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CHEMICAL CHARACTERISATION, BIOLOGICAL ACTIVITY AND EMULSION DOSAGE FORM DEVELOPMENT OF *SPERANSKIA TUBERCULATA (BUNGE) BAILL*

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Abstract

Establishing the chemical composition of *Speranskia tuberculata (Bunge) Baill.* extracts, their analytical characterisation, and assessment of pharmacological activity for further pharmaceutical development. Methods. Extraction of the aerial parts of the herb with four solvents (ethyl acetate, petroleum ether, n-butanol, and water) was performed. Antibacterial activity was assessed by agar diffusion method against *Staphylococcus aureus*, *Escherichia coli* and *Propionibacterium acnes*. Antioxidant activity was determined using DPPH and SARS tests. Cytotoxicity was assessed in four human cancer cell lines (A549, HEPG2, A375, and HeLa) using the MTT assay and IC_{50} calculations. The chemical composition was investigated by LC/MS and spectrophotometrically to determine the total content of phenols, flavonoids, terpenoids and alkaloids. FTIR was used to control the compatibility of components during the development of the dosage form. An EA extract was used to create an emulsion cream, and the critical technological parameters were assessed. The results. The chemical profile and biological activity of the extracts depend significantly on the solvent, with EA extracts showing the best indicators. The dominant plant compounds belong to phenols, flavonoids, terpenoids and alkaloids. Based on extraction and analytical screening results, the EA extract was used as an active pharmaceutical ingredient in the development of an emulsion cream. The optimal composition was determined using the orthogonal planning method, with assessment of critical quality indicators (pH, dynamic viscosity, and technological stability). FTIR testing confirmed the chemical compatibility of the components and the preservation of marker sites of polyphenolic compounds in the finished product. The cream showed pronounced antibacterial activity against *Propionibacterium acnes* and *Staphylococcus aureus*, with the optimal composition achieving the maximum effect. Conclusions. The study demonstrates an analytically controlled and technologically justified strategy for integrating plant extracts into dosage forms within the framework of chemical engineering of pharmaceutical systems.

Keywords: Speranskia tuberculata (Bunge) Baill.; plant extracts; analytical determination and control; LC/MS; spectrophotometry; FTIR; antibacterial and antioxidant activity; cytotoxicity; pharmaceutical development; emulsion dosage form.

ХІМІЧНА ХАРАКТЕРИСТИКА, БІОЛОГІЧНА АКТИВНІСТЬ ТА РОЗРОБКА ЕМУЛЬСІЙНОЇ ЛІКАРСЬКОЇ ФОРМИ НА ОСНОВІ ЕКСТРАКТІВ *SPERANSKIA TUBERCULATA (BUNGE) BAILL*

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Анотація

Метою роботи було встановлення хімічного складу екстрактів *Speranskia tuberculata (Bunge) Baill.*, їх аналітична характеристика та оцінка фармакологічної активності для подальшої фармацевтичної розробки. Методи. Використано екстракцію аерільних частин трави чотирма розчинниками (етилацетат, петролейний ефір, n-бутанол, вода). Антибактеріальну активність оцінювали методом дифузії в агарі щодо *Staphylococcus aureus*, *Escherichia coli* та *Propionibacterium acnes*. Антиоксидантну активність визначали за допомогою DPPH та SARS тестів. Цитотоксичність оцінювали на чотирьох лініях людських ракових клітин (A549, HEPG2, A375, HeLa) методом MTT з розрахунком IC_{50}. Хімічний склад досліджували LC/MS та спектрофотометрично для визначення загального вмісту фенолів, флавоноїдів, терпеноїдів і алкалоїдів. FTIR застосовано для контролю сумісності компонентів при розробці лікарської форми. ЕА-екстракт використовували для створення емульсійного крему з оцінкою критичних технологічних параметрів. Результати показали, що хімічний профіль та біологічна активність екстрактів суттєво залежить від розчинника, причому ЕА-екстракти характеризуються найкращими показниками. Домінуючі сполуки рослини належать до фенолів, флавоноїдів, терпеноїдів і алкалоїдів. Відповідно до результатів екстракційно-аналітичного скринінгу ЕА-екстракт був використаний як активний фармацевтичний інгредієнт для розробки емульсійного крему. Оптимальний склад визначено методом ортогонального планування з оцінкою критичних показників якості (pH, динамічна в'язкість, технологічна стабільність). FTIR-перевірка підтвердила хімічну сумісність компонентів та

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збереження маркерних ділянок поліфенольних сполук у готовому продукті. Крем проявив виражену антибактеріальну активність щодо *Propionibacterium acnes* та *Staphylococcus aureus*, максимальної дії досягнуто при обраному оптимальному складі. Висновки. Дослідження демонструє аналітично контрольовану та технологічно обґрунтовану стратегію інтеграції рослинних екстрактів у лікарські форми в межах хімічної інженерії фармацевтичних систем.

Ключові слова: Speranskia tuberculata (Bunge) Baill.; рослинні екстракти; аналітичне визначення та контроль; LC/MS; спектрофотометрія; FTIR; антибактеріальна та антиоксидантна активність; цитотоксичність; фармацевтична розробка; емульсійна лікарська форма.

Introduction

Humanity is constantly faced with serious diseases, particularly those caused by bacterial infections. Modern synthetic antibiotics often lose their effectiveness, so the development of medicinal products based on plant materials that can provide a synergistic effect from multiple biologically active compounds simultaneously requires greater attention [1; 2]. Phenolic compounds, alkaloids, flavonoids and terpenoids show significant antibacterial potential through various mechanisms – membrane disruption, protein binding, interference with intermediate metabolism [1; 3]. However, insufficient systematic research and limited study of their synergistic effects hinder the development of medicinal products based on them.

Medicinal plants remain an important source of discovery of new active pharmaceutical ingredients (APIs). Every year, a significant number of works devoted to the development of herbal preparations appear. However, the potential for developing medicinal products from medicinal plant raw materials has not yet been fully realised. In some cases, their implementation requires appropriate adaptation of technological approaches to production.

Large areas with rich flora, particularly in Asia, still contain a significant number of plants not included in modern pharmacopoeias, although they are widely used in traditional medicine [4]. The Chinese endemic herb *Speranskia tuberculata (Bunge) Baill*. (also known as Tou Gu Cao or TGC) is a prime example of this situation. The plant has been used in traditional Chinese medicine for centuries, but scientific data on its mechanisms of action and potential as a source of APIs are lacking. The first studies of flavonoids in this plant appeared almost 30 years ago [5], and to date, research has focused on medicinal properties [6–10].

According to the literature, drugs derived from Speranskia tuberculata exhibit antibacterial, antioxidant, and antitumor activity [11–15], and have also been described as analgesic and anti-inflammatory agents [6]. At the same time, despite significant practical experience, the mechanisms of action and the composition of the active pharmaceutical ingredients remain insufficiently studied. Except for individual works confirming the presence of flavonoids in Speranskia tuberculata preparations [5; 16], systematic studies of the chemical composition and identification of marker compounds are practically absent.

Since plant extracts are multicomponent systems with numerous potential targets, their full understanding requires the development of multilevel models and the avoidance of misconceptions. Integration of analytical data with network models of interactions within the "component–target–biological pathway" framework enables the prediction of synergistic effects and the clarification of mechanisms of action. In this context, the use of systems simulation tools, particularly agent-oriented platforms, which enable modelling of complex, multifactorial interactions in biological systems, is promising [17-19]. Such an integrated approach is important for developing a scientifically sound system to assess the quality and safety of medicinal plant raw materials, as well as for establishing relationships among chemical composition, Q-markers, and pharmacological activity.

Studies of the chemical composition, identification of compounds acting as APIs, and manifestations of synergistic effects remain at an early stage [9]. Information on the concentration of flavonoids, terpenoids, and other secondary metabolites capable of acting as APIs is particularly limited [7]. In this study, *Speranskia tuberculata* was chosen as the object of a comprehensive study. The work was carried out in accordance with the concept of "characteristics of medicinal raw materials — chemical quality — prediction of activity". It included determining the chemical composition of the extracts, assessing their biological activities (antibacterial, antioxidant, and anticancer), and selecting the most promising extract for further development of a dosage form.

One example of practical application was the chemical support for the creation of a cream for local therapy of skin affected by *Propionibacterium acnes* and *Staphylococcus aureus*. This approach

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allows combining traditional experience with modern analytical and pharmacological methods and helps fill scientific gaps regarding the plant's bioactive components and their possible mechanisms of action.

Objective of the work. The work aimed to establish the chemical composition of *Speranskia tuberculata* extracts, their analytical characterisation, and assessment of pharmacological activity to substantiate further pharmaceutical development.

Experimental

Sample preparation. The study examines extracts of the herb *Speranskia tuberculata (Bunge) Bail*. Traditionally, the above-ground (aerial) part of this plant in the first place is used for medicinal purposes both in the market of medicinal plants and in the scientific literature [20]. These parts are ripe stems, leaves, flowers, and shoots in the flowering or vegetative phase, rather than the rhizome or roots. The herb is commercially available from suppliers of traditional Chinese medicinal materials. Dried aerial parts of *Speranskia tuberculata (Bunge) Bail.* were purchased from Tongrentang Pharmacy Ltd. (Beijing, China). The plant material was cleaned of visible foreign impurities before use. Dr Liwen Han, an expert in Traditional Chinese Herbal Medicine and botanist, confirmed the botanical identity (genus and species). A voucher specimen (No. 2017-012-TGC) of the material used in this study has been deposited at Qilu University of Technology and is available for future reference and verification. At present, TGC has not been included in the Pharmacopoeia of the People's Republic of China. Consequently, officially established quality control parameters for this herb remain limited.

In previous research [9], extraction methods are critical for determining the extract composition and quantitative content of active components. The dried herbal materials were ground into a fine powder. Separate portions of the powdered material were extracted using a Soxhlet apparatus with one of the following four extractants: petroleum ether (PE), ethyl acetate (EA), n-butanol (n-but), and deionised water (aqueous solution or AS extractant), yielding the corresponding extracts. The solid-to-solvent ratio was 1:10 (w/v). Each extract was concentrated under reduced pressure, vacuum dried (at -20°C). The dried extracts were reconstituted in dimethyl sulfoxide (DMSO) to the required concentrations for further experiments. DMSO (final concentration 0.1%) was used as the blank control.

Instrumentation. The antibacterial, antioxidant and anticancer properties of the prepared extracts were investigated in the work.

To assess the antibacterial activity, strains of *Staphylococcus aureus* (SA), *Escherichia coli* (EC) and *Propionibacterium acnes* (PA) were used. Bacterial cultures were stored at −80°C. Before the study, the cultures were thawed at room temperature (25°C). 50 μL of bacterial suspension was inoculated into a test tube, and 5 mL of nutrient medium was added (1:100). The cultures were incubated at 37°C for 12 hours. 100 μL of bacterial suspension was applied to the surface of Petri dishes containing solid nutrient medium and evenly distributed. After incubation for 10–15 minutes, sterile filter paper discs were placed on the agar surface, ensuring they were in close contact with the medium. The antibacterial activity of four types of extracts was determined by the agar diffusion method. The test samples were divided into a control group (distilled water) and groups of extracts at concentrations of 10, 50, 100, 200 and 500 μg/ml. 5 μL of the corresponding solution was applied to paper discs and left to evaporate completely at room temperature. Incubation was carried out at 37°C for 12 hours, after which the diameter of the zone of inhibition of bacterial growth (D) was measured by the following equation:

$$\text{Antibacterial rate } (\%) = \frac{D_{samples} - D_{control}}{D_{control}} 100 \quad (1)$$

$D_{samples}$ – 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.

The antioxidant activity of the extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [21], a widely recognised spectrophotometric method that measures the reduction of DPPH free radicals by antioxidants. The assay was performed in 96-well microplates. Extract solutions were prepared to obtain final concentrations of 5, 10, 20, 50, and 100 μg/mL in the reaction mixture. In each well, 180 μL of extract solution was mixed with 90 μL of 0.1 mM DPPH solution. The control group received the corresponding solvent instead of the extract, with 90 μL of 0.1 mM DPPH solution added to each well. After thorough mixing, the plates were incubated in the dark at room temperature for 15 min with gentle shaking. Absorbance of the sample (OD_A) and control (OD_{A0}) was measured at 520 nm using a SpectraMax M5

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spectrophotometer. The DPPH radical scavenging activity (%) was calculated as:

$$\text{DPPH rate (\%)} = \frac{OD_{A0} - OD_A}{OD_{A0}} 100 \quad (2)$$

Another parameter, known as superoxide anion radical scavenging (SARS) activity, was evaluated using the pyrogallol autoxidation method [22]. Pyrogallol undergoes rapid autoxidation in alkaline conditions, generating superoxide anions. The blank solution consisted of 600 μL of 0.1 M Tris–HCl buffer (pH 8.2) and 600 μL of distilled water and was equilibrated in a 25 °C water bath for 20 min. For the reaction system (Solution I), 600 μL of 0.1 M Tris–HCl buffer (pH 8.2) and 580 μL of distilled water were mixed. Extracts were added to obtain final concentrations of 5, 10, 20, 50, and 100 μg/mL. The control group contained solvent instead of the extract. The reaction was initiated by adding 20 μL of 5 mM pyrogallol solution (Solution II). The mixtures were incubated at 37 °C for 10 min. Absorbance was measured at 325 nm. Measurements were recorded every 1 min starting from 0 min, and five consecutive readings were collected.

The superoxide anion radical scavenging activity (%) was calculated as:

$$\text{SARS rate (\%)} = \frac{\Delta A_0 - \Delta A_x}{\Delta A_0} 100, \quad (3)$$

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

Anticancer activity was estimated by measuring IC_{50} values, which characterise the amount of inhibitor required to inhibit a biological process by 50 %, and were calculated from graphs plotting scavenging percentage against sample concentration.

Two TGC extracts, based on EA and PE, were used. Four commercially available cell lines of different types of human cancer, namely A549 (lung adenocarcinoma), HEPG2 (hepatocellular carcinoma of the liver), A375 (malignant melanoma) and HeLa (uterine neck carcinoma), were selected to test the effectiveness of working solutions against cancer cells. All cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS (Fetal Bovine Serum) in 10 cm culture dishes. Other details of cell culture are given in [9].

The generally recognised MTT test (manufacturer: Sigma-Aldrich) was used to assess the effects of EA and PE extracts on the viability of cancer cells of various origins [23]. It is based on the ability of a mitochondrial membrane enzyme to reduce the yellow salt of 3-[e4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to purple formazan crystals. The colour intensity of the accumulated formazan (measured by spectrophotometry) is proportional to cell viability.

All MTT concentration-dependent cell viability experiments were repeated three times for each cell and extract type. The obtained data were expressed as means with standard deviations. To determine the significance of the difference between the average indicators across individual experiments, the Student's t-test was used (p=0.05 was set as the threshold). The experimental curves in all cases had a quasi-linear character. Concentration curves of viability obtained from MTT tests allowed one to calculate the value of the half-maximum inhibitory concentration IC_{50}), the concentration of the solution required to inhibit the biological process by half, for each cell type and both solutions. For this, linear approximations of the experimental viability-concentration curves were used. Calculations were made using OriginPro 2017 (Origin Lab Co, USA).

The study and identification of individual compounds in the extracts were carried out using LC/MS with a quadrupole time-of-flight tandem liquid chromatograph/mass spectrometer (QTOF-LC/MS, Agilent Technologies, USA). 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. 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; flow rate, 12 L/min; capillary voltage, 3200 V.

Mass spectrometric studies revealed 4 dominant classes of compounds across all studied extracts: phenolic compounds, flavonoids, terpenoids, and alkaloids. It allowed one to determine the total content of all compound classes using conventional spectrophotometric methods. Total phenolic content was expressed in mg of glucose equivalent (GE) per gram [24], total flavonoid content – in mg of rutin equivalent (RE) per gram [25; 26], total terpenoids content – in mg of ursolic acid equivalent (UAE) per gram [27] and total alkaloids content – in mg of tuberostemonine equivalent (TE) per gram [28].

Fourier-transform infrared (FTIR) spectroscopy was applied at the stage of medicinal cream development. It was used to identify

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potential molecular interactions between the active pharmaceutical ingredients and excipients. All spectra were obtained in the wavenumber range of 4000-500 cm^{-1} in transmission mode. For each sample, the spectra from three repetitions were automatically averaged, reducing the influence of random errors and improving the analysis accuracy.

Results and discussion

Therapeutic activity of extracts obtained with different extractants. Antibacterial activity was evaluated by determining the antibacterial rate (formula (1)) for systems containing different concentrations of TGC extracts (50-500 μg/mL) prepared with four solvents. Figure 1a shows the antibacterial activity indicators for inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli* and *Propionibacterium acnes* for four types of extracts (at a concentration of 500 μg/mL). Only extracts obtained using EA demonstrated a pronounced antibacterial effect. For these extracts, IC_{50} values were determined at 120, 135, and 112 μg/mL for SA, EC, and PA, respectively. For all other extracts, the IC_{50} was not reached within the studied concentration range.

Among the studied bacteria, EC was the most resistant to EA extracts. PE extracts showed the least antibacterial activity against all three bacterial strains. For them, the antibacterial index ranged from 1 % to 10 % across all concentrations, indicating a weak antibacterial effect. The indices of other extracts ranged from minimal to moderate, typically 10–30 %. In no case was the IC_{50} value achieved.

The antioxidant activity was determined by assessing the free radical scavenging rate for different concentrations of extracts (from 5 to 100 μg/mL) using the DPPH test (DPPH radical scavenging rate, formula (2)) and the superoxide anion radical scavenging rate (SARS, formula (3)).

Figure 1b shows the scavenging rate (%) for DPPH and SARS for four types of extracts (at a concentration of 100 μg/mL). According to both methods, EA and PE extracts showed the highest antioxidant activity, while n-butanol extracts showed the lowest. Converting the experimental DPPH and SARS inactivation rates to IC_{50} values enabled us to quantitatively assess the antioxidant activity of individual extracts. Compounds with IC_{50} values below 50 μg/mL are considered to have high antioxidant activity, while those in the range of 50–100 μg/mL are considered to have moderate activity [29]. Except for the DPPH test results for the n-butanol extract, all other IC_{50} values indicate a high level of antioxidant activity of the tested extracts. However, the correlation between the results of the two tests is not entirely consistent (Pearson correlation coefficient r = 0.795; two-sided significance level p = 0.2025), suggesting differences in the mechanisms of interaction between the extracts and different types of radicals.

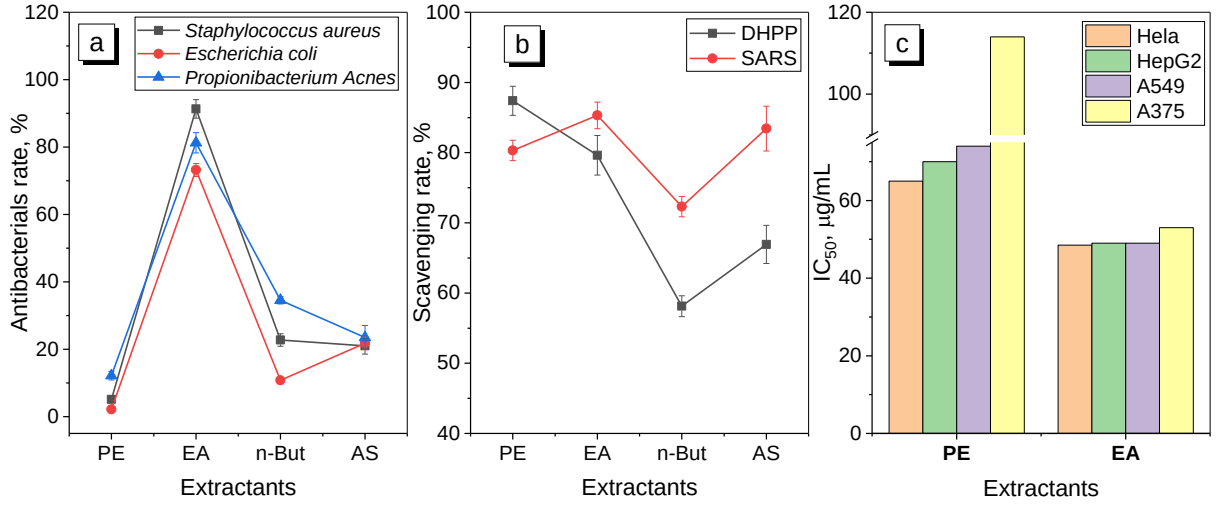

Fig. 1. Antibacterial (a), antioxidant (b), and anticancer (c) activities of extracts of TGC obtained using four different extractants

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Anticancer activity was determined for extracts of two types (EA and PE) against four cancer cell lines: A549, HepG2, A375 and HeLa. Figure 1c shows a comparison of IC_{50} values obtained by calculation, namely by approximating the experimental cell viability curves as a function of extract concentration. It is shown that for EA-based extracts, the IC_{50} values across all studied cell lines are similar (49–53 μg/mL). The lowest IC_{50} value is observed in HeLa cells, while the highest is in A375 cells. A similar trend is observed for PE extracts; however, their IC_{50} values across all cell lines are higher than those of EA extracts and increase in the following order:

HeLa → HepG2 → A549 → A375.

For A375 cells, the IC_{50} value for the PE extract was determined by linear extrapolation beyond the studied concentration range (12.5–75 μg/mL). However, it can be confidently stated that the IC_{50} of PE-based extracts for these cells is notably lower than in other cases.

Thus, among the studied cell lines, HeLa cells are the most sensitive to the action of extracts, particularly EA, as evidenced by the most pronounced inhibition of their viability. The greater effectiveness of EA extracts is also confirmed by the greater decrease in cell viability across the studied concentration range (12.5–75 μg/mL). In particular, in HeLa and HepG2 lines, viability decreased from 100 % to 13–15 %, whereas in A549 and A375 lines, it decreased to 19–28 %. For PE extracts, residual cell viability was 35–62 %, which was 22–35 % higher than for EA extracts.

Extraction efficiency. As shown in the previous section, different TGC extracts demonstrate different therapeutic activity and efficacy. Therefore, it is important to determine their composition, develop and subsequently validate methods for the quality control system of medicinal products, and develop technologies based on these extracts.

Mass spectrum analysis allowed one to assess the composition of the extracts and determine differences in extraction efficiency when using different extractants. The conditions for recording the spectra were chosen so that the total ionic currents of each sample were almost the same. The amplitude of the maximum peaks of the extracts was about 10^6 arbitrary units (a.u.). The smallest peaks fluctuated around 10^2 a.u. The most intense peaks were of greatest interest at the beginning of the work, since the intensity of the pe

aks, as a rule, correlates with the concentration of the substance. Therefore, analysing the most intense peaks was considered equivalent to studying the substances with the highest concentrations in the extracts. It provides reason to hope that among the studied substances, some may have the greatest pharmacological effect. Obviously, this approach is only the first step in investigating a little-studied plant.

At the same time, the probability of incorrect identification increases as peak amplitude decreases, due to increased noise and the masking of smaller satellite peaks by interference. Therefore, only peaks with amplitudes exceeding 10^4 a.u., i.e. approximately 1 % of the amplitude of the maximum peak, were considered.

Although samples of the same plant were studied, the recorded spectra differed in the number of peaks. In some extracts (e.g., PE), up to 300 peaks were detected; in others (e.g., EA), about 100 of the most pronounced peaks were detected. The previous section showed that among the 4 extracts studied, the greatest therapeutic effect is associated with EA-based extracts. Accordingly, the greatest practical interest lies in studying the composition of these extracts.

As already mentioned, about 100 substances are recorded in TGC-EA extracts, of which 50 of the most intense peaks were identified and classified (Table 1).

Table 1

Classification by classes of 50 compounds with the most intense peaks in TGC-EA extracts

Class name	Phenolics	Flavonoids	Terpenoids	Alkaloids	Others	Total
Number of detected metabolites	14	7	5	12	12	50

The data obtained show that only 4 classes of compounds dominate, accounting for more than 75 % of the total number of studied compounds. According to the literature, each of the dominant classes can influence the medicinal properties of plants [30; 31]. Another 12 compounds are classified as amino acids, peptides and proteins, carbohydrates and lipids, which usually perform a

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supporting function. In particular, lipids facilitate the penetration of other active ingredients through the skin, as they are partially lipophilic, while carbohydrates provide energy and serve as building blocks for tissue repair.

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 is obvious that, given the availability of almost four dozen potential active biological substances and the impossibility at this stage to determine the contribution of each substance separately, from a practical point of view, it seems advisable to shift the focus from studying the structure and role of each substance to the synergistic effect of 40 compounds belonging to different classes of substances in extracts [32].

Such a criterion should be used as the basis for selecting the most promising extract for further development of an emulsion dosage form based on it. Therefore, it seems quite logical at this stage to proceed to the analysis of the total content of substances belonging to a particular chemical class. For each selected class of substances, well-developed analytical methods are available, mainly based on spectrophotometry. The results of the determination are given in Table 2.

Table 2

Total contentis of extracted compounds for 4 chemical classes

Samples	Total Phenolic Content (mg GE/g)	Total Flavonoid Content (mg RE/g)	Total Terpenoids Content (mg UAE/g)	Total Alkaloids Content (mg TE/g)
Petroleum Ether extract	6.31 ± 1.89	6.85 ± 1.67	364.51 ± 29.36	29.11 ± 5.89
Ethyl Acetate extract	280.29 ± 25.44	165.72 ± 12.31	226.69 ± 34.93	65.14 ± 17.62
n-Butanol extract	46.45 ± 5.29	17.83 ± 4.11	115.36 ± 21.41	20.35 ± 8.37
Aqueous solution extract	51.87 ± 6.32	15.96 ± 7.24	26.43 ± 6.38	11.58 ± 3.45

The results in Table 2 indicate the concentration ratios for the most influential classes of substances between extracts prepared with different solvents. The most important results include 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 concentration across different solvents is 44, whereas for flavonoids it is 26. The smallest dependence is for alkaloids, the ratio of maximum to minimum is only 5.6.
3. The ranking of compound concentrations in the extracts, from highest to lowest, is:

terpenoids → phenols → flavonoids → alkaloids.

4. If one conditionally sums the total content across all 4 classes for different extracts, the 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 other two extracts.

The most promising herbal medicine for further development. The previous section summarised the experimental results obtained on the therapeutic efficacy of the studied extracts. Such results could receive additional support if a drug were developed from the most promising extract and its efficacy were tested. It would significantly strengthen the argument in favour of using Speranskia tuberculata in the development of dosage forms.

Table 2 shows that two extracts are significantly richer in potential biologically active compounds than the other two, which became the first criterion for their selection. For the final selection, the results from Fig. 1 were compared. Only the extracts obtained using ethyl acetate (TGC-EA) demonstrate a clearly pronounced antibacterial activity. For these extracts, IC_{50} values were determined at 120, 135, and 112 μg/mL for the bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Propionibacterium acnes*, respectively. For all other extracts, IC_{50} values were unattainable.

In terms of anticarcinogenic activity, the TGC-EA extract has a small (20–30%) but stable advantage over TGC-PE. In terms of antioxidant activity, the indicators of both extracts are close. Thus, the advantage of TGC-EA over TGC-PE is obvious in two directions, while their effectiveness in the third direction is approximately equal. This fact supports further research on the TGC-EA extract.

Based on the experimental data, the TGC-EA extract demonstrated the most pronounced antibacterial activity. In particular, it showed the maximum inhibitory effect on gram-positive microorganisms, primarily *Staphylococcus aureus*, as well as on the anaerobic bacterium *Propionibacterium acnes* (also known as

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Cutibacterium acnes), which is a key etiological factor in acne development. Considering that these microorganisms are the main causative agents of infectious and inflammatory skin lesions (folliculitis, impetigo, acne, and other dermatological conditions), it is advisable to develop a drug for topical use that provides high concentrations of the active substance directly at the lesion site and minimises systemic exposure and the risk of side effects.

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 ethyl acetate extracts, compounds of medium polarity are usually concentrated; i.e., part of the flavonoids are presented 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.

Given the physicochemical properties of TGC-EA (the presence of lipophilic biologically active compounds - flavonoids, terpenoids and other secondary metabolites identified by LC/MS), 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;
- is characterised by better sensory properties and patient compliance;
- promotes sufficient penetration of lipophilic components into the superficial layers of the epidermis;
- does not create excessive occlusion, which is important in the treatment of acne and other inflammatory dermatoses.

The emulsion system of the cream helps dissolve the lipophilic components of the extract, maintains their contact with the skin and creates conditions for effective local delivery to the surface and follicular structures. 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. This approach complies with the principles of rational pharmacotherapy and ensures the appropriate level of biological safety of the developed medicinal product.

Formulation of the selected medicinal product. To develop the optimal cream composition, the Orthogonal Design method [33] was used, with 9 samples with different component ratios selected at the initial stage.

Subsequently, the determination of 5 pharmaco-technological parameters in products at different stages of pharmaceutical development was carried out: Appearance, Colour, Transmittance, Texture, and Spreadability. Technological stability was also assessed by three indicators: Centrifugal Stability, Cold-resistant Stability, and Hot-resistant Stability. pH and Dynamic Viscosity (DV) were measured for each sample.

Each determined parameter was assessed on a 10-point scale for each of the 9 studied compositions. Based on the total indicator across all parameters, the optimal cream composition with the highest score was identified (Table 3).

Compatibility of components by FTIR. To control product quality, FTIR spectroscopy was used at each stage of production. The FTIR study aims to address a classic pharmaceutical development problem: Drug-Excipient Compatibility Studies. FTIR is an ideal tool for this [34; 35], as it allows changes in chemical bonds to be seen without destroying the sample. If the spectrum of a mixture is simply the mathematical sum of the spectra of the components, they are compatible. If new peaks appear, old ones disappear, or there is a significant shift in the main functional groups, this indicates a chemical interaction. FTIR is extremely sensitive to physicochemical interactions between components. FTIR reacts when mixing different substances leads 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 of the starting substances) and the appearance of new peaks (bands of new functional groups).

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Table 3

The formulation of the antibacterial medical cream based on TGC-EA Dry Extracts

Names of active and auxiliary substances	Content of components in one tube, g		The function of each component of the dosage form
TGC-EA Dry Extracts	0.1	Oil Phase	Active ingredient
Stearic acid	6.0	Oil Phase	Viscosity and consistency regulator
Cetearyl alcohol	8.0	Oil Phase	Viscosity and consistency regulator
Apricot kernel oil PEG-6 esters	4.0	Oil Phase	Basis
Petrolatum	2.0	Oil Phase	Basis
Polysorbate 80	8.0	Water Phase	Emollient
Acacia Senegal Gum	3.6	Water Phase	Emulsifier
Astragalus Gummifer Gum	1.2	Water Phase	Emulsifier
Glycerin	10	Water Phase	Humectant
Ethyl Paraben	0.1	Water Phase	Antimicrobial preservative
Methyl Paraben	0.1	Water Phase	Antimicrobial preservative
EDTA	0.4	Water Phase	Chelating agent
Tocopherol	0.2	Oil Phase	Antioxidant
Purified water	56.3		
Mass of cream	100.0		

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, if it is necessary to confirm the absence of changes, the spectrum of the mixture must be a perfect additive mathematical sum of the spectra of the individual components.

The cream is made by combining the aqueous and oil phases with purified water, which are then mixed to form the final ointment. 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 active ingredient, a viscosity and consistency regulator, an antioxidant, and an excipient (base) are placed, which serves as a "carrier" for the active ingredient, ensuring its stability and delivery to the tissues (Fig. 2).

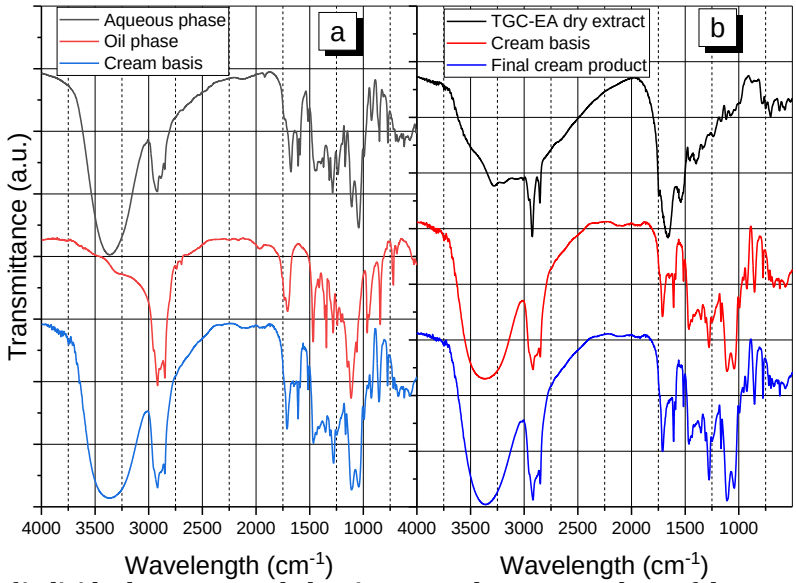

Fig. 2. FTIR spectra of individual components belonging to: a – the aqueous phase of the cream, b – the oil phase of the cream

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. 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. The peak at about 1740 cm^{-1} indicates

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the presence of ester groups (C=O) of triglycerides or oils.

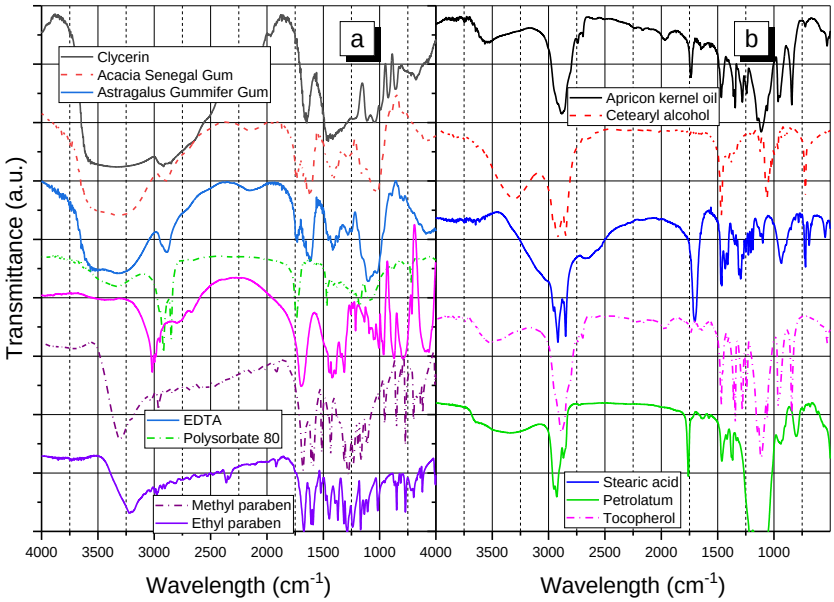

Fig. 3. FTIR spectra: a – total spectrum of the aqueous, oily phases and the cream base as a whole, b – of the herb extract, cream base and the final product

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 emulsion stability (Fig. 3a). 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.

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. 3b).

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 (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 extremely difficult to isolate alkaloids in the final product by FTIR alone.

In summary, the most pronounced (specific markers) visually in terms of peak size 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, since it is their specific "fingerprints" in the region of 1000–1650 cm^{-1} that most noticeably change 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. 3b). In the final product, it is seen that 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 repeats the profile of the cream base, but with the imposition of characteristic 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 appeared in the spectrum of the final cream product (which were absent in both the base and the extract), confirming the chemical compatibility of the components. That is, 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.

Antibacterial efficiency of the developed cream. The effectiveness of the developed cream was tested in experiments on the cultivation of *Propionibacterium acnes* and *Staphylococcus aureus* on a dense nutrient medium in the presence of different concentrations of TGA-EA extract (Fig. 4).

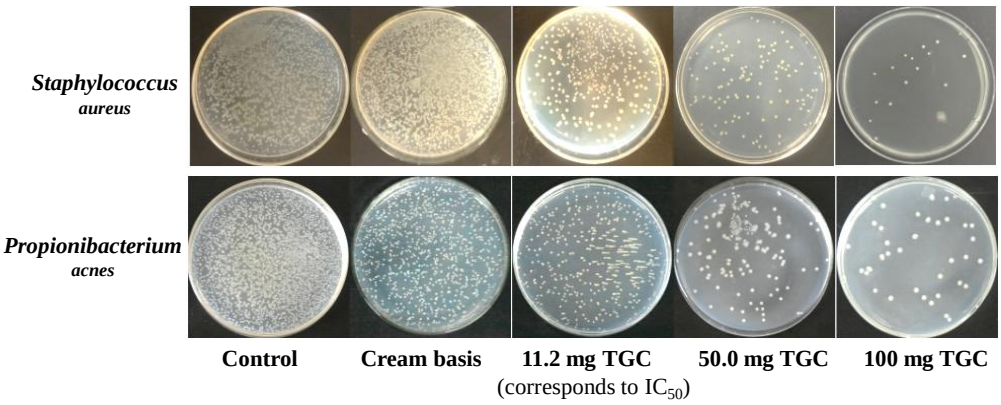

Fig. 4. Results of cultivation of *Propionibacterium acnes* and *Staphylococcus aureus* bacteria on dense nutrient medium in the presence of different concentrations of TGA-EA extract

As we can see, the IC_{50} value is 11.2 mg of TGC (i.e., a concentration almost 10 times lower than the determined optimum). At 100 mg TGC (corresponding to the selected optimal cream composition), the effect on bacterial colonies is maximal, with a relatively greater effect on

Staphylococcus aureus than on *Propionibacterium acnes*.

Conclusions

1. A significant dependence of the biological activity of the extracts on the nature of the extractant was established, which confirms the decisive role of the technological stage of extraction in the formation of the qualitative and quantitative composition of the product. EA extracts exhibit the lowest IC_{50} values (112–135 μg/mL) in antibacterial activity tests, whereas IC_{50} values are not reached with other solvents. Quantitative assessment of antioxidant activity using the DPPH and SARS methods showed high radical-scavenging activity for the EA and PE extracts. The cytotoxic activity is the highest and stable for EA extracts (49–53 μg/ml) for all studied cell lines.

2. Mass spectrometric analysis confirmed the significant dependence of the qualitative and quantitative profile of the extracts on the nature of the solvent. In TGC-EA extracts, more than 75% of the intensive components belong to four classes: phenols, flavonoids, terpenoids, and alkaloids, which contribute to their biological activity. EA provides the maximum total content of phenols (280.29 mg GE/g), flavonoids (165.72 mg RE/g) and alkaloids (65.14 mg TE/g), while the PE extract is characterised by the highest content of terpenoids (364.51 mg UAE/g). By integral saturation with dominant classes, the extracts are ranked in line EA → PE → n-but → AS. The results confirm that the choice of solvent is a key factor in shaping the chemical profile and can be used as a controlled parameter in developing and controlling the extraction technology.

3. The TGC-EA extract, selected according to the results of extraction and analytical screening, was used as an API for the development of an emulsion dosage form. The optimal cream composition was determined using an orthogonal planning method, with assessment of critical quality indicators (pH, dynamic viscosity, technological stability and organoleptic parameters). The selected composition is characterised by reproducible rheological properties and physical stability, indicating the controllability of the system-formation process. FTIR control confirmed the chemical compatibility of the components: the spectrum of the finished product is an additive superposition of the spectra of the base and the extract without the appearance of new absorption bands. The preservation of marker areas 1000–1700 cm^{-1} indicates the stability of polyphenolic compounds and the absence of degradation during technological processing.

4. The antibacterial activity of the developed cream was confirmed in experiments on dense nutrient medium against *Propionibacterium acnes* and *Staphylococcus aureus*. At a concentration of 100 mg of TGC (selected composition), the maximum inhibitory effect was observed, more pronounced against *Staphylococcus aureus* than against *Propionibacterium acnes*. Thus, a scalable, analytically controlled strategy for integrating PGC-EA extracts into a dosage form with defined critical quality parameters was demonstrated.

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