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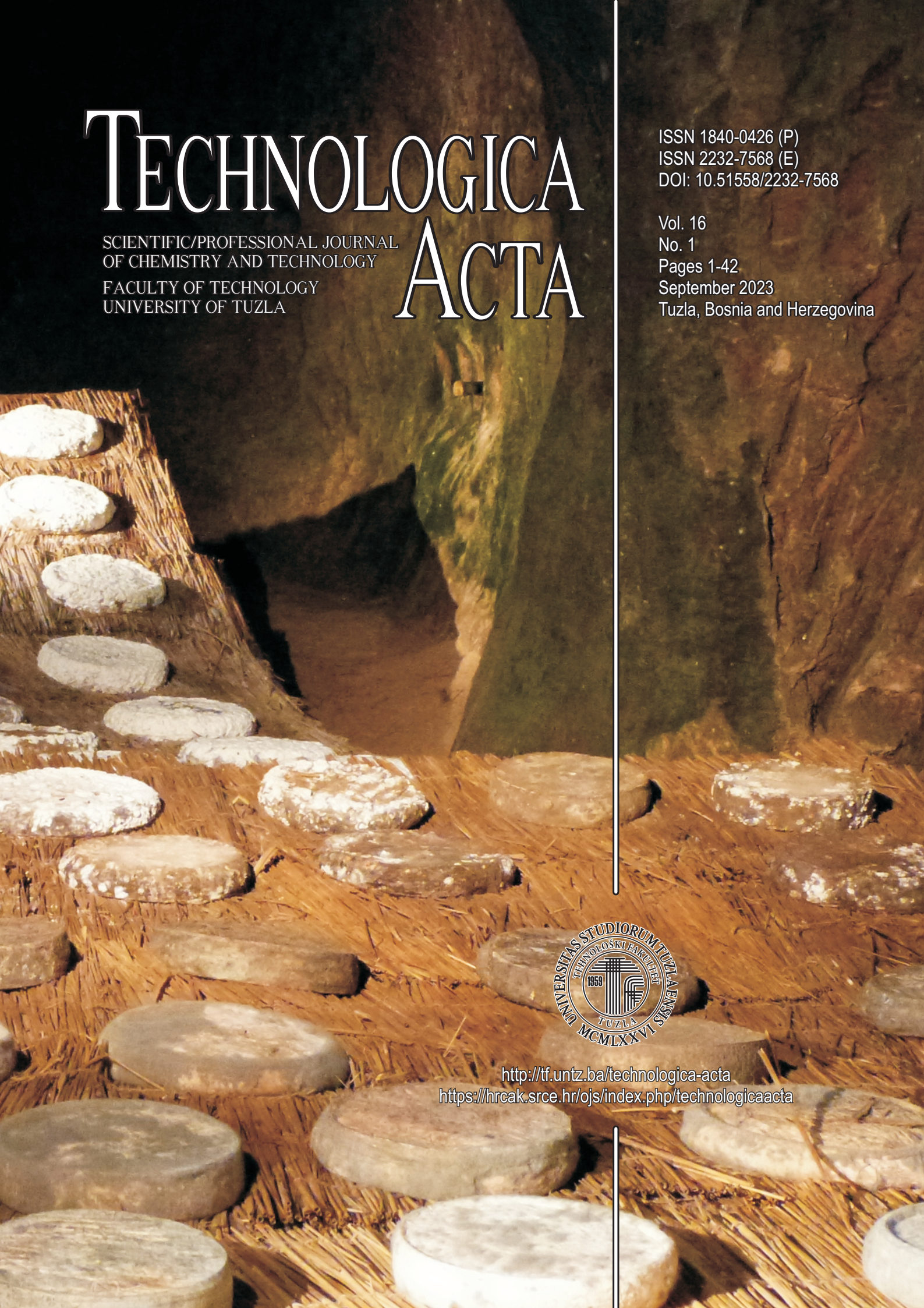

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CONTENT


UNIVERSITAS STUDIORUM TUZLAENSIS
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A COMPARATIVE STUDY OF ANTIMICROBIAL ACTIVITY OF *MELALEUCA ALTERNIFOLIA*, *ACHILLEA MILLEFOLIUM* AND *CINNNAMOMUM CAMPHORA* VAGINAL SUPPOSITORIES

ORIGINAL SCIENTIFIC PAPER

Merima Ibišević[1]✉, Saša Pilipović[2], Darja Husejnagić[3], Fadila Malohodžić[4], Alma Kulanić[4], Lejla Mustafić[4], Ermina Cilović Kozarević[5], Emir Horozić[6], Enida Karić[1]

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ABSTRACT:
Vaginal inflammation represents a heterogeneous group of disorders caused by infection, inflammation, or disruption of vaginal microflora. The most common causes of vaginal infection are *Staphylococcus aureus, Enterococcus faecalis, Streptococcus agalactiae, Escherichia coli* and *Candida albicans*. Antibiotic resistance is a major global problem, which can be mitigated by using natural antimicrobial substances such as essential oils. Each essential oil has an extremely complex composition (some essential oils have over 200 components), which prevents microorganisms from developing resistance. Therefore, essential oils retain their effects. The aim of our study was to investigate antibacterial activity *Melaleuca alternifolia, Achillea millefolium* and *Cinnamomum camphora* vaginal suppositories, and see which essential oil has the strongest potential to be used as active ingredient for vaginal infections. The antimicrobial activity of the vaginal suppositories was examined using the disk diffusion method. Standard bacterial strains were used for the ATCC collection: *Staphylococcus aureus (S. aureus)* ATCC 25923, *Enterococcus faecalis (E. faecalis)* ATCC 51299, *Escherichia coli (E. coli)* ATCC 25922, *Candida albicans (C. albicans)* ATCC 10231. The results showed that Melaleuca alternifolia essential oil has an antimicrobial effect on all tested strains, with the strongest effect on *Candida albicans* (ZI 22.7 mm). *Achillea millefolium* essential oil had no effect on *Enterococcus faecalis*, whereas *Cinnamomum camphora* essential oil did not show zones of inhibition of *Candida albicans.*

KEYWORDS: vaginal suppository, *Melaleuca alternifolia, Achillea millefolium, Cinnamomum camphora,* antimicrobial activity

INTRODUCTION

Vaginitis is defined as a spectrum of conditions that cause vaginal and sometimes vulvar symptoms, such as itching, burning, irritation, unpleasant odor and vaginal discharge. Vaginitis is diagnosed based on a combination of symptoms, physical examination, vaginal fluid pH, and microscopy. Vaginal infections are caused by the exogenous intake of the causative agent or the endogenous growth of a facultative pathogenic agent when the vaginal defense system is weak [1,2].

The most common causes of vaginal infections are: *Staphylococcus aureus, Enterococcus faecalis, Streptococcus agalactiae, Escherichia coli, Candida albicans.* [3,4].

Tea tree oil, *Melaleuca alternifolia* (Myrtaceae), is a therapeutical and often used agent in the treatment of various conditions. It possesses antibacterial, antifungal, and antiviral properties [5]. Owing to its medicinal properties, tea tree oil is used to prepare different preparations, such as vaginal suppositories, shampoos, lotions, skin creams, and tooth paste. Tea tree oil may have clinical applications, especially for the clearance of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage or as a hand disinfectant to prevent cross-infection with Gram-positive and Gram-negative epidemic organisms [6].

Achillea millefolium (Asteraceae) is one of the most important medicinal and aromatic plants used in the food, pharmaceutical, cosmetic, and perfume industries [7]. The essential oil of yarrow consists mainly of monoterpenes (30-80%), sesquiterpenes (8-62%), and in smaller quantities, other compounds (1-3%), such as alcohols, esters, and aldehydes. Previous research has shown that this medicinal plant has a wide range of effects, including antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, and stimulant effects [8].

Camphor tree (*Cinnamomum camphora*) is a member of the Lauraceae family, and is known to be native to India, China, and South Korea, and is now distributed in many other regions such as Australia and the Himalayas [9]. The main component of *Cinnamomum camphora* oil is 1,8-cineole (approximately 60%), while sabinene, alpha, and beta-pinene are also present at approximately 10%. It is used to treat viral infections of the respiratory system, viral hepatitis, HPV, herpes, viral warts, and numerous other viral infections. It also has antibacterial properties [9,10] and it is a good expectorant.

Vaginal suppositories [11] are dosed drug preparations intended for vaginal applications. They are usually spherical or conical shape. They are solid at room temperature, but dissolve in vaginal secretions at body temperature.

Cocoa butter and similar fatty substances, macrogols, or mixtures of gelatin, glycerol, and water are most often used as bases. If necessary, the carrier may contain emulsifiers, excipients, and preservatives. Suppositories and vaginal suppositories are made in the main practice by melting and pouring them into molds. This method implies that the drug substances are dissolved, uniformly suspended, or emulsified in a dissolved base, and the mixture is poured into appropriate molds. The most commonly used bases are different types of Witepsol, which are semi-synthetic lipophilic bases. They have advantages over cocoa butter because they have a defined melting point and are more chemically stable [12].

The aim of our study was to investigate antibacterial activity *Melaleuca alternifolia, Achillea millefolium* and *Cinnamomum camphora* vaginal suppositories, and see which essential oil has the strongest potential to be used as active ingridient for vaginal infections.

MATERIALS AND METHODS

The commercial essential oils used in this study were as follows:

- *Melaleuca alternifolia* essential oil (BIOETERICA, Zagreb, Croatia)
- *Achillea millefolium* essential oil (BIOHalilović d.o.o., Ilijaš, Bosnia and Herzegovina)
- *Cinnamomum camphora* essential oil (Volimo prirodno d.o.o., Mostar, Bosnia and Herzegovina

Table 1. Basic informations about essential oils

Botanical name	*Melaleuca alternifolia*	*Achillea millefolium*	*Cinnamomum camphora*
Origin	Croatia	Bosnia and Herzegovina	Belgium
Part of the plant	Leaves and twigs	Flowers	Leaves
Out of date	05/2023	03/2023	06/2025

[a] (taken from the Quality Specification)

According to the manufacturer's specifications, the main components of *Melaleuca alternifolia* essential oil are terpinen-4-ol (39.8%), γ-terpinene (20.3%), α-terpinene (10.1%), and 1,8-cineole (4.2%). The main components of *Achillea millefolium* essential oil were β-pinene (11.29%), linalool (8.58%), camphor (8.10%), and α-pinene (7.14%), whereas the main components of *Cinnamomum camphora* essential oil were1,8-cineole (62.6%), sabinene (12.2%), α-terpineol (7.3%), and α-pinene (5.0%).

Witepsol consists of glycerol esters of saturated vegetable fatty acids, mainly lauric acid, and is derived from coconut and palm kernel oils. Cera alba is a purified wax that comes from bee honeycombs that the bees make by converting the nectar they gather from flowers.

PREPARATION OF VAGINAL SUPPOSITORIES

Vaginal suppositories were prepared using different essential oils as active ingredients (*Melaleuca alternifolia, Achillea milefolium,* and *Cinnamomum camphora*). The method of pouring into the molds was used. Witepsol and cera alba were melted in a water bath and essential oils were added to the melted medium (38-40°C). A homogeneous mixture of appropriate consistency was poured into the molds. The concentrations of essential oils in vaginal suppositories were determined on the basis of preparations present in the market of Bosnia and Herzegovina; for example, vaginal suppositories with *Melaleuca alternifolia* essential oil (Cydonia d.o.o.) contain 200 mg of essential oil in one vagitorium. During preparation, 200 mg per vagitorium was used for other essential oils to compare the antimicrobial effect.

Table 2. Characteristics of Witepsol

Composition	Glycerides, coco mono-, di- and tri-, hydrogenated
Additives	-
Function	Lipophilic base
Hydroxyl value	5-15
Solidification point	34.5 ± 1 ºC

Table 3. Formulations of vaginal suppositories

Components	***Melaleuca alternifolia***	***Achillea milefolium***	***Cinnamomum camphora***
***M. alternifolia* essential oil**	1.0 g	-	-
***A. millefolium* essential oil**	-	1.0 g	-
***C. camphora* essential oil**	-	-	1.0 g
Cera alba	2.50 g	2.50 g	2.50 g
Witepsol	6.50 g	6.50 g	6.50 g

[a] (quantities for 5 vaginal suppositories)

The vaginal suppositories were stored in a refrigerator at 4 °C. After production, visual inspection was performed, and the following parameters were recorded: shape, color, and presence or absence of fissures.

WEIGHT VARIATION OF VAGINAL SUPPOSITORIES

The recommended mass of vaginal suppositories with respect to the available molds was 2 g. The prepared vaginal suppositories were evaluated for weight variation according to the British Pharmacopoeia [13,14]. Twenty vaginal suppositories from each series were weighed, and the average and standard deviation values were calculated.

DISINTEGRATION TEST

The disintegration test determines whether vaginal suppositories disintegrate at the prescribed time under certain experimental conditions in a suitable medium. This test was performed using a magnetic stirrer [12,15] set at 100 rpm. Phosphate buffer pH 4.5 was used as a medium and the temperature was 37 ± 0.5 ºC, which, according to the given conditions, mimics the vaginal environment. According to the recommendations of the British Pharmacopoeia, the disintegration time of vaginal suppositories should not exceed 60 min [14].

EXAMINATION OF ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AS ACTIVE INGREDIENTS

The antimicrobial activity of the essential oils was determined using the disk diffusion method [16,17]. Mueller Hinton (HiMedia, India) agar plates were inoculated with bacterial and fungal suspensions. There were used standard bacterial strains from ATCC collection: *Staphylococcus aureus (S. aureus)* ATCC 25923, *Enterococcus faecalis (E. faecalis)* ATCC 51299, *Escherichia coli (E. coli)* ATCC 25922, *Candida albicans (C. albicans)* ATCC 10231. Depressions with metal cylinders were made on each plate and 100 µL of the solution was introduced. The plates were then incubated at 37 °C for 24 h. After incubation, the sizes of the inhibition zones (in millimeters) were measured in triplicate. These measured inhibition zones of essential oil were used to compare the antimicrobial effects of vaginal suppositories, and ciprofloxacin was used as a positive control.

RESULTS AND DISCUSSION

Vaginal suppositories with *M. alternifolia* and *C. camphora* were white color, and vaginal suppositories with *A. millefolium* were bright blue, solid at room temperature.

Figure 1. Vaginal suppositories (own photo)

Table 4. Physical properties and characterization of prepared vaginal suppositories

Properties	**Vaginal suppositories with *M. alternifolia***	**Vaginal suppositories with *A. millefolium***	**Vaginal suppositories with *C. Camphora***
Shape	Conical	Conical	Conical
Colour	White	Bright blue	White
Weight variation (g)	2.04±0.05	2.00±0.042	1.95 ±0.05
Disintegration time (min)	14.2	12	13.3

All vaginal suppositories had on average weight about 2 g. The weight variation test complied with the regulations of the British Pharmacopoeia, which states that the standard deviation should be less than 5%. All vaginal suppositories had similar disintegration times owing to the same base of suppositories (Witepsol, Cera alba).

After incubation for 24 h, inhibition zones (mm) were measured. The obtained results showed the best antimicrobial activity for *M. alternifolia* essential oil, which was equally effective against all the strains. Inhibition zones greater than 20 mm indicate high sensitivity of microorganisms [18,19]. A slightly lower sensitivity was observed if the inhibitory zone was in the range of 15-19 mm, whereas the sensitivity was very weak for inhibition zones below 14 mm.

Table 5. Antimicrobial activity of prepared vaginal suppositories

Name of the organism	*M. alternifolia* ZI (mm)	*A. millefolium* ZI (mm)	*C. camphora* ZI (mm)	Ciprofloxacin (5 µg) ZI (mm)
E. coli ATCC 25922	15.5 ± 0.70	12.1 ± 0,32	20.3 ± 0.57	34
E. faecalis ATCC 51299	15.0 ± 1.0	0	14.3 ± 0.98	18
S. aureus ATCC 25923	19.3 ± 0.57	14.6 ± 0.30	20.0 ± 1.0	26
C. albicans ATCC 10231	22.7 ± 0.72	17.0 ± 1.0	0	15

[a] mean ± SD (n=3)

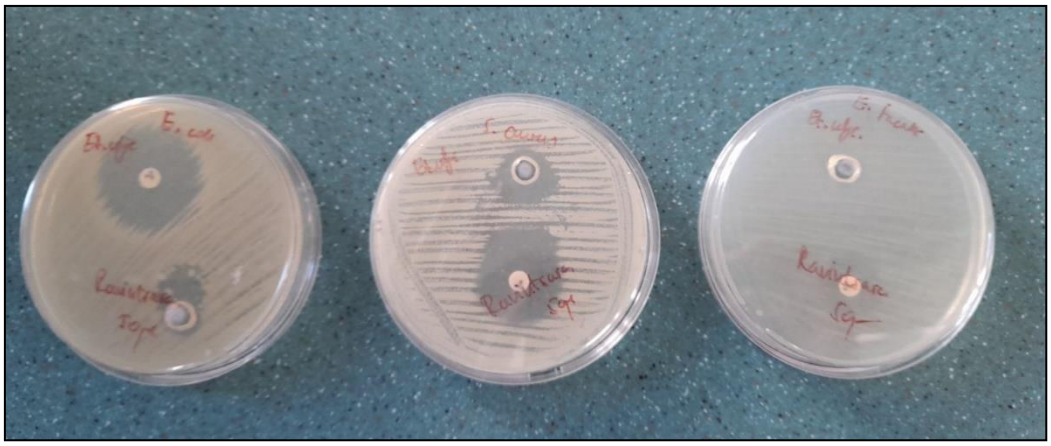

Figure 2. Inhbition zones (*C. camphora* essential oil)

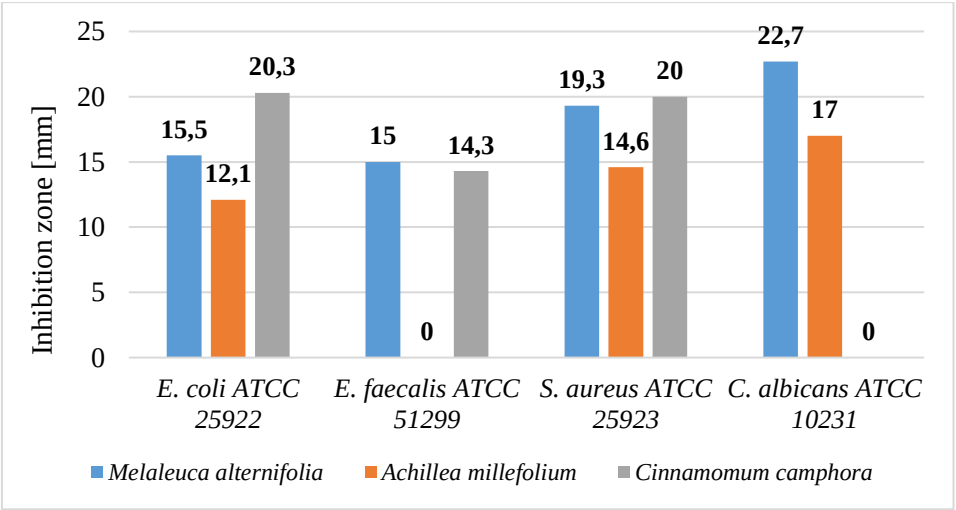

Figure 3. Antibacterial activity of Melaleuca alternifolia, Achillea millefolium and Cinnamomum camphora

The results presented in the table show that *Melaleuca alternifolia* essential oil has an antimicrobial effect on all tested strains, and the strongest effect on *Candida albicans* (ZI 22.7 mm) [20]. Puvača et al. [21] observed the antimicrobial activity of *Melaleuca alternifolia* essential oil against *E. coli* with an inhibition zone of 21 mm. Esmael et al. [22] detected that this essential oil was also active against antibiotic-resistant *S. aureus* with an inhibition zone of 15.5 mm.

Melo et al. [23] observed strong inhibition activity of *M. alternifolia* against *E. coli, S. aureus* and *E. faecalis* in the range of 23.43–50.80 mm. Ergin et al. [24,25] measured the inhibition zones of six *Candida* species with inhibition zones of 14–42 mm.

Achillea millefolium essential oil has no effect on *E. faecalis*, while *Cinnamomum camphora* essential oil did not show zones of inhibition on *Candida albicans*. El-Kalamouni et al [26] detected that *Achillea millefolium* essential oil was active against *S. aureus* wth inhibition zone of 12,8 mm, and has no activity against *E. coli.*

Cinnamomum camphora had inhibition zones over 20 mm on *E. coli* and *S. aureus* strains. As expected, ciprofloxacin showed larger zones of inhibition than the essential oils.

The size of the inhibition zones can be influenced by various factors, such as the thickness of the substrate and the genotypes of the bacteria, as well as differences in the chemical composition of the essential oils. The chemical composition of essential oils depends on the geo-climatic location and growing conditions, such as the concentration of nutrients, temperature, type of soil, length of day, climate, altitude, amount of water, season or vegetative period of the plant, as well as the method of extraction itself.

CONCLUSIONS

Based on the results of this study, the following conclusions were drawn:

- *Based on this study, it can be concluded that essential oils could be very effective in the treatment of vaginal infections.*
- *Melaleuca alternifolia essential oil showed antimicrobial activity against all strains, with inhibition zones greater than 15 mm.*
- *Vaginal suppositories with Melaleuca alternifolia essential oil could be used in the treatment of bacterial and fungal vaginal infections, thereby reducing the use of antibiotic therapy and reducing antibiotic resistance.*

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CHOLINESTERASE INHIBITION POTENTIAL OF ENDEMIC *SATUREJA SUBSPICATA* L. ESSENTIAL OIL AND EXTRACTS

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:
Medicinal plants are potential sources of bioactive compounds. One of the medicinal plants used in the traditional medicine of Bosnia and Herzegovina is endemic *Satureja subspicata* L. In this work, we examined the ability of *Satureja subspicata* L. essential oil and hot water and methanol extracts to inhibit the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) using Ellman's method. The ability of *Satureja subspicata* L. essential oil in concentration of 1 mg/mL and 2 mg/mL to inhibit enzymes was moderate: 72.82%, and 76.89% for AChE, and 51.51%, and 27.15% for BChE, respectively. Analyzed hot water and methanol extracts in concentration of 1 mg/mL showed weak ability of cholinesterase inhibition. Extracts were additionally analyzed regarding to ability to protect proteins from oxidation, during 1 h and 24 h incubation periods. After incubation for 1 h hot water extract showed a very good protective effect (10.61%), while the methanolic extract showed prooxidative activity. After incubation for 24 h, both extracts showed prooxidative activity. The obtained results show that the examined essential oil and extracts of *S. subspicata* L. contain compounds with cholinesterase inhibition and antioxidant potential, and therefore can be useful in treatment of Alzheimer's disease.

KEYWORDS: *Satureja subspicata*, essential oil, extracts, cholinesterase inhibition, protein oxidation.

INTRODUCTION

In the central nervous system, the stimulation of nicotine receptors by the action of the neurotransmitter acetylcholine is related to learning and memory processes, while the stimulation of receptors on muscle cells leads to their contractions. Acetylcholine hydrolysis controls the transmission of nerve impulses in the cholinergic synapse of the central and peripheral nervous system. Decomposition of acetylcholine in the synaptic cleft by the action of the enzyme acetylcholinesterase (AChE) establishes the polarization of the postsynaptic membrane and the transmission of impulses stops. Released ACh has a short half-life, since AChE is one of the most efficient enzymes; the rate of hydrolysis for human AChE, *kcat*, is approximately 400 000 min^{-1} [1]. Two types of ChE are currently known; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE is also called "true cholinesterase", while BChE is also known as "pseudocholinesterase", because it hydrolyzes numerous choline esters and other non-choline esters (butyrylcholine, succinylcholine, acetylcholine, acetylsalicylic acid, cocaine, and heroin). In the normal brain, BChE is present – in relation to total ChE – with a share of 10%, while it is more abundant in the liver, lungs, heart tissue, and blood plasma.

AChE inhibition prevents the hydrolysis of ACh, thus prolonging its activity in the transmission of nerve impulses. This concept is applied in the treatment of diseases characterized by low ACh levels, such as Alzheimer's disease. It is also studied in toxicology due to disease conditions and deaths caused by increased cholinergic stimulation [2].

Alzheimer's dementia (AD) is the most common neurodegenerative disorder and the cause of dementia in elders. It affects about 2% of the population in industrialized countries. In the next fifty years, the number of patients is expected to increase by about three times. Inhibition of the enzyme cholinesterase is one of the strategies in the treatment of Alzheimer's disease and other similar disorders. The use of most of the ChE inhibitors tested so far was followed by side effects, such as fatigue, sleep disorders, cardiorespiratory, gastrointestinal disorders, and low bioavailability. This was the impetus for further research with the aim of finding new ChE inhibitors of natural origin, with greater efficiency and bioavailability and fewer side effects [3]. Since plants are considered potential sources of inhibitors, many of

them, as well as various phytochemicals, are the subject of numerous research [4].

Oxidative stress is related to the etiology and pathogenesis of numerous diseases, so it is considered possible to prevent or delay the occurrence of pathological changes and to reduce the occurrence of diseases by removing the cause of oxidative stress. In a state of oxidative stress, excess free radicals can damage lipids, proteins, carbohydrates, and nucleic acids. Oxidative modification of proteins, both reversible and irreversible, occurs during redox signaling and other cellular processes. It also occurs as a result of oxidative stress. Exposure of proteins to OH• and/or $O_2^{\bullet-}$ leads to their structural modifications. Modified proteins may further undergo spontaneous fragmentation and cross-linking, or show a significant increase in proteolysis. In addition to fragmentation, oxidation of lysine, arginine, proline, and threonine can increase the concentration of carbonyls. Thus, the presence of protein carbonyl groups can be used as an indicator of protein oxidation caused by reactive oxygen species [5], [6].

Oxidative modification of proteins is present in diseases and changes associated with the aging process, such as: atherosclerosis, tumor, neurodegenerative diseases, and the aging process itself. Natural antioxidants, individually or in the composition of extracts, can be useful in the therapy of such diseases, which is also the reason for the great interest in researching the antioxidant activity of aromatic, medicinal, and edible plants [7].

Plants from the Lamiaceae family show numerous biological activities; antimicrobial, anti-inflammatory, antioxidant, antiviral, cytotoxic, and neuroprotective activity [8]. Being rich in essential oils, many of them are used as spices and medicinal plants. Lamiaceae (Labiatae) is a family of flowering plants belonging to the order Lamiales with opposite leaves. They are easy to grow, so many plants from this family are cultivated [9]. This family also includes *Satureja* species, which are known as aromatic and medicinal. Many of them are used in the perfume and cosmetic industry, as herbs, and in traditional medicine [10].

Satureja subspicata L. from the Lamiaceae family is a shrub from 8 to 20 cm in height (**Figure 1)**. The plant blooms from August to October. It is found in the higher localities of the Dinaric Karst, on exposed ridges or peaks, from 200 to 800 m above sea level [11]. In the traditional medicine of Bosnia and Herzegovina, the aerial parts of this plant are used in the form of infusions in the treatment of lymphatic system diseases; in the treatment of respiratory system diseases, as well as for improving the complete blood count (CBC). Honey from *S. subspicata* L. is used in the treatment of heart conditions [10].

Figure 1. Medicinal plant *Satureja subspicata* L.

The aim of the paper was to test essential oils, as well as water and methanol extracts in terms of inhibition of the enzymes acetylcholinesterase and butyrylcholinesterase, and to test the extracts in terms of protein protection against oxidation. Inhibition of the cholinesterase enzyme was tested using the Ellman's method, [12] while protein oxidation was tested using the method of measuring the formation of carbonyls [5], [13].

EXPERIMENTAL

PLANT MATERIAL

For the purposes of this work, plant material was used, i.e. aerial parts of the *Satureja subspicata* L. It was collected in the period of full flowering in the area of the Una-Sana Canton (Bosnia and Herzegovina). Confirmation of the identification of plant species was carried out by botanical identification methods, through morphology, by the botanist Mirko Ruščić, Associate professor at the Department of Biology at the Faculty of Science, University of Split. Aerial parts of fresh plants were used for essential oil isolation and preparation of water and methanol extracts.

CHEMICALS

Used reagents and solvents with analytical grade were: Acetylcholinesterase from *Electrophorus electricus* L. – electric eel, type V-S; Butyrylcholinesterase from equine serum; Acetylthiocholine iodide (ATChI); Butyrylthiocholine iodide (BTChI); 5,5-Dithiobis(2-nitrobenzoicacid) DTNB, Ellman's reagent,

guanidine-HCl (Sigma-Aldrich, GmbH, Steinheim, Germany); BHA (Butylated hydroxyanisol); BHT (Butylated hydroxytoluene), BSA (Bovine serum albumin) (Darmstadt, Germany); Ascorbic acid (Sigma-Aldrich, USA); Methanol (J.T. Baker, USA), Ethanol, H_2O_2 (Chemica, Croatia); DNPH, $FeCl_3$ (Acros Organics, New Jersey, USA).

METHOD OF ESSENTIAL OIL EXTRACTION FROM PLANT MATERIAL

Fifty grams of fresh, roughly chopped plant material and 0.5 liters of distilled water heated to boiling temperature were added to the round-bottom flask. With further heating, the volatile components of the essential oil and water vapor from the flask passed through the tube of the apparatus, cooled and condensed in a cooler made according to Allihn. A mixture of organic solvents pentane and diethylether, the so-called "trap", was also in the tube of the apparatus in a ratio of 1 : 3. The "trap" has the task of retaining volatile ingredients, while the water fell to the bottom of a graduated tube. After 150 minutes of hydrodistillation, the mixture of essential oil and organic solvents from the "trap" in the graduated tube was transferred to a dry bottle. Water remains from the essential oil were removed by adding anhydrous sodium sulfate, which was then removed by decantation. Upon drying, the mixture was transferred to a dry, weighed bottle in which the oil was hermetically sealed and stored in a freezer at -20 °C for further analysis.

To measure the biological activity of the essential oil, each essential oil was individually dissolved in ethanol, and its initial mass concentration was determined.

METHODS OF PREPARATION OF WATER AND METHANOL EXTRACTS

Water extracts – infusions

A measured mass of fresh plant material was poured with boiling water and left at room temperature for 24 hours, with occasional stirring (~ 7 mL of deionized water per 1 g of plant material). The extracts were filtered and then evaporated under vacuum at 40 °C (**Figure 2**). Upon drying in a desiccator for 48 h, the extracts were lyophilized, the mass was measured, and the yield was calculated. For the purpose of analyses, the dried extracts were dissolved in water. The samples were stored in glass vials at - 20 °C.

Methanol extracts

A measured mass of fresh plant material was macerated in 70% methanol w/w (~7mL MeOH per 1 g of plant material) at room temperature, with occasional stirring over 48 h. The extracts were filtered and then evaporated under vacuum at 40 °C (**Figure 3**). After drying in a desiccator for 48 h, the extracts were lyophilized, the mass was measured, and the yield was calculated. For the purpose of analyses, the dried extracts were dissolved in 86% ethanol. The samples were stored in glass vials at - 20 °C.

Figure 2. Evaporation of the aqueous extract

Figure 3. Evaporation of the MeOH extract

Method of determining the ability to inhibit the cholinesterase enzyme

The Ellman's method was used to determine the ability to inhibit the enzyme cholinesterase, which is based on the reaction of Ellman's reagent (DTNB) and

thiocholine, resulting in a yellow-colored product [12].

The reaction mixture consisted of 180 μL of phosphate buffer (0.1 M; pH = 8), 10 μL of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 10 μL of acetylthiocholine iodide (ATChI), 10 μL of acetylcholinesterase (AChE) from electric eel electrophorus, and 10 μL of samples of a certain concentration. Samples of essential oil and methanol extracts were dissolved in 86% ethanol, while water was used to dissolve aqueous extracts. Water and 86% ethanol were used for the control measurement. The total volume of the reaction mixture was 220 μL.

A stock solution of DTNB (6.6 mM) was prepared using phosphate buffer (0.1 M; pH = 7) supplemented with sodium bicarbonate (0.12 M). Stock solutions of AChE (0.66 U/mL) and ATChI (11 mM) were prepared using phosphate buffer (0.1 M; pH = 8).

A multichannel microtiter plate reader "Sunrise" (Tecan GmbH, Austria) was used for measurements, with automatic sample mixing and computerized data recording. Measurements were conducted at 25 °C and a wavelength of 412 nm.

Enzyme activity measurements were carried out under conditions of a constant amount of substrate, lasting 6 minutes, with three repetitions each. The percentage of AChE enzyme inhibition by essential oils or extracts was calculated according to the formula:

When testing the ability to inhibit the enzyme butyrylcholinesterase (BChE) by the action of essential oils or extracts, synthetic butyrylthiocholine iodide (BTChI) and the enzyme butyrylcholinesterase (BChE) from horse serum were used as a substrate. Stock solutions of BTChI (11 mM) and BChE (0.66 U/mL) were prepared using phosphate buffer (0.1 M; pH = 8). The other components of the reaction mixture, as well as the reaction mechanism, were the same as when testing the inhibition of the AChE enzyme. The percentage of inhibition of enzymes AChE and BChE by extracts was calculated using the formula: % inhibition = {[(Ae - Abe) - (Au - Abu)] / (Ae - Abe)} x 100; where: Ae – enzyme solution without sample (inhibitor) absorbance; Abe – blank sample of enzyme solution without substrate absorbance; Au – enzyme solution with sample (inhibitor) absorbance; Abu – absorbance of a blank sample of enzyme solution without a sample (inhibitor).

Method of testing the ability to protect proteins from oxidation

The method is based on monitoring the oxidation of bovine serum albumin (BSA) protein in a metal-catalyzed reaction with or without the presence of the tested extract. The reaction of the resulting aldehyde or keto functional groups with the dinitrophenyl hydrazine (DNPH) reagent produces DNP-hydrazone, with a maximum absorbance at a wavelength of 370 nm [5], [13].

The reaction mixture contained BSA (4 g/L), $FeCl_3$ (0.05 mM), ascorbic acid (0.1 mM), H_2O_2 (1 mM), and a plant extract with a concentration of 167 mg/L. The total volume of the reaction mixture was 600 μL. Water was used as a blind test for infusions, and 86% ethanol for MeOH extracts. The mixture was incubated for 1 hour, i.e. 24 h at 37 °C.

The amount of carbonyls formed was determined spectrophotometrically. After incubation, 1 mL of DNPH solution (10 mM) was added to the mixture and the solution was incubated for 60 minutes at room temperature. After that, 1 mL of 10% trichloroacetic acid (TCA) solution was added to the sample and the solution was kept on ice for 15 minutes, after which the samples were centrifuged for 40 minutes at 5,000 RPM (*Rotation Per Minute*).

The supernatant was discarded; the precipitate was washed 3 times with 2 mL of a solvent mixture of ethanol/ethyl acetate (1:1 v/v). After the last centrifugation, the precipitates were dissolved in 6M guanidine-HCl in 2 mL of 2 M HCl (pH 2) by vortexing and incubating at 37 °C.

After the sediments were resuspended, they were transferred to cuvettes suitable for measurement in the ultraviolet part of the spectrum, and the absorbance was measured at a wavelength of 370 nm. The spectrophotometer photoLab 6600 UV-VIS was used for the measurement. The results are expressed in % of oxidation inhibition compared to the blind testing, according to formula: % inhibition of oxidation = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$.

Statistical data processing

Measurements were performed in triplicates and the results are expressed as mean value ± standard deviation (SD). Statistical processing was performed using the Microsoft Office Excel 2010 program, upgraded with the statistical program XLStat 2018, Addinosoft.

RESULTS AND DISCUSSION

INHIBITION OF CHOLINESTERASE ENZYME WITH ESSENTIAL OILS AND EXTRACTS OF SELECTED PLANT SPECIES

The ability to inhibit the enzymes cholinesterase (ChE), acetylcholinesterase and butyrylcholinesterase (AChE and BChE) with essential oils and extracts of

the plant *Satureja subspicata* L. was tested by the Ellman's method.

The initial concentration of the extracts was 1 mg/mL, while the essential oils were tested in initial concentrations of 1 and 2 mg/mL. The obtained results were compared with the results for the well-known inhibitor eserine, which was tested at a ten or twenty times lower concentration (0.1 mg/mL). The results of testing the ability of essential oils to inhibit the enzymes AChE and BChE are shown in **Table 1**.

Table 1. Ability to inhibit AChE and BChE enzymes with essential oils and extracts

Sample		**Inhibition %**	
		AChE	BChE
Essential oil	1 mg/mL	72.82 ± 20.71*	51.51 ± 11.71
	2 mg/mL	76.89 ± 13.36	27.15 ± 9.84
Hot water extract	1 mg/mL	32.09 ± 3.66	0.00
Methanol extract	1 mg/mL	8.59 ± 1.15	11.9 ± 0.71
Eserine	0.1 mg/mL	95.92 ± 1.47	79.12 ± 0.00
* Mean value ± standard deviation (n = 3)			

The essential oils of the four tested plants in concentration of 1 and 2 mg/mL (45 and 90 μg/mL in the reaction system) showed inhibition of AChE 72.8% and 76.9%, and BChE 51.5% and 11.7%. The well-known ChE inhibitor eserine, at an initial concentration of 0.1 mg/mL, showed inhibition of AChE of 95.9% and BChE of 79.1%, so compared to it, the essential oil of *S. subspicata* L. showed moderate inhibitory activity.

The inhibition efficiency of the AChE enzyme was 32.1% for the water extract, and 8.6% for the methanol extract. Compared to the previously mentioned results for eserine, the extracts showed weak inhibition of the enzyme acetylcholinesterase. When inhibiting the enzyme BChE, the methanol extract with 11.9% showed a weak ability, while the aqueous extract did not inhibit the enzyme butyrylcholinesterase.

With regard to existing literature data, testing of methanolic and aqueous extracts and essential oil for *S. subspicata* L. in terms of AChE and BChE inhibition is the first of its kind.

As expected, the results for the inhibition ability of essential oils and water and methanol extracts were generally weaker compared to the less specific BChE.

The tested essential oil showed a moderate inhibitory potential on AChE and a weaker inhibitory potential on BChE. Such results may be due to the fact that among the main components of the examined essential oil, of the known cholinesterase inhibitors published so far, only α-pinene (10.2%) and β-caryophyllene (14%) was found [14], while the others are absent or found in low concentrations. The chemical composition of the examined essential oil and extracts of the plant *S. subspicata* L. was presented in a previously published study by Bektašević et al. (2017) [14].

Numerous plant extracts and essential oils, as well as their components, are studied in terms of ChE inhibitory activity [2], [15], [16]. In terms of testing the ability to inhibit ChE, most of them refer to the research of monoterpenes [17]. Among the monoterpenes, 1,8-cineole and α-pinene are the most effective in inhibiting AChE. Besides them, δ-2-carene (2-carene), δ-3-carene (3-carene) and myrtenal [15], [17] as well as geraniol, 3-carene, α-caryophyllene and limonene show their ability to inhibit AChE [4]. Carvone also showed good AChE inhibitory activity [2].

In various studies, the monoterpenes α-pinene, 1,8-cineole, linalool, terpinen-4-ol, linalyl acetate, thymol, γ-terpinene, and the phenylpropanoid eugenol showed good to moderate BChE inhibitory potential [17].

The most frequently investigated sesquiterpenes in terms of AChE inhibition are β-caryophyllene and α-humulene. At the same time, β-caryophyllene had a good inhibition ability, in contrast to α-humulene (α-caryophyllene), which showed a weak ability to inhibit AChE. In several studies, β-caryophyllene also showed good to moderate BChE inhibitory potential [17].

The tested water and methanol extracts of the selected plant species showed a weak inhibition of the AChE enzyme. The methanol extract showed a weak ability to inhibit the BChE enzyme, while the aqueous extract did not show inhibitory activity. In a previous study, Bektašević et al. (2017) [14] found that the following ChE inhibitors are present in the mentioned water and methanol extracts, respectively: naringenin 0.2% and 0.4%, eriodictyol 0.2% and 7.7%, ellagic acid 0.3% and 1%, rosmarinic acid 45% and 49%, kaempferol 0.1 and 1.5%, and luteolin 0.3% and 0.4%. The inhibitors apigenin and diosmetin were present in smaller amounts. The higher content of the mentioned inhibitors in the methanol extract explains the better

ChE inhibitory activity of the methanol extract compared to the water extract obtained in this work.

Several *in vivo* studies have been conducted examining the effect of polyphenol-rich extracts on AChE activity, which have shown inhibition of hippocampal AChE activity and greater cognitive ability, which may be a result of the presence of apigenin, kaempferol, and luteolin [16]. Natural phenolic components that are AChE inhibitors are also chlorogenic acid, rutin, and rosmarinic acid. The results show that rosmarinic acid easily crosses the blood-brain barrier and acts as an AChE inhibitor in the brain [16]. Ellagic acid also inhibits the action of AChE [18].

The ability to inhibit AChE with ethanol extracts of *S. subspicata* plant in initial concentration 0.25; 0.5 and 1 mg/mL was examined in the study by Vladimir-Knežević et al. (2014) and results were 16, 28 and 52%. The same study tested hydroxycinnamic acids (caffeic, chlorogenic, rosmarinic, ferulic acid), which in concentration of 0.25 and 0.50 mg/mL inhibited AChE activity in values from 30.0% to 48.9%, and from 73.6% to 86.6% respectively. Their effectiveness in inhibition was as follows: caffeic acid > ferulic acid > rosmarinic acid > chlorogenic acid [19].

PROTECTION OF PROTEINS FROM OXIDATION

Aqueous and methanolic extracts of the tested plant in an initial concentration of 1 g/L were tested in terms of protein protection against oxidation. Inhibition of BSA carbonylation (expressed as % of inhibition), in the presence of tested extracts and commercial antioxidants, is shown in **Table 2**. The incubation period, at 37 ºC with metal-induced oxidation, was 1 and 24 h.

Table 2. Activity of the extracts regarding the protection of proteins from carbonylation

Sample	Inhibition %	
	1 h	24 h
Hot water extract	10.61 ± 3.67*	- 69.81 ± 4.18
Methanol extract	- 6.06 ± 2.02	- 79.05 ± 3.43
BHT	11.12 ± 4.29	13.54 ± 3.45
BHA	-29.91 ± 6.27	8.04 ± 3.63
* Mean value ± standard deviation (n = 3)		

The obtained results, after incubation for 1 h, show a very good protective activity of the aqueous extract of *S. subspicata* L. (10.61%), compared to the standard antioxidant BHT, which showed an inhibition of carbonylation of 11.12%. The methanolic extract of *S. subspicata* showed prooxidation activity, as did BHA. After incubation for 24 h, all extracts showed prooxidation activity, while BHT and BHA inhibited BSA carbonylation by 13.54% and 8.04%.

Regarding the examination of the protective role of extracts of the investigated plant, in terms of protecting proteins from oxidation, there is no literature data available. The data for plants from the same family (Lamiaceae) from the territory of Bosnia and Herzegovina regarding the inhibition of protein carbonylation, after incubation for 1 h, show a better inhibition ability for the aqueous extract of the plant *Mentha pulegium* (14.6%), and a weaker inhibition ability for the *Mentha pulegium* methanol extract (5.6%). Aqueous and methanolic extracts of *Clinopodium vulgare* showed pro-oxidation activity. After incubation for 24 h, all extracts showed prooxidative activity [20], [21].

The prooxidative activity of the extracts can be explained by the following literature data. Polyphenols from food act as prooxidants catalyzing oxidative damage to DNA, proteins and carbohydrates, despite their role in protecting lipids from oxidation. Dorman and Hiltunen's research (2011) [7] shows the complexity, i.e. the paradox of certain phytochemicals with antioxidant activity, which can have a protective role by protecting some biologically important molecules from oxidative damage, e.g. lipids, but also show prooxidative activity towards other biologically important molecules, e.g. proteins. In the aforementioned study, gallic acid showed prooxidative activity, as did extracts of juniper (*Juniperus communis* L.), basil (*Ocimum basilicum* L.), caraway (*Carum carvi* L.), and laurel (*Laurus nobilis* L.). In Mayo et al.'s research (2003) [5] on the protection of proteins from oxidative damage, trolox and ascorbic acid showed prooxidative activity. Ascorbic acid is known to be a strong reducing agent, and is used to reduce transition metals, such as Fe^{3+} or Cu^{2+}, as well as to generate $^{\bullet}OH$ radicals. The combination of vitamin C and Cu^{2+} causes significant protein damage. In the same study, resveratrol did not show a protective effect [5].

The results obtained in this work related to protein protection from oxidation can be explained by the reaction mechanism. Namely, metal-catalyzed protein oxidation is a process that mainly takes place at specific metal binding sites on the protein, where one or several amino acids are oxidized. In this regard, the amino acid residues of histidine, proline, arginine, and lysine are most subject to oxidation. The reaction takes place as a "closed" process in which iron undergoes a redox cycle from Fe^{2+} to Fe^{3+} in reaction with H_2O_2 and again by protein-dependent reduction to Fe^{2+}. At the same time, the oxygen radical, which occurs as an intermediate product, does not diffuse into the

surrounding medium, because it reacts with the mentioned amino acid residues of the protein [6]. Since potential antioxidants cannot come into contact with free radicals, they are unable to compete with amino acids for them, and thus they lack protective activity.

CONCLUSIONS

The essential oil of *S. subspicata* L. showed a moderate ability to inhibit the enzymes AChE and BChE, compared to the known inhibitor eserine. Inhibition of AChE was more successful than inhibition of the less specific BChE. The components of this essential oil, known for their anticholinesterase effect, are probably responsible for this type of activity: α-pinene and ß-caryophyllene. The tested extracts showed a weak ability to inhibit the enzymes AChE and BChE. The obtained results show that the examined essential oil and extracts of *S. subspicata* L. contain active phenolic and terpene compounds, which show activity in terms of cholinesterase inhibition. They can be of importance in the prevention and therapy of Alzheimer's disease and other neurodegenerative disorders, which implies additional research. The results of testing the protection of proteins from oxidation, after incubation for 1 h, in comparison with the standard antioxidants BHT and BHA, showed a very good protective effect of the aqueous extract, while the methanolic extract of *S. subspicata* showed prooxidative activity. After incubation for 24 h, both aqueous and methanolic extracts showed prooxidative activity, which can be attributed to the protein oxidation reaction mechanism itself.

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COLOR, TOTAL PHENOLS AND ANTIOXIDANT ACTIVITY OF HONEY FROM NORTHWESTERN BOSNIA AND HERZEGOVINA

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:
Thanks to the climatic and geographical conditions, the area of the Northwestern part of Bosnia and Herzegovina has a long tradition of producing honey and other bee products. However, there is little or no literature data on the physico-chemical properties and biological activity of different types of honey and other bee products from Bosnia and Herzegovina. Five different types of honey were analyzed: monofloral honey (acacia, chestnut, linden), meadow honey and forest honey. Physico-chemical parameters, sensory analysis, color of honey, antioxidant activity, and content of total phenols were analyzed in five types off collected honey samples. The analyzes performed showed that chestnut honey contains the highest and acacia honey has the lowest content of total phenolic compounds. The forest honey showed the best antioxidant activity. The color of the honey was measured according to the CIELab system and the estimated L^*, a^*, b^* parameters show that all types of honey from this area can be characterized as dark types of honey ($L^* < 50$) with the presence of a yellow color. The obtained results show that the analyzed samples of five different types of honey are rich in polyphenolic components and represent a good source of antioxidants in the human diet.

KEYWORDS: honey, physico-chemical parameters, color, antioxidant activity, total phenols

INTRODUCTION

The positive influence of honey on human health is attributed to its antibacterial, antiseptic, and antioxidant properties. The physical and chemical properties of honey depend on its botanical origin, climatic conditions, and the area where bees collect nectar and pollen [1], so we can say that no two honey samples are identical. The therapeutic potential of honey is also connected with its antioxidant capacity [2]. So far, more than 150 polyphenolic compounds in honey have been investigated, including phenolic acids and flavonoids. It has been noted that the content of polyphenols is significantly correlated with the color of honey, the content of mineral substances, and electrical conductivity, which indicates that dark-colored honey shows a higher content of phenolic compounds, which in turn indicates increased antioxidant activity [2,3]. Examinations of phenolic compounds content and flavonoids in honey have shown that there is a correlation with botanical and geographical origin on the one hand and antimicrobial activity on the other [4].

One of the important properties of honey is the color, which is primarily important for customer preference. According to research by Szabo et al. [5], by using the Minolta Chroma Meter, very detailed data on the color of honey can be obtained, and due to the stability of the circumstances, accurate and objective results can always be obtained. The most popular color distance is based on the CIELab method, where the values are L* (brightness), a* (degree of greenness/redness), and b* (degree of blueness/yellowness) [6,7]. This color system is practical because each color can be defined by a mixture of red, blue, and green. For some types of honey, chemical-physical analyzes do not provide enough characteristic values, therefore sensory analysis has become an indispensable process in assessing the quality of honey and defining the overall properties of honey [8].

The quality of honey from northwestern Bosnia and Herzegovina was confirmed in a study by Alibabić et al. [9]. However, there is a lack of data on the effect of different geographical and floral origins on the color, total phenolic content, and antioxidant activity of honey.

In this research, multivariate statistical techniques were used to evaluate the influence of botanical origin of honey, physicochemical properties and color intensity using principal component analysis (PCA).

Statistical analysis showed significant correlations between the antioxidant activity tested by the DPPH method and the total phenolic content. In addition, a sensory analysis of honey samples was performed, as an indispensable parameter in the assessment of honey quality.

MATERIAL AND METHODS

SAMPLES

In this research, a total of fifty honey samples were analyzed and classified into five groups: acacia honey (*Robinia pseudoacacia* L.), chestnut honey (*Castanea sativa* Mill.), linden honey (*Tilia* spp.), meadow honey, and forest honey. The analyzed samples were collected in 2019, and they are representative samples from the Second International Honey Evaluation in Cazin (2019), for samples from the area of Una-Sana Canton: ten samples each of chestnut, acacia, and forest honey, five samples of linden honey and fifteen samples of meadow honey. The honey samples were stored in a dark place in a glass container at +4 °C until analyses.

In order to determine the antioxidant activity, in addition to the mentioned honey samples, a sugar analogue solution was also prepared, so that the obtained values could be corrected. The sugar analogue, which consisted of 40% fructose, 30% glucose, 10% maltose and 20% water, was prepared by dissolving 4 g of fructose, 3 g of glucose, 1 g of maltose in 2 mL of distilled water using an ultrasonic bath. The sugar analogue sample was further analyzed in the same way as the honey samples.

CHEMICALS AND INSTRUMENTS

All used chemicals in this work were analitical grade: 5,5-Dithiobis(2-nitrobenzoic acid) DTNB, 2,4,6-Tris(2-pyridyl)-*s*-triazine TPTZ, glucose, fructose, sucrose, maltose (Sigma-Aldrich, GmbH, Steinheim, Germany); BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene), Sodium carbonate (Acros Organics, USA); Gallic acid (Carl Roth, GmbH); Folin-Ciocalteu's reagent (Darmstadt, Germany); ethanol 96%, HCl, $FeCl_3$ x $6H_2O$, $FeSO_4$ x $7H_2O$ (Kemika, Zagreb). The spectrophotometric measurements were performed on a photoLab 6600 UV-VIS spectrophotometer.
Colorimeter LCC-A11 (LABTRON, Japan) was used to determine the color parameters, equipped with a standard light source D65, the color characteristics are expressed in the CIE $L^*a^*b^*$ system.

PHYSICAL-CHEMICAL AND SENSORY ANALYSIS OF HONEY

The sensory analysis was carried out according Golob et al. 2008 [10].The honey botanical origin was determined by the beekeepers, and it was additionally confirmed by sensory analysis by 5 sensory assessors, who evaluated the appearance, smell, taste, and aroma of the honey samples. All honey samples were analyzed for water content and electrical conductivity as part of the *Second International Honey Evaluation*. Water content was determined using a refractometer at 20ºC, using official AOAC methods (1995) [11]. Electrical conductivity was measured according to the method proposed by the International Honey Commission (IHC) [12]. All samples were analyzed in triplicate.

INSTRUMENTAL DETERMINATION OF HONEY COLOR

The honey samples color was measured using a LABTRON colorimeter LCC-A11 (LABTRON, Japan). The samples were measured in Petri dishes (diameter 6.5 cm and height 1.5 cm) on a white background indicating the sum of the colors [13]. The resulting L* value refers to the brightness of the sample (0=black; 99=white), the a* value refers to the redness of the sample (in the +60 direction red, in the -60 direction green) and the b* value gives the yellowness of the sample (in the + 60 yellow, in the direction -60 blue).

ANALYSIS OF TOTAL PHENOLIC CONTENT

The total phenolic content was determined by the Folin-Ciocalte method, and the results were expressed in mg of gallic acid/kg of honey [3]. For the purposes of determining the total phenolics content in honey samples, individual samples were prepared as follows: 5 g of honey were weighed and dissolved in about 20 mL of water using an ultrasonic bath. The dissolved samples were quantitatively transferred into 50 mL volumetric flasks and topped up with distilled water up to the mark. A sample of the sugar analogue was prepared in the same way.

For the analysis, a volume of 100 μL of the sample was measured, to which 1 mL of FC reagent (diluted with distilled water in a ratio of 1:10) was added, after which the contents in the test tube were intensively mixed for 2 minutes. After holding the sample for 20 minutes at room temperature, the absorbance was measured at a wavelength of 750 nm. A sugar analogue was used as a control, which was analyzed in the same way. Measurements for each sample were made 3 times. The concentration of total phenols was read from the calibration curve of gallic acid, which was analyzed in the concentration range of 8 to 120

mg/L. The results are expressed as mg gallic acid/kg of honey.

DPPH METHOD

The antiradical activity of honey samples was determined according to the procedure of Brand-Williams et al. [14] with certain modifications made by Beretta et al. [15]. 800 μL of acetate buffer (100 mM, pH 5.5) and 1900 μL of DPPH reagent (130 μM DPPH radical solution in 96% ethanol) were added to 300 μL of honey solution (10%). A control sample (sugar analogue) was also prepared. Vitamin C, BHA and BHT (0.1%) were used as standards. Absolute alcohol was used to set the zero on the apparatus, and the absorbance of the DPPH reagent was also measured. For blank tests, acetate buffer was added instead of DPPH for each sample. The prepared samples were left in the dark for 60 minutes, after which the absorbance was read at 517 nm.

The ability of tested samples in radical scavenging was calculated using the following formula: % inhibition = $[(A_0 - A_{sample}) / A_0] \times 100$; A_0 – absorbance of the DPPH ethanol solution measured at the beginning at 517 nm; A_{sample} – absorbance of the sample measured after 60 minutes. Obtained results were expressed as a percentage of inhibition of DPPH radicals. For measurement, the spectrophotometer Perkin Elmer Lambda EZ 201 UV-VIS was used.

FRAP METHOD

The antioxidant impact of honey was described by Bertoncelj et al. [3]. The principle of this method is based on the reduction of the iron complex 2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ) into its colored form (Fe^{2+}-TPTZ) in the presence of antioxidants. The FRAP reagent contained 2.5 ml of a 10 mM solution of TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3 \times 6H_2O$ and 25 ml of 0.3 M acetate buffer (300 mM, pH 3.6). The FRAP reagent was prepared immediately before use and was kept in a water bath at t 37 °C during the analysis. Aliquots of 200 μL of the sample were mixed with 1.8 ml of FRAP reagent and the absorbance of the reaction mixture was measured spectrophotometrically at 593 nm after incubation at 37°C for 10 minutes against the sugar analogue. Aqueous standard solutions of $FeSO_4 \times 7H_2O$ (0.1 to 1 mM) were used for the calibration curve, and the results were expressed as FRAP value μM Fe(II) of 10% honey solution.

STATISTICAL ANALYSIS OF THE RESULTS

The results of the analyzed samples are presented as mean value ± standard deviation. One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's Post Hoc Test) were used to assess the significant difference in data at the $p<0.05$ significance level. Statistics were implemented using Microsoft Office 2014 and the demo version of the statistical package MS Office XLSTAT-Pro 2014 [16]. A principal component analysis (PCA) was also performed. Measurements for all methods used in this research were done in three repetitions for each sample.

RESULTS AND DISCUSSION

PHYSICO-CHEMICAL PARAMETERS AND SENSORY ASSESSMENT

Table 1. Physico-chemical parameters and sensory assessment of analyzed honey samples

Parameter	Statistics	Honey type				
		Acacia (n=10)	Linden (n=5)	Chestnut (n=10)	Forest (n=10)	Meadow (n=15)
Water content (%)	Mean ± SD	(16.08 ±0.98)[a]	(16.36±0.45)[a]	(15.94±1.04)[a]	(16.17±0.85)[a]	(16.67±0.69)[a]
	Range	15.10-17.06	15.90-16.80	14.89-16.98	15.31-17.02	15.98-17.36
Electrical conductivity (mS/cm)	Mean ± SD	(0.19±0.07)[e]	(0.68±0.09)[c]	(1.46±0.04)[a]	(1.06±0.02)[b]	(0.54±0.06)[d]
	Range	0.12-0.25	0.59-0.77	1.42-1.49	1.04-1.07	0.48-0.59
Sensory evaluation	Mean ± SD	(28.55±3.05)[a]	(28.02±2.45)[a]	(28.08±2.49)[a]	(27.97±1.04)[a]	(27.39±0.60)[a]
	Range	25.50-31.60	25.58-30.47	25.59-30.56	26.94-29.01	26.79-27.99

[a,b,c,d,e] – Mean values in the same row marked with different letters are statistically significantly different according to Duncan's test ($p<0.05$).

The values of physico-chemical parameters and sensory evaluations of all honey samples are given in Table 1. In general, the values for water content and electrical conductivity for all tested honey samples were within the established legal requirements [17]. The honey samples from this study did not show

statistically significant differences ($p<0.05$) in terms of water content in the examined types of honey (Table 1). Electrical conductivity values ranged from 0.19 (mS/cm) in acacia honey to 1.46 (mS/cm) in chestnut honey and statistically significant differences ($p<0.05$) were found between all honey samples.

Honey sensory analysis evaluated the appearance of the sample (purity, color, smell, taste, and aroma), which also enabled the identification of the honey's botanical origin. The values for all types of honey ranged from 25.00 to 31.60 points, while the value for acacia honey was the highest and amounted to 32.4 points. Sensory analysis of honey showed that there are no statistically significant differences between different types of honey (Table 1).

HONEY COLOR INSTRUMENTAL ANALYSIS

The color characteristics are shown in Table 2, where the mean values, standard deviations, and range of parameters L*, a*, b* are shown. The samples of acacia and linden honey have the highest average L* values, 41.78 and 39.25 (Table 2). The color of the analyzed honey determined with a colorimeter showed that there are statistically significant differences between the measured values, among the samples.

Table 2. Color characteristics of the analyzed types of honey

Parameter	Statistics	Honey type				
		Acacia (n=10)	Linden (n=5)	Chestnut (n=10)	Forest (n=10)	Meadow (n=15)
L*	Mean ± SD	(41.78±1,26)^{a}	(39.25±1.95)^{a,b}	(34.22±2.00)^{c,d}	(31.57±3.07)^{d}	(35.69±2.00)^{b,c}
	Range	40.51-43,03	37.30-41.20	32.22-36.22	28.13-34.03	33.70-37.70
a*	Mean ± SD	(-5.90±1.15)^{b}	(-4.60±1.43)^{a,b}	(-2.03±0.15)^{a}	(-1.99±0.24)^{a}	(-1.47±3.27)^{a}
	Range	-7.10 to -4.80	-35.98 to 3.12	-2.20 to -1.90	-2.23 to -1.75	-4.75 to 1.80
b*	Mean ± SD	(11.48±4.50)^{a}	(13.04±1.96)^{a}	(10.63±3.99)^{a}	(4.96±0.97)^{b}	(12.32±1.91)^{a}
	Range	7.01-16.01	11.08-15.01	6.64-14.62	3.98-5.93	10.42-14.23

^{a,b,c,d} – Mean values in the same row marked with different letters are statistically significantly different according to Duncan's test ($p<0.05$).

The obtained values of L∗ for monofloral and multifloral types of honey are lower than the values obtained in the research of Szabó et al. [5] and Bertocelj et al. [3] for the same Hungarian and Slovenian types of honey, while the values are similar to those in the study by Flanjak et al. [18] for chestnut and linden honey and honeydew from Croatia. All types of honey from the area of the Una-Sana Canton can be characterized as dark types of honey based on the obtained value of L (L∗<50). Smetanska et al. [19] reported a positive correlation between the honey color and the content of phenols, flavonoids, and carotenoids. In all the analyzed samples, except for one meadow honey sample that had a positive a∗ value, negative a∗ values were obtained, which indicates a green shade in all analyzed types of honey. In the research by Flanjak et al. [18] the negative a∗ value is explained by the greater presence of chlorophyll in the nectar. The values of the parameter b∗ in the tested types of honey ranged in a positive range from 4.96 for honeydew to 13.04 for linden honey, which indicates the presence of a yellow color in all samples that depends on the presence of carotenoids and flavonoids in honey [18]. The color of each honey is the result of the presence of pigments such as flavonoids and carotenoids, which depends on the botanical and geographical origin of the product. Storage conditions can also affect color intensity. The color of honey also depends on the content of water, pollen, and saccharides [20].

TOTAL PHENOLS AND ANTIOXIDANT CAPACITY

The results of the content of total phenolics and antioxidant activity in samples of different types of honey are shown in Table 3. Samples of different types of honey were analyzed to evaluate their antioxidant activity and to find a correlation between antioxidant activity and the content of total phenolics. The obtained results showed (Table 3) that all the analyzed samples were antioxidant active, and that the total phenolics content and antioxidant activity varies significantly among the types of honey.

The total phenolics content in honey samples ranged between 264.89-406.02 mg of gallic acid/kg of honey for chestnut honey, 210.44-353.78 mg of gallic acid/kg of honey for forest honey, meadow honey 192.67-354.89 mg of gallic acid/kg of honey, linden honey 137.11-220.44 mg of gallic acid/kg of honey and acacia honey 120.44-189.33 mg of gallic acid/kg of honey . Analysis of variance revealed that there were statistically significant differences in the total phenolics content between different types of honey (Table 3). The total phenolics content in chestnut, forest, and meadow honey was significantly higher than in other types of honey. In general, our chestnut honey samples show a higher content of total phenols

compared to Slovenian chestnut honey 199.9 mg of gallic acid/kg of honey [3] and Croatian chestnut honey 162.1 mg of gallic acid/kg of honey [18]. The obtained values for forest honey were similar to the values for forest honey from Slovenia [3] and lower than the total phenolics content for forest honey from Croatia and Serbia [18], [20]. The total phenolics content in meadow honey was higher compared to the research conducted on honey by Bertoncelj et al. [3], Beretta et al. [15], and Srećković et al. [21], except for polyfloral honey from Poland, where the obtained values were higher [2]. Also, the total phenolics content in the samples of acacia honey was higher compared to several recent studies [3], [15], [18] and [21]. The high content of phenol in the tested types of honey from the Una-Sana Canton area confirmed the good quality of the honey. Certain variations in the phenolic content of our honey samples compared to those from different countries may be due to different geographical and botanical origins of honey.

Table 3. Total phenol content and antioxidant activity of honey samples

Parameter	Statistics	Honey type				
		Acacia (n=10)	Linden (n=5)	Chestnut (n=10)	Forest (n=10)	Meadow (n=15)
Total phenols mg of Gallic acid/kg of honey	Mean ± SD	(142.29± 40.77)^{c}	(166.37± 46.88)^{b,c}	(343.03± 71.77)^{a}	(282.67± 71.67)^{a,b}	(261.96± 83.67)^{a,b,c}
	Range	120.44-189.33	137.11-220.44	264.89-406.02	210.44-353.78	192.67-354.89
FRAP µM Fe(II) of 10% solution	Mean ± SD	(304.89± 95.85)^{b}	(488.67± 113.26)^{a,b}	(551.89± 313.96)^{a,b}	(874.11± 353.3)^{a}	(338.67± 94.39)^{b}
	Range	209.04-400.74	375.41-601.92	237.93-865.85	520.81-1227.41	244.27-433.06
DPPH % inhibition of 10% solution	Mean ± SD	(12.97± 3.67)^{c}	(35.12± 7.47)^{b}	(48.38± 1.07)^{a}	(59.15± 8.80)^{a}	(49.61± 5.95)^{a}
	Range	9.29-16.65	27.65-42.59	47.31-49.46	50.35-67.96	43.66-55.56

^{a,b,c} – Mean values in the same row marked with different letters are statistically significantly different according to Duncan's test (p<0.05).

DPPH AND FRAP

DPPH and FRAP methods were chosen to measure antioxidants in honey because they are simple, precise, and accurate methods. The obtained values for the tested samples of honey from the Una-Sana Canton area using the DPPH method showed inhibition of DPPH radicals in the range of 59.15% for forest honey and 12.97% for acacia honey (Table 3). The inhibition ability decreased in the following order: forest > meadow > chestnut > linden > acacia. This order differed slightly from the results obtained for the FRAP method. The results for forest honey, chestnut honey, and linden honey showed that they differ statistically significantly compared to honey from linden and acacia (Table 3). Several types of compounds may contribute to the antioxidants in honey, including carotenoids, ascorbic acid, tocopherols, and polyphenolic compounds. The obtained values for the ability of the reduction potential of five types of honey from the Una-Sana Canton area ranged from 874.11 ± 353.3 µM Fe(II) of 10% honey solution for forest honey to 304.89 ± 95.85 µM Fe(II) of 10% honey solution for acacia honey. The reduction potential decreased as follows: forest > chestnut > linden > meadow > acacia, which is in accordance with the results for different types of honey from Slovenia [3]. Analysis of variance revealed that there were statistically significant differences (Duncan test, p<0.05) when it comes to the antioxidant activity of the samples obtained by the FRAP method. As can be seen from the following literature data, the values obtained for honey from the Una-Sana Canton area were higher than those obtained by the same method for honey from the areas of Nigeria, Malaysia, Italy, Algeria, Slovenia, and Croatia.

CORRELATION OF COLOR, TOTAL PHENOLS, AND ANTIOXIDANTS

The correlation established between color, total phenols, and antioxidant activity is presented in Fig. 1 and Fig. 2, in order to investigate possible relationships between these values. There was a

positive correlation between color and total phenols (0.76) and total phenols and antioxidant activity (0.67).

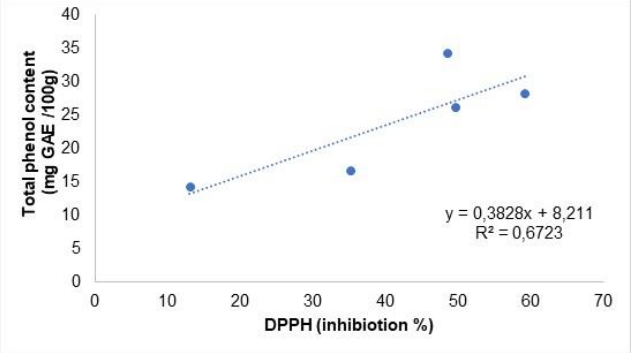

Figure 1. Correlation between total phenol content and total antiradical activity (DPPH) of honey samples

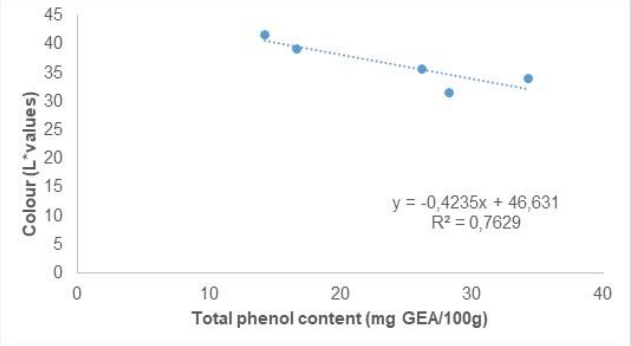

Figure 2. Correlation between total phenolic content and color (L*value) of honey samples

The positive correlation between color and total phenols (0.76) indicates that darker types of honey can be attributed to the presence of a greater amount of flavonoids and phenols that increase the number of antioxidants. For example, the average L* color value was the lowest in forest and chestnut honey (31.57 and 34.22), which indicates that it is dark honey, and has a higher content of total phenolics (forest 282.67 mg of gallic acid/kg of honey and chestnuts; 343.04 mg of gallic acid/kg of honey) and higher antioxidant activity (551.89 and 874.11 μM Fe(II) of 10% honey solution).

PRINCIPAL COMPONENT ANALYSIS

Variances Analysis of physico-chemical parameters and sensory evaluation showed that there are statistically significant differences between the tested types of honey. Principal component analysis (PCA) was performed with the aim of studying the interrelationship between different variables, which in this case are physico-chemical parameters and sensory evaluation. PCA was performed on the results obtained with different types of honey from the Una-Sana Canton area. The first principal component (PC1) included 60.76% of the total data variability, and the second principal component (PC2) was 26.18%. The value of PCA, i.e. their mutual projections for the first two components are presented in Fig. 3.

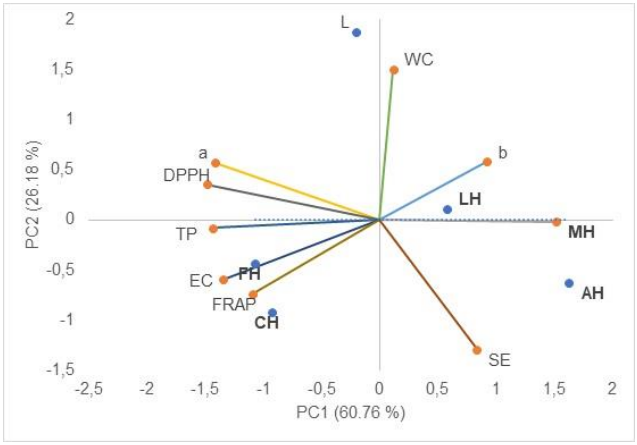

Figure 3. Principal component analysis (PCA) of the chemical composition and sensory analysis of honey samples
FH-Forest honey; CH-Chestnut honey; LH- Linden honey; MH- Meadow honey; AH Acacia honey. TP-Total phenols; EC-Electrical conductivity; WC-Water content; SE-Sensory evaluation.

According to the obtained honey samples results with the first factor PC1, the physico-chemical parameters that correlate best are the amounts of DPPH, TP, EC, FRAP, L* (forest and chestnut honey). According to PC2, the physico-chemical parameters that are positively correlated are the results of the sensory evaluation and the composition of the water (acacia and linden).

CONCLUSIONS

These are the first studies that classify the properties of color, total phenolics, and antioxidant activity of honey from the area of northwestern Bosnia and Herzegovina. The mentioned parameters varied in different types of honey: among the examined samples, forest and chestnut honey were the richest in color, total phenols, and antioxidant activity. Compared to honey from other countries, the analyzed honey from northwestern Bosnia was rich in total phenols and showed noticeable antioxidant activity, which is an important feature that should be taken into account when it comes to human health. Based on the obtained results of the physico-chemical parameters of different types of honey, the justification for using honey in human nutrition as a functional product with a high content of bioactive components, mineral elements, and significant nutritional and sensory

properties can be explained. In further research, it would be desirable to identify and quantify individual flavonoids and phenolic acids, the compounds that contribute the most to the antioxidant activity of honey.

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ANTIMICROBIAL ACTIVITY OF SELECTED SPICE EXTRACTS ON *ESCHERICHIA COLI*, *SALMONELLA SPP.*, AND *LISTERIA MONOCYTOGENES*

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:
The study determined antimicrobial activity using the Disc-diffusion method and extracts of three plants: garlic (*Allium sativum* L.), turmeric (*Curcuma longa* L.), and parsley (*Petroselinum crispum* L.) on *Escherichia coli*, *Salmonella ssp*. and *Listeria monocytogenes*. These pathogens have attracted the attention of numerous agencies and researchers, because of the negative impact on food and human health - and because of the impact of disease development on the economy. These studies are necessary and represent potential natural antimicrobial drugs, and due to the resistance of bacteria to known antibiotics, there is a constant increase in global consumer demand for natural ingredients. The tested plant extracts showed excellent antibacterial activity in all three replicates on *Escherichia coli* with an average inhibition zone of 21,86 mm and were characterized as ***S - sensitive for the mentioned bacterium. With an average zone of inhibition of 19,052 mm, *Curcuma longa* showed the strongest effect on the tested bacterium *Salmonella enteritidis*, which tells us that this bacteria is very sensitive to the extract of the mentioned plant. The study identified zones of inhibition of very low values (> 8 mm) in all three tested extracts: *Allium sativum* L., *Curcuma longa* L. *Petroselinum crispum* L., and *Listeria monocytogenes*.

KEYWORDS: Antimicrobial activity, extract, Disc-diffusion method

INTRODUCTION

One of the leading problems of the food industry is the microbiological spoilage of food and food poisoning, and as a result, various diseases are appearing. According to (Anand and Sati [1] food preservation is the main concern of the food industry today due to the existing preservatives that are based on synthetic chemicals, but they also show a negative effect on human health. Spices are mainly of plant origin, with a specific smell and taste, they are added to food or food products and drinks in order to achieve the appropriate smell, taste and color, but they also exhibit significant antioxidant and antimicrobial activity, which is why they are increasingly used as natural preservatives [2]. They can be consumed fresh, dried, chopped, turned into powder and as extracts of aromatic ingredients [3]. Due to the presence of the mentioned substances, they also show an effect on the digestive tract, and achieve the secretion of saliva, that is, the secretion of the glands, and thus a greater secretion of digestive enzymes, thus improving digestion.

Research carried out by Melvin et al. [4] the antimicrobial and antifungal activity of spices is also based on the presence of essential oils that contain sulfur and alkaloids, and it is very important to use fresh spices in the daily diet, as an addition to meals, since older spices, in addition to being poorer in essential oils, can cause some side effects after use. Due to their important properties, spices have become indispensable for culinary world-wide, and spice trade has been an important commercial activity since ancient times and a means of economic development [5]. Between the year 2000. and 2019., the value of spice imports increased by 2,9% per year, and the volume increased by 5,9%. In 2019, the market of spices was about 1,547 million tons worth 2,97 billion US dollars, which reflects the importance of spices in the world and their demand [6].

The main goal of the conducted experimental research is to determine the antimicrobial activity of extracts of three selected types of herbs: garlic (*Allium sativum* L.), turmeric (*Curcuma longa* L.) and parsley (*Petroselinum crispum* L.) against the following bacteria: *Escherchia coli*, *Salmonella ssp*., *Listeria*

monocytogenes. The assumption is that the antimicrobial activity of the mentioned extracts will have a negative effect on the survival ability of pathogenic strains of *Salmonella ssp*., *Listeria monocytogenes* and *Escherchia coli* in foods, because the extracts from spices, in addition to providing meat and dairy products with a characteristic taste, also show an antimicrobial effect and in this way they prevent the development of bacteria, and thus the process of food spoilage.

MATERIAL AND METHODS

For the conducted research, methanol extracts of herbs were used as samples: Parsley - *Petroselinum crispum*/leaf; Garlic - *Allium sativum* L./bulb and Turmeric - *Curcuma longa*/tuber-powder, and the bacterial isolates used are: *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATOC 13076 and *Listeria monocytogenes* ATCC 19115. Herbs parsley (*Petroselinum crispum* L.) and garlic (*Allium sativum* L.) were homogenized and chopped, and turmeric powder per 50 g sample was extracted with 100 ml of 80% methanol for 24 h at room temperature. Extracts of these spices were obtained by filtering on a Bihners funnel. After filtering the extracts, methanol was separated using a rotary evaporator (Rotavapor R-210/215 BÜCHI, Buchi AG, Flawil, Switzerland) at 40°C. The extracts prepared in this way represent the samples used to determine the antimicrobial activity.

Isolation of Salmonella enteritidis - after preparating the dilution of the sample (sample positive for *Salmonella ssp*. from the Veterinary Institute of USK), isolation of *Salmonella enteritidis* ATOC 13076 was carried out according to the directives BAS EN ISO 6579:2005, BAS EN ISO 6579/cor1 (2010).

Additional identification was done by sowing isolated bacteria on XLD agar (xylose lysine deoxycholate agar; Torlak, Belgrade, Serbia) on a non-selective medium. Isolated Salmonella enteritidis ATOC 13076 was seeded on Salmonella Chromogenic Agar (Torlak, Belgrade, Serbia). Additional identification was done by sowing isolated bacteria on XLD agar (xylose lysine deoxycholate agar; Torlak, Belgrade, Serbia) on a non-selective medium. Isolated *Salmonella enteritidis* ATOC 13076 was seeded on *Salmonella Chromogenic* Agar (Torlak, Belgrade, Serbia).

Isolation of *Listeria monocytogenes* - detection of *Listeria monocytogenes* was performed using the Horizontal method BAS EN ISO 11290-1:2018 (sample positive for Listeria monocytogenes) from the Veterinary Institute of the USK. Detection of *L. monocytogenes* takes place successively, by primary and secondary enrichment, and by seeding on selective substrates Aloe and Oxford agar. Typical colonies on Aloe agar are green-blue surrounded by an opaque halo, while on Oxford agar typical colonies are small (1 mm) and grayish colonies with a black halo, which after 48 h become darker with a greenish sheen and have black halos and a sunken center. Colonies suspected of being L. monocytogenes go for further confirmation, which includes catalase test, Gram stain, hemolysis test, acid formation by decomposition of carbohydrates and Camp test .

Isolation of *Escherichia coli* - by the Membrane Filtration method - a very popular test in microbiology of water. The presence and number of *Escherichia coli* can be determined by using membrane filtrates. The selected volume of water is filtered (water sample suspected of *E.coli*), and any bacteria present remain on the filter. The filter is then removed from the funnel, placed on a solid selective medium in a petri dish (most often EMB or endoagar), and then incubated at 35 to 37°C or at 44,5°C. Colonies of *E.coli* present on the filter will grow within 24 to 48 hours.

The disc-diffusion method is the most frequently used qualitative method for determining resistance in microorganisms. It is performed by applying an inoculum containing approximately 1 to 2 × 108 log CFU/mL of the tested microorganism to the surface of a Petri plate with a suitable nutrient medium, and then paper discs impregnated with a fixed concentration of antibiotic and sample are placed on it. The results are read after 16 of 24 h, by measuring the growth inhibition zone of the tested microorganisms [7]. Although the disk-diffusion method is most often used with clinical isolates, because it is cheap, simple and well standardized, with it isolates can only be classified as sensitive or resistant based on the size of the inhibition zones [8]. The method is based on the principle of diffusion of plant extract into a solid nutrient medium,whereby it has a more or less inhibitory effect on the bacteria previously seeded on that substrate. Cellulose discs are applied, on which the sample extracted from the plants is applied. Cultures of microorganisms are seeded in Petri dishes. 1 ml of the bacterial culture suspension is applied evenly on the substrate with a stick according to Drigalski. Then cellulose discs soaked with extracts of the plants Parsley - *Petroselinum crispum* L., Garlic - *Allium sativum* L. and Turmeric - *Curcuma longa* L. are placed. The samples prepared in this way were incubated (Incubator Selecta: 2000209, Spain). The extract diffuses from the application site into the substrate in all directions. Preventing the growth of the tested microorganism occurs if it is sensitive to the effect of the tested sample. The width of the inhibition

zone (inhibition diameter) is proportional to the degree of sensitivity of the microorganism to the applied agent, that is, to the antimicrobial effect of the examined extract (table 1). After incubation at 37°C, the first inhibition reading was taken after 24 hours, and the final results after 48 hours. If there is no inhibition zone, it means that the microorganism is not resistant to the effect of the tested substance. The experiment was performed in replicate with two types of control: positive and negative.

The negative control implies that water is applied to the disc instead of the sample in a certain dilution. Positive control implies the use of antibiotics. An antibiotic (Penicillin) was used as a control to compare the activity of the extracts.

Table 1. Resistance of bacteria towards microorganisms

Inhibition zone	Sensitivity marks	Interpretation
do 10 mm	R*	Resistant bacteria
10 - 17 mm	I, 2**	Moderately resistant bacteria
> 17 mm	S, 3***	Resistant bacteria

*R – resistant, **I – intermediate, ***S - sensitive

Data collected through experimental research were processed in a statistical program (PAST 4.0) and SAS 9.4 (SAS, 2012).

RESULTS AND DISCUSSION

The excessive use of antibiotics has led to the selection of new strains of bacteria resistant to antibiotics, a situation that is often encountered in practice, especially in the case of *Escherichia coli*. Mos et al. [9] investigated the sensitivity of different strains of Escherichia coli to different groups of antibiotics, including penicillin. They used two methods for the research: the disc diffusion method and the agar dilution method, and 113 strains of *Escherichia coli* were isolated from the external wounds of patients in the period from 2006 to 2008 and were used as the test material. The results confirm the low sensitivity of the bacteria to pencillins, and resistance of over 50%, which is in line with our research. The data that testifies to the increase of various phenotypes of bacteria resistant to multiple drugs in enterobacteriaceae, including *Escherichia coli*, are worrying, such as research [10]; [11]; [12]; [13]; [14]; [15]; especially third-generation cephalosporins, as well as colistin (used as an antibiotic of last resort to treat carbapenem-resistant enterobacteriaceae).

Statistical processing of the collected data from our research and the use of extracts of three plants (parsley, turmeric and garlic) determined their antibacterial effect on the tested bacteria. which characterizes it as an extract to which the tested bacterium is very sensitive because the identified inhibition zones are higher compared to the aforementioned studies. (table 2), but also a significant reaction of turmeric extract with a medium inhibition zone of 20,26 and garlic extract 19,63 mm.

Table 2. Results of inhibition zones - *Escherichia coli* ATCC 25922

Escherichia coli	Min.	Max.	Mean	Variance	Stan.dev.	Coeff.var.	Std.error
Petroselinum crispum	17,20	23,70	21,67	9,161	3,026	13,96	1,513
Curcuma longa	15,72	23,48	20,26	10,981	3,313	16,35	1,656
Alliuma sativum	17,82	23,17	19,63	5,947	2,438	12,42	1,219
ANOVA	Sum of sqrs		df	Mean squere		F	p (≤0,01)
Between groups	8,68805		4	4,34402		0,4995	0,062*
Within the group	78,9586		30	8,69673			0,9015

Kruskal - Walis test

$H(ch^2)$: 1,192

H_c (tie corrected): 0,892

p (≤0,01): 0,05509

Statisticaly significant diference beetwen samples is present.

Fereshteh et al. [16] studying the antimicrobial activity of parsley (*Petroselinum crispum*) against pathogenic bacteria in food, conducted experimental research with the aim of determining the antimicrobial activity of the essential oil from the leaves and seeds of parsley.

Antimicrobial activity was tested using the paper disk diffusion method and the by micro dilution technique against five pathogenic bacteria (*Escherichia coli*, *Salmonella ssp.*, *Staphylococcus aureus*, *Yersinia* and *Vibrio cholera*).

The results show the high efficiency of essential oils in the control of pathogenic bacteria and the need for their use in the development of new systems for the prevention of bacterial growth in accordance with their role in extending the expiration date and

increasing the safety of processed food. Parsley seed essential oil showed an inhibition zone diameter of 11 mm against Staphylococcus aureus, 10 mm against Escherichia coli and Yersinia, 10,5 mm against *Vibrio cholera*, while the diameter of the inhibition zone was low against *Salmonella* (9 mm). The diameter of the zone of inhibition of the essential oil from the leaves varied from 12 to 14,5 mm and is equal to the resistance of microorganisms to antibiotics currently in use. A large number of side effects during application, as well as the high price of synthetic compounds, resulted in the tendency of many researchers to search for efficient natural products. Kasta [17] investigating the necessary concentration and antimicrobial activity of the ethanolic extract of the rhizome of turmeric (*Curcuma Longa* L.) for the growth of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* used Disk diffusion method to measure the zone of inhibition with different concentrations of turmeric rhizome extract (500 mg/mL, 400 mg/mL, 300 mg/mL, 200 mg/mL, 100 mg/mL, 50 mg/mL and 25 mg/mL). The results of phytochemical screening showed that the ethanolic extract of turmeric rhizomes contains alkaloids, flavonoids, saponins, tannins and triterpenoids/steroids, while the antimicrobial inhibition of the ethanolic extract of turmeric rhizomes against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* at a concentration of 500 mg/mL was 15,88 mm, 15,63 mm and 15,22 mm. Investigating the comparative assessment of the antimicrobial effects of garlic (*Allium sativum*) and antibiotics on diarrhoeagenic organisms, Egbobor [18] performed antimicrobial susceptibility tests on *Escherichia coli*, *Shigella sp*., *Salmonella sp*. and *Proteus mirabilis* using standard procedures.

Significant statistical differences at the level of significance ($p<0,01$) were observed in the action of antimicrobial agents (garlic, ciprofloxacin and ampicillin), and in the sensitivity of microbial species ($p<0,01$) to antimicrobial agents, which is in accordance with our conducted research where the average zone of inhibition was determined to be 19,65 mm. From the three tested cultures, the lowest inhibition zone on *Escherichia coli* was recorded with the use of garlic extract, but still sufficient to characterize that extract as favorable and significant in the fight against the mentioned bacteria with an inhibition zone greater than 17 mm and characterized as ***S - sensitive .

Salmonella can be obtained from any food that has not been handled properly and hygienically or that has not been properly processed. Symptoms can appear six hours to six days after infection and can last four to seven days, and include: abdominal cramps and pain, sudden fever, nausea, vomiting, headache, dehydration, and blood in the stool.

Microorganism resistance is still a challenge for health and food production. Based on the demand for natural products for the control of microorganisms, and the re-evaluation of potential medicinal plants for the control of diseases, the aim of this research was, among other things, to determine the antibacterial activity of parsley extract against the mentioned pathogen, but also the activity of *Curcuma longa* and *Allium sativum* both on *Salmonella enteriditis*, and the results are presented in table 2.

Table 3. Results of inhibition zone of *Salmonella enteritidis* ATCC 13076

Escherichia coli	Min.	Max.	Mean	Variance	Stan.dev.	Coeff.var	Std.error
Petroselinum crispum	15,79	16,96	16,375	0,294	0,542	3,344	0,2713
Curcuma longa	17,41	22,47	19,944	5,429	2,330	12,228	1,1665
Alliuma sativum	15,53	21,54	18,535	6,841	2,615	14,734	1,3078
ANOVA	Sum of sqrs		df	Mean squere		F	p (≤0,01)
Between groups	16,0806		4	8,04031		1,92	0,020**
Within the group	37,6962		30	4,18847			0,105

Kruskal - Walis test

$H(ch^2)$: 3,092

H_c (tie corrected): 1,179

p (≤0,01): 0,03209

Statisticaly significant diference beetwen samples is present.

Curcumin is the most important fraction of turmeric that is responsible for its biological activity. In the research of [19] the isolation and biological evaluation of turmeric and curcumin in relation to the standard and mycobacterial strains such as: *E. coli, S. aureus, E. feacalis, P. aeuroginosa, M. smegmatis, M. simiae, M. kansasii, M. terrae, M. szulgai* and fungi *Candida albicans*. All isolated turmeric extracts and curcumin showed very weak activity against the studied mycobacteria *M. smegmatis, M. simiae, M.*

kansasii, M. terrae and M. szulgai, and moderate antibacterial activity of the extract and pure curcumin against the gram-negative bacteria *E. coli* and *P. aeuroginosa*. The common onion, *Allium cepa*, in addition to its nutritional effects, has antibacterial and antifungal activity, while compounds obtained from onions have shown anti-inflammatory and antihistaminic effects in vitro and by applying them to animals [20]; [21]. The first studies prove antibacterial activity (including *H. pylori*), that is, antiparasitic and antifungal activity, which is in accordance with numerous studies, such as [23]; [24]; [25]; [26]. Conducted research by a group of Asian scientists on the topic: Antibacterial activity of parsley (*Petroselinum crispum*) and ethanolic extract of *Prunus mahaleb* seeds [27] where the antimicrobial activity against eleven bacteria (*Bacