Chemical composition and application of flowers of false acacia (*Robinia pseudoacacia* L.)

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Abstract

Introduction. The study was carried out to determine the chemical composition and biological activity of false acacia flowers and their products (concrete and syrups) grown in Bulgaria.

Materials and methods. Concrete was obtained by extraction with n-hexane and its chemical composition was determined by GC-MS. Syrups with different concentrations have been obtained and their polyphenols content, antimicrobial and antioxidant activity were established.

Result and discussion. The yield of concrete was 1.06% and the major constituents of the concrete are as follows: n-nonacosane (25.18%), n-heptacosane (20.10%), α-linolenic acid (5.97%), n-pentacosane (4.98%), palmitic acid (4.92%), diisooctyl phthalate (4.05%), hexahydrofarnesyl acetone (3.86%), linoleic acid (3.64%), isopropyl myristate (3.47%), and n-hentriacontane (3.39%). The total aliphatic hydrocarbons constituted the highest percentage of the component of the concrete constituting 61.50%. *The minerals identified as biggest content in the tested samples of false acacia flowers are* nickel, copper, calcium and chromium.

Higher values of phenolic compounds in the flowers (0.77 mg GAE/mL) than those of the syrup 60 °Brix (0.06 mg GAE/mL) and *R. pseudoacacia* syrup 70 °Brix (0.14 mg GAE/mL) were found. The syrup samples exhibit antimicrobial properties against foodborne pathogenic bacteria as *Salmonella, E. coli* and *L. monocytogenes.*

Conclusion. Based on the chemical and biochemical properties of false acacia flowers, it could be recommended as a potential raw material for food, pharmaceutical and cosmetic products.

Introduction

Robinia pseudoacacia L. (black locust, false acacia), belonging to the family of Fabaceae, originally native to the south-eastern USA, is widely distributed as wild and cultivated species growing in temperate regions throughout the world [1]. The genus *Robinia* commonly known as locust comprises 10 species of trees and shrubs characterized by white or pink flowers with intensive, distinctive, sweet aroma [1, 2]. The flowers are white, borne in pendulous racemes of 10-15 cm long and are edible, with high nutrient and functional values. The flowers of false acacia are used in traditional medicine as diuretic, spasmolytic, sedative and cholagogic agents and relieve inflammation of the kidneys and biliary ducts [3, 4].

The investigation for chemical composition of *R. pseudoacacia* showed that flowers were rich in proteins and microelements which could be used for additives in foods [5, 6]. Its flowers also contained an important bioactive compound called robinin which had a lot of medicinal usage.

The review of the scientific literature indicated that flowers of false acacia, have been attracting a special interest due to their gentle fragrance, containing essential oil which composition depends on geographical region. Volatile compounds of flowers have been studied by several researchers, as well as aroma profiles in honey made from these flowers. In 1994, Kandem et al. [7] used Tenax tube cartridges to trap the floral fragrance of fresh false acacia and analyzed these volatile compounds using GC-MS. δ-3-carene (54.6%), linalool (21%), (Z)-*β*-farnesene (3.0%) and anthranilate aldehyde (3.9%) were found to be the major components. Xie et al. [8] analyzed the chemical constituents of top fragrance from fresh flowers of false acacia growing in China using solid phase microextraction followed by GC-MS analysis and the main components were linalool (33.1%), (E)-*β*-ocimene (26.6%), (E)-*α*-bergamotene (8.9%) and formanilide (7.4%).

The main bioactive components existed in false acacia flowers include flavonoids, phenolics, ascorbic acid, *etc.* [9, 10, 11, 12]. Flowers of false acacia contain volatile compounds, flavonoids, proteins, robinin, polysaccharide and some microelements [13].

Flavonoids content of false acacia is also of increasing interests to researchers. In 2000, five flavonoids including acacetin, secundiflorol, mucronulatol, isomucronulatol and isovestitol were isolated from ethanolic extracts of acacia, following an activity-guided fractionation [14, 15]. Different parts of acacia have wide application in different areas. Its flowers could be eaten and are generally used for honey production. The flowers are also used for preparation of cakes. Series of findings support the consumption of edible flowers of the false acacia as functional food and their usage as sources of natural antioxidants in the food industry.

