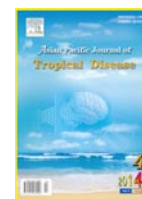




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Phenolic profile and antimicrobial activities to selected microorganisms of some wild medical plant from Slovakia

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Comments

The described research fits the global trend of looking for natural medicines with antibiotic properties. Tested plants are known in folk and traditional medicine but not as antibacterial agents. Study has been carried out properly and the description of the results is concise and clear.

Details on Page 273

ABSTRACT

Objective: To investigate the chemical composition and antimicrobial activity of the methanol extracts of *Tussilago farfara* (*T. farfara*), *Equisetum arvense*, *Sambucus nigra* (*S. nigra*) and *Aesculus hippocastanum*.

Methods: The antimicrobial activities of the extracts against *Enterococcus raffinosus*, *Escherichia coli*, *Lactobacillus rhamnosus*, *Pseudomonas aeruginosa*, *Serratia rubidaea*, *Saccharomyces cerevisiae* and *Staphylococcus epidermis* were determined by the microbroth dilution method according to Clinical and Laboratory Standards Institute, while the concentrations of main phenolic acids and flavonoids in the form of trimethylsilyl ethers were analysed using gas chromatography–mass spectrometry. The probit analysis was used for statistical evaluation.

Results: Of the 4 plant tested, all extracts showed a significant antimicrobial activity against one or more species of examined microorganisms. The most active antimicrobial plant extract was gathered from *T. farfara*, followed by *Aesculus hippocastanum* and *Equisetum arvense*. The extract from *S. nigra* showed no antimicrobial effects. The flavonoids quercetin and kaempferol, as well as several phenolic acids (*p*-hydroxybenzoic acid, gallic acid, ferulic acid and caffeic acid) were identified in all extracts. The highest concentrations of bioactive compounds were detected in the extracts of *T. farfara* (9587.6 µg/mg quercetin and 4875.3 µg/mg caffeic acid) as well as *S. nigra* (4788.8 µg/mg kaempferol).

Conclusions: We can state that the methanolic plant extract of *T. farfara* showed the strongest antimicrobial activity against *Saccharomyces cerevisiae* as well as other tested microorganisms. At the same time, a good antimicrobial activity was found in the other medical plant extracts as well. No antimicrobial effect of the *S. nigra* extract was found with respect to the growth of *Pseudomonas aeruginosa*, *Enterococcus raffinosus* and *Saccharomyces cerevisiae*.

KEYWORDS

Wild medical plants, Antimicrobial activity, Minimum inhibitory concentration, Chemical composition

1. Introduction

The research interest on the production of biologically active compounds from natural resources has been increasing over the past decade^[1–9]. Many efforts have been made to discover new antimicrobial substances from

different sources such as microorganisms, animals and plants, which have found to be useful in various traditional and folk medicines^[10,11]. Natural products of higher plants may provide a variety of antimicrobial agents with possibly novel mechanisms of action^[12–15]. Medical plants represent such a rich source of antimicrobial substances, and many

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of them have been already used in different countries as central components of potent and powerful drugs[16]. Furthermore, infections have increased to a great extent in recent years with the downside of antibiotics resistance as an essential therapeutic concern[17]. The extensive use of synthetic drugs as well as unwanted medication, will cause increasing side effects to the body; sometimes, the toxic effects caused by the administration of drugs may be much more serious than the disease itself. In recent years, pharmaceutical companies have invested significantly in testing natural products extracted from plants, to produce more cost effective remedies that are affordable to common people. Plant extracts have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory conditions[18,19]. The selection of crude plant extracts for screening programs has the potential of being more successful in its initial steps than the screening of pure compounds that are isolated from natural products[20]. The present study was designed to determine the role of methanolic extracts from *Tussilago farfara* (*T. farfara*), *Equisetum arvense* (*E. arvense*), *Sambucus nigra* (*S. nigra*) and *Aesculus hippocastanum* (*A. hippocastanum*) for potential antibacterial and antifungal activity against selected microorganisms, namely, *Escherichia coli* (*E. coli*), *Serratia rubidaea* (*S. rubidaea*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus epidermis* (*S. epidermis*), *Lactobacillus rhamnosus* (*L. rhamnosus*), *Enterococcus raffinosus* (*E. raffinosus*) and *Staphylococcus epidermis* (*S. cerevisiae*).

2. Materials and methods

2.1. Plant materials

The plant materials used in this experiment consist of flowers from *S. nigra* and *A. hippocastanum*, flowers and stems from *T. farfara*, leaves and stems from *E. arvense*. The plants were collected from Nitra and Gelnica outskirts (Slovakia) during the spring season of 2012. The material was initially dried at the room temperature in the dark. More detailed information is available in Table 1.

Table 1

Detailed information about the plant extracts.

Plants	Plant parts	Yield*	Area
<i>T. farfara</i>	flower + stem	757.6	Gelnica
<i>E. arvense</i>	leaf + stem	392.5	Nitra
<i>S. nigra</i>	flower	442.4	Nitra
<i>A. hippocastanum</i>	flower	509.5	Nitra

*Yield (in mg) from 50 g dried plants per 400 mL methanol.

2.2. Microbial tests

Seven strains of microorganisms were tested in this research, including three Gram-negative bacteria (*E.*

coli CCM 3988, *S. rubidaea* CCM 4684, *P. aeruginosa* CCM 1960), three Gram-positive bacteria (*S. epidermis* CCM 4418, *L. rhamnosus* CCM 1828, *E. raffinosus* CCM 4216) and one yeast strain (*S. cerevisiae* CCM 8191). All tested strains were collected from the Czech Collection of Microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37 °C and the yeast suspension was cultured in the malt extract broth (Biomark, India) at 30 °C.

2.3. Preparation of plant extracts

After drying, the plant materials were crushed, weighed out to 50 g and soaked separately in 300 mL of methanol p.a. (99.5%, Sigma, Germany) during two weeks at room temperature. Exposure to sunlight was avoided in order to prevent the degradation of active components. Then, methanolic plant extracts were filtered through the Whatman No. 1 filter paper. The obtained extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the methanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany). For the antimicrobial assays, the crude plant extracts were dissolved in dimethyl sulfoxid (DMSO) (Penta, Czech Republic) to 102.4 mg/mL as stock solution, while for chemical analysis methanol was used as solvent. Stock solutions of plant extracts were stored at -16 °C in refrigerator until use.

2.4. Antimicrobial assay

The minimum inhibitory concentration (MIC) is the lowest concentration of the sample that will inhibit the visible growth of microorganisms. Plant extracts dissolved in DMSO were prepared to a final concentration of 1024 µg/mL by dissolving stock solution with 102.4 mg/100 mL. MICs were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation 2009[21] in Mueller Hinton broth (Biolife, Italy) for bacteria and Sabouraud broth (Biolife, Italy) for yeast. Briefly, the DMSO plant extracts solutions were prepared as serial two-fold dilutions, in order to obtain a final concentration ranging between 0.5–512 µg/mL. Each well was then inoculated with microbial suspension at the final density of 0.5 McFarland. After 24 h incubation at 37 °C for bacteria and 30 °C for yeast, the inhibition of microbial growth was evaluated by measuring the well absorbance at 450 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 micro-well plates were measured before and after experiment. Differences between both measurements were evaluated as growth. Measurement error was established for 0.05 values from absorbance. Wells without plant extracts were used as positive controls of growth. Pure DMSO was used as negative control. This experiment was done in eight-replicates for a higher accuracy of the MICs of used medical plant extracts.

2.5. Extraction, hydrolysis conditions and sample preparation for GC–MS

The procedure used for acid hydrolysis of flavonoid glycosides has previously been described by Hertog *et al.*[22]. Acidified methanol (25 mL) containing 1% (v/v) HCl was added to plant methanol extracts (25 mL). Hydrochloric acid (1.2 mol/L, 5 mL) was added and the mixture was stirred at 90 °C under reflux for 2 h to obtain the aglycons by hydrolysis of the flavonoid glycosides. The hydrolysates were cooled to room temperature and extracted with ethyl acetate (1:1, v/v). Then obtained extracts were evaporated to dryness under reduced pressure, redissolved in ethyl acetate, and dried overnight over sodium sulphate. The dried solutions (400 µL) were transferred to vials, 100 µL bis(trimethylsilyl) trifluoroacetamide (Sigma, Germany) were added, and the vials were heated at 70 °C for 15 min.

Phenolic constituents of four medicinal plants were determined by gas chromatography–mass spectrometer (GC–MS) operating either in SCAN (total ion current) or selective ion monitoring (SIM) mode.

An aliquot (1 µL) of the derivatized sample was injected into the gas chromatograph at a split ratio 1:20. An Agilent 6890N coupled with a 5975B (Agilent Technologies, USA), split–splitless injector and an HP 7683 autosampler were employed. Mass selective (MS) detector was operated under electron impact ionisation (70 eV) and MS scan range was 50–800 Da. Analysis of the samples was performed using an HP–5 MS capillary column (5% phenyl–95 % methylsiloxane, 30 m×0.25 mm×250 µm). The carrier gas was helium at a flow rate of 0.7 mL/min, injector and MS detector transfer line temperatures were set at 220 °C and 300 °C, respectively. To obtain the total ion chromatograms (GC–MS operating in SCAN mode) of derivatized samples, the following temperature program was applied: oven was initially set to 100 °C, next the temperature increased at 5 °C/min to 310 °C, then 310 °C were held for 8 min. Under these conditions, the phenolic constituents in the form of their trimethylsilyl ethers were identified using their mass spectra and by reference to the Wiley7Nist mass spectra libraries, as well as by analyzing pure standards.

In this way, the qualitative analysis of plant methanolic extracts was achieved.

Under the same conditions as extracts, the standard compounds were transformed to their trimethylsilyl ethers. Quantitative analyses were performed from the calibration curves of trimethylsilyl derivatives of standard compounds (Extrasynthese, France).

2.6. Statistical analysis

Using obtained absorbance before and after the analysis,

we were able to express the differences in absorbance between the measurements as a set of binary values. These values were assigned to exact concentrations. The following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05. For this statistical evaluation probit analysis in Statgraphics software was used.

3. Results

All the plants tested in this research are commonly used as medical plants in different areas of Europe and world. Their medical properties and some other information were described by Krejča and Kresánek[23] and are presented in Table 2. The antimicrobial activity (expressed as µg/mL) of four methanolic extracts from *T. farfara*, *E. arvense*, *S. nigra* and *A. hippocastanum* against various strains of bacteria and yeast are summarized in Table 3. The organism *S. cerevisiae* CCM 8191 was found to be more susceptible to the *T. farfara* extract with a MIC₅₀ value of 24 µg/mL. *S. rubidaea* CCM 4684 was less susceptible to *T. farfara* with MIC₅₀ value of 48.01 µg/mL. The organisms *E. coli* CCM 3988, *S. epidermis* CCM 4418, *L. rhamnosus* CCM 1828, *P. aeruginosa* CCM 1960 and *E. raffinosus* CCM 4216 were less susceptible to the *T. farfara* extract with higher MIC₅₀ values (MIC ≥64 µg/mL). The organism *P. aeruginosa* CCM 1960 was found to be more susceptible to the *E. arvense* extract with a MIC₅₀ value of 92.09 µg/mL. The organisms *E. coli* CCM 3988, *S. rubidaea* CCM 4684, *L. rhamnosus* CCM 1828, *S. epidermis* CCM 4418, *E. raffinosus* CCM 4216 and *S. cerevisiae* CCM 8191 were less susceptible to the *E. arvense* extract with higher MIC₅₀ values (MIC₅₀ ≥143.73 µg/mL). The organism *E. coli* CCM 3988 was found more susceptible to the *S. nigra* extract with MIC₅₀ value of 64 µg/mL. *S. rubidaea* CCM 4684, *L. rhamnosus* CCM 1828 and *S. epidermis* CCM 4418 were less susceptible to the *S. nigra* extract with higher MIC₅₀ values (MIC₅₀ ≥191.85 µg/mL). The antimicrobial effect of the *S. nigra* extract against *E. coli* isolated from ecological cattle breeding was not found. *P. aeruginosa* CCM 1960, *E. raffinosus* CCM 4216 and *S. cerevisiae* CCM 8191 were found not to react to the extracts. The organism *E. coli* CCM 3988 was found more susceptible to the *A. hippocastanum* extract with a MIC₅₀ value of 48.01 µg/mL. *S. rubidaea* CCM 4684, *P. aeruginosa* CCM 1960, *L. rhamnosus* CCM 1828, *S. epidermis* CCM 4418, *E. raffinosus* CCM 4216 and *S. cerevisiae* CCM 8191, were less susceptible to the *A. hippocastanum* extract with higher MIC₅₀ values (MIC₅₀ ≥96.05 µg/mL). The MIC₉₀ and MIC₅₀ values are summarized in Table 3.

Table 2

Descriptions of medical plants prepared by Kresánek and Krejča[23].

Plants	Common name	Systematic position	Botanical description	Medical properties	Chemical compounds	
					Stems and leaves	Flowers
<i>T. farfara</i>	Coltsfoot	Asterales: Asteraceae	Perennial herb, 10–20 cm long stem, yellow flowers, in summer leaves has heart shape, growing on the glade etc.	Respiratory diseases, suppressant of cough, diuretics, antiallergic, accelerates wound healing, etc.	Mucus, sterins, tannins, galic acid, small amount of bitters, small amount of silica, inulin, paraffin, sugar and ash contain high amount of K and Zn. Dried stems and leaves	Silica, high amount of xanthophyll, mucus, tannins, glycosidic bitter.
<i>E. arvense</i>	Common horsetail	Equisetales: Equisetaceae	Perennial herb, 10–20 cm stem in spring, 10–60 cm stem in summer, without flower, ear of stem in spring has spores, in summer stem without gametes, growing on the field etc.	Diuretics, stops bleeding, against fever for tuberculosis, antisclerotics, gargle in bacterial inflammation, etc.	The dried stems contain high amount of silica acid (6%–8%), flavonoids glycosides (aglycones–quercetin, luteolin and kempherol), saponins (equisetonin), bitters, small amount of silica and alkaloides (nicotin), acids (oxalic, malic, aconitic), small amount of fats, ash contains higher amount of Al and K.	
<i>S. nigra</i>	Elder	Dipsacales: Loniceraeae	Bush with opposite leaf, inflorescence of white small flowers, fruits are small round drupes, growing on the slightly moist opinion etc.	Laxative effect, herbal wrap, urological tea	Flowers Flowers contain 3% of silica (mainly rutin), tannins, amines (ethyamin, isobuthylamine, isoamylamine), sugars, organic acid and Vitamin C. Flowers, leaves, seeds	Fruits Except compounds from flowers, fruits contain also some vitamins, sugars and anthocyanins.
<i>A. hippocastanum</i>	Horse Chestnut	Sapindales: Aesculaceae	Its a tree with sticky buds, fan-shaped leaves, flowers are bisexual, fruits are spiny capsules, trees are growing in the park as decorative solitaire.	Suppressant of cough, circulatory disorders	Triterpenoids saponins, mixture of these saponins is called aescin (aglycones–protoescigenin, angelic acid, tiglic acid, acetic acid, isobutyric acid or alfa methyl butyric acid), huge amount of starch, flavonoids glycosides, aglycones–quercetin and kempherol, derivates of purins, adenin, adenosin, uric acid, fatty oil (7%–8%), crude protein, catechin, kumarin, aesculin.	

Table 3

The antimicrobial activity of medical plant extracts (MIC, µg/mL).

Microorganisms	<i>T. farfara</i>		<i>E. arvense</i>		<i>S. nigra</i>		<i>A. hippocastanum</i>	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Gram-negative bacteria								
<i>E. coli</i> CCM 3988	64.00	71.53	>512.00	>512.00	64.00	71.53	48.01	51.26
<i>S. rubidaea</i> CCM 4684	48.01	51.26	254.27	428.13	>512.00	>512.00	96.05	102.30
<i>P. aeruginosa</i> CCM 1960	64.00	71.53	92.09	>512.00	n.d.	n.d.	279.21	>512.00
Gram-positive bacteria								
<i>L. rhamnosus</i> CCM 1828	64.00	71.53	>512.00	>512.00	>512.00	>512.00	96.05	102.30
<i>S. epidermis</i> CCM 4418	191.85	203.97	237.19	455.60	191.85	203.97	>512.00	>512.00
<i>E. raffinosus</i> CCM 4216	64.00	71.53	143.73	291.71	n.d.	n.d.	141.77	>512.00
Yeast								
<i>S. cerevisiae</i> CCM 8191	24.00	25.69	383.00	407.06	n.d.	n.d.	222.26	413.91

n.d.–antimicrobial effect not determined.

The phenolic compound *p*-hydroxybenzoic acid, caffeic acid, gallic acid, ferulic acid, kaempferol, quercetin, luteolin and apigenin were quantitatively determined. The target and qualifier ions for the trimethylsilyl ethers of the eight compounds are given in Table 4.

Table 4

Polyphenols quantified by GC–MS.

Compound	GC–MS run mode	Target ion (m/z) ^a	Qualifier ions (m/z) ^a
<i>p</i> -Hydroxybenzoic acid	SIM ^b	267	223, 193
Gallic acid	SIM	281	458, 443
Ferulic acid	SIM	338	323, 308
Caffeic acid	SIM	396	219, 381
Kaempferol	SIM	559	560
Quercetin	SIM	647	559, 575
Luteolin	SCAN ^c		
Apigenin	SCAN		

^aTrimethylsilyl ether derivatives of the compounds; ^bSIM–selective ion monitoring; ^cSCAN–total ion current monitoring.

The concentrations of specific compounds identified in plant methanol extracts are summarized in Table 5. In all plant extracts the following phenolic acids were found: *p*-hydroxybenzoic acid, gallic acid, ferulic acid and caffeic acid. Among the identified phenolic acids, gallic acid was identified with the highest amounts in the case of *T. farfara* and *S. nigra* (4875.3 and 1123.3 µg/mg, respectively). Quercetin and kaempferol were identified in all samples, while luteolin and apigenin were not detected in the extract of *T. farfara*. Also, GC–MS analysis in SCAN mode does not show presence of apigenin in the methanol extract of *S. nigra*. The most abundant flavonoid in *T. farfara* and *E. arvense* extracts was quercetin (9587.6 µg/mg and 2587.6 µg/mg, respectively). On the other hand, the main flavonoid in methanol extracts of *S. nigra* and *A. hippocastanum* was kaempferol (4788.8 µg/mg and 2874.6 µg/mg, respectively).

Table 5

Retention times and concentration of identified compounds.

RT ^a	Compound	Concentration (µg/g of extract)			
		<i>T. farfara</i>	<i>S. nigra</i>	<i>A. hippocastanum</i>	<i>E. arvense</i>
7.52	<i>p</i> -Hydroxybenzoic acid	112.6	221.4	158.8	7.9
24.05	Gallic acid	2.8	5.8	13.9	4.8
25.21	Ferulic acid	896.8	28.5	547.3	58.6
34.95	Caffeic acid	4875.3	1123.3	328.6	25.6
41.12	Kaempferol	3896.5	4788.8	2874.6	987.6
41.56	Luteolin	n.d. ^b	258.9	856.6	n.d. ^b
41.99	Apigenin	n.d. ^b	n.d. ^b	25.8	1103.6
42.18	Quercetin	9587.6	876.8	1447.4	2587.6

^aRT–retention times in min on a HP–5 MS column; ^bn.d.–not detected.

4. Discussion

Kokoska et al. researched an antimicrobial activity

of ethanolic extract from *T. farfara* determining an antimicrobial effect against *Bacillus cereus* (MIC=15.63 mg/mL) and *Staphylococcus aureus* (*S. aureus*) (MIC=62.5 mg/mL)[24]. Stanojković *et al.* studied a similar antimicrobial activity of *T. farfara* ethanolic extract by disc diffusion method, and unlike our experiment they showed a very small antimicrobial activity of *T. farfara* ethanol extract[25]. Radulović *et al.*[26] used *E. arvense* essential oil for antimicrobial susceptibility testing and they determined that essential oil of *E. arvense* in 1:10 dilution was shown to possess a broad spectrum of a very strong antimicrobial activity against all tested strains (*S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *Salmonella enteritidis*; fungi: *Aspergillus niger* and *Candida albicans*). Also Sandhu *et al.*[27] discussed in their review about the good antimicrobial activity of *E. arvense* essential oil against the following bacteria: *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Salmonella enterica*, and against fungi *A. niger* and *C. albicans*. Hleba *et al.*[28] studied a similar extract by the disc diffusion method and they determined strong antimicrobial effect of the *S. nigra* extract against the antibiotic resistant *E. coli* isolated from conventional cattle breeding. Furthermore, they studied the antimicrobial activity of *T. farfara*, *E. arvense* and *A. hippocastanum* extracts, determining very strong antimicrobial effects against *E. coli* isolated from conventional cattle breeding. According to these authors, the *T. farfara* extract had the strongest antimicrobial effect. Similar results were found in our study as well. Hearst *et al.*[29] claimed that the ethanolic *S. nigra* extract was more effective to all bacteria than the other used plant extracts. Roy *et al.*[30] tested the ethanolic extract of *A. hippocastanum* to some oral microbes by the disc diffusion method and they determined a very good antibacterial activity (100 µg/disc=range from 8 to 14 mm inhibition zone, 200 µg/disc=range from 12 to 20 mm inhibition zone). Heleno *et al.*[31] tested *p*-hydroxybenzoic acid against *S. aureus*, *Bacillus cereus*, *Myotis flavus*, *Listeria monocytogenes*, *P. aeruginosa*, *Salmonella typhimurium*, *E. coli* and *Enterobacter cloacae* and they determined strong antimicrobial effect with MIC ranging from 0.003 to 0.03 mg/mL. Also gallic acid as antimicrobial agent was described by Chanwitheesuk *et al.* in their research study[32]. They determined MIC values of 2 500 and 1 250 µg/mL against *Salmonella typhi* and *S. aureus*. In the case of ferullic acid, Lemos *et al.*[33] detected good antimicrobial activity. They tested ferullic acid against *Bacillus cereus* and *Pseudomonas fluorescens* and they determined MIC value of 500 µg/mL for both bacteria. Also antibacterial synergy between quercetin and other polyphenolic acids was determined by Prasad *et al.*[34].

In conclusion, we can state that the methanolic plant extract of *T. farfara* showed the strongest antimicrobial activity against *S. cerevisiae* as well as other tested microorganisms. At the same time, a good antimicrobial activity was found in the other medical plant extracts as well. No antimicrobial effect of the *S. nigra* extract was found with respect to the growth of *P. aeruginosa*, *E. raffinosus* and *S. cerevisiae*.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Regarding the fact that the extensive use of synthetic drugs as well as unwanted medication, will cause increasing side effects to the body, and sometimes, the toxic effects caused by the administration of drugs may be much more serious than the disease itself, natural products extracted from plants have been invested significantly by pharmaceutical companies in recent years.

Research frontiers

Suitable methods for this kind of research have been applied, *e.g.* commonly accepted microbroth dilution method for antimicrobial examination as well as GC–MS method for phenolic compounds determination. The used methods are written in details.

Related reports

In the present study, the references to laboratory method were listed. The obtained results were compared with other authors' experiments, earlier observations were cited and discussed in this research work.

Innovations & breakthroughs

Generally for preparing extracts of herbal plants, ethanol as solvent is used. In this paper authors chose the methanol and they stated the different properties of obtained extract. GC–MS analysis was used for phenolic compounds examination.

Applications

The results of this research work could be used by pharmaceutical and cosmetic industry.

Peer review

The described research fits the global trend of looking for natural medicines with antibiotic properties. Tested plants are known in folk and traditional medicine but not as antibacterial agents. Study has been carried out properly and the description of the results is concise and clear.

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