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INVESTIGATION OF THE ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF THE *PYROLA ROTUNDIFOLIA* L. LEAVES DRY EXTRACT

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Abstract

Pyrola rotundifolia L. commonly known as round-leaved wintergreen, belongs to the family *Ericaceae* is a well-known plant in many countres of the world. The raw material of this plant using in various diseases. The dry extract of leaves of round-leaved wintergreen was researched to antibacterial and antifungal activity. Phytochemical analysis of the dry extract of leaves of round-leaved wintergreen indicated the presence of hydroxycinnamic acids, that have antibacterial and antifungal properties. The total content of hydroxycinnamic acids, in recalculation at rosmarinic acid, was 5.16%. Antibacterial and antifungal activity of the dry extract of leaves of round-leaved wintergreen was evaluated by two-fold serial dilutions in a liquid nutrient and agar diffusion ("wells" method). It was the most effective against *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans*, so it may be used for the development of new medicins as a promising component.

Keywords: *round-leaved wintergreen, dry extract, leaves, antibacterial activity, antifungal activity, Pyrola rotundifolia L.*

Introduction

The use of medicinal plants in scientific and folk medicine has a centuries-old tradition [1, 2]. In recent years, methods of treatment using medicinal plants have become increasingly common [3]. 25 % of herbal remedies in the modern pharmacopeia are plant-derived [4]. Medicinal plants have high tolerability and minor side effects [5, 6]. Plant metabolites are nearly to metabolites of the human body, and the head effect of the use of plant medicines is to regulate impaired processes [1]. The importance of medicinal plants did not reduce the yearly increase in the number of synthetic remedies, which often model their biologically active substances [7, 8]. The plants often use in the struggle against many diseases [9, 10]. These plants include *Pyrola rotundifolia* L.

Pyrola rotundifolia L. is a perennial herbaceous plant [11], commonly known as round-leaved wintergreen, belongs to the family *Ericaceae*. This species occurs in North America, western and central Asia, Europe and China [12]. The round-leaved wintergreen using for the treatment of various inflammatory diseases, kidney deficiency, urogenital disease rheumatic pain, hypertension, tuberculosis, and cancer. These properties are the result of many groups of biologically active compounds in plant. The plant contains various secondary metabolites as flavonoids (luteolin, taxifolin, quercetin, hyperoside, isoquercitrin, guajaverin, rhamnetin and their glycosides), triterpenoids (oleanolic and ursolic acids) and quinones (renifolin, chimaphilin). Whereas the plant has been used for years in medicine the reports regarding of their pharmacological activities are limited [12-15].

Despite the wide arsenal of antimicrobials drugs, scientists invest their searches looking for new antimicrobials, because pathogenic microorganisms have developed resistance to ordinary antibiotics. Further studies should be carried out in order to clarify the relationship between the usage of these biologically active substances and their benefic impact on the human body. In this perspective, different studies propose hydroxycinnamic acids as antimicrobial agents [16]. Consequently, the purpose of the study was to investigate antimicrobial and antifungal activity of the dry extract obtained from leaves of round-leaved wintergreen.

Methods

Plant Materials

The object of the study was to the round-leaved wintergreen leaves, which was harvested during a mass flowering period in the Kovel district, Volyn region (N 51°46'80.47" E 25°01'37.12"), in 2016 [17]. The study raw material was dried using conventional method and stored in paper bags in a dry, protected from direct sunlight place [18, 19].

Preparation of extract. About 2000 g of dried round-leaved wintergreen leaves were powdered with the help of a suitable crusher. It was taken in an extractor and extracted at a temperature of 20±2° C using 80% ethanol as a solvent. The extract was concentrated under vacuum to half under volume and dried at a temperature of 50±2° C. It was using remaceration as the extraction method. During remaceration, the amount of extractant was divided into portions and each portion was being infused with the raw material [20].