Recently, many investigations have been concerned with antioxidant properties of different nutritional products [16]. Antioxidant ability has usually been attributed to the activity of antioxidant enzymes as well as to the content of low-molecular antioxidants such as carotenoids, tocopherols, ascorbic acid, phenolic substances [3, 17, 18].

According to Marinas et al. [19], the alcoholic extracts of false acacia showed antimicrobial activity towards the tested strains belonging to the Gram-positive (*Staphylococcus aureus, Bacillus subtilis* and *Enterococcus faecalis*) and Gram-negative (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii*) bacterial and the yeasts (*Candida* sp.) strains. Talas-Ogras et al. [20] studied the in vitro antibacterial activity of isolated from *R. pseudoacacia* seed and it had been found that the *Staphylococcus aureus* was the most sensitive strain compared with others strains

(*Corynebacterium michiganense, Bacillus subtilis, Erwinia carotovora, Pseudomonas syringae, Xanthomonas campestris and Escherichia coli*).

Bhalla and Bajpai [21] reported significant antimicrobial effects against some of the selected foodborne pathogens such as *Staphylococcus aureus* KCTC 1621, *Bacillus subtilis* KCTC 3569, *Listeria monocytogenes* KCTC 3569, *Escherichia coli* O157:H7 and *Salmonella enterica* ATCC 4731 with diameters of the zones of inhibition (15.2±0.3-17.3±2.0 mm).

As unpretentious species regarding the environmental requirements, it has also adapted successfully to a diverse range of natural habitats in Bulgaria. Data relating to the pharmacological and botanical features of the species have been found in the literature. No detailed data for the composition and application of acacia flowers in the food industry have been found.

The aim of this study is to determine the chemical composition of false acacia (*Robinia pseudoacacia* L.) flowers and their potential application in the food, cosmetic and pharmacological industries.

Materials and methods

Plant material

False acacia flowers (*R. pseudoacacia*) were collected in May 2018 from a ten-fifteen - years - old black locust tree, naturally growing in the foot of the Eastern Balkan Mountains, in the lands of the village of Topolchane, Sliven, Bulgaria. Samples were identified by an expert in Agricultural University of Plovdiv, Bulgaria.

The moisture content of the raw material (83.7±0.03%) was determined by drying up to constant weight, at 105 °C and all results have been presented on a dry weight basis [22].

Obtaining and GC-MS analysis of concrete

Concrete was obtained by two-stage, static batch extraction of 75 g flowers with n-hexane under the following conditions: hydro module (raw material: solvent) – 1:10 (w/v); duration of the first and second extraction stage – 1 h and 0.5 h; temperature 40 °C. The solvent was evaporated on a rotary vacuum evaporator at water bath temperature of 35 °C [23].

The GC-MS analysis was carried out with an Agilent 5975C MSD system coupled to an Agilent 7890A gas chromatograph (Agilent Technologies Inc., Santa Clara, CA). Agilent J&W HP-5MS column (0.25 μm, 30 m x 0.25 mm) was used with helium as a carrier gas (1.0 mL min^{-1}). The operational conditions were: oven temperature 35 °C/3 min, 5 °C/min to 250 °C for 3 min, total run time 49 min; injector temperature 260 °C; ionization voltage 70 eV; ion source temperature 230 °C; transfer line temperature 280 °C; solvent delay 4.25 min and mass range 50 - 550 Da. The MS was operated in scan mode. One μL of the sample diluted with n-hexane (10%, v/v) was injected into the GC/MS system at split ratio 30:1. The GC analysis was carried out using an Agilent 7890A GC system; FID temperature 270 °C. In order to obtain the same elution order with GC/MS, simultaneous triplicate injections were done by using the same column and the same operational conditions.

