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THE ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF THE EXTRACT OF *GENTIANA CRUCIATA* L. HERB

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Abstract

Gentiana cruciata L. belongs to the *Gentianaceae* family and is a well-known plant, that has long been used in folk medicine in many countries of the world. The herb and roots of *Gentiana cruciata* L. are used for many diseases. The dry extract obtained of *Gentiana cruciata* L. herb was studied to antibacterial and antifungal properties. Phytochemical investigation of the dry extract indicated the presence of tannins and polyphenols, which have antibacterial and antifungal activities. The total content of tannins and polyphenols, in recalculation at pyrogallol, was 1.84% and 8.52% respectively. Antibacterial and antifungal activity of dry extract of *Gentiana cruciata* L. herb was determined "wells" method (agar diffusion) by measuring of inhibition zone diameters (mm). The native extract was very active against *Staphylococcus aureus* (25.2 mm), *Bacillus subtilis* (22 mm), *Escherichia coli* (20.1 mm), and *Candida albicans* (17.9 mm). Also, the extract of *Gentiana cruciata* L. herb in N:2 dilution was very active against *Staphylococcus aureus* (19 mm), active to *Bacillus subtilis* (15 mm), *Escherichia coli* (13 mm), and less active to *Candida albicans* (11.6 mm). Consequently, extract of *Gentiana cruciata* L. herb may be used for the development of new medicines as an active pharmaceutical ingredient.

Keywords: *Gentiana cruciata L., herb, dry extract, antibacterial activity, antifungal activity*

Introduction

The medicinal plants have been used in traditional and folk medicine use for many centuries [1, 2]. Today, the global pharmaceutical industry widely uses herbal raw materials as a basis for the creation of medicines. 25 % of remedies in the modern pharmacopeia are plant-derived [3, 4]. The plant medicines are well tolerable, often used in the fight against many diseases, and have minor side effects [5-8]. In addition, plant drugs have a wide range of pharmacological properties, which are realized through many groups of phytochemicals [9, 10]. Since 1981, 38 molecules have been derived from medicinal plants, out of which 1,130 new therapeutic agents have been approved as pharmaceutical drugs [11, 12]. The current nomenclature of herbal medicines according to data of specialists in different countries is 30-50% of the total number of drugs produced. According to the WHO, the market of herbal medicines is 60 billion US dollars. Combinations of different medicinal plants deserve special attention as such herbal mixtures have many biologically active substances [13, 14]. Given the growing needs of the pharmaceutical industry in plant raw materials for the manufacture of drugs, an important task of pharmaceutical science is to widen existing and searching for new sources [15-17]. Ukraine has sufficient raw material resources of wild and cultivated medicinal plants, the necessary industrial and scientific potential to ensure the further development of the creation and production of phytopreparations.

Such a promising source of biologically active substances is a plant of the *Gentianaceae* family – *Gentiana cruciata* L., which has long been used in folk medicine and according to previous studies contains many biologically active substances.

The *Gentiana cruciata* L. roots and herb are a source of secoiridoids and their glycosides namely gentiopicrin, sweroside, gentiopicroside, amarogentin, and swertiamarine [18-21].

The plant is a source of important pharmacologically active phytochemicals that have many properties. Thus, during the hydrolysis of gentiopicroside by β-glycosidases gentiogenol is formed, which exhibits antifungal properties.

The various parts of *Gentiana cruciata* L. have been used in folk medicine as anticholinesterase, antimicrobial, antioxidant, and antigenotoxic remedies [22]. In addition, the roots have stomachic, sedative effects and stimulate the production of white blood cells [23]. Researchers was determined the inhibitory effect of ethyl acetate extract of *Gentiana cruciata* L. root on butyrylcholinesterase, which is contained in structures placed usually outside the central nervous system [24].

Further studies should be carried out to clarify the relationship between biologically active substances plant and their benefic effect on the human body. Different researches propose tannins and polyphenols as antimicrobial means [25]. Therefore, the purpose of the study was to investigate antimicrobial and antifungal activity of dry extract obtained from the herb of *Gentiana cruciata* L.