Chemicals and reagents

Sodium nitrite, Sodium molybdate, Sodium hydroxide, Hydrochloric acid were of the highest purity available and purchased from the Ltd. Sfera Sim (Lviv, Ukraine).

Microorganisms

An experiment used a standardized daily suspension of testing strains of the following microorganisms: *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, and *Candida albicans* ATCC 885- 653; cell concentration was 0.5 McFarland.

Total hydroxycinnamic acids content

The quantitative content of the hydroxycinnamic acids was determined out by the spectrophotometric method according to the European Pharmacopoeia [21]. The investigation of the content of these biologically active substances was carried out in a five-fold repetition with subsequent statistical processing of results [22].

The quantitative content of hydroxycinnamic acids was determined on a spectrophotometer Lambda 25 UV Perkin Elmer (USA) [23].

Sample preparation:

To 0.125 g (exact weight) of the extract was added 80 ml of ethanol *(50% (vol/vol)) R*, heat in a water bath under reflux for 30 min, cooled, and filtered. The filter is rinsed with 10 ml of ethanol *(50% (vol/vol)) R*, the filtrate and washings are combined in a 100 ml volumetric flask and added ethanol *(50% (vol/vol)) R* to the mark and stirred. (*Stock solution*).

Test solution.

1 ml of the original solution was placed in a 10 ml volumetric flask, sequentially added, stirring after each addition, 2 ml of 0.5 M hydrochloric acid solution, 2 ml of a solution prepared by dissolving 10.0 of sodium nitrite R and 10.0 of sodium molybdate R, in 100 ml of water R, 2 ml of sodium hydroxide solution of diluted R, and was brought the volume of the solution with water R to 10 ml and mixed.

Compensatory solution.

1 ml of the stock solution was placed in a 10 ml volumetric flask, 2 ml of 0.5 M hydrochloric acid solution and 2 ml of dilute sodium hydroxide solution R were successively added, the volume of the solution was brought to mark with water R and mixed.

Used a specific absorption of rosmarinic acid equal to 400 [21].

Antibacterial and antifungal test

The antimicrobial action of the obtained extract was studied *in vitro* according to the State Pharmacopoeia of Ukraine [23]. The study used a dry extract of round-leaved wintergreen obtained from wild leaves of this culture.

Antibacterial and antifungal action of the obtained extract on microorganisms was studied by two-fold serial dilutions in a liquid nutrient (meat infusion broth) and agar diffusion ("wells" method).

A test was repeated ten times. Standardization of "well" studies of the agar diffusion was secured by 10 mm medium thickness and 6 mm "well" diameter. After infusion of testing strains into a nutrient, "wells" were filled with droplets of a dry extract diluted in meat infusion broth. Then Petri dishes were placed into a heating block at 37 °C. Results were evaluated in 24 hours by measuring the diameter of the inhibition zone around a "well" in millimeters. The serial dilutions method used the concentration of dried round-leaved wintergreen from 1:2 to 1:128 (volume ratio). All test tubes containing 2 ml of diluted substance and a control tube (filled with a liquid nutrient medium) were inoculated with 0.2 ml one-day inoculum of a testing culture of microorganisms. Results were evaluated by the degree of growth inhibition for a given testing culture of the studied substance in a certain dilution.

The antimicrobial activity of the extract was evaluated by successive serial dilutions, which allow determining the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations. To determine MIC, they prepared serial twofold dilutions of the substance in a liquid nutrient medium; later on, it was identified by the lowest concentration of a substance not giving rise to the culture's growth. The bactericidal concentration of the studied substances was determined by seeding dilutions into a dense nutrient media.

Statistical analysis

Statistical analysis of the results was performed by variational statistics [25]. Data were expressed as mean ± SEM [26]. Statistical significance of differences between mean values during the analysis was assessed by the Student test (t). The difference between the values was considered reliable if the likelihood was $p \leq 0.05$.

Results and Discussion

Experimental research revealed that the dry extract of round-leaved wintergreen leaves showed indication for the presence of hydroxycinnamic acids at the phytochemical screening which is correlated with the activity of this extract.

Hydroxycinnamic acids, among them, rosmarinic acid play an important role in nature [27].

The result of the study of total hydroxycinnamic acids content the dry extract of round-leaved wintergreen leaves is shown in Figure 1.

The content of the total of hydroxycinnamic acids in the dry extract of study raw material, in recalculation at rosmarinic acid, which was (5.16 ± 0.08)%.

Rosmarinic acid is the result of 3,4-dihydroxyphenyl lactic acid and caffeic acid esterification. This hydroxycinnamic acid interesting antioxidant, antimicrobial, antitumor and anti-inflammatory properties. Rosmarinic acid weakens allergic diseases like allergic rhinitis and asthma, and atopic dermatitis-like symptoms, slow the development of Alzheimer's disease, attenuates T-cell receptor-mediated signaling, and protects from neurotoxicity [28]. Since of its medicinal properties, it is attracting interest from cosmetic and pharmaceutical manufacturers [29]. *Suriyarak et al.* confirmed that rosmarinic acid concentration up to 500 mM and dodecylrosmarinate concentration up to 12.8 mM inactivated *Staphylococcus carnosus* LTH1502. The anti-microbial efficacies of these compounds are affected by the type and concentration of salts, and pH values [28]. *Abedini et al.* reported that rosmarinic acid (0.3 to 1.3 mg/mL) also showed inhibitory andbactericidal functions against pathogenic bacteria like *Stenotrophomonas maltophilia*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Corynebacterium*, *Pseudomonas aeruginosa* and *Mycobacterium smegmatis* [30].

Results of the study of antimicrobial and antifungal activity of a dry extract of round-leaved wintergreen by serial dilutions are given in Table 1.

As shown in Table 1, a dry extract of round-leaved wintergreen exhibits antibacterial action in a minimal dilution (1:2) against all testing cultures of microorganisms. The most sensitive were Gram-positive cocci *Staphylococcus aureus* (Fig. 2) and yeast fungi *Candida albicans* (Fig. 3).

Pseudomonas aeruginosa (Fig. 4) and *Escherichia coli* (Fig. 5) demonstrated low sensitivity to the studied extract.

Results of the study of antimicrobial effect of a dry extract of round-leaved wintergreen by "wells" method are given in Table 2.

This method confirmed results obtained by serial dilutions. Given the diameter of inhibition zones, *Staphylococcus aureus* (Fig. 6) is sensitive to dried round-leaved wintergreen in a dilution of 1:2 and 1:4, low sensitive to dilution of 1:8, 1:16.

Bacillus subtilis (Fig. 7) is sensitive to the dry extract in dilutions of 1: 2, 1: 4, 1: 8, and low sensitive to dilution of 1:16.

Candida albicans (Fig. 8) is sensitive to wintergreen extract at dilutions of 1: 2 and 1: 4.

Pseudomonas aeruginosa and *Escherichia coli* are not sensitive to the dry extract of round-leaved wintergreen in a dilution of 1: 2; the inhibition zone was only 10 mm.

Consequently, experimental research revealed that the dry extract of round-leaved wintergreen leaves contains hydroxycinnamic acids which are correlated with the activity of this extract.

Conclusions

Test results showed that the dry extract of round-leaved wintergreen has anti-microbial properties. It was the most effective against *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans*, so the dried wintergreen extract may be used for the development of new drugs as a promising component. These properties of the dry extract of round-leaved wintergreen are the result of hydroxycinnamic acids in the plant. The total content of hydroxycinnamic acids, in recalculation at rosmarinic acid, was determined by the spectrophotometric method. In round-leaved wintergreen content of these biologically active compounds contentained in the dry extract of leaves was 5.16%.

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Table 1. Analysis of antibacterial and antifungal activity of dried round-leaved wintergreen by serial dilutions

Testing culture of microorganisms	Dilutions						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Bacillus subtilis	—	—	—	—	+	+	+
Escherichia coli	—	—	+	+	+	+	+
Pseudomonas aeruginosa	—	+	+	+	+	+	+
Staphylococcus aureus	—	—	—	—	—	±	±
Candida albicans	—	—	—	—	—	±	±

Notes: + – positive growth;

— – no growth;

± – a slight growth.

Table 2. Quantitative analysis of antibacterial activity of a dry extract of round-leaved wintergreen by "wells" method (M ± SEM, n = 5)

	Diameter of delayed microbial growth exposed to various dilutions of a dry extract of round-leaved wintergreen, mm						
Testing culture of microorganisms	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Staphylococcus aureus	22	15	12	11	—	—	—
Escherichia coli	10	—	—	—	—	—	—
Bacillus subtilis	20	16	15	13	—	—	—
Pseudomonas aeruginosa	10	—	—	—	—	—	—
Candida albicans	18	15	10	10	—	—	—

Note.: — – not delayed microbial growth.

Figure 1: UV spectrum for rosmarinic acid of dry extract of round-leaved wintergreen leaves

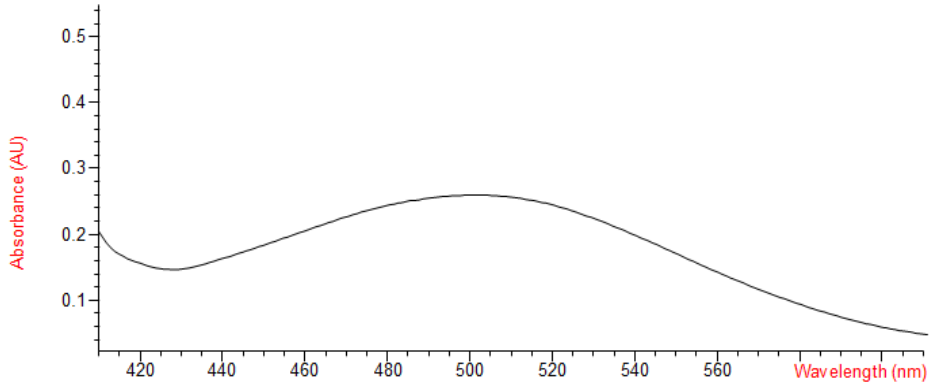

Figure 2: Staphylococcus aureus

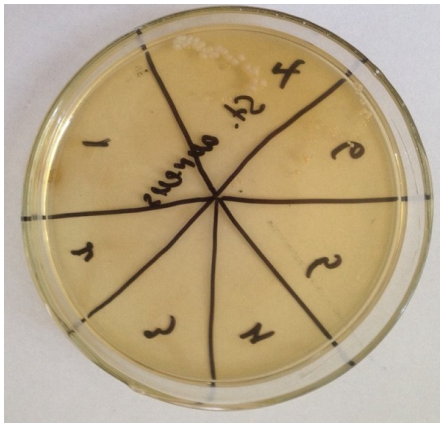

Figure 3: Candida albicans

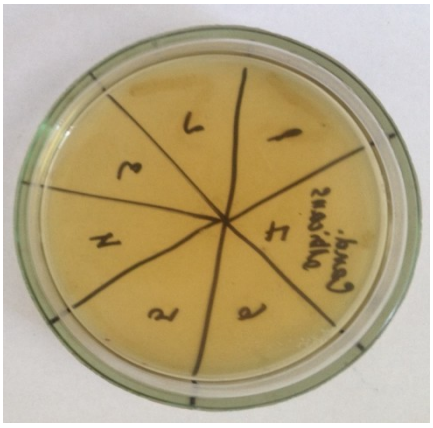

Figure 4: Pseudomonas aeruginosa

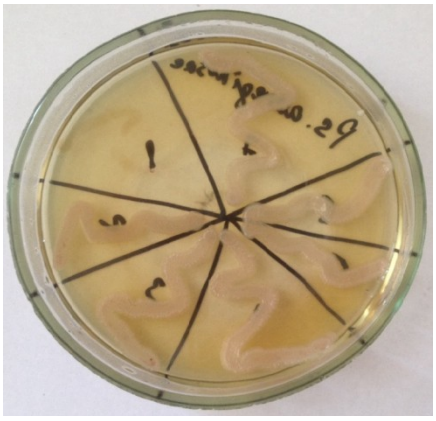

Figure 5: Escherichia coli

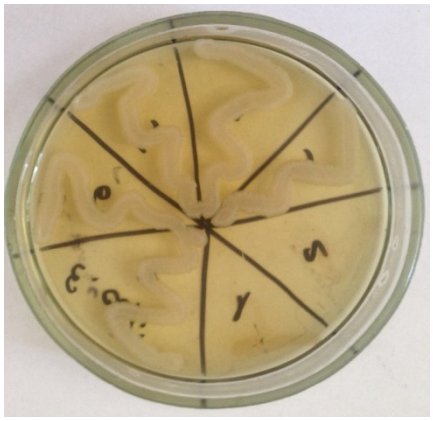

Figure 6: Staphylococcus aureus

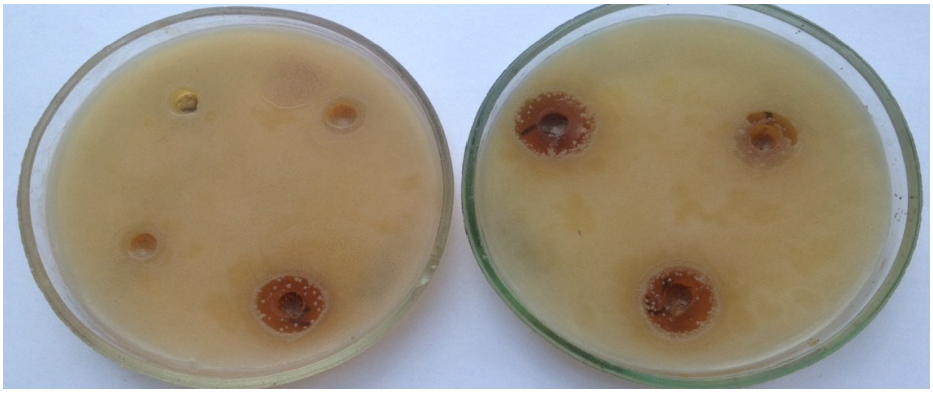

Figure 7: Bacillus subtilis

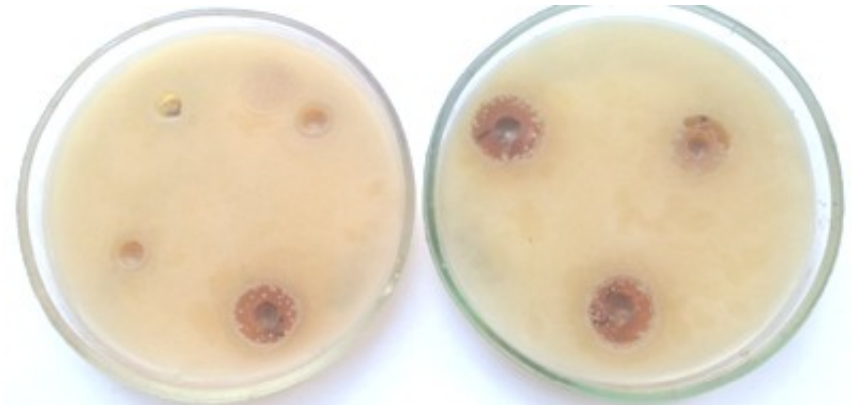

Figure 8: Candida albicans