The identification of compounds was made by comparing their mass spectra with those from mass spectra libraries [24] and by comparing the literature and estimated Kovat's (retention) indices that were determined using mixtures of homologous series of normal

alkanes from C_8 to C_{40} in hexane, under the conditions described above. The percentage ratio of volatile components was computed using the normalization method of the GC/FID peak areas.

Mineral composition of flowers

Content of selected elements was measured by ICP-AES spectrometer: SPECTROFLAME MODULA-FTMOA81A. 1 g of air dried sample flowers was weighed with precision up to 0.001 g, and was poured on with 2 cm^2 concentrated nitric acid. Mixture was cooled in freezer until end of foaming process, homogenized on vortex at 3000RPM, and was heated on thermoreactor KUTESZ type 656 at 120 °C until dissolution of solid phase, and clarification of solution. After mineralization, the samples were filled up to 20 mL with 2% solution of nitric acid.

Acacia syrup preparation

For the preparation of the acacia syrups with different concentrations, anhydrous citric acid Parafarm (Saporiti, Argentina) was used to regulate the pH, food grade sucrose, potable bottled water, each from the same batch, were bought from the local market.

The acacia flowers were stored at 5 °C for 24 h. Acacia syrups are produced in two different concentrations.

For the obtaining of the lower concentration syrup the acacia flowers were extracted with a boiling 50% aqueous sugar solution for 10 minutes. Then, cooled in a cold water bath to 5 °C. The extraction takes 24 hours under refrigeration conditions (4 °C) in the presence of flowers in the sugar syrup. Citric acid (1% by the weight of the final product) was added to the syrup. Total soluble solids (TSS) were up to 60 °Brix; water activity – 0.69±0.01 (LabSwift-a_w, Novasina, Switzerland); pH – 3.23±0.02.

For the obtaining of the higher concentration syrup from acacia flowers, the common steps for preparation of extract were followed with the difference that they were subjected to extraction with a boiling aqueous solution of sugar (50%) for 30 min. Citric acid (1% by the weight of the final product) was added to the syrup. Total soluble solids (TSS) content reached 70 °Brix; water activity – 0.67±0.01 (LabSwift-aw, Novasina, Switzerland); pH – 3.19±0.02.

The prepared syrups were filtered, cooled properly and poured into sterile glass bottles.

Total phenolic contents

Total phenolic content was measured using a Folin-Ciocalteu reagent. Briefly, 1 mL five times diluted Folin-Ciocalteu reagent was mixed with 0.2 mL sample and 0.8 mL 7.5% disodium carbonate. The reaction was performed for 20 min at room temperature in dark. Then the absorbance was measured at 765 nm against blank. The results were expressed as mg equivalent of gallic acid (GAE) per mL extract, according to calibration curve [25].

Total flavonoids content

The total flavonoids content was analyzed by aluminum trinitrate ($Al(NO_3)_3$) reagents [26]. The absorbance was measured at 415 nm against blank. The results were presented as mg equivalents quercetin (QE) per g dry extract according to the calibration curve with quercetin as a standard.

DPPH radical scavenging ability

To conduct the assay, 0.15 mL from extract was mixed with 2.85 mL freshly prepared 0.1 mol solution of DPPH in methanol. The sample was incubated for 15 min at 37 °C in darkness. The reduction of absorbance was measured at 517 nm in comparison to the blank containing methanol and% inhibition were calculated [25].

Ferric reducing antioxidant power (FRAP) assay

The assay was performed according to Benzie and Strain [27] with slight modification by Kivrak et al. [26]. The FRAP reagent was freshly prepared by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 1 part 20 mM $FeCl_3{}^x6H_2O$ in distilled water. The reaction was started by mixing 3.0 mL FRAP reagent with 0.1 mL of investigated extract. The reaction time was 10 min at 37 °C in darkness and the absorbance was measured at 593 nm against blank prepared with methanol. Antioxidant activity was expressed as mM Trolox® equivalents (TE) per mL extract [25].