Methods

Text *Plant Materials*

The object of the study was the herb of *Gentiana cruciata* L., which was collected at the territory of Volove, Ternopil region (Western Ukraine, N 49°21'13.0" E 26°05'24.1") during the flowering period in 2017 [26]. The raw material was authenticated by professor Svitlana Marchyshyn (I. Horbachevsky Ternopil National Medical University, Ukraine) [27, 28]. A voucher specimen of *Gentiana cruciata* L. no. 135 is kept at the Department of Pharmacognosy and Medical Botany, TNMU, Ternopil, Ukraine. The study plant material was dried using the conventional method and stored in paper bags in a dry, protected from direct sunlight place [29, 30].

Preparation of extract. About 5000 g of dried *Gentiana cruciata* L. herb were powdered with the help of an appropriate grinder. It was taken in an extractor and extracted using 70% ethanol as a solvent. Extraction was performed by the maceration method at a temperature of 20 ± 2° C. The obtained extract was concentrated under a rotary vacuum evaporator at a temperature of 60±2° C. During remaceration, the amount of extractant was divided into portions and each portion was being infused with the raw material.

Chemicals reagents

Ammonium iron (III) sulfate, gelatin, quinine hydrochloride, chloroform, sodium molybdate dehydrate, Phosphotungstic acid hydrate, sodium carbonate, pyrogallol were purchased from Ltd. Sfera Sim (Lviv, Ukraine), were of the highest purity available.

Microorganisms

In research used a standardized diumal suspension of testing strains American Type Culture Collection (ATCC) of the microorganisms, namely: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 885-653. Cell concentration was 0.5 McFarland.

Determination of tannins and polyphenols

Identification reactions

In the presence of tannins, the water extract of the herb of the studied plant was investigated. To do this, 1 g of crushed dry material was placed in a 250 ml flask, poured into 100 ml of purified water, and heated in a boiling water bath for 20 min, cooled and filtered. Lipophilic substances were removed from the aqueous extract, shaking it with chloroform in a separating funnel in a ratio of 1:1. The chloroform layer was separated and three volumes of alcohol were added to the aqueous extract. The precipitate was filtered off and discarded.

The presence of tannins in the study extract was confirmed by the following reactions:

1. with a solution of ammonium iron (III) sulfate. 2-3 drops of a solution of ammonium iron (III) sulfate was added to 2-3 ml of the extract. The appearance of black-green residue indicated the presence of tannins.

2. with 1 % gelatin solution. 1 % gelatin solution was added dropwise to 2 ml of purified extract. There was a dreg, which disappeared with excess gelatin;

3. with a 1 % solution of quinine hydrochloride. A few drops of 1 % quinine hydrochloride solution were added to 2 ml of the extract. An amorphous precipitate appeared [31].

The quantitative content of biologically active substances was determined out by the spectrophotometric method according to the European Pharmacopeia and State Pharmacopoeia of Ukraine. The investigation of the content of polyphenols and tannins was carried out in a five-fold repetition.

Determination of quantitative content of tannins. 0.50 g of powdered raw material was placed in a 250 ml round flask, 150 ml of water R was added to the flask; heated for 30 minutes in a water bath, cooled under running water R, and quantitatively transferred to a 250 ml graduated flask. The round-bottomed flask was rinsed with water, the flushing waters were transferred to a measuring flask and the solution volume was diluted with water R to 250 ml. The precipitate was allowed to settle and the liquid was filtered through filter paper with a diameter of 125 mm. The first 50 ml of the filtrate was discarded.

The amount of polyphenols. 5 ml of the filtrate was diluted with water R to 25 ml. A mixture of 2 ml of the obtained solution 1 ml of phosphomolybdotungstic reagent R and 10 ml of water R was brought to a solution of 290 g/l sodium carbonate R to a volume of 25 ml. After 30 min, the optical density of the solution was measured at a wavelength of 760 nm, using water R as a compensatory solution.

Polyphenols not adsorbed on the hide powder. 0.10 g of the pharmacopoeial reference standard of cutaneous powder was added to 10 ml of the filtrate and shaken vigorously for 60 min. The mixture was filtered and dilute 5 ml of filtrate with water R to a volume of 25 ml.

A mixture of 2 ml of the obtained solution, 1 ml of phosphomolybdotungstic reagent R, and 10 ml of water was diluted with a solution of 290 g/l sodium carbonate R to a volume of 25 ml. After 30 min, the optical density of the solution was measured at a wavelength of 760 nm, using water as a compensatory solution.

Standard solution. Immediately before trial, 50.0 mg of pyrogallol R was dissolved in water R and diluted the volume of the solution with the same solvent to 100 ml. 5 ml of the resulting solution were diluted to 100 ml with water R.

A mixture of 2 ml of the obtained solution, 1 ml of phosphomolybdotungstic reagent R, and 10 ml of water R was diluted to a solution of 290 g/l sodium carbonate R to a volume of 25 ml. After 30 min, the optical density of the solution was measured at a wavelength of 760 nm, using water R as a compensatory solution [32, 33].

The quantitative content of tannins and polyphenols was determined on a spectrophotometer Lambda 25 UV Perkin Elmer (USA) [34].

Antibacterial and antifungal test

The antimicrobial action of the obtained dried extract of *Gentiana cruciata* L. herb was studied *in vitro* according to the State Pharmacopoeia of Ukraine [35].

Antibacterial and antifungal action was studied by agar diffusion ("wells" method).

Two layers of dense nutrient medium poured into Petri dishes were used in the study. In the lower layer, "hungry" media were used, which were not sown. Thin-walled cylinders made of stainless steel were installed on the lower layer. Standardization of "well" studies of the agar diffusion was secured by 6 mm "well" diameter and 10 mm medium thickness. The top layer was filled around the cylinders, namely a sterile agar medium, melted and cooled to 40 °C, in which the corresponding standard of the daily test culture of the microorganism (Escherichia coli ATCC 25922, Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 6538, Candida albicans ATCC 885-653). After solidification of the upper layer, the cylinders were removed with sterile tweezers, and 0.06 ml of the substance was added to the formed "wells". The plates were dried for 30-40 minutes at room temperature and placed in a thermostat to incubate the seed at 37 ° C for 24 hours. The diameter of the growth retardation zone of the test cultures was measured in millimeters [3].

The antibacterial activity was determined by measuring of inhibition zone. Results were evaluated according to the parameters suggested by Alves et al. (2000) [36]:

- <9 mm, inactive ;
- 9–12 mm, less active ;
- 13–18 mm, active;
- >18 mm, very active.

Statistical analysis

All the assays were carried out five times. Obtained results were expressed as mean value ± SEM [37]. Statistical significance of differences between mean values was assessed by the Student's t-test [38, 39]. The level of significance was set at *p<0.05 for all statistical analyses [40].

Results and Discussion

Experimental studies revealed that the dry extract of *Gentiana cruciata* L. herb showed indication for the presence of tannins, polyphenols at the phytochemical screening which is correlated with the activity of the obtained extract.

The detection of tannins in dry extracts from the *Gentiana cruciata* L. herb was carried out with the help of generally known qualitative reactions.

Due to the reaction with a solution of ammonium iron (III) sulfate, as a result of which a dark green color appeared, the presence of condensed tannins was established.

The appearance of white turbidity as a result of the reaction with 1 % gelatin solution indicated the presence of tannins in the extracts under study.

As a result of the reaction with a 1% quinine hydrochloride solution, a white amorphous precipitate was observed.

The above qualitative reactions testify to the presence of tannins in the studied extract.

Polyphenols and their derivatives are widespread in nature and are practical mandatories components of many plants. Tannins are water-soluble polyphenols that are present in higher herbaceous and woody plants [41]. It is known that these compounds can affect the course of various physiological processes, react with free radical compounds, manifest a tendency to specific interaction with proteins that perform regulatory functions. The spectrum of biological action of polyphenols and tannins is extremely wide. They

have antimicrobial, anti-inflammatory, antifungal, antihistamine, anticarcinogenic, antimutagenic, and anti-edema properties, stabilize cell membranes [42, 43]. The high physiological activity and low toxicity of plant phenols make them particularly important for the pharmaceutical, cosmetic, and food industries. The wide range of pharmacological action, their physicochemical properties cause the widespread use of polyphenols in medicine and pharmacy.

The results of determining the quantitative content of tannins and polyphenols in the dry extract of *Gentiana cruciata* L. herb are shown in Table 1.

