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STUDY OF ANTIBACTERIAL PROPERTIES OF EXTRACTS OF THE HERB *SPERANSKIA TUBERCULATA* (BUNGE) BAILL

Key words: *Speranskia tuberculata*, antibacterial activity, *Staphylococcus aureus*, *Escherichia coli*, *Propionibacterium acnes*, ethyl acetate extract

A B S T R A C T

The article deals with the problem of developing antibacterial drugs of plant origin, focusing on the properties of the herb *Speranskia Tuberculata* (Bunge) Baill, which is still understudied.

The aim was to determine the antibacterial activity of preparations of the herb *Speranskia Tuberculata* (Bunge) Baill, extracted with different solvents.

Materials and methodology. Crude extracts were obtained using four solvents: petroleum ether, ethyl acetate, n-butanol, and aqueous solutions. Subsequently, the dried extracts were weighed and dissolved in dimethylsulfoxide to prepare working solutions with 10 to 500 μg/mL concentrations. Colonies of three types of bacteria, Staphylococcus aureus, Escherichia coli, and Propionibacterium Acnes, were used as the object of the study. The degree of antibacterial activity was determined by measuring the antibacterial zone formed after treating bacteria with different concentrations of extracts compared to the control group (treatment with distilled water). In addition, the total content of phenolic compounds and flavonoids was determined by chemical methods. Antioxidant activity, volume of the dry residue of crude extracts, and content of flavonoids and phenolic compounds were compared with the results of liquid chromatography-mass spectrometry on the presence of antibacterial substances in the extracts known from the literature.

It was shown that the ethyl acetate extract among the 4 studied extracts had a clearly expressed antibacterial effect. The calculated value of IC_{50} for this extract varied between 112–135 μg/mL for different bacteria. Other solvents did not show a pronounced antibacterial effect. The weakest effect was associated with petroleum ether extracts. At the same time, the mass of the dry residue, when extracted with petroleum ether, was the maximum among all solvents; on the contrary, with ethyl acetate, it was the minimum. Regarding the content of phenolic compounds and flavonoids, the results of the studies were opposite; namely, the maximum concentrations were characteristic of the ethyl acetate extract, and the minimum concentrations were characteristic of the petroleum ether extract. According to the literature, twelve compounds found in the extracts have pronounced antibacterial activity. Suppose the presence of an aromatic ring and a carbon-linked hydroxyl group OH^- is taken as the main sign of belonging to phenolic compounds. In that case, 8 of 12 invented antibacterial compounds belong to phenols and flavonoids.

Experimentally proven high antibacterial activity of preparations of the herb *Speranskia Tuberculata* (Bunge) Baill based on ethyl acetate extracts. This property is related to the extract's high content of phenolic compounds and flavonoids.

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ВИВЧЕННЯ АНТИБАКТЕРІАЛЬНИХ ВЛАСТИВОСТЕЙ ЕКСТРАКТІВ ТРАВИ *SPERANSKIA TUBERCULATA* (BUNGE) BAILL

Ключові слова: сперанскія туберкулята, антибактеріальна активність, стафілокок золотистий, кишкова паличка, пропіонова бактерія акне, екстракт етилацетату

АНОТАЦІЯ

Стаття торкається проблеми розроблення антибактеріальних препаратів рослинного походження із фокусом на властивості трави *Speranskia tuberculata* (Bunge) Baill, яка досі є малодослідженою.

Метою було визначення антибактеріальної активності препаратів трави *Speranskia tuberculata* (Bunge) Baill, екстрагованих різними розчинниками.

Сирі екстракти одержували за допомогою чотирьох різних розчинників, а саме петролейного ефіру, етилацетату, н-бутанолу, водних розчинів. У подальшому висушені екстракти було зважено та потім розчинено в диметилсульфоксиді для приготування робочих розчинів із концентраціями від 10 до 500 мкг/мл. Як об'єкт дослідження використано колонії трьох видів бактерій – стафілокока золотистого, кишкової палички, пропіонової бактерії акне. Ступінь антибактеріальної активності визначали в процесі вимірювання антибактеріальної зони, що утворювалася після оброблення бактерій різними концентраціями екстрактів, відносно контрольної групи (оброблення дистильованою водою). На додаток, загальний вміст фенольних сполук та флавоноїдів було визначено хімічними методами. Антиоксидантну активність, розмір сухого залишку сирих екстрактів, вміст флавоноїдів та фенольних сполук порівняно з результатами рідинної хроматографії-мас-спектрометрії щодо наявності в екстрактах антибактеріальних речовин, відомих із літератури.