Microbiological analyses

For the microbial analyses a crude extract was obtained under aseptic surroundings by mashing fresh flowers after preliminary washing under tap water, sterilized distilled water and soaking for 5 min with ethanol and exposing under the influence of ultraviolet illumination for 20 min.

Antibacterial activity was tested against Gram-positive bacteria - *Listeria monocytogenes* NCTC 11994 and *Staphylococcus aureus* ATCC 25093, and Gram-negative bacteria – *Escherichia coli* ATCC 8739 and *Salmonella enterica* subsp. *enterica* serovar Abony NCTC 6017. The selective growth media, were: *Listeria* Oxford Agar Base /Merck/; Baird Parker Agar Base with Egg Yolk Tellurite emulsion supplement /Merck/, Rapid' E.coli 2 Agar /BioRad/ and Mac CONKEY Agar /Merck/, respectively. The media were inoculated with 24-hour suspension of the bacterial species.

Antimicrobial assay by agar diffusion method

The used inoculums have resulted as actual concentration cells of - *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella enterica* into the responding selective medium about 1.10^4 CFU/mL. Melted and cooled to 45 °C selective media were inoculated with the tested microorganisms and next equally dispensed into Petri dishes. After setting of the media, sterile rings (Ø 6 mm) were placed on, and different amounts of each sample (0.05; 0.10 and 0.15 mL) were put into the rings. Petri dishes were incubated at 37 °C for 24 or 48 h according to the bacterial spices, and then the distinct zone of growth inhibition around the rings was measured. The total plate count was estimated by the conventional plate-counting technique using appropriate dilution.

Statistical analysis

All experiments were performed in triplicate. All data were presented as mean±standard deviation (SD).

Results and discussion

Chemical composition of concrete

The yield of concrete was 1.06% (in abs. dry mass). The concrete was yellow waxy pastes and had sharp odor. Chemical composition of the concrete was shown in Table 1.

The concrete was composed by 34 components representing 99.02% of its total content. Sixteen of them were in concentrations over 1% and the rest 18 constituents were in concentrations under 1%.

Table 1

Chemical composition of concrete

№	Compound	RI	Content,%
1	*trans*-Linalyl Oxide	1074	0.48
2	*cis*-Linalyl Oxide	1088	0.65
3	Methyl salicylate	1175	1.06
4	*p*-Anisic acid	1399	0.81
5	n-Pentadecane	1500	1.12
6	n-Hexadecane	1600	0.37
7	Methyl veratrate	1602	0.78
8	*cis*-Methyl dihydrojasmonate	1654	0.69
9	n-Heptadecane	1700	0.58
10	(E,E)-farnesol	1722	0.41
11	(E,Z)-farnesol	1744	0.51
12	n-Octadecane	1800	0.33
13	Isopropyl myristate	1814	3.47
14	Hexahydrofarnesyl acetone	1833	3.86
15	Diisobutyl phthalate	1862	1.52
16	Methyl isopalmitate	1891	0.59
17	Dibutyl phthalate	1914	1.38
18	Palmitic acid	1942	4.92
19	Isopropyl hexadecanoate	1989	0.72
20	Linoleic acid	2098	3.64
21	α-Linolenic acid	2106	5.97
22	Tributyl acetylcitrate	2259	0.84
23	n-Pentacosane	2500	4.98
24	Diisooctyl phthalate	2589	4.05
25	n-Hexacosane	2600	0.63
26	n-Heptacosane	2700	20.10
27	n-Octacosane	2800	1.05
28	all-trans-Squalene	2835	0.59
29	n-Nonacosane	2900	25.18
30	n-Triacontane	3000	1.86
31	n-Hentriacontane	3100	3.39
32	n-Dotriacontane	3200	0.72
33	*β*-Amyrin	3290	0.84
34	*α*-Amyrin	3320	0.93

As could be seen the major constituents (up 3%) of the concrete are as follows: n-nonacosane (25.18%), n-heptacosane (20.10%), α-linolenic acid (5.97%), n-pentacosane (4.98%), palmitic acid (4.92%), diisooctyl phthalate (4.05%), hexahydrofarnesyl acetone (3.86%), linoleic acid (3.64%), isopropyl myristate (3.47%), and n-hentriacontane (3.39%). The difference in chemical composition of our investigations and the reported data may be due to environmental conditions under which the plant has grown as well as the variation in conditions of the analysis.

The classification of the identified compounds, based on functional groups, is presented in Figure 1.

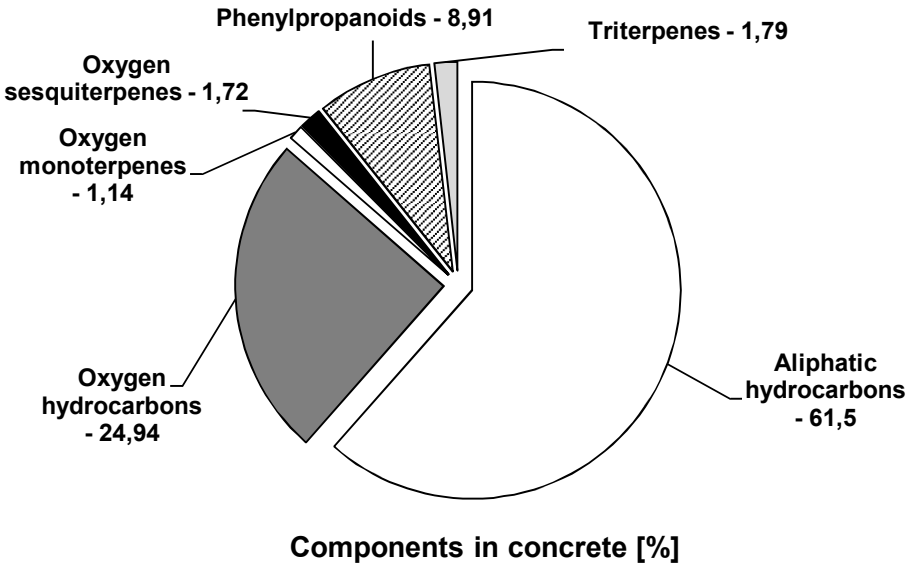


Figure 1. Groups of components in concrete

The total aliphatic hydrocarbons constituted the highest percentage of the component of the concrete constituting 61.50%. The concrete consisted above 10% concentration oxygen hydrocarbons (24.94%). The percentage of phenylpropanoids, triterpenes, oxygen sesquiterpenes and oxygen monoterpenes are under 9%.

Mineral composition of flowers

The minerals identified in the tested samples of false acacia flowers are as follows: boron – 182.64 nm, arsenic – 188.98 nm, zinc – 213.86 nm, manganese – 257.61, iron – 259.54 nm, and magnesium – 279.81 nm, and chromium – 283.56 nm, calcium – 317.93 nm, copper – 324.75 nm and nickel – 341.48 nm.

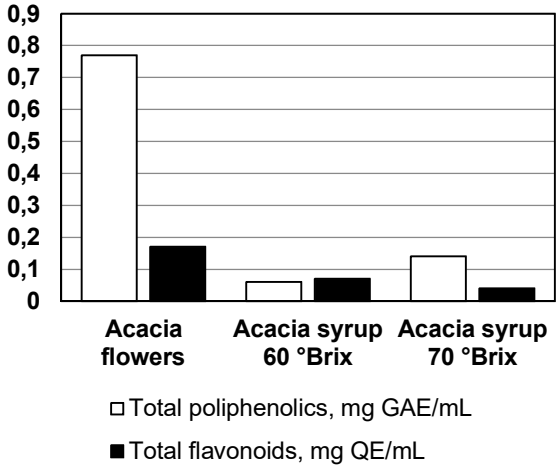
Differences in identified elements relative to other researchers may be due to environmental factors (geographical, climatic and seasonal). The relatively high levels of microelements, iron 259.54 nm is found in the active sites of many redox enzymes and

electron carriers such as hemoglobin and myoglobin, and therefore may have potential functional benefits in the human body. Copper (324.75 nm) has an important role in the active site of many redox enzymes and electron carriers, the production of hemoglobin or bone formation. Manganese (257.61 nm) activates many enzymes.

Content of polyphenolics and flavonoids

The extract of flowers presented the highest phenolic content (0.77 mg GAE/mL), followed by the acacia syrup 70 °Brix (0.14 mg GAE/mL) (Figure 2). Among the samples, false acacia flowers have the highest value for flavonoids (0.17mg QE/mL), the total phenolic content was 0.77 mg GAE/mL. With regard to the heating in the extract and syrup could rapidly flow inactivation of polyphenol oxidases, presented in flowers. Cooking with heat may lead to a higher extraction efficiency of total phenolics through destroying of the cell structure, which may lead to better solvent access and extraction.

Acacia flowers presented the highest flavonoids content (0.17 mg QE/mL), followed by the acacia syrup 60 °Brix (0.07 mg QE/mL), and syrup 70 °Brix (0.04 mg QE/mL), respectively (Figure 2).



*[1]GAE - Gallic Acid Equivalents, [2]QE - Quercets Equivalents

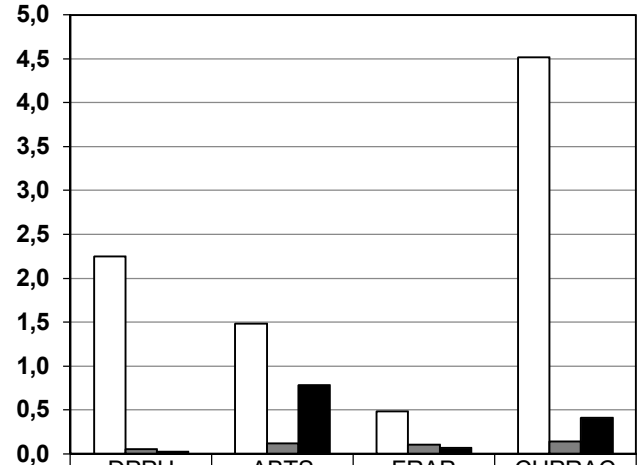
Figure 2. The total polyphenolics and flavonoids on *R. pseudoacacia* flowers and syrups

The results obtained in this study showed that the flowers are rich in phenolic compounds and have significant antioxidant activity. Flowers of false acacia as a natural additive in food, cosmetic and pharmaceutical products could be used as effective strategy to improve their nutritional and medical value while ensuring consumer safety.

Antioxidant activity

There have been few reports on the antioxidant activity of false acacia extracts. The antioxidant activity of lyophilized extracts of acacia leaves had a lower antioxidant capacity (1940 μmol Trolox equivalent g^{-1}) compared with *Rhus typhina* (4651 μmol Trolox

equivalent g^{-1}), *Acer rubrum* (3805 μmol Trolox equivalent g^{-1}), and *Rosa multiflora* (2533 μmol Trolox equivalent g^{-1}) [28]. Recently, Marinas et al. [19] reported that the highest content of polyphenols (GAE) was found in the leaf extract (266.7 μg GAE mL^{-1} extract), followed by the extract of the seeds (232.2 μg GAE mL^{-1} extract), respectively. In addition, the content of polyphenols presented in the flowers creates a strong antioxidant potential [29]. Differences in the distribution of the polyphenols and flavonoids arise from various factors that can be biological e.g., the part analyzed and the vegetative stage of the plant [30] and technical such as the extraction method, the solvents and their concentrations [31, 32] and there are also differences in the structure and properties of the phenolic compounds presented in the different samples analysed. False acacia flowers have the highest antioxidant activities – radical scavenging activity (DPPH – 2.25 mM TE/ml) and metal reducing ability (CuPRAC – 4.52 mM TE/mL) (Figure 3).



	DPPH	ABTS	FRAP	CUPRAC
Acacia flowers, mM TE/ml	2,25	1,48	0,48	4,52
Syrup 60 °Brix, mM TE/ml	0,05	0,12	0,10	0,14
Syrup 70 °Brix, mM TE/ml	0,02	0,78	0,07	0,41

Figure 3. Antioxidant activity of acacia flowers and syrups

Higher values of isolated metals as copper – 324.75 nm and nickel – 341.48 nm are directly related to the antioxidant properties of acaci flowers.

Microbiological analyses

The results of antibacterial testing are presented in Table 2.

Table 2

Diameter of zones of growth inhibition (mm) of tested pathogenic bacteria

Sample \ Bacteria		*E.coli*	*Salmonella*	*L. mono cytogenes*	*S. aureus*
		Zones of growth inhibition (mm)			
Flowers	0.15 mL	25.00	16.00	21.00	0.00
	0.10 mL	24.00	8.00	19.00	0.00
	0.05 mL	4.00	4.00	0.00	0.00
Syrup 60 °Brix	0.15 mL	18.00	13.00	0.00	0.00
	0.10 mL	10.00	0.00	0.00	0.00
	0.05 mL	0.00	0.00	0.00	0.00
Syrup 70 °Brix	0.15 mL	20.00	15.00	20.00	0.00
	0.10 mL	14.00	7.00	12.00	0.00
	0.05 mL	5.00	6.00	5.00	0.00

The crude extract of false acacia, applied in an amount of 0.15 mL, possessed the most pronounced antibacterial activity with inhibition zones: 25.00 mm against *E. coli*, 21.00 mm against *L. monocytogenes* and 16.00 mm against *Salmonella enterica*. False acacia crude extract had no inhibitory activity against *S. aureus.*

It is obvious that *S. aureus* was most resistable bacterium. False acacia syrup 60 °Brix, applied in an amount of 0.15 mL showed highest antibacterial potential against *E. coli* with a zone of inhibition of 18 mm and at the lowest sample concentration (0.05 mL) is ineffective. False acacia syrup 60 °Brix was effective against Gram-negative bacteria and didn't inhibit the growth of the Gram-positive. False acacia syrup 70 °Brix showed antibacterial activity against *E. coli, Salmonella* and *L. monocytogenes* in all applied concentrations. The antibacterial activity of false acacia syrups with different Total Soluble Solids content is considered to be due to the high sugar content in their composition.

It is obvious from the results that the activity of the false acacia extract depended on its concentration and the tested bacteria. This affirmation could be confirmed by the concluded results of Cioch et al. [33] reported that the type of the extract (ethanol, methanol or water) and the concentration of black locust displayed distinction in the level of inhibition the growth of microorganisms used in their experiments. In the case of most microorganisms, it is reported that their growth was inhibited by concentration of 2.00 mg/mL. Rosu et al. [34] reported that extracts from different parts of the plant had different antibacterial activities. Extracts of false acacia flowers and seeds were efficient antibacterials for Gram positive cocci. Bark and leaf extracts were active against *E. coli, Pseudomonas, Proteus, Salmonella choleraesuis, Candida albicans*.

Conclusion

The present study shows that flowers of false acacia (*R. pseudoacacia* L.) were with high levels of phenolic compounds and minerals that have pronounced antioxidant properties. Probably, most of the phytochemicals have preserved during the heat treatment of the syrup.

Higher values of phenolic compounds in the flowers (0.77 mg GAE/mL) than those of the false acacia syrup 60 °Brix (0.06 mg GAE/mL) and false acacia syrup 70 °Brix (0.14 mg GAE/mL) were found. The aromatic substances and phenolic compounds passing through the extracts exhibit antimicrobial properties against foodborne pathogenic bacteria as *Salmonella, E. coli* and *L. monocytogenes.* This is a reason for more thorough examination and extensive research of the chemical and biochemical properties of false acacia flowers as a potential raw material for usage in the food, pharmaceutical and cosmetic industries.

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