collected, adjusted to 5.0 mL, and filtered through a 0.45 μm

membrane filter. It was placed in a lyophiliser and lyophilised to a solid powder at -10°C. The obtained TGC cream powders were used to determine total alkaloids.

Preparation of Test Solution. Weigh 1 g of TGC dry extracts or TGC cream powders into a conical flask. Add 50 mL of anhydrous ethanol and subject to ultrasonic extraction for 30 min. Vacuum-filter off, then dilute the filtrate to 50 mL with anhydrous ethanol. Take 0.5 mL of the sample solution, add 10 mL of chloroform and 2 mL of 0.04% bromocresol green buffer solution, shake for 3 min, and leave for 30 min. Collect the lower chloroform layer and dilute to 10 mL to obtain the test solution.

Determination of Total Terpenoid Contents in TGC Extracts of different polarities and TGC cream. At the maximum absorption wavelength of 416 nm, use chloroform as the blank control for the colourimetric determination of the total alkaloid content in the test solution [103].

2.3.7 Determination of solvent residues

The values of residual quantities of organic solvents were determined in accordance with regulations of SPhU, 2nd ed., v. 1, 5.4, p. 930.

2.4 Pharmaco-technological parameters of cream at different stages of pharmaceutical development

The development of semi-solid formulations, particularly multiphase cream systems, is a highly complex pharmaceutical engineering process. Establishing a standardised, quantifiable, and scientifically robust evaluation system at each developmental stage—from initial excipient screening and cream base formulation to the final API-loaded cream (TGC cream)—is paramount. This section meticulously delineates the methodologies and evaluation criteria used to assess the physicochemical properties, component compatibility, technological stress stability, and the completeness of dissolution of the active extract within the formulation.

Before experimenting, a 10-point evaluation system for cream bases was developed as an author-designed integrated tool for the comparative ranking of experimental formulations at the stage of pharmaceutical development; it is not a pharmacopoeial method or a regulatory acceptance criterion. The selection of parameters and the establishment of scoring ranges were carried out taking into account

the principles of ICH Q8(R2) Pharmaceutical Development and the corresponding Ukrainian guideline ST-N MoH Ukraine 42-3.0:2011 "Medicinal Products. Pharmaceutical Development", which provide for the scientifically justified identification of quality attributes affecting the characteristics of the medicinal product, as well as the general pharmacopoeial and technological requirements for semi-solid medicinal products for cutaneous use. Details of the regulatory documents used are provided in Tables 2.4–2.7. The evaluation system included parameters that, according to the results of preliminary experimental studies, were found to be the most informative for selecting a cream base: pH, dynamic viscosity, organoleptic characteristics, spreadability, homogeneity, and technological stability. Scores were assigned according to the degree of compliance of each sample with the technological requirements pre-established within this study, while the categories "bad", "satisfactory", "good", and "very good" were used exclusively for the comparative evaluation of the experimental formulations within this experiment. The division of the overall 10-point score into four levels made it possible to assign a corresponding score range to each level and to provide a more differentiated ranking of the experimental samples.

2.4.1 Criteria for the quality characteristics of a cream base

The organoleptic properties, rheological behaviour, and physicochemical stability of a semi-solid cream base directly dictate patient compliance, the API's release kinetics, and the product's overall shelf-life. To transpose subjective organoleptic evaluations into reproducible quantitative data, a rigorous 10-point semi-quantitative scoring scale was used. This evaluation matrix assesses the cream basis across four primary dimensions: Description Standards, Technological Stability, pH, and Dynamic Viscosity. The scores are stratified into four distinct classifications: Bad (0-2), Satisfactory (3-5), Good (6-8), and Very Good (9-10). The specific determination methods and evaluation criteria are as follows:

1) **Determination and Scoring of Descriptive Criteria**. The descriptive attributes are assessed by trained personnel through organoleptic and physical testing under standardised illumination (D65 daylight illuminant). They include five

parameters: **appearance**, which evaluates the structural state of the basis after cooling to room temperature; **colour**, which reflects the light-scattering properties of its internal droplets and the system's homogeneity; **transmittance** of light, which closely correlates with emulsion droplet size and density; **texture**, which is evaluated by placing a small aliquot of the cream between two clean glass slides, applying gentle pressure, and observing the spread edge and internal structure; and **spreadability**, which simulates clinical application. A description of the possible values for each parameter, along with the corresponding scores, is given in Table 2.4.

Table 2.4

Description criteria (ICH Q8(R2) [218]; ST-N MoH Ukraine 42-3.0:2011 [217]; SPhU, 2nd ed., Vol. 1 [76]; Ph. Eur. 11th ed., Mon. 0132 [87])

Description	Bad (0-2)	Satisfactory (3-5)	Good (6-8)	Very Good (9-10)
Appearance	Inhomogeneous liquid upon cooling	Homogeneous solid upon cooling, but thin	Homogeneous solid upon cooling, but hard	Homogeneous viscous solid upon cooling
Colour	Transparent and colourless	Transparent and white	Milky white but variegated	Homogeneous milky white
Transmittance	Easy light transmission	Slight light transmission	No obvious light transmission	No light transmission
Texture	Very bad roundness and rough texture	Bad roundness and rough texture	Good roundness and ordinary texture	Good roundness and very good texture
Spreadability	Difficult to apply, and no graininess	It can be applied, but there is graininess	Easy to apply, but there is graininess	Easy to apply, and no graininess

2) Determination and Scoring of Technological Stability. Physical stability is the critical metric for evaluating the cream's resistance to environment-induced phase separation (coalescence, flocculation or creaming) in three directions (Table 2.5).

Centrifugal Stability. A 5.0 g sample is placed in a centrifuge tube and subjected to a specified rotational speed (e.g., 3000-4000 rpm) for 15-30 min.

Cold-resistant Stability. A 5.0 g sample is stored at a low temperature (-5°C to 4°C) for 24 h, then allowed to equilibrate to room temperature.

Heat-resistant Stability. A 5.0 g sample is incubated in a thermostatic chamber (40°C or 45°C) for 24 h.

Table 2.5

Technological stability (ICH Q8(R2) [218]; ST-N MoH Ukraine 42-3.0:2011 [217]; ICH Q1A(R2) [222]; Ph. Eur. 11th ed., Mon. 0132 [87])

Classification	Bad (0-2)	Satisfactory (3-5)	Good (6-8)	Very Good (9-10)
Centrifugal Stability	Obvious stratification	Slight stratification	No obvious stratification	No changes & homogeneous cream
Cold-resistant Stability	Obvious changes & inhomogeneous cream	Slight changes & homogeneous cream	No obvious changes	No changes & homogeneous cream
Hot-resistant Stability	Obvious water-oil separation	Slight water-oil separation	No obvious water-oil separation	No changes & homogeneous cream

3) Determination and Scoring of pH. The human stratum corneum is covered by a slightly acidic mantle (pH~4.5-5.5). Topical formulations should align with this physiological pH to prevent skin irritation, barrier disruption, or alterations in API permeation. For the assay, 1.0 g of the cream is weighed and dispersed in 9.0 mL of freshly boiled and cooled deionised water by sonication. The pH is measured with a high-precision glass electrode pH meter calibrated with standard buffers (Table 2.6).

Table 2.6

pH (at 25°C) (ICH Q8(R2) [218]; ST-N MoH Ukraine 42-3.0:2011 [217]; SPhU, 2nd ed., Vol. 1, 2.2.3 [76]; Ph. Eur. 11th ed., 2.2.3 [87])

Bad (0-2)	Satisfactory (3-5)	Good (6-8)	Very Good (9-10)
pH＞ 9.0 or pH＜3.0	8.0＜pH＜9.0 or 3.0＜pH＜4.0	7.0＜pH ＜8.0 or 4.0＜pH＜5.0	5.0＜pH ＜7.0

4) Determination and Scoring of Dynamic Viscosity (DV). As with typical non-Newtonian fluids, the dynamic viscosity of creams directly reflects the mechanical strength of the internal three-dimensional network. It governs the formulation's rheological profile (e.g., thixotropy and pseudoplasticity) and its clinical extrudability. Measurements are conducted using a rotational viscometer at a strictly controlled temperature of 25±0.5°C. An appropriate spindle is selected, and the shear rate is set to 10 rpm. Readings are recorded once the spindle rotation stabilises. Viscosities that are either excessively high or low are detrimental to the application (Table 2.7).

Table 2.7

Dynamic Viscosity (DV) (25°C, 10 rpm) (ICH Q8(R2) [218]; ST-N MoH Ukraine 42-3.0:2011 [217]; SPhU, 2nd ed., Vol. 1, 2.2.10 [76]; Ph. Eur. 11th ed., 2.2.10 [87])

Bad (0-2)	Satisfactory (3-5)	Good (6-8)	Very Good (9-10)
DV＞60 Pa·s or DV＜5 Pa·s	5Pa·s＜DV ＜10 Pa·s or 50 Pa·s＜DV ＜60 Pa·s	10 Pa·s＜DV ＜20 Pa·s or 40 Pa·s＜DV ＜50 Pa·s	20 Pa·s＜DV ＜40 Pa·s

2.4.2 Chemical compatibility of components

During the preformulation phase, ensuring that no deleterious chemical reactions occur between excipients or between excipients and the API is a prerequisite for establishing formulation viability. Particularly in multicomponent cream bases, water-phase excipients (aqueous solutions of humectants, thickeners, and preservatives) and oil-phase excipients (long-chain fatty alcohols, esters, mineral oils, and emulsifiers) are susceptible to hydrolysis, esterification, oxidation, or cross-linking during high-temperature emulsification and long-term storage. This study innovatively employs Fourier Transform Infrared Spectroscopy (FTIR) as the primary analytical modality for assessing chemical compatibility. Unlike traditional High-Performance Liquid Chromatography (HPLC), FTIR enables direct, in situ probing of intramolecular and intermolecular vibrational states without destroying the semi-solid microarchitecture.

1) Sample Preparation and Instrumental Parameters. The infrared spectra of four parallel sample groups, namely the pure water phase mixture (all water phase excipients), the pure oil phase mixture (all oil phase excipients), the blank cream basis (the physical mixture formed by emulsification, devoid of API), and the final cream product (containing the TGC extract), are acquired to validate compatibility. An FTIR spectrometer was equipped with an Attenuated Total Reflectance (ATR) accessory. A highly durable and high-refractive-index diamond or Zinc Selenide (ZnSe) ATR crystal is utilised. Prior to each measurement, the crystal surface is cleaned with isopropanol and air-dried to collect a blank background spectrum. An appropriate aliquot (approx. 0.1g) of the semi-solid sample is evenly spread across the ATR crystal, ensuring intimate contact without entrapped air bubbles. The scanning range is set from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1}, and 32 co-added scans are collected

per sample to optimise the signal-to-noise ratio. Upon completion, the raw instrumental transmittance (%) data are exported as CSV files for downstream processing.

2) Spectral Interpretation Logic and Evaluation Principles. Rigorous spectral comparison is used to substantiate the absence of interactions between components. The idea relies on the principle of spectral superposition: assuming no covalent chemical reactions occur between the aqueous and lipophilic phases during emulsification (i.e., solely physical mixing), the FTIR spectrum of the cream basis should be a linear superposition of the pure water and oil spectra. During data processing, the principal characteristic peaks of each sample are extracted:

Water Phase Signatures. Emphasis is placed on the broad absorption band near 3300-3400 cm^{-1} (attributed to O-H stretching vibrations of water molecules or polyols) and the strong absorption bands between 1000-1100 cm^{-1} (attributed to C-O stretching of primary or secondary alcohols).

Oil Phase Signatures. The "fingerprint" signals of the long-chain aliphatic hydrocarbon backbone are rigorously monitored. These include the asymmetric stretching vibration (~2917 cm^{-1}, Asym. Stretching, -CH_2-), the symmetric stretching vibration (~2850 cm^{-1}, Sym. Stretching, -CH_2-), the methylene scissoring bending (~1465 cm^{-1}), and the long-chain planar rocking vibration (~720 cm^{-1}). If esters are present in the oil phase, the ester carbonyl (C=O) stretching vibration peak near 1711-1750 cm^{-1} must be closely scrutinised.

3) Acceptance Criteria for Chemical Compatibility. Every prominent characteristic peak present in the cream basis spectrum must be unequivocally traced back to its origin in either the pure water phase or the pure oil phase. The strict criterion for concluding excellent chemical compatibility (i.e., the absence of chemical reactions) is defined as follows: Compared to the spectra of the raw components (water and oil phases), absolutely no additional peaks should appear in the cream basis spectrum, nor should any original characteristic peaks disappear. A critical caveat in this evaluation is the influence of solvents. Since emulsification disperses the oil phase as microdroplets within the water phase, the polarity of the molecular microenvironment is altered, and extensive new hydrogen bond networks may form

(e.g., between water molecules and the polar heads of emulsifiers). These physical environmental alterations can induce extremely small wavenumber shifts (typically $\Delta\nu < 10\ cm^{-1}$) in certain characteristic peaks, particularly hydroxyl or carbonyl bands. In spectral interpretation, this study explicitly classifies such minor shifts as intermolecular physical interactions rather than covalent bond cleavage or formation. Provided the premise of "no new functional groups generated" and "no original groups lost" is satisfied, FTIR provides definitive proof of excellent chemical compatibility among the excipient components within the cream formulation.

2.4.3 Technological stability and homogeneity

This subsection was designed to evaluate whether the TGC cream could maintain an acceptable degree of compositional uniformity and structural integrity when exposed to the main technological variables involved in manufacturing, namely temperature, stirring, homogenisation, and cooling. In accordance with the study design, a combination of process observation and FTIR monitoring was employed in order to assess both the macroscopic and molecular responses of the system to these process-related stresses. The purpose of this methodological approach was not only to determine whether the formulation remained visually homogeneous throughout manufacture, but also to verify that the applied technological conditions did not induce detectable chemical changes in the formulation components.

Samples were collected at the critical stages of the manufacturing process. These sampling points included: (1) the aqueous phase after preparation; (2) the oil phase after complete melting; (3) the cream basis formed by complete homogenization of the aqueous and oil phases; (4) the final cream product obtained following the complete homogenization of the API with the cream basis; (5) the final cream product subjected to a centrifugal stability test; (6) the cream product subjected to a heat-resistant stability test; (7) the final product subjected to a cold-resistant stability test. This sampling scheme was intended to cover the full sequence of technological transitions: dissolution and melting, dispersion, emulsification, and final structuring during cooling.

At the macroscopic level, each sample was visually examined for its appearance, the presence or absence of oil droplets or aqueous exudation, the occurrence of local

agglomerates or undispersed particles, and the overall uniformity of the system. Special attention was paid to the changes occurring at different stages of processing. The aqueous phase was examined for clarity and the absence of undissolved material; the oil phase was checked to confirm complete melting and the absence of residual solid particles; the oil phase containing the extract was observed for visible agglomeration; the post-homogenisation sample was evaluated for the formation of a homogeneous cream rather than a streaked or phase-separated intermediate; and the cooled final product was assessed for evidence of oiling-out, water separation, coarsening, or non-uniform thickening. If all samples remained consistent with the intended technological objective at each stage, the formulation was considered to possess an acceptable degree of resistance to process-related external influences.

In parallel, FTIR spectra were recorded for the samples obtained at the critical process stages and compared with those of the final cream product. The principal criterion was not that all spectra should be identical in absolute intensity, but that no new characteristic absorption bands should appear and no original bands should disappear after heating, stirring, homogenisation, or cooling. Any spectral differences were interpreted primarily in terms of physical variations, such as changes in baseline, relative intensity, or band broadening, rather than as evidence of chemical transformation. Particular emphasis was placed on comparing spectra before and after the high-temperature stage and before and after high-shear homogenisation, since these process steps are most likely to affect the system's physical state. If the spectra remained essentially unchanged in terms of characteristic bands, it was concluded that the manufacturing process altered only the dispersion state and microstructure of the cream, but not the chemical structure of the formulation components.

To further assess homogeneity, additional sampling of the final cream was performed at different positions within the batch, including the upper, middle, and lower portions of the vessel, as well as at different locations before filling. These samples were compared by visual observation and FTIR analysis. If no differences in macroscopic characteristics were observed and the FTIR spectra remained comparable, the cream was considered sufficiently homogeneous, indicating that the mixing and

homogenisation procedures were adequate and that no significant spatial segregation of components had occurred during production.

It should be emphasised that the term “technological stability” in this subsection refers specifically to the stability of the system under the external influences encountered during manufacture, rather than to long-term storage stability. Accordingly, this methodological approach was intended to answer whether the manufacturing process could reproducibly generate a homogeneous cream without inducing process-related instability or chemical incompatibility. In this sense, the present subsection provides a process-oriented methodological basis for the subsequent evaluation of the final product's quality and storage stability.

2.4.4 Completeness of dissolution of components

Visual process observation was adopted as the primary method for evaluating the completeness of component transformation, and the assessment was performed based on the physicochemical class of each formulation constituent. For water-soluble components, certain preservatives, EDTA, polysorbate 80, and glycerin, completeness was assessed during aqueous phase preparation. Satisfactory completion was achieved if, after the prescribed heating and mixing period, the system appeared clear or at least uniformly free of visible particles, with no sediment, floating material, or local turbidity. The persistence of white particles, sediment, or cloudy regions was regarded as evidence of incomplete dissolution. Accordingly, for these components, completeness was defined primarily in terms of clarity and visual uniformity.

For oil-phase structural excipients, including petrolatum, stearic acid, cetearyl alcohol, and apricot kernel oil PEG-6 esters, the relevant process was not molecular dissolution but complete melting and formation of a uniform oil phase. During oil phase preparation, the system was considered acceptable only when all solid or semi-solid excipients had been transformed into a visually homogeneous liquid phase, free of residual crystals, unfused fragments, or local solid domains. Thus, the observational criteria were based on flow behaviour, visual uniformity, and the absence of residual solid material. Complete melting at this stage was considered essential for reproducible incorporation of the active extract and for subsequent emulsification.

For TGC-EA Dry Extract, the expected state in the oil phase is more appropriately described as dispersion or suspension rather than true molecular-level dissolution. Consequently, its completeness was evaluated by comparing the cream base without the extract with that containing the extract. If, following the addition of the extract, the system remained homogeneous and showed no visible agglomerates, sediment, coloured specks, fibrous residues, or perceptible graininess, the extract was considered to have achieved an acceptable degree of dispersion under the selected processing conditions. Conversely, the presence of obvious particles, local colour inhomogeneity, spread-film residues, or rapid settling after short standing was taken to indicate incomplete dispersion or insufficient deagglomeration.

For the natural gums (*Acacia Senegal Gum* and *Astragalus Gummifer Gum*), the critical process was wetting and hydration, rather than dissolution in the strict sense. These materials are known to be prone to the formation of “fish-eye” structures, in which an outer hydrated layer encloses an incompletely wetted dry core. Therefore, the evaluation focused on whether the system remained free of gel lumps, fish-eyes, thread-like agglomerates, or dry powder inclusions after the prescribed mixing and hydration period, and whether the viscosity appeared uniform throughout the sample. The presence of transparent or semi-transparent gel nuclei, or a visibly uneven viscosity distribution, was interpreted as evidence of incomplete hydration.

Finally, the completeness of the component transformation was verified again at the final emulsion level. Even if each constituent had reached its appropriate state in its respective phase, the finished cream still had to be examined for overall homogeneity, the absence of visible oil droplets or aqueous exudation, the absence of white or coloured particles, and the absence of perceptible graininess. Thus, the final endpoint of this subsection was not the isolated completion of an individual component, but the successful integration of all constituents into a homogeneous, finely textured, and physically coherent cream system**.**

Accordingly, the methodological principle of this subsection may be summarised as follows: the completeness of dissolution, melting, dispersion, and hydration of the different formulation components was assessed by stage-specific visual observation;

for the plant extract, dispersion completeness was evaluated by comparing the cream basis without the dry extract with the corresponding system after extract incorporation; and the final acceptance criterion was the formation of a homogeneous cream free from visible particles, graininess, and phase separation.

2.5 Quality control and standardisation of bulk and tube cream

2.5.1 Control of the antibacterial activity in bulk and tube cream

Research Objective. The *in vitro* antibacterial activity of the TGC cream was evaluated against two selected test microorganisms, *Staphylococcus aureus* and *Cutibacterium acnes*. *S. aureus* was included as a representative skin-associated bacterial pathogen, whereas *C. acnes* was selected because of its involvement in the multifactorial pathogenesis of acne. This experiment aims to overcome the diffusion limitations of the traditional agar well diffusion method for lipophilic creams by employing a "quantitative time-kill assay" to quantitatively evaluate the *in vitro* antibacterial activity of the formulation under defined contact conditions. Concurrently, the intra-batch homogeneity of the emulsification and scaling filling processes is validated during biological activity studies by parallel measurements of samples from different production stages.

Bulk intermediate product (Bulk cream). Following completion of high-shear emulsification in the homogenisation reactor, 5 g of sample is precisely extracted from three spatial levels within the reactor: the top, middle, and bottom.

Finished tube product (Tube cream). Five finished tubes are randomly sampled from the front, middle, and back temporal phases of the automated filling line (samples are collected after discarding the initial extruded portion).

Control Sample. A blank cream base, formulated using the same manufacturing process as the test samples but without the 0.1% TGC-EA extract, is used to eliminate background interference caused by the matrix's physical encapsulation effect.

Test Strains. The Gram-positive aerobic/facultative anaerobic bacterium, *Staphylococcus aureus* (ATCC 25923), and the Gram-positive obligate anaerobic bacterium, *Cutibacterium acnes* (ATCC 6919 or 11827).

Culture Media. For *S. aureus*: Conventional Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA). For *C. acnes*: Brain Heart Infusion (BHI) broth and anaerobic agar supplemented with 5% defibrinated sheep blood.

Chemical Neutraliser and Diluent. Sterile Phosphate-Buffered Saline (PBS, pH 7.0) containing 3% Polysorbate 80 and 0.3% lecithin, utilised to quench the activity of the preservative system (parabens) in the cream and emulsify the lipids. Sterile 0.9% saline is used for serial bacterial dilutions.

Instruments and Equipment. Aerobic thermostatic incubator and full-temperature orbital shaker incubator (temperature accuracy ±0.5℃); Obligate anaerobic jar and accessory system (equipped with anaerobic gas-generating sachets and redox indicators to establish a microenvironment of O_2<1% and CO_2 ≤10%); UV-Vis spectrophotometer; Clean bench (ISO Class 5); Automated colony counter.

Operating Procedures.

Step 1: Pre-dispersion of cream samples. Precisely weigh 1.0 g of the cream samples from each sampling point (as well as the blank basis) into sterile test tubes. Add a small volume of sterile PBS containing Polysorbate 80, and vigorously vortex at 37℃ to completely disintegrate and disperse the sample into a homogeneous primary oil-in-water (O/W) dispersion, thereby maximising interfacial contact between the TGC-EA extract and the pathogenic cell membranes.

Step 2: Preparation of high-titer bacterial suspensions. Cultivate *S. aureus* under aerobic conditions (18- 24 h) and *C. acnes* under strict anaerobic conditions (48- 72 h) until the logarithmic growth phase is reached. Harvest the cells, wash and resuspend them in sterile saline, then adjust the concentration to 1.0×10^6 to 5.0×10^6 CFU/mL.

Step 3: Dynamic co-culture contact. Precisely pipette 9.0 mL of the standardised bacterial suspension into each test tube containing 1.0 g of the pre-dispersed cream. Place the *S. aureus* test tubes in a 37℃ aerobic orbital shaker (150 rpm); place the *C. acnes* test tubes inside the nitrogen-flushed anaerobic incubation jar and incubate in a 37℃ shaker. A uniform contact time of 24 hours is established.

Step 4: Preservative neutralisation and serial dilution. Upon completion of the 24-hour contact period, immediately pipette 1.0 mL of the mixture from each tube into

9.0 mL of the chemical neutraliser (Tween-lecithin system) and vigorously vortex for 1 minute to completely terminate the antibacterial reaction. Subsequently, perform 10-fold serial dilutions (down to 10^{-4}) using sterile saline.

Step 5: Pour 1.0 mL of each diluted sample into Petri dishes. For *SA*, use molten TSA and incubate aerobically at 37℃ for 24 h. For *PA*, use blood-supplemented anaerobic agar and incubate within the anaerobic jar at 37℃ for 48-72 h.

Viable Count and Antibacterial Rate Calculation. Following incubation, select plates with 30-300 colonies to precisely calculate the surviving Colony Forming Units per gram (CFU/g) of the cream sample after the 24-hour contact period. The mathematical formula for the Antibacterial Rate (R) is defined as follows:

$$R(\%) = \frac{N_{blanck} - N_{sample}}{N_{blanck}} \times 100\%, \quad (2.6)$$

where N_{blank} represents the average viable bacterial count of the blank cream basis control group after the identical contact time, and N_{sample} represents the viable bacterial count of the API-loaded experimental cream group.

2.5.2 Description of the appearance of the cream

Research Objective. The description of organoleptic and appearance characteristics serves as the primary evaluation metric in the quality control system for semi-solid formulations. It provides a critical basis for preliminary judgments on the thermodynamic stability, excipient compatibility, and process reproducibility of the emulsified system. In accordance with the SPhU, 2nd ed., suppl. 7, v. 2, p. 283, which outlines general requirements for the description of soft dosage forms (including appearance, homogeneity, and consistency), a semi-quantitative sensory scoring system was developed based on approaches commonly used in cosmetic and pharmaceutical research. This study aims to establish a 10-point (0–10) semi-quantitative scale to convert subjective visual and tactile evaluations into more reproducible quantitative data. Given that the 0.1% TGC-EA extract is completely soluble in the lipid phase without interfering with the background colour, an optimal final cream should exhibit a pure, homogeneous milky white appearance driven by the Mie scattering effect of the internal phase microdroplets.

Test of Bulk intermediate product (Bulk cream). Following completion of high-shear emulsification in the homogenisation reactor, 5 g of sample is precisely extracted from three spatial levels within the reactor: the top, middle, and bottom.

Test of Finished tube product (Tube cream). Five finished tubes are randomly sampled from the front, middle, and back temporal phases of the automated filling line (samples are collected after discarding the initial extruded portion).

Reagents and Materials. Colourless, transparent advanced optical glass slides and coverslips (for microscopic texture observation via the glass slide compression method); Non-reflective pure black and pure white standard contrast background plates (for comparative evaluation of colour purity and transmittance); Standard biomimetic membranes (for the spreadability test).

Instruments and Equipment. The colour assessment cabinet is equipped with a D65 standard daylight illuminant. The environmental illuminance must be strictly calibrated and maintained between 1000 and 1500 Lux to eliminate subjective visual biases among operators and colour differences caused by variations in natural lighting conditions. A high-magnification stereomicroscope is equipped with cold light illumination and a digital imaging system, utilised to magnify and inspect for insoluble particulates and phase separation phenomena at the microscopic level.

Operating Procedures. Under the D65 standard light source, trained personnel specialising in pharmaceutical organoleptic evaluation sequentially inspect the five core dimensions of the samples using standardised methods:

Step 1 (Appearance): Extrude the cream onto the observation plate and focus on evaluating its phase structure. Observe whether it maintains a homogeneous semi-solid state with optimal viscosity (the optimal state) or exhibits process defects such as an inhomogeneous liquid state, excessive hardness, or excessive thinness.

Step 2 (Colour): Place the samples on both white- and black-contrast plates. Observe whether the cream presents a homogeneous, pure milky white colour (the optimal state), and meticulously examine for any transparency, localised pigmentation, or variegated colours.

Step 3 (Transmittance): Spread a defined thickness of the cream into a thin layer

on a transparent glass plate for back-lit transmission observation. Assess the system's density; a dense, high-quality emulsified system will induce total internal reflection and multiple scattering, resulting in no light transmission (the optimal state).

Step 4 (Texture): Employ the glass slide compression method by precisely weighing approximately 0.1 g of the cream between two glass slides and applying uniform pressure to flatten it. Observe the roundness of the spreading edge and the fineness of the internal structure of the flattened film.

Step 5 (Spreadability): Simulate clinical topical administration by applying 0.5 g of the sample onto standard biomimetic membranes and gently spreading it in circular motions with constant pressure. Carefully evaluate the smoothness during application, checking for any frictional resistance or minute graininess.

Based on the physical phenomena observed during the operating procedures, map the results to four predefined quality tiers and assign scores: Bad (0-2), Satisfactory (3-5), Good (6-8), and Very Good (9-10). Record the scores of the 3 independent samples and calculate the average score for each dimension.

2.5.3 pH of the cream

Research Objective. The pH value is a core parameter for evaluating the quality and safety of topical semi-solid formulations. According to SPhU, 2nd ed., suppl. 1, 2.2.4, p. 31, the pH must be strictly controlled to align with the slightly acidic physiological "acid mantle" of human skin (pH:4.5-5.5) to prevent epidermal barrier disruption and irritation. Furthermore, an optimal pH is vital for maximising the antimicrobial efficacy of the preservative system (methyl and ethyl parabens) and for maintaining the rheological properties of the natural macromolecular thickeners (*Acacia Senegal* and *Astragalus Gummifer* gums) in the aqueous phase.

Test of Bulk intermediate product (Bulk cream). Following completion of high-shear emulsification in the homogenisation reactor, 5 g of sample is precisely extracted from three spatial levels within the reactor: the top, middle, and bottom.

Test of Finished tube product (Tube cream). Five finished tubes are randomly sampled from the front, middle, and back temporal phases of the automated filling line (samples are collected after discarding the initial extruded portion).

Reagents and Materials. Freshly boiled and cooled ultra-pure deionised water (conductivity <1.0 μS/cm); Certified standard pH buffer solutions (e.g., pH 4.00 and pH 6.86/7.00) with established traceability.

Instruments. High-precision benchtop pH meter equipped with a glass electrode; Automatic Temperature Compensation (ATC) system (accuracy ±0.01 pH units); Thermostatic ultrasonic water bath; Magnetic stirrer with Teflon-coated stir bars.

Operating Procedures.

Step 1: Calibration. Perform a two-point calibration of the pH meter using the standard buffer solutions to ensure the theoretical slope falls within the acceptable operational range of 95.0% to 105.0%.

Step 2: Sample Dispersion. Accurately weigh 1.0±0.05 g of the cream sample into a clean 50 mL glass beaker. Add 9.0 mL of the CO_2-free ultra-pure water. Place the beaker in an ultrasonic water bath set to 35-40℃ for 10 minutes to facilitate dispersion of lipid microdroplets, until a uniform milky suspension forms. Subsequently, strictly equilibrate the dispersion to 25.0±0.5℃.

Step 3: Measurement. Immerse the thoroughly rinsed and dried pH electrode in the 10% cream dispersion. Initiate magnetic stirring at 100 rpm to ensure uniform ion exchange at the liquid junction. Record the pH reading once the "Stable" indicator on the display remains constant for at least 15 seconds.

Step 4: Electrode Maintenance. Post-measurement, cleanse the electrode with a mild non-ionic surfactant solution to remove adhered emulsified lipids, thoroughly rinse with copious amounts of ultra-pure water, and store in a 3M KCl solution.

2.5.4 Dynamic viscosity of the cream

Research Objective. Dynamic viscosity is a pivotal pharmaco-technological parameter characterising the rheological profile of a semi-solid formulation. As a typical non-Newtonian fluid exhibiting pseudoplasticity and thixotropy, the viscosity of this cream is governed by the dense three-dimensional macromolecular network formed by hydrated *Acacia* and *Astragalus gums*, synergised with the liquid crystalline interfacial film constructed by the oil phase (stearic acid, cetearyl alcohol). Accurate quantification of dynamic viscosity is crucial, as it directly dictates the clinical

extrudability from the tube, tactile spreadability on the skin, and the thermodynamic resistance to phase separation (creaming or coalescence) over the product's shelf life.

Test of Bulk intermediate product (Bulk cream). Following completion of high-shear emulsification in the homogenisation reactor, 5 g of samples are precisely extracted from three spatial fluid levels: the top, middle, and bottom of the reactor.

Test of Finished tube product (Tube cream). Five finished tubes are randomly sampled from the front, middle, and back temporal phases of the automated filling line (samples are collected after discarding the initial extruded portion).

Reagents and Materials. Standardised viscosity calibration fluids (Newtonian silicone oils) are used for periodic instrument verification and calibration.

Instruments and Equipment. Rotational viscometer complying with pharmacopoeial specifications (Brookfield RVT and DV digital series); High-precision circulating thermostatic water bath system connected to the sample chamber.

Operating Procedures.

Step 1: Equilibration and Thixotropic Recovery. Immerse the sample cup containing the loaded cream in the thermostatic water bath and let it rest undisturbed for at least 30 min. This mandatory resting period serves a dual purpose: it ensures the sample's core temperature precisely equilibrates with the standard testing temperature of 25.0±0.1℃, and it allows the polymer network and emulsion microstructure, which were sheared during extrusion, to undergo sufficient thixotropic recovery.

Step 2: Instrument Setup. Based on the preformulation rheological data, select an appropriate spindle (Spindle No.7 of the RV system). Set the instrument's shear rate to a standardised low rotational speed (10 rpm) to simulate the gentle shear stress experienced during topical application.

Step 3: Measurement Execution. Lower the viscometer head smoothly and vertically, immersing the rotating spindle into the cream sample until the liquid level aligns with the immersion mark on the spindle shaft. Wait for the spindle to rotate for a strictly specified duration (e.g., 60 s) or until the torque percentage reading stabilises within the optimal valid range of 10%-90%, and record the dynamic viscosity value.

Extract 3 independent samples from the same batch for parallel determination. Since the rotational viscometer measures apparent viscosity at a constant shear rate, report the final result as the Mean ± Standard Deviation (SD) in Pascal-seconds (Pa·s).

2.5.5 Microbiological purity

Research Objective. Although the cream formulation incorporates the antibacterial TGC-EA extract and a preservative system (0.2% methyl paraben and 0.1% ethyl paraben), the substantial aqueous phase (68.2%) and natural macromolecular gums (*Acacia Senegal* and *Astragalus Gummifer*) provide an optimal carbon source for environmental microorganisms and opportunistic pathogens. In accordance with the regulatory specifications of the SPhU, 2nd ed., suppl. 6, 5.1.4, p. 123, for non-sterile topical preparations, this experiment aims to quantitatively determine the Total Aerobic Microbial Count (TAMC) and Total Yeasts and Moulds Count (TYMC) in the finished cream. Furthermore, it rigorously screens for specific objectionable microorganisms (*S. aureus*, *C. acnes*, and *E. coli*) that pose a high risk of dermal infection and systemic toxicity. This methodology validates the cleanliness control level of the manufacturing environment and confirms the microbiological safety of the final product.

Test of Bulk intermediate product (Bulk cream). Following completion of high-shear emulsification in the homogenization reactor, 5 g of samples are precisely extracted from three spatial fluid levels: the top, middle, and bottom of the reactor.

Test of Finished tube product (Tube cream). Five finished tubes are randomly sampled from the front, middle, and back temporal phases of the automated filling line (samples are collected after discarding the initial extruded portion).

The sampling, weighing, and pretreatment processes must be conducted under strict aseptic conditions to prevent secondary contamination from exogenous sources.

Reagents. Sterile buffered sodium chloride-peptone solution (pH 7.0).

Non-selective Enumeration Media. Tryptic Soy Agar (TSA) for TAMC; Sabouraud Dextrose Agar (SDA) for TYMC;

Specific Pathogen Isolation/Identification Media. Tryptic Soy Broth (TSB, for the non-selective enrichment of pathogens) and MacConkey Broth (for the specific

enrichment of enteric *EC*); Mannitol Salt Agar (MSA) for screening *SA*; Cetrimide Agar (CET) for screening *CA*; MacConkey Agar (MAC) for screening *EC*.

Instruments and Equipment. Biological safety cabinet or clean bench meeting ISO Class 5 environment standards; Vortex mixer and automated colony counter; Biochemical thermostatic incubators; Thermostatic water bath or sterile homogeniser.

Operating Procedures.

Step 1: Preservative neutralisation and test solution preparation. Within the aseptic workspace, precisely weigh 10 g of the cream sample and add it to 90 mL of the buffer containing the "Tween-lecithin" neutraliser. Place the mixture in a sterile water bath at 40-45℃ for brief heating, then vigorously vortex (or homogenise to ensure the preservatives are completely encapsulated and quenched by micelles, yielding a uniform 1:10 initial test solution.

Step 2: Quantitative determination of TAMC and TYMC (Pour plate method). Pipette 1.0 mL of the aforementioned 1:10 test solution into four separate sterile Petri dishes. Pour the molten TSA into two dishes, cooled to approximately 45℃, and SDA into the remaining two dishes. Gently swirl the plates to evenly mix the sample. Once solidified, invert the TSA plates and incubate at 30-35℃ for 3-5 days; invert the SDA plates and incubate at 20-25℃ for 5-7 days.

Step 3: Enrichment cultivation of objectionable microorganisms. Inoculate 10 mL of the 1:10 test solution (equivalent to 1 g of the absolute cream sample) into 90 mL of TSB and incubate at 30-35℃ for 18-24 h for the resuscitation and enrichment of *S. aureus* and *C. acnes*. Concurrently, inoculate another 10 mL of the test solution into 90 mL of MacConkey Broth and incubate for 18-24 h to enrich for *E. coli.*

Step 4: Selective streak isolation and identification.

Screening for *S. aureus*: Use an inoculating loop to streak the TSB enrichment broth onto an MSA plate. Incubate at 30-35℃ for 18-72 hours. The absence of yellow/white colonies surrounded by a yellow halo indicates a negative result.

Screening for *C. acnes*: Streak the TSB enrichment broth onto a CET plate. Incubate at 30-35℃ for 18-72 hours. The absence of characteristic colonies producing green pyocyanin indicates a negative result.

Screening for *E. coli*: Streak the MacConkey enrichment broth onto a MAC agar plate. Incubate at 30-35℃ for 18-72 hours. The absence of brick-red/purplish-red colonies surrounded by a bile salt precipitate zone indicates a negative result.

Following the incubation, count the CFU on the TSA and SDA plates using a colony counter. Calculate the arithmetic mean of the two parallel plates and multiply by the dilution factor (10) to determine the TAMC and TYMC per gram of cream.

2.5.6 Assessment of Irritation Potential by the HET-CAM Test

Research Objective. Since the developed antibacterial cream is intended for topical application, its potential to induce acute local irritation was evaluated as part of the preclinical characterisation of the formulation. The Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) was used as an alternative experimental model for preliminary assessment of irritation potential. The method is based on observation of acute vascular responses of the chorioallantoic membrane following exposure to the test sample, particularly haemorrhage, vascular lysis and coagulation. These endpoints were monitored within a 300-s observation period and used to calculate the Irritation Score (IS). The HET-CAM test was used in the present study specifically to characterise irritation-related responses. It was not considered a comprehensive assessment of the biological safety or overall biocompatibility of the medicinal product. The HET-CAM procedure was performed using a protocol adapted from the method of Luepke [254] and the ICCVAM-recommended HET-CAM procedure, with modifications appropriate for the semi-solid cream formulation.

Test of Bulk intermediate product (Bulk cream). Following completion of high-shear emulsification in the homogenization reactor, 5 g of samples are precisely extracted from three spatial fluid levels: the top, middle, and bottom of the reactor.

Test of Finished tube product (Tube cream). Five finished tubes are randomly sampled from the front, middle, and back temporal phases of the automated filling line (samples are collected after discarding the initial extruded portion).

Reagents and Materials.

Biological Model. Fertilised and normally developed Specific Pathogen-Free (SPF) White Leghorn chicken eggs (weight strictly controlled between 50-60 g).

Control Reagents. Negative Control: Sterile 0.9% sodium chloride solution (physiological saline), utilised to exclude physical interference with the blood vessels caused by the experimental manipulation itself.

Positive Control: 0.1 mol/L sodium hydroxide (NaOH) solution, utilised to induce standard, severe irritant vascular damage.

Consumables. Teflon O-rings with an internal diameter of approximately 8 mm are utilised to confine the exposure area of the semi-solid cream on the CAM.

Instruments and Equipment. Automated Egg Incubator: Equipped with high-precision temperature and humidity control systems and an automatic egg-turning function. High-Magnification Stereomicroscope: Equipped with cold light illumination (to prevent thermal radiation damage to the blood vessels) and a high-resolution digital continuous imaging system (10x - 40x magnification).

Precision Surgical Instruments: High-frequency toothless dental micro-drill, precision ophthalmic micro-forceps. Precision Timer (accuracy of 1 second).

Operating Procedures.

Step 1: Egg incubation. Place fresh SPF eggs in the incubator, setting the temperature to 37.5±0.5℃ and relative humidity to 60-70%. From day 1 to day 8, set the automatic turning to once every 2 h to prevent embryonic adhesion. At the end of day 8, confirm embryonic viability (identifiable by a distinct vascular network) using a candling lamp and mark the air cell end (blunt end).

Step 2: Windowing. On day 9 or 10 of incubation, perform the windowing procedure. After disinfecting the shell at the air cell end with 75% ethanol, use the micro-drill to remove a circular section approximately 2.0 cm in diameter, exposing the inner shell membrane. Instil 2-3 drops of warm physiological saline for hydration. Under the stereomicroscope, gently peel away the inner shell membrane using precision microforceps to completely expose the underlying chorioallantoic membrane (CAM), which is brimming with microvessels.

Step 3: Sample application. Select healthy embryos with uniformly developed vascular networks and randomly divide them into the TGC cream group, negative control group, and positive control group. Six eggs were used per group, and 0.1 g of

the semi-solid cream (or 0.1 mL of NaOH solution control) was applied within a Teflon O-ring to standardise the exposed area.

Step 4: Dynamic monitoring and recording. Initiate timing at the exact moment the sample is applied. Under the cold light of the stereomicroscope, continuously observe changes in the CAM microvascular network for 5 min (300 s). Record the exact time of the initial occurrence of the following three core pathological endpoints: 1) Haemorrhage (T_H): Blood extravasates from the capillary walls, forming diffuse red macules; 2) Vascular Lysis (T_L): Blood vessel walls rupture, vessel diameters abruptly contract or sever, and blood flow ceases; 3) Coagulation (T_C): Dark proteinaceous clots appear intra- and extra-vascularly, leading to blood flow stagnation.

Calculation of Irritation Score (IS): Upon completion of the observation, calculate the Irritation Score (IS) for each egg based on the occurrence times of the three pathological endpoints. The mathematical formula is as follows:

$$IS = \frac{301-T_H}{300} \times 5 + \frac{301-T_L}{300} \times 7 + \frac{301-T_C}{300} \times 9, \quad (2.7)$$

where T_H, T_L, and T_C are the times (in seconds) observed for haemorrhage, vascular lysis, and coagulation, respectively. If a specific endpoint does not occur within the 300 seconds, its corresponding time T is recorded as 301 seconds, resulting in a score of 0 for that term. The maximum possible score is 21.

Standardised Evaluation Limits: Calculate the arithmetic mean and standard deviation of the IS for the 6 eggs in each group. Perform qualitative toxicity discrimination based on the internationally recognised Luepke classification criteria:

IS = 0.0 ~ 0.9: Non-irritant; IS = 1.0 ~ 4.9: Slight/Mild irritant;

IS = 5.0 ~ 8.9: Moderate irritant; IS = 9.0 ~ 21.0: Severe irritant.

The calculated IS was interpreted exclusively as an indicator of irritation potential within the HET-CAM model. According to the adopted classification, lower IS values indicate a lower irritation response. The HET-CAM result was not used as evidence of overall biological safety or biocompatibility, since other toxicological endpoints were outside the scope of the present assay. The final classification therefore reflects irritation potential in the HET-CAM model only.

2.5.7 Content of heavy metals

Research Objective. Natural botanical extracts (TGC-EA) and natural macromolecular thickeners (*Acacia Senegal* and *Astragalus Gummifer*) are highly susceptible to accumulating heavy metals from soil and water sources during their natural growth and primary processing. In accordance with the SPhU 2nd ed., v. 1, 2.4.8, p. 184 and 2.4.27, p. 206, it is mandatory to conduct rigorous quantitative screening for highly toxic Class 1 elemental impurities (Lead, Cadmium, Arsenic, Mercury) and transition metals capable of compromising product stability (Copper, which actively catalyses lipid oxidation leading to rancidity). The analytical procedures were performed for the determination of Pb, Cd, and Cu according to the method on p. 184 and for the determination of As and Hg according to the method on p. 206.

Test of Finished tube product (Tube cream). Five finished tubes are randomly sampled from the front, middle, and back temporal phases of the automated filling line (samples are collected after discarding the initial extruded portion).

Reagents and Materials. All reagents used in the present study were of analytical grade or higher purity, and the water used was ultrapure or deionised water. The principal reagents and materials included: Nitric acid; Hydrogen peroxide; Ultrapure or deionised water; Single-element stock standard solutions of cadmium (Cd), copper (Cu), lead (Pb), arsenic (As), and mercury (Hg); Working standard solutions of the respective elements; Reagent blank solution; Polytetrafluoroethylene digestion vessels or other acid-resistant digestion containers; Volumetric flasks, pipettes, polyethene centrifuge tubes, and other trace-element analysis containers.

Instruments and equipment. Analytical balance; Microwave digestion system or temperature-controlled wet digestion apparatus; Atomic absorption spectrophotometer or the corresponding elemental analysis instrument specified by the pharmacopoeial method; Hydride generation system or the corresponding accessory for the determination of arsenic and mercury; Ultrapure water purification system; Temperature-controlled heating plate or auxiliary digestion device.

All samples, blanks, and standards were analysed under the same calibration conditions to ensure comparable results.

Operating Procedures.

Step 1: Microwave digestion pretreatment. Accurately weigh 0.2 g of the homogeneously mixed cream sample into a PTFE-TFM microwave digestion vessel, and add 6.0 mL of concentrated HNO_3 and 2.0 mL of H_2O_2. To prevent violent instantaneous reactions from the organic lipids in the cream, allow the open vessel to undergo cold pre-digestion in a fume hood for 30 minutes. Subsequently, seal the vessel and run the microwave program: ramp to 190℃ and hold for 25 minutes (maximum power 1600 W). After cooling, quantitatively transfer the digestate and dilute to 50 mL with 2% nitric acid to obtain the test solution.

The following preparations were made simultaneously with each digestion batch: (a) reagent blank solution; (b) parallel sample preparations; and (c) spiked recovery samples. The resulting digests were used to determine Pb, Cd, Cu, As, and Hg.

Step 2: Lead (Pb), Copper (Cu), and Cadmium (Cd) were determined according to the method specified in SPhU 2nd ed., v. 1, 2.4.8, p. 184. Arsenic (As) and mercury (Hg) were determined by the method specified in SPhU 2nd ed., v. 1, 2.4.27, p. 206.

Dried and cleaned specimens of *Speranskia tuberculata* (Bunge) Baill. were crushed to 20 mesh using a crusher to prepare crude extracts. Weighed 50 g of powder; 500 mL of analytically pure ethyl acetate (EA), petroleum ether (PE), n-butanol and water were added to a Soxhlet extraction flask in sequence. Extraction was performed at 72℃ for EA, 85℃ for PE, 80℃ for n-butanol, and 90℃ for water for 4 hours in each case. After cooling to room temperature, the 4 crude extracts were centrifuged at 6000 rpm for 5 min. The resulting liquid crude extracts were dried, and the dried extracts were weighed. A stock solution at 50 mg/mL was prepared from these extracts (solvent: dimethyl sulfoxide). Later, this solution was used to prepare working solutions at concentrations of 10-1000 μg/mL for experimentation.

2.5.8 Tube cream shelf life

Long-term stability, or shelf life, is certified by the consistency of the cream's FTIR spectrum, which should remain unchanged throughout the observation period. Also, the following cream parameters are controlled during 12 months (Table 2.8).

Table 2.8

Cream parameters for long-term monitoring during 12 months

Parameter	0 m	3 m	6 m	12 m
Appearance				
Colour				
Transmittance				
Texture				
Spreadability				
Total contents of flavonoids, phenols, terpenoids, & alkaloids				
pH				
Dynamic viscosity				
The amount of aerobic bacteria and mould & fungus				
Availability of S. aureus, C. acnes and E. coli				
Antibacterial efficiency against *C. acnes and S. aureus*				

2.6 Experiment planning and statistical data analysis

To optimise the number of experiments while achieving reliable results, the work used mathematical planning methods for the chemical-technological process and applied a Design of Experiments approach for formulation optimisation. A parsimonious "key-variable" strategy was implemented using three formulation factors to build the L9 orthogonal array.

Statistical processing of experimental results was carried out in accordance with the requirements of the articles of the SPhU: 2nd ed., v. 1, p. 840, 5.3 "Statistical analysis of biological tests and quantitative determinations" and 2nd ed., v. 1, p. 881, 5.3.N.1 "Statistical analysis of chemical experiment results".

2.7 List of laboratories involved in the experimental research

The following studies were conducted in the laboratory of School of Chemistry and Chemical Engineering, Qilu University of Technology: Sequential Soxhlet Extraction with Solvents of Different Polarity; Preparation and Yield Determination of Dry TGC Extracts; MTT Cytotoxicity Assay in Human Tumour Cell Lines; MTT Cytocompatibility Assay in HaCaT and HFF-1 Cells; DPPH Free-Radical Scavenging Assay; Pyrogallol Autoxidation / Superoxide Anion Radical Scavenging Assay; Agar Disk Diffusion Antibacterial Assay; Quantitative Liquid Co-incubation Assay with CFU Enumeration; Validation of Spectrophotometric Analytical Methods; L9 Orthogonal Experimental Design for Cream-Base Optimisation; Comprehensive 10-

Point Evaluation of Cream Bases; Optimisation of TGC-EA Concentration in the Cream; Evaluation of Dissolution, Dispersion, Hydration and Homogeneity; Organoleptic Evaluation of the Cream; pH Determination of the Cream; Microbiological Purity Test; Antibacterial Activity and Intra-batch Reproducibility of Bulk and Tube Cream; 12-Month Long-Term Stability and Shelf-Life Study.

The following studies were conducted in the Shandong Analysis and Test Centre: QTOF-LC/MS Phytochemical Profiling; Determination of Total Polyphenol Content; Determination of Total Flavonoid Content; Determination of Total Terpenoid Content; Determination of Total Alkaloid Content; Heavy Metal / Elemental Impurity Analysis of TGC-EA; Residual Solvent Determination by Gas Chromatography;

The following studies were done in the laboratory of the School of Pharmacy and Pharmaceutical Sciences, Shandong First Medical University & Shandong Academy of Medical Sciences: HET-CAM Irritation Potential Test. FTIR Compatibility Analysis of Cream Components; Dynamic Viscosity / Rheological Evaluation; Technological Stability Tests; Elemental Impurity Analysis of the Finished Cream.

Conclusions to Chapter 2

Chapter 2 aims to describe the study material and the research methods used. The main focus is on the prospects for using the endemic herb *Speranskia tuberculata* from China as a medicinal plant. This plant has long been known in traditional Chinese medicine, but has been relatively little studied by official science.

1. Since extracts are the most acceptable form for physicochemical and pharmacopoeial studies, the effect of four different extractants (ethyl acetate, petroleum ether, n-butanol and deionised water) was investigated. Methods for obtaining liquid extracts, followed by their drying over a wide concentration range, were developed.

2. Methods for the *in vitro* evaluation of the biological activities of the prepared extracts were established and systematised. The MTT assay was used to assess concentration-dependent cytotoxic activity in four human tumour cell lines (A549, A-375, HeLa and HepG2). Supplementary MTT experiments using HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts were employed to assess the

in vitro cytocompatibility of TGC-PE and TGC-EA in human skin-related cellular models. Antioxidant activity was evaluated using the DPPH radical-scavenging and superoxide-anion radical-scavenging assays, whereas antibacterial activity was investigated using the microbiological methods described in Section 2.2.3.

3. In parallel with the study of therapeutic properties, an approach to standardising the developed extracts from the viewpoint of their chemical composition was developed and implemented. This approach accounted for indicators of the content of biologically active organic compounds, as well as heavy metals and residual solvents. The content of biologically active compounds was considered an important factor associated with the experimentally observed *in vitro* biological activity of the extracts. The last two parameters, compared with the known requirements for medicines, were used to assess the safety of the developed extracts.

4. To determine the content of individual organic compounds, the method of tandem high-performance liquid chromatography - mass spectrometry (HPLC/MS) was used. The total content of individual classes of biologically active compounds was determined using established spectrophotometric methods. To ensure correct determination, methods for analysing the liquid extract and the separately prepared cream were developed. The content of heavy metals was determined using ICP-AES and AAS. Gas chromatography was used to determine residual solvents.

5. Pharmacotechnological characteristics of the developed cream, namely organoleptic (Appearance, Colour, Transmittance, Texture, Spreadability), physicochemical (pH and dynamic viscosity), and technological stability (colloidal, cold and hot) were determined according to the SPhU recommendations.

6. Microbiological characteristics were investigated in three different aspects. Microbiological purity was determined in accordance with the recommendations of the SPhU by measuring TAMC and TYMC in the finished cream and by detecting *S. aureus*, *C. acnes,* and *E. coli*. To assess irritation potential, the Hen's Egg Test on the Chorioallantoic Membrane was used. Antibacterial activity was assessed against two strains of *S. aureus* and *C. acnes* by measuring the zone of inhibition.

The results of Chapter 2 are published in [104-109].