Antibacterial and antifungal activity

Results of research of antimicrobial and antifungal activity of a 50% water extract of *Gentiana cruciata* L. herb by "wells" method are given in Table 2.

The obtained results indicate the sensitivity of museum strains of *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 885-653) to 50% water extract of *Gentiana cruciata* L. herb (Fig. 1-4). The native extract was very active against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*. Also, the extract of *Gentiana cruciata* L. herb in N:2 dilution was very active against *Staphylococcus aureus*, active to *Bacillus subtilis*, *Escherichia coli*, and less active to *Candida albicans*. Consequently, experimental researches revealed that the dry extract of *Gentiana cruciata* L. herb contains tannins and polyphenols which are correlated with the activity of the study extract.

Conclusions

The results of the research indicate that extract of *Gentiana cruciata* L. herb has anti-microbial properties. The native extract was very active against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans*. Also, the extract of *Gentiana cruciata* L. herb in N:2 dilution was very active against *Staphylococcus aureus*, active to *Bacillus subtilis*, *Escherichia coli*, and less active to *Candida albicans*. The properties of extract of *Gentiana cruciata* L. herb are the result of tannins and polyphenols in the plant. The content of these biologically active substances was determined by the spectrophotometric method. The total content of tannins and polyphenols was 1,84% and 8,52% respectively.

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Table 1. The quantitative content of tannins and polyphenols in the dry extract of *Gentiana cruciata* L. herb (M ± SEM, n = 5)

	Content of biologically active compounds, %	
Dry extract of *Gentiana cruciata* L. herb	tannins	polyphenols
	1.84±0.06	8.52±0.09

Table 2. Analysis of the antibacterial activity of a 50% water extract of *Gentiana cruciata* L. herb by "wells" method (M ± SEM, n = 5)

	Diameter of delayed microbial growth exposed to various dilutions of a 50% water extract of *Gentiana cruciata* L. herb, mm			
Dilution of extract	*Staphylococcus aureus* (ATCC 6538)	*Escherichia coli* (ATCC 25922)	*Bacillus subtilis* (ATCC 6633)	*Candida albicans* (ATCC 885-653)
N	25.2 ± 0.7	20.1 ± 0.7	22.0 ± 0.6	17.9 ± 0.5
N:2	19.0 ± 0.9	13.0 ± 0.8	15.0 ± 0.8	11.6 ± 0.8

Note: N – native extract.

Figure 1. The results of determining the sensitivity of *Staphylococcus aureus* to 50% water extract of *Gentiana cruciata* L. herb by "wells" method

Figure 2. The results of determining the sensitivity of *Escherichia coli* to 50% water extract of *Gentiana cruciata* L. herb by "wells" method

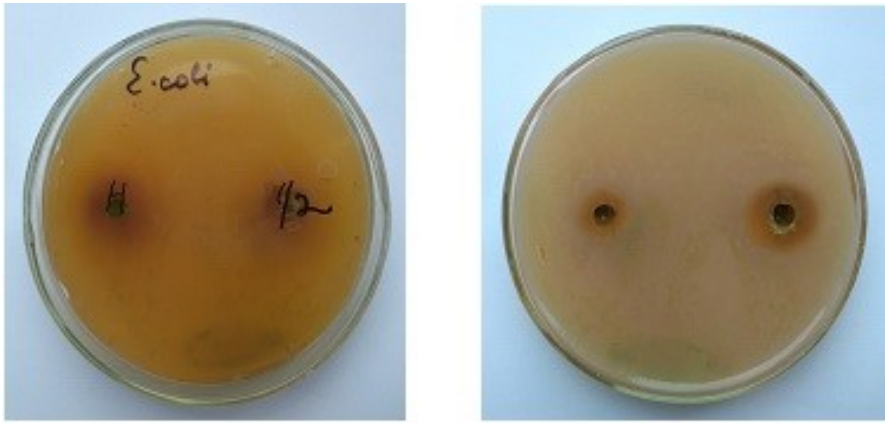

Figure 3. The results of determining the sensitivity of *Bacillus subtilis* to 50% water extract of *Gentiana cruciata* L. herb by "wells" method

Figure 4. The results of determining the sensitivity of *Candida albicans* to 50% water extract of *Gentiana cruciata* L. herb by "wells" method