Показано, що екстракт етилацетату серед 4-х досліджених екстрактів має чітко виражений антибактеріальний ефект. Розрахована величина IC_{50} для цього екстракту варіюється в межах 112–135 мкг/мл для різних бактерій. Екстракти інших розчинників не проявляють вираженої антибактеріальної дії. Найслабший ефект пов'язаний з екстрактами петролейного ефіру. Одночасно маса сухого залишку у разі екстрагування петролейним ефіром була максимальною серед усіх розчинників, а етилацетатом – навпаки, мінімальною. Щодо вмісту фенольних сполук та флавоноїдів, результати досліджень зворотні, а саме максимальні концентрації характерні для етилацетатного екстракту, а мінімальні – для екстракту петролейного ефіру. В екстрактах виявлено 12 сполук, які, за даними літератури, мають виражену антибактеріальну активність. Якщо наявність ароматичного кільця та пов'язаної з вуглецем гідроксильної групи OH^- прийняти за основну ознаку належності до фенольних сполук, тоді 8 із 12 винайдених антибактеріальних сполук належать до фенолів та флавоноїдів.

Експериментально доведено високу антибактеріальну активність препаратів трави *Speranskia tuberculata* (Bunge) Baill на основі екстрактів етилу ацетату. Ця властивість пов'язана із високим вмістом в екстракті фенольних сполук та флавоноїдів.

Introduction

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. In widespread and often excessive consumption of synthetic antibiotics, bacteria have acquired the ability to adapt to the drugs and mutate quickly. Therefore, the use of known antibiotics and the development of new synthetic agents is gradually losing importance as they quickly become ineffective against multiresistant bacteria. 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 transferred to the active involvement of medicinal plants capable of providing the simultaneous synergistic effect of several biologically active substances.

Medicinal plants can serve as a promising alternative to ineffective synthetic antibiotics in the fight against infectious diseases [1, 2]. Phenolic compounds, alkaloids, saponins, and terpenoids have shown significant antibacterial potential, primarily through mechanisms of membrane disruption, protein binding, interference with intermediate metabolism, and so on [1, 3]. However, such a transition is restrained by insufficient study of the potential of medicinal plants, including the synergistic effect of the simultaneous action of several active pharmaceutical ingredients (APIs). Many new works on developing herbal medicines appear yearly [4–6]. However, the potential for the development of herbal medicine remains undiscovered. Moreover, in some cases, the production of herbal medicines requires certain changes in the technological cycle [7].

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 Chinese traditional medicine for centuries. Almost 30 years ago, this plant's first studies of flavonoids appeared [8]. 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 following years, *Speranskia tuberculata*, remaining among the popular means of traditional Chinese medicine, did not undergo significant coverage in the world's scientific literature. Only a few works appeared in recent years and decades [9–13]. It should be noted that most of the publications aim to study medicinal properties. Research of the chemical composition, detection of compounds acting as APIs, and manifestations of synergistic effect remain at the initial stage [13].

Nevertheless, much needs to be done for further progress, especially bearing in mind the complex chemical composition of the plant, the ambiguity of its interaction with solvents, and the degree of manifestation of possible antibacterial properties when interacting with various bacteria.

The work aimed to determine the antibacterial activity of preparations of the herb *Speranskia Tuberculata* (Bunge) Baill, extracted in crude form with various solvents, in the environment of three different widespread bacteria.

Materials and Methods

Dried *Speranskia tuberculata* (Bunge) Baill herbs were purchased from Tongrentang Pharmacy Ltd. (Beijing, China). 4 solvents, namely petroleum ether (hereafter PE extract), ethyl acetate (EA), n-butanol (n-But) and aqueous solution (AS), respectively, were used for the production of crude extracts, as described in detail in [13]. Dry residues at the stage of crude extraction preparation were carefully

weighed. Further dissolution in dimethyl sulfoxide (DMSO) prepared working solutions [13] with 10, 50, 100, 200 and 500 μg/mL concentrations.

Three kinds of bacteria were used to evaluate the antibacterial activity: Staphylococcus aureus (SA), Escherichia coli (EC), and Propionibacterium Acnes (PA). Bacterial colonies were prepared for the study as follows. Bacteria were taken from a refrigerator at -80 °C. After thawing at room temperature (25 °C), 50 μL of bacterial liquid was inoculated into a test tube. Then, 5 mL of culture medium was added to it at a ratio of 100:1. The next step was incubation at 37 °C for 12 h. 100 μL of the bacterial solution was placed on a plate with a solid culture medium, covering it evenly. After standing for 12 min, the sterilised filter paper was attached to adhere to the culture medium's surface.

Determination of the antibacterial capacity of 4 types of extracts took place as follows. Experimental samples were divided into 2 groups: control group (distilled water) and sample groups (4 types of extracts, each with concentrations of 10, 50, 100, 200 and 500 μg/mL). 5 μL of solutions of different concentrations of extract samples were taken and applied to filter paper for further drying in the solvent naturally. The sample obtained this way was placed in a shaking incubator with a constant temperature to observe the experiment's results and measure after 12 h diameter of the antibacterial zone (D).

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

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

In addition, the total content of flavonoids and phenolic compounds was determined chemically. 0, 0.4, 0.8, 1.2, 1.6 and 2.0 mL of the standard rutin solution were carefully pipetted into a 10 mL volumetric flask, and 2.0, 1.6, 1.2, 0.8, 0.4, and 0 mL of 60% ethanol solution were added respectively. After that, 0.5 mL of 5% sodium nitrite solution was added, and the solution was shaken well. Then, it was left to stand for 6 min; 0.5 mL of 10% aluminium nitrate solution was added and kept for 6 min. 4.0 mL of 4% sodium hydroxide solution and 60% ethanol were added to the volume, shaken well and left for 15 min. The absorbance value at 510 nm was measured using a SpectraMax M5 spectrophotometer. The content of flavonoids in each extract was checked according to the absorbance value. Total flavonoid content was expressed in mg of rutin acid equivalents (RE) per gram of extract.

For phenolic compounds, 25 mg of gallic acid was accurately weighed, dissolved in water, and made up to volume in a 250 mL volumetric flask to obtain a 0.1 mg/mL standard stock solution. 0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mL of standard gallic acid solution were taken and placed in 25 mL stoppered test tubes. 1 mL of folinol was added, shaken well, and 2 mL of 12% Na_2CO_3 solution was added. It was brought to 25 mL with water and shaken well. Absorbance values were measured at

a wavelength of 765 nm using a SpectraMax M5 spectrophotometer after reaction in a dark environment at room temperature for 2 h. Then, the content of phenolic compounds in each extract was checked according to the absorbance value. The total content was expressed in mg of gallic acid equivalents (GE) per gram of extract.

Liquid chromatography-mass spectrometry using a tandem QTOF-LC/MS (Agilent Technologies, USA) was applied to characterise the chemical composition of extracts. The separation of compounds was achieved on Waters Cortecs C18 2.1*50 mm 1.7 µm column in gradient mode. 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 4 min balance back to 90%A–10% B. The injection volume was 20 µL, and the flow rate was 0.3 ml/min. The mass spectra were acquired in ESI negative mode (100–1 500 m/z). The parameters were as follows: drying gas (nitrogen) with a flow rate of 15 L/min; sheath gas temperature 350 °C, flow rate 12 L/min; voltage 3200 V.

Results and Discussion

The values of the antibacterial rate measured and calculated according to equation (1) depending on the concentration of extracts of different origins are shown in Fig. 1 to suppress the bacteria *Staphylococcus aureus* (*a*), *Escherichia coli* (*b*) and *Propionibacterium acnes* (*c*). Only the extracts obtained with the help of EA show a clearly expressed antibacterial effect. For these extracts, the IC_{50} values can be determined, which were equal to 120, 135 and 112 µg/ml, respectively, for SA, EC and PA bacteria. For all other extracts, the IC_{50} value was unattainable. The EC species was the most resistant to EA extracts among the studied bacteria.

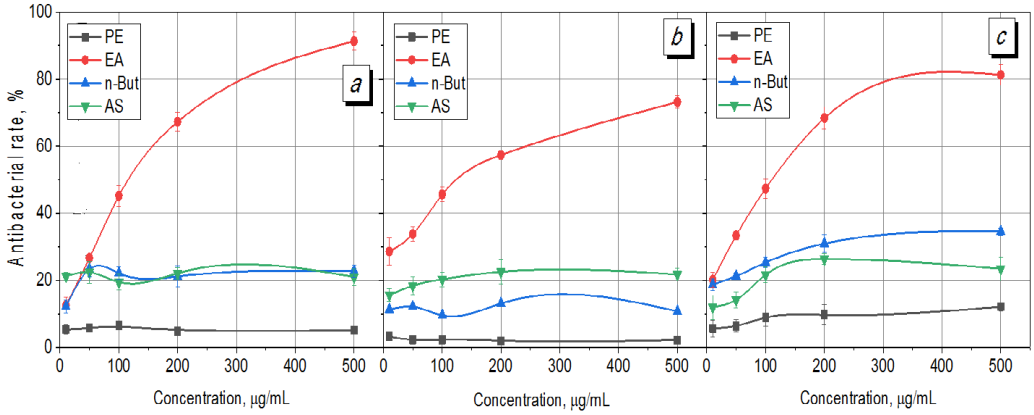

Fig. 1. **Antibacterials rates for 4 kinds of extracts in the different bacteria media**: *a – Staphylococcus aureus*, *b – Escherichia coli*, *c – Propionibacterium acnes*

At the opposite pole (with minimal antibacterial effect), PE extracts were found on all three types of bacteria. The antibacterial rates ranged from a few units to 10%

for all bacteria and PE concentrations, indicating their negligible antibacterial effect. The figures for other extracts varied between a minimum and a maximum, usually between 10 and 30%. In no case was the IC_{50} achieved.

Research on the composition of *Speranskia tuberculata* extracts is at the very initial stage [13], so there is no clear answer as to which processes are behind the better antibacterial activity of EA extracts. Nevertheless, Fig. 2 contains additional information, where experimental data on the value of the dry residue of raw extracts and the measured values of the total flavonoids and phenolic compounds in them are given.

It is noticeable that the amount of dry residue at the preparation stage of raw extracts was maximum when PE was used. However, it did not provide antibacterial activity. The data on the dry residue (Fig. 2, *a*) contradict the results of the study of the content of phenols (Fig. 2, *b*) and flavonoids (Fig. 2, *c*). According to these indicators, EA extracts were in the lead, which at the same time had the highest antibacterial activity. Conversely, PE extracts were characterised by a large dry residue and, at the same time, low amounts of flavonoids and phenols and the lowest antibacterial activity.

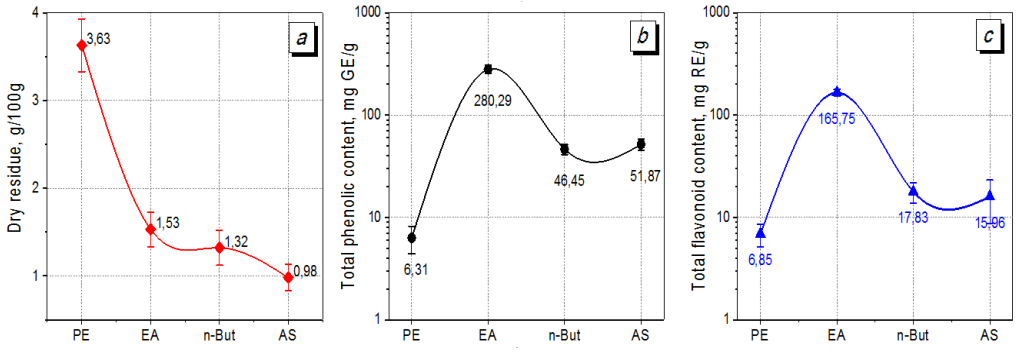

Fig. 2. **The dry residues (*a* – in g/100g), total contents of phenolic compounds (*b* – in mg/GE/g) and flavonoids (*c* – in mg/RE/g) extracted with 4 different solvents**

The results of tandem liquid chromatography-mass spectroscopy provide more information. The samples of PE and EA extracts were studied. 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 10^6 arbitrary units (au). The smallest peaks in amplitude varied at the level of 10^3 au. The probability of false identification increases as the peak amplitude decays due to the increased influence of noise and the masking of smaller satellite peaks by interference. Therefore, only the most intense peaks, the intensity of which varied within 10^4–10^6 au, were further analysed.

A characteristic feature was approximately twice the number of PE peaks of the extract in the given intensity interval, which generally correlates with almost twice

the volume of the dry residue. Substances, especially APIs, inherent in the herb *Speranskia tuberculata* have practically not been studied in the literature. Therefore, attention was drawn to the literature to answer the question of which substances form antioxidant activity. The analysis of the obtained spectra made it possible to identify 12 compounds, which, on the one hand, had intense peaks in the extracts. On the other hand, as evidenced by literature data, these compounds showed antibacterial, antimicrobial and antifungal effects in preparations of other plants. Identified compounds are shown in Table 1.

Table 1

Compounds were detected in PE and EA extracts by LC/MS with apparent antibacterial activity, as shown in [Source].

Chemical classification was done by [14]

Compound	Detected in extracts	Superclass	Class	Subclass	Source
Linoleyl acetate	EA	Lipids & lipid-like molecules	Fatty Acyls	Fatty alcohol esters	[15]
Aschantin	EA, PE	Lignans, neolignans & related compounds	Furanoid lignans	Na	[16]
3,5-Dimethyl-4-methoxybenzoic acid	EA, PE	Benzenoids	Benzene & substituted derivatives	Benzoic acids & derivatives	[17]
(E)-3-Methoxy-4,5-methylenedioxycinnamic aldehyde	PE	Organoheterocyclic compounds	Benzodioxoles	Na	[18]
14-Methyl hexadecanoic acid	EA	Lipids & lipid-like molecules	Fatty Acyls	Fatty acids & conjugates	[19]
(E)-4-(1,5-Dimethyl-3-oxo-1-hexenyl) benzoic acid	PE	Lipids & lipid-like molecules	Prenol lipids	Sesquiterpenoids	[20]
Filicinic acid	EA, PE	Organic acids & derivatives	Vinylogous acids	Na	[21]
Ethyl heptadecanoate	PE	Lipids & lipid-like molecules	Fatty Acyls	Fatty acid esters	[22]
(2R,3R)-(+)-4',5,7-Trimethoxydihydroflavonol	PE	Phenylpropanoids & polyketides	Flavonoids	Flavones	[23]
Haploperine	PE	Organoheterocyclic compounds	Quinolines & derivatives	Furanoquinolines	[24]
Alpha-Narcotine	PE	Alkaloids & derivatives	Phthalide isoquinolines	Na	[25]
Mycosinol	PE	Organic oxygen compounds	Organooxygen compounds	Ethers	[26]

Organic compounds, as you know, differ in diversity, so it is not easy to classify them. The work [14] proposed the principles of classification, which made it possible to build 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 point of view of their pharmaceutical action, have similar structures and properties. In pharmaceutical chemistry, such compounds are often clustered together.

The determining factor of phenolic compounds is a hydroxylated aromatic ring and a hydroxy group attached directly to phenyl. Note that most of the compounds from Table 1 have an aromatic ring and an attached OH^- group. Accordingly, structurally and from the viewpoint of critical characteristics, they can be attributed to phenolic compounds. More precisely, 8 out of 12 compounds with high antibacterial activity are related to phenolic compounds and flavonoids. This situation explains the experimental fact that EA extracts, which have the maximum total content of phenolic compounds and flavonoids, are simultaneously characterised by the highest antibacterial activity.

It should be said that at this stage, it is impossible to say which compound has a vital role in the formation of antibacterial activity. Other than that, there are several such compounds, and their synergistic effect dominates practice. The latter is a typical phenomenon in herbal medicines, so further research should clarify this issue more precisely.

Conclusions

1. Four solvents were studied for the extract with the greatest antibacterial effect. Ethyl acetate extract shows high antibacterial activity (for different bacteria, the value of IC_{50} varies in the range of 112–135 μg/mL), while the other three studied extracts remain inactive.

2. The antibacterial activity of different solvent extracts is proportional to the total content of flavonoids and phenolic substances. It shows an inverse dependence on the volume of the dry residue of the crude extract.

3. In analysing the most intense mass peaks of the extracts using liquid chromatography-mass spectroscopy, 12 substances were identified, which, according to literature data, have antibacterial properties. If belonging to phenolic compounds is assessed by an aromatic group with a hydroxyl group OH^- connected to carbon, then 8 out of 12 compounds found belong to phenols and flavonoids.

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