Chapter 3. Research on TGC extracts and selection of dry extract for further technological development of a medicinal product

3.1 Characterisation of prepared extracts

As already mentioned in the previous chapter, the effectiveness of different solvents in the preparation of herbal extracts was investigated using petroleum ether (PE), ethyl acetate (EA), n-butanol (n-but) and deionised water (aqueous solution or AS). The conditions for obtaining the solutions were as close to each other as possible. The powder-to-solvent ratio was 1:10 m/v. Each extract was concentrated under reduced pressure, vacuum-dried (-20°C), and then dissolved to the required concentrations in dimethyl sulfoxide (DMSO) at 0.1%. Thus, for each solvent, several extract samples with varying concentrations of dissolved plant material were obtained, typically up to 100 or 500 μg/mL. It further allowed one to investigate the concentration-dependent effects of different types of *in vitro* biological activity of the extracts.

3.2 Determination of medicinal properties of TGC extracts

The *in vitro* biological activities of the developed TGC extracts were investigated with respect to cytotoxicity and cytocompatibility, antioxidant activity, and antibacterial activity.

3.2.1 In vitro cytotoxicity and cytocompatibility of TGC extracts

In this section, the effects of the obtained extracts of *Speranskia tuberculata* (Bunge) Baill. on cell viability were investigated. Four human tumour cell lines, A549, A-375, HeLa and HepG2, were initially used to evaluate the concentration-dependent cytotoxic activity of the extracts. In addition, because TGC-EA was subsequently selected as the active pharmaceutical ingredient for the development of a topical antibacterial cream, HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts were further included to evaluate the *in vitro* cytocompatibility of TGC-PE and TGC-EA in human skin-related cell models. Figure 3.1 illustrates the concentration-dependent effects of *Speranskia tuberculata* extracts on the viability of the four human tumour cell lines. Comparative data on the effects of PE- and EA-based

extracts are provided for A549, A-375, HeLa and HepG2 cells. In all four investigated human tumour cell lines, exposure to the extracts resulted in a decrease in cell viability with increasing extract concentration.

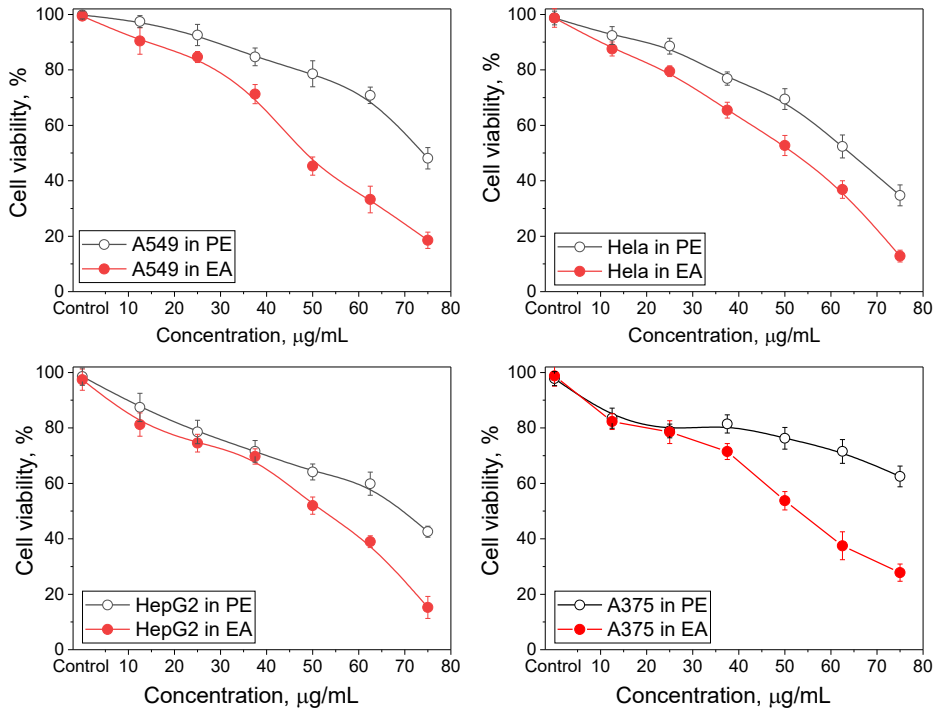


Fig. 3.1 Dependence of the viability of four human tumour cell lines on the concentrations of PE- and EA-based extracts of *Speranskia tuberculata*

In all investigated cases, the decline in viability in EA solutions was higher than in PE. In other words, EA extracts proved to be more effective in reducing the viability of the investigated tumour cells. It is worth noting that for some cells, the effect of EA is significantly accelerated at certain stages of increasing concentration. For example, in A549 cells, this acceleration begins at 25 μg/mL and becomes evident at 37.5 μg/mL. For A-375 and HepG2, the pictures look similar, with additional acceleration observed at 37.5 μg/mL. The picture is less apparent for the HeLa line since the acceleration is more monotonous. Still, as concentration increases, the extract's action always occurs at a higher rate.

For PE, the influence of *Speranskia tuberculata* concentration appears closer to linear. That is, it depends less on the concentration interval. If there is any acceleration, it occurs only in the last concentration interval (62.5–75 μg/mL). The enhanced extract effect is most likely due to increased concentrations of certain bioactive compounds.

Therefore, EA can extract more compounds from *Speranskia tuberculata* than PE. It is known that solvent polarity influences the extraction of bioactive compounds, and the effect may be opposite across different classes of compounds [110]. Thus, the extraction of flavonoids and phenolic compounds decreases with increasing solvent polarity. Conversely, with increasing polarity, there is an increase in total extraction yield, antioxidant activity, and free radical scavenging activity, which can be explained by the high affinity of antioxidant compounds to more polar solvents than nonpolar ones. One may suggest that phytochemical compounds extracted with polar solvents are pharmaceutically more important, at least because of their comparatively higher antioxidant, reducing, and free radical-scavenging activities.

The polarity index, a relative measure of the degree of interaction of the solvent with various polar test solutes, is significantly higher for EA than for PE: 4.4 versus 0.1 [111]. Accordingly, the superior extractive capabilities of EA do not contradict the above literature. The greater cytotoxic activity of EA is reflected in the extent to which it reduced the viability of the investigated human tumour cells across the given concentration interval. Thus, for the HeLa and HepG2 lines, viability dropped from 100% to approximately 13–15%, and for the A549 and A-375 lines, it fell to approximately 19–28%. There was consistently a more substantial decrease in viability with EA than with PE, for which the residual level of viability remained approximately 35–62%, i.e., about 22–35% higher than that observed for EA. The half-maximal inhibitory concentration (IC_{50}) is commonly used to quantitatively characterise the concentration-dependent activity of a test substance. The IC_{50} values calculated by approximation of the experimental curves shown in Fig. 3.1 are presented in Fig. 3.2.

For EA, the IC_{50} values were relatively weakly dependent on the investigated tumour cell line, ranging from approximately 49 to 53 μg/mL. A slightly lower IC_{50} was observed for HeLa, whereas the highest value was observed for A-375. Therefore, among the four investigated human tumour cell lines, HeLa cells were the most sensitive to the crude ethyl acetate extract. For PE-based extracts, IC_{50} values across the investigated tumour cell lines were higher than those determined for EA extracts, and they increased further from HeLa to A549 and then to A-375. For A-375 cells, the

IC_{50} value of the PE extract was conditionally calculated by extrapolation beyond the experimentally investigated concentration range of 12.5–75 μg/mL.

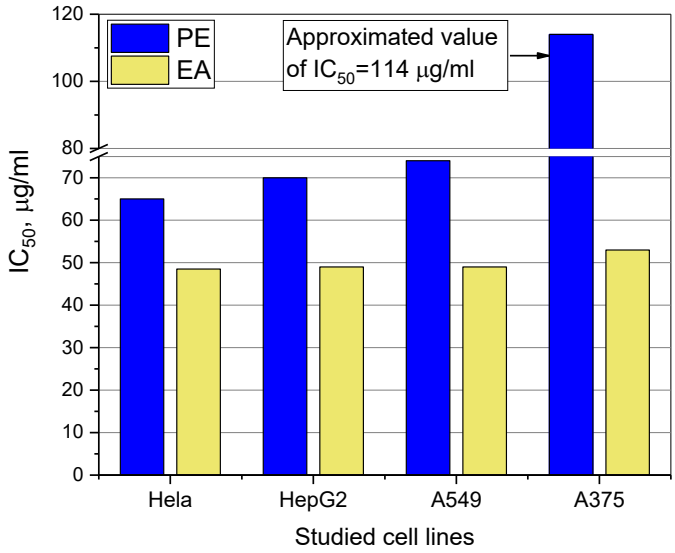


Fig. 3.2 Experimental IC_{50} values of EA and PE extracts determined in the four investigated human tumour cell lines (the IC_{50} value for PE in A-375 cells was estimated beyond the investigated concentration interval)

In summary, two series of extracts of the medicinal plant *Speranskia tuberculata*, prepared using petroleum ether and ethyl acetate, at concentrations ranging from 12.5 to 75 μg/mL, were investigated using four human tumour cell lines: A549, A-375, HeLa and HepG2. For both extracts, cell viability decreased with increasing concentration, with the reduction being more pronounced for the ethyl acetate extract.

In particular, the IC_{50} values for EA extracts were relatively stable at approximately 49–53 μg/mL. The IC_{50} for PE gradually increased from approximately 65 to 74 μg/mL across HeLa and A549 cells. For A-375 cells, the IC_{50} of the PE extract fell outside the experimentally investigated concentration range and was estimated at approximately 114 μg/mL. Among the investigated tumour cell lines, HeLa showed the greatest sensitivity to the extracts. Overall, these results indicate that TGC-EA demonstrated stronger *in vitro* cytotoxic activity than TGC-PE in the four investigated human tumour cell models. These findings characterise the *in vitro* cytotoxic activity of the extracts and should not be interpreted as evidence of antitumour or clinical therapeutic efficacy.

Following the comparative assessment of cytotoxic activity in the human tumour cell lines, additional MTT experiments were performed using HaCaT and HFF-1 cells. This supplementary assessment was introduced because TGC-EA was subsequently selected as the active pharmaceutical ingredient for the development of a topical antibacterial cream. HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts were therefore used as human skin-related cellular models to evaluate the *in vitro* cytocompatibility of TGC-PE and TGC-EA.

For this assessment, the concentration range was extended beyond that used in the tumour-cell experiments. HaCaT and HFF-1 cells were exposed to TGC-PE and TGC-EA at concentrations of 12.5, 25, 37.5, 50, 62.5, 75, 100, 200, 500 and 1000 µg/mL. The extended range enabled evaluation of cytocompatibility at concentrations that overlapped with and exceeded those associated with the *in vitro* antibacterial activity of the extracts.

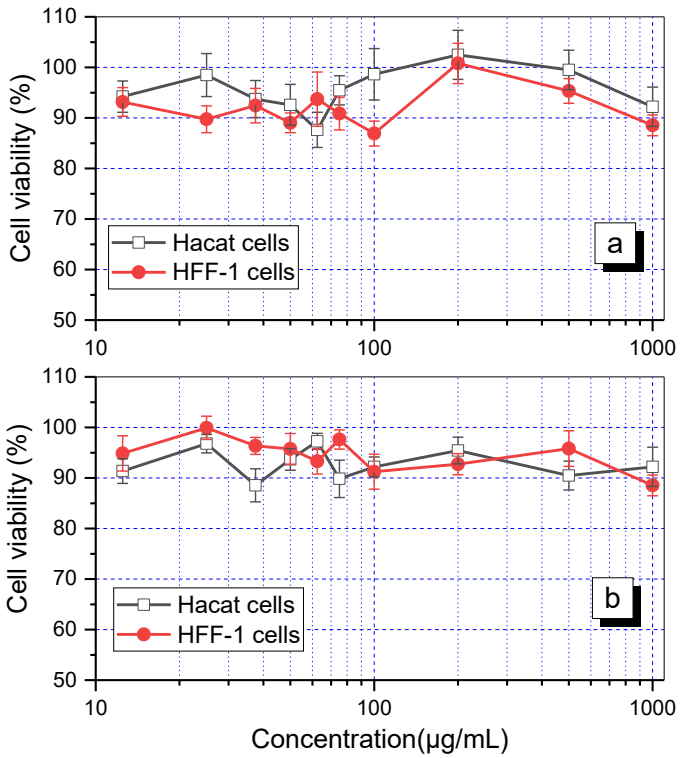


Fig. 3.3 Effects of TGC-PE and TGC-EA extracts on the viability of HaCaT and HFF-1 cells at concentrations of 12.5–1000 µg/mL

The results demonstrated that neither TGC-PE nor TGC-EA caused a marked reduction in the viability of HaCaT or HFF-1 cells over the investigated concentration range. In all experimental groups, mean cell viability remained above 85%.

For TGC-PE, the viability of HaCaT cells ranged from 87.63% to 102.47%, whereas HFF-1 cell viability ranged from 86.92% to 100.78%. At the highest investigated concentration of 1000 μg/mL, HaCaT and HFF-1 cell viability remained at 92.20 ± 3.88% and 88.53 ± 2.05%, respectively. No evident monotonic concentration-dependent reduction in cell viability was observed over the investigated concentration range. The values slightly exceeding 100% at individual concentrations were regarded as fluctuations around the untreated-control level within the variability of the MTT assay and were not interpreted as stimulation of cell proliferation.

Similarly, TGC-EA demonstrated high cell viability in both human skin-related cell models. HaCaT cell viability ranged from 88.54% to 98.10%, whereas HFF-1 cell viability ranged from 91.24% to 99.92% across concentrations from 12.5 to 1000 μg/mL. At the highest investigated concentration of 1000 μg/mL, cell viability was 98.10 ± 1.42% for HaCaT and 97.33 ± 2.90% for HFF-1 cells. As with TGC-PE, no evident concentration-dependent decline in cell viability was observed.

A 50% reduction in cell viability was not reached for either extract in either of the human skin-related cell models. Therefore, under the applied experimental conditions, the CC_{50} values of both TGC-PE and TGC-EA for HaCaT and HFF-1 cells were above the highest investigated concentration (>1000 μg/mL).

The concentration range used in these supplementary experiments is also relevant to the subsequent pharmaceutical development of TGC-EA. As demonstrated in Section 3.2.3, the IC_{50} values associated with the *in vitro* antibacterial activity of TGC-EA were approximately 112–135 μg/mL. Therefore, the concentration range investigated in HaCaT and HFF-1 cells encompassed and substantially exceeded the concentrations associated with the observed antibacterial activity. Nevertheless, cell viability remained above 85% throughout the investigated range.

Taken together, the supplementary HaCaT and HFF-1 results indicate low *in vitro* cytotoxicity of TGC-PE and TGC-EA toward the investigated human skin-related

cellular models under the applied experimental conditions. In particular, the maintenance of high viability in both HaCaT and HFF-1 cells at TGC-EA concentrations of up to 1000 μg/mL provides additional experimental support for the favourable *in vitro* cytocompatibility of the ethyl acetate extract selected for subsequent topical formulation development.

These findings should nevertheless be interpreted within the limitations of the experimental models. HaCaT is an immortalised human keratinocyte cell line rather than a primary normal keratinocyte culture, and conventional two-dimensional HaCaT and HFF-1 cultures do not reproduce all structural, barrier, metabolic and immunological properties of intact human skin. Consequently, the present results support favourable *in vitro* cytocompatibility of TGC-EA under the investigated conditions but do not constitute evidence of overall dermal or clinical safety.

3.2.2 Antioxidant effect

The chemistry of free radicals has become very popular in research over the past few decades, as it studies reactive forms of oxygen and nitrogen generated by the body under the influence of various external or pathological conditions. Free radicals negatively alter lipids, proteins and DNA and cause many human diseases. Interest in antioxidants is growing, primarily because of their role in neutralising free radicals or reducing oxidation-related damage [112].

A diet high in antioxidants can reduce the risk of many diseases (including heart disease and some cancers). For such applications, antioxidants of natural origin are preferred over synthetic ones, because some known synthetic antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole and others, pose a health hazard. Some plants have great potential for use as functional ingredients or as additives in the food industry. Research on determining the antioxidant activity continues to grow rapidly, while emphasising the need to find new sources of plant raw materials. In this section, the antioxidant effects of different concentrations of *Speranskia Tuberculata* (Bunge) Baill. Herb extracts were studied for four different extractants.

The results of determining the DPPH and SARS rates as a function of solution concentration and extract type are shown in Fig. 3.4.

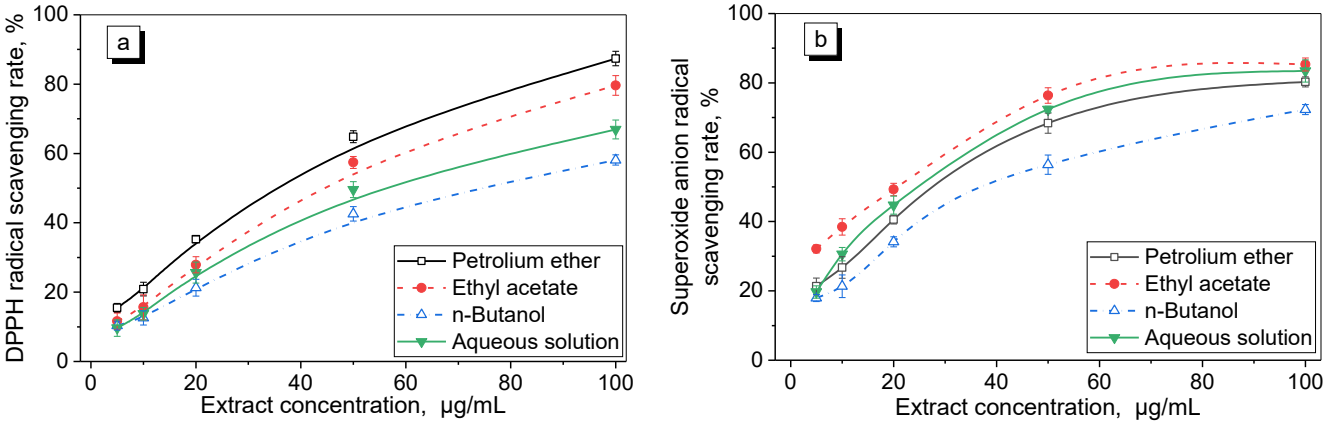


Fig. 3.4 Relative values (%) of DPPH (a) and SARS rates (b) as a function of solute concentrations extracted with 4 different solvents

For both methods, n-butanol extracts exhibit the lowest antioxidant activity, while EA and PE extracts exhibit the highest. For other solutions, the two methods used give mixed results. The results of determining the total content of phenolic compounds and flavonoids are demonstrated in Fig. 3.5. The obtained results for the two substances correlate well. The high Pearson correlation coefficient (0.9945) is unequivocal and significant at the 2-sided p-value of 0.005. Extracts based on ethyl acetate (EA extracts) show the highest content of extracted phenolic compounds and flavonoids, while petroleum ether extracts (PE extracts) have the lowest content.

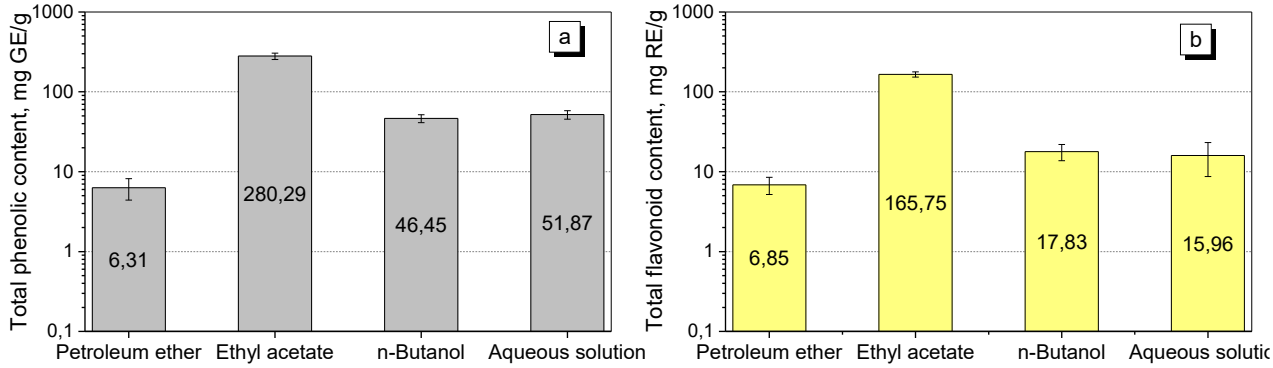


Fig. 3.5 The total contents of phenolic compounds (a – in mg/GE/g) and flavonoids (b – in mg/RE/g) extracted with 4 different solvents

This observation may have a straightforward explanation. Flavonoids are natural phenolic compounds with a 15-carbon skeleton consisting of two aromatic rings and a cyclohexane ring fused to one of these aromatic rings. Based on their structure, they

can be classified into anthocyanins, catechins, flavones, flavonols, flavanols, flavanones, and isoflavonoids [113]. The potential closeness of the extracted compounds' chemical structures may underlie interactions with different solvents.

The recalculation of the experimental DPPN and SARS values into IC50 values enabled the estimation of the antioxidant activity of individual extracts. Although there is no agreed criterion, it is often assumed that substances with an $IC_{50} < 50$ µg/mL have high antioxidant activity. In contrast, those with an IC50 in the range of 50-100 µg/mL have medium activity [114]. Except for the DPPN test for n-butanol, all other results indicate high extract activity (Fig. 3.6a). However, the correlation between the two activity tests is not ideal (Pearson's coefficient = 0.795, two-sided p = 0.2025).

Phenolic compounds, especially flavonoids, are known to exhibit strong antioxidant properties and play an essential role in neutralising free radicals [116, 112]. The antioxidant mechanism of flavonoid action involves transferring a hydrogen atom to free radicals – the faster and easier the hydrogen transfer, the greater the antioxidant power. However, when we compare the determined levels of phenols and flavonoids (Fig. 3.5) with their corresponding IC_{50} values (Fig. 3.6a), the lack of correlation is striking. Evidently, the mechanisms of the formation of antioxidant activity of phenols and flavonoids are more complicated than a simple linear correlation.

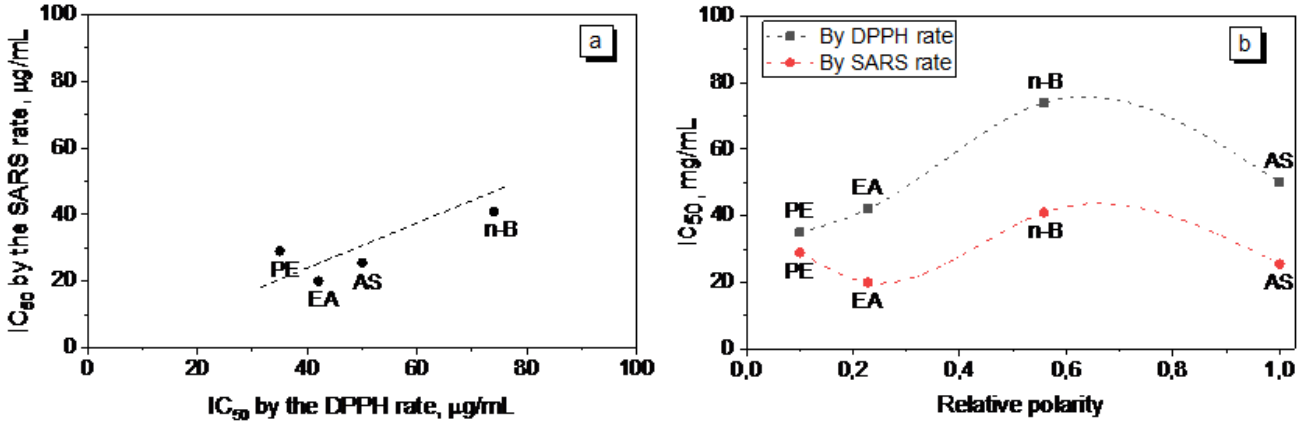


Fig. 3.6 IC_{50} measured by DPPH and SARS rates (a) and shown as a function of the relative polarity of solvents (b). The relative polarities were taken from [115]

The solvent polarity has a complex effect on the extraction. The extraction of flavonoids and phenols may decrease with increasing solvent polarity, whereas

antioxidant and free radical-scavenging activities increase [110]. The IC_{50} first increases (i.e., activity decreases) with increasing polarity (Fig. 6b). In strongly polar solutions, the activity trend changes and begins to increase. The non-monotonous dependence of activity and extractions on solvent polarity may explain the complex correlation between the phenol and flavonoid contents and the solutions' antioxidant activity. In conclusion, EA-based extracts (IC50 by two methods: 20-42 μg/mL) and PE-based extracts (29-35 μg/mL) showed the highest antioxidant activity; those based on n-but showed the lowest (41-74 μg/mL). The total content of phenols and flavonoids ranged from 7 mg/GE/g and 7 mg/RE/g (PE) to 280 mg/GE/g and 166 mg/RE/g (EA).

3.2.3 Antibacterial effect

The invention of antibiotics was a real breakthrough in saving human life. However, the events of recent years provide increasing evidence of a gradual decline in the role of antibiotics in some areas of treatment. With widespread, often excessive, consumption of synthetic antibiotics, bacteria have acquired the ability to adapt to the drugs and mutate quickly. Therefore, the increasing prevalence of multidrug-resistant bacteria may limit the effectiveness of some existing antibiotics and highlights the need for the development of new antimicrobial agents. Medicinal plants may represent a promising source of compounds with antimicrobial activity for further pharmaceutical research. Sooner or later, humanity will be unable to resist serious diseases, especially those caused by bacterial infections, if the focus on creating new drugs is not shifted to the active use of medicinal plants that provide simultaneous synergistic effects from multiple biologically active substances.

It is widely recognised that medicinal plants can serve as a promising alternative to ineffective synthetic antibiotics in the fight against infectious diseases [117, 118]. Phenolic compounds, alkaloids, saponins, and terpenoids have shown significant antibacterial activity, primarily through mechanisms such as membrane disruption, protein binding, and interference with intermediary metabolism [119]. However, such a transition is constrained by insufficient study of the potential of medicinal plants, including the synergistic effects of the simultaneous action of multiple active APIs. Many new works on the development of herbal medicines appear each year [120-122].

However, the potential for the development of herbal medicine remains undiscovered. Moreover, in some cases, the production of herbal medicines requires adjustments to the technological cycle [123].

The Chinese endemic herb *Speranskia Tuberculata* is a typical example of this lack of study, even though the plant has been widely and successfully used in traditional Chinese medicine for centuries. Almost 30 years ago, the first studies of this plant's flavonoids appeared [124]. Accordingly, these results can be considered the first and limited evidence of the promising application in areas where official medicine recognises flavonoids as essential. Unfortunately, in the years that followed, *Speranskia tuberculata*, which remained a popular treatment in traditional Chinese medicine, received little coverage in the world's scientific literature. Only a few works appeared in recent years and decades [125-128]. Most of the publications aim to study medicinal properties. Research on the chemical composition, detection of APIs, and manifestations of the synergistic effect is still in its initial stages [75].

However, much remains to be done, especially given the plant's complex chemical composition, the ambiguity of its interactions with solvents, and the extent of its potential antibacterial activity against various bacteria. In this section, the antibacterial activity of various crude extracts of the herb *Speranskia Tuberculata* (Bunge) Baill. was evaluated against three widespread bacteria.

The antibacterial rates measured and calculated according to equation (1) for extracts of different origins, depending on concentration, are shown in Fig. 3.7 to suppress the bacteria *S. aureus* (a), *E. coli* (b), and *C. acnes* (c).

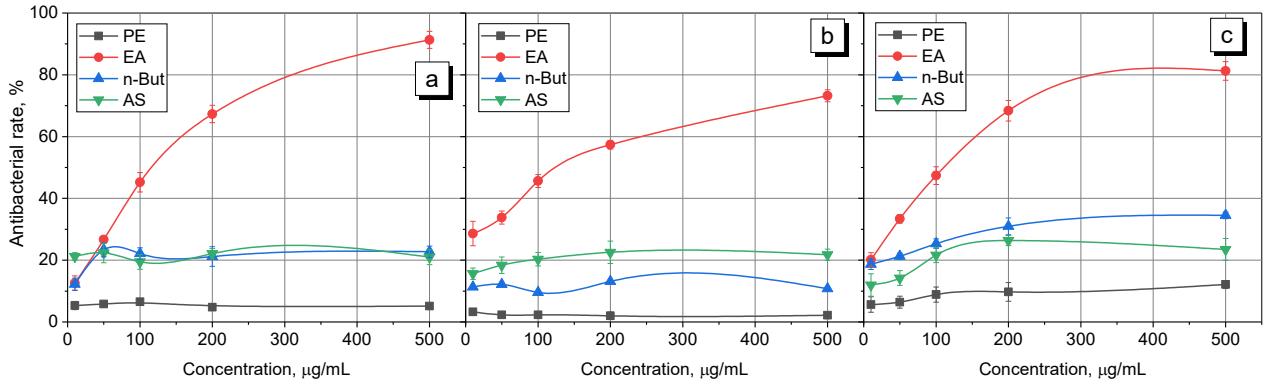


Fig. 3.7 Antibacterial rates for 4 kinds of extracts in the different bacterial media: a – *Staphylococcus aureus*, b –*Escherichia coli*, c –*Cutibacterium acnes*

Only the extracts obtained using EA show a clearly expressed antibacterial effect. For these extracts, the IC_{50} values were 120, 135, and 112 μg/mL for *SA*, *EC*, and *CA* bacteria, respectively. For all other extracts, the IC_{50} could not be determined. The *E. coli* species was the most resistant to EA extracts among the studied bacteria.

At the other end (with minimal antibacterial effect), PE extracts did not affect any of the three types of bacteria. The antibacterial rates ranged from 0 units to 10% across all bacteria and PE concentrations, indicating negligible antibacterial activity. The figures for other extracts ranged from 10% to 30%. In no case was the IC_{50} achieved.

Research on the composition of *Speranskia tuberculata* extracts is at a very early stage, so there is no clear answer as to which processes underlie the better antibacterial activity of EA extracts. Figure 3.8 provides additional information, including experimental data on the dry residue of raw extracts and the measured levels of total flavonoids and phenolic compounds. The amount of dry residue at the extract preparation stage was maximum when PE was used. However, it did not provide antibacterial activity. The data on the dry residue (Fig. 3.8a) contradict the results of the study of the content of phenols (Fig. 3.8b) and flavonoids (Fig. 3.8c). EA extracts showed the highest antibacterial activity. Conversely, PE extracts were characterised by a large dry residue, low amounts of flavonoids and phenols, and the lowest activity.

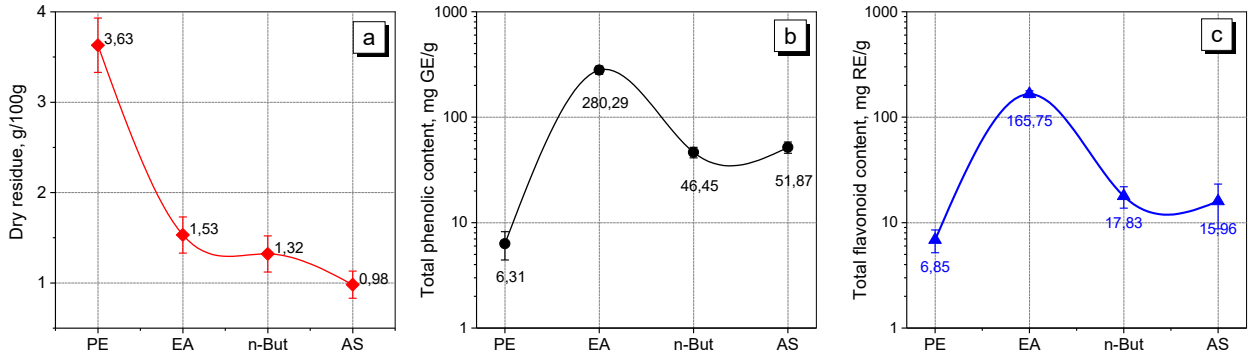


Fig. 3.8 The dry residues (a – in g/100g), total contents of phenolic compounds (b – in mg/GE/g) and flavonoids (c – in mg/RE/g) extracted with 4 different solvents

Therefore, the study identified the solvent with the greatest antibacterial effect. The EA extract shows high antibacterial activity (IC_{50} values range from 112 to 135 µg/mL across different bacteria), while the other three extracts remain inactive.

3.3 Chemical composition of the most promising extracts

The results presented in the previous section demonstrated that the investigated extracts exhibited different profiles of *in vitro* cytotoxic, antioxidant, and antibacterial activity. At the same time, supplementary HaCaT and HFF-1 experiments provided additional information on their cytocompatibility in human skin-related cellular models. Since different extractants were used to dissolve the same medicinal plant, the obvious reason is the different degrees of extraction of active biological substances from the herb, which depend on the particular solvent. In other words, an important first step is to study the chemical composition of the liquid extracts. Then, in the second stage, when the most effective of the four studied is determined, the results of the study of residual solvents, as well as the heavy metal content in the dry extract, should be added to the content of biologically active substances. Knowledge of all these factors is necessary to assess the safety of the developed extracts for human use.

The most powerful instrument for studying individual organic compounds in extracts is LC/MS, thanks to its high sensitivity and resolution. As shown earlier, among the 4 solvents, EA and PE are the most promising. For example, using MTT assays to assess the effects of concentrations of EA and PE extracts on cancer cell viability enabled the determination of IC_{50} values for cells of different origins. The efficiency of the solutions increased with increasing concentration ($p < 0.05$). The results of IC_{50} calculations are shown in Fig. 3.9.

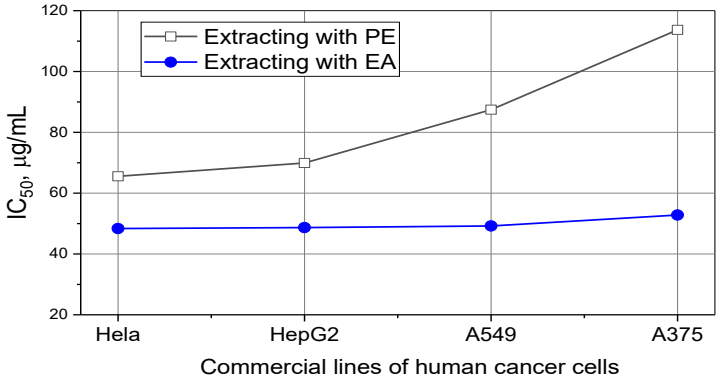


Fig. 3.9 IC_{50} values of TGC-EA and TGC-PE extracts determined in the four investigated human tumour cell lines.

Both extracts demonstrated *in vitro* cytotoxic activity in all four investigated human tumour cell lines. TGC-EA consistently showed lower IC_{50} values and therefore stronger cytotoxic activity under the applied experimental conditions. Thus, the IC_{50} for them increases only slightly, from 48 to 53 μg/mL, when switching from HeLa to A375 cells. A similar indicator for preparations based on PE extracts increases more noticeably from 65 to 114 μg/mL (the last value is approximated by calculation, as it exceeds the studied experimental concentration range). As a result, the difference in cytotoxic activity between the extracts remains relatively moderate in HeLa and HepG2 cells (35-45% in favour of preparations based on the EA extract). For A549 and A375 cells, the advantage increases significantly (up to 80-100%).

3.3.1 HPLC-MS results

The results of tandem liquid chromatography-mass spectrometry (LC/MS provide more information. The PE and EA extracts were selected as the most promising. The spectrum separation and registration conditions were as similar as possible; the same instrument was used in both cases. The amplitude of the most intense peaks of EA and PE extracts was about 5×10^6 arbitrary units (a.u.). The smallest peaks in amplitude varied at the level of 10^3 a.u. Analysis of the mass spectra of both extracts enabled comparison of their compositions and the determination of their differences.

The PE extract shows almost 300 peaks of organic compounds (Fig. 3.10). The distribution of compound mass spectra in the PE extract is shown in Fig. 3.10a, with peaks ordered by decreasing intensity. An overview of the mass spectrum, with the 56 most intense peaks ranging from 136 to 737 Da, is shown in Fig. 3.10b. The compounds are most densely located in the 180-220 Da mass range.

All peaks with the smallest amplitudes will not be considered further, as the composition and medical properties of the compounds are unknown, and the probability of misidentification increases with peak amplitude decay due to greater noise and the masking of smaller satellite peaks by interference. Only peaks with amplitudes > 10^4 a.u. (1% of the maximum peak amplitude) will be further analysed.

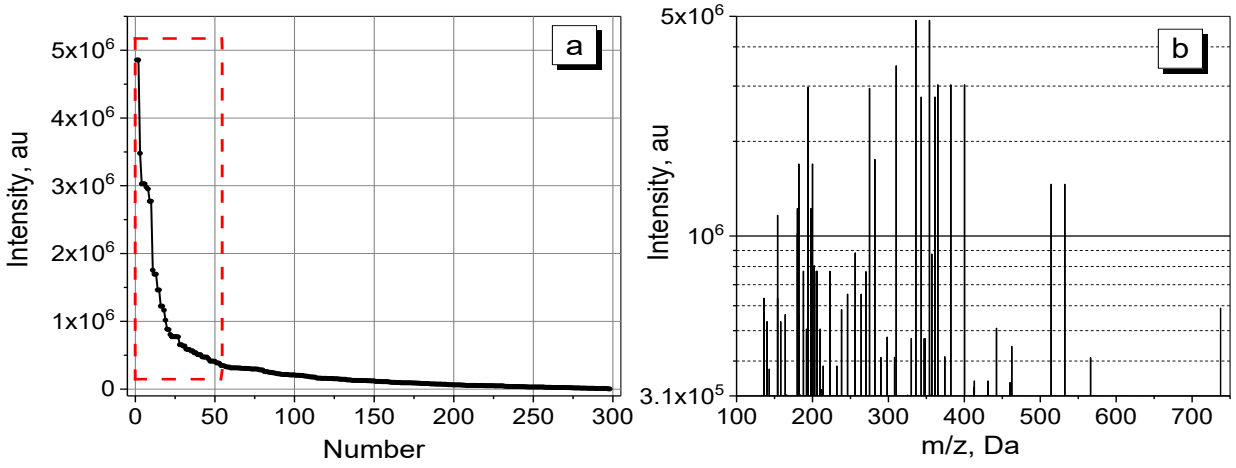


Fig. 3.10 300 mass spectra detected in PE extracts arranged following their peak intensities (a) and the intensities of the 56 most intense peaks (b) as a function of m/z

Figure 3.11 illustrates the number of compounds with peaks of 10^4-10^6 a.u. identified either in one of the PE and EA preparations or both at once.

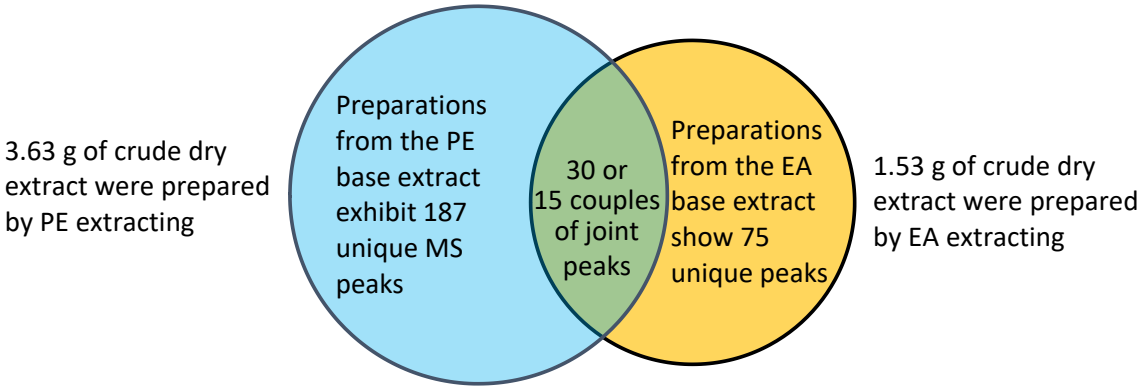


Fig. 3.11 The number of unique peaks with an amplitude of at least 10^4 a.u. identified either in only one of the extracts or present in both extracts simultaneously

The recorded spectra of EA and PE extracts from the same plant were surprisingly different in the number of peaks and the identified compounds (Fig. 3.12). The number of unique intensive peaks in the sample extracted using PE was almost 2.5 times more than in EA (187 versus 75 peaks). This observation correlates with PE extraction, yielding approximately 3.63 g of crude dried extract from 100 g of herb powder, while EA extraction yields only 1.53 g (Fig. 3.12).

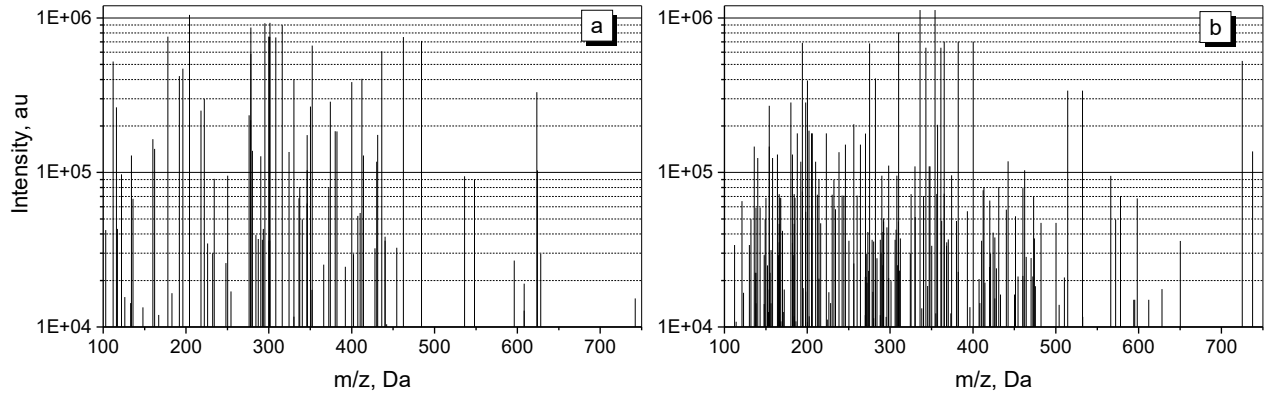


Fig. 3.12 Peaks of unique compounds with intensities 10^4-10^6 a.u. as a function of m/z (Da) in *Speranskia tuberculata* preparations extracted with EA (a) and PE (b)

At first glance, the relatively small number of compounds simultaneously present in both samples is unexpected. For peaks with 10^4-10^6 a.u., only 15 compounds present in both samples were identified; their peaks are shown in Fig. 3.13. Among them, the peaks' amplitudes increased significantly from PE to EA extract in only 10 cases. For others, the changes were either close to zero or negligible in favour of PE extracts.

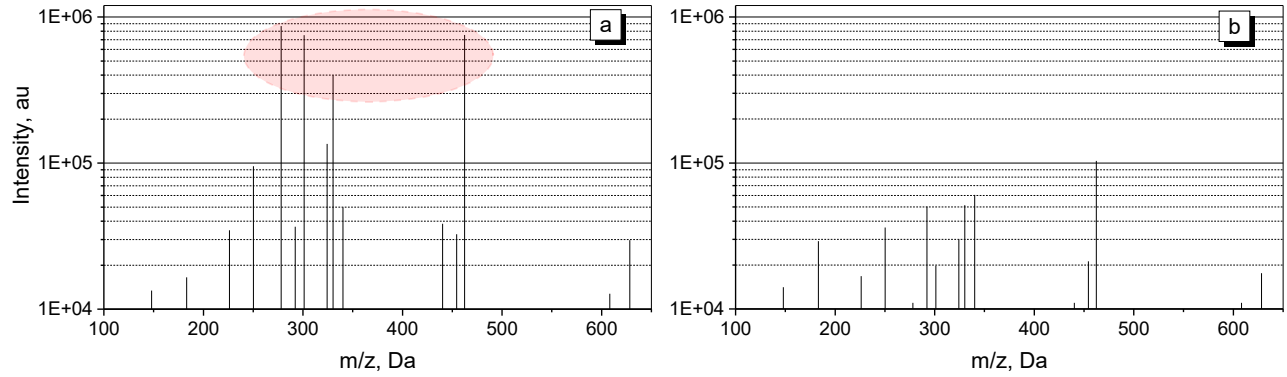


Fig. 3.13 Peaks of joint compounds with intensities 10^4-10^6 (a.u.) as a function of m/z (Da) in preparations extracted with EA (a) and PE (b) solvents

Figure 3.14 illustrates the difference between solvents. The studied mass range (m/z) was divided into segments with a step of 50 Da. For each range, the total ion current from all peaks was calculated and plotted as a function of the range. The maximum peak falls in the 300-350 Da and 350-400 Da ranges for EA and PE extracts, respectively.

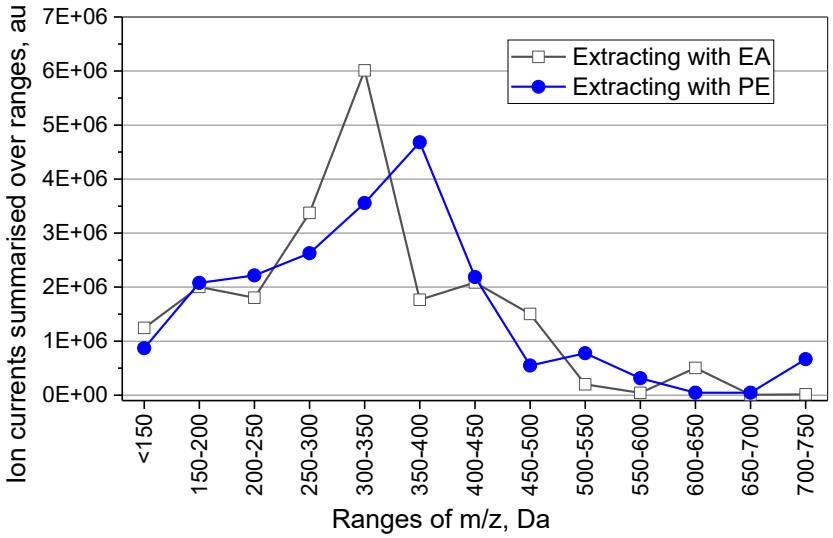


Fig. 3.14 The total intensity of all peaks in each 50 m/z range as a function of Da in *Speranskia tuberculata* medicines extracted with EA and PE

3.3.2 Individual compounds by classes

Since the ethyl acetate extracts are more effective against almost all the studied diseases, it was reasonable to start the analysis with the most intense peaks observed in the EA extracts. The most intense peaks are easier to register and identify. Let us set 2×10^5 a.u. as the lower threshold for consideration. Then, in the range of 2×10^5-5×10^6 a.u., approximately 50 compounds were detected. The literature showed that most of the identified compounds exhibit at least some medicinal properties. *Speranskia tuberculata* (Bunge) Baill. has still not been studied much. Therefore, there is virtually no information on the role of individual compounds in this herb. However, such compounds and their bioactivity have been studied in other plants. The available literature is used to study potential clinical practice areas for the identified compounds.

The data obtained show that only 4 chemical classes of dominant compounds account for more than 75% of the total number of studied compounds, as illustrated in Table 3.1. They are phenolics, flavonoids, alkaloids, and terpenoids. According to the literature, each of the above dominant classes can influence the medicinal properties of plants [129, 130]. At this stage, it is impossible to say which compound has a vital role in the formation of therapeutic activity. There are several compounds whose synergistic effect dominates in practice. The latter is a typical phenomenon in herbal medicines, so further studies should clarify this issue more precisely.

Table 3.1

By class classification of 50 compounds with the most intense peaks in TGC-EA extracts

Class name	Phenolics	Flavonoids	Terpenoids	Alkaloids	Others	Total
Detected metabolite number	14	7	5	12	12	50

Organic compounds are diverse, so classifying them is not easy. The work [81] proposed classification principles that enable the construction of a single chemical classification system for tens of millions of organic compounds. At the same time, the proposed system assigns different chemical classes to some compounds that, from the standpoint of their pharmaceutical action, have similar structures and properties. In pharmaceutical chemistry, such compounds are often clustered together.

The other 12 compounds are classified as amino acids, peptides and proteins, carbohydrates, and lipids, which usually perform a supporting function. Lipids facilitate the penetration of active ingredients into the skin because they are partially lipophilic. Carbohydrates provide energy and serve as building blocks for tissue repair.

The most representative group is phenolic compounds (Table 3.2).

Table 3.2

Phenolic compounds with the largest peak amplitudes (in a.u.) were identified in TGC-EA. According to the literature [Source], they displayed therapeutic properties

Compound	Amplitude	Formulae	MWeight	Source
(+)-Sesamin	4854176	$C_{20}H_{18}O_6$	354.1	[131, 132]
4-Prenyldihydropinosylvin	1753168	$C_{19}H_{22}O_2$	282.2	[133, 134]
Jacaranone	1694648	$C_9H_{10}O_4$	182.1	[135, 136]
3,5-Dimethyl-4-methoxybenzoic acid	1222949	$C_{10}H_{12}O_3$	180.2	[137]
3,5-Dihydroxybenzoic acid	1163814	$C_7H_6O_4$	154.1	[138]
Plumbagin	773130	$C_{11}H_8O_3$	188.2	[139, 140]
(E)-3-Methoxy-4,5-methylenedioxycinnamicaldehyde	773130	$C_{11}H_{10}O_4$	206.1	[141]
(E)-4-(1,5-Dimethyl-3-oxo-1-hexenyl)benzoic acid	652802	$C_{15}H_{18}O_3$	246.3	[142, 143]
Coumarinic acid	562703	$C_9H_8O_3$	164.16	[144]
D-8Acetoxycarvotanacetone	505935	$C_{12}H_{18}O_3$	210.1	[145]
Irisquinone 64	413186	$C_{24}H_{38}O_3$	374.3	[146]
Mycosnol	385868	$C_{13}H_{10}O_3$	214.0	[147]

It includes 14 compounds, and therapeutic properties were reported in 19 scientific articles. It is obvious that over time, the number of such publications will only increase. The areas of biological activity for compounds of this group, as well as others, are, as a rule, quite broad. They include antibacterial, anticancer, antimicrobial, antioxidant, anti-inflammatory, antifungal, antitumor, and antiproliferative actions. Alkaloids are also widely represented (Table 3.3).

Table 3.3

Alkaloids with the largest peak amplitudes (in a.u.) were identified in TGC-EA. According to the literature [Source], they displayed therapeutic properties

Compound	Amplitude	Formulae	MWeight	Source
Tylophorinidine	3027625	$C_{22}H_{23}NO_4$	365.4	[148]
Corynoxeine	3027625	$C_{22}H_{26}N_2O_4$	382.2	[149, 150]
Anti-isorhynchophylline N-oxide	3027625	$C_{22}H_{28}N_2O_5$	400.2	[151-153]
Annotine	2950868	$C_{16}H_{21}NO_3$	275.3	[154]
Codamine 15 in the list	2770196	$C_{20}H_{25}$ NO_4	343.4	[155, 156]
Cephalofortuneine	2770196	$C_{20}H_{27}NO_5$	361.4	[157, 158]
1-Ethyl-4,8-dimethoxy-beta-carboline	884227	$C_{15}H_{16}N_2O_2$	256.1	[159]
Protostephanine	876922	$C_{21}H_{27}NO_4$	357.4	[160, 1611]
Gentianamine	773130	$C_{11}H_{11}NO_3$	205.2	[162]
Wilfordic acid	773130	$C_{11}H_{13}NO_4$	223.1	[163, 164]
Haploperine (Evoxine)	472057	$C_{18}H_{21}NO_6$	347.4	[165]
alpha-Narcotine	345918	$C_{22}H_{2}3N$ O_7	413.4	[166]

The number of identified compounds in two other classes is lower: 7 flavonoids (from 10 sources) (Table 3.4) and 5 terpenoids (from 6 papers) (Table 3.5).

Table 3.4

Flavonoids with the largest peak amplitudes (in a.u.) were identified in TGC-EA. According to the literature [Source], they displayed therapeutic properties

Compound	Amplitude	Formulae	MWeight	Source
Glabrone	4854176	$C_{20}H_{16}O_5$	336.3	[167]
Rehmaglutin C	1694648	$C_9H_{12}O_5$	200.07	[168, 169]
delta-Solamarine	590326	$C_{39}H_{63}NO_{12}$	737.4	[170]
(2R,3R)-(+)-4',5,7-Trimethoxydihydroflavonol	472057	$C_{18}H_{18}O_6$	330.1	[171-173]
Irisoquin F	445547	$C_{29}H_{50}O_4$	462.4	[174]
(-)-Epicatechin	411095	$C_{15}H_{14}O_6$	290.0	[147]
Aucubigenin	312329	$C_9H_{12}O_4$	184.0	[175]

It is important to note that only about half of the most intense peaks and even fewer (about a quarter) of all peaks of substances registered in the TGC-EA extract were studied. It means that the identified biologically active substances represent only a subset of the substances that may be present in the extract. Therefore, it should not be surprising that such traditional flavonoids as rutin or quercetin are absent from the list. Most likely, their peak intensities are slightly lower than those of the strongest peaks.

Table 3.5

Terpenoids with the largest peak amplitudes (in a.u.) were identified in TGC-EA. According to the literature [Source], they displayed therapeutic properties

Compound	Amplitude	Formulae	MWeight	Source
(+)-Myrtenyl acetate	2979411	$C_{12}H_{18}O_2$	194.1	[180, 181]
9-Deacetyl-9-benzoyl-10-debenzoyl brevifoliol	1461323	$C_{29}H_{38}O_8$	514.6	[182]
Paeonilactone A	1222949	$C_{10}H_{14}O_4$	198.2	[183]
Filicinic acid	634302	$C_8H_{10}O_3$	154.2	[184]
tert-O-Methyl byakangelicin	472057	$C_{18}H_{20}O_7$	348.0	[185]

The classification used is conditional, since raw materials often contain multiple groups of biologically active compounds, and it is not always known which of them have a therapeutic effect in a particular herb. However, it helps determine the dominant compound classes in *Speranskia tuberculata*. In addition, the analysis enabled the creation, based on [176-179], of a list of generally recognised phytochemical screening methods relevant to *Speranskia tuberculata* extracts.

The natural conclusion from the results is the complexity of the extracts' composition, due to the presence of many compounds. Under such conditions, determining which substance can serve as the API seems inappropriate, since there are dozens of candidates for this role in the plant. Moreover, the idea that the most important role belongs to the compounds with the highest concentration is not a fact but only a hypothesis. Also, the presence of many biologically active ingredients does not rule out the possibility of synergistic interactions among them. Under such conditions, it seems appropriate, having received primary information about the potential active ingredients, to move from the analysis of the content of individual compounds to the preliminary analysis of the content of classes of compounds. The methods for this type of analysis are well known; most are based on spectrophotometry, and the results are quite easy to obtain and interpret.

3.3.3 Total phenolic, flavonoid, terpenoid and alkaloid contents

Thus, the obtained data indicate the complex nature of herbal medicines. In the most effective TGC-EA preparations, up to 40 compounds claim the role of API, while synthetic preparations usually have only one API. It is still unclear which compounds

are vital for the formation of therapeutic properties. It seems quite logical at this stage to determine the total content of substances belonging to a particular chemical class using well-developed analytical methods, mainly spectrophotometric. The results of the determination are given in Table 3.6, showing the following.

1. In 3 of the 4 classes, TGC-EA shows the highest concentrations of phenols, flavonoids, and alkaloids. Only the terpenoid content is higher in the TGC-EA extract, while TGC-EA remains in second place.

2. The dependence on solvent type is greatest for phenols: the ratio of the maximum to the minimum phenol concentrations across different solvents is 44, whereas it is 26 for flavonoids. The smallest dependence is for alkaloids; the above ratio is only 5.6.

3. The ranking of compound concentrations in the extracts, from highest to lowest, is as follows: terpenoids → phenols → flavonoids → alkaloids.

4. If one conditionally sums the total content across all 4 classes for different extracts, the concentration ranking (in descending order) would be: EA → PE → n-but → AS. Moreover, two extracts, TGC-EA and TGC-PE, appear to be 2-7 times more saturated than the rest. The therapeutic activity of different solvent extracts is proportional to the total content of flavonoids, phenolics, terpenoids and alkaloids.

Table 3.6

Total Phenolic, Flavonoid, Terpenoid, and Alkaloid Content in 4 kinds of extracts

Extracts	Phenolics (mg GE/g)	Flavonoids (mg RE/g)	Terpenoids (mg ursolic acid /g)	Alkaloids (mg Tuberostemonine /g)
Petroleum Ether	6.31±1.89	6.85±1.67	364.51±29.36	29.11±5.89
Ethyl Acetate	280.29±25.44	165.72±12.31	226.69±34.93	68.49±6.14
n-Butanol	46.45±5.29	17.83±4.11	115.36±21.41	20.35±8.37
Aqueous solution	51.87±6.32	15.96±7.24	26.43±6.38	11.58±3.45

In general, the chemical composition of herbal preparations is susceptible to various factors, including plant origin [119]. To the best of the authors' knowledge, no universally accepted theory establishes an unambiguous relationship between solvent type and the extracted compounds. Specific known facts are difficult to extend to other experimental conditions. The effect of solvent polarity on extraction is well known, but it is complex and depends on both the extraction conditions and the compounds being

extracted. The total extraction yield of antioxidant reagents and free radical scavenging activity increased. Similar findings were reported in studies of *Thymelaea hirsuta (L.). Endl.* In [186], the suitability of polar solvents for extracting phytochemical compounds from *T. hirsuta* aerial parts and their antioxidant activity against several radicals and ions is discussed. The lipids extracted by polar solvents were higher than those by non-polar ones [187]. The polarity of solvents also affected the composition of fatty acid methyl esters. Under such conditions, experiments to optimise solvents and extraction conditions will become a priority.

3.3.4 Contents of heavy metals and solvent residuals

The detection and quantification of metals is performed by inductively coupled plasma atomic emission spectrometry (ICP-AES), a modern pharmacopoeial method with high sensitivity [188, 76, 79]. In the context of this work, the most important thing is the content of four heavy metals: Pb, Hg, Cd and As. These metals, due to their toxicity, are among the most strictly controlled in all pharmaceutical, food, and other medical applications—different documents set slightly different permissible limits [188, 189, 190, 191]. The results of extract studies, along with the current restrictions on heavy metal levels in medicines and pharmaceutical raw materials, are presented in Table 3.7. Copper is usually not strictly regulated; its data gives a general idea of extract purity.

The SPhU is based on the expediency of using gas chromatography to determine the concentration of residual solvents in dry extracts. The results of chromatographic studies for EA (a) and, for comparison, n-but dry extract (b) are shown in Fig. 3.15.

Table 3.7

Heavy metals in dry TCG-EA extracts measured by ICP-AES in ppm and permissible limits for medicines and pharmaceutical raw materials according to various sources

	Cu	Cd	Pb	As	Hg	Total for 4 heavy metals
Measured by ICP-AES	0.962± 0.127	0.012± 0.008	0.514± 0.083	0.286± 0.065	0.011± 0.003	0.823± 0.203
Limits for medicinal products and pharmaceutical raw materials (in ppm)						
By [192]		0.5-2.0	1-10.0	1.0-5.0	0.1-0.2	
By [193]	25.0	2.5	≤1.0	≤1.5	≤1.5	
By [194]			3-10			
By [195]			≤1.0			

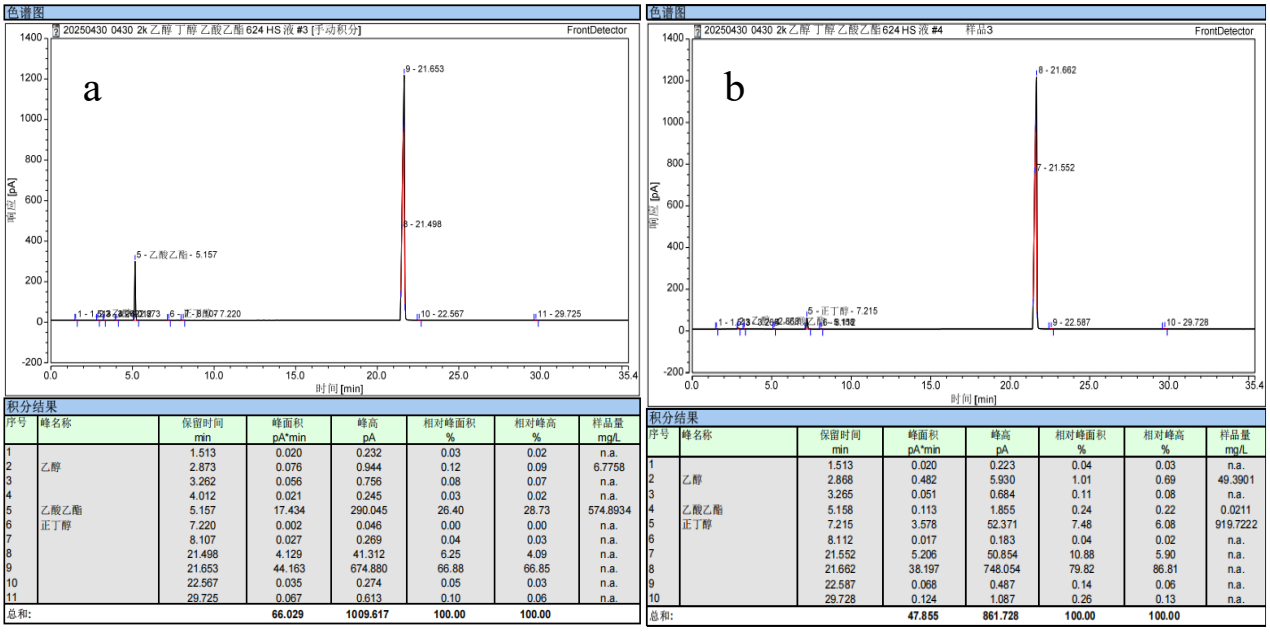


Fig. 3.15 Gas chromatograms for determining solvent residues in dry extracts: a – TGC-EA, b – TGC-n-but

All three organic solvents used are class 3 (low toxicity) according to the ICH guidelines and the SPhU. The content limits for each are set at the total residual quantity limit — no more than 5000 ppm (0.5%). All studied extracts meet the SPhU requirements across all parameters (Table 3.8).

Table 3.8

Concentrations of residues in dry extracts, mg/L

Extracts \ Residues	Ethanol	Ethyl Acetate	N-butanol
TGC-EA extract	6.7758	579.8934	-
TGC-n-but extract	49.3901	0.0211	919.7222
ICH Q3C (R9) limits for Class 3 residuals	≤ 5000	≤ 5000	≤ 5000

3.4 Extraction and application field for further technological development

The previous sections summarised the experimental results obtained on the experimental biological activity of the studied extracts. Such results could receive additional support if a medicine were developed from the most promising extract and its biological activity were tested. It would significantly strengthen the argument in favour of using *Speranskia tuberculata* in the development of dosage forms.

Among the effects studied across the three medicinal directions, the biological activity profiles of the two extracts, EA and PE, appear well established and prevail over those of the other extracts in virtually all cases studied. Table 3.6 shows that these two extracts are significantly richer in potential biologically active compounds than the other two, which became the first criterion for their selection.

The second criterion was based on the results of the *in vitro* biological activity studies. In terms of antibacterial activity and *in vitro* cytotoxic activity in the investigated human tumour cell lines, TGC-EA showed a consistent advantage over TGC-PE across the studied experimental models. In terms of antioxidant activity, the indicators for both extracts are similar, as the comparison results depend on the method used. Thus, the advantage of TGC-EA over TGC-PE is obvious in two directions, while their antioxidant activities in the third direction are approximately comparable. This fact supports further focus on the TGC-EA extract.

The supplementary cytocompatibility assessment provided an additional criterion supporting the selection of TGC-EA for topical formulation development. In HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts, cell viability remained above 85% throughout the investigated concentration range of 12.5–1000 μg/mL following exposure to TGC-EA, and the CC_{50} was not reached (>1000 μg/mL). Importantly, this concentration range encompassed and substantially exceeded the concentrations associated with the observed *in vitro* antibacterial activity of TGC-EA. These findings indicate favourable *in vitro* cytocompatibility of TGC-EA in the investigated human skin-related cellular models and provide additional experimental support for its selection as the API for subsequent topical formulation development.

Overall, TGC-EA demonstrated the most pronounced antibacterial activity

among the investigated extracts. It showed marked inhibitory activity against *S. aureus* and *C. acnes*. *C. acnes* is a member of the normal skin microbiota and one of the microorganisms involved in the multifactorial pathogenesis of acne, a chronic inflammatory disease of the pilosebaceous unit. Therefore, the observed *in vitro* antibacterial activity against *C. acnes* provides a rationale for further investigation of TGC-EA-containing topical formulations in the context of acne treatment.

As demonstrated, the extract contains numerous biologically active compounds, most of which are grouped into 4 chemical classes. Each of these classes can play a positive role in shaping the medicinal properties of the dosage form under development. **Terpenoids**, due to their high lipophilicity (affinity for fats), will enhance penetration, ensuring passage through the skin and solubility. In other words, they act as natural enhancers. They loosen the lipid layer of the skin, helping other components (alkaloids, phenols) to penetrate deeper. They also usually mix well with the fatty bases of the skin ointment. **Phenols** have antioxidant and antiseptic effects and can form hydrogen bonds. In addition to the antibacterial effect, phenols act as stabilisers. They protect the cream from oxidation and ensure the reliable fixation of active substances within the cream base. **Flavonoids** have anti-inflammatory effects and promote skin regeneration. That is, in the composition of the ointment, they provide therapeutic support: they relieve swelling and irritation associated with bacterial lesions, improving tissue trophism at the site of application. Flavonoids are polyphenolic compounds by their chemical nature. Their lipophilicity (the ability to dissolve in lipids and interact with lipid structures) depends on the structure: aglycones of flavonoids (without sugar residues) are more lipophilic, while glycosides (with attached sugars) are more hydrophilic. In EA extracts, compounds of medium polarity are usually concentrated; i.e., part of the flavonoids is present as aglycones or low-polarity derivatives, which increases their ability to interact with the lipid membranes of microorganisms. It is of fundamental importance for the skin because the stratum corneum of the epidermis forms a lipid barrier through which lipophilic substances penetrate more readily, and many antibacterial mechanisms involve disruption of bacterial cell membranes. **Alkaloids** provide a targeted antibacterial attack. The

effectiveness of the action depends on the pH of the medium, so that the alkaloids remain in a form that can be released from the base and penetrate bacterial cells.

Thus, the results obtained once again confirmed that the use of phytopreparations activates simultaneous pharmacological effects, primarily anti-inflammatory, reparative, antioxidant, antibacterial, antifungal, and antiviral. This effect provides a significant advantage of phytopreparations over synthetic drugs [196, 197].

It should be emphasised that acne is a chronic inflammatory disease of the pilosebaceous unit with a multifactorial pathogenesis involving sebum production, follicular keratinisation, inflammatory responses and microbial factors. Therefore, the observed activity of the developed cream against *C. acnes* should be interpreted as evidence of *in vitro* antibacterial activity rather than evidence of clinical efficacy in acne treatment. These findings provide an experimental basis for further investigation of the developed cream in the context of acne management.

To develop an effective drug with a complex API, it is necessary to ensure a combination of hydrophilic and lipophilic components, which require an emulsifier to make the cream homogeneous. Given the physicochemical properties of TGC-EA (the presence of lipophilic biologically active compounds - flavonoids, terpenoids and other secondary metabolites), the optimal dosage form is a soft emulsion form - cream. Among the possible options (ointment, gel or cream), the cream was chosen because:

- it ensures uniform distribution of the active substance on the skin surface;
- better sensory properties and patient compliance characterise it;
- it promotes sufficient penetration of lipophilic components into the superficial layers of the epidermis;
- it does not create excessive occlusion, which is a relevant consideration in the development of topical formulations intended for further investigation in acne and other inflammatory skin conditions.

The emulsion system of the cream helps dissolve the lipophilic extract components, maintains their contact with the skin and creates conditions for effective local delivery to niduses. At the same time, the local route of administration allows one to achieve therapeutically effective concentrations directly at the lesion, minimising

systemic absorption and the risk of undesirable effects [198, 199]. At the same time, the local route of administration is intended to limit systemic exposure and reduce the potential for systemic adverse effects. However, the overall biological safety of the developed medicinal product was not evaluated within the scope of the present study.

3.5 Specification and permissible contaminations in dry TGC-EA extract

Individual compounds cannot be used for specification because too many compounds were found in the extract. Also, it is really unknown which compounds are the most important. So, it is better to go from the contents of individual substances to the total contents of 4 essential classes of substances.

Specifying a dry extract means that

1) the plant raw material was identified and certified by the vendor.
2) The dry extract produced by the developed technology is characterised by a permissible level of heavy metals (Cd, Hg, As, and Hg) to ensure its safe use, as confirmed by ICP-AES analysis in accordance with SPhU recommendations.
3) The dry extract has permissible levels of residual Class 3, low-toxicity solvents.
4) The biologically active concentration intervals of flavonoids, phenols, alkaloids, and terpenoids required for antibacterial activity were experimentally determined.

The lower limit of these concentration ranges will be considered as the specification requirement for the minimum acceptable content of the API components.

Based on the above-listed specification requirements, Table 3.9 illustrates the minimum required values for total phenols, flavonoids, terpenoids, and alkaloids, as well as the permitted concentrations of heavy metals and solvent residues.

Table 3.9

Minimum required values for total phenols, flavonoids, terpenoids, and alkaloids, and permitted concentrations of heavy metals and solvent residues in dry TGC-EA extracts

Biologically active compounds	Phenols (mg GE/g)	Flavonoids (mg RE/g)	Terpenoids (mg UAE /g)	Alkaloids (mg TE /g)
Total content in TGC-EA extracts	≥ 255	≥ 153	≥ 192	≥ 62
Heavy metals	Cd (ppm)	Pb (ppm)	As (ppm)	Hg (ppm)
Limits in medicinal products*	≤ 0.5-2.5	≤ 1.0-10.0	≤ 1.0-5.0	≤ 0.1-1.5
Class 3 residuals in dry extracts	Ethanol	Ethyl Acetate	N-butanol	
ICH Q3C (R9) limits for residuals	≤5000 mg/L	≤ 5000 mg/L	≤ 5000 mg/L	

*as shown in Table 3.7

Given the critical importance of ensuring the required API content, spectrophotometric determination techniques will be validated in the next section.

3.6 Validation of spectrophotometric methods to determine the total contents of bioactive compounds

In the standardisation of medicinal plant materials, spectrophotometric [200-205] and chromatographic methods [206, 207] are widely employed for the quantitative determination of the total content of biologically active substances. These approaches are characterised by adequate accuracy, relatively short analysis time, and cost-effective use of reagents and samples, which makes them suitable for quality control. Among these, spectrophotometry has been widely applied and validated for the analysis of a broad range of compounds in pharmaceutical research, particularly for herbal substances and preparations [208, 202, 209].

From a regulatory perspective, analytical method validation has traditionally been guided by the principles outlined in ICH Q2(R1) [201], which established core validation characteristics, including specificity, linearity, precision, recovery, range, robustness, and sensitivity. However, in response to the evolving complexity of analytical technologies and regulatory expectations, this approach has been substantially revised in the updated ICH Q2(R2) guideline [210]. In parallel, ICH Q14 introduces a modern, science- and risk-based framework for analytical procedure development, incorporating the concepts of lifecycle management and enhanced method understanding. Together, ICH Q2(R2) and ICH Q14 form a complementary, integrated approach that reflects the current state of the art in analytical method development and validation [211].

In this study, flavonoids, phenols, terpenoids, and alkaloids were identified as the main biologically active components in the dry extract of the herb *Speranskia tuberculata*. Absorption-based molecular spectrophotometry techniques were used to quantify the total content of each compound class and were validated. The procedures for sample preparation from dry extracts and cream were described in Chapter 2.

The validation steps and results are discussed in detail for the determination of total flavonoids in extracts. These descriptions serve as suitable examples of a validation procedure for other compounds. The validation procedures for the other three classes of compounds are very similar to those for flavonoids. For this reason, they are not always detailed in this study, and only the final validation results and the required sample characteristics are presented. The principles for selecting the analytical wavelength for each compound class are taken from the literature.

The difference in the quantification of dry extracts and creams is primarily due to differences in the pre- and final sample treatments; this does not apply to spectrophotometric measurements. After pre-treatment procedures, the samples are subjected to identical processing at the final treatment stage for analysis using the same reagents. The spectrophotometric analysis appears the same for both the prepared extract and cream samples. Accordingly, it can be assumed that many validation parameters, such as specificity, linearity, range, precision, and sensitivity, remain unchanged when switching from extract samples to cream sample analysis.

However, such a conclusion regarding the accuracy (or trueness) parameter requires additional justification, since the results of the spike-recovery procedure are not guaranteed to be identical. Accordingly, the accuracy parameter will be validated separately for extracts and creams for the case of flavonoid determination.

Following [212], the following parameters were validated: specificity, linearity, precision, accuracy, range, robustness, and sensitivity.

3.6.1 Specificity

Specificity is the ability of an analytical method to unambiguously assess the quantitative content of the analyte in the presence of other components. In the present study, the specificity of the method was evaluated in accordance with both the Ukrainian State Pharmacopoeia (SPhU, 2nd ed., v. 1, 5.3.N.2, p. 910) and the modern ICH guidelines [210, 211], which define specificity as the capacity of the method to accurately measure the analyte without interference from excipients, degradation products, or other endogenous components.

To validate the spectrophotometric methods according to the parameter "Specificity", an example of a series spectra used for flavonoid quantification is shown in Fig. 16. The absorption spectra of a mixture of all reagent without rutin or extract (Fig. 3.16a), the standard solution of rutin after the reaction with a mixture of the reagents ($NaNO_2$, $Al(NO_3)_3$, NaOH, and methanol) (Fig. 3.16b), and the solution of the extract (analyte) in dimethyl sulfoxide without reagents (Fig. 3.16c) were recorded.

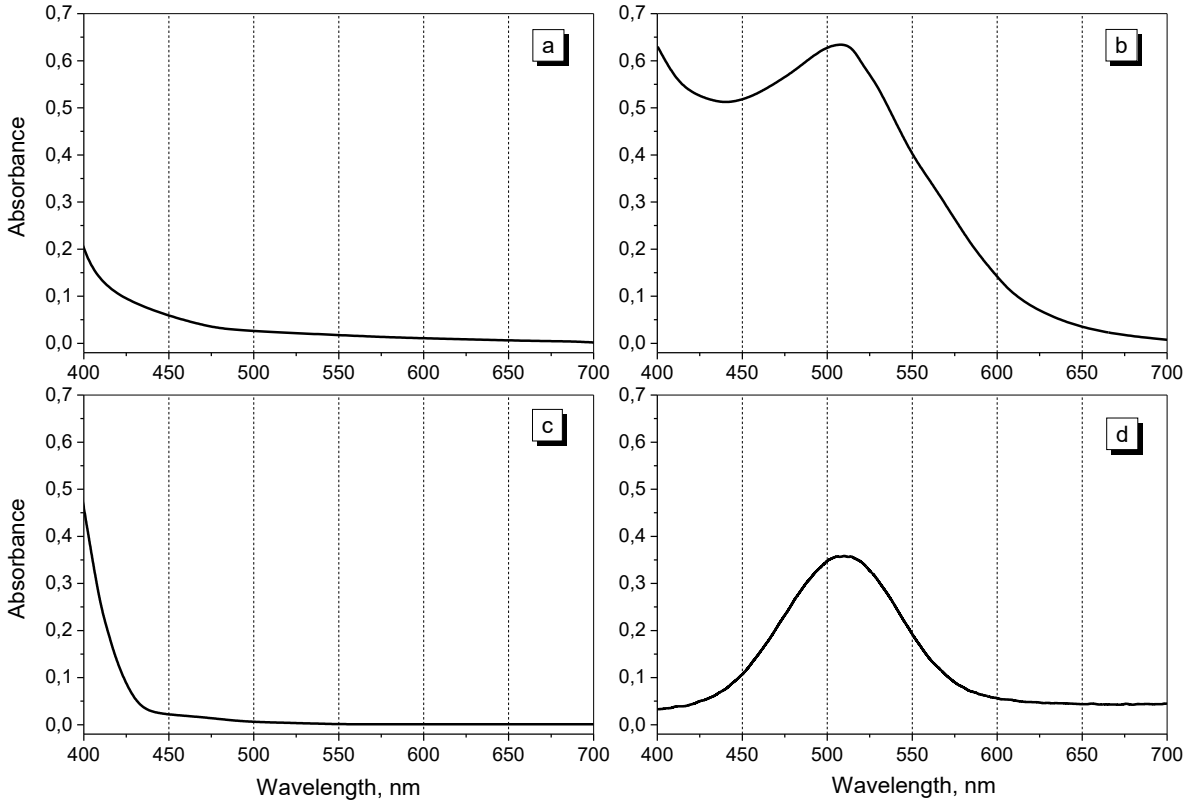


Fig. 3.16 Optical absorptions of: a) a blank solution containing all reagents but without rutin and extract; b) the product of the interaction of a standard rutin solution and a mixture of reagents; c) a dimethyl sulfoxide solution of TGC-EA; d) the product of the interaction of an extract solution of TGC-EA after reaction with reagents

As shown in Fig. 3.16a, the reagent mixture does not exhibit absorption peaks at 510 nm. It is a blank solution containing all reagents, except rutin and the TGC-EA extract. For comparison, the so-called "reference" spectrum was recorded over 400–00 nm (Fig. 3.16b). During the full scan of the solution containing the product of the interaction between the standard rutin solution and the reagent mixture, a clearly

pronounced absorption maximum at 510 nm was observed. The 510 nm wavelength was chosen as the analytical wavelength for determining the total flavonoid content. Fig. 3.16c shows the spectrum of a dimethyl sulfoxide solution of the TGC-EA. As is seen, the extract without reagents does not absorb light at the working wavelength of 510 nm. Absorption at this wavelength arises precisely from the formation of a complex with Al^{3+}. Figure 3.16d confirms this conclusion, showing the spectrum of the TGC-EA after reaction with the reagents (λ_{max} = 510 nm).

Thus, no absorption or spectral band overlap from excipients or reagents was detected in the vicinity of 510 nm. The maximum absorption at 510 nm is due to the formation of a flavonoid-aluminium(III) complex. Thus, the method is specific for the determination of total flavonoids, such as rutin and meets the specificity criterion.

3.6.2 Linearity

The linearity of the technique was established following the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Q2(R1) by assessing the relationship between rutin concentration and optical density over the established range. The linearity of the spectrophotometric method is the ability to ensure direct proportionality between the detector signal (optical absorption) and the analyte concentration in solution. The construction of the calibration curve is detailed for flavonoids; for the other three classes, only the measurement and calculation results are presented in tabular form.

A standard rutin solution at 0.2 mg/mL was used to construct a calibration curve for the determination of total flavonoids. 0.2; 0.8; 1.4; 2.0; 2.6, and 3.2 mL of the standard solution were added to the reaction system, which corresponded to the final concentration range of 0.004–0.064 mg/mL (Table 3.10).

For each concentration, at least three parallel measurements were performed. The following parameters were calculated:

mean value of the absorbance (mean optical density) $\bar{Z} = \frac{\sum Z_i}{n}$; (3.1)

standard deviation (*SD*) $SD = \sqrt{\frac{\sum(Z_i - \bar{Z})^2}{n-1}}$; (3.2)

relative standard deviation (*RSD*, %) $RSD = \frac{SD}{\bar{Z}} \times 100\%$; (3.3)

Table 3.10

Conversion of the added volume of standard rutin solution to the final rutin concentrations in the calibration graph solutions

Volume of standard rutin solution, mL	Amount of rutin in a 10 mL flask, mg	Final rutin concentration, mg/mL
0.2	0.04	0.004
0.8	0.16	0.016
1.4	0.28	0.028
2.0	0.40	0.040
2.6	0.52	0.052
3.2	0.64	0.064

The optical density was measured at 510 nm, and then a calibration graph was plotted (optical density along the Y-axis as a function of concentration along the X-axis). The linear regression equation was determined as $y=ax+b$, where y — optical density, x — rutin concentration (mg/mL), a — slope, b — intercept. Additionally, the coefficient of determination (R^2) and residual standard deviation (S_{res}) were calculated by:

$$S_{res} = \sqrt{\frac{\sum(Y_{obs}-Y_{calc})^2}{n-2}} \quad (3.4)$$

where *(n-2)* is the number of degrees of freedom, $Y_{obs} - Y_{calc}$ is the sum of squares of the residuals, which illustrates the dispersion of the points observed relative to the line.

Absorbance measurements were repeated three times for each rutin concentration. The calculation results as a function of rutin concentration are shown in Table 3.11. The calibration curve for flavonoids is plotted in Fig. 3.17.

Table 3.11

Data for constructing a calibration graph and calculating RSD

Rutin concentration, mg/mL	A1	A2	A3	Mean	SD	RSD (%)
0.004	0.039	0.0375	0.036	0.0375	0.0015	4.00
0.016	0.167	0.163	0.169	0.1663	0.0031	1.84
0.028	0.295	0.288	0.298	0.2937	0.0051	1.75
0.040	0.44	0.43	0.45	0.4400	0.0100	2.27
0.052	0.58	0.56	0.56	0.5667	0.0115	2.04
0.064	0.69	0.71	0.68	0.6933	0.0153	2.20

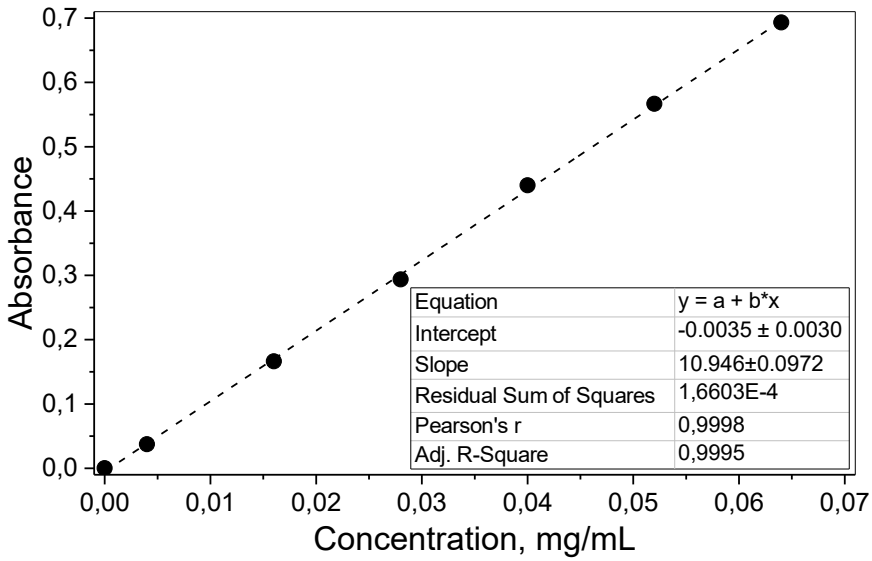


Fig. 3.17 Standard curve for the determination of flavonoids in terms of rutin

Calibration curves for other compounds were constructed similarly; their equations are presented in Table 3.12.

Table 3.12

Parameters of linear calibration curves for flavonoids, phenols, alkaloids and terpenoids

Class	Equation: $y = ax + b$	Linear range, mg/mL	R^2	λ, nm
Flavonoids	y = 10.946x - 0.0035	0.04 to 0.8	0.9995	510
Phenols	y = 179.01x - 0.1042	0.0011 to 0.0066	0.9993	760
Alkaloids	y = 0.0216x - 0.0034	0.005 to 0.05	0.9997	416
Terpenoids	y = 0.0784x + 0.0003	0.002 to 0.012	0.9995	540

The total content (C_i) of compound i in the extracts is determined as follows:

$$C_i = \frac{x_i \times V_i \times N_i}{m_i} \tag{3.5}$$

where V_i is the initial volume of the extract sample (mL), N_i is the dilution factor, m_i is the mass of the TGC-EA (g), and index "i" refers to a class of compounds. The parameters m, V, and N from (3.5) are listed in Table 3.13. The values of x_i (mg/mL) are the concentrations of the test solutions measured using the standard curves from Table 3.12. The total contents of biocompounds in the extracts, including average values, are calculated and listed in Table 3.14.

Table 3.13

Parameters for the calculation of the total contents of bioactive compounds

Class	*m*, g	*V*, mL	*N*
Flavonoids	0.2	50	10
Phenols	0.2	50	500
Alkaloids	1	10	200
Terpenoids	0.02	50	10

Table 3.14

Measurement results for calculating the total contents of bioactive compounds

Compound class	Sample No	Mass of TGC-EA, *m* (g)	The absorbance (measured at), *y*	Concentration, *x* (mg/mL)	Total content, C (mg Equivalent /g)
Flavonoids	No. 1	0.200	0.662 (at 510 nm)	0.0608	152.03
	No. 2	0.200	0.738	0.0677	169.27
	No. 3	0.200	0.766	0.0703	175.86
	Mean				165.72 ± 12.31
Phenols	No. 1	0.200	0.258 (at 706 nm)	0.00202	252.72
	No. 2	0.200	0.304	0.002282	285.29
	No. 3	0.200	0.330	0.002423	302.86
	Mean				280.29 ± 25.44
Alkaloids	No. 1	1.000	0.670 (at 416 nm)	0.0312	62.31
	No. 2	1.000	0.737	0.0343	68.58
	No. 3	1.000	0.802	0.0373	74.59
	Mean				68.58 ± 6.14
Terpenoids	No. 1	0.020	0.590 (at 540 nm)	0.00753	188.16
	No. 2	0.020	0.739	0.00943	235.63
	No. 3	0.020	0.804	0.01025	256.28
	Mean				226.69 ± 34.93

3.6.3 Precision of the instrument

Precision of the instrument is the ability to produce the same results when measuring the same sample multiple times. The sources of error are the electronic noise and the stability of the instrument's lamp or detector. Usually, this indicator is much better (i.e., lower) than others; for example, *RSD* <0.5%. Parameters necessary for the

instrument precision experiment are described in Table 3.12 for calibration curves and wavelengths used, and in Table 3.15 for the samples used.

Table 3.15

Parameters of the extract samples to check the instrument precision and technique robustness

Class	*m*, g	*V*, mL	N
Flavonoids	0.1	50	10
Phenols	0.05	50	100
Alkaloids	0.05	50	10
Terpenoids	0.05	50	50

Using the identical TGC-EA sample chromogenic solutions, the absorbances of four compound classes were measured 6 times each consecutively at 510, 760, 416, and 540 nm (Table 3.16). The outcomes revealed average absorbances of 0.359, 0.406, 0.145, and 0.370, with very low relative standard deviations ranging from 0.473% to 0.872%, indicating favourable instrument precision.

Table 3.16

Estimates of the instrument precision in experiments with four classes of bioactive compounds

Test number	The absorbance *A* at 510 nm (for flavonoids)	The absorbance *A* at 760 nm (for phenols)	The absorbance *A* at 416 nm (for alkaloids)	The absorbance *A* at 510 nm (for terpenoids)
No. 1	0.362	0.404	0.146	0.369
No. 2	0.357	0.409	0.144	0.373
No. 3	0.361	0.406	0.143	0.371
No. 4	0.357	0.405	0.147	0.368
No. 5	0.359	0.408	0.145	0.370
No. 6	0.358	0.404	0.144	0.371
Mean *A*	0.359	0.406	0.145	0.370
SD	0.0021	0.0021	0.0013	0.0018
RSD, %	0.584 (acceptable)	0.517 (acceptable)	0.872 (acceptable)	0.473 (acceptable)

3.6.4 Precision of the technique

Precision of the technique is the reproducibility of results throughout the entire analysis cycle, from the very beginning. In such experiments, the results of measurements performed with different weights and samples are analysed. The sources of error include instrument error, scale error, errors during dilution in the flasks, filtration and human error. This indicator is always higher than the instrumental one.

The precision of an analytical technique is expressed by the degree of dispersion of the values obtained when measuring several samples of the same homogeneous material under the same conditions, around the distribution's centre (arithmetic mean). The precision is determined at two levels: reproducibility and convergence.

The convergence (repeatability) is a measure of precision when measuring under the same conditions over a short period. To validate the method under the "precision – convergence" item, nine repeated measurements of optical density were performed according to the proposed method (Table 3.17) [201].

Table 3.17

The results of the estimate of the convergence for two series of measurements

Parameter	Analyst 1	Analyst 2
Measurement 1	0.363	0.356
Measurement 2	0.361	0.365
Measurement 3	0.354	0.351
Measurement 4	0.358	0.357
Measurement 5	0.359	0.375
Measurement 6	0.356	0.354
Measurement 7	0.361	0.364
Measurement 8	0.354	0.345
Measurement 9	0.364	0.344
Mean, *Z*	0.3589	0.3568
Standard deviation, *SD*	0.0038	0.0102
Relative standard deviation, *RSD* %	1.0280	2.7951
Confidence interval, Δ*Z* (95%)	0.0028	0.0077

The confidence interval was calculated as

$$\Delta Z = \frac{t(P,f)\times SD}{\sqrt{n}} \tag{3.6}$$

where *t(P, f)* is the critical value of the Student's t-distribution with *f* degrees of freedom (*t(P, f)* = 2.3060 for f = 8), *SD* is the standard deviation, and *n* is the sample size.

The reproducibility of the technique is defined as intra-laboratory convergence; i.e., the entire procedure described for "convergence" is performed every other day by a different operator (analyst 2). This characteristic indicates how stable the analysis results remain when external conditions change. While repeatability focuses on keeping everything the same, reproducibility assesses the method's robustness under

different conditions. In the context of comparing two samples, the absolute difference between the means of two groups is calculated as

$$d = |\overline{Z_1} - \overline{Z_2}| \quad (3.7)$$

After that, the technique's reproducibility is calculated using the confidence interval of the average measurements of the first and second analysts (ΔZ) as:

$$\Delta Z_{dif} = t(P, f) \times \sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}; \quad (3.8)$$

where indexes 1 and 2 refer to the 1st and 2nd experimental series. Since the number of degrees of freedom in this case is $f = 16$, the value of the t-distribution $t(P, f) = 2.120$.

If $d < \Delta Z_{dif}$, the discrepancy between analysts is considered statistically unpredictable (random). The results from Table 3.18 demonstrate the technique's good precision. The difference between the two analysts' means (0.0021) falls within the 95% confidence interval (±0.0075), indicating no statistically significant difference and consistent results. The reproducibility of 0.59% (<1.0%) is a good indicator, confirming the analytical method's precision.

Table 3.18

The results of the estimate of the reproducibility between the two analysts' results

Reproducibility (between analysts)	Value
Z overall average for both analysts together.	0.3578
Difference of means	0.0021
Confidence interval of difference (95%)	±0.0075
Reproducibility, confidence interval, ΔZ, % (relative difference of means)	0.59%

The data can also be checked using the Dixon criterion (Q-test) at the 95% confidence level (P=0.95) (SPhU, 5.3.N.1 Statistical analysis of the results of a chemical experiment) to detect and exclude gross errors in a small sample (9 measurements in our case). To do this, one needs to calculate the experimental value Q_{exp} for the most "suspicious" result x_a in comparison with the closest result in the series x_b and the sample range ($R = x_{max} - x_{min}$) using the following formula:

$$Q_{exp} = \frac{|x_a - x_b|}{x_{max} - x_{min}}. \quad (3.9)$$

Then, it is necessary to compare the calculated Q_{exp} with the tabulated Q_{crit} for the number of measurements n and the level P. If $Q_{exp} > Q_{crit}$, the "suspicious" value

is a gross error, and it should be excluded from further calculations. If the opposite is true, the value remains in the sample as statistically valid.

For the sample size $n = 9$, $Q_{crit} = 0.493$. For analysts 1 and 2, the sample ranges are $R_1 = 0.01$ and $R_2 = 0.031$. The absorbance maxima of both samples are 0.364 and 0.375, respectively, and the corresponding Q_{exp} are calculated as 0.100 and 0.323. So, in both cases $Q_{exp} < Q_{crit} = 0.493$, i.e. the data obtained are free from errors. Both full samples can be used reliably to calculate confidence intervals or means.

3.6.5 Spiking recovery experiments in extracts and cream

A spiking recovery experiment is a method for assessing the accuracy (trueness) of the technique used. It is based on adding a precisely known amount of a substance to a sample, followed by checking how much of this "added" substance the applied technique was able to "see" (find). The calculation formula is as follows:

$$Recovery, \% = \frac{C_{found} - C_{blank}}{C_{added}} \times 100\% , \qquad (3.10)$$

C_{found} - this is what is measured (found) after adding,

C_{blank} - this is what was in the sample from the beginning,

C_{added} - this is what was added to the sample.

Flavonoids in extracts. Calculations of the *Recovery* value will start with the study of flavonoids in TGC-EA extracts. As is known, a recovery experiment consists of an experimental check of the completeness of the extraction of a substance (e.g., flavonoids) at all stages of the analysis. Its essence is to compare the actually found amount of a component with its real (true) amount in the sample. The parameters required for calculations are described in Tables 3.12 (calibration curves and wavelengths) and Table 3.19 (parameters *m*, *V* and *N* used).

Table 3.19

Parameters of the extract and cream samples to check the technique recovery

Class	*m*, g	*V*, mL	*N*
Flavonoids	0.05	50	10
Flavonoids in cream	10	50	1
Phenols	0.05	50	100
Alkaloids	0.05	50	10
Terpenoids	0.05	50	50

The original flavonoid content in the TGC-EA sample is ≈8.30 mg. Employing the standard addition recovery method, 9 samples of TGC-EA with known flavonoid contents were precisely weighed. Subsequently, rutin reference standards were added at 80% (6.64mg), 100% (8.30 mg), and 120% (9.96 mg) of the labelled amounts, respectively. Using the calibration curve equations ($y = ax + b$), the calculated total flavonoid amount is computed for each experimental point as follows:

$$C_{found} = V \times N \times x = V \times N \times \frac{y-b}{a} \quad (3.11)$$

where y is the absorbance at 510 nm, a *and* b are the slope and intercept of the calibration curve, V is the initial volume of the extract sample, and N is the dilution factor.

The recovery rates were then determined by (3.10) and shown in Table 3.20.

Table 3.20

Recovery test data for the validation of total flavonoid analysis in the extract

Percentage of addition	Sample No.	Mass of TGC-EA (g)	Original flavonoids (blank) (mg)	Added flavonoids (mg)	Absorbance measured at 510 nm.	Calculated total flavonoids (found) (mg)	Recovery (%)
80 %	No.1	0.0504	8.352	6.64	0.321	14.823	97.45
	No.2	0.0495	8.203	6.64	0.324	14.960	101.76
	No.3	0.0512	8.485	6.64	0.327	15.097	99.58
100 %	No.4	0.0508	8.419	8.30	0.362	16.695	99.71
	No.5	0.0498	8.253	8.30	0.355	16.376	97.87
	No.6	0.0510	8.452	8.30	0.365	16.833	100.98
120 %	No.7	0.0501	8.303	9.96	0.395	18.203	99.40
	No.8	0.0515	8.535	9.96	0.400	18.431	99.36
	No.9	0.0492	8.153	9.96	0.388	17.883	97.69

Analysing the recovery values obtained in individual experiments, the following values were calculated: the mean recovery = 99.31%, recovery range = 97.45 - 100.98, *SD* = 1.466, *RSD* = 1.476% (acceptable), standard error of the mean = 0.49, confidence interval (95%) = 99.31±1.13. Statistical analysis of the recovery experiment data showed that the standard error of the mean is only 0.49%. The confidence interval for the mean recovery is within [98.18%; 100.44%]. Since the ideal value of 100% falls within this interval, there is no statistically significant systematic error, and the method

is highly reliable. It exhibits high accuracy, and the extract matrix does not significantly interfere with the colourimetric determination of total flavonoids.

Phenols in extracts. The original phenol content in the TGC-EA sample is ≈14.01 mg. Employing the standard addition recovery method, 9 samples of TGC-EA with known phenol contents were precisely weighed. Subsequently, phenol reference standards were added at 80% (11.2 mg), 100% (14.00 mg), and 120% (16.80 mg) of the labelled amounts, respectively. Using initial data from Tables 3.12 and 3.19, and formulas (3.10) and (3.11), the recovery rates for phenols are calculated in Table 3.21.

Table 3.21

Recovery test data for the validation of total phenol analysis in the extract

Percentage of addition	Sample No.	Mass of TGC-EA (g)	Original phenols (blank) (mg)	Added phenols (mg)	Absorbance measured at 706 nm	Calculated total phenols (found) (mg)	Recovery (%)
80 %	No.1	0.0501	14.043	11.20	0.790	24.976	97.62
	No.2	0.0494	13.846	11.20	0.798	25.200	101.37
	No.3	0.0505	14.155	11.20	0.801	25.284	99.37
100 %	No.4	0.0502	14.071	14.00	0.894	27.878	98.62
	No.5	0.0498	13.958	14.00	0.901	28.074	100.82
	No.6	0.0507	14.211	14.00	0.904	28.169	99.70
120 %	No.7	0.0496	13.902	16.80	0.983	30.367	98.01
	No.8	0.0503	14.099	16.80	1.007	31.036	100.82
	No.9	0.0510	14.295	16.80	1.005	30.986	99.35

The following values were calculated for phenol recovery: the mean recovery = 99.519%, recovery range = 97.62 - 100.82, *SD* = 1.304, *RSD* = 1.311% (acceptable). Thus, the technique used is highly reliable. It exhibits high accuracy; the extract matrix does not interfere with the colourimetric determination of total phenols.

Alkaloids in extracts. The original alkaloid content in the TGC-EA sample is ≈3.42 mg. Employing the standard addition recovery method, 9 samples of TGC-EA with known alkaloid contents were precisely weighed. Subsequently, alkaloid reference standards were added at 80% (2.74 mg), 100% (3.42 mg), and 120% (4.12 mg) of the labelled amounts, respectively. Using initial data from Tables 3.12 and 3.19,

and formulas (3.10) and (3.11), the recovery rates for alkaloids were calculated in Table 3.22.

Table 3.22

Recovery test data for the validation of total alkaloid analysis in the extract

Percentage of addition	Sample No.	Mass of TGC-EA (g)	Original alkaloids (mg)	Added alkaloids (mg)	Absorbance measured at 416 nm.	Calculated total alkaloids (mg)	Recovery (%)
80 %	No.1	0.0492	3.374	2.74	0.258	6.051	97.70
	No.2	0.0506	3.470	2.74	0.264	6.190	99.26
	No.3	0.0498	3.415	2.74	0.260	6.097	97.89
100 %	No.4	0.0503	3.450	3.42	0.291	6.815	98.39
	No.5	0.0495	3.395	3.42	0.294	6.884	102.03
	No.6	0.0508	3.484	3.42	0.293	6.861	98.75
120 %	No.7	0.0491	3.367	4.12	0.318	7.440	98.85
	No.8	0.0505	3.463	4.12	0.323	7.556	99.33
	No.9	0.0496	3.402	4.12	0.316	7.394	96.88

The following parameters were found for alkaloid recovery: the mean recovery = 98.787%, recovery range = 96.88-102.03, *SD* = 1.45, *RSD* = 1.468% (acceptable). The technique used is highly reliable and highly accurate.

Terpenoids in extracts. The original terpenoid content in the TGC-EA sample is ≈11.33 mg. Employing the standard addition recovery method, 9 samples of TGC-EA with known terpenoid contents were precisely weighed. Subsequently, terpenoid reference standards were added at 80% (9.06 mg), 100% (11.33 mg), and 120% (13.60 mg) of the labelled amounts, respectively.

As for other compounds, the results of the recovery calculation, obtained from initial data in Tables 3.12 and 3.19 and formulas (3.10) and (3.11), are summarised in Table 3.23.

The following parameters were calculated for terpenoid recovery: the mean recovery = 98.979%, recovery range = 97.32 - 100.81, *SD* = 1.295, *RSD* = 1.308% (acceptable). These parameters prove the high reliability of the technique used. It exhibits high accuracy, and the extract matrix does not significantly interfere with the colourimetric determination of total terpenoids.

Table 3.23

Recovery test data for the validation of total terpenoid analysis in the extract

Percentage of addition	Sample No.	Mass of TGC-EA (g)	Original terpenoids (blank) (mg)	Added terpenoids (mg)	Absorbance measured at 540 nm.	Calculated total terpenoids (found) (mg)	Recovery (%)
80 %	No.1	0.0487	11.040	9.06	0.623	19.857	97.32
	No.2	0.0511	11.584	9.06	0.650	20.717	100.81
	No.3	0.0493	11.176	9.06	0.632	20.143	98.97
100 %	No.4	0.0509	11.539	11.33	0.711	22.663	98.18
	No.5	0.0484	10.972	11.33	0.701	22.344	100.37
	No.6	0.0515	11.675	11.33	0.719	22.918	99.23
120 %	No.7	0.0492	11.153	13.60	0.765	24.385	97.29
	No.8	0.0504	11.425	13.60	0.786	25.053	100.21
	No.9	0.0488	11.062	13.60	0.767	24.449	98.43

Flavonoids in cream. The recovery test was applied to cream samples after pretreatment. The determined flavonoid content in the cream sample was 1.52±0.006 (mg RE/g). The standard curve for the routine was prepared in the same way as for the extracts: $y = 10.946x - 0.0035$. $R^2 = 0.9995$ (Table 3.12). The mass of the cream was 10 g; the initial volume of the sample, V (mL), and the dilution factor, N, were 50mL and 1, respectively (Table 3.19).

Employing the standard addition recovery method, 9 samples of TGC cream with known flavonoid contents were precisely weighed. Subsequently, rutin reference standards were added at 80% (1.22 mg), 100% (1.52 mg), and 120% (1.82 mg) of the labelled amounts, respectively. The recovery rates were then determined by (3.10) and (3.11). The experimental results are summarised in Table 3.24. Analysing the recovery results obtained in individual experiments, the following values were calculated: mean recovery = 99.70%; recovery range = 97.42-102.3; *SD* = 1.816; *RSD* = 1.822% (acceptable); standard error of the mean = 0.61.

Statistical analysis of the recovery experiment data showed that the standard error of the mean is only 0.61%. The confidence interval for the mean recovery is within [97.91%; 100.71%]. Since the ideal value of 100% falls within this interval, there is no statistically significant systematic error, and the method is highly reliable.

It exhibits high accuracy, and the extract matrix does not significantly interfere with the colourimetric determination of total flavonoids.

Table 3.24

Recovery test data for the validation of total flavonoid analysis in the cream

Percentage of addition	Sample No.	Mass of TGC cream (g)	Original flavonoids (blank) (mg)	Added flavonoids (mg)	Absorbance measured at 510 nm.	Calculated total flavonoids (found) (mg)	Recovery (%)
80 %	No.1	10.02	1.523	1.22	0.591	2.716	97.79
	No.2	9.98	1.517	1.22	0.599	2.752	101.23
	No.3	10.06	1.529	1.22	0.595	2.734	98.77
100 %	No.4	10.04	1.526	1.52	0.671	3.081	102.30
	No.5	9.95	1.512	1.52	0.654	3.003	98.09
	No.6	10.10	1.535	1.52	0.664	3.049	99.61
120 %	No.7	9.97	1.515	1.82	0.734	3.369	101.87
	No.8	10.08	1.532	1.82	0.720	3.305	97.42
	No.9	10.01	1.522	1.82	0.729	3.346	100.22

3.6.6 Range

The working range of the technique was determined, for example, based on the results of the linearity, accuracy, and precision study described above. It was found that the linear relationship between rutin concentration and optical density is maintained over the range of 0.04–0.064 mg/mL, as confirmed by the high coefficient of determination ($R^2 = 0.9995$) and the absence of systematic deviations. To confirm the suitability of the method for the analysis of real samples, linearity was additionally assessed over 80–120% of the expected rutin content. The expected content of the ethyl acetate extract dissolved in dimethyl sulfoxide was set at 100% (not less than 50 mg RE/g). Accordingly, model solutions were prepared at 80%, 100%, and 120%,

In the specified range, the following parameters are ensured:

- compliance with the linearity criterion ($R^2 \geq 0.9995$);
- acceptable precision ($RSD \leq 2\%$);
- absence of interference at the analytical wavelength of 510 nm;
- adequacy of the linear regression model.

Since the specified parameters meet the established criteria, the selected range is reasonable for quantification.

3.6.7 Robustness

Robustness is the ability of an analytical method to remain insensitive to small changes in analysis conditions and provide reliable results. For spectrophotometric methods, in accordance with the SPhU, robustness is assessed by applying small intentional changes to analysis conditions that may occur during laboratory work and determining their impact on total flavonoid determination using rutin as the standard. The assessment was carried out in accordance with the recommendations of the SPhU and the ICH Q2(R1) harmonised approach, Validation of Analytical Procedures. The following conditions were changed to check robustness:

- the time for the colour development of the reaction mixture after adding the reagent was up to 120 min for flavonoids and up to 8 h for other compounds;
- measurement wavelength (λ = 509, 511, 512 nm);
- pH of the reaction medium was ±0.2 from the nominal value (pH 8.5).

For each condition, the complete analysis was repeated: a new sample solution was prepared, and three parallel optical density measurements were performed. Such an approach allows one to assess the impact of changes in methodological parameters on the result, rather than only on the instrument's measurement.

The sample concentration for the robustness test corresponded to 100% of the calibration level (50 mg RE/g). For each point, the mean optical density (A), the relative standard deviation (RSD, %), the concentration of total flavonoids in mg RE/g, and the deviation from the nominal value (%) were calculated. The method was considered robust if the $RSD \leq 2\%$ and the deviation of the results was ≤ 2%.

Time stability. To investigate whether the red complex formed by the combination of flavonoids and aluminium ions fades after long-term storage at room temperature, a test solution was prepared, with NaOH added to trigger colour development and start timing.

The determined flavonoid content of TGC-EA was 165.72±12.31 (mg RE/g). Rutin standard curve: $y = 10.946x - 0.0035$. $R^2 = 0.9995$. For three other compounds,

calibration curves from Table 3.12 were used. Other initial parameters were taken from Table 3.15 and Table 3.16. The test solutions were prepared according to the aforementioned methodology, and absorbances at the selected wavelengths were measured (Table 3.25) after standing at room temperature for 0 to 120 min for flavonoids (or for 0-8 h for other compounds).

Table 3.25

The results of the stability experiment (effect of colour development time)

Test Time	Flavonoid *A* at 510 nm	Test Time	Phenols *A* at 706 nm	Alkaloids *A* at 416 nm	Terpenoids *A* at 540 nm
0 min	0.363	0 h	0.399	0.144	0.371
15 min	0.362	1 h	0.404	0.146	0.370
30 min	0.360	2 h	0.409	0.145	0.368
60 min	0.358	4 h	0.410	0.143	0.369
90 min	0.355	6 h	0.406	0.141	0.366
120 min	0.353	8 h	0.402	0.139	0.364
Mean *A*	0.359		0.405	0.143	0.368
SD	0.0039		0.0042	0.0026	0.0026
RSD, %	1.098 (accept)		1.036 (accept)	1.824 (accept)	0.709 (accept)

The outcomes for all 4 compound classes demonstrated *RSDs* of 0.709% to 1.818%, indicating that the chromogenic solution maintained favourable stability at room temperature for the studied time periods, thereby meeting the temporal requirements for batch testing.

Wavelength robustness. In analogy with the time-stability experiments, the robustness of the technique was investigated by varying the wavelength, using flavonoids as an example. The absorbance results at three neighbouring wavelengths were compared. The aim is to ensure that a small deviation in the instrument settings does not significantly affect the analysis result within the error limits.

For this, the absorbance for one prepared extract solution was measured at three wavelengths (509, 511 and 512 nm) three times in succession. For each measurement, the concentration was calculated using a calibration graph. The results were compared based on the RSD (%) values. The main indicator is the relative standard deviation between the results obtained at different wavelengths. For robustness, it should usually

be less than 2%. The data for the calculations are in Tables 3.12, 3.15, and 3.16. The calculation results are in Table 3.26.

Table 3.26

Robustness: Effect of wavelength on the detection results

λ	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean	SD	RSD
509 nm	0.358	0.354	0.359	0.355	0.354	0.360	0.3567	0.0026	0.744
511 nm	0.357	0.361	0.356	0.359	0.355	0.358	0.3577	0.0022	0.603
512 nm	0.356	0.352	0.358	0.353	0.356	0.351	0.3543	0.0027	0.771

The study found that small changes in the reaction medium's wavelength and pH did not significantly affect the determination of total flavonoids. In all cases, the RSD did not exceed 1%.

pH robustness. As in wavelength-robustness studies, the pH-related robustness of the technique was investigated using flavonoids as an example. The absorbance results at three neighbouring pH levels of the studied media were compared. The aim was to ensure that a small deviation in the pH of the media does not significantly affect the analysis result within the error limits.

For this, the absorbance for one prepared extract solution was measured at three pH levels (8.3, 8.5 and 8.7) three times in succession. For each measurement, the concentration was calculated using a calibration graph. The results were compared based on the *RSD* (%) values. The main indicator is the relative standard deviation between results obtained at different pH values. For robustness, it should usually be less than 2%. The data for the calculations are in Tables 3.12, 3.15, and 3.16. The calculation results are in Table 3.27.

Table 3.27

Effect of pH of the reaction medium on detection results

pH	Optical absorption			Average	RSD, %	Content, mg RE/g
	No. 1	No. 2	No. 3			
8.3	0.352	0.356	0.345	0.3511	1.586	161.93 ± 2.54
8.5	0.349	0.354	0.357	0.3533	1.143	162.99 ± 1.84
8.7	0.355	0.361	0.358	0.3580	0.838	165.13 ± 1.37

The study found that small changes in the reaction medium's wavelength and pH did not significantly affect the determination of total flavonoids. In all cases, the *RSD* did not exceed 1.5 %.

Therefore, the spectrophotometric method for determining total flavonoids (rutin) in the dry extract is robust to these three parameters (storage time, wavelength and pH fluctuations) and suitable for analytical applications.

3.6.8 Sensitivity

Important validation parameters are the minimum amount of a substance that can be detected and accurately measured. These are, respectively, *LOD* (Limit of Detection) and *LOQ* (Limit of Quantitation). These parameters can be determined from the calibration graph. For spectrophotometry, the calculation is carried out according to the formulas:

$$LOD = \frac{3.3 \times \sigma}{S} \quad (3.12)$$

$$LOQ = \frac{10 \times \sigma}{S} \quad (3.13)$$

where σ is the standard deviation of the response (most often it is the standard deviation of the free term of the regression - intercept or the standard deviation of the blank sample), and S is the slope of the calibration curve (the coefficient a in the equation $y = ax + b$). The estimated *LOD* and *LOQ* values are shown in Table 3.28.

Table 3.28

Estimates of *LOD* and *LOQ* for spectrophotometric detection and quantification of biocompounds

Compounds	LOD, µg/mL	LOQ, µg/mL
Flavonoids	1.1	3.2
Phenols	1.9	5.8
Alkaloids	51.9	157.4
Terpenoids	12.6	38.3

Conclusions to Chapter 3

1. The study confirmed the potential of the studied extracts, TGC, for use as antitumor, antioxidant and antibacterial agents. The extracts significantly reduce the viability of cancer cells in most of the studied cancer lines and exhibit a high ability to

neutralise free radicals, making them promising for the treatment of oncological and inflammatory diseases.

2. Among all the studied samples, the ethyl acetate extract (EA) showed the most pronounced antibacterial activity. It effectively inhibits the growth of *Staphylococcus aureus (S. aureus*) and acne-causing bacteria, *C. acnes*, with an IC_{50} of 112–135 μg/mL, whereas other extracts were not sufficiently active.

3. Mass spectrometry analysis revealed a complex chemical composition of the prepared extracts. About 300 peaks of organic compounds were found in the petroleum ether extract (PE), and 75 unique intense peaks in the ethyl acetate extract. The majority of identified substances in the extracts (over 75%) belong to four dominant classes of compounds with proven biological activity in the literature. Two extracts, TGC-EA and TGC-PE, appear to be 2-7 times more saturated than the rest. The therapeutic activity of different solvent extracts is proportional to the total content of flavonoids, phenolics, terpenoids and alkaloids.

4. The total content of polyphenols, flavonoids, terpenoids and alkaloids was determined by the spectrophotometric method. When applied to all 4 classes of compounds, the method was validated for specificity, linearity within the working range, precision, and robustness with respect to wavelength, colour development time, and pH. The validation results were found to be in line with the SPhU requirements.

5. Ethyl acetate provides the highest yield of phenolic compounds and flavonoids among other extracts. In order of decreasing concentrations in the most promising EA extract, the classes of compounds were distributed in the following order: phenols, terpenoids, flavonoids and alkaloids. Analysis of the content of biologically active substances in comparison with the results of the study of the antibacterial activity of the EA extract allowed one to determine the minimum permissible levels of their contents, which provide the desired antibacterial effect: phenols – ≥255 mg GE/g, flavonoids – ≥154 mg RE/g, terpenoids – ≥192 mg UAE/g, alkaloids – ≥62 mg TE/g.

6. Verification of the safety of the chemical composition of the dry extract TGC-EA confirmed its compliance with the standards of the SPhU. The content of heavy metals (lead, mercury, cadmium and arsenic) is within the permissible limits, and the

residual amounts of 3rd class solvents do not exceed the limit of 0.5%, which classifies the product as low-toxicity.

7. For quality control of TGC-EA, it is proposed to use a comprehensive system that includes certification of plant raw materials, testing for the absence of contaminants, determination of minimum levels of the four main classes of bioactive substances, control of heavy metals and residual solvents. It allows one to ensure the stability of the antibacterial effect and the safety of the TGC-EA dry extract.

8. The validation of the spectrographic method and equipment used to determine the total content of biologically active substances in extracts and cream proved that the experimental values met the validation requirements in terms of specificity, linearity, precision, accuracy, range, robustness with respect to time stability, wavelength and pH, and sensitivity.

The results of the research in Chapter 3 are contained in the following published articles: [75, 213-216].

Chapter 4. Pharmaceutical development and technological scheme for producing an antibacterial cream with a dry Tou Gu Cao extract

Pharmaceutical development is carried out in accordance with Guideline 42-3.0:2011, Medicinal products. Pharmaceutical development (ICH Q8) [217]. This guideline is the adopted (English version) regulatory document of the European Medicines Agency EMEA/CHMP/167068/2004 (ICH Topic Q8 (R2) Pharmaceutical Development) and Annex to EMEA/CHMP/167068/2004 (ICH Topic Q8 Annex Pharmaceutical Development) [218-220].

The purpose of pharmaceutical development is to create a high-quality medicinal product and optimise its production process, ensuring stable product production with the necessary functional characteristics. Scientific research conducted within the framework of pharmaceutical development, as well as practical production experience, contribute to a deeper understanding of the processes, which, in turn, serve as the basis for determining design parameters, specifications, and production control methods.

The data obtained during the research are key to managing quality-related risks. It is important to consider that the quality of the medicinal product is established during development and is not determined solely by testing the finished product. It is necessary to provide information confirming that the selected dosage form and its composition correspond to the intended purpose. Each aspect of the "Pharmaceutical Development" section should contain sufficient information to fully understand the process of creating a medicinal product and its production. Special attention should be paid to critical factors affecting product quality, in particular the characteristics of the active substance, excipients, packaging, and production parameters. Justification of the quality control strategy is mandatory. Critical composition and process parameters are determined by analysing their impact on the final product.

Terms used. A critical process parameter (CPP [218]) is a process parameter whose variability can affect a critical quality parameter and, as a result, must be monitored and controlled to ensure the required product quality. **A critical quality attribute** (CQA [218]) is a physical, chemical, biological or microbiological property or characteristic that must be within the appropriate limits, range or distribution to

ensure the required quality of the product. **Drug product** - a medicinal product in a specific dosage form, placed in a final package and intended for placement on the market. **Material** is a general term that encompasses raw materials (starting materials, reagents, solvents), excipients and other materials, intermediates, active pharmaceutical ingredients, packaging, and labelling materials. Official experimental plans; formal experimental designs (also known as design of experiments [218]) - a structured, organised method for determining the relationship between factors affecting a process and the products obtained due to that process.

4.1 Justification of cream components and composition

4.1.1 Principles of selection of drug formulation, API and excipients

The development of a therapeutic antibacterial cream necessarily involves balancing the stability of the active substance, its ability to penetrate the skin, and patient comfort. The following principles allow one to achieve such a balance:

1. The correct choice of the active substance API with an optimal spectrum of activity, stability (taking into account sensitivity to hydrolysis or oxidation when changing the pH of the medium), sufficient lipophilicity and low affinity for the base

2. The optimal choice of the type of emulsion (oil-in-water O/W or water-in-oil W/O) is guided by the following:

3. The correct choice of excipients, primarily compatible with the API. When developing the formulation of an antibacterial cream, it is worth excluding or minimising excipients with high comedogenicity indicators. To do this, one should avoid the use of animal fats and their derivatives, fatty acids and oils, heavy oils and butters, and some surfactants. Instead, it is better to use safe alternatives, such as moisturisers, emollients, and silicones.

4. The excipients should be selected to fulfil their functional duties, namely: not to be inert, but to support the therapeutic effect; serve as emulsifiers and structurants to provide the necessary viscosity; act as solvents and penetration enhancers; act as preservatives and antioxidants; be less irritating to damaged skin; and maintain physiological pH. Based on the formulated requirements, the following excipients are planned for use in an antibacterial cream (Table 4.1).

Table 4.1

Main properties of excipients included in the antibacterial cream product

Excipient name	Functional role	Main Typical properties
EDTA (Disodium Edetate) (Ph. Eur. Monograph 0232: *Disodium edetate*)	Chelating Agent & Preservative Synergist	1. Description: White or almost white, crystalline powder. 2. Solubility: Soluble in water, practically insoluble in ethanol (96 %). 3. pH: From 4.0 to 5.5 (Determined on a 50 g/L solution). 4. Appearance of solution: The solution is clear and colourless. 5. Iron: Maximum 80 ppm. 6. Impurity A: Nitrilotriacetic acid: Maximum 0.1 %. 7. Water: From 8.7 % to 11.4 %. 8. Assay: 98.5 % to 101.0 % (dried substance).
Glycerin (Ph. Eur. Monograph 0496: Glycerol)	Humectant	1. Description: Clear, colourless, syrupy liquid, oily to the touch, hygroscopic. 2. Solubility: Miscible with water and with ethanol (96 %), slightly soluble in acetone, practically insoluble in fatty oils and in essential oils. 3. Refractive index: From 1.470 to 1.475. 4. Relative density: Not less than 1.260. 5. Acidity or alkalinity: Not more than 0.2 mL of 0.1 M NaOH is required to change the colour of the indicator. 6. Water: Not more than 2.0 %. 7. Sulphated ash: Not more than 0.01 %. 8. Aldehydes: Not more than 10 ppm. 9. Impurity A and related substances - Impurity A (Diethylene glycol): No more than 0.1% - Impurity B (Ethylene glycol): No more than 0.1% 10. Esters: Requires not less than 8.0 mL of 0.1 M HCl for titration (indicating limit of esters).
Apricot Kernel Oil PEG-6 Esters (Corresponds to Ph. Eur. Monograph 1429: Glycerol monolinoleate)	Hydrophilic Emollient & Co-surfactant	1. Description: Amber, oily liquid. 2. Solubility: Dispersible in water, freely soluble in methylene chloride and in ethanol (96 %). 3. Physical state / Freezing point: Liquid at room temperature. (Viscous oily liquid). 4. Acid value: No more than 2.0. 5. Hydroxyl value: From 45 to 65. 6. Iodine value: From 75 to 95. 7. Saponification value: From 150 to 170. 8. Peroxide value: No more than 12.0.
Cetearyl Alcohol		1. Description: White or pale yellow, wax-like mass, plates, flakes or granules.

Excipient name	Functional role	Main Typical properties
(Ph. Eur. Monograph 0702: Ph. Eur. Monograph 0702: Cetostearyl alcohol)		2. Solubility: Practically insoluble in water, soluble in ethanol (96 %) and in light petroleum. When melted, it is miscible with fatty oils, liquid paraffin, and melted wool fat. 3. Melting point: From 49°C to 56°C. 4. Acid value: No more than 1.0. 5. Hydroxyl value: From 208 to 228. 6. Iodine value: No more than 2.0. 7. Saponification value: No more than 2.0.
Petrolatum (Ph. Eur. Monograph 1799: Paraffin, white soft)		1. Description: White or almost white, translucent, soft, unctuous mass, slightly fluorescent in daylight when melted. 2. Solubility: Practically insoluble in water and in ethanol (96%), soluble in methylene chloride. 3. Drop point: From 35°C to 70°C. 4. Cone penetration: From 130 to 210 (0.1 mm). 5. Acidity or alkalinity: Not more than 0.5 mL of 0.01 M NaOH is required to change the colour of the indicator. 6. Polycyclic aromatic hydrocarbons: Absorbance at 260-420 nm is not greater than that of the reference solution (naphthalene standard). 7. Sulphated ash: No more than 0.05 %. 8. Appearance: The substance is white. The melted mass is not more intensely coloured than the reference solution mixture.
Stearic Acid (Ph. Eur. Monograph 1474: Stearic acid)		1. Description: White or almost white, waxy, flaky crystals, white hard masses or white or yellowish-white powder. 2. Solubility: Practically insoluble in water, soluble in ethanol (96 %) and in light petroleum (50-70°C). 3. Freezing point: From 53°C to 59°C. 4. Acid value: From 194 to 212. 5. Iodine value: No more than 4.0. 6. Assay: - Stearic acid ($C_{18}H_{36}O_2$): 40.0% to 60.0% - Sum of Stearic & Palmitic acids (C_{18}+C_{16}): Not less than 90.0% 7. Appearance: The substance, when melted, is not more intensely coloured than reference solution Y_5 or BY_5. 8. Nickel: Not more than 1 ppm.
DL-α-Tocopheryl Acetate (Corresponds to Ph. Eur. Monograph 0439: all-rac-alpha-Tocopheryl acetate)		1. Description: Clear, slightly greenish-yellow, viscous, oily liquid. 2. Solubility: Practically insoluble in water, freely soluble in acetone, in anhydrous ethanol and in fatty oils. 3. Refractive index: From 1.494 to 1.498. 4. Optical rotation: From -0.01° to +0.01° (Angle of rotation). 5. Absorbance: The specific absorbance at the maximum (284 nm) is from 40.0 to 42.0. 6. Acid value: No more than 2.0.

Excipient name	Functional role	Main Typical properties
		7. Free tocopherol: No more than 1.0 %. 8. Sulphated ash: No more than 0.1 %. 9. Related substances: - Impurity A (Free tocopherol): No more than 1.0% - Impurity B: No more than 0.5% - Impurity C: No more than 0.5% - Sum of impurities D and E: No more than 1.0%
Polysorbate 80 (Ph. Eur. Monograph 0428: Polysorbate 80)		1. Description: Oily, yellowish or brownish-yellow, clear or slightly opalescent liquid. 2. Solubility: Dispersible in water, in anhydrous ethanol, in ethyl acetate and in methanol. Practically insoluble in fatty oils and in liquid paraffin. 3. Relative density: About 1.08. 4. Viscosity: About 400 mPa·s at 25°C. 5. Acid value: No more than 2.0. 6. Hydroxyl value: From 65 to 80. 7. Peroxide value: No more than 10.0. 8. Saponification value: From 45 to 55. 9. Water: No more than 3.0%. 10. Composition of fatty acids: - Myristic acid: Max 5.0% - Palmitic acid: Max 16.0% - Palmitoleic acid: Max 8.0% - Stearic acid: Max 6.0% - Oleic acid: Minimum 58.0% - Linoleic acid: Max 18.0% - Linolenic acid: Max 4.0% 11. Impurities: - Ethylene oxide: No more than 1 ppm - Dioxane: No more than 10 ppm
Acacia Senegal Gum (Corresponds to Ph. Eur. Monograph 0307: Acacia)		1. Description: Yellowish-white, yellow or pale amber-coloured, thin, glass-like flakes, tears, or powder. 2. Solubility: Very soluble in water, giving a mucilaginous liquid that is colourless or yellowish, dense, viscous, adhesive, translucent and weakly acidic to blue litmus paper. Practically insoluble in ethanol (96 %). 3. Loss on drying: No more than 15.0%. 4. Total ash: No more than 4.0%

Excipient name	Functional role	Main Typical properties
		5. Acid-insoluble ash: No more than 0.5%. 6. Optical rotation: Aqueous solution is levorotatory (optical rotation is negative) for *Acacia Senegal*. 7. Microbial contamination: - TAMC: Not more than 10^3 CFU/g - TYMC: Not more than 10^2 CFU/g - *Escherichia coli*: Absence in 1 g - Salmonella: Absence in 10 g 8. Impurities: - Glucose and fructose: Absence / Trace - Starch, dextrin and agar: Absence - Tannins: Absence
Astragalus Gummifer Gum (Tragacanth) (Ph. Eur. Monograph 0532: Tragacanth)		1. Description: Flattened, lamellated, frequently curved fragments, or straight or spirally twisted ribbon-like pieces; white or pale yellowish-white; horny fracture. (Powder is white or almost white). 2. Solubility: When treated with water, it forms a mucilaginous gel. 1 g in 50 ml of water forms a homogeneous, thick, opalescent mucilage. Practically insoluble in ethanol (96 %). 3. Total ash: No more than 4.0%. 4. Foreign matter: No more than 1.0%. 5. Flow time: Not less than 10 seconds (Determined on a 10 g/L mucilage using a specific flow cup, indicating high viscosity). 6. Microbial contamination: - TAMC: Not more than 10^4 CFU/g - TYMC: Not more than 10^2 CFU/g - *Escherichia coli*: Absence in 1 g - Salmonella: Absence in 10 g 7. Identification: - Reaction with iodine: Yellowish colour (distinction from starch). - Methyl red reaction: The mucilage is acidic.

One of the most important characteristics of excipients, in addition to consumer properties, is their compatibility with the associated risks (Table 4.2).

Table 4.2

Chemical compatibility of cream excipient components

Substance Name	Functional Category	Incompatibility & Rationale for Formulation Suitability
Stearic Acid	A component of the cream base	Incompatible with metal hydroxides due to the risk of saponification (formation of soaps). May form insoluble precipitates with Zn or Ca salts. The formulation is devoid of strong alkalis and primarily utilises non-ionic emulsifiers. It is incorporated as a viscosity regulator; the negligible risk of saponification ensures physical stability.
Cetearyl Alcohol	A component of the cream base	Generally stable but incompatible with strong oxidising agents. Functioning as a co-emulsifier and consistency agent, its chemical stability is safeguarded by the inclusion of DL-α-Tocopheryl Acetate, which prevents oxidative degradation.
Apricot Kernel Oil PEG-6 Esters	A component of the cream base	Susceptible to hydrolysis in the presence of strong acids or bases. As a non-ionic surfactant mixture, it exhibits good compatibility. Stability is maintained as the cream's pH is controlled within the physiological range (weakly acidic to neutral), preventing hydrolytic degradation.
Petrolatum	A component of the cream base	Inert nature; incompatible only with strong oxidising agents. Selected for its occlusive properties and chemical inertness. It does not react with APIs, ensuring the formulation's structural integrity.
Polysorbate 80	A component of the cream base	May reduce the antimicrobial efficacy of Parabens via micellar entrapment (sequestration of the preservative within surfactant micelles). The formulation remains rational provided the Parabens concentration is optimised to ensure the free concentration in the aqueous phase exceeds the Minimum Inhibitory Concentration (MIC).
Acacia Senegal Gum	A component of the cream base	Natural grades may contain peroxidases, which can catalyse the oxidation of susceptible drugs. Incompatible with heavy metals and high concentrations of ethanol. The inclusion of 0.4% EDTA is a strategic formulation decision. EDTA inhibits potential enzymatic activity and chelates destabilising metal ions. Furthermore, the use of enzyme-free (heat-treated) grades effectively mitigates oxidation risks.
Astragalus Gummifer Gum (Tragacanth)	A component of the cream base	Viscosity decreases significantly outside the pH range of 4–8. The formulation is designed to maintain a physiological pH (4.5–7.0), which aligns with the optimal stability window of this rheological modifier, ensuring consistent texture.
Glycerin	A component of the cream base	Potential for violent reaction with strong oxidisers (e.g., potassium permanganate). Included as a humectant and cosolvent. It enhances the solubility of Parabens in the aqueous phase, contributing to the robustness of the preservative system.
Ethyl Paraben / Methyl Paraben	Antimicrobial preservative	Antimicrobial activity is compromised by non-ionic surfactants (e.g., Polysorbate 80, PEG esters) due to micellar partitioning. Susceptible to adsorption by plastic packaging. The use of a binary paraben system provides synergistic antimicrobial protection. The formulation

Substance Name	Functional Category	Incompatibility & Rationale for Formulation Suitability
		rationality is supported by calculating a sufficient "preservative excess" to compensate for the fraction sequestered by micelles, ensuring compliance with efficacy standards (e.g., Ph.Eur. 5.1.3 or pp.379, 604 in [234]).
EDTA (Disodium Edetate)	A component of the cream base	Incompatible with strong bases and heavy metal ions. A critical stabiliser. Its inclusion at 0.4 % provides a robust chelation capacity to sequester trace metal ions that could otherwise catalyse the oxidation of *Acacia gum* or lipids. EDTA destabilises the cell walls of Gram-negative bacteria, potentiating the efficacy of the parabens.
DL-α-Tocopheryl Acetate	A component of the cream base	Sensitive to oxidation by air and light (auto-oxidation). Incompatible with metal salts. Functions as a sacrificial antioxidant. Its high susceptibility to oxidation is intentional; it is oxidised in preference to the API and lipid excipients, thereby preserving the potency and organoleptic properties of the final product.

4.1.2 Mathematical modelling for optimisation of cream base

To establish a robust cream base that serves as a stable platform prior to API incorporation, this study adopted a Design of Experiments (DoE) approach for formulation optimisation following preliminary single-factor investigations. The overall performance of a cream base comprises multiple, interrelated quality attributes.

In the present work, the principal critical quality attributes (CQAs) of the cream basis were defined to include: (i) appearance and macroscopic uniformity (smooth and homogeneous, free of visible particulates, and without signs of oiling-out, aqueous exudation, or phase separation); (ii) rheological behaviour (an appropriate apparent viscosity with a measurable yield character and thixotropic recovery, enabling easy extrusion/spreadability under shear yet shape retention at rest); (iii) usability (spreadability, extrudability, and acceptable skin feel); and (iv) physical stability (resistance to separation under storage or accelerated stress conditions). To avoid an unmanageably large design space and to ensure interpretability of statistical outcomes, a parsimonious "key-variable" strategy was implemented. Three formulation factors with high controllability and strong mechanistic relevance to the CQAs were selected:

A: ratio of *Acacia Senegal Gum* to *Astragalus Gummifer Gum*;

B: total amount of rheology modifier (combined amount of the two gums);

C: mass ratio of the aqueous phase to the oil phase (water-to-oil ratio).

The three factors and their three corresponding levels are presented in Table 4.3. The L9 orthogonal array used is given in Table 4.4 (L9 orthogonal design matrix).

Table 4.3

Factors and levels

	Factors		
Levels	(A) The Ratio of Acacia Senegal Gum and Astragalus Gummifer Gum	(B) The Mass of the rheology modifier (*Acacia Senegal Gum* and *Astragalus Gummifer Gum*)	(C) The mass ratio of the water phase and the oil phase.
1	5:1	1 %	4:1
2	3:1	5 %	2:1
3	2:1	10 %	1:1

Table 4.4

L9 orthogonal design matrix

	Factors			
No	The Ratio of Acacia Senegal Gum and Astragalus Gummifer Gum	The Mass of the rheology modifier (*Acacia Senegal Gum* and *Astragalus Gummifer Gum*)	The mass ratio of the water phase and the oil phase.	Score
1	5:1	1 %	4:1	
2	5:1	1 %	2:1	
3	5:1	1 %	1:1	
4	3:1	5 %	4:1	
5	3:1	5 %	2:1	
6	3:1	5 %	1:1	
7	2:1	10 %	4:1	
8	2:1	10 %	2:1	
9	2:1	10 %	1:1	

(1) Scientific basis for selecting Factor A (gum ratio) and setting its levels

As a typical multi-phase dispersed system, an O/W cream relies critically on the properties of the aqueous continuous phase, which not only serves as the dispersion medium but also forms a viscoelastic background through polymer hydration and network formation, thereby providing "structural support" for the dispersed oil droplets. Plant-derived polysaccharide gums exhibit distinct hydration kinetics, swelling behaviour, chain entanglement, and weak network formation; consequently, varying the ratio of two gums can alter the network architecture, the thixotropic recovery dynamics, and the shear-dependent flow curve of the continuous phase.

Importantly, for a cream base, "good performance" is not equivalent to simply

maximising viscosity. Rather, it requires a balanced rheological profile that supports low resistance under application shear (extrusion and rubbing) while enabling rapid structural recovery at rest (shape retention and reduced tendency to creaming or separation). Therefore, the gum ratio was treated as an independent factor to expand the tunable formulation space without increasing the overall number of variables, facilitating the concurrent optimisation of spreadability and structural integrity.

For level selection, the ratios 5:1, 3:1, and 2:1 were chosen to span a representative range from *Acacia Senegal Gum*-dominant compositions toward more balanced combinations. This range was selected to ensure (i) a sufficiently large contrast to generate measurable structural responses, and (ii) maintenance of manufacturability by avoiding extreme ratios that may increase the risk of incomplete hydration, lumping/fish-eye formation, and poor processability.

(2) Scientific basis for selecting Factor B (total gum content) and setting its levels

The physical stability of creams (e.g., resistance to creaming, oiling-out, or phase separation) is associated with the rheological background of the cream phase. The total gum content determines the baseline viscosity**,** yield behaviour, and structural resilience of the aqueous network, thereby modulating droplet mobility, collision frequency, and structural rearrangement under gravitational or thermal stress. At low gum content, the network may be insufficient to restrain droplet migration, leading to a thin or flowable base with a higher propensity to separate. In contrast, excessive gum may enhance stability yet compromise extrudability and spreadability, increasing the risk of macroscopic non-uniformity due to incomplete dispersion/hydration (e.g., lumps or fish-eyes). Thus, the total gum content is best conceptualised as a window-type parameter rather than a monotonically beneficial factor.

Accordingly, 1%, 5%, and 10% were selected as low-to-high gradient levels, representing a low-structure exploratory region, an intermediate region expected to better balance usability and stability, and an upper boundary region to probe potential stability gains and associated usability trade-offs.

(3) Scientific basis for selecting Factor C (water-to-oil ratio) and setting its levels

The water-to-oil mass ratio governs the phase volume fraction, droplet crowding,

and the mechanical and thermodynamic stresses placed on interfacial stabilisation and continuous-phase structuring. Increasing the oil fraction increases dispersed-phase volume fraction, shortens droplet–droplet distances, and raises collision frequency, thereby elevating the risk of coalescence and structural rearrangement; under such conditions, stronger interfacial films and more robust continuous-phase yield/viscoelastic support are required to maintain stability. Conversely, a water-dominant system provides a larger continuous phase, which generally facilitates gum hydration and network development but may shift sensory attributes (e.g., reduced occlusivity or a "waterier" skin feel). Critically, changing the water-to-oil ratio also alters the effective continuous-phase volume, thereby influencing the effective gum concentration and hydration environment, which can couple with Factors A and B. To establish a representative and manufacturable formulation space, 4:1, 2:1, and 1:1 were selected as the three levels of water-to-oil ratio (see Table 4.3), reflecting water-dominant, intermediate, and oil-enriched regimes.

(4) Selection of orthogonal design and experimental efficiency

A full factorial design for three factors at three levels would require 27 experiments (3^3). To reduce experimental burden while retaining balanced level representation and comparability of main effects, an L9 (3^3) orthogonal array was employed (Table 4.4), resulting in nine experimental runs that support subsequent range analysis and/or analysis of variance (ANOVA) using a composite score (Score) as the response. To quantitatively compare the overall quality of nine cream-based samples (without the active ingredient), a weighted scoring system was established comprising Description (50%), Stability (30%)**,** pH (10%)**,** and Dynamic Viscosity (25°C, 10 rpm) (10%)**.** The Description domain consisted of five sub-indicators—Appearance, Colour, Transmittance, Texture, and Spreadability (10% each).

In contrast, the Stability domain consisted of Centrifugal and Cold- and Hot-resistant Stability (10% each). All indicators were scored on a 0–10 scale using a four-level rating framework **(**Bad, Satisfactory, Good, Very Good**)** and/or predefined numerical intervals. The overall score (0–100) was calculated as the sum of Description

(0–50), Stability (0–30), pH (0–10), and Dynamic Viscosity (0–10)**.** Tables 4.5 – 4.8 show the results of the comparative assessment for samples No. 1-9 from Table 4.4.

For Description (50%)**,** scores spanned 30–48, representing one of the primary contributors to between-sample differences (Table 4.5). The "Very Good" criteria emphasised a homogeneous, viscous, semi-solid appearance; a uniformly milky-white colour; low transmittance; a smooth, fine texture without grittiness; and good spreadability. Sample 4 scored 9–10 across all five sub-indicators (Appearance 10, Colour 9, Transmittance 9, Texture 10, Spreadability 10), indicating superior macroscopic uniformity, refined texture, and optimal spreadability.

Table 4.5

Scores of Description

Sample No.	Appearance	Colour	Transmittance	Texture	Spreadability
1	5	5	6	6	8
2	6	7	6	7	8
3	7	8	8	7	9
4	10	9	9	10	10
5	10	8	8	9	9
6	9	8	8	8	9
7	8	7	9	7	8
8	8	7	10	6	6
9	8	6	10	6	6

As shown in Table 4.6, for Stability (30%)**,** total scores ranged from 23 to 30**.** Centrifugal stability was generally high, and cold-resistant stability was also consistently strong. Hot-resistant stability provided greater discrimination, indicating that thermal stress more readily revealed weaknesses in structural robustness.

Table 4.6

Scores of Stability

Sample No.	Centrifugal Stability	Cold-resistant Stability	Hot-resistant Stability	Total
1	9	10	8	27
2	9	10	7	26
3	8	8	7	23
4	10	10	10	30
5	10	10	8	28
6	10	9	7	26
7	10	8	7	25
8	10	8	8	26
9	10	8	6	24

Regarding physicochemical indices (Table 4.7), the pH scoring criteria defined pH 5.0–7.0 as "Very Good." The measured pH values for all samples ranged from 5.08±0.20 to 5.97±0.22, placing all within the optimal window (10/10). So, pH served a compliance/acceptability function rather than providing discriminatory power.

Table 4.7

Scores of pH and Dynamic Viscosity (25°C, 10 rpm)

Sample No.	pH	Scores of pH	Dynamic Viscosity (Pa·s)	Scores of Dynamic Viscosity
1	5.71±0.23	10	13.45±3.91	7
2	5.55±0.19	10	15.76±1.87	8
3	5.14±0.27	10	21.34±3.16	10
4	5.62±0.15	10	28.53±4.23	10
5	5.33±0.26	10	33.21±3.79	10
6	5.08±0.20	10	38.95±2.20	10
7	5.48±0.31	10	42.73±4.82	8
8	5.26±0.17	10	48.59±4.30	8
9	5.97±0.22	10	56.77±3.68	5

For dynamic viscosity, the grading standard defined 20–40 Pa·s as "Very Good." The observed dynamic viscosity values ranged from 13.45±3.91 to 56.77±3.68 Pa·s, demonstrating appreciable sensitivity to formulation differences. Samples 3–6 (21.34–38.95 Pa·s) fell within the optimal DV window. The total scores ranged from 74 to 98, demonstrating meaningful discrimination among formulations (Table 4.8).

Table 4.8

Total Scores of the Nine Studied Samples

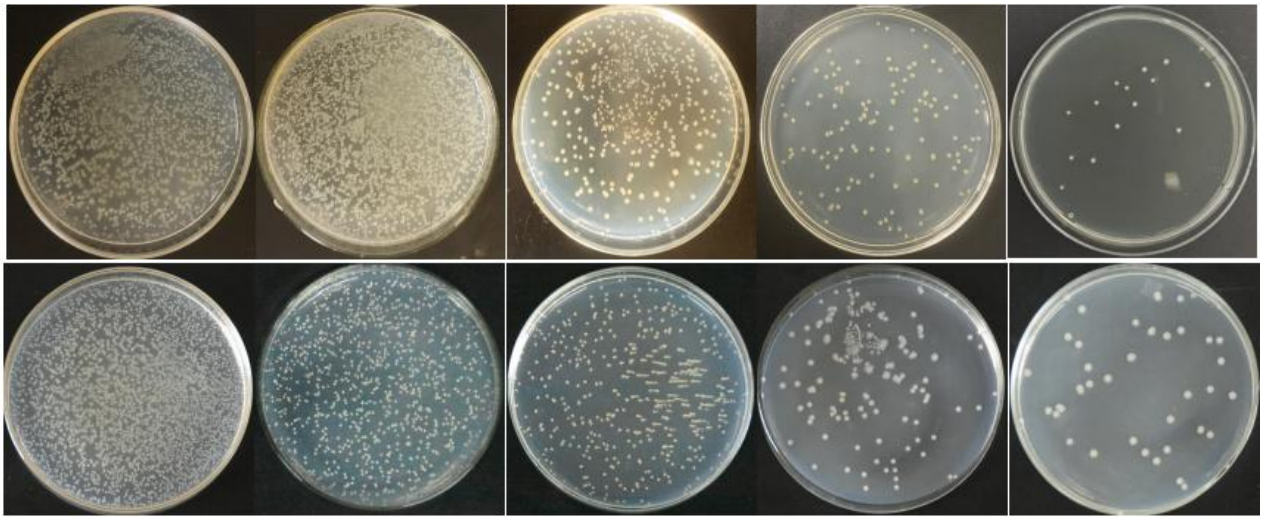
Sample No.	Description	Stability	pH	Dynamic Viscosity	Total
1	30	27	10	7	74
2	34	26	10	8	78
3	39	23	10	10	82
4	48	30	10	10	98
5	44	28	10	10	92
6	42	26	10	10	88
7	39	25	10	8	82
8	37	26	10	8	81
9	36	24	10	5	75

Sample 4 achieved the highest total score **(**98**),** with sub-scores of 48/50 (Description), 30/30 (Stability), 10/10 (pH), and 10/10 (DV)**,** and was considered the best-performing candidate. Samples 5 (92) and 6 (88) formed the second tier.

4.1.3 API content providing maximal antibacterial activity

As mentioned in the previous Chapter, the IC_{50} of the antibacterial was 112 µg/mL, but this data applied only to the TGC-EA Dry Extract, not the cream product. An additional antibacterial experiment was performed on the final cream products in bulk to estimate the antibacterial activity of the developed cream (Fig. 4.1).

Control Cream basis 11.2 mg TGC 50.0 mg TGC 100 mg TGC



Samples	Cream basis	11.2 mg TGC	50.0 mg TGC	100 mg TGC
Antibacterial rate for *S. aureus* (%)	3.28 ± 0.32	48.64 ± 2.67	93.51 ± 3.80	99.72 ± 0.15
Antibacterial rate for *C. acnes* (%)	4.29 ± 0.17	44.52 ± 3.86	96.76 ± 2.95	99.58 ± 0.24

Fig. 4.1 Cultivation of *S. aureus* (upper line) and *C. acnes* (lower line) bacteria on dense nutrient medium in the presence of different concentrations of TGA-EA extract

The *in vitro* antibacterial activity of the developed cream was evaluated against *Cutibacterium acnes* and *Staphylococcus aureus* using the applied microbiological assay in the Agar Dilution Method on a dense nutrient medium in the presence of different concentrations of TGA-EA Dry Extract. When 11.2 mg (IC_{50} value for the extract alone) of TGC-EA was added to 100 g of the final cream products, the antibacterial effect was suboptimal. When 50 mg of TGC-EA was added, the cream reduced the levels of these two bacteria by 90%. At 100 mg of TGC-EA, the cream achieved an inhibition rate of approximately 99% against both tested bacterial strains. So, 100 mg of TGC-EA dry extract was optimal because it had the greatest impact.

The studied TGC-EA dry extracts showed > 80% inhibition at 500µg/mL in solution. To achieve a comparable inhibition, the cream required a higher loading at 0.10% (w/w), corresponding to 1.0 mg of TGC-EA per gram of cream, for two reasons.

First, because TGC-EA was incorporated into the oil phase of an O/W cream, the extract constituents are expected to reside predominantly within the lipid domains and/or interfacial microenvironments. In an undiluted, semi-solid system, mass transfer from these domains into the aqueous phase surrounding bacterial cells is governed by partitioning and diffusion, which are intrinsically slower than in a fully mixed solution. Consequently, the bacterial population assessed by CFU plating is likely exposed to a release-limited dose. In other words, only the fraction that can partition out of the oil phase and diffuse across the cream–aqueous interface within the assay timeframe contributes to immediate antibacterial action. The cream, therefore, behaves as a reservoir system, where a substantial proportion of the nominally present extract remains sequestered and is not instantaneously bioavailable.

Second, the specific structural features of the TGC cream are expected to further constrain the mobility of the extract. The formulation contains a lipid structuring system (e.g., petrolatum and fatty alcohol/fatty acid components) and a gum-based rheological network in the aqueous phase. Such semi-solid matrices increase viscosity and create a mechanically stable microstructure, reducing convective mixing and increasing the effective diffusion path length. Moreover, non-ionic surfactants (e.g., polysorbate 80 and PEG-6 ester-based excipients) can form interfacial microdomains that may non-specifically sequester polyphenolic and flavonoid constituents. These matrix-retention effects reduce the free fraction available to interact with bacterial cells, even when the formulation itself has negligible intrinsic antibacterial activity.

Taken together, the most parsimonious interpretation is that the cream dosage form imposes a partitioning–diffusion–retention limitation, so that the nominal extract concentration in the cream overestimates the concentration immediately accessible to bacteria. This results in an apparent requirement for a higher extract loading to achieve the same CFU reduction observed in a solution system. Importantly, this phenomenon is consistent with well-recognised “dosage-form effects” for semi-solid O/W systems and should be interpreted as a difference in bioavailability at the microbiological interface, rather than as an intrinsic loss of antibacterial potency of TGC-EA.

The observed shift in the required antibacterial concentration from 0.05% (w/w)

(pure TGC-EA extracts) to 0.10% (w/w) (0.1% formulated TGC cream) represents a classical pharmaceutical matrix effect. The lipophilic TGC-EA is incorporated and dispersed within the inner lipid core (petrolatum/stearic acid) of the lipid phase and structurally shielded by the dense polysaccharide interface (Acacia/Tragacanth gums); its release into the aqueous testing environment is thermodynamically and kinetically decelerated. This matrix-associated retention may contribute to a more gradual release of TGC-EA constituents from the semisolid formulation. However, the present *in vitro* experiments were not designed to establish an *in vivo* release profile, follicular delivery, or maintenance of antibacterial concentrations at the application site. Therefore, these findings should be interpreted primarily as evidence of formulation-dependent *in vitro* antibacterial performance.

4.1.4 Optimal cream formulation

Based on the results of the L9 orthogonal experiment, the following formulation of the antibacterial TGC-EA cream is considered promising for further research and technological development (Table 4.9).

4.2 Production process

4.2.1 Technological scheme

Developing a technological process is one of the most important components of pharmaceutical development for high-quality, effective, and safe medicines. The technological process of drug production must ensure the quality of medicines from batch to batch and guarantee the release of products with specified functional characteristics. When developing a cream production technology, it is necessary to assess the system's structural and mechanical properties, as well as its ability to recover during preparation, packaging, and storage. When studying rheological properties, it is necessary to take into account the mutual influence of both the constituent components that determine the consistency of the cream and external factors (temperature, method and duration of processing of the dispersed system, conditions and time of storage, transportation, etc.), which affect the consistency of the finished product.

Given the consistency of the recipe, technology, regime, and shelf life of the cream, it is necessary to obtain an identical, stable picture of the microstructure and

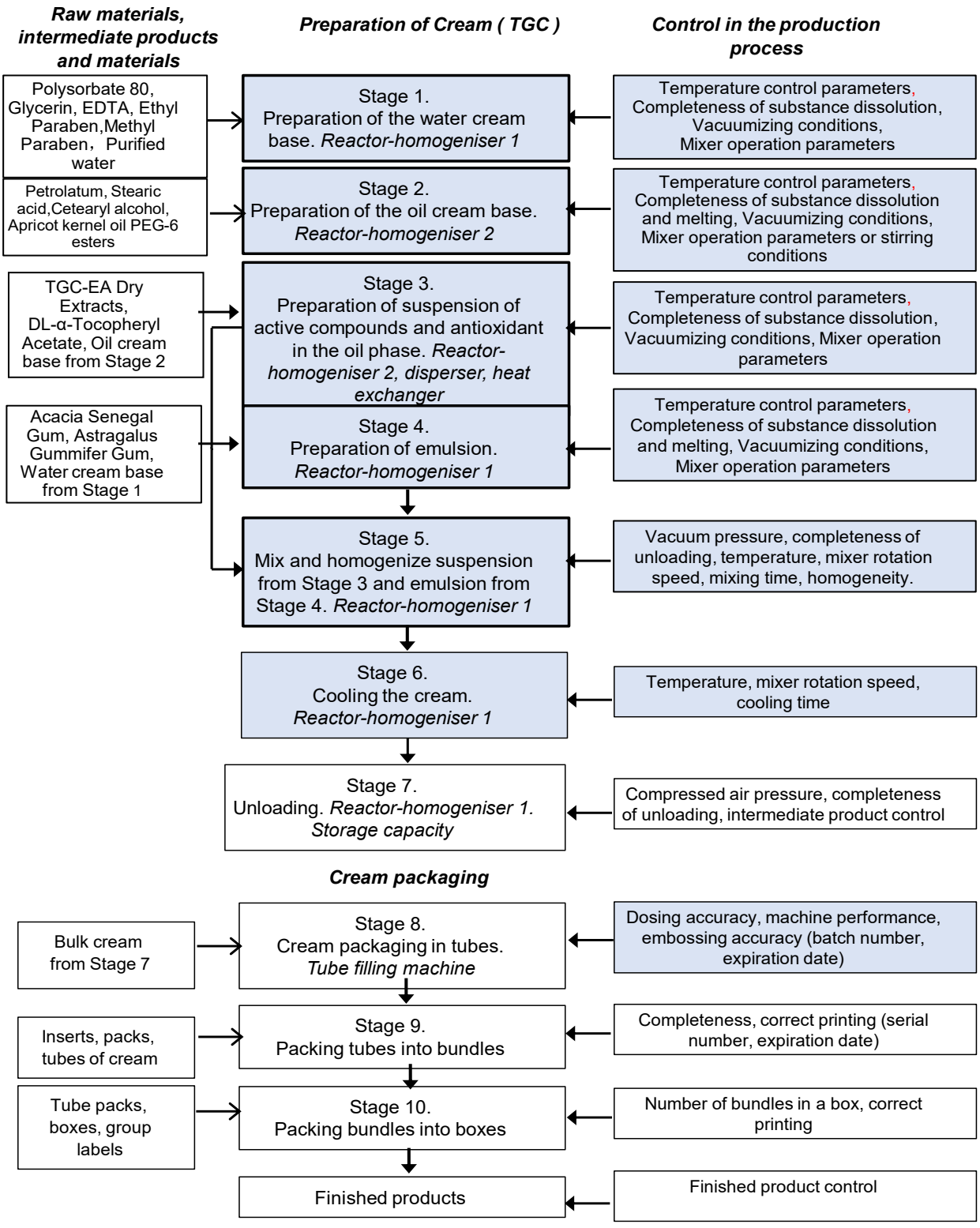
structural-mechanical properties, which can then be used to organise the technological process and determine optimal conditions.

Table 4.9

The optimal formulation of the antibacterial medical cream with TGC-EA dry extract

Names of active and auxiliary substances	Component contents g per 100 g cream		The function of each component of the dosage form	Component content (g) per 30 g tube
TGC-EA Dry Extracts	0.1	Oil Phase	Active Pharmaceutical Ingredient	0.03
Stearic acid	3.0	Oil Phase	Consistency Factor & Structure-forming Agent	0.9
Cetearyl alcohol	3.0	Oil Phase	Co-emulsifier & Viscosity Builder	0.9
Apricot kernel oil PEG-6 esters	5.0	Oil Phase	Hydrophilic Emollient & Co-surfactant	1.5
Petrolatum	8.0	Oil Phase	Occlusive Emollient	2.4
Polysorbate 80	2.0	Water Phase	Primary Emulsifier & Solubiliser	0.6
Acacia Senegal Gum	3.6	Water Phase	Rheology Modifier & Physical Stabiliser	1.08
Astragalus Gummifer Gum	1.2	Water Phase	Rheology Modifier & Thickening Agent	0.36
Glycerin	5.0	Water Phase	Humectant	1.5
Ethyl Paraben	0.1	Water Phase	Antimicrobial preservative	0.03
Methyl Paraben	0.2	Water Phase	Antimicrobial preservative	0.06
EDTA	0.4	Water Phase	Chelating Agent & Preservative Synergist	0.12
DL-α-Tocopheryl Acetate	0.2	Oil Phase	Antioxidant	0.06
Purified water	68.2		Solvent (Continuous Phase)	20.46
Mass of cream	100.0			30.0

Based on previously conducted research, the composition of the cream with antibacterial activity was developed under the working name "TGC". The dry extract of TGC was selected as the API. Other components, such as Stearic acid, Cetearyl alcohol, Apricot kernel oil PEG-6 esters, Petrolatum, Polysorbate 80, *Acacia Senegal* and *Astragalus Gummifer Gum,* Glycerin, Ethyl/Methyl Paraben, EDTA and DL-α-Tocopheryl Acetate were taken as the base due to their physicochemical properties, as well as the solubility of the drug substance. The developed scheme for cream production, with its stages and control points, is shown in Fig. 4.2.



Notes. *1. Critical process parameters (CPP) & stages in the production are shown in grey.*
2. The main equipment used in the stages of the technological process is shown in italics.

Fig. 4.2 Technological scheme for the production of the developed antibacterial medical cream TGC

In the weighing room (Cleanliness Grade D), various raw materials for a single

batch of the pharmaceutical product are precisely weighed utilising industrial and analytical balances. The technological processes for preparing, melting, and mixing the cream components are conducted exclusively in the production room (Cleanliness Grade D). All samples are prepared in glass containers.

Stage 1: Preparation of the aqueous cream base

Loading involves weighing the excipients of the aqueous phase on an electronic scale: polysorbate 80 (2.0 g), glycerin (5.0 g), EDTA (0.4 g), ethyl paraben (0.1 g), methyl paraben (0.2 g), and the appropriate volume of purified water. The weighed components are then transferred to the homogeniser reactor 1. The process parameters regulate the heating temperature (70–75°C) to ensure the dissolution of paraben-based preservatives while minimising water loss, with mixing at 30–50 rpm for 15–20 min.

In-process control (IPC). The temperature is strictly controlled. The transformation of the aqueous phase into a clear, homogeneous liquid is visually confirmed, indicating complete dissolution of all introduced substances.

Stage 2: Preparation of the oil base of the cream

The loading begins with weighing the excipients of the oil phase: petrolatum (8.0 g), stearic acid (3.0 g), cetearyl alcohol (3.0 g) and PEG-6 apricot kernel oil esters (5.0 g). These components are then put into a separate reactor-homogeniser 2. The process parameters are a heating temperature (75–80°C) above the melting point of stearic acid (72°C) to ensure complete liquefaction, and stirring at 30–50 rpm for 20–30 min.

IPC: Confirm complete melting of the system and the absence of solid particles; a homogeneous transparent oily liquid should form.

Stage 3: Preparation of API and antioxidant suspension in the oil phase

During the loading process, the active pharmaceutical ingredient (TGC-EA dry extract, 0.1 g) and antioxidant (DL-α-tocopheryl acetate, 0.2 g) are added to the reactor-homogeniser 2, which contains the oil base prepared in Stage 2. The system temperature must be precisely controlled and maintained at 58–65°C to avoid cooling below 55°C, which could cause recrystallisation/solidification of stearic acid and cetearyl alcohol, while minimising thermal effects on the plant extract. Mixing and dispersion are carried out at a speed of 25–30 rpm with simultaneous activation of the high shear disperser at

2500–4000 rpm for intensive deagglomeration of the dry extract powder for 15–20 min.

IPC: Visually confirm that the dry extract and DL-α-tocopheryl acetate form a uniformly dispersed suspension, completely free of agglomerates.

Stage 4: Preparation of the aqueous hydrocolloid phase

While loading, the emulsion stabilisers and thickeners—*Acacia Senegal gum* (3.6 g) and *Astragalus gum* (1.2 g)—are gradually added to the aqueous cream base (from Stage 1), which is maintained at 60–65°C. The internal circulation homogeniser operates at 1500–2500 rpm (high shear promotes rapid hydration of natural plant hydrocolloids and prevents lump formation). The anchor stirrer with scrapers operates at 30–40 rpm. Homogenisation and mixing in the reactor-homogeniser 1 are carried out for 8–12 min (dispersion), then 30–45 min (hydration at 60–65°C).

IPC: Confirm complete hydration and swelling of the colloidal system; the aqueous phase should appear as a homogeneous, viscous, gel-like mass.

Stage 5 Homogenisation and emulsification: Mixing and homogenising the oil suspension and the aqueous hydrocolloid phase (emulsification)

Before emulsification, both phases are adjusted to 70–75°C. Using a vacuum transfer system, the API-antioxidant oil suspension prepared in reactor-homogeniser 2 at Stage 3 is transferred into reactor-homogeniser 1, which contains the aqueous hydrocolloid phase prepared at Stage 4. The system is maintained at an emulsification temperature of 70–75°C (target temperature 72±3°C). A vacuum of −0.06 to −0.08 MPa is applied to minimise air entrainment during emulsification.

The high-shear homogeniser of reactor-homogeniser 1 is operated at 3000–6000 rpm, while the scraper/anchor mixer operates at 25–30 rpm. Homogenisation is continued for 10–15 min or until a uniform emulsion is obtained.

IPC: Samples are periodically taken through the sampling valve to assess the appearance, gloss, dispersion quality and macroscopic homogeneity of the cream. The system should show no visible phase separation, oiling-out, agglomerates or air bubbles.

Stage 6: Cream Cooling

The cooling parameters are achieved by switching off the reactor-homogeniser 1 and feeding chilled water into the reactor jacket to cool the cream to ~25°C at a

controlled rate of 1–1.5°C/min. The scraper mixer operates continuously throughout the cooling phase, maintaining a stable rotation of 25–30 rpm to prevent local supercooling and subsequent emulsion delamination—controlled cooling time: 40–60 min.

IPC: After reaching equilibrium at 30–35°C, the system stabilises into a structurally stable semi-solid cream.

Stage 7: Unloading

The temperature-controlled bulk cream is unloaded from reactor-homogeniser 1 into a special stainless steel storage tank. As an auxiliary unloading mechanism, oil-free, sterile-filtered compressed air (or nitrogen) at 0.10–0.20 MPa is used to ensure complete emptying. A representative pooled sample is taken for intermediate quality control (including parameters such as appearance, viscosity, pH and API content).

Stage 8: Filling the cream into tubes

After confirming compliance with the quality indicators, the agitator in the tank is turned on for 15 min (15–20 rpm) to reduce thixotropic viscosity. The cream is vacuum-fed (−0.05 MPa) into the hopper of the tube-filling machine under cleanliness class D conditions. Filling is carried out in aluminium tubes with an internal coating. The accuracy of dosing, equipment operation and labelling is monitored.

Stages 9 and 10: Packing the packs into boxes

The tubes with instructions are packed into cardboard boxes. The correctness of the labelling, expiration dates and the number of units in the box are checked. The products are moved to the quarantine zone. After confirming the quality results, the batch may be sold. Standard procedures for cleaning and sanitising the equipment are applied.

The data obtained allowed substantiating the method of manufacturing "TGC" cream, adapting it to industrial conditions, developing a technological and production equipment scheme, and determining the equipment specification.

4.2.2 Critical process parameters and quality attributes

In the pharmaceutical industry (according to GMP and ICH Q8 standards), two different types of control are usually distinguished.

CPPs (Critical Process Parameters) are process variables (e.g., temperature, pH, mixing speed) that must be controlled to ensure product quality. CPPs must be strictly

monitored, as exceeding the established range can lead to the failure of the entire batch. During the production process, CPPs can be influenced by adjusting the equipment.

Critical parameters for the quality of the original medicinal cream are associated with the stages of raw material preparation, preparation of water and oil bases, addition of API and antioxidant to the oil base, preparation of an emulsion, as well as mixing and homogenization of water and oil components and cooling the cream (stages 1-6 of the technological scheme). Finally, the critical parameter concerns the packaging stage in tubes, with attention to accurate packaging and the application of the initial data (stage 8). All CPPs are highlighted by hatching in Fig. 4.2.

According to ICH Q8(R2) recommendations, the term CPP is inextricably linked to product quality indicators, in particular critical quality attributes (CQAs). CQAs are physical, chemical, biological or microbiological properties or characteristics that must be within specified limits to ensure product quality. These concepts are inextricably linked because CPPs influence CQAs. The CQA specification states what is desired (product quality), while CPPs determine how the equipment should be operated.

In the cream manufacturing process, CQAs focus on emulsion/base stability, uniform distribution of the API, and microbiological purity. According to Guideline 42-3.1:2014 for pharmaceutical development, the main CQAs are as follows:
i) Content Uniformity - depends on the time and speed of homogenisation. ii) Particle Size. iii) Rheological properties (viscosity and consistency) - depend on the temperature regime of the prepared cream cooling. iv) pH. v) Microbiological purity

Another type of control in pharmaceuticals concerns the CP (Control Point) indicator. CP is any step or point in the technological process at which an inspection, measurement, or observation is carried out. Not every CP is “critical”. CPs are used for general production control to ensure that the process is going according to plan. Going beyond the limits in a typical CP may require process adjustments, but usually does not pose a direct risk to drug safety, unlike a CPP. Individual steps in a developed technological scheme are associated with either a CPP or a CP, as shown in Table 4.10.

Table 4.10

Description of Critical Process Parameters and Control Points

Process stages	Status	Process parameters
Weighing raw materials (Stage 0)	CP	Mass of each component (weight method; reconciliation within predefined limits); status of balances (calibrated/within due date); environmental/line status (Cleanliness Grade D & line clearance completed); operations performed by trained personnel according to approved SOPs.
Stage 1. Preparation of the water cream base	CPP	Heating temperature: 70–75°C (temperature probe); agitator speed (anchor/wall-scraper): 30–50 rpm; heating & mixing time: 15–20 min (after set-point reached); completeness of dissolution of parabens/EDTA/surfactant (visual clarity; no undissolved particles); water quality: purified water compliant with pharmacopeial requirements.
Stage 2. Preparation of the oil cream base	CPP	Heating temperature: 75–80°C (≥ stearic acid melting point); agitator speed: 30–50 rpm; holding/melting time: 20–30 min; completeness of melting (visual; no solids; homogeneous, clear oily liquid).
Stage 3. Suspension of TGC-EA Dry Extracts and DL-α-Tocopheryl Acetate in the oil phase	CPP	Processing temperature: 58–65°C (avoid <55°C to prevent wax/fat recrystallization; minimize API heat exposure); anchor agitation: 25–30 rpm; high-shear disperser speed: 2500–4000 rpm; dispersion time: 15–20 min; suspension quality (visual): uniform, no agglomerates/grittiness; IPC: uniform distribution of API in suspension.
Stage 4. Preparation of aqueous hydrocolloid phase (*Acacia Senegal Gum* + *Astragalus Gummifer Gum*)	CPP	Charging temperature: 60–65°C; homogeniser speed (internal circulation): 1500–2500 rpm; anchor agitation: 30–40 rpm; dispersion time: 8–12 min; hydration time: 30–45 min at 60–65°C; hydrocolloid quality (visual): no “fish eyes”, uniform viscous gel-like phase.
Stage 5. Emulsification: combine oil	CPP	Phase temperatures before combining: both phases adjusted to 70–75°C (with controlled reheating of the oil phase if required), temperature difference between phases ≤ 5°C; emulsification temperature: 70–75°C (target 72±3°C);

Process stages	Status	Process parameters
suspension and aqueous hydrocolloid phase; homogenise		vacuum: -0.06 to -0.08 MPa (to limit air entrapment/foaming); high-shear homogeniser speed: 3000–6000 rpm; anchor agitation: 25–30 rpm; homogenization time: 10–15 min (or until uniform); IPC: confirmation of emulsion type (o/w), absence of phase inversion, fine and uniform dispersion of the internal phase; absence of phase separation and visible air bubbles.
Stage 6. Cooling the cream	CPP	Cooling mode: homogeniser OFF; jacket cooling water ON; cooling rate: 1.0–1.5°C/min (controlled); anchor agitation during cooling: 25–30 rpm (continuous); cooling end-point: 30–35°C (suitable for discharge/filling); cooling time: ~40–60 min; IPC: no creaming/oiling-out; consistent texture upon reaching end-point temperature.
Stage 7. Unloading / intermediate storage (bulk cream)	CP	Product temperature at discharge: 30–35°C; transfer mode: closed discharge; minimise aeration; auxiliary gas (if used): oil-free, sterile-filtered compressed air or N_2; 0.10–0.20 MPa; intermediate product control: appearance, apparent viscosity/rheology, pH, assay of API (per QC specification).
Stage 8. Filling into tubes	CPP	Pre-filling conditioning: gentle mixing 15–20 rpm × 15 min (to reduce thixotropic viscosity); vacuum transfer set-point: -0.05 MPa; bulk temperature at filling: 30–35°C (target window); tube filler settings: dosing accuracy (net weight), sealing/crimp integrity, embossing accuracy (batch/expiry); IPC: net weight check (net weight control, visual inspection, container tightness/leak test.
Stage 9–10. Secondary packaging (cartoning / case packing)	CP	Completeness of packaging components (tube + insert + carton); correctness of printed information (batch number/expiry; visual); count accuracy per bundle/carton/shipper; final disposition: quarantine release after the compliant finished product testing is complete.
Post-batch cleaning (equipment)	CP	Execution of validated CIP/COP and sanitisation cycle (per SOP); critical checks: cleaning agent concentration/temperature/time as specified; final rinse acceptance (e.g., conductivity/TOC, visual cleanliness); line clearance before the next batch.

Controlled cooling was applied to ensure gradual crystallisation of the lipid phase and formation of a stable emulsion microstructure. The cooling rate (1.0–1.5°C/min) was selected as a critical process parameter influencing the cream's rheological properties and physical stability.

4.2.3 Equipment for the developed production line

This section describes the selected equipment for cream production, based on the technology shown in Fig. 4.2. The equipment was selected for the developed production line, which produces a batch of 300 kg of cream, packed into 30 g tubes. Only the key units were shown for production in Stages 1-8 (Table 4.11).

Table 4.11

Key equipment and tools for the technological line of cream production

Parameter	Value
METTLER TOLEDO MA32001L precision balance	
Maximum capacity	32.2 kg
Readability	0.1 g
Typical minimum weight (U=1%, k=2)	8.2 g
Adjustment	Internal
Interfaces (optional)	Bluetooth; RS232; USB-A
Reactor-homogeniser: ZJR vacuum emulsifying mixer, Type 500 (vacuum emulsification vessel)	
Emulsifying pot design volume, L	500
Rated pot capacity (supplier), L	400
Recommended working fill for 300 kg batch, L	≈300
Scraper/anchor stirring power, kW	3.0
Scraper/anchor stirring speed, rpm	10–120
High-shear (cut-off) mixing power, kW	7.5
High-shear mixing speed, rpm	1000–3300
Heating type	Jacket heating system (steam or electric, depending on utilities)
Material (product-contact parts)	Stainless steel; SUS316L recommended for GMP contact parts
Primary function in the line	Heating, vacuum deaeration, emulsification, homogenisation and cooling prior to filling
Reactors (pre-mix vessels): Matched water pot and oil pot for the Type 500 system	
Water pot design volume, L	300
Water pot capacity (supplier), L	250
Water pot stirring power, kW	1.1
Water pot stirring speed, rpm	60–1000

Parameter	Value
Oil pot design volume, L	250
Oil pot capacity (supplier), L	200
Oil pot stirring power, kW	1.1
Oil pot stirring speed, rpm	60–1000
Primary function in the line	Separate preparation of aqueous and oil phases; gum hydration/dispersing (water pot) and melting/dispersion (oil pot) before emulsification
Disperser: Quadro/YTRON ZC-3 inline powder disperser (powder inductor)	
Liquid capacity	39–79 gpm (≈9–18 m³/h)
Powder capacity	165 lb/min (≈75 kg/min)
Motor power (example configuration)	20 hp
Operating mode	Single-pass or recirculation
Primary function in the line	Fast wetting and dispersion of difficult-to-wet powders (gums) and optional pre-dispersion of powder in the oil
Heat exchanger: Contherm® scraped-surface heat exchanger (SSHE)	
Working temperature range	−35 °C to +150 °C
Max. working pressure	Product side 20 bar (300 psig); media side 17 bar (250 psig)
Product connections	2" or 3" sanitary (union)
Primary function in the line	Efficient heating/cooling of viscous cream with reduced fouling
Storage capacity (buffer/holding tank): 500 L jacketed stainless-steel mixing tank (filling buffer)	
Tank capacity, L	500
Motor power, kW	1.5
Mixing speed options, rpm	25 / 36 / 80 / 200 (adjustable; low-speed recommended for holding)
Jacket function	Heating and/or cooling for temperature maintenance
Material	Stainless steel; 316L recommended for product-contact parts
Primary function in the line	Short-term holding and gentle agitation to maintain homogeneity and temperature prior to tube filling
Tube filling machine MAKWELL MWAF-2-80 aluminium tube filling and sealing machine	
Filling range	5–180 mL
Suitable tube diameter	13–35 mm
Filling speed	50–75 tubes/min
Filling accuracy	±1%
Working pressure	0.5–0.6 MPa
Compressed air consumption	1.5 m³/min
Power	1.5 kW
Machine dimensions (L×W×H), mm	2200 × 1200 × 1900
Machine weight, kg	1200
Target tube specification	30 g per tube (≈10,000 tubes per 300 kg batch)
Estimated filling time per batch	≈2.2–3.3 h (based on 75–50 tubes/min)

4.3 Quality and stability control of bulk medicinal product

4.3.1 Chemical compatibility of cream components by FTIR

To control product quality, FTIR spectroscopy was used at each stage of production to control the mixing process, as it is extremely sensitive to interactions between components. The peak intensities are directly proportional to the concentrations of the corresponding functional groups.

FTIR reacts when mixing different substances to:

1. Formation of hydrogen bonds, i.e. if components A and B begin to interact when mixed, this will instantly be reflected in the spectrum in the form of peak shifts, absorption bands of groups to the lower frequency region, and peak broadening (peaks become less clear, indicating a change in the electron density of bonds).

2. Chemical incompatibility (reactions). FTIR is the main tool for checking the stability of a mixture. If the components react, this can lead to the disappearance of peaks (bands) and the appearance of new peaks (bands of new functional groups).

3. Change in crystal form (polymorphism): with intensive mixing or grinding, the energy of mechanical action can lead to either amorphisation (clear, sharp peaks become broad "hills") or to polymorphic transitions (change in the position of peaks in the "fingerprint region", even if the chemical formula remains unchanged.

4. Sensitivity to homogeneity. FTIR has a very shallow penetration depth into the sample (usually 0.5-5 μ) and only sees the surface of the particles. If the mixture is heterogeneous, the results will vary greatly from point to point. Thus, the spectrum of the mixture must be a perfect additive mathematical sum of the component spectra.

The cream is made by combining the aqueous and oil phases with purified water, then mixing them to form the final product. In the prepared aqueous phase, an antimicrobial agent, an emollient, an emulsifier, a humectant and a chelating agent are dissolved in certain proportions. In the oil phase, the API, a viscosity and consistency regulator, an antioxidant, and an excipient (base) are placed; the base serves as a "carrier" for the API, ensuring its stability and delivery to tissues (Fig. 4.3).

The aqueous phase is characterised by an intense broad band in the region of 3200–3500 cm^{-1}, which corresponds to the stretching vibrations (-OH) of water groups

(Fig. 4.3a). A peak around 1640 cm^{-1} (deformation vibrations (H-O-H)) is also noticeable. The oil phase has clear, sharp peaks in the region of 2850–2950 cm^{-1}, which correspond to stretching vibrations (C-H) of the bonds of aliphatic chains of fatty acids or paraffins (Fig. 4.3b). The peak at about 1740 cm^{-1} indicates the presence of ester groups (C=O) of triglycerides or oils.

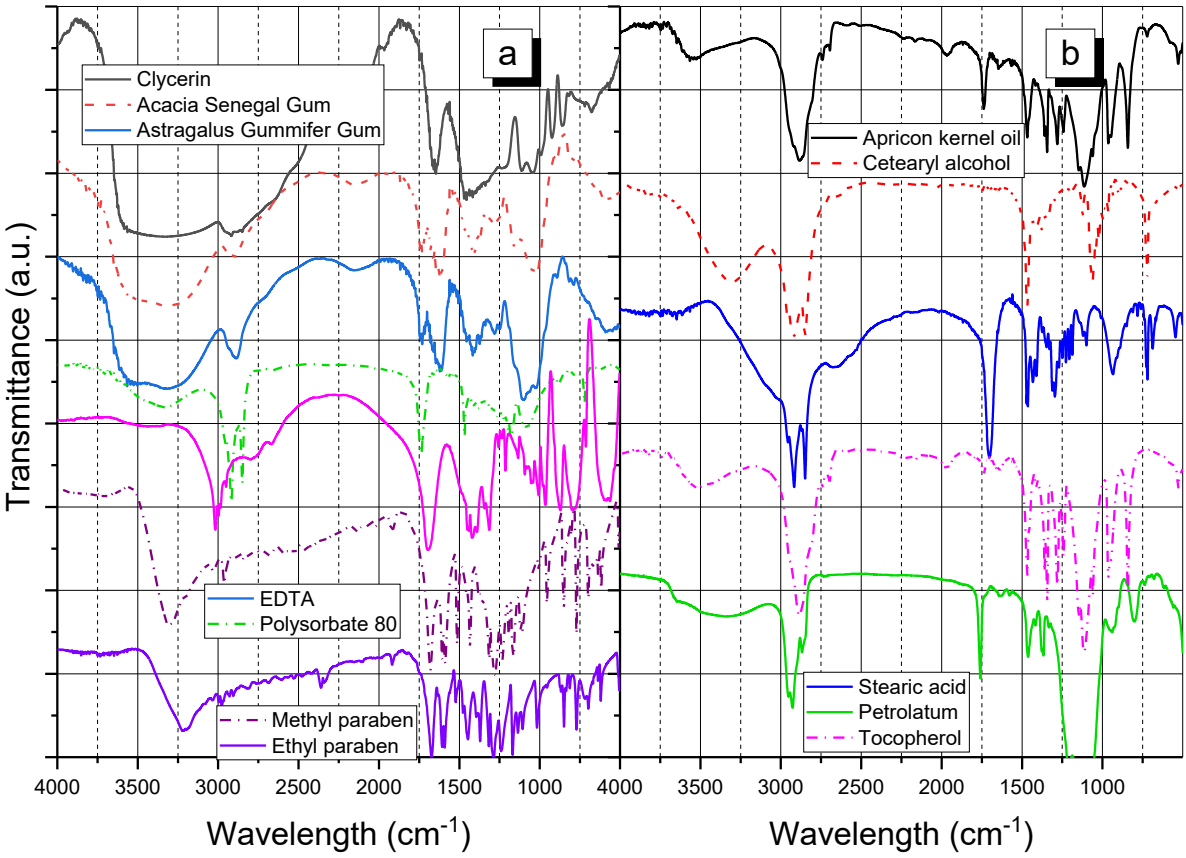


Fig. 4.3 FTIR spectra of individual components belonging to: a - the aqueous phase of the cream, b - the oil phase of the cream

Comparison of the FTIR spectra of the cream components (oil and aqueous phases) with those of the finished cream base allows determination of the chemical composition, interactions among components, and the emulsion's stability (Fig. 4.4a).

The formation of the cream base indicates a combination of the above-mentioned features, i.e. it is a superposition (overlay) of the spectra of both phases. The spectrum simultaneously contains a broad band (-OH) from the water phase and characteristic "teeth" (C-H) from the oil phase. In addition, there are no visible chemical changes, i.e. no new peaks appeared in the spectrum that were not present in the individual

phases. Therefore, no chemical reactions occurred between the components during mixing; they coexist physically as an emulsion. The shift or broadening of the (-OH) peak in the cream base compared to the pure aqueous phase indicates the formation of new hydrogen bonds, which is important for stabilising the emulsion with emulsifiers. Thus, the comparison confirms the successful inclusion of both phases in a stable cream system without signs of chemical degradation of the components.

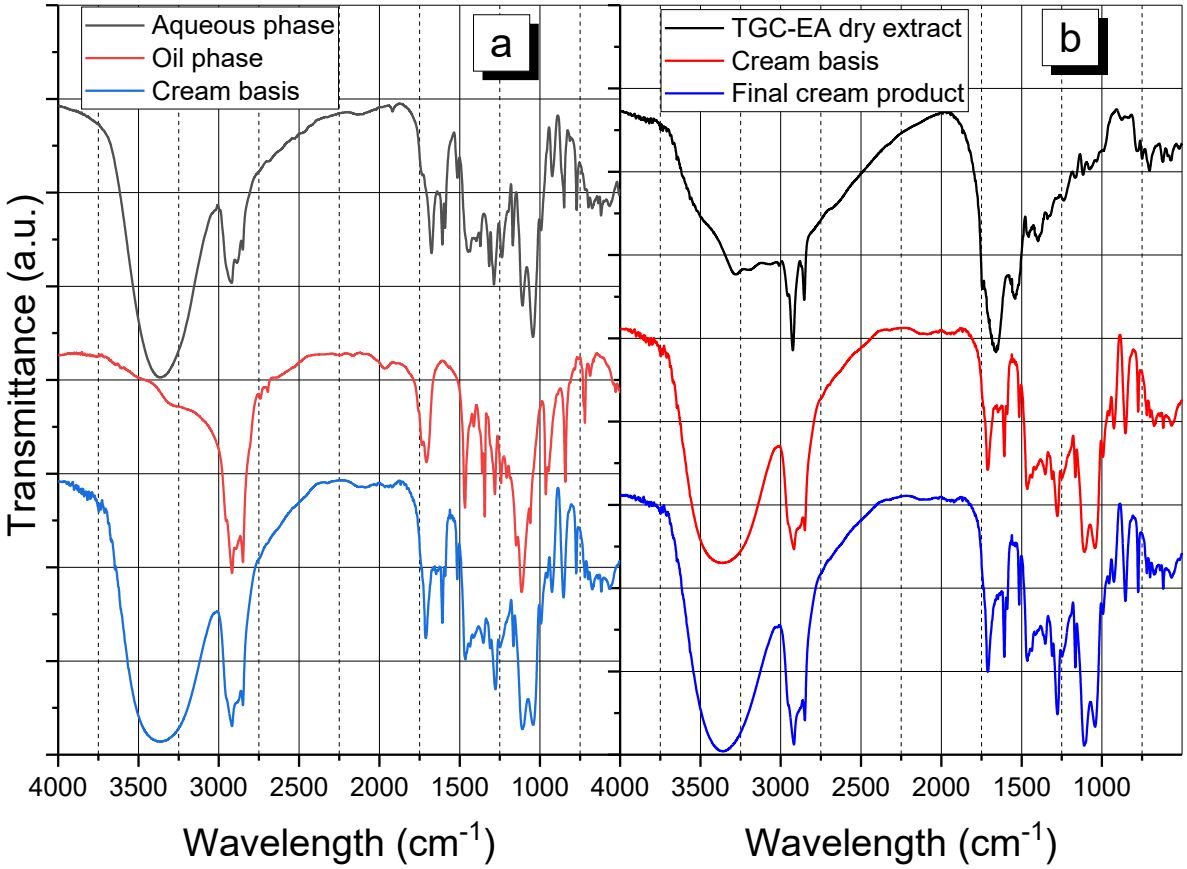


Fig. 4.4 FTIR spectra: a – total spectrum of the aqueous, oily phases and the cream base as a whole, b – of the herb extract, cream base and the final product

The spectrum of the TGC-EA extract shows the most pronounced functional groups of biologically active substances and allows tracing their transfer to the final product (Fig. 4.4b).

1. Polyphenols and flavonoids. It is the most pronounced fraction in the extract. The hydroxyl region (~3200–3400 cm^{-1}) is characteristic of phenolic (-OH) groups. In the extract, this band is less pronounced than in the base, but in the final product, it can broaden due to hydrogen bonding between the extract and the base. Aromatic rings

(~1580–1615 cm^{-1} and 1450–1510 cm^{-1}) are the key zone for flavonoids. The peaks here correspond to bond stretching (C=C) in aromatic structures. Carbonyl groups (~1640–1690 cm^{-1}) indicate the presence of ketone or ester groups, characteristic of the structure of many flavonoids. In the region of 1000–1200 cm^{-1}, there are intense peaks (C-O stretching (C-O)), indicating a high content of phenolic compounds and glycosides. In the final product, these peaks are largely preserved and even overlap with the base, confirming the cream's high polyphenol concentration.

2. The presence of terpenoids is confirmed by specific peaks reflecting the features of the hydrocarbon structure. Due to the branched hydrocarbon skeleton, terpenoids cause intense absorption in the region of stretching vibrations of C-H bonds (methyl and methylene groups) at 2850-2960 cm^{-1}. It manifests as clear peaks that often overlap or enhance the signals of other components of the base. The presence of unsaturated double bonds characterises many terpenoids. Their signals in the region (1650-1670 cm^{-1}) can overlap with the aromatic ring absorption bands of flavonoids, requiring careful differential spectral analysis. Oxygen-containing functional groups form additional intense peaks in the spectrum. Suppose alcohols, aldehydes or ketones represent terpenoids. These peaks are formed by hydroxyl groups (-CHN), a broad band in the area of 3200-3500 cm^{-1}. Carbonyl groups (C=C) give a narrow, intense peak in the range of 1680-1750 cm^{-1}. Since the cream base itself is rich in oils, these peaks in the final product are summed up, making the "hydrocarbon trace" very intense.

3. The alkaloid group is present, but visually less pronounced. Alkaloids contain nitrogen, which gives specific signals. Amine groups (N-H) can appear in the region of 3300–3500 cm^{-1}, often overlapping with a broad band (-OH). Therefore, a small bend or shoulder in the region of 3300–3400 cm^{-1} in the spectrum of the extract can indicate an (N-H) bond of the alkaloids. (C-N) bonds (~1000–1350 cm^{-1}) can introduce complex peaks in the "fingerprint" region, which helps distinguish alkaloids from pure polyphenols. However, due to the large water peak (-OH) in the same region in the cream base, it is challenging to isolate alkaloids in the final product by FTIR alone.

In summary, the most visible markers are polyphenols/flavonoids: 1600–1640 cm^{-1} (rings) and 1000–1200 cm^{-1} (C-O). Terpenoids demonstrate medium/high peaks

at 2850–2930 cm^{-1} (C-H) and 1450 cm^{-1}. Alkaloids are characterised by moderate signals at 1630–1650 cm^{-1} (overlapping) and 3300–3400 cm^{-1}.

Thus, according to the FTIR results, the extract most strongly enriches the cream with flavonoids and polyphenols, as their specific "fingerprints" in the 1000-1650 cm^{-1} region most noticeably alter the spectrum of the pure cream base.

In the region of 3200–3500 cm^{-1}, the extract has a broad band corresponding to hydroxyl (-OH) or amine (-NH_2) groups, which are often found in phenolic compounds or polysaccharides of plant origin. The presence of peaks in this region of 1600–1750 cm^{-1} indicates carbonyl groups (C=O), which may belong to flavonoids or other active components of the extract. In the "fingerprint" region (below 1500 cm^{-1}), the extract adds several small peaks that are unique to its molecular structure.

When comparing the spectra of the final product with the combination of the spectra of the base and the extract, the preservation of the structure is clearly seen (Fig. 4b). In the final product, the cream base "absorbs" most of the signals, but the extract adds a fine structure (additional "shoulders" and small peaks) in the range of 1000–1700 cm^{-1}. Therefore, the spectrum of the final product almost completely reproduces the profile of the cream base, while incorporating features of the extract.

Such an observation confirms that all active substances remained stable and did not enter into an aggressive reaction with the cream base. The extract was successfully integrated into the base. No new absorption bands, absent in both the base and the extract, appeared in the spectrum of the final product, confirming the chemical compatibility of the components. No undesirable chemical reactions occurred between the cream base and biologically active substances. The extract enriches the cream with active functional groups without compromising its stability or chemical integrity.

4.3.2 Dissolution completeness and component homogeneity at production stages

Guaranteeing the completeness of solubility and the homogeneity from bulk to a tube throughout the manufacturing stages is essential for the physical stability, microbiological resilience, and content uniformity of TGC Cream. In the case of this semi - solid formulation, the concept of “completeness” is intrinsically stage-specific and should encompasses: (i) the thorough dissolution/solubilisation of low - dose

hydrophilic excipients within the aqueous phase; (ii) the complete melting of waxy/fatty structuring agents in the oil phase; (iii) the full deagglomeration and uniform dispersion of the botanical dry extract (TGC-EA Dry Extract) within the oil phase (existing in a suspension state rather than a true solution); (iv) the complete wetting and hydration of natural hydrocolloids to attain reproducible rheological properties; and (v) the complete emulsification and homogenisation after phase combination, followed by controlled cooling to solidify a uniform microstructure.

At Stage 0, process homogeneity is conditioned by upstream material handling. Given the presence of low-dose constituents (e.g., preservatives, chelating agents, and TGC-EA Dry Extract), small weighing or charging deviations can disproportionately affect local concentrations and, in turn, compromise unit-dose uniformity. Accordingly, reconciliation of weighed quantities and verification of charging sequence constitute prerequisite controls before thermal processing begins.

At Stage 1, polysorbate 80, glycerin, EDTA, and parabens are incorporated into purified water and processed at 70–75°C under anchor agitation (30–50 rpm) for 15–20 min to facilitate dissolution and/or solubilisation of low-dose auxiliaries. The selected thermal window supports dissolution performance while limiting avoidable evaporation-driven concentration drift. Operationally, completeness is evidenced by a clear and uniformly transparent aqueous phase, without visible undissolved particles or sediment. Any persistent turbidity or particulates indicate incomplete dissolution/solubilisation and may lead to non-uniform preservative distribution and downstream sensory defects.

At Stage 2, petrolatum, stearic acid, cetearyl alcohol, and apricot kernel oil PEG-6 esters are heated to 75–80°C with anchor agitation (30–50 rpm) and held for 20–30 min to ensure complete liquefaction of the highest-melting structuring components. Here, “completeness” denotes the absence of residual solid domains, as incomplete melting may act as crystallisation centres during cooling, broadening the microstructural distribution and increasing the risk of heterogeneity in the final cream.

At Stage 3, TGC-EA Dry Extract (0.1 g) and DL-α-Tocopheryl Acetate (0.2 g) are introduced into the oil phase prepared at Stage 2. Since TGC-EA is incorporated as

a solid botanical extract, the principal process-control objective is dispersion completeness rather than molecular dissolution. The system temperature is maintained at 58–65°C to preserve sufficient fluidity of the oil phase while minimising thermal stress on the extract. Dispersion is performed using anchor agitation at 25–30 rpm combined with high-shear processing at 2500–4000 rpm for 15–20 min. The endpoint of this stage is the formation of a uniformly dispersed API-antioxidant suspension without visible agglomerates or perceptible graininess. Incomplete dispersion at this stage may result in local non-uniformity of the API and persistent particulate defects that are difficult to correct after emulsification.

At Stage 4, *Acacia Senegal Gum* and *Astragalus Gummifer Gum* require controlled wetting and hydration to deliver reproducible viscosity and network formation. The hydrocolloids are gradually introduced into a warmed aqueous base at 60–65°C, under combined anchor agitation (30–40 rpm) and homogeniser-assisted circulation (1500–2500 rpm). The operation is intentionally partitioned into: (i) high-shear dispersion for 8–12 min to minimise “fish-eye” formation (incompletely wetted cores protected by an externally hydrated layer), followed by (ii) hydration for 30–45 min at 60–65°C to complete swelling and viscosity development. Hydration completeness results in a uniform, lump-free hydrocolloid phase with no visible gel clots or dry inclusions; insufficient hydration manifests as time-dependent viscosity drift and an elevated risk of phase instability after emulsification.

At Stage 5, prior to mixing, both phases are aligned at 70–75°C to reduce viscosity mismatch and mitigate premature crystallisation during emulsification. The oil suspension is incorporated into the hydrocolloid-containing aqueous phase under vacuum (−0.06 to −0.08 MPa) to limit air entrapment. High-shear homogenisation runs at 3000–6000 rpm for 10–15 min, while maintaining wall-scraping agitation (25–30 rpm) to prevent localised overheating/cooling and promote macroscopic uniformity. At this stage, homogeneity completeness is defined by: (i) absence of visible oiling-out or phase streaking, (ii) a uniformly glossy and consistent appearance indicative of adequate droplet-size reduction, and (iii) no visible particulate agglomerates. Insufficient homogenization increases droplet size polydispersity, increasing the

likelihood of agglomeration/coalescence during subsequent cooling and storage.

At Stage 6, following emulsification, the homogeniser is stopped, and the batch is cooled using jacket cooling at ~1.0–1.5°C/min, while maintaining continuous wall-scraping agitation (25–30 rpm) to suppress temperature gradients and ensure uniform crystallisation of the fatty structuring agents. Controlled cooling is essential for consolidating a coherent microstructure and minimising heterogeneity arising from localised overcooling. Homogeneity during cooling is evidenced by the absence of creaming/oiling-out and by uniform texture development. Cooling is continued to 30–35°C (typically ~40–60 min), a practical endpoint that balances rheological stability and fillability. Excessive cooling rates or inadequate agitation are treated as contributors to non-uniform crystal networks and potential graininess.

At Stage 7, the bulk cream is discharged at 30–35°C under closed transfer conditions to minimise aeration. If propellant assistance is applied, oil-free, sterile-filtered compressed air or nitrogen is used to avoid contamination and oxidative stress. During any interim holding, homogeneity is preserved by preventing temperature stratification and avoiding prolonged quiescent residence that may permit density-driven separation. Representative sampling of the bulk (appearance, viscosity, pH, and assays, if necessary) provides evidence that the batch remains uniform prior to filling.

At Stage 8, mixing (15–20 rpm for 15 min) is performed immediately before filling to restore uniformity in thixotropic systems without introducing air. A vacuum transfer (~−0.05 MPa) is used to further mitigate air entrainment. Maintaining the bulk at 30–35°C supports consistent flow behaviour and accurate dosing. In-process monitoring (e.g., net weight checks and continuous visual inspection for texture uniformity) provides practical assurance that bulk homogeneity is appropriately translated into unit-dose consistency. There are no black powders (TGC-EA extracts) in the cream background (Fig. 4.5).

In conclusion, the above definitions and controls establish a coherent, stage-appropriate framework for demonstrating “completeness” of dissolution, dispersion, hydration, emulsification, and final homogeneity for TGC Cream. Importantly, each completeness criterion is anchored to an observable manufacturing endpoint (e.g.,

clarity, absence of solids/agglomerates, lump-free hydration, no oiling-out, consistent texture), thereby enabling operational verification without over-claiming molecular solubility where the formulation science supports a dispersed/suspended state.

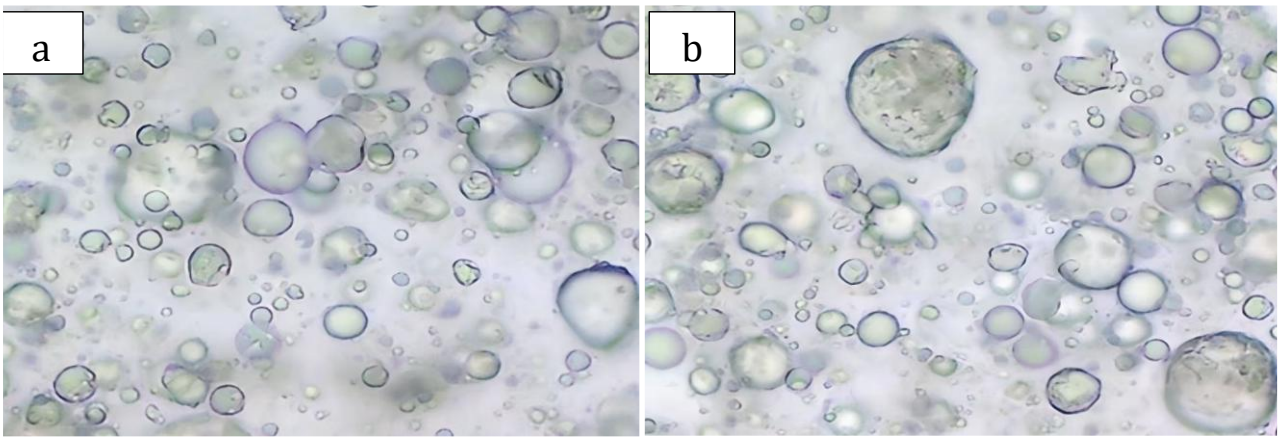


Fig. 4.5 Micrographs confirming homogeneity of bulk cream at different production stages: before (a) and after (b) the TGC-EA extracts were added

4.3.3 Technological stability at intermediate stages

In pharmaceutical production, technological stability indicators are checked at the intermediate product stage (bulk cream) and the final product stage (cream in a tube) [221, 222]. At the intermediate stage (usually stage 7 of the technological scheme), indicators are checked to confirm the correctness of the technological preparation process and its completion before packaging. At the final stage, the main focus is on checking indicators that may have arisen or changed during packaging and storage, and on assessing the cream's long-term stability throughout its life.

At the intermediate stage, the following were investigated:

1. Colloid Stability tests the system's resistance to stratification under the influence of gravitational forces.

2. Heat Stability assesses the ability of the cream base to maintain its structure at elevated temperatures, which is important for the production and packaging processes.

3. Cold Stability determines the cream's resistance to water crystallisation or fat phase solidification, which can destroy the emulsion.

Centrifugation, thermostating, and freezing were performed using appropriate equipment to study these parameters. Stability is considered achieved if the indicators

studied remain unchanged when comparing samples before and after testing.

The FTIR method was the primary tool for detecting changes in the cream during or after testing. Bulk cream at stage 7 of the technological scheme, after cream structure formation and cooling, was selected as the initial state.

In Colloid Stability tests, FTIR determines the stability of the emulsion to separation into fat and water phases; i.e., the state of emulsifiers and their interactions at the O/W interface is analysed by monitoring changes in the intensities of the (–OH) and carbonyl (–C=O) peaks. If colloidal stability is disturbed, the energy of hydrogen bonds changes, and FTIR shows shifts in peak positions.

In Heat Stability tests, when the bulk cream is heated in a reactor or when kept in a thermostat, destruction or phase transition may occur. FTIR spectra are recorded at different temperatures. If at +40°C the peaks blur or new ones appear, this indicates the destruction of the base structure (e.g. melting of the paraffin lattice).

In Cold Stability tests, low temperatures can cause the API to precipitate (crystallise) or freeze water in the emulsion, thereby forming spectral fingerprints of crystalline phases. After the freeze/thaw cycle, the FTIR spectrum is compared with the original one. If sharp peaks appear in the "fingerprint" zone, it means the API or base components have entered an irreversible crystalline state, a sign of instability.

The FTIR results of the Colloid-, Cold-, and Heat-stability studies of the buckwheat cream are shown in Fig. 4.6 and Fig. 4.7, respectively. In all three tests, no changes were observed in the FTIR spectra, proving a high level of cream stability.

The high stability of the developed creams is confirmed by the data examining the characteristics of 3 samples (#4.3.4) with different TGC-EA contents (Table 4.12).

Table 4.12

Stability control of bulk creams（Samples 1-3）

Quality Control	Sample 1	Sample 2	Sample 3
Centrifugal Stability	No changes and homogeneous cream	No changes and homogeneous cream	No changes and homogeneous cream
Cold-resistant Stability	No changes and homogeneous cream	No changes and homogeneous cream	No changes and homogeneous cream
Hot-resistant Stability	No changes and homogeneous cream	No changes and homogeneous cream	No changes and homogeneous cream

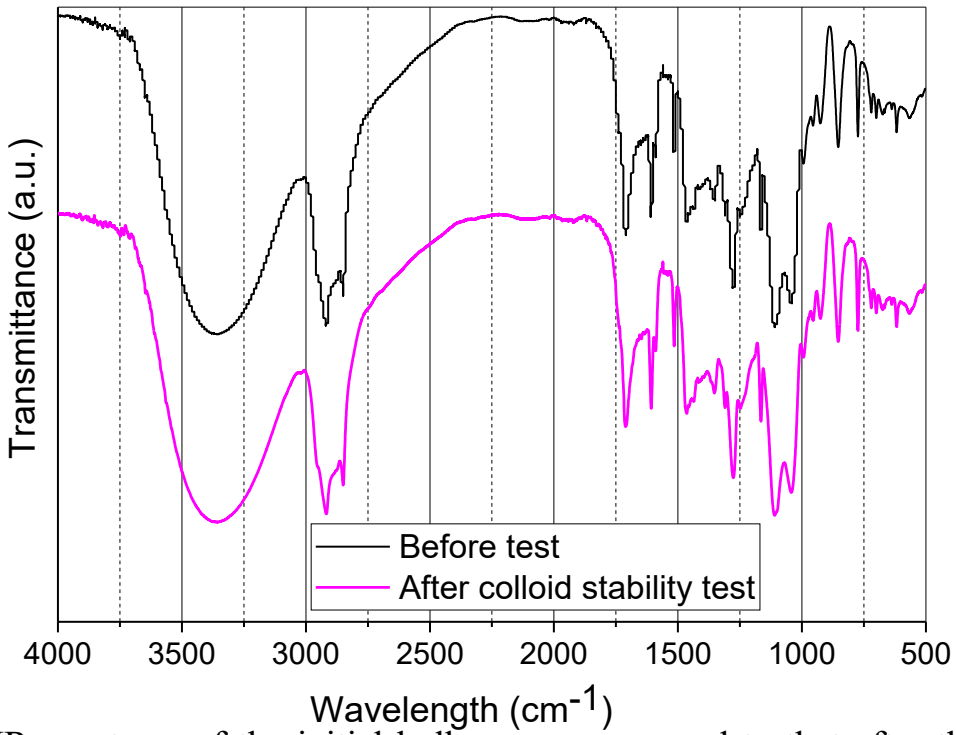


Fig. 4.6 FTIR spectrum of the initial bulk cream compared to that after the colloid stability test

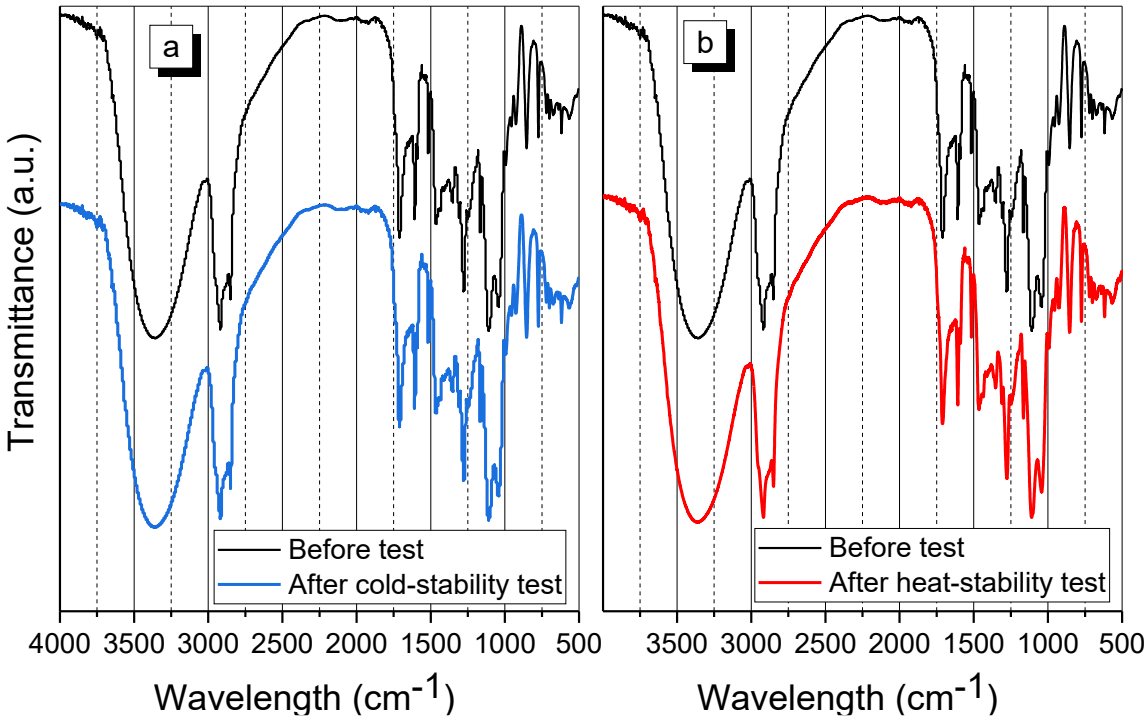


Fig. 4.7 FTIR spectrum of the initial bulk cream compared to those after cold- (a) and heat-stability (b) tests

In addition to stability testing, several characteristics of the bulk cream are investigated at the intermediate stage. These include:

• Homogeneity: Visual absence of particles, agglomerates or delamination.

• Emulsion type and particle size to confirm microstructure stability and particle distribution.

• pH: Compliance with the specified limits for the stability of the API.

•Viscosity, which affects the subsequent ability of the mass to be packaged.

• Active ingredient content (Assay): Quantitative determination of the concentration of antibacterial components in the total volume of the reactor.

• Antibacterial activity.

The content of heavy metals, which is often included in the characteristics of mandatory control, is not controlled if, as in this case, the control of the incoming raw materials (API and excipients) was carried out, and if the equipment (stainless steel reactors) is certified and inert and is not a source of metal contamination. The results of these studies are presented in the following sections.

4.3.4 Organoleptic properties

To study the organoleptic properties, three cream samples were selected that differed in API content. Sample 1 is the cream base, with added 11.2 mg active compounds (TGC-EA Dry Extracts); Sample 2 is the cream base with added 50 mg active compounds (TGC-EA Dry Extracts); Sample 3 is the cream base with added 100 mg active compounds (TGC-EA Dry Extracts). For all formulations (Table 4.13), various raw materials for a single batch of the pharmaceutical product are precisely weighed utilising industrial and analytical balances.

Table 4.13

Organoleptic properties of the finished cream in bulk

Quality Control	Sample 1	Sample 2	Sample 3
Appearance	Homogeneous viscous solid upon cooling	Homogeneous viscous solid upon cooling	Homogeneous viscous solid upon cooling
Colour	Homogeneous milky white	Homogeneous milky white	Homogeneous milky white
Transmittance	No light transmission	No light transmission	No light transmission
Texture	Good roundness and very good texture	Good roundness and very good texture	Good roundness and very good texture
Spreadability	Easy to apply, and there is no graininess	Easy to apply, and there is no graininess	Easy to apply, and there is no graininess

The technological processes for preparing, melting, and mixing the cream components are conducted exclusively in the production room (Cleanliness Grade D). All samples are prepared in glass containers. The cream bulk is a homogeneous, viscous solid, indicating that the system has retained a coherent semi-solid structure after cooling without macroscopic phase separation or localised thinning, and therefore meets all requirements.

4.3.5 pH and Dynamic viscosity

Physicochemical properties of Samples 1-3 are given in Table 4.14. Figure 4.8 helps assess whether a significant difference in pH and DV exists between indicators across Samples 1-3, given the achieved standard errors of the averages. As shown, no significant difference was observed for either pH or DV.

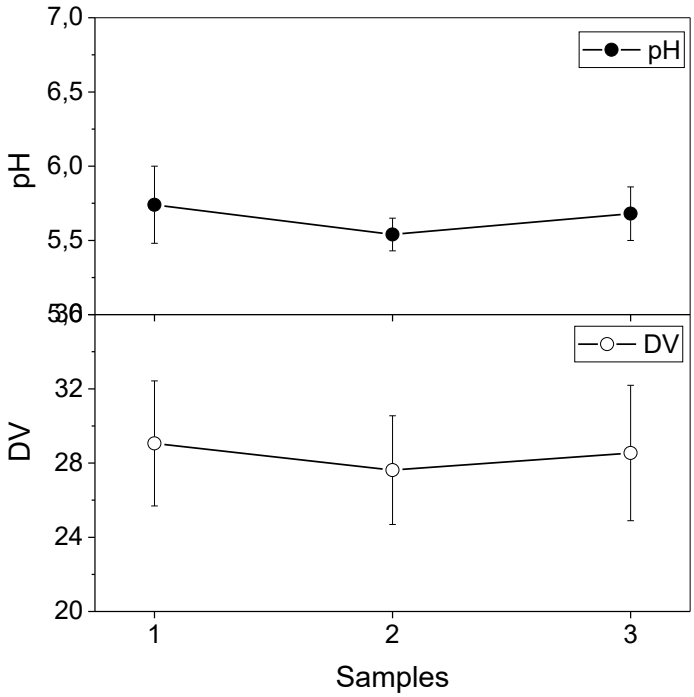


Fig. 4.8 Average pH and DV values in Samples 1-3 sampled from bulk creams with different TGC-EA contents

Table 4.14

pH and Dynamic Viscosity of the finished cream in bulk

Quality Control	Sample 1	Sample 2	Sample 3
pH	5.74±0.26	5.54±0.11	5.68±0.18
Dynamic Viscosity (Pa·s)	29.16±3.37	27.62±2.93	28.54±3.65

4.3.6 Microbiological purity and irritation potential

For both tests, three cream samples with different TGC-EA Dry Extracts content were studied. Sample 1 was the cream base with added 11.2 mg active compounds (TGC-EA Dry Extracts); Sample 2 was the cream base with added 50 mg active compounds (TGC-EA Dry Extracts); Sample 3 was the cream base with added 100 mg active compounds (TGC-EA Dry Extracts).

To assess microbiological purity, the direct inoculation method (plate method) was used to count mesophilic bacteria and fungi in 1 g or 1 mL of product, and to detect the absence of specific unacceptable pathogens. A certain volume of the diluted sample was mixed with agar in a Petri dish. After the incubation period, colonies were counted (CFU - colony-forming units) and, if necessary, biochemical tests were performed to identify pathogens. Table 4.15 presents the results of microbiological purity testing: all three samples (Nos. 1-3) complied with the existing standards.

Table 4.15

Microbiological purity of the finished cream in bulk

Types of microorganism	Standards of Ph. Eur.	No 1	No 2	No 3
The amount of aerobic bacteria	$\leq 10^3$ (CFU/g or CFU/mL)	None	None	None
The amount of mould and fungus	$\leq 10^2$(CFU/g or CFU/mL)	None	None	None
Staphylococcus aureus	Must not be detected	None	None	None
Cutibacterium acnes	Must not be detected	None	None	None
Escherichia coli	Must not be detected	None	None	None

The HET-CAM test was applied as a preliminary experimental method to assess the irritation potential of the bulk cream formulations. During the 300-s observation period, no haemorrhage, vascular lysis or coagulation was observed for any of the tested samples. According to the adopted HET-CAM evaluation criteria, these findings indicate a low irritation potential under the conditions of the applied experimental model. The HET-CAM results are limited to irritation-related vascular responses and are not interpreted as evidence of the overall biological safety or biocompatibility of the cream. The results of HET-CAM experiments are summarised in Table 4.16 for three cream samples with different TGC-EA contents, with more details in Fig. 4.9.

Table 4.16

Results of the HET-CAM irritation-potential assessment of the bulk cream samples

Sample	**Haemorrhage**	**Vascular lysis**	**Coagulation**	**IS**	**Interpretation**
Sample 1	Not observed	Not observed	Not observed	0.0	Non-irritant under HET-CAM conditions
Sample 2	Not observed	Not observed	Not observed	0.0	Non-irritant under HET-CAM conditions
Sample 3	Not observed	Not observed	Not observed	0.0	Non-irritant under HET-CAM conditions

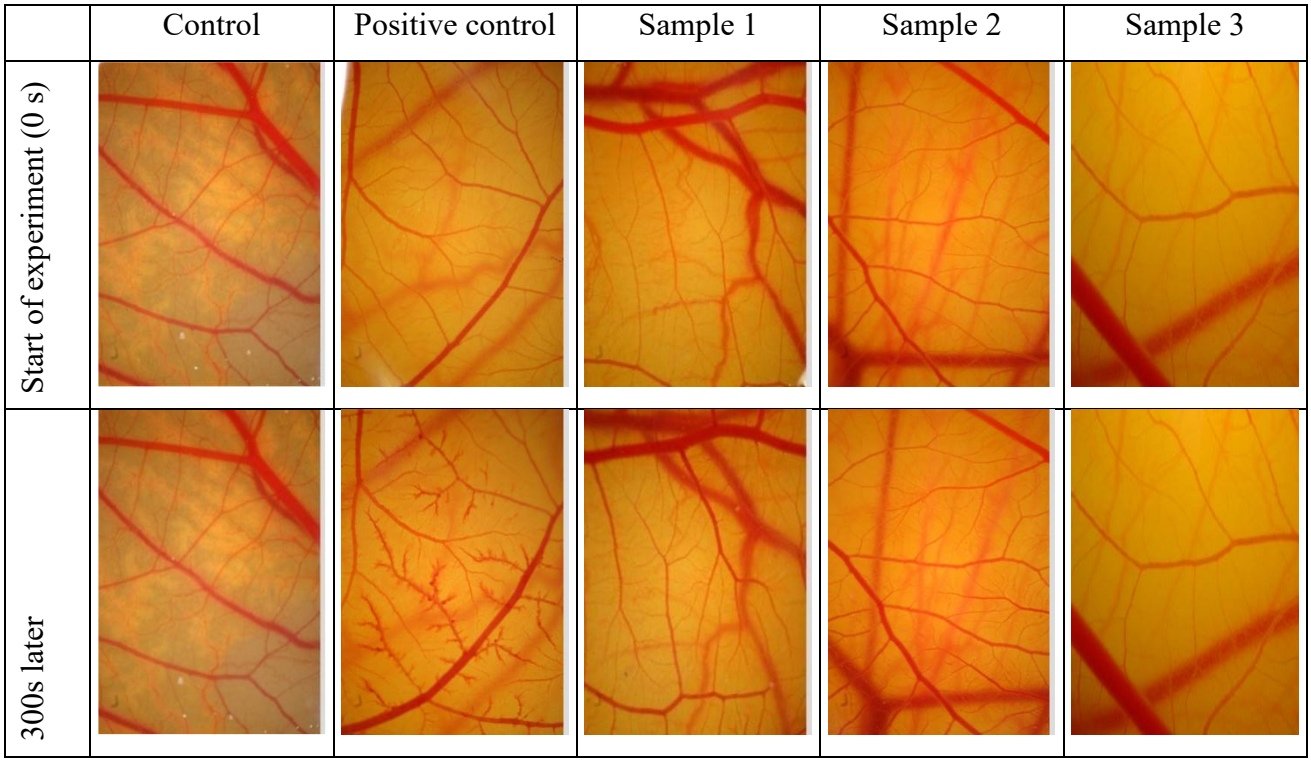


Fig. 4.9 HET-CAM assessment of the irritation potential of bulk cream samples containing different amounts of TGC-EA

4.3.7 Antibacterial activity

The antibacterial activity of the cream was tested twice: once at the bulk cream stage and again in the next chapter at the tube cream stage. Even with parameter similarities, antibacterial activity at these stages has different meanings. Thus, the activity of the bulk cream is an element of process control. That is, this parameter checks whether the API was not "killed" during mixing and heating at production. The cream activity in the tubes is part of the stability control. That is, they indicate whether the tube material does not affect the drug's activity during its shelf life. The results of the antibacterial activity determination are given in Table 4.17.

Table 4.17

Antibacterial rate of TGC bulk cream

Staphylococcus aureus	99.42 ± 0.33 %
Cutibacterium acnes	98.86 ± 0.51 %

Conclusions to Chapter 4

1. In accordance with existing ICH guidelines, the pharmaceutical development of the antibacterial cream was based on a risk-based approach. The selection of the API, in particular the dry extract of Tou Gu Cao, was based on its targeted antibacterial activity and stability profile. Excipients were selected based on their chemical compatibility and functional roles as emulsifiers, structuring agents, penetration enhancers, antioxidants, and antimicrobial preservatives. Key attention was paid to ensuring the base's non-comedogenicity and physiological compatibility with the skin's pH, creating a stable delivery system for the herbal extract.

2. A structured Design of Experiments methodology was implemented to optimise the cream base before incorporating the API. Using the L9 orthogonal matrix, the study systematically evaluated the influence of three critical factors: the ratio of *Acacia Senegal gum* and *Astragalus gummifer gum*, the total concentration of these rheological modifiers, and the water-oil phase ratio. The applied modelling method, followed by experimental verification, enabled the determination of the optimal quantitative composition of the cream, ensuring a stable viscoelastic structure. The final ratio provides reliable "structural support" for the dispersed oil droplets, achieving the desired balance between stability and functional characteristics.

3. A comprehensive technological scheme for the production of TGC cream was developed. The scheme covers all 10 stages from the preparation of raw materials to the final primary packaging of the manufactured product. Critical process parameters, such as homogenisation intensity, phase-addition order, and temperature-time regimes, were established to ensure batch consistency. The integration of process control methods enables real-time monitoring of Quality Critical Attributes, ensuring the production process is not only reproducible but also meets modern pharmaceutical quality standards. The developed scheme was supported by the selection of key technological equipment that enables the production of 300 kg of cream per cycle, which is then packaged in 30 g tubes. A pilot batch of cream was successfully manufactured according to the optimised recipe and technological scheme. This stage served as a practical verification of theoretical and laboratory models.

4. A full range of organoleptic characteristics was investigated to ensure the sensory quality of the product. The developed cream exhibits a homogeneous, smooth texture, with no visible particles or phase separation. The appearance is a homogeneous semi-solid mass, usually milky white in colour due to the natural extract, with a slight characteristic odour. These parameters were identified as critical indicators of the cream's macroscopic homogeneity, which is important for ensuring the uniform distribution of the active substance after application.

5. The key physicochemical characteristics of the antibacterial cream were determined and standardised. The pH value was optimised to remain within the physiological range (5.5 to 6.5) and to prevent skin irritation, ensuring the stability of the extract. Rheological studies confirmed that the cream is easily distributed under shear forces and maintains its shape at rest. The measured viscosity ranged from 27.6 to 29.2 Pa·s, indicating successful optimisation of the cream base.

6. The stability of the organoleptic and physicochemical properties of the cream was thoroughly tested under various stress conditions (thermal and mechanical). These primarily mimic the production conditions. Parameters such as resistance to oil release, water exudation and phase separation (syneresis) were monitored. The results show that the synergistic combination of *Acacia gum* and *Astragalus* creates a stable emulsion that maintains its integrity over time. The established limits for these stability markers (e.g., colloidal stability at 100%) serve as a guideline for the product's shelf life and ensure the cream maintains its consumer appeal throughout its use.

7. The cream was evaluated with respect to irritation potential, microbiological purity and *in vitro* antibacterial activity. The obtained results met the predefined development criteria for these investigated parameters.

The results of Chapter 4 are in the following articles: [223-228].

Chapter 5. Packaging of cream in tubes, its quality and shelf life

Long-term stability experiments were designed and performed taking into account the following regulatory documents:

1) ICH Q1A(R2) — Stability Testing of New Drug Substances and Products. It is the main document for long-term, accelerated and intermediate stability studies (Guideline ST-N MOZU 42-3.3:2004 "Medicinal products. Stability testing" — harmonised with ICH Q1A)
2) ICH Q1E — Evaluation of Stability Data. For evaluating results and justifying shelf life.
3) ICH Q6A — Specifications: Test Procedures and Acceptance Criteria. For justifying specifications and acceptance criteria.
4) EMA Guideline on quality and equivalence of topical products. It needs to justify the quality of a topical preparation, rheology, microstructure, *in vitro* release, etc.

5.1 Selection of packaging

In the development of pharmaceutical products, the primary packaging material comes into direct contact with the dosage form. Its permeability, light-shielding properties, mechanical compliance, and chemical inertness directly determine the microenvironmental homeostasis of the preparation during its shelf life. Tailored to the formulation composition and clinical application scenarios of the TGC antibacterial cream, this study ultimately selected pharmaceutical-grade aluminium-plastic laminated tubes (APT) as its primary commercial packaging. This selection is based on the following in-depth pharmaceutical and materials science considerations:

1) Extremely Low Moisture Vapour Transmission Rate and Rheological Network Maintenance. As an O/W emulsion, the TGC cream utilises purified water as its continuous phase (accounting for up to 68.2% of the formulation). The constancy of moisture is the fundamental prerequisite for maintaining the rheological properties of this preparation. If ordinary single-layer low-density polyethene (LDPE) tubes or wide-mouth jars are used, moisture can easily evaporate and diffuse outward through the packaging wall, driven by osmotic and vapour pressure gradients. The loss of

moisture not only causes the cream to shrink and harden but also triggers an abnormal increase in the local concentration of water-soluble components (e.g., glycerin, EDTA, and preservatives) in the continuous phase. It may even induce the "salting out" or precipitation of the aqueous phase polymers (3.6% *Acacia Senegal Gum* and 1.2% *Astragalus Gummifer Gum)* due to solvent depletion, ultimately leading to the collapse of the microscopic structure. The middle layer of the aluminium-plastic laminated tube contains a dense aluminium foil core, which endows the packaging with a moisture vapour transmission rate (MVTR) of nearly zero, thereby creating an absolutely sealed physical hydration microenvironment for the high-water-content cream.

2) Dual Physical Defence Lines of Light-Shielding and Anti-Oxidation. This formulation contains the core API, TGC-EA Dry Extract, as well as the co-surfactant Apricot kernel oil PEG-6 esters and the antioxidant DL-α-Tocopheryl Acetate. These molecules, which contain unsaturated bonds or conjugated polyene structures, are highly sensitive to the high-energy radiation of ultraviolet and visible light, making them highly susceptible to photo-catalysed lipid peroxidation chain reactions. The aluminium foil layer of the laminated tube acts not only as a moisture barrier but also as an absolute light-shielding barrier (completely opaque). This physical isolation from light and oxygen, synergised with the chemical antioxidant mechanism of 0.2% DL-α-Tocopheryl Acetate in the formulation, effectively severs the initiation pathway of free radical reactions. During the 12-month stability study, the cream consistently maintained a "homogeneous milky white" appearance and a highly constant pH (without the formation of oxidative degradation products such as short-chain fatty acids), demonstrating the excellent antioxidant efficacy of this packaging system.

3) Anti-Contamination Operational Ergonomics and Preservative Adsorption Evaluation. Compared to traditional jar packaging, tube packaging offers significant anti-contamination advantages during clinical application. Opening a jar exposes a large surface area of the cream, making it highly susceptible to airborne spores and pathogenic bacteria from the patient's hands. In contrast, a tube dispenses unidirectionally through a narrow orifice, and the aluminium-plastic composite material undergoes irreversible deformation upon squeezing. It eliminates the "suck-

back effect" commonly observed in ordinary plastic tubes, thereby preventing the ingress of external air and contaminants. This physical isolation at the packaging level substantially alleviates the antimicrobial burden on the internal preservative system (Methyl Paraben/Ethyl Paraben). Furthermore, the inner wall of the laminated tube directly contacting the cream is typically composed of chemically inert high-density polyethene (HDPE) or a specialised polymer coating. Experiments have demonstrated that this inner layer material exerts no adsorption or partitioning effects on the preservatives (Parabens) in the formulation, ensuring an effective inhibitory concentration of preservative molecules in the aqueous phase.

5.2 Quality control of the final product in tubes

Quality control of the final product is directly related to the acceptance control stage (Final/Acceptance inspection) in QCM. It first includes checking the parameters that may have appeared or changed during filling and packaging. They are:

- Average weight of the package contents: Checking the accuracy of dosing (deviation from the nominal weight).
- Packaging tightness: Checking the tube's sealing quality to prevent leakage or oxidation.
- Organoleptic indicators: Colour, odour and appearance of the mass immediately after squeezing from the tube.
- Microbiological purity: Confirmation of the absence of contamination during the packaging process.
- Storage stability during stability testing using the tubes to assess the drug's interaction with the packaging material (aluminium, laminate, etc.).
- Marking: Checking the presence of the batch, manufacture date, and expiration date.

Some of these parameters, such as packaging weight and tightness, and marking, are more likely to depend on the equipment rather than on the cream's quality. Others, on the contrary, directly characterise the product's quality, so they are considered first.

5.2.1 Organoleptic properties (colour, odour, texture)

For semi-solid preparations filled into tubes, organoleptic properties represent not only patient/consumer acceptability but also a practical macroscopic “fingerprint” reflecting formulation robustness and process consistency. In this study, the finished cream was evaluated after cooling and setting for appearance, colour, optical behaviour, texture, and spreadability (Table 5.1).

Table 5.1

Organoleptic properties of the finished cream in tubes

Quality Control	Cream Product
Appearance	Homogeneous viscous solid upon cooling
Colour	Homogeneous milky white
Transmittance	No light transmission
Texture	Good roundness and very good texture
Spreadability	Easy to apply, and there is no graininess

The product formed a homogeneous, viscous solid upon cooling, indicating that the system retained a coherent semi-solid structure after filling, without macroscopic phase separation or localised thinning. The colour was described as homogeneous milky white, suggesting uniform light scattering and a visually consistent distribution of dispersed domains at the macroscopic level, with no visible mottling or stratification. Regarding optical appearance, the cream showed no light transmission, consistent with an opaque cream-type product and indirectly supportive of the absence of local clarification or oil/water segregation that could otherwise alter optical uniformity.

Texture-related observations further supported the overall acceptability of the tube product: the cream exhibited good roundness and very good texture, and it was recorded as easy to apply with no graininess. These findings imply a fine, smooth tactile profile and an appropriate balance between structural integrity and ease of spreading, with no perceptible particulate aggregates that could compromise the user experience. Collectively, the organoleptic profile—uniform appearance and colour, complete opacity, refined texture, and smooth application—provides a coherent

macroscopic basis for interpreting the physicochemical and microbiological outcomes in subsequent sections as part of an integrated quality-evidence chain.

5.2.2 pH and Dynamic Viscosity

pH is a critical physicochemical attribute for topical creams, with direct relevance to skin compatibility, formulation stability, and batch consistency. From a quality-control perspective, pH serves multiple functions: (i) it contributes to the assessment of tolerability under intended conditions of use; (ii) it reflects the chemical microenvironment that may influence the performance of excipients associated with preservation and overall product stability; and (iii) it provides an indirect indicator of manufacturing consistency and within-batch uniformity, because pronounced pH drift or high variability can signal inadequate mixing, instability or other process deviations.

The pH of the finished tube product was measured at 5.68±0.18, which falls within the mildly acidic range and shows limited dispersion across replicates (Table 5.2). The combination of an appropriate mean value and a relatively small variability suggests a homogeneous pH distribution within the tested batch and the absence of marked acid–base imbalance. Importantly, this pH result is consistent with the favourable macroscopic observations reported in Section 5.2.1 (homogeneous, opaque, refined texture, easy application without graininess), which together imply that the system maintains a stable physicochemical state after filling and cooling.

Table 5.2

pH of the finished cream in tubes

Quality Control	**Cream Product**
pH	5.68±0.18

In addition, the pH value is best interpreted not merely as a pass/fail datum but as a baseline parameter for subsequent comparability and stability monitoring. Should future batches or storage conditions lead to systematic pH shifts, the present value (5.68±0.18) provides a traceable reference for investigating potential causes within the formulation–process–packaging system. Overall, the measured pH supports the acceptability and consistency of the tube product and provides a key quantitative component of the release-oriented quality assessment.

Dynamic viscosity (DV) is among the most representative rheological attributes for topical creams and is directly linked to tube extrusion behaviour, spreadability, film formation, and post-application shape retention. From a product-use standpoint, viscosity that is too low may lead to dripping or poor dose control and may be associated with an increased risk of instability; conversely, excessive viscosity may require higher extrusion forces and poor spreadability, negatively affecting adherence. Therefore, DV measured under fixed, reproducible conditions provides a core quantitative basis for release testing and for batch-to-batch comparability.

The value of DV was measured at 25°C and 10 rpm, yielding 28.54±3.65 Pa·s for the finished tube product (Table 5.3).

Table 5.3

Dynamic Viscosity of the finished cream in tubes

Quality Control	**Cream Product**
Dynamic Viscosity (Pa·s)	28.54±3.65

A clear semi-solid "cream-like" consistency is observed rather than a low-viscosity fluid, supporting practical usability for tube dispensing and controlled spreading. The DV outcome aligns internally with the organoleptic profile reported for the same product—specifically, a homogeneous, viscous solid with very good texture and easy application, free of graininess—thereby linking subjective sensory judgement to an auditable physicochemical metric. The reported dispersion (±3.65 Pa·s) suggests acceptable repeatability and within-batch consistency under the specified measurement conditions. While the present work does not extend beyond the reported results to attribute variability to specific microstructural causes, the quantitative DV value and its limited spread collectively support the conclusion that the batch demonstrates a stable rheological profile suitable for tube packaging and topical use. Accordingly, DV is a key control parameter for future manufacturing control, scale-up comparability, and stability follow-up of the final tube product.

5.2.3 Microbiological purity

Microbiological purity is a high-priority quality and safety element for tube-filled topical creams, particularly for products containing natural-origin materials

where baseline bioburden risks may be higher. Insufficient control of initial microbial load may pose safety risks and can adversely affect stability during storage and use. In this study, microbiological quality was assessed against Ph.Eur. limits, with the test outcomes presented alongside them, enabling direct compliance-based interpretation.

The results demonstrated that the total aerobic microbial count limit (≤10^3 CFU/g or CFU/mL) was met, with the product recorded as none, and the combined mould/yeast limit (≤10^2 CFU/g or CFU/mL) was likewise recorded as none (Table 5.4).

Table 5.4

Microbiological purity of the finished cream in tubes

Types of microorganism	**Standards of Ph. Eur.**	**Cream Product**
The amount of Aerobic bacteria	≤10^3 (CFU/g or CFU/mL)	None
The amount of mould and fungus	≤10^2(CFU/g or CFU/mL)	None
Staphylococcus aureus	Must not be detected	None
Cutibacterium acnes	Must not be detected	None
Escherichia coli	Must not be detected	None

The absence of specified objectionable microorganisms is particularly relevant to topical safety. The results showed None for *Staphylococcus aureus*, *Cutibacterium acnes*, and *Escherichia coli*, meeting the requirement of "must not be detected." From a tube product quality-control perspective, these findings indicate that the finished cream exhibits an extremely low microbiological burden and meets the stated pharmacopeial criteria. This outcome supports the adequacy of hygiene controls across manufacturing and filling operations, and it provides a critical safety rationale for release. Moreover, a low initial microbial level constitutes a robust starting point for subsequent stability evaluation, because microbial growth—if present—can be a major confounder affecting appearance, odour, and physicochemical parameters over time. Thus, the microbiological results form an essential component of the integrated safety and quality evidence for the final cream product in tubes.

5.2.4 Antibacterial activity

Antibacterial activity is a function-related quality attribute of the final TGC antibacterial cream and provides direct evidence of performance consistency at the

finished product level. Unlike purely physicochemical indices, *in vitro* antibacterial activity reflects whether the product, under its final dosage form and packaging, maintains a reproducible functional response under the applied assay conditions. Five randomly selected samples in a tube demonstrated an overall high level and a limited range across samples (minimum 96.87%; maximum 98.85% for *Cutibacterium acnes* and minimum 99.49%; maximum 99.75% for *Staphylococcus aureus*) (Table 5.5).

Table 5.5

Antibacterial activity of five random samples of the developed cream

Sample	Antibacterial rate (%) (*Cutibacterium acnes*)	Antibacterial rate (%) (*Staphylococcus aureus*)
Cream Product (Random sampling 1)	96.87±1.16	99.56±0.27
Cream Product (Random sampling 2)	98.41±0.89	98.68±0.80
Cream Product (Random sampling 3)	98.85±0.53	99.53±0.21
Cream Product (Random sampling 4)	98.28±0.45	98.93±0.31
Cream Product (Random sampling 5)	97.95±1.07	98.77±0.94

Such an observation suggests that the functional output was maintained consistently across independently sampled units within the batch. The primary interpretation of these data is quality-control-oriented: the relatively tight distribution supports intra-batch functional uniformity of the tube product. Additionally, the antibacterial activity outcomes are not contradictory to, but rather compatible with, the favourable macroscopic and physicochemical attributes observed.

Together, these results suggest that the finished product demonstrates a coherent quality profile in which sensory acceptability and stable rheological/chemical baselines coexist with stable antibacterial performance, thereby strengthening the overall evidence base supporting product quality release and subsequent comparability studies.

5.2.5 Assessment of Irritation Potential by the HET-CAM Test

Since the developed cream is intended for topical application, its potential to induce acute local irritation was assessed using the HET-CAM model. The evaluation focused specifically on acute vascular reactions of the chorioallantoic membrane during the defined observation period, namely haemorrhage, vascular lysis and coagulation, focusing on acute reactions of the membrane/vasculature as primary

readouts within the defined observation window (Fig. 5.1). A Positive Control Sample was used to confirm the expected action of the substance.

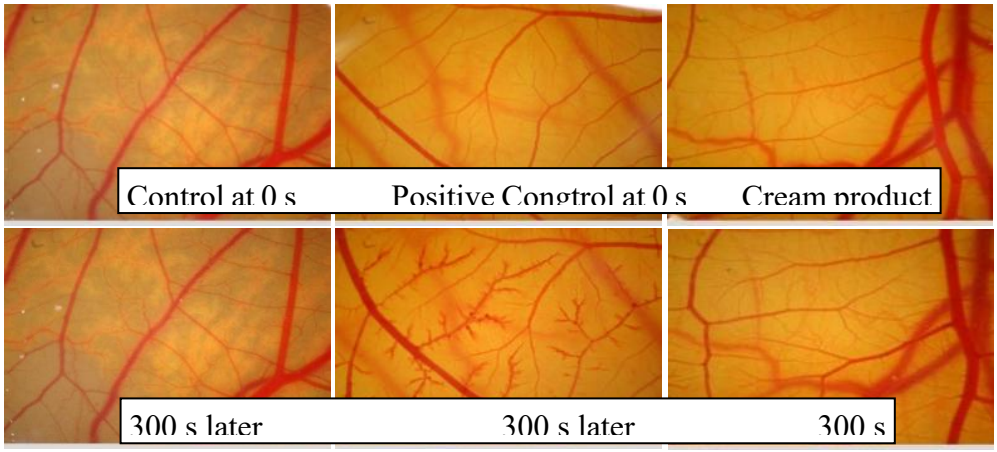


Fig. 5.1 HET-CAM assessment of irritation potential of the finished cream

At the 300 s observation time point, the negative control was recorded as "Secure, no bleeding, blood clots, inflammation". Under the same conditions, the finished cream product showed the same outcome, with no observable bleeding, clotting, or inflammatory response in the model (Table 5.6). These findings prove experimentally that the finished tube product in this assay did not elicit detectable acute irritation-related phenomena and provide preliminary information specifically concerning acute irritation-related vascular responses.

Table 5.6

Results of the HET-CAM irritation-potential assessment of the finished cream in tubes

Sample	Haemorrhage	Vascular lysis	Coagulation	IS	Interpretation
Final Cream Product in tubes	Not observed	Not observed	Not observed	0.0	Non-irritant under HET-CAM conditions

5.2.6 Heavy metal content

Since natural-origin materials may introduce elemental impurities, heavy metal monitoring is essential for risk management and release-oriented quality assurance of topical products. In this study, heavy metal testing and compliance interpretation were conducted in accordance with the current highest-level national guidance in China, the Safety and Technical Standards for Cosmetics (2015 edition) [229].

Under this standard, the maximum limits for hazardous elements are specified as mercury (Hg) ≤ 1 mg/kg (with an exception for eye-area cosmetics containing organomercury preservatives), lead (Pb) ≤ 10 mg/kg, arsenic (As) ≤ 2 mg/kg, and cadmium (Cd) ≤ 5 mg/kg. In addition, the standard provides dedicated physicochemical test methods for the determination of Hg, Pb, As, and Cd.

The measured heavy metal contents in the finished cream product are shown in Table 5.7. These values are substantially below the corresponding regulatory limits. Consequently, based on the Safety and Technical Standards for Cosmetics (2015) limit requirements, the tube product satisfies the control targets for the four heavy metals.

Table 5.7

Heavy metal content (Cd, Pb, As, and Hg in ppm) in the developed cream in tubes

Heavy metals	Cu	Cd	Pb	As	Hg	Total for 4 heavy metals
Permissible limits for hazardous metals by [229]		≤ 5	≤ 10	≤ 2	≤ 1	
Measured contents in the cream	1.556± 0.472	0.762± 0.138	1.279± 0.313	0.417± 0.091	0.068± 0.024	2.526± 1.038

In addition to the regulated elements, Cu 1.556±0.472 ppm was monitored as supplementary information for metal impurity trending and potential source tracing (Cu is not part of the core hazardous-element limit table). Overall, the heavy metal dataset provides a quantitative baseline for batch release documentation, future batch-to-batch comparability, and upstream control of risks within a risk-based quality framework for the final cream product in tubes.

In Chapter 3, heavy metals were determined in the dry TGC-EA extract. The comparative results for the heavy metals in the extract and cream are shown in Fig. 5.2.

The Hg and As content did not change noticeably, whereas the Cd, Pb, and Cu content in the cream increased significantly compared to the API. The difference in the content of these three impurities became statistically significant. The main interest is the increase in the content of four heavy metals, among which the effect is manifested only for lead and cadmium. The equipment used in the technological process is relatively new, and its cleaning procedures are fully performed. Therefore, although

contact with the equipment cannot be completely excluded from the main sources of contamination, the probability of such an event is limited.

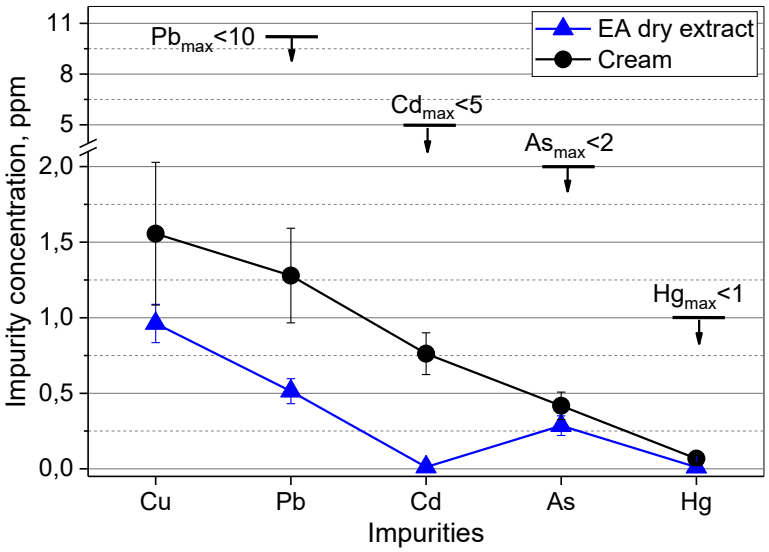


Fig. 5.2 Concentrations of heavy metals in dry TGC-EA extracts and cream in tubes

At the same time, another factor cannot be ignored. The main difference between the extract and cream is the presence in the cream of 13 excipients in addition to the API, each of which can, in principle, introduce additional impurities.

Possible sources of excipient contamination with metals include the following. In addition to the active ingredient TGC-EA, certain excipients in the TGC cream may also contribute trace elemental impurities, among which Pb, Cd, As, Hg, and Cu should be regarded as the principal elements of concern [92, 93].

From the perspective of potential sources, the presence of Cu and Pb in purified water is more likely associated with the pharmaceutical water system, including piping, valves, weld points, and the system's overall maintenance status [230]. Natural gums, such as *Acacia Senegal gum* and *Astragalus Gummifer gum*, are natural raw materials. Metals like Pb, Cd, and As may have a natural origin: harvesting/processing water, soil uptake or ash/inorganic salts [231, 232]. Apricot kernel oil PEG-6 esters, polysorbate 80, petrolatum, stearic acid, and cetearyl alcohol may introduce Cu, Pb, and other trace metallic impurities as a consequence of the background composition of vegetable-oil- or petroleum-derived feedstocks, the extent of refining, ethoxylation or esterification processes, and contact with manufacturing equipment/catalysts [233].

Glycerin, methyl paraben, ethyl paraben, EDTA, and DL-α-tocopheryl acetate are generally more highly refined excipients. However, very low Pb and Cu, as well as other elements at trace levels, arising from raw material purity, process water, EDTA chelation of metals, or equipment contact, cannot be completely excluded [92, 93].

Practice shows that not all manufacturers indicate the content of heavy metals on their labels. The most comprehensive, generally recognised source of information on Pharmacopeial specifications, including permitted values for heavy metal content, is the Handbook of Pharmaceutical Excipients [234]. The Handbook is based on the US, European and Japanese Pharmacopoeias. The available information from this source is collected in Table 5.8. In most cases, the total permissible content of heavy metals is 20 ppm, though it can be 10 ppm. For individual impurities, the limit is 2-3 ppm. The composition of purified water is regulated most strictly.

Table 5.8

Permissible levels of heavy metal content by [234]

Excipient (Handbook, 8th Ed., page)	Limits to heavy metals
Purified water (p. 1012)	Ph.Eur. 9.2: Heavy metals <0.1 ppm
Glycerin (p. 401)	USP40-NF 35 SI: Heavy metals <5 ppm; JP XVII: As <2 ppm
Polysorbate 80 (p. 738)	USP40-NF 35 SI: Heavy metals <10 ppm; JP XVII: Heavy metals <20 ppm, As <2 ppm
Apricot kernel oil PEG-6 esters (p. 748)	USP40-NF 35 SI: <0.001%
Petrolatum (p. 659)	JP XVII: Heavy metals <30 ppm, As <2 ppm
Stearic acid (p. 930)	USP40-NF 35 SI: Heavy metals <10 ppm; JP XVII: Heavy metals <20 ppm
Cetearyl alcohol (p. 211)	Not applicable
Acacia Senegal Gum (p. 27)	USP 40-NF 35 SI: As <3 ppm, Pb <10 ppm, Heavy metals <4 ppm
Astragalus Gummifer Gum (p. 983)	USP 40-NF 35 SI: Pb <10 ppm, Heavy metals <20 ppm
Methyl paraben **(**p. 604**)**	JP XVII: Heavy metals <20 ppm
Ethyl paraben (p. 379)	JP XVII: Heavy metals <20 ppm
EDTA (p. 345)	USP40-NF 35 SI: Heavy metals <20 ppm; JP XVII: Heavy metals <20 ppm
DL-α-Tocopheryl acetate (p. 58, 1010)	JP XVII: Heavy metals <20 ppm

The norming of heavy metals, on the one hand, is evidence of the existence of a potential threat of excipient contamination. On the other hand, the acceptable level

coincides with the levels accepted in pharmacology for a majority of medicinal products. Thus, one may believe that the increase in heavy metal content during the transition from API to the finished cream is most likely due to excipient effects. The tested cream samples never exceeded the maximum permissible contamination levels. Clearly formulated limit values give hope that compliance with the standards will serve as a barrier to critical contamination. The experiments confirm this assumption.

5.2.7 Total contents of biologically active compounds by classes

Samples of the developed cream in tubes were selected and analysed for the total content of biologically active substances using the pre-treatment procedures and methods described in Chapter 2. The results of the determination, together with similar indicators for the dry extract obtained in Chapter 3, are given in Table 5.9. The API content in the dry extracts was measured in mg equivalents per 1 g of dry extract. In contrast, the developed cream contains only 0.1 g of dry extract per 100 g of the cream. In other words, one gram of the cream should contain 0.001 g of dry extract. Under such conditions, to better visualise the contents of biologically active substances, the cream results should be multiplied by 10^3 to align with those of the dry extracts.

Table 5.9

The content of total flavonoids, polyphenols, terpenes and alkaloids in the dry TGC-EA extract and tube cream compared to the minimum permissible values

Biologically active compounds	Phenolics (mg GE/g)	Flavonoids (mg RE/g)	Terpenoids (mg UAE /g)	Alkaloids (mg TE /g)
Total content in dry TGC-EA extracts	280±25	166±12	227±35	68±6
Total content in tube TGC-EA cream	0.267 ± 0.011	0.152 ± 0.006	0.218 ± 0.013	0.062 ± 0.004
Taken for the minimum permissible values	≥ 255	≥ 154	≥ 192	≥ 62

Figure 5.3 illustrates the API contents measured in the dry extract and those measured and recalculated in the cream (i.e., multiplied by 1000). Permissible API values are shown as red columns.

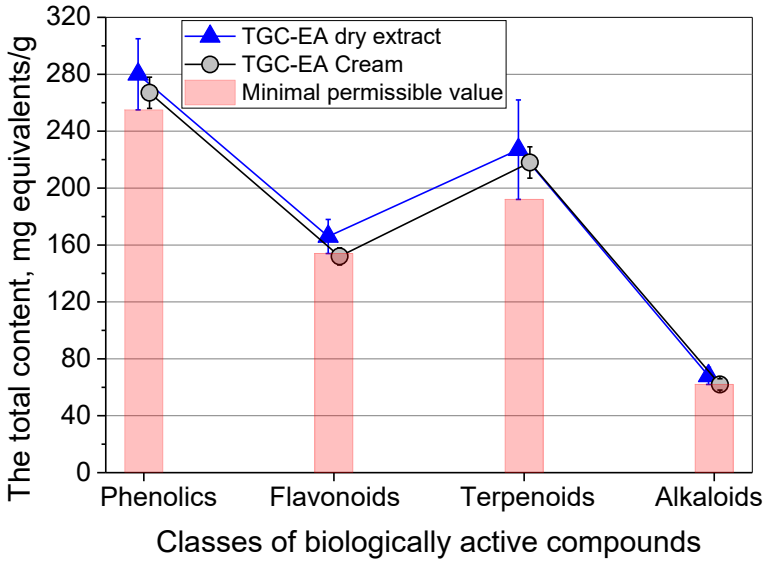


Fig. 5.3 The total content of biologically active compounds by classes in tube cream (magnified by 1000) and dry extract compared to values taken as minimally permissible limits

The minimum permissible values of the total content for each class of compounds that provide a desired antibacterial effect are indicated by the bars of the bar chart. In almost all cases, the measured average concentrations in both the extract and the cream exceed the minimum permissible levels. The only exception is the cream's flavonoid content. Its measured average value is approximately 7% less than the permissible limit. However, given the achieved measurement accuracy, the interval of values overlaps the normalised minimum content.

On the one hand, the average concentrations in the extract are always 7-14% higher than those in the cream. However, the difference is not statistically significant for any compounds. Therefore, there is no reason to believe that there is a reliably established difference in the content of the studied substances between the extract and cream. Since the measured concentrations remain within the minimum permissible limits, the developed cream meets the voiced requirements for antibacterial activity.

On the other hand, the small but stable decrease in the API content in the cream suggests the presence of a continuously acting responsible factor in the production technology and control points. It is most likely, although not proven, that this factor is associated with the use of a more complex, multi-stage sample preparation method for cream analysis, which may contribute to partial extraction or analyte loss. As already mentioned, the recorded difference does not exceed the standard error of the average content; therefore, from a practical point of view, it is not important.

5.3 Storage conditions, shelf life and stability of cream in tubes

As a complex, thermodynamically metastable system composed of multiple phases, the long-term physical stability, chemical stability, and microbiological safety of an oil-in-water (O/W) soft dosage form depend not only on the anti-coalescence barrier and preservative system within the formulation but also heavily on the physical isolation efficacy of the primary packaging system and the strict control of external storage conditions. This section aims to systematically elucidate the rationale for selecting packaging materials based on the physicochemical characteristics of the TGC antibacterial cream, and to scientifically define its storage conditions and commercial shelf life based on long-term experimental data.

In accordance with the EMA Guideline on declaration of storage conditions for medicinal products [235], clearly defined storage conditions are essential to maintain the stability, efficacy, and safety of medicinal products throughout their intended shelf life. This recommendation is particularly relevant for creams, where both formulation and packaging play critical roles in preserving product quality.

5.3.1 Storage conditions

Based on the physicochemical structural characteristics of the TGC cream and its long-term performance in sealed aluminium-plastic tubes, this study established strict labelled storage conditions: "Store in a tightly closed container, protected from light, at room temperature not exceeding 25°C. Do not freeze." These conditions are based on rigorous pharmaceutical thermodynamic principles that dictate shelf life.

1) Thermodynamic and Rheological Basis for the Upper Limit of Storage Temperature (≤ 25°C). The anti-coalescence capability of an O/W cream is highly dependent on the lamellar liquid crystalline gel network constructed by structure-forming agents (3.0% stearic acid and 3.0% cetearyl alcohol) at the oil-water interface, alongside the steric hindrance provided by macromolecular polysaccharides in the aqueous phase. These lipid skeletons and non-covalently cross-linked networks are extremely thermosensitive. When the storage temperature remains elevated (e.g., accelerated ageing conditions of >30°C or 40°C) for a prolonged period, the system's molecular kinetic energy increases significantly.

Elevated temperatures induce a sharp decline in the dynamic viscosity of *Acacia Senegal Gum* and *Astragalus Gummifer Gum* in the continuous phase, thereby weakening the fluid resistance that restricts the Brownian motion of droplets. Also, an increase in environmental temperature may approach or exceed the melting points of the lipid components (especially the petrolatum-higher fatty alcohol complex), causing the originally rigid crystalline network to soften or even partially melt. The collapse of the lipid skeleton removes steric hindrance, leading to an exponential increase in the collision frequency of oil droplets, which in turn accelerates Ostwald ripening and droplet coalescence. Therefore, restricting storage to room temperature (≤25°C) aims to maintain the system's thermodynamic energy at a lower ground state, ensuring that the dynamic viscosity of ~32 Pa·s and the crystalline network do not disintegrate.

2) Physical-Chemical Destructive Mechanisms Underlying "Do Not Freeze". For an O/W cream with a water content of up to 68.2%, freezing constitutes a devastating physical process. If the cream is exposed to sub-zero temperatures (< 0°C), the free water in the continuous phase undergoes a phase transition, crystallising into ice. The freezing of water is accompanied by a volume expansion of approximately 9%. This severe crystallisation stress acts as microscopic blades, physically piercing and rupturing the interfacial film formed by Polysorbate 80 and auxiliary emulsifiers, while simultaneously severing the macromolecular cross-linked network of the gums. Upon temperature elevation and subsequent melting of ice crystals back into liquid water (a freeze-thaw cycle), the compromised interfacial film cannot spontaneously repair itself. The oil and aqueous phases, devoid of surfactant protection and structural support, rapidly undergo irreversible macroscopic phase separation. Consequently, a mandatory "Do not freeze" warning must be prominently displayed on the tube label to prevent damage from extremely low temperatures during logistics and storage.

5.3.2 TGC cream shelf life

Topical semi-solid preparations of the oil-in-water (O/W) type are thermodynamically unstable systems. During long-term storage, creams are highly susceptible to environmental factors and the interactions among internal multiphase components, which can lead to flocculation, coalescence, Ostwald ripening, and even

phase separation. Consequently, this results in destruction of the product's physical structure, alterations in drug release behaviour and a potential reduction in product performance, including *in vitro* antibacterial activity. Therefore, systematically evaluating the Critical Quality Attributes (CQAs) of the cream within the proposed shelf life and elucidating its stability mechanisms from a formulation design perspective is an indispensable step to verify the rationality of the formula and the robustness of the manufacturing process.

This section aims to evaluate the evolution of the sensory characteristics, microscopic physical structure, rheological behaviour, chemical environment, microbial limits, and *in vitro* antibacterial activity of the cream at 0, 3, 6, and 12 months, through a 12-month long-term stability study. The research findings will reveal the synergistic mechanisms between the excipients and the API at both the molecular and macroscopic levels, providing robust experimental and theoretical support for establishing the preparation's commercial shelf life.

Macroscopic Sensory and Physical Stability Analysis Based on the Matrix Composition. The macroscopic sensory characteristics of a cream not only directly determine patient compliance but also serve as the primary intuitive indicator of the emulsion system's macroscopic physical stability. According to the 12-month retention sample observation data, the TGC antibacterial cream demonstrated excellent physical barrier stability throughout the investigation period (Table 5.10).

Table 5.10

Properties of the cream in tubes as a function of lifetime

Parameter	0 months	In 3 months	In 6 months	In 12 months
Appearance	Homogeneous viscous solid upon cooling	Homogeneous viscous solid upon cooling	Homogeneous viscous solid upon cooling	Homogeneous viscous solid upon cooling
Colour	Homogeneous milky white	Homogeneous milky white	Homogeneous milky white	Homogeneous milky white
Transmittance	No light transmission	No light transmission	No light transmission	No light transmission
Texture	Good roundness and very good texture	Good roundness and very good texture	Good roundness and very good texture	Good roundness and very good texture
Spreadability	Easy to apply, and there is no graininess	Easy to apply, and there is no graininess	Easy to apply, and there is no graininess	Easy to apply, and there is no graininess

In terms of appearance, the cream maintained a "Homogeneous viscous solid upon cooling" state at the 0, 3, 6, and 12-month testing intervals. This excellent resistance to phase separation is directly attributed to the rational combination of structure-forming agents in the oil phase. The formulation contains 3.0 g of stearic acid and 3.0 g of cetearyl alcohol. Acting as a consistency factor and co-emulsifier, respectively, these two higher aliphatic compounds crystallise at the oil-water interface and within the continuous phase during cooling after high-temperature homogenization, forming a robust lamellar liquid-crystalline gel network. This 3D network acts as a structural skeleton, effectively locking water and oil within the system and preventing the exudation of free water or the precipitation of the oil phase.

Regarding colour and transmittance, the preparation consistently maintained a "Homogeneous milky white" colour and exhibited "No light transmission" over the 12 months. This typical opaque appearance corresponds to the optical scattering properties of a macroemulsion with a high concentration of the dispersed phase. Furthermore, the absence of discolouration over a year indirectly confirms that 0.2 g of DL-α-Tocopheryl Acetate functioned as an antioxidant, effectively protecting the API and unsaturated lipids from lipid peroxidation-induced destruction.

In terms of texture and spreadability, the cream still maintained "Good roundness and very good texture," and was "Easy to apply, and there is no graininess" on the skin after 12 months of storage. Although the formulation contains up to 8.0 g of the occlusive emollient petrolatum, macroscopic recrystallisation (i.e., the formation of a rough, gritty feel) did not occur during long-term storage. This stability is due to the highly efficient synergy between 2.0 g of the primary emulsifier, Polysorbate 80, and 5.0 g of the co-surfactant, Apricot kernel oil PEG-6 esters. This combination reduces interfacial tension, perfectly encapsulating the complex semi-solid lipids within the emulsified microdroplets and the gel network, ensuring a long-term, smooth skin feel.

Figure 5.4 illustrates the absence of changes in the FTIR spectra of cream samples in the tube over 12 months of observation, which indicates high long-term stability of the product under storage conditions.

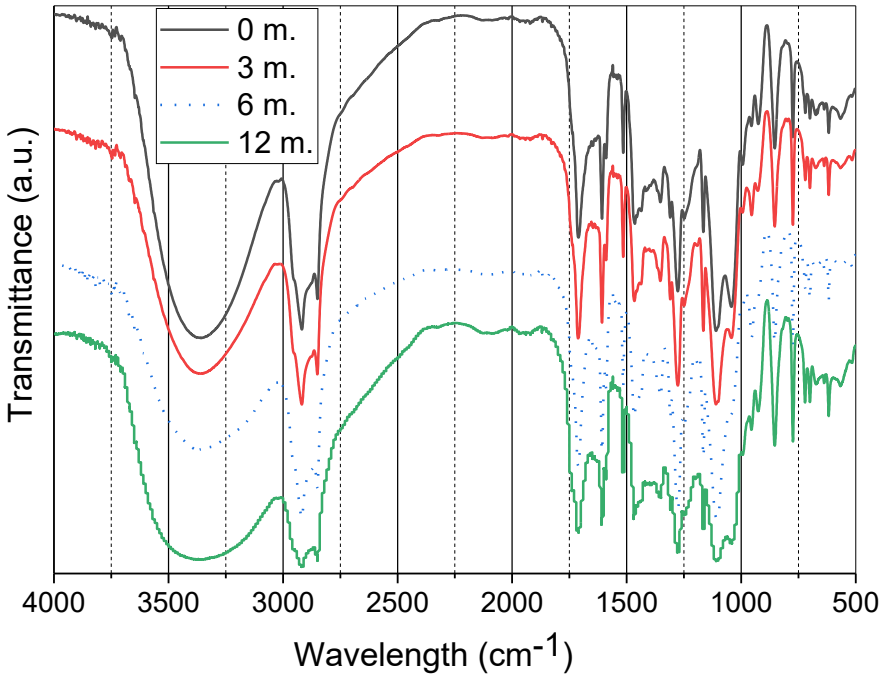


Fig. 5.4 FTIR spectra of the cream in tubes as a function of storage in months

Steric Hindrance and Microscopic Particle Size Dynamics. Although the macroscopic state remains homogeneous, as a thermodynamically unstable system, the cream droplet size will inevitably evolve at the microscopic level. Through particle-size analysis and optical microscopy, the dynamics of the dispersed-phase droplets were detailed, and the anti-coalescence mechanism was verified from a formulation-design perspective. Experimental data show that upon completion of the initial preparation (0 months), the cream had an average particle size of 922.98±12.74 nm and a Polydispersity Index (PDI) of 0.39±0.12. So, the preparation process successfully dispersed the oil phase into submicron- to micron-sized fine droplets.

Combined with the cream droplet morphology picture, it can be observed that the droplets at 0 months presented a perfect spherical shape and were evenly distributed in the continuous phase, without significant large-scale aggregation (Fig. 5.5).

With prolonged storage time, the particle size exhibited extremely slow, controllable growth. At 3, 6, and 12 months, particle sizes increased slightly to 956.42±10.30 nm, 1014.98±16.53 nm, and 1028.79±14.86 nm, respectively. Meanwhile, the PDI value gradually increased from the initial 0.39±0.12 to 0.56±0.14 at 12 months. However, a small number of slightly larger droplets appeared in the field

of view; the vast majority of droplets maintained independent microscopic morphology without destructive demulsification or large-scale phase fusion.

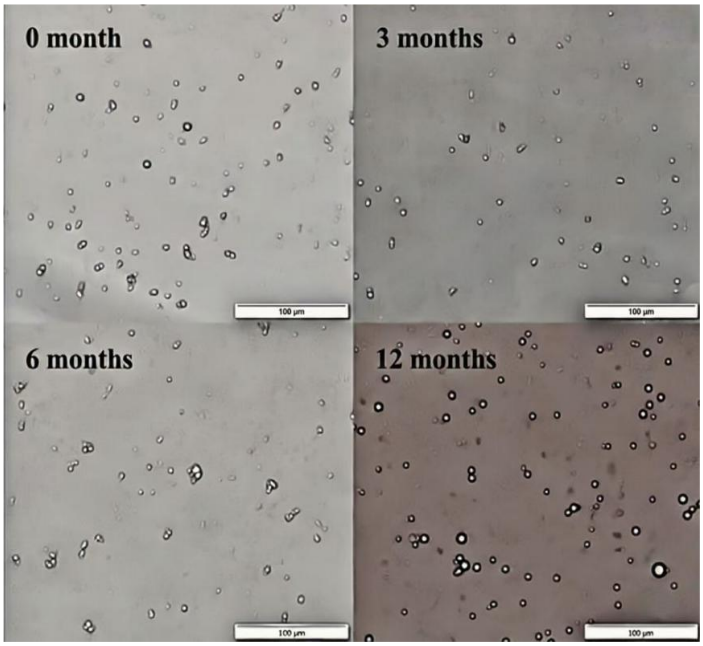


Fig. 5.5 Cream droplet morphology pictures as a function of storage time

This sluggish particle-size growth (only an approximately 11% increase over 12 months) clearly demonstrates that the formulation possesses a remarkably strong physical stability barrier. According to the formulation design, this highly effective barrier primarily originates from the steric hindrance effect of the macromolecular polysaccharides in the aqueous phase. The formulation incorporated 3.6 g of *Acacia Senegal Gum* as a physical stabiliser and 1.2 g of *Astragalus Gummifer Gum* as a thickening agent. These two natural high-molecular-weight polymers fully hydrate and swell in 68.2 g of purified water, which not only significantly increases the viscosity of the continuous phase and reduces the collision frequency of oil droplets under Brownian motion, but also, more importantly, they are capable of forming a thick and rigid mechanical multinuclear adsorption film on the surface of the oil droplets. This macromolecular adsorption layer greatly increases the repulsive force between droplets, effectively blocking Ostwald ripening and droplet coalescence, thereby protecting the long-term integrity of the cream's microscopic structure.

Rheological Evolution and Structural Network Maturation. Dynamic viscosity is a core rheological parameter for evaluating the internal structural strength,

batch reproducibility, and application skin feel of topical semi-solid preparations. Over 12 months, the viscosity evolution trajectory of this cream perfectly corroborated the scientific validity of the rheology modifiers employed in the formulation (Table 5.11).

At 0 months, the cream's initial dynamic viscosity was 28.54±3.65 Pa·s. After 3 months of storage, the viscosity exhibited a statistically significant modest increase, reaching 31.29±2.97 Pa·s. This viscosity buildup phenomenon is entirely consistent with the classical physicochemical behaviour of O/W creams that use higher aliphatic alcohols as structure-forming agents. Within the months following homogenisation and cooling, the 3.0 g of cetearyl alcohol (viscosity builder) and 3.0 g of stearic acid in the formulation continue to undergo microscopic rearrangement and crystallisation, promoting further thickening and maturation of the lamellar liquid-crystalline gel network. Furthermore, the macromolecular polymer chains of *Acacia Senegal Gum* and *Astragalus Gummifer Gum* (totalling 4.8 g) in the aqueous phase undergo a slow post-hydration uncoiling and entanglement, which jointly dominate the macroscopic viscosity increase during the initial storage period of the preparation.

Table 5.11

Physicochemical and biological properties of the cream in tubes as a function of lifetime

Parameter	0 months	In 3 months	In 6 months	In 12 months
Dynamic Viscosity in Pa·s	28.54±3.65	31.29±2.97	30.85±3.44	32.92±2.16
pH	5.68±0.18	5.62±0.23	5.70±0.11	5.66±0.14
The amount of Aerobic bacteria	$\leq 10^3$ (CFU/g or CFU/mL)	$\leq 10^3$ (CFU/g or CFU/mL)	$\leq 10^3$ (CFU/g or CFU/mL)	$\leq 10^3$ (CFU/g or CFU/mL)
The amount of mould and fungus	$\leq 10^2$(CFU/g or CFU/mL)	$\leq 10^2$(CFU/g or CFU/mL)	$\leq 10^2$(CFU/g or CFU/mL)	$\leq 10^2$(CFU/g or CFU/mL)
Staphylococcus aureus	None	None	None	None
Cutibacterium acnes	None	None	None	None
Escherichia coli	None	None	None	None
Antibacterial rate (%) – *C. acnes*	98.85±0.51	97.63±0.82	98.42±0.66	98.74±0.39
Antibacterial rate (%) – *S. aureus*	99.63±0.25	98.96±0.39	98.21±0.78	98.17±0.70
Particle Size, nm	922.98±12.74	956.42±10.30	1014.98±16.53	1028.79±14.86
Polydispersity Index	0.39±0.12	0.43±0.08	0.52±0.17	0.56±0.14

Following the initial structural reorganisation, the system's thermodynamic state tended toward equilibrium. At the subsequent 6-month and 12-month testing points, the dynamic viscosity stabilised at 30.85±3.44 Pa·s and 32.92±2.16 Pa·s, respectively. The stable fluctuation of data between approximately 30 and 33 Pa·s represents a reasonable rheological plateau. This long-term, stable rheological behaviour confirmed that the polymer-lipid gel network within the cream is robust enough to withstand the internal yield stress induced by long-term gravitational forces. It ensures that the product maintains a consistent application consistency and residence time throughout its shelf life, fully complying with the stringent quality control requirements for topical preparations in mainstream international pharmacopoeias.

Chemical Microenvironment Monitoring and System pH Homeostasis. The pH value of a topical preparation is directly related to the tolerance of the skin barrier and to the chemical stability of the API and preservatives in the formula. Natural herbal extracts and oils are highly prone to hydrolysis or oxidation during long-term storage, potentially releasing trace amounts of acids and causing an irreversible decline in the system's pH. The cream's pH at 0 months was 5.68±0.18, and at 3, 6, and 12 months, it was 5.62±0.23, 5.70±0.11, and 5.66±0.14, respectively (Table 5.11). Over the 1-year observation, the system's pH fluctuated almost horizontally and consistently remained within a weakly acidic range, close to the physiological pH of human skin.

This extremely stable chemical environment results from multiple formulation mechanisms working in concert. Firstly, the addition of 0.4 g of EDTA (a chelating agent) effectively complexed trace metal ions potentially present in the system, thereby blocking lipid peroxidation reactions catalysed by these ions. Simultaneously, EDTA itself imparted an extremely weak buffering capacity to the aqueous phase. Secondly, the incorporation of 5.0 g of glycerin as a humectant not only reduced the system's water activity but also, to a certain extent, inhibited the drastic hydrolysis of components in the continuous phase. The constancy of the pH value, both physically and chemically, demonstrated that the chemical microenvironment of the main drug, TGC-EA Dry Extract (0.1 g), was effectively protected, eliminating the risk of API degradation or increased skin irritation from severe pH fluctuations.

Microbial Limit Monitoring and Multi-Efficacy Preservative System Verification. An O/W type cream contains up to 68.2 g of purified water as the continuous phase, and is rich in plant extracts, glycerin, and polysaccharide colloids; such a matrix serves as a natural breeding ground for aerobic bacteria and fungi. Therefore, constructing an effective preservative system capable of lasting beyond 12 months is a fundamental prerequisite for establishing the shelf life.

Throughout the entire 0-12-month monitoring cycle, the levels of aerobic bacteria in the cream were strictly controlled at $\leq 10^3$ CFU/g, and those of mould and fungus were consistently controlled at $\leq 10^2$ CFU/g (Table 5.11). For highly pathogenic specific indicator bacteria (*Staphylococcus aureus*, *Cutibacterium acnes*, *Escherichia coli*), an ideal state of "None" was observed at all testing time points.

These outstanding microbiological indicators compellingly validated the scientific validity and durability of the combined preservative strategy employed in the formulation. The formula utilised a broad-spectrum antimicrobial preservative system comprising 0.1 g of Ethyl Paraben and 0.2 g of Methyl Paraben. The different oil-water partition coefficients of methyl and ethyl parabens ensured that the preservative molecules maintained sufficient effective inhibitory concentrations in both the aqueous continuous phase and at the oil-water interface. In addition, the incorporation of 0.4 g of EDTA as a preservative synergist can disrupt the integrity of the bacterial outer membrane structure, further amplifying the preservative efficacy of the parabens. During the one-year matrix partitioning and storage process, this specific combination successfully prevented extensive migration of preservatives into the oil phase and subsequent inactivation, thereby ensuring the preparation's microbiological safety.

Pharmacodynamic Core: Persistence Evaluation of *In vitro* Antibacterial Activity. For a cream intended for topical antibacterial therapy, physical, chemical, and microbiological stability merely signifies that the carrier system is qualified. The ultimate criterion for its shelf life is whether the core API, namely 0.1 g of TGC-EA Dry Extract, retains the expected pharmacological activity and release capability after long-term encapsulation within a complex multiphase matrix.

This study selected two test microorganisms, *Staphylococcus aureus* and *Cutibacterium acnes*, to evaluate the *in vitro* antibacterial activity of the cream during storage. *S. aureus* was included as a representative skin-associated bacterial pathogen, whereas *C. acnes* was included because of its involvement in the multifactorial pathogenesis of acne. Experimental data for *C. acnes* showed that the antibacterial rate was 98.85 ± 0.53% at 0 months and 97.63 ± 0.81%, 98.42 ± 0.66%, and 98.74 ± 0.39% after 3, 6, and 12 months, respectively. Thus, the *in vitro* antibacterial activity against *C. acnes* remained above 97% throughout the investigated storage period. Similarly, the antibacterial rate against *S. aureus* was 99.63 ± 0.25% at 0 months and 98.96 ± 0.39%, 98.21 ± 0.78%, and 98.17 ± 0.70% after 3, 6, and 12 months, respectively, indicating maintenance of the *in vitro* antibacterial activity during storage.

The retention of sustained and potent *in vitro* antibacterial activity provides the strongest endorsement of the preparation's formulation and process design. It not only confirms that the API, TGC-EA Dry Extract, encapsulated within micron-sized oil droplets, possesses high chemical stability and resists degradation during storage, but also demonstrates that the release channels mediated by excipients such as Polysorbate 80 remain unobstructed. The stable particle size distribution and appropriate viscosity network enabled the cream to release the drug stably, as predicted by the established drug release kinetic model post-application. The preparation maintained reproducible *in vitro* antibacterial activity throughout the investigated 12-month storage period.

5.3.3 Variation of bioactive compound contents in the cream over time

During the 12-month long-term stability tests, the variation trajectories of the four core active markers (total polyphenols, flavonoids, terpenes, and alkaloids) within the TGC cream are shown in Fig. 5.6 in milligram-equivalents per gram of the cream.

Although botanical extracts are typically subject to significant oxidation and hydrolysis in semi-solid formulations with high water content, the developed cream system exhibited exceptional chemical protective efficacy. At the end of the 12-month storage period, the contents of highly oxidizable total polyphenols and total flavonoids decreased marginally from the initial levels of 0.267 mg GE/g and 0.152 mg RE/g to 0.239 mg GE/g and 0.131 mg RE/g, respectively, maintaining retention rates of

approximately 90% and 86%. The retention rate of the highly lipophilic total terpenes reached an impressive 87% (decreasing from 0.218 to 0.189 mg UAE/g). The total alkaloids are less stable; they exhibit a retention rate of 74%, corresponding to 0.062 and 0.046 mg TE/g.

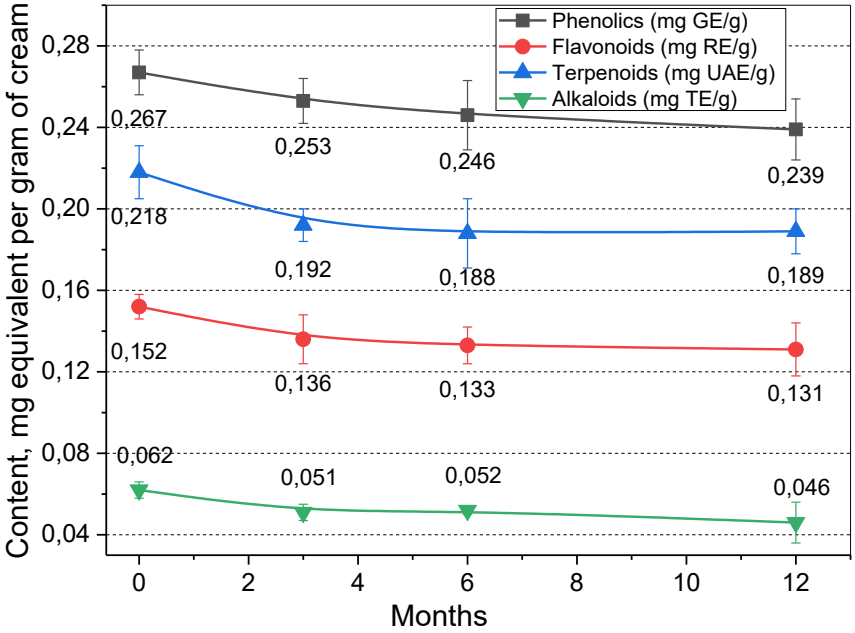


Fig. 5.6 The total contents of flavonoids, polyphenols, terpenes, and alkaloids with the standard deviations in TGC cream as a function of storage time (0-12 months)

Overall, the degradation margins of three core pharmacodynamic components did not exceed the 15% (25% for alkaloids) significant variation limit, thereby demonstrating the formulation's good chemical stability and shelf-life.

This superior chemical stability is primarily attributed to the precise design of the synergistic "antioxidant and metal-chelating" system within the formulation. The molecular structures of total flavonoids and total polyphenols are rich in phenolic hydroxyl groups, which are highly susceptible to autoxidation at the oil-water (O/W) interface or to catalytic degradation driven by trace transition metals. In this formulation, the incorporation of 0.4% EDTA in the aqueous phase acts as a potent metal ion chelating agent. It effectively sequesters trace Cu^{2+} and Fe^{3+} ions that may be introduced by the natural macromolecular excipients (*Acacia Senegal* and *Astragalus Gummifer* gums) or by the stainless-steel manufacturing equipment, thereby fundamentally blocking Fenton-like catalytic oxidation chain reactions. Concurrently,

0.2% Vitamin E (DL-α-Tocopheryl Acetate) dissolved in the oil phase serves as a lipophilic free-radical scavenger, providing a direct chemical barrier for the TGC-EA localised within the lipid core. The synergy between these two agents minimises the rate of oxidative degradation of the phenolic active substances.

Furthermore, from the perspectives of process kinetics and microscopic physical structure, the formulation's isolation effect is another core mechanism that delays the API's degradation. During the industrialised manufacturing process, the TGC-EA extract is first molecularly dissolved at 75℃ within a highly lipophilic matrix composed of petrolatum, stearic acid, and cetearyl alcohol. Subsequently, it is formed into an O/W emulsion via high-shear homogenization in a vacuum reactor.

During the gradient cooling phase, the higher fatty acids and fatty alcohols self-assemble at the periphery of the oil droplets to form a dense liquid crystal interfacial film (characterised by Maltese crosses under polarised light). This structural film, coupled with the high-viscosity three-dimensional gel network formed by the hydration of high-concentration gums (3.6% *Acacia gum* and 1.2% *Tragacanth gum*) in the continuous aqueous phase, constructs a robust physical steric hindrance. This "microencapsulation"-like stable structure significantly restricts the diffusion coefficients of free oxygen molecules and moisture within the system. Consequently, the lipophilic terpenes and flavonoids are firmly anchored and enclosed within the oil droplets, effectively resisting external environmental stressors.

In conclusion, the 12-month quantitative analysis data not only confirm the robustness of the pharmacological material basis of the TGC cream, but also retrospectively validate the profound rationality of the formulation design and emulsification process—specifically, the strategy of "lipid-targeted dissolution, antioxidant synergy, and dense liquid crystal membrane encapsulation".

5.4 Standardisation of the developed product

Standardisation of the cream in tubes was carried out to ensure reproducible quality, performance, and safety. Key quality attributes (parameters), the measured values for the investigated batch, and the acceptable limits are shown in Table 5.12.

Table 5.12

Parameters of standardisation of the developed TGC cream

Parameter	**Real value**	**Acceptable limits**
Description (colour, odour, consistency)	Homogeneous viscous solid upon cooling; milky white; no light transmission; good roundness and very good texture; easy to apply; no graininess	Homogeneous, milky-white, opaque cream; no phase separation or surface oiling; smooth and uniform texture; free from visible particles/grittiness; no abnormal odour; easy spreading with continuous film formation
Identification and quantitative determination of the total content of API components	Not determined; to be established using validated assays for the selected marker(s) (e.g., total flavonoids as rutin equivalents (RE))	Provisional: 90.0–110.0% of the target content for each marker; assay RSD ≤ 5.0% across sampling points (tube top/middle/bottom). Final limits to be confirmed using data from ≥3 consecutive batches (mean ± 3 SD)
Homogeneity	Macroscopically homogeneous; no graininess observed during smear test	Homogeneous throughout; no visible aggregates. Smear test on a glass plate: smooth film without grit; no creaming, coalescence, or phase separation
pH value	5.68±0.18 (25°C).	5.5 to 6.0 (25°C).
Net content (weight of container contents)	Not determined	Complies with applicable net content requirements: average net content not less than the labelled amount; no individual tube below the maximum permissible value
Microbiological purity	TAMC: none detected; TYMC: none detected; *S. aureus*, *C. acnes* and *E. coli*: none detected	Ph. Eur. (non-sterile products for cutaneous use): TAMC ≤ 10^3 CFU/g; TYMC ≤ 10^2 CFU/g; absence of *S. aureus*, *C. acnes* and *E. coli* in 1 g
Dynamic viscosity (25°C, 10 rpm)	28.54±3.65 Pa·s	24–32 Pa·s (25°C, 10 rpm)
Antibacterial activity of *CA* (random sampling, n = 5)	96.87±1.16; 98.41±0.89; 98.85±0.18; 98.28±0.45; 97.95±1.07. (≥ 95 %)	Provisional: not less than 90.0% and consistent across units (range within 5.0 units). To be confirmed with multi-batch data
Antibacterial activity of *SA* (random sampling, n = 5)	99.56±0.27; 98.68±0.80; 99.53±0.21; 98.93±0.31; 98.77±0.94. (≥ 95 %)	Provisional: not less than 90.0% and consistent across units (range within 5.0 units). To be confirmed with multi-batch data
Heavy metal content [229]	Cd 0.762±0.138 ppm; Pb 1.279±0.313 ppm; As 0.417±0.091 ppm; Hg 0.068±0.024 ppm; Cu 1.556±0.472 ppm	Hg ≤ 1 mg/kg; Pb ≤ 10 mg/kg; As ≤ 2 mg/kg; Cd ≤ 5 mg/kg (ppm ≈ mg/kg). Cu: monitored for trend control
Packaging integrity (tube seal/leakage)	Not determined	No leakage or seal failure after defined stress testing (e.g., centrifugation and thermal cycling); crimp/seal intact
Irritation potential (HET-CAM test, 300 s)	Secure; no bleeding, blood clots or inflammation (300 s)	No bleeding, clotting or inflammatory reaction; outcome consistent with the negative control

5.5 Risk assessment

An integral part of pharmaceutical development is Quality Risk Management (QRM), which involves a systematic process for identifying, analysing, and evaluating risks that may affect the quality of the medicinal product, the efficiency of the technological process, and the safety of the finished product. This approach is an important component of the Quality by Design (QbD) concept. It is regulated by the international guidelines ICH Q8 (Pharmaceutical Development), ICH Q9 (Quality Risk Management) and ICH Q10 (Pharmaceutical Quality System) [236-238].

According to ICH Q9, risk assessment includes identifying potential hazards, analysing their risks, and assessing their significance, taking into account the probability of occurrence and the severity of possible consequences for product quality [237, 239]. The results of the risk assessment form the basis for further identification of critical process parameters (CPPs) and critical quality attributes (CQAs), as well as for the development of control and monitoring strategies for the production process.

To systematise risk factors in pharmaceutical development, various risk management tools are used, such as cause-and-effect analysis methods. One common tool is the Ishikawa diagram, which identifies potential sources of risk and their relationships [237, 240, 239]. As part of the risk analysis, all influencing factors were systematised according to the 6M model and grouped into six categories: Manpower, Machines, Materials, Methods, Measurement, and Mother Nature (or Environment), identifying key sources of deviations in the technological process and assessing their impact on the product quality. The Ishikawa diagram (Fig. 5.7) shows the relationships between the influencing factors and causes of deviations in the technology.

For the **Materials** category, the main source of risk is the non-compliance of starting materials with established specifications or acceptance criteria. This category includes API, excipients, purified water, and primary packaging materials. Potential risk factors may include instability or variability in API characteristics, imbalance between the cream emulsion's hydrophilic and lipophilic phases, oxidation of the cream base due to deviations from the optimal composition, insufficient preservative content, microbiological contamination of water, or packaging tightness issues.

For the **Machines & Equipment** category, potential risks include the equipment's technical and sanitary condition, as well as its settings. In particular, risks may arise from equipment wear, deviations in operating parameters (temperature, mixing speed, pressure), insufficient efficiency of cleaning systems, or violations of the tightness of technological units. Key technological process units associated with risks in this category include the homogeniser, heat exchanger, storage capacity, vacuum system, dosing equipment, and the overall tightness of the production system.

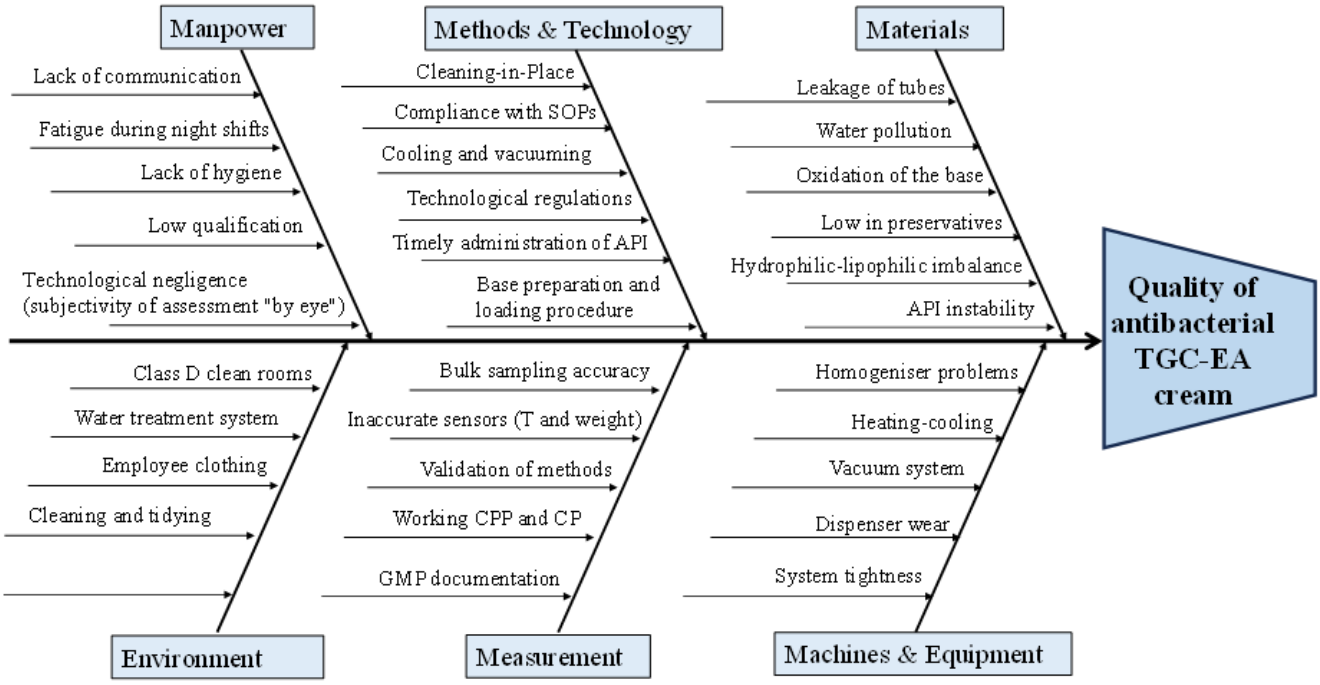


Fig. 5.7 Ishikawa (fishbone) diagram of obtaining antibacterial TGC-EA cream

The **Methods & Technology** category covers risks associated with process parameters and operation sequence. Problems can arise from violations of the component introduction sequence, incorrect ingredient weighing, or deviations from established process parameters. Compliance with technological regulations is also key, including control of the melting temperature, homogenisation speed and duration, mass holding time, cooling and vacuuming parameters. Possible cross-contamination due to insufficient sanitation efficiency (Cleaning-in-Place) is also a risk.

For the **Measurement** category, potential risks relate to measurement accuracy and the effectiveness of process control. Sources of risk may include sampling errors,

insufficient analytical measurement accuracy, the use of uncalibrated measuring equipment, or the application of unvalidated analytical methods. Particular attention is paid to the control of critical process parameters (CPPs) and critical control points (CCPs), which directly impact the quality of the medicinal product [237, 241]. An important element of the quality assurance system is maintaining production documentation in accordance with good manufacturing practice (GMP) requirements.

The **Manpower** category includes risks associated with human factors, such as personnel qualifications, compliance with hygiene requirements, correct performance of technological operations, and the accuracy of interpretation of measurement results. Insufficient communication between employees and staff overwork, or violations of production discipline can also negatively impact the production process.

The **Environment** category covers factors of production premises, such as microclimate parameters, air cleanliness and microbiological contamination. In the production of creams, it is particularly important to maintain appropriate levels of cleanliness on production premises, control air parameters, and ensure compliance with GMP requirements for personnel's protective clothing [240, 242].

The results of the risk analysis, including the main risk categories and factors, their potential impact, and possible control strategies, are shown in Table 5.13.

For each identified risk factor, corresponding critical process parameters (CPPs) are established that affect the critical quality attributes (CQAs). Control of temperature, homogenisation speed and time, the sequence of phase introduction, and equipment cleaning parameters minimises the risk of cream instability and cross-contamination.

Table 5.13

Main risk factors, their potential impact, CPP/CQA and control strategy

Risk category	**Potential risk factor**	**Potential impact on quality**	**CPP / CQA**	**Control strategy**
Materials	Variability of API characteristics	Change in the medicine activity and instability	API content, potency, stability	Incoming control of raw materials and stability testing
Materials	Violation of the emulsion phase ratio	Phase inversion, cream instability	Emulsion stability	Control of recipe, mixing process
Materials	Water contamination	Microbiological product contamination	Bioburden	Control of water quality, process sterility
Machines & Equipment	Unstable operation of the homogeniser	Cream heterogeneity	Homogenisation speed, time	Equipment qualification, parameter monitoring
Machines & Equipment	Deviation of the temperature regime	Base melting disorder, cream stability	Temperature	Monitoring, heating/cooling control, maintenance
Methods & Technology	Violation of the component introduction sequence	Phase inversion, instability	Process sequence, phase addition order	Process standardisation, SOP
Methods & Technology	Insufficient efficiency of equipment cleaning	Cross contamination	Cleaning validation, residuals	Validation of cleaning processes (CIP/SIP)
Measurement	Uncalibrated equipment, inaccurate methods	Quality control deviation	CPP monitoring, assay	Calibration and validation of analytical methods
Manpower	Insufficiently qualified personnel	Operation errors	Personnel competency	Training, personnel certification, SOP
Environment	Violation of cleanliness class, microbiological contamination	Product contamination	Cleanroom classification	Control of cleanrooms, microbiological monitoring, and technological clothing

The risk analysis results were the basis for further determination of CQAs and CPPs and were applied in developing a process control strategy in accordance with the principles of QbD [236-238, 241, 242] (Table 5.14).

Each CPP directly affects the CQA, i.e., it controls critical quality attributes. IPC/control methods are linked to each CQA and meet GMP requirements. Table 5.14 is consistent with Ishikawa diagram categories.

Table 5.14

Correlation between CPP, CQA and IPC at each stage of production

Stage	Critical Process Parameter (CPP)	Related CQA	Control / IPC
0. Weighing raw materials	Mass of each component; balance calibration; line/environmental status	Accuracy of ingredient dosage; uniformity	Equipment calibration; cleanliness verification
1. Water cream base preparation	Heating T (70–75°C); agitator speed; mixing time; dissolution completeness	Complete dissolution of preservatives/EDTA/surfactant; solution clarity	Temperature monitoring; visual inspection
2. Oil cream base preparation	Heating T (75–80°C); agitator speed; holding time	Homogeneity of oil base; melting completeness	Temperature probe; visual inspection
3. API dispersion in oil	Processing T (58–65°C); anchor and high-shear speed; dispersion time	Uniform dispersion of TGC-EA and vitamin E; no agglomerates	Visual inspection; particle size check if available
4. Aqueous hydrocolloid phase	Charging T; homogeniser speed; anchor agitation; dispersion & hydration time	Homogeneous hydrocolloid gel; absence of "fish eyes"	Visual inspection; viscosity check
5. Emulsification	Phase T before combining (70–75°C); homogeniser speed; vacuum; anchor agitation; emulsification time	Emulsion type (O/W); fine and uniform droplet distribution; absence of phase separation or air bubbles	IPC: visual inspection, droplet size monitoring if feasible
6. Cooling	Cooling rate (1.0–1.5°C/min); anchor agitation	Cream texture & rheology; stability of emulsion	T recording; visual/tactile inspection
7. Unloading / intermediate storage	Product T; transfer method; auxiliary gas	Quality of bulk cream: texture, viscosity, pH, API content	IPC: sampling and analysis per QC
8. Filling	Bulk T at filling; vacuum transfer; tube filler settings	Filling accuracy; container integrity; cream uniformity	Net weight check; leak test; visual inspection
9–10. Secondary packaging	Completeness of packaging components; correctness of printed info; count accuracy	Final product integrity; batch traceability	Visual inspection; count verification
Post-batch cleaning	CIP/SIP cycle parameters (agent C, T, t)	Absence of residues; prevention of cross-contamination	Validation of cleaning: final rinse checks

Conclusions to Chapter 5

1. The product formed a homogeneous, viscous solid upon cooling, was completely opaque, had a fine texture, was easy to apply and was free of grittiness. Thus, the cream is fully acceptable in terms of appearance and user experience, both of which are required quality attributes for semi-solid topical preparations. The favourable organoleptic profile confirms the structural uniformity and the required consistency of the produced batch.

2. pH and dynamic viscosity constitute the core physicochemical dual indices for quality control of the tube product. The measured pH was 5.68±0.18, indicating a mildly acidic environment with limited variability across samples. pH stability is relevant to both skin compatibility and the stability of the chemical microenvironment in the final dosage form. Dynamic viscosity (28.54±3.65 Pa·s) confirms a semi-solid structure capable of supporting controllable tube extrusion and spread-film formation. Importantly, the DV result is consistent with the sensory observations of homogeneity and very good texture, with easy application, thereby converting subjective tactile impressions into an auditable rheological parameter and enhancing the objectivity and examinability of the quality-control conclusions.

3. The microbiological findings demonstrated compliance with the applied microbiological quality requirements for aerobic bacteria, moulds/yeasts, as well as *Staphylococcus aureus*, *Cutibacterium acnes*, and *Escherichia coli*. All were absent and thus corresponded to "must not be detected" requirements. For topical antibacterial creams, microbial control has direct risk significance because a high initial bioburden may increase contamination and proliferation risk during use.

4. The antibacterial activity, among five randomly selected samples, varied within a narrow range (96.87–98.85). This fact indicates the product's stability and satisfactory homogeneity within the batch. Thus, the product is described not only as aesthetically and physicochemically acceptable, but also as functionally reproducible.

5. The HET-CAM results showed no observable haemorrhage, vascular lysis or coagulation during the 300-s observation period. Under the conditions of the applied model, the tested cream therefore demonstrated low irritation potential. This

conclusion provides experimental support for a low risk of acute irritation within the context of this assay.

6. The investigated heavy metal contents (Cd 0.762±0.138, Pb 1.279±0.313 ppm, As 0.417±0.091 ppm and Hg 0.068±0.024 ppm) did not exceed the specified limits, indicating controlled levels of the investigated elemental impurities in the manufactured product. These results provide quantitative evidence that the investigated elemental impurity levels were adequately controlled in the manufactured product but also serve as a quantitative guideline for future batch comparisons and for changes in the role of raw material risk associated with process modifications. For products with naturally occurring components, heavy metal monitoring is valuable not only for release decisions but also for creating a database of long-term quality trends.

7. Antibacterial cream with dry extract TGC-EA demonstrated exceptional comprehensive stability during 12 months of observation. This conclusion is based on the results of the study of appearance, dynamics of microscopic particle size, rheological characteristics, pH value, microbiological safety and antibacterial activity *in vitro*. All test parameters met the relevant pharmaceutical quality control requirements. The stability of the cream is ensured by the rational selection of structuring agents (stearic acid/cetearyl alcohol), rheological modifiers (*Acacia Senegal* and *Astragalus Gummifer Gum*) and an antioxidant/preservative system in the composition of the preparation. Thus, the obtained stability data support a shelf life of at least 12 months under the investigated conditions and provide a basis for further pharmaceutical development, scale-up, and subsequent preclinical and clinical evaluation.

The results of the research in Chapter 5 are contained in the following published articles: [243-246].

General conclusions

The dissertation addresses a relevant scientific and applied problem concerning the pharmaceutical development of a topical antibacterial cream based on *Speranskia tuberculata* (Bunge) Baill. The feasibility of using TGC-EA as the API was substantiated through phytochemical, microbiological, cytocompatibility and pharmaceutical-technological investigations. The obtained results provide a scientific and technological basis for further scale-up, preclinical investigation and subsequent clinical evaluation of the developed formulation.

1. Based on the analysis and systematisation of these literary sources, the prospects of using *Speranskia tuberculata* as a source of biologically active substances were substantiated. Information on the botanical features, traditional use and pharmacological properties of the plant was summarised. *Speranskia tuberculata* exhibits pronounced anti-inflammatory, analgesic, antitumor, and antibacterial activities, confirming the feasibility of further pharmacognostic and pharmaceutical studies. The difference between the botanical species and the term Tou Gu Cao in traditional Chinese medicine is clarified.

2. Methods for obtaining dry extracts of *Speranskia tuberculata* (Bunge) Baill. using solvents of different polarity—ethyl acetate (EA), petroleum ether (PE), n-butanol and purified water—were developed. The *in vitro* biological activities of the obtained extracts were investigated with respect to cytotoxicity, antioxidant activity and antibacterial activity. TGC-EA demonstrated the strongest cytotoxic activity in four investigated human tumour cell lines (A549, A-375, HeLa and HepG2), with IC_{50} values of approximately 49–53 μg/mL. These results describe *in vitro* cytotoxic activity and were not interpreted as evidence of antitumour therapeutic efficacy. The cytocompatibility of TGC-PE and TGC-EA was additionally evaluated in HaCaT human immortalised keratinocytes and HFF-1 human fibroblasts. Cell viability remained above 85% throughout the investigated concentration range of 12.5–1000 μg/mL, and CC_{50} was not reached for either extract (>1000 μg/mL), indicating favourable *in vitro* cytocompatibility under the applied experimental conditions. Antioxidant activity was evaluated using the DPPH radical-scavenging and superoxide-anion radical-scavenging assays, with the highest activity observed for EA (IC_{50} = 20–42 μg/mL) and PE extracts (IC_{50} = 29–35 μg/mL). Antibacterial activity was investigated against *Staphylococcus aureus*,

Escherichia coli and *Cutibacterium acnes*; TGC-EA demonstrated the strongest antibacterial activity, with IC_{50} values of approximately 112–135 μg/mL, whereas the other extracts did not reach IC_{50} within the investigated concentration range.

3. The composition of *Speranskia tuberculata* extracts was investigated by LC/MS with identification of components with peak intensities ≥1% of the maximum. About 300 peaks of organic compounds were detected in the PE extract, and 75 unique, intense components were detected in the EA. A correlation was established with the yield of dry extracts (3.63 g/100 g of raw material for PE and 1.53 g/100 g for EA); at the same time, TGC-EA and TGC-PE extracts show 2–7 times higher saturation compared to others. The key importance of establishing the relationship between the composition of extracts and their experimentally studied *in vitro* biological activity lies in the detailed classification of individual substances. In the most promising EA extracts, more than 75% of the identified components belong to four dominant classes with proven biological activity: flavonoids, phenols, terpenoids, and alkaloids. Their total content was determined by spectrophotometry. The method was validated for dry extracts and the finished dosage form with respect to specificity, linearity, precision, recovery, and robustness. Despite the higher dry-residue content in PE extracts, EA extracts have higher levels of these bioactive compounds. Ethyl acetate provides the highest yield of phenolic compounds, flavonoids and alkaloids. In EA extracts, the classes of compounds are distributed in decreasing concentration as follows: phenols, terpenoids, flavonoids, and alkaloids.

4. The minimum required content of biologically active substances with pronounced antibacterial effect in the EA extract has been established: phenols - ≥255 mg GE/g, flavonoids - ≥153 mg PE/g, terpenoids - ≥192 mg UAE/g, alkaloids - ≥62 mg TE/g. The obtained values justify the use of these indicators as critical parameters of API quality in the development of dosage form technology. Specification requirements for the quality of the active pharmaceutical ingredient - dry EA extract of the medicinal plant *Speranskia tuberculata* have been substantiated and established.

5. A rational technology and composition of an antibacterial cream containing dry EA extract of the *Speranskia tuberculata* plant as an active pharmaceutical ingredient have been developed. The optimal composition of the cream has been determined through mathematical modelling and the construction of the L9 orthogonal design matrix. The

API content was experimentally determined and justified at 0.10 g of TGC-EA dry extract per 100 g of cream, corresponding to 0.10% (w/w), based on the results of the *in vitro* antibacterial activity studies against *Staphylococcus aureus* and *Cutibacterium acnes*. The optimal balance of hydrophilic components, the total mass fraction of emulsifiers, and the rational ratio of water to oil phases were established, ensuring the formation of a stable o/w (oil-in-water) emulsion system with satisfactory rheological properties, an optimal pH, and a homogeneous texture. According to microbiological, physicochemical and organoleptic indicators, the developed cream meets the requirements of the regulatory and technical documentation regarding the specification characteristics.

6. A technological scheme for the production of antibacterial cream containing dry EA extract of the *Speranskia tuberculata* plant as an active pharmaceutical ingredient has been developed. The proposed technological cycle for cream production comprises 10 stages. The technological scheme indicates Critical Process Parameters and Control Points. Appropriate equipment was selected to produce 300 kg of cream per cycle, which was subsequently packaged in 30 g tubes. A risk analysis of the quality of the medicinal product during production was conducted, and an Ishikawa cause-and-effect diagram was constructed to identify critical points in the finished product production process.

7. The quality of the developed cream was assessed at the stages of the release of the bulk product and the finished dosage form. The cream is a homogeneous, semi-solid, milky-white mass with a smooth texture, free of phase separation or graininess, confirming the uniform distribution of active substances and the stability of the system. Physicochemical indicators are standardised by pH and dynamic viscosity values. The pH value (5.68±0.18) falls within the physiological pH range of the skin (5.5–6.5), indicating the drug's compatibility with the skin. The dynamic viscosity (28.54±3.65 Pa·s) confirms the cream's optimal rheological properties, including ease of extrusion and application. The contents of the investigated elemental impurities (Cd, Pb, As and Hg) did not exceed the specified limits, indicating that these selected elemental impurity levels were adequately controlled in the developed product. Stability studies under thermal and mechanical loads showed no syneresis, separation of the oil phase, or emulsion delamination. The combination of acacia gum and astragalus provides colloidal stability of the cream at 100% and preserves its consumer properties during storage.

8. The microbiological purity, irritation potential and *in vitro* antibacterial activity of the developed cream were investigated. Microbiological testing confirmed compliance with the established requirements for the tested microbial quality parameters. The irritation potential of the bulk and tube-filled cream was assessed using the HET-CAM method. During the 300-s observation period, no haemorrhage, vascular lysis or coagulation was observed, indicating a low irritation potential under the conditions of the applied experimental model. The developed cream also demonstrated reproducible *in vitro* antibacterial activity against *Staphylococcus aureus* and *Cutibacterium acnes*. For antibacterial activity, five randomly selected samples were examined for both bulk and packaged cream. Typical antibacterial rates varied within a narrow range (96.8–98.8%). This relatively narrow distribution indicates that the *in vitro* antibacterial activity remained reproducible across the investigated samples. The antibacterial activity results were also consistent with the observed macroscopic homogeneity and stable pH and dynamic viscosity values. Thus, the product demonstrated reproducible *in vitro* antibacterial performance under the applied experimental conditions.

9. The 12-month tests confirmed the high stability of the antibacterial cream in tubes. Throughout the entire observation period, the pH and dynamic viscosity indicators remained stable, with fluctuations of no more than 7%. Organoleptic properties (colour, odour, uniformity) and the particle size distribution did not change, indicating the absence of demulsification or chemical degradation. The high stability of the system is ensured by a rational combination of structure-forming agents (stearic acid/cetearyl alcohol) and rheological modifiers (Acacia Senegal and Astragalus Gummifer Gum), along with an antioxidant/preservative system. The product meets pharmaceutical quality standards, including microbiological purity and *in vitro* antibacterial activity. The obtained stability results support a shelf life of at least 12 months under the investigated storage conditions and provide a basis for further pharmaceutical development, scale-up and subsequent preclinical and clinical evaluation. So, the product meets the comprehensive quality control objective, characterised as “acceptable physicochemical properties + microbiological quality compliance + low irritation potential in the HET-CAM model + controlled elemental impurity levels”.

References

1. Dai, Z., Liao, X., Wieland, L. S., Hu, J., Wang, Y., Kim, T.-H., Liu, J.-P., Zhan, S., Robinson, N. (2022). Cochrane systematic reviews on traditional Chinese medicine: What matters-the quantity or quality of evidence? Phytomedicine: Int. J. Phytoth. Phytopharm., 98(153921), 153921. https://doi.org/10.1016/j.phymed.2021.153921

2. Fu, Y., Ju, M.-M., Ma, H.-C., Xin, P.-Y., He, C.-Z., Jia, D.-R., & Tian, B. (2016). Development and characterization of novel EST-SSR markers for *Speranskia tuberculata* (Euphorbiaceae). Applications in Plant Sciences, 4(10), 1600067. https://doi.org/10.3732/apps.1600067

3. Ye, J.-W., Wu, H.-Y., Fu, M.-J., Zhang, P., & Tian, B. (2021). Insights into the significance of the Chinese loess plateau for preserving biodiversity from the phylogeography of *Speranskia tuberculata* (Euphorbiaceae). Frontiers in Plant Science, 12, 604251. https://doi.org/10.3389/fpls.2021.604251

4. Yang, C., Ren, J., Xu, L., & Wang, M. (2013). Research of pharmacognostical authentication of herba *Speranskia*e *tuberculata*e. *Journal of Shanxi Medical College for Continuing Educatio*n, 23(2), 1–3. https://doi.org/10.13863/j.issn1001-4454

5. Lu, R. (2020). Study on the quality standard and its ecological suitability analysis of *Speranskia tuberculata*. *Chemical Engineering Design Communications*, 49(2), 197–199. https://doi.org/10.16155/j.0254-1793.2020 -0717

6. Jin, Y., Li, S., & Lou, Z. (1982). Morphological and histological studies of the Chinese herbal medicine Tougucao. *Acta Pharmaceutica Sinica*, 17(12). https://doi.org/10.16438/j.0513-4870.1982.12.009

7. Wu, T. H., Li, F. Y., & Wu, Y. C. (2026). Pharmacological effects of *Tougucao* and progress in its application in traditional Chinese traumatology and orthopaedics. *Zhongguo Minjian Liaofa, 34*(5), 111–115. https://doi.org/10.19621/j.cnki.11-3555/r.2026.0534

8. Zhao, W. X., Jiang, H. B., Wang, Y., Jiang, L., & Wen, P. H. (2011). Research progress on the chemical constituents of *Tougucao*. *Yingyong Huagong, 40*(4), 696–697, 703. https://doi.org/10.3969/j.issn.1671-3206.2011.04.039

9. Zhang, Y. F., Zeng, C. Q., Huang, W., & Gao, S. (2010). Research progress on

the chemical constituents and pharmacological effects of TGC. *Zhonghua Zhongyiyao Xuekan, 28*(9), 1960–1962. https://doi.org/10.13313/j.issn.1673-4890.2010.09.5006

10. Suo, J. H., & Yi, X. F. (2009). Chemical constituents and pharmacological effects of Zhenzhu *Tougucao*. *Nei Menggu Minzu Daxue Xuebao (Ziran Kexue Ban), 24*(5), 506–508. https://doi.org/10.3969/j.issn.1671-0185.2009.05.010

11. Wang, X., Cui, J. R., Xiao, Z. P., Zhang, Y. H., Li, C. L., Zhao, Y. Y., & Cai, S. Q. (1998). Comparison of the anti-inflammatory and analgesic effects of *Tougucao* medicinal materials. *Beijing Yike Daxue Xuebao, 30*(2). https://doi.org/10.3321/j.issn:1671-167X.1998.02.017

12. Shen, G., Jing, Y. F., Chen, J., Ge, R., & Yang, G. E. (2019). Correlation analysis between HPLC fingerprint and anti-inflammatory activity of *Speranskiae Tuberculatae Herba*. *Chinese Journal of Experimental Traditional Medical Formulae, 12*(25), 174–180. https://doi.org/10.13422/j.cnki.syfjx.20191215

13. Li, J. J., Zhang, X. F., Xu, Z. R., Zhang, X., Shi, X. H., & Duan, Y. T. (2026). Evaluation of quality differences in Zhenzhu *Tougucao* from different producing areas by multi-index quantification and chemometric analysis combined with the CRITIC-TOPSIS method. *Zhongcaoyao, 57*(2), 665–674. https://doi.org/10.7501/j.issn.0253-2670.2026.02.025

14. Bao, X. H., Zhao, H. Y., Wei, Y. M., Wang, S. L., Xiao, W. Y., & Lei, Y. (2019). Study on the microwave extraction process and antioxidant activity of total flavonoids from Zhenzhu *Tougucao*. *Guangzhou Huagong, 47*(19), 63–65. https://doi.org/10.3969/j.issn.1001-9677.2019.19.027

15. Li, C., Zhang, C. Z., & Hu, F. D. (2000). Chemical constituents of *Tougucao*. *Zhongguo Zhongyao Zazhi, 25*(5), 291. https://doi.org/10.3321/j.issn:1001-5302.2000.05.014

16. Zeng, C. Q., Gao, S., Shen, Y., & Zhang, Y. F. (2009). Effect of *Tougucao* extracts on the *in vitro* proliferation of bacteria. *Liaoning Zhongyi Zazhi, 36*(11), 1952–1954. https://doi.org/10.3969/j:issn.2095-1191.2015.08.1522

17. Wang, F., Gao, S., Qiu, S. S., Yan, F., & Yang, X. W. (2011). Study on the antibacterial effect of Tougucao extract. Liaoning Zhongyi Zazhi, 38(4), 709–710.

https://doi.org/10.20087/j.cnki.1672-8114.2011.10.027

18. Lin, H. B., Yu, M., Guan, R. W., Guo, R. Q., Jiang, L., Shao, X., Liu, G. X., Wang, X. X., Zhan, Z. L., & Lin, J. Q. (2025). Herbal textual research on *Tougucao* in classical famous prescriptions. *Chinese Journal of Experimental Traditional Medical Formulae, 31*(23), 211–222. https://doi.org/10.13422/j.cnki.syfjx.20250761

19. Xiang, G. F. (2000). A brief discussion on *Tougucao*. *Heilongjiang Yiyao, 13*(1), 39–40. https://doi.org/10.3969/j.issn.1006-2882.2000.01.037

20. Han, Z. X., Tao, X. Q., Cheng, L., & Zhang, C. G. (2024). Research progress in the quality control of traditional Chinese medicine formula granules. *Shenyang Yaoke Daxue Xuebao, 41*(7), 847–854. https://doi.org/10.14066/j.cnki.cn211349/r.2022.1051

21. Zhag, Z. Q., Shen, J. M., Wang, X. Y., Ma, Z. L., Du, W. B., Chen, J. R., & Li, X. (2022). *A quality control method for a Zhenzhu Tougu Cao formula granule* (Chinese Patent No. CN115420844B). China Nat. Intellectual Property Administration

22. Xu, M. (2017). *Preparation process of fresh-pressed spray-dried granule decoction pieces of Tougucao* (Chinese Patent No. CN107485639A). China Nat. Intellectual Property Administration

23. Guo, D. X., Yu, S. S., Guo, Y. H., Cui, X. F., Qin, H., Cui, W. L., Lin, Y. Q., & Liu, H. C. (2022). Analysis of technical issues and countermeasures in the development of standards for traditional Chinese medicine formula granules. *Chinese J. Exp. Tradit. Medical Formulae, 28*(17), 188–194. https://doi.org/10.13422/j.cn ki. syfjx. 20220453

24. Wang, M., Li, Y., Pan, T., & Jia, N. (2024). Plant natural compounds in the cancer treatment: A systematic bibliometric analysis. *Heliyon*, 10(14), e34462. https://doi.org/10.1016/j.heliyon.2024.e34462

25. Shaik, B. B., Katari, N. K., & Jonnalagadda, S. B. (2022). Role of Natural Products in Developing Novel Anticancer Agents: A Perspective. *Chemistry & Biodiversity*, 19(11), e202200535. https://doi.org/10.1002/cbdv.202200535

26. Wani, M. C., & Horwitz, S. B. (2014). Nature as a remarkable chemist: a personal story of the discovery and development of Taxol. *Anti-Cancer Drugs*, 25(5), 482–487. https://doi.org/10.1097/CAD.0000000000000063

27. Lai, J. I., Chao, T. C., Liu, C. Y., Huang, C. C., & Tseng, L. M. (2022). A

systematic review of taxanes and their side effects in metastatic breast cancer. *Frontiers in Oncology*, 12, 940239. https://doi.org/10.3389/fonc.2022.940239

28. Škubník, J., Pavlíčková, V., Ruml, T., & Rimpelová, S. (2021). Current Perspectives on Taxanes: Focus on Their Bioactivity, Delivery and Combination Therapy. *Plants* (Basel, Switzerland), 10(3), 569. https://doi.org/10.3390/plants10030569

29. Schiller, J. H., Harrington, D., Belani, C. P., Langer, C., Sandler, A., Krook, J., Zhu, J., Johnson, D. H., & Eastern Cooperative Oncology Group (2002). Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. The *New England Journal of Medicine*, 346(2), 92–98. https://doi.org/10.1056/NEJMoa011954

30. Tong, Y., Luo, Y., & Gao, W. (2021). Biosynthesis of paclitaxel using synthetic biology. *Phytochemistry Rev.* 21, 863–877. https://doi.org/10.1007/s11101-021-09766-0

31. Wang, X., Zhuang, Y., Wang, Y., Jiang, M., & Yao, L. (2023). The recent developments of camptothecin and its derivatives as potential anti-tumour agents. *Eur. J. Medicinal Chemistry*, 260, 115710. https://doi.org/10.1016/j.ejmech.2023.115710

32. Garg, N., Farhat, J., Dhankhar, S., Chauhan, S., Bala, R., Madaan, R., Sharma, H., Saini, M., Singh, T. G., Aldahish, A. A., Almarhoon, Z. M., Al-Omari, B., & Sharifi-Rad, J. (2025). Vincristine in Cancer Therapy: Mechanisms, Efficacy, and Future Perspectives. *Current Medicinal Chemistry*, 32(32), 6941–6959. https://doi.org/10.2174/0109298673319496240911060138

33. Montecucco, A., Zanetta, F., & Biamonti, G. (2015). Molecular mechanisms of etoposide. *EXCLI Journal*, 14, 95–108. https://doi.org/10.17179/excli2015-561

34. de Weger, V. A., Beijnen, J. H., & Schellens, J. H. (2014). Cellular and clinical pharmacology of the taxanes docetaxel and paclitaxel--a review. *Anti-Cancer Drugs*, 25(5), 488–494. https://doi.org/10.1097/CAD.0000000000000093

35. Zoi, V., Kyritsis, A. P., Galani, V., Lazari, D., Sioka, C., Voulgaris, S., & Alexiou, G. A. (2024). The Role of Curcumin in Cancer: A Focus on the PI3K/Akt Pathway. *Cancers*, 16(8), 1554. https://doi.org/10.3390/cancers16081554

36. Chaudhary, P., Janmeda, P., Docea, A. O., Yeskaliyeva, B., Abdull Razis, A. F., Modu, B., Calina, D., & Sharifi-Rad, J. (2023). Oxidative stress, free radicals and antioxidants: potential crosstalk in the pathophysiology of human diseases. *Frontiers in*

Chemistry, 11, 1158198. https://doi.org/10.3389/fchem.2023.1158198

37. Muscolo, A., Mariateresa, O., Giulio, T., & Mariateresa, R. (2024). Oxidative Stress: The Role of Antioxidant Phytochemicals in the Prevention and Treatment of Diseases. *Int. J. Molecular Sciences*, 25(6), 3264. https://doi.org/10.3390/ijms25063264

38. Tufail, T., Fatima, S., Bader Ul Ain, H., Ikram, A., Noreen, S., Rebezov, M., Al-Farga, A., Saleh, R., & Shariati, M. A. (2025). Role of Phytonutrients in the Prevention and Treatment of Chronic Diseases: A Concrete Review. *ACS Omega*, 10(13), 12724–12755. https://doi.org/10.1021/acsomega.4c02927

39. Tighe, S. P., Akhtar, D., Iqbal, U., & Ahmed, A. (2020). Chronic Liver Disease and Silymarin: A Biochemical and Clinical Review. *Journal of Clinical and Translational Hepatology*, 8(4), 454–458. https://doi.org/10.14218/JCTH.2020.00012

40. Jaffar, H. M., Al-Asmari, F., Khan, F. A., Rahim, M. A., & Zongo, E. (2024). Silymarin: Unveiling its pharmacological spectrum and therapeutic potential in liver diseases-A comprehensive narrative review. *Food Science & Nutrition*, 12(5), 3097–3111. https://doi.org/10.1002/fsn3.4010

41. Mohammadi, S., Ashtary-Larky, D., Asbaghi, O., Farrokhi, V., Jadidi, Y., Mofidi, F., Mohammadian, M., & Afrisham, R. (2024). Effects of silymarin supplementation on liver and kidney functions: A systematic review and dose-response meta-analysis. *Phytoth. Res. PTR*, 38(5), 2572–2593. https://doi.org/10.1002/ptr.8173

42. Dai, J., Qiu, L., Lu, Y., & Li, M. (2024). Recent advances of traditional Chinese medicine against cardiovascular disease: overview and potential mechanisms. *Frontiers in Endocr.*, 15, 1366285. https://doi.org/10.3389/fendo.2024.1366285

43. Zhao, J., Liang, H., Li, Y., Xu, Z., Li, X., Zhao, L., Zhang, M., & Duan, X. (2024). Impact of Traditional Chinese Medicine Antioxidants on Oxidative Stress and Drug-Induced Liver Injury: A Review. *Medical Sci. Monitor: Int. Med. J. Experimental and Clinical Research*, 30, e945147. https://doi.org/10.12659/MSM.945147

44. Ren, Q., Lin, J., Wang, H., Huang, M., Tan, X., Huang, W., & Xu, Y. (2023). Effects of ginseng consumption on the biomarkers of oxidative stress: A systematic review and meta-analysis. *Phytoth. Res.: PTR*, 37(8), 3262–3274. https://doi.org/10.1002/ptr.7893

45. Meim, X. D., Cao, Y. F., Che, Y. Y., Li, J., Shang, Z. P., Zhao, W. J., Qiao, Y. J., & Zhang, J. Y. (2019). Danshen: a phytochemical and pharmacological overview. *Chin. J. Nat. Medicines*, 17(1), 59–80. https://doi.org/10.1016/S1875-5364(19)30010-X

46. Shi, Y., & Ma, P. (2024). Pharmacological effects of Astragalus polysaccharides in treating neurodegenerative diseases. *Frontiers in Pharmacology*, 15, 1449101. https://doi.org/10.3389/fphar.2024.1449101

47. Chi, F., Cheng, C., Liu, K., Sun, T., Zhang, M., Hou, Y., & Bai, G. (2025). Baicalein disrupts the KEAP1-NRF2 interaction to alleviate oxidative stress injury by inhibiting M1 macrophage polarisation. *Free Radical Biology & Medicine*, 227, 557–569. https://doi.org/10.1016/j.freeradbiomed.2024.12.036

48. Faghihzadeh, F., Adibi, P., Rafiei, R., & Hekmatdoost, A. (2014). Resveratrol supplementation improves inflammatory biomarkers in patients with nonalcoholic fatty liver disease. *Nutrition Research* (New York, N.Y.), 34(10), 837–843. https://doi.org/10.1016/j.nutres.2014.09.005.

49. Kim, J. K., & Park, S. U. (2018). Quercetin and its role in biological functions: an updated review. *EXCLI J.*, 17, 856–863. https://doi.org/10.17179/excli2018-1538

50. Zhou, Q., Zhang, N., Hu, T., Xu, H., Duan, X., Liu, B., Chen, F., & Wang, M. (2022). Dietary phenolic-type Nrf2-activators: implications in the control of toxin-induced hepatic disorders. *Food & Function*, 13(10), 5480–5497. https://doi.org/10.1039/D1FO04237H

51. Forni, C., Facchiano, F., Bartoli, M., Pieretti, S., Facchiano, A., D'Arcangelo, D., Norelli, S., Valle, G., Nisini, R., Beninati, S., Tabolacci, C., & Jadeja, R. N. (2019). Beneficial Role of Phytochemicals on Oxidative Stress and Age-Related Diseases. *BioMed Research Int.*, 2019, 8748253. https://doi.org/10.1155/2019/8748253

52. Woo, S., Marquez, L., Crandall, W. J., Risener, C. J., & Quave, C. L. (2023). Recent advances in the discovery of plant-derived antimicrobial natural products to combat antimicrobial-resistant pathogens: insights from 2018-2022. *Natural Product Reports*, 40(7), 1271–1290. https://doi.org/10.1039/d2np00090c

53. Achan, J., Talisuna, A. O., Erhart, A., Yeka, A., Tibenderana, J. K., Baliraine, F. N., Rosenthal, P. J., & D'Alessandro, U. (2011). Quinine, an old anti-malarial drug in

a modern world: role in the treatment of malaria. *Malaria Journal*, 10, 144. https://doi.org/10.1186/1475-2875-10-144

54. Kairey, L., Agnew, T., Bowles, E. J., Barkla, B. J., Wardle, J., & Lauche, R. (2023). Efficacy and safety of Melaleuca alternifolia (tea tree) oil for human health: A systematic review of randomised controlled trials. *Frontiers in Pharmacology*, 14, 1116077. https://doi.org/10.3389/fphar.2023.1116077

55. Duda-Madej, A., Viscardi, S., Bazan, H., & Sobieraj, J. (2025). Exploring the Role of Berberine as a Molecular Disruptor in Antimicrobial Strategies. *Pharmaceuticals* (Basel), 18(7), 947. https://doi.org/10.3390/ph18070947

56. Nakamoto, M., Kunimura, K., Suzuki, J. I., & Kodera, Y. (2020). Antimicrobial properties of hydrophobic compounds in garlic: Allicin, vinyldithiin, ajoene and diallyl polysulfides. *Experimental and Therapeutic Medicine*, 19(2), 1550–1553. https://doi.org/10.3892/etm.2019.8388

57. Bhatwalkar, S. B., Mondal, R., Krishna, S. B. N., Adam, J. K., Govender, P., & Anupam, R. (2021). Antibacterial Properties of Organosulfur Compounds of Garlic (*Allium sativum*). *Frontiers in Microbiology*, 12, 613077. https://doi.org/10.3389/fmicb.2021.613077

58. White, N. J., & Chotivanich, K. (2024). Artemisinin-resistant malaria. *Clinical Microbiology Reviews*, 37(4), e0010924. https://doi.org/10.1128/cmr.00109-24

59. Yu, M., Jin, X., Liang, C., Bu, F., Pan, D., He, Q., Ming, Y., Little, P., Du, H., Liang, S., Hu, R., Li, C., Hu, Y. J., Cao, H., Liu, J., Fei, Y. (2020). Berberine for diarrhea in children and adults: a systematic review and meta-analysis. *Therap. Adv. in Gastroent*. 13, 1756284820961299. https://doi.org/10.1177/1756284820961299

60. Hao, S., Yang, D., Zhao, L., Shi, F., Ye, G., Fu, H., Lin, J., Guo, H., He, R., Li, J., Chen, H., Khan, M. F., Li, Y., & Tang, H. (2021). EGCG-Mediated Potential Inhibition of Biofilm Development and Quorum Sensing in *Pseudomonas aeruginosa*. *Int. Journal of Molecular Sciences*, 22(9), 4946. https://doi.org/10.3390/ijms22094946

61. Li, J., Feng, S., Liu, X., Jia, X., Qiao, F., Guo, J., & Deng, S. (2022). Effects of Traditional Chinese Medicine and Its Active Ingredients on Drug-Resistant Bacteria. *Frontiers in Pharmacology*, 13, 837907. https://doi.org/10.3389/fphar.2022.837907

62. Zhou, H., Wang, W., Cai, L., & Yang, T. (2023). Potentiation and Mechanism of Berberine as an Antibiotic Adjuvant Against Multidrug-Resistant Bacteria. *Infect. & Drug Resist.*, 16, 7313–7326. https://doi.org/10.2147/IDR.S431256

63. El-Tarabily, K. A., El-Saadony, M. T., Alagawany, M., Arif, M., Batiha, G. E., Khafaga, A. F., Elwan, H. A. M., Elnesr, S. S., & E Abd El-Hack, M. (2021). Using essential oils to overcome bacterial biofilm formation and their antimicrobial resistance. *Saudi J. Biological Sci.*, 28(9), 5145–5156. https://doi.org/10.1016/j.sjbs.2021.05.033

64. Pang, D., Huang, Z., Li, Q., Wang, E., Liao, S., Li, E., Zou, Y., & Wang, W. (2021). Antibacterial Mechanism of Cinnamaldehyde: Modulation of Biosynthesis of Phosphatidylethanolamine and Phosphatidylglycerol in *Staphylococcus aureus* and *Escherichia coli. Journal of Agricultural and Food Chemistry*, 69(45), 13628–13636. https://doi.org/10.1021/acs.jafc.1c04977

65. Hengge R. (2019). Targeting Bacterial Biofilms by the Green Tea Polyphenol EGCG. *Molecules* (Basel), 24(13), 2403. https://doi.org/10.3390/molecules24132403

66. Alum, E. U., Gulumbe, B. H., Izah, S. C., Uti, D. E., Aja, P. M., Igwenyi, I. O., & Offor, C. E. (2025). Natural product-based inhibitors of quorum sensing: A novel approach to combat antibiotic resistance. *Biochemistry and Biophysics Reports*, 43, 102111. https://doi.org/10.1016/j.bbrep.2025.102111

67. Si, Z., Pethe, K., & Chan-Park, M. B. (2023). Chemical Basis of Combination Therapy to Combat Antibiotic Resistance. *JACS Au*, 3(2), 276–292. https://doi.org/10.1021/jacsau.2c00532

68. Dhanda, G., Acharya, Y., & Haldar, J. (2023). Antibiotic Adjuvants: A Versatile Approach to Combat Antibiotic Resistance. *ACS Omega*, 8(12), 10757–10783. https://doi.org/10.1021/acsomega.3c00312

69. Carson, C. F., Hammer, K. A., & Riley, T. V. (2006). Melaleuca alternifolia (Tea Tree) oil: a review of antimicrobial and other medicinal properties. Clinical *Microbiology Reviews*, 19(1), 50–62. https://doi.org/10.1128/CMR.19.1.50-62.2006

70. Noviana, E., Indrayanto, G., & Rohman, A. (2022). Advances in Fingerprint Analysis for Standardisation and Quality Control of Herbal Medicines. *Frontiers in Pharmacology*, 13, 853023. https://doi.org/10.3389/fphar.2022.853023

71. Yang, S., Su, P., Li, L., Liu, S., & Wang, Y. (2025). Advances and mechanisms of traditional Chinese medicine and its active ingredients against antibiotic-resistant *Escherichia coli* infections. *Journal of Pharmaceutical Analysis,* 15(2), 101117. https://doi.org/10.1016/j.jpha.2024.101117

72. Zhang, M., Han, W., Gu, J., Qiu, C., Jiang, Q., Dong, J., Lei, L., & Li, F. (2022). Recent advances on the regulation of bacterial biofilm formation by herbal medicines. *Front. Microbiol.*, 13, 1039297. https://doi.org/10.3389/fmicb.2022.1039297

73. Bhunia, S., Mallick, S., Mondal, A. I., Saha, A., Ray, P., Roy, S., & Chakraborty, T. (2025). Exploring the scope of traditional Chinese medicinal plants in battle of antibiotic resistance – A comprehensive review. *Pharmacolog. Res. - Modern Chinese Medicine*, 14, 100574. https://doi.org/10.1016/j.prmcm.2025.100574

74. Chung, M. C., Su, L. J., Chen, C. L., & Wu, L. C. (2024). Revealing the antimicrobial potential of traditional Chinese medicine through text mining and molecular computation. *Brief. Bioinformatics*, 26(1), bbaf077. https://doi.org/10.1093/bib/bbaf077

75. Sun, Z., & Derkach, T. M. (2024). Biologically active compounds in the extracts from the *Speranskia Tuberculata (Bunge) Baill* herb and their effect on the viability of cancer cells of five different lines. *Pharmaceutical Review*, (1), 23–34. https://doi.org/10.11603/2312-0967.2024.1.14441

76. Volume 1: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2015). State pharmacopoeia of Ukraine (2nd ed., Vol. 1). RIREG.

77. Volume 2: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2014). State pharmacopoeia of Ukraine (2nd ed., Vol. 2). RIREG.

78. Volume 3: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2014). State pharmacopoeia of Ukraine (2nd ed., Vol. 3). RIREG.

79. Supplement 2.1: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2016). State pharmacopoeia of Ukraine: Suppl. 1 (2nd ed.). SPhU.

80. Supplement 2.2: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2018). State pharmacopoeia of Ukraine: Supplement 2 (2nd ed.), SPhU

81. Supplement 2.3: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2019). State pharmacopoeia of Ukraine: Suppl. 3 (2nd ed.). SPhU.

82. Supplement 2.4: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2020). State pharmacopoeia of Ukraine: Suppl. 4 (2nd ed.). SPhU.

83. Supplement 2.5: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2021). State pharmacopoeia of Ukraine: Suppl. 5 (2nd ed.). SPhU.

84. Supplement 2.6: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2023). State pharmacopoeia of Ukraine: Suppl. 6 (2nd ed.). SPhU.

85. Supplement 2.7: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2024). State pharmacopoeia of Ukraine: Suppl. 7 (2nd ed.). SPhU.

86. Supplement 2.8: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". (2025). State pharmacopoeia of Ukraine: Supplement 8 (2nd ed.). SPhU.

87. European Pharmacopoeia, 11th ed. (2023). Strasbourg, France: Council of Europe. https://www.webofpharma.com/

88. Alley, M. C., Scudiero, D. A., Monkes, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R.H., Boyd, M. R. (1988). Feasibility of drug screening with panel of human tumor cell lines using a microculture tetrazolium assay. *Cancer Research,* 48(3), 589–601.

89. Gulcin, İ. & Alwasel, S. H. (2023). DPPH Radical Scavenging Assay. *Processes*. 11(8), 2248. https://doi.org/10.3390/pr11082248

90. Andrés, C. M. C., Pérez de la Lastra, J. M., Andrés Juan, C., Plou, F. J. & Pérez-Lebeña, E. (2023). Superoxide Anion Chemistry—Its Role at the Core of the Innate Immunity. *Int. J. Molecul. Sci*., 24(3), 1841. https://doi.org/10.3390/ijms24031841

91. Djoumbou Feunang, Y., Eisner, R., Knox, C., Chepelev, L., Hastings, J., Owen, G., Fahy, E., Steinbeck, C., Subramanian, S., Bolton, E., Greiner, R., & Wishart, D. S. (2016). ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. *J. Cheminf*., 8, 61. https://doi.org/10.1186/s13321-016-0174-y

92. Food and Drug Administration. (2022). Q3D(R2) elemental impurities: Guidance for industry. U.S. Department of Health and Human Services.

93. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2022). Q3D(R2): Guideline for elemental impurities.

94. Chiarentin, L., Moura, V., Pais, A. A. C. C., & Vitorino, C. (2025). AQbD

approach applied to NIR in a complex topical formulation: Bifonazole as case study. *Pharmaceutics*, 17(7), 835. https://doi.org/10.3390/pharmaceutics17070835

95. Karavalasi, A., Almpani, S., Tserkezou, P., Chachlioutaki, K., Kamaris, G., & Markopoulou, C. K. (2024). Application of a validated HPLC method for the determination of resveratrol, ferulic acid, quercetin, retinol, and α-tocopherol in a cold cream—Permeability study. *Appl. Sci.*, 14(24), 11843. https://doi.org/10.3390/app142411843

96. Mapoung, S., Semmarath, W., Arjsri, P., Umsumarng, S., Srisawad, K., Thippraphan, P., Yodkeeree, S., & Limtrakul, P. (2021). Determination of phenolic content, antioxidant activity, and tyrosinase inhibitory effects of functional cosmetic creams available on the Thai market. *Plants*, 10(7), 1383. https://doi.org/10.3390/plants10071383

97. Wang, P., Wang, Y.-H., Li, H., Lai, P.-H., & Gao, H. (2020). Correlation between the content of total flavonoids and total polyphenols in loquat nuclei with different extracts and their antioxidant activities. *Huaxue Shiji*, 42(9), 1067–1072. https://doi.org/10.13822/j.cnki.hxsj. 2020007520

98. Xu, G., & Li, B. (2012). Extraction of total flavonoids from loquat seed and its scavenging effect on hydroxyl radical. *Guangzhou Chemical Industry*, 40(21), 75–77. https://doi.org/10.57239/ pjlss-2024-22.1.00169

99. Ginwala, R., Bhavsar, R., Chigbu, D. I., Jain, P., & Khan, Z. K. (2019). Potential role of flavonoids in treating chronic inflammatory diseases with a special focus on the anti-inflammatory activity of apigenin. *Antioxidants* (Basel, Switzerland), 8(2), 35. https://doi.org/10.3390/antiox8020035

100. Lee, J. S., Nam, Y. R., Kim, H. J., & Kim, W. K. (2023). Quantification and validation of an HPLC method for low concentrations of paclitaxel and attemisinin in Agrimonia pilosa aqueous ethanol extract topical cream by liquid-liquid extraction. *Molecules*, 28(2), 713. https://doi.org/ 10.3390/molecules28020713

101. Yang, Y., Peng, X.-M., Ju, R.-J., Yang, S.-M., You, C.-C., & Guan, J. (2024). Antioxidant activity of different polar parts of Paeonia Lactiflora and correlation with total terpene content. *Modern Chemical Industry*, 44(2), 109–112.

https://doi.org/10.16606/j.cnki.issn0253-4320.2024.02.021

102. Mabhiza, D., Chitemerere, T., & Mukanganyama, S. (2016). Antibacterial Properties of Alkaloid Extracts from *Callistemon citrinus* and *Vernonia adoensis* against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *International Journal of Medicinal Chemistry*, 2016, 6304163. https://doi.org/10.1155/2016/6304163

103. Tian, F., Li, Q., & Xie, X. (2021). Study on the determination method for total alkaloids in the traditional Chinese medicine Stemona. *Heilongjiang Medicine Journal*, 34(4), 810–812. https://doi.org/10.14035/j.cnki.hljyy.2021-810

104. Ren, Y., Sun, Z., Huang, Y., An, X., Bian, X., Cao, Z., Liu, Y., Javed, K., Derkach, T., Li, X. (2024). Preparation of fluorinated chitosan-based multifunctional materials with rapid hemostasis, anti-infection, and non-adhesive properties for wounds. *Journal of Polymer Science,* 62(12), 2762–2779. https://doi.org/10.1002/pol.20240038

105. Chen, Z., Ge, C., Zhu, X., Sun, P., Sun, Z., Derkach, T., Zhou, M., Wang, Y., & Luan, M. (2024). A novel nanoprobe for visually investigating the controversial role of miRNA-34a as an oncogene or tumor suppressor in cancer cells. *Analytical Methods*, 16(5), 667–675. https://doi.org/10.1039/D3AY02270F

106. Wu, T., Liu, X., Sun, Z., Xing, S., Han, L., Li, X., Pan, X., Chen, J., Zhou, M., Derkach, T. & Bielicki, J. K. (2022). Fruit of Phyllanthus emblica L. suppresses macrophage foam-cell genesis and vascular lipid deposition using in vivo and *in vitro* models of early atherosclerosis development. *Food Science and Technology Research*, 28(4), 317–328. https://doi.org/10.3136/fstr.FSTR-D-22-00002

107. Sun, Z., Wu, T., Xing, S., Mingyang, Z., Derkach, T. M. (2021). Anti-hepatocellular carcinoma: effect of bioactive components of *Speranskia Tuberculata (Bunge) Baill.* In: *Innovation in Education, Science and Business: Challenges and Opportunities. The II All-Ukrainian Conference of Higher Education Students and Young Scientists*, Nov 18, 2021, vol. 1. Kyiv: KNUTD, pp. 189–193.

108. Sun, Z., Derkach, T. (2023). Study on Evaluation of Vitro Coagulation Effect of Extracts of Mugwort. In: *KyivLvivPharma-2023. Pharmaceutical Technol. & Pharmacol. in Ensuring Active Longevity*. Kyiv-Lviv, Ukraine, November 16-18, 2023.

109. Ren, Y., Sun, Z., Huang, Y., An, X., Bian, X., Cao, Z., Liu, Y., Javed, K.,

Derkach, T., Li, X. (2024). Preparation of TiO2 Nanorods Composites Doped with Silver Nanoparticles and Their Bactericidal Properties under Visible Light Irradiation. *Particles & Particle Systems Characterisation*. 2300191. https://doi.org/10.1002/ppsc.202300191.

110. Nawaz, H., Shad, M. A., Rehman, N., Andaleeb, H., & Ullah, N. (2020). Effect of solvent polarity on extraction yield and antioxidant properties of phytochemicals from bean (*Phaseolus vulgaris*) seeds. *Braz. J. Pharm. Sci.*, 56. https://doi.org/10.1590/s2175-97902019000417129

111. Snyder, L. R. (1978). Classification of the solvent properties of common liquids. *J. Chromatographic Science*, 16(6), 223–234. https://doi.org/10.1093/chromsci/16.6.223

112. Parcheta, M., Świsłocka, R., Orzechowska, S., Akimowicz, M., Choińska, R. & Lewandowski, W. (2021). Recent Developments in Effective Antioxidants: The Structure and Antioxidant Properties. *Materials*, 14(8), 1984. https://doi.org/10.3390/ma14081984

113. Mutha, R. E., Tatiya, A. U., & Surana, S. J. (2021). Flavonoids as natural phenolic compounds and their role in therapeutics: an overview. *Future Journal of Pharmaceutical Sciences*, 7(1), 25. https://doi.org/10.1186/s43094-020-00161-8

114. Lang, Y., Gao, N., Zang, Z., Meng, X., Lin, Y., Yang, S., Yang, Y., Jin, Z. & Li, B. (2024). Classification and antioxidant assays of polyphenols: a review. *Journal of Future Foods*, 4(3), 193–204. https://doi.org/10.1016/j.jfutfo.2023.07.002

115. Reichardt, Ch. *Solvents and Solvent Effects in Organic Chemistry*. 3rd ed. Weinheim: Wiley-VCH, 2003.

116. Mustafa, R. A., Hamid, A. A., Mohamed, S., & Bakar, F. A. (2010). Total Phenolic Compounds, Flavonoids, and Radical Scavenging Activity of 21 Selected Tropical Plants. *Journal of Food Science*, 75, C28–C35. https://doi.org/10.1111/j.1750-3841.2009.01401.x

117. Abdallah, E. M., Alhatlani, B. Y., de Paula Menezes, R. & Martins, C. H. G. (2023). Back to Nature: Medicinal Plants as Promising Sources for Antibacterial Drugs in the Post-Antibiotic Era. *Plants,* 12, 3077. https://doi.org/10.3390/plants12173077

118. Nwozo, O. S., Effiong, E. M., Aja, P. M., & Awuchi, C. G. (2023).

Antioxidant, phytochemical, and therapeutic properties of medicinal plants: a review. *Int. J. Food Properties*, 26(1), 359–388. https://doi.org/10.1080/10942912.2022.2157425

119. Derkach, T. M. and Starikova, O.O. (2019). Variation of chemical composition of medicinal herbs of different producers. *Journal of Chemistry and Technologies*, 27(1), 79–91. https://doi.org/10.15421/081909

120. Nikitina, O. A., Roik, O. N., Orlov, D. Y. & Tsarova, O. N. (2024). Study of antimicrobial properties of liquid plaster containing of oregano essential oil (*Origanum vulgare L.*). *Farmatsevt. Zhurn.*, (2), 58–72. https://doi.org/10.32352/0367-3057.2.24.05

121. Yatsiuk, K. M., Feodorovska, M. I., & Kutsyk, R. V. (2018). The investigation of the cranberry (*Vaccinium oxycoccos L.*) concentrated juice antimicrobial activity. *Farmatsevt.i Zhurn.*, (1), 84–92. https://doi.org/10.32352/0367-3057.1.17.11

122. Vrubel, O. R., Zin, A. R., and Antonyuk, V. O. (2019). Investigation of lipophilic substances of the leaves and flowers of spindle tree (*Euonymus Europaea L.*). *Farmatsevt. Zhurn.*, (2), 73–80. https://doi.org/10.32352/3057.2.19.08

123. Slipchenko, G. D. (2019). Determination of the critical parameters of the technological process of obtaining solid dosage forms with dry extract and crushed roots and roots of Scutellaria baicalensis. *Farmatsevt. Zhurn.* (3), 56–64. https://doi.org/10.32352/0367-3057.3.19.07

124. Li, C., Zhang, C. Z., Hu, F. D. and Shi, J. G. (2000). Chemical constituents from *Speranskia tuberculata* (Bge.) Baill. *China J. Chin. Materia Medica*, 25(5), 291–292.

125. Li, Y.-M., Zhao, Y.-Y., Fan, Y.-B., Wang, X. & Cai, L. N. (1997). Flavonoids from *Speranskia Tuberculata*. *Journal of Chinese Pharmaceutical Sciences*, 6, 70–74.

126. Kim, G., Gan, R.-Y., Zhang, D., Farha, A.K., Habimana. O., Mavumengwana, V., Li, H.-B., Wang, X.-H., Corke, H. (2020). Large-Scale Screening of 239 Traditional Chinese Medicinal Plant Extracts for Their Antibacterial Activities against Multidrug-Resistant *Staphylococcus aureus* and Cytotoxic Activities. *Pathogens*, 9(3), 185. https://doi.org/10.3390/pathogens9030185

127. Zhou, Y. X., Wang, S. J., Li, Y., Xia, W., Meng, X. Y., Peng, C. (2015). Evaluation of analgesic, anti-inflammatory and antipyretic activities of the ethanol extract

from *Speranskia* tuberculate. *African Journal of Traditional, Complementary and Alternative Medicines*, 12(3) 4954. http://dx.doi.org/10.4314/ajtcam.v12i3.6

128. Mazzio, E., Badisa, R., Mack, N., Deiab, S. and Soliman, K. F.A. (2014). High Throughput Screening of Natural Products for Anti-mitotic Effects in MDA-MB-231 Human Breast Carcinoma Cells. *Phyt.Res.*, 28, 856–867. https://doi.org/10.1002/ptr.5065

129. Casciaro, B., Mangiardi, L., Cappiello, F., Romeo, I., Loffredo, M. R., Iazzetti, A., … Quaglio, D. (2020). Naturally-occurring alkaloids of plant origin as potential antimicrobials against antibiotic-resistant infections. *Molecules*, 25(16), 3619. https://doi.org/10.3390/molecules25163619

130. Zhang, G., Yang, Y., Memon, F. U., Hao, K., Xu, B., Wang, S., Wang, Y., Wu, E., Chen, X., Xiong, W., Si, H. (2021). A natural antimicrobial agent: Analysis of antibacterial effect and mechanism of compound phenolic acid on *Escherichia coli* based on tandem mass tag proteomics. *Frontiers in Microbiology*, 12, 738896. https://doi.org/10.3389/fmicb.2021.738896

131. Yoder, S. C., Lancaster, S. M., Hullar, M. A. J., & Lampe, J. W. (2015). Gut microbial metabolism of plant lignans. In: Diet-Microbe Interactions in the Gut (pp. 103–117). https://doi.org/10.1016/b978-0-12-407825-3.00007-1

132. Bournival, J., Plouffe, M., Renaud, J., Provencher, C., & Martinoli, M.-G. (2012). Quercetin and sesamin protect dopaminergic cells from MPP+-induced neuroinflammation in a microglial (N9)-neuronal (PC12) coculture system. *Oxidative Medicine and Cellular Longevity*, 2012, 921941. https://doi.org/10.1155/2012/921941

133. Lee, C. M., Wong, H. N., Chui, K. Y., Choang, T. F., Hon, P. M., & Chang, H. M. (1991). Miltirone, a central benzodiazepine receptor partial agonist from a Chinese medicinal herb Salvia miltiorrhiza. *Neuroscience Letters*, 127(2), 237–241. https://doi.org/10.1016/0304-3940(91)90802-z

134. Zhou, X., Wang, Y., Lee, W. Y. W., Or, P. M. Y., Wan, D. C. C., Kwan, Y. W., & Yeung, J. H. K. (2015). Miltirone is a dual inhibitor of P-glycoprotein and cell growth in doxorubicin-resistant HepG2 cells. *Journal of Natural Products*, 78(9), 2266–2275. https://doi.org/10.1021/acs.jnatprod.5b00516

135. Dávid, C. Z., Kúsz, N., Pinke, G., Kulmány, Á., Zupkó, I., Hohmann, J., &

Vasas, A. (2022). Jacaranone derivatives with antiproliferative activity from Crepis pulchra and relevance of this group of plant metabolites. *Plants*, 11(6), 782. https://doi.org/10.3390/plants11060782

136. Loizzo, M. R., Tundis, R., Statti, G. A., & Menichini, F. (2007). Jacaranone: a cytotoxic constituent from Senecio ambiguus subsp. ambiguus (biv.) DC. against renal adenocarcinoma ACHN and prostate carcinoma LNCaP cells. *Archives of Pharmacal Research*, 30(6), 701–707. https://doi.org/10.1007/bf02977631

137. Rajendran, A., Sagadevan, S., Lett, J. A., Saravanan Kaliaraj, G., Fatimah, I., Mohammad, F., Al-Lohedan, H. A., Alshahateet, S. F., & Podder, J. (2021). Synthesis, growth, supramolecularity and antibacterial efficacy of 3,4-dimethoxybenzoic acid single crystals. *Chem. Phys. Let.*, 764, 138269. https://doi.org/10.1016/j.cplett.2020.138269

138. Liu, C., Kuei, C., Zhu, J., Yu, J., Zhang, L., Shih, A., Mirzadegan, T., Shelton, J., Sutton, S., Connelly, M. A., Lee, G., Carruthers, N., Wu, J., & Lovenberg, T. W. (2012). 3,5-Dihydroxybenzoic acid, a specific agonist for hydroxycarboxylic acid 1, inhibits lipolysis in adipocytes. *The Journal of Pharmacology and Experimental Therapeutics*, *341*(3), 794–801. https://doi.org/10.1124/jpet.112.192799

139. Tabata, M. (1988). Naphthoquinones. In: Phytochemicals in Plant Cell Cultures (pp. 99–111). https://doi.org/10.1016/b978-0-12-715005-5.50013-3

140. Petrocelli, G., Marrazzo, P., Bonsi, L., Facchin, F., Alviano, F., & Canaider, S. (2023). Plumbagin, a natural compound with several biological effects and anti-inflammatory properties. *Life*, 13(6), 1303. https://doi.org/10.3390/life13061303

141. Guzman, J. D. (2014). Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobial activity. *Molecules* (Basel, Switzerland), 19(12), 19292–19349. https://doi.org/10.3390/molecules191219292

142. Siriwardhana, A., Wijesundara, S., & Karunaratne, V. (2015). A review of studies on bioactive compounds isolated from Sri Lankan flora. *Journal of the National Science Foundation of Sri Lanka*, 43(1), 11. https://doi.org/10.4038/jnsfsr.v43i1.7912

143. Jayasinghe, L., Kumarihamy, B. M. M., Jayarathna, K. H. R. N., Udishani, N. W. M. G., Bandara, B. M. R., Hara, N., & Fujimoto, Y. (2003). Antifungal constituents of the stem bark of Bridelia retusa. *Phytochemistry*, 62(4), 637–641.

https://doi.org/10.1016/s0031-9422(02)00623-4

144. Kaur, J., & Kaur, R. (2022). p-coumaric acid: A naturally occurring chemical with potential therapeutic applications. *Current Organic Chemistry*, 26(14), 1333–1349. https://doi.org/10.2174/1385272826666221012145959

145. Moço, G., Sousa, C., Capitão, A., MacKinnon, S. S., Leitão, A. J., & Mendes, A. F. (2023). Synthesis of carvone derivatives and in silico and *in vitro* screening of anti-inflammatory activity in Murine macrophages. *International Journal of Molecular Sciences*, 24(3), 2263. https://doi.org/10.3390/ijms24032263

146. Xu, B. (1991). Recent advances in pharmacologic study of natural anticancer agents in China. *Memorias Do Instituto Oswaldo Cruz*, 86 Suppl. 2, 51–54. https://doi.org/10.1590/s0074-02761991000600014

147. Marshall, P. S., Harborne, J. B., & King, G. S. (1987). A spiroketalenol ether phytoalexin from infected leaves and stems of Coleostephus myconis. *Phytochemistry*, 26(9), 2493–2494. https://doi.org/10.1016/s0031-9422(00)83861-3

148. Silveira, D., & Boylan, F. (2023). Medicinal plants: Advances in Phytochemistry and Ethnobotany. *Plants*, 12(8), 1682. https://doi.org/10.3390/plants12081682

149. Ravipati, A. S., Reddy, N., & Koyyalamudi, S. R. (2014). Biologically active compounds from the genus Uncaria (Rubiaceae). In: *Studies in Natural Products Chemistry* (pp. 381–408). https://doi.org/10.1016/b978-0-444-63430-6.00013-8

150. Chen, L., Huang, Y., Yu, X., Lu, J., Jia, W., Song, J., … Li, M. (2021). Corynoxine protects dopaminergic neurons through inducing autophagy and diminishing neuroinflammation in rotenone-induced animal models of Parkinson's disease. *Frontiers in Pharmacology*, 12, 642900. https://doi.org/10.3389/fphar.2021.642900

151. Wang, L. H., Gu, Z. W., Li, J., Yang, W. Q., Li, Y. L., Qi, D. M., Wang, D. Y., & Jiang, H. Q. (2023). Isorhynchophylline inhibits inflammatory responses in endothelial cells and macrophages through the NF-κB/NLRP3 signaling pathway. *BMC Complement Med. & Therapies*, *23*(1), 80. https://doi.org/10.1186/s12906-023-03902-3

152. Zhou, Z., Su, Y., & Fa, X.-E. (2019). Isorhynchophylline exerts anti-inflammatory and anti-oxidative activities in LPS-stimulated murine alveolar macrophages. *Life Sciences*, 223, 137–145. https://doi.org/10.1016/j.lfs.2019.03.017

153. Zeng, P., Wang, X.-M., Ye, C.-Y., Su, H.-F., & Tian, Q. (2021). The main alkaloids in Uncaria rhynchophylla and their anti-Alzheimer's disease mechanism determined by a network pharmacology approach. *International Journal of Molecular Sciences*, 22(7), 3612. https://doi.org/10.3390/ijms22073612

154. Saito, T., Awad, J. M., & Zhang, W. (2023). Synthetic studies on tetracyclic diquinane Lycopodium alkaloids Magellanine, magellaninone and paniculatine. *Molecules* (Basel, Switzerland), 28(3), 1501. https://doi.org/10.3390/molecules28031501

155. Brochmann-Hanssen, E., Nielsen, B., & Utzinger, G. E. (1965). Opium alkaloids. II. Isolation and characterization of codamine. *Journal of Pharmaceutical Sciences*, 54(10), 1531–1532. https://doi.org/10.1002/jps.2600541030

156. Tang, W., & Eisenbrand, G. (1992). Cephalotaxus spp. In: Chinese Drugs of Plant Origin (pp. 281–306). https://doi.org/10.1007/978-3-642-73739-8_38

157. Hao, D.-C. (2021). Biodiversity, chemodiversity, and pharmacotherapy of Cephalotaxus medicinal plants. In: Taxaceae and Cephalotaxaceae (pp. 243–305). https://doi.org/10.1016/b978-0-12-823975-9.00007-9

158. Liu, Z., Du, Q., Wang, K., Xiu, L., & Song, G. (2009). Completed preparative separation of alkaloids from Cephaltaxus fortunine by step-pH-gradient high-speed counter-current chromatography. *Journal of Chromatography* A, 1216(22), 4663–4667. https://doi.org/10.1016/j.chroma.2009.03.083

159. Lee, J., Gong, Y.-X., Jeong, H., Seo, H., Xie, D.-P., Sun, H.-N., & Kwon, T. (2021). Pharmacological effects of Picrasma quassioides (D. Don) Benn for inflammation, cancer and neuroprotection (Review). *Experimental and Therapeutic Medicine*, 22(6), 1357. https://doi.org/10.3892/etm.2021.10792

160. Protostephanine. (1972). In: *Organic Chemistry* (pp. 418–425). https://doi.org/10.1016/b978-0-12-638250-1.50026-5

161. Islam, M. T., Hossain, M., Azad, M. A. K., & Tareq, S. M. (2017). Botanical and phyto-pharmacological reports on Stephania japhonica. *Int. J. Applied Pharm. Sciences and Research*, 2(01), 1–7. https://doi.org/10.21477/ijapsr.v2i1.6980

162. Buza, V., Niculae, M., Hanganu, D., Pall, E., Burtescu, R. F., Olah, N. K., Matei-Lațiu, M. C., Vlasiuc, I., Iozon, I., Szakacs, A. R., Ielciu, I., & Ștefănuț, L. C.

(2022). Biological Activities and Chemical Profile of *Gentiana asclepiadea* and *Inula helenium* Ethanolic Extracts. *Molecules (Basel, Switzerland)*, 27(11), 3560. https://doi.org/10.3390/molecules27113560

163. Spivey, A. C., Weston, M., & Woodhead, S. (2002). Celastraceae sesquiterpenoids: biological activity and synthesis. *Chemical Society Reviews*, 31(1), 43–59. https://doi.org/10.1039/b000678p

164. Whitson, E. L., Mala, S. M. V. D., Veltri, C. A., Bugni, T. S., de Silva, E. D., & Ireland, C. M. (2006). Oppositines A and B, sesquiterpene pyridine alkaloids from a Sri Lankan Pleurostylia opposita. *Journal of Natural Products*, 69(12), 1833–1835. https://doi.org/10.1021/np060459s

165. Cuca Suarez, L. E., Pattarroyo, M. E., Lozano, J. M., & Delle Monache, F. (2009). Biological activity of secondary metabolites from Peltostigma guatemalense. *Natural Product Research*, 23(4), 370–374. https://doi.org/10.1080/14786410802228439

166. Thawabteh, A., Juma, S., Bader, M., Karaman, D., Scrano, L., Bufo, S. A., & Karaman, R. (2019). The biological activity of natural alkaloids against herbivores, cancerous cells pathogens. *Toxins*, 11(11), 656. https://doi.org/10.3390/toxins11110656

167. Guo, Q., Wu, S., Liang, W., Tan, J., Liu, X., Yuan, Y., Li, X., & Zhao, H. (2022). Glabrol impurity exacerbates glabridin toxicity in zebrafish embryos by increasing myofibril disorganization. *Journal of ethnopharmacology*, 287, 114963. https://doi.org/10.1016/j.jep.2021.114963

168. Kitagawa, I., Fukuda, Y., Taniyama, T., & Yoshikawa, M. (1996). ChemInform Abstract: Chemical studies on crude drug processing. Part 8. On the constituents of rehmannniae Radix. Part 2. Absolute stereostructures of rehmaglutin C and glutinoside isolated from Chinese rehmanniae Radix, the dried root of *Rehmannia glutinoasa libosch*. *ChemInform*, 27(13). https://doi.org/10.1002/chin.199613207

169. Jia, J., Chen, J., Wang, G., Li, M., Zheng, Q., & Li, D. (2023). Progress of research into the pharmacological effect and clinical application of the traditional Chinese medicine Rehmanniae Radix. *Biomedecine & Pharmacotherapie*, 168(115809), 115809. https://doi.org/10.1016/j.biopha.2023.115809

170. Milner, S. E., Brunton, N. P., Jones, P. W., O'Brien, N. M., Collins, S. G., &

Maguire, A. R. (2011). Bioactivities of glycoalkaloids and their aglycones from Solanum species. *J. Agricult. Food Chem.*, 59(8), 3454–3484. https://doi.org/10.1021/jf200439q

171. Dias, M. C., Pinto, D. C. G. A., & Silva, A. M. S. (2021). Plant Flavonoids: Chemical Characteristics and Biological Activity. *Molecules (Basel, Switzerland)*, *26*(17), 5377. https://doi.org/10.3390/molecules26175377

172. Chagas, M. D. S. S., Behrens, M. D., Moragas-Tellis, C. J., Penedo, G. X. M., Silva, A. R., & Gonçalves-de-Albuquerque, C. F. (2022). Flavonols and Flavones as Potential Anti-Inflammatory, Antioxidant, and Antibacterial Compounds. *Oxidative medicine and cellular longevity*, *2022*, 9966750. https://doi.org/10.1155/2022/9966750

173. Tiwari, V. J. (2017). Assessment of validity of ethnopharmacological uses of medicinal plants used by tribal people of Gadchiroli district of Maharashtra state, India. *Res. J. Pharm. Phytochem.*, 9(1), 8. https://doi.org/10.5958/0975-4385.2017.00002.4

174. Mahmood, U., Kaul, V. K., & Jirovetz, L. (2002). Alkylated benzoquinones from Iris kumaonensis. *Phytochemistry*, 61(8), 923–926. https://doi.org/10.1016/s0031-9422(02)00474-0

175. Kartini, K., Irawan, M. A., Setiawan, F., & Jayani, N. I. E. (2023). Characteristics, Isolation Methods, and Biological Properties of Aucubin. *Molecules*, 28(10), 4154. https://doi.org/10.3390/molecules28104154

176. Shah, B. N., Seth, A. K. (2009). *Textbook of Pharmacognosy and Phytochemistry*. 1st ed. Elsevier India.

177. Bhavikatti, S. K., Karobari, M. I., Zainuddin, S. L. A., Marya, A., Nadaf, S. J., Sawant, V. J., … Scardina, G. A. (2021). Investigating the antioxidant and cytocompatibility of Mimusops elengi Linn extract over human gingival fibroblast cells. *Int. J. Environ. Res. Public Health*, 18(13), 7162. https://doi.org/10.3390/ijerph18137162

178. Shaikh, J. R., & Patil, M. K. (2020). Qualitative tests for preliminary phytochemical screening: An overview. *International Journal of Chemical Studies*, 8(2), 603–608. https://doi.org/10.22271/chemi.2020.v8.i2i.8834

179. Evans, W. C. (2009). *Trease and Evans Pharmacognosy*, 16th ed. Edinburgh, London, New York: Saunders Elsevier. 603 p.

180. Mimica-Dukić, N., Bugarin, D., Grbović, S., Mitić-Culafić, D., Vuković-

Gacić, B., Orcić, D., … Couladis, M. (2010). Essential oil of Myrtus communis L. as a potential antioxidant and antimutagenic agents. *Molecules* (Basel, Switzerland), 15(4), 2759–2770. https://doi.org/10.3390/molecules15042759

181. Maggio, A., Loizzo, M. R., Riccobono, L., Bruno, M., Tenuta, M. C., Leporini, M., … Tundis, R. (2019). Comparative chemical composition and bioactivity of leaves essential oils from nine Sicilian accessions of Myrtus communis L. *J. Essential Oil Research*, 31(6), 546–555. https://doi.org/10.1080/10412905.2019.1610089

182. Kaur, R., Chattopadhyay, S. K., Chatterjee, A., Prakash, O., Khan, F., Suri, N., … Saxena, A. K. (2014). Synthesis and *in vitro* anticancer activity of brevifoliol derivatives substantiated by in silico approach. *Medicinal Chemistry Research: Int. J. for Rapid Communications on Design and Mechanisms of Action of Biologically Active Agents*, 23(9), 4138–4148. https://doi.org/10.1007/s00044-014-0980-6

183. Hayashi, T., Shinbo, T., Shimizu, M., Arisawa, M., Morita, N., Kimura, M., … Kikuchi, T. (1985). Paeonilactone-A, -B, and -C, new monoterpenoids from paeony root. *Tetrahed. Let.*, 26(31), 3699–3702. https://doi.org/10.1016/s0040-4039(00)89227-8

184. Jayasuriya, H., Clark, A. M., & McChesney, J. D. (1991). New antimicrobial filicinic acid derivatives from Hypericum drummondii. *Journal of Natural Products*, *54*(5), 1314–1320. https://doi.org/10.1021/np50077a013

185. Kimura, Y., Ohminami, H., Arichi, H., Okuda, H., Baba, K., Kozawa, M., & Arichi, S. (1982). Effects of various coumarins from roots of Angelica dahurica on actions of adrenaline, ACTH and insulin in fat cells. *Planta medica*, 45(3), 183–187. https://doi.org/10.1055/s-2007-971370

186. Ounis, R., Benchikh, F., Amira, S., Benabdallah, H., Amira, H., Mamache, W., Bensouici, C., and Hellal, K. (2023). Impact of Extraction Solvent Polarity: Antioxidant Activity of Methanolic, Hydromethanolic and Aqueous Decocted Extracts of Algerien *Thymelaea hirsuta (L.) Endl.* Areal Parts. *Turk. J. Agric. - Food Sci. Technol*., 11(6), 1161–1167. https://doi.org/10.24925/turjaf.v11i6.1161-1167.5931

187. Zarrinmehr, M. J., Daneshvar, E., Nigam, S., Gopinath, K. P., Biswas, J. K., Kwon, E. E., … Bhatnagar, A. (2022). The effect of solvents polarity and extraction conditions on the microalgal lipids yield, fatty acids profile, and biodiesel properties.

Bioresource Technol. (B), 344, 126303. https://doi.org/10.1016/j.biortech.2021.126303

188. ICH Guideline Q3D (R1) on elemental impurities, 2019. 82 p.

189. Britsun, V. M., Levin, M. G., Suvorova, I. M., Ostanina, N. V. (2023). Determination of the content of heavy metals (As, Cd, Hg, Pb) in the presence of Al, Ca, Cu, Fe, K, Mg, Na, Zn by inductively coupled plasma atomic emission spectrometry. *Pharmacology and Drug Toxicology*, 17(6), 420–430. https://doi.org/10.33250/17.06.420

190. Britsun, V. M., Kuznetsova, O. M., Ocheretyanaya, N. M., Suvorova, I. M., Ostanina, N. V. (2025). Determination of metals content (Ag, Al, As, Ba, Ca, Cd, Cr, Co, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Sn, V, Zn) in thyme herb by the method of atomic emission spectrometry with inductively coupled plasma. *Pharmacology and Drug Toxicology*, 2025, 19 (1), 91–104. https://doi.org/10.33250/19.01.091

191. Britsun, V. M., Kuznetsova, O. M., Ocherethyana, N. M., Levin, M. G., Ostanina, N. V. (2023). Determination of the heavy metals (As, Cd, Hg, Pb) content in dietary supplements, pharmaceutical raw and medicinal plant materials by inductively coupled plasma atomic emission spectrometry. *Pharmacology and Drug Toxicology*, 17(1), 59–65. https://doi.org/10.33250/17.01.059

192. Inada, I., Kiuchi, F., & Urushihara, H. (2023). Comparison of regulations for arsenic and heavy metals in herbal medicines using pharmacopoeias of nine countries/regions. *Therapeutic Innovation & Regulatory Science*, 57(5), 963–974. https://doi.org/10.1007/s43441-023-00532-2

193. Abernethy, D. R., Destefano, A. J., Cecil, T. L., Zaidi, K., Williams, R. L., & USP Metal Impurities Advisory Panel. (2010). Metal impurities in food and drugs. *Pharmaceutical Research*, 27(5), 750–755. https://doi.org/10.1007/s11095-010-0080-3

194. Kauffman, J. F., Westenberger, B. J., Robertson, J. D., Guthrie, J., Jacobs, A., & Cummins, S. K. (2007). Lead in pharmaceutical products and dietary supplements. *Regul. Toxicol. & Pharmac.*, 48(2), 128–134. https://doi.org/10.1016/j.yrtph.2007.03.001

195. Wani, A. L., Ara, A., & Usmani, J. A. (2015). Lead toxicity: a review. *Interdisciplinary Toxicology*, 8(2), 55–64. https://doi.org/10.1515/intox-2015-0009

196. Kiselyova, K., Vyshnevska, L., Yudkevych, T., Bodnar, L., Skybitska, M., Ivanauskas, L., Mykhailenko, O., Kukhtenko, O., & Georgiyants, V. (2025).

Development of the composition of a dermatological product in the form of a cream with extracts of the aerial part of Lepedeza bicolour. *ScienceRise: Pharmaceutical Science*, (1 (53), 26–40. https://doi.org/10.15587/2519-4852.2025.322855

197. Shmalko, O., Filimonova, H., Dzhoraieva, S., & Vyshnevska, L. (2024). Study of antimicrobial activity of dry phytoextract. *Annals of Mechnikov's Institute*, (2), 54–58. https://doi.org/10.5281/zenodo.11636381

198. Buryak, M. V., Bodnar, L., Shtuchna, N., Oliinyk, S. V., Семченко, К. В., & Vyshnevska, L. I. (2025). Development of the composition and technology of preparation of a suspension with adapalene for the treatment of acne, taking into account the biopharmaceutical factors. *ScienceRise: Pharmaceutical Science*, 2 (54), 12–21. https://doi.org/10.15587/2519-4852.2025.321250

199. Shmalko, O. O., Filimonova, N. I., & Vyshnevska, L. I. (2024). Microbiological research on microemulsion composition development with anti-inflammatory and antimicrobial action. *Farmatsevtychnyi Zhurnal*, (3), 66-78. https://doi.org/10.32352/0367-3057.3.24.06

200. Gryzodub, O. I. (2016). *Standardised validation schemes for drug quality control procedures*. Kharkiv: State Enterprise «Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines». — 396 p.

201. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. (2005). Validation of analytical procedures: text and methodology Q2(R1)

202. Burmaka, O. V., Gureyeva, S. M., & Margitich, V. M. (2017). Development and validation of the spectrophotometric method of quantitative determination of enisamium iodide in capsules. *Pharmaceutical Review*, (2), 64–71. https://doi.org/10.11603/2312-0967.2017.2.7894

203. Frimpong, E. and Logoyda, L. (2020). Development and Validation of Methods for Identification and Quantification of Biological Active Substances in the Fruit of Tetrapleura tetraptera. *American Journal of Analytical Chemistry*, 11, 335–348. https://doi.org/10.4236/ajac.2020.119027.

204. Logoyda, L., Horyn, M., Kryskiw, L., Kucher, T., Zarivna, N., Shulyak, N., &

Ivanchuk, I. (2025). Spectrophotometric determination of nimodipine, nitrendipine, lacidipine in tablets via derivatisation with para-dimethylaminobenzaldehyde. ScienceRise: *Pharm. Sci.*, 3(55), 30–37. https://doi.org/10.15587/2519-4852.2025.332156

205. Georgiyants, V. A., Evtifeeva, O. A. (2007). Validation of analytical methods in pharmacy: theory, normative aspects, problems of practice. *Pharmaceutical Review*, (2), 13–18. https://doi.org/10.11603/2312-0967.2007.2.3137

206. Typlynska, K., Kondratova, Y., Horyn, M., & Logoyda, L. (2024). Ultra-performance LC/MS methods for the determination of residual amounts of ramipril and hydrochlorothiazide to control equipment cleaning. ScienceRise: *Pharm. Sci.*, 4(50), 35–43. https://doi.org/10.15587/2519-4852.2024.310759

207. Havrylenko, O., Kondratova, Y., Typlynska, K., & Logoyda, L. (2026). Development and Validation of HPLC Methods for the Quantitative Determination and Related Impurities of Naftifine Hydrochloride in Solution and Cream Dosage Forms. *Scientia Pharmaceutica*, 94(1), 5. https://doi.org/10.3390/scipharm94010005

208. Lisovyi, V., Bessarabov, V., Goy, A., & Kostiuk, V. (2024). Spectrophotometric method for determining the assay of hesperidin in the composition of polymer composite material obtained by centrifugal fibre formation. *Herald of Khmelnytskyi National University. Technical Sciences*, 335(3(1), 135-141. https://doi.org/10.31891/2307-5732-2024-335-3-19

209. Alfred-Ugbenbo, D., Zdoryk, O. A., & Georgiyants, V. A. (2017). Validation of analytical method for determination of furosemide in extemporaneous syrup. *Medical & Clinical Chem.*, (2), 5–11. https://doi.org/10.11603/mcch.2410-681X.2017.v0.i2.7859

210. Int. Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2023). ICH Q2(R2): Validation of analytical procedures.

211. Int. Council for Harmonisation of Techn. Requirements for Pharmaceuticals for Human Use. (2023). ICH Q14: Analytical procedure development.

212. Zarivna, N. O. (2023). Development of projects of quality control methods for finished medicinal products depending on the type of medicinal form in the study of the

discipline "Standardisation of Medicinal". *Medical Education*, (1), 29–34. https://doi.org/10.11603/m.2414-5998.2023.1.13823

213. Sun, Z., Derkach, T. M. (2024). Anti-Atherosclerosis Effect of the Herb *Speranskia Tuberculata* (Bunge) Baill. *Health & Education,* (2), 142–148. https://doi.org/10.32782/health-2024.2.18

214. Sun, Z., Derkach, T. M. (2024). Bioactive Compounds Extracted from the Herb *Speranskia Tuberculata* (Bunge) Baill. *Journal of Chemistry and Technologies,* 32(3), 580–590. https://doi.org/10.15421/jchemtech.v32i3.305125

215. Sun, Z., Derkach, T. M. (2024). Antioxidant Activities of Petroleum Ether, Ethyl Acetate, N-butanol and Aqueous Extracts from *Speranskia Tuberculata* (Bunge) Baill. herb. *Modern Medicine, Pharmacy and Psychological Health*, No. 2 (16), 101–105. https://doi.org/10.32689/2663-0672-2024-2-16

216. Sun, Z., Derkach, T. M. (2024). Antibacterial Properties of Extracts of the Herb *Speranskia Tuberculata* (Bunge) Baill. *Farmatsevtychnyi zhurnal,* (4), 72–82. https://doi.org/10.32352/0367-3057.4.24.07

217. Guidelines. (2011). Medicinal products. Pharmaceutical development (ICH Q8). Guideline 42-. 3.0:2011. Kyiv: Ministry of Health of Ukraine.

218. EMEA/CHMP/167068/2004 – ICH. – Part I. – ICH Topic Q8 (R2). – Step 5: Note for Guidance on Pharmaceutical Development, 2009.

219. ICH. – Part II. (2009). ICH Topic Q8 Annex. – Step 5: Annex to Note for Guidance on Pharmaceutical Development (EMEA/CHMP/167068/2004).

220. Int. Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2012). ICH Q11: Development and manufacture of drug substances.

221. Committee for Medicinal Products for Human Use. (2003). Note for guidance on stability testing: Stability testing of existing active substances and related finished products (CPMP/QWP/122/02 Rev 1).

222. CPMP/ICH/2736/99 (ICH Topic Q1A (R2)). (2003). – ICH Topic Q1A (R2) Stability Testing of New Drug Substances and Products

223. Sun, Z., Derkach, T. M. (2026). Development of a Technology for Producing

an Antibacterial Cream Based on The Herb *Speranskia Tuberculata* (Bunge) Baill. *Farmatsevtychnyi zhurnal.* 81(4) 84–96. https://doi.org/10.32352/0367-3057.3.26.07

234. Sun, Z., Derkach, T. (2024). Ternopil Anti-Atherosclerosis Effect Of The Herb *Speranskia Tuberculata* (Bunge) Baill. In: Scientific and Technical Progress and Optimization of Technological Processes for the Creation of Medicinal Products. Xth Scientific and Practical Conf. Eds.: M. M. Korda et al. I. Ya. Gorbachevsky Ternopil Nat. Medical University, Ternopil, Oct. 17-18, 2024, pp. 148–149.

225. Sun, Z., Derkach, T. (2024). The study of the free radical scavenging rate of one kind of microbial fermentation product based on Chinese traditional medicine. In: *Collection of Materials of the XIIth Scient. and Practical Conf. "Science, Innovation and Quality in Modern Pharmaceutical Production*". Kyiv: JSC "Farmak", pp. 8–10.

226. Sun, Z., Derkach, T. (2025). Research on the antibacterial efficacy and stability of a kind of medical cream based on the extracts of the herb *Speranskia Tuberculata* (Bunge) Baill. *Chemical and Biopharmaceutical Technologies in 2025: The collection of abstracts of VII Int. Scientific and Practical Conf. "KyivLvivPharma-2025.* Pharmaceutical Technology and Pharmacology in Ensuring Active Longevity", November 20-21, 2025 (Kyiv-Lviv)/ Tallinn: Nordic Sci Publisher, 2026, pp. 181-183.

227. Sun, Z., Derkach, T. (2025). Study Of Antibacterial Properties Of Extracts of The Herb *Speranskia Tuberculata* (Bunge) Baill. In: *Advanced Materials and Technologies: Biotechnology, Applied Chemistry and Ecology.* Kyiv, Kyiv National University of Technologies and Design, Faculty of Chemical and Biopharmaceutical Technologies. October 30-31, pp. 26.

228. Xu, X., Xu, J., Sun, Z., & Derkach, T. (2024). Cyclodextrin-grafted redox-responsive hydrogel mediated by disulfide bridges for regulated drug delivery. *Designed Monomers and Polymers*, 27(1), 21–34. https://doi.org/10.1080/15685551.2024

229. Safety and Technical Standards for Cosmetics (2015 edition). Beijing, China.

230. Stigter, J.B., de Haan, H. P. A., Guicherit, R., Dekkers, C. P. A., & Daane, M. (2000). Determination of Cd, Zn, Cu, Cr, and As in crude oil cargoes. *Environ. Pollut.*, 107(3), 451–464. https://doi.org/10.106/S0269-7491 (99) 00123-2

231. Li, X., Xu, X., & Yao, Y. (2024). Determination of six heavy metal elements

in acacia by ICP-MS after microwave digestion. *Journal of Shanxi Medical University*, 55(6), 763–766. https://doi.org/10.13753/j.issn.1007-6611.2024.06.013

232. Salducci, M.-D., Folzer, H., Issartel, J., Rabier, J., Masotti, V., Prudent, P., Affre, L., Hardion, L., Tatoni, T., & Laffont-Schwob, I. (2019). How can a rare protected plant cope with the metal and metalloid soil pollution resulting from past industrial activities? Phytometabolites, antioxidant activities and root symbiosis are involved in the metal tolerance of *Astragalus tragacantha*. *Chemosphere*, 217, 887–896. https://doi.org/10.1016/j.chemosphere. 2018.11.078

233. Akhtar, M. S., Ali, S., & Zaman, W. (2024). Recent advancements in catalysts for petroleum refining. *Catalysts*, 14(12), 841. https://doi.org/10.3390/catal14120841

234. Sheskey, P. J., Cook, W. G., and Cable, C. G. (Eds). (2017). *Handbook of Pharmaceutical Excipients* (8th edn). London, England: Pharmaceutical Press.

235. Committee for Medicinal Products for Human Use. (2007). Guideline on declaration of storage conditions: A – in the product information of medicinal products; B – for active substances (CPMP/QWP/609/96/Rev. 2). European Medicines Agency.

236. Int. Council for Harmonisation of Techn. Requirements for Pharmaceuticals for Human Use. (2009). *ICH Q8(R2): Pharmaceutical development.*

237. Int. Council for Harmonisation of Techn. Requirements for Pharmaceuticals for Human Use. (2022). *ICH Q9(R1): Quality risk management.*

238. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2015). *ICH Q10: Pharmaceutical quality system.*

239. European Commission. (2022). EudraLex – Volume 4 – Good Manufacturing Practice (GMP) guidelines: Annex 20. Quality risk management.

240. European Commission. (2022). EudraLex – Volume 4 – EU guidelines for good manufacturing practice for medicinal products for human and veterinary use.

241. European Commission. (2022). EudraLex – Volume 4 – Good manufacturing practice (GMP) guidelines: Annex 15. Qualification and validation.

242. International Organization for Standardization. (2003). ISO 14698-1:2003. Cleanrooms and associated controlled environments – Biocontamination control.

243. Sun, Z., Derkach, T. M. (2026). Chemical Characterisation, Biological

Activity and Emulsion Dosage Form Development of *Speranskia tuberculata* (Bunge) Baill. *Journal of Chemistry and Technologies,* 34(2), 499–511. https://doi.org/10.15421/jchemtech.v34i2.352308

244. Sun, Z., Derkach, T. M. (2026). Phytochemical Profile and Antibacterial Activity of Secondary Metabolites from *Speranskia Tuberculata* (Bunge) Baill. *BIO Web of Conferences,* 258 (2026) 03002. https://doi.org/10.1051/bioconf/202625803002

245. Sun, Z., Derkach, T. (2025). Bioactive Compounds and Standardisation Extracted from The Herb *Speranskia Tuberculata* (Bunge) Baill. In: *Zaporizhzhia Pharmaceutical Forum – 2025: Pharmacy of the Future – From the Current State to Global Challenges*, 20-21 November 2025, Zaporizhzhia, ZDMFU, pp. 167.

246. Sun, Z., Derkach, T. (2026). Phytochemical Profile and Antibacterial Activity of Secondary Metabolites from *Speranskia Tuberculata* (Bunge) Baill. In: *BioChemSecurity 2026 - International Conference on Biosecurity, Chemical Safety, and Global Cooperation.* Kryvyi Rih: Ukraine. March 20 2026. https://lnk.ua/ZpsCCTOLp

247. Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The Determination of Flavonoid Contents in Mulberry and Their Scavenging Effects on Superoxide Radicals. *Food Chemistr*y, 64, 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

248. Pękal, A., Pyrzynska, K. (2014). Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. *Food Anal. Methods*, 7, 1776–1782. https://doi.org/10.1007/s12161-014-9814-x

249. Sun, Z.-H., Zhang, S., Wang, L., Gong, H., Tian, S. (2024). Determination of total flavonoids and total polyphenols in *Abelmoschus corolla* extracts and their *in vitro* antibacterial and antioxidant activities. *Chemistry & Bioengineering*, 41(2), 26–32. https://doi.org/10.3969/j.issn.1672-5425.2024.02.004

250. Fadhil, S., Reza, M. H., Rouhollah, G., and Reza, V. R. M. (2007). Spectrophotometric Determination of Total Alkaloids in *Peganum harmala L*. Using Bromocresol Green. *Res. J. Phytochem.*, 1, 79–82. https://doi.org/10.3923/rjphyto.2007.79.82

251. Sreevidya, N., & Mehrotra, S. (2003). Spectrophotometric method for estimation of alkaloids precipitable with Dragendorff's reagent in plant materials. Journal

of AOAC International, 86(6), 1124–1127. https://doi.org/10.1093/jaoac/86.6.1124

252. Du, M., Guo, S., Zhang, J., Hu, L. and Li, M. (2018). Quantitative Analysis Method of the Tea Saponin. *Open Journal of Forestry*, 8, 61–67. https://doi.org/10.4236/ojf.2018.81005.

253. Guo, Y., Han, Z., Zhang, J., Lu, Y., Li, C., & Liu, G. (2024). Development of a high-speed and ultrasensitive UV/Vis-CM for detecting total triterpenes in traditional Chinese medicine and its application. *Heliyon*, 10(11), e32239. https://doi.org/10.1016/j.heliyon.2024.e32239

254. Luepke, N. P. (1985). Hen's egg chorioallantoic membrane test for irritation potential. *Food & Chem. Toxicol.*, 23(2), 287–291. https://doi.org/10.1016/0278-6915(85)90030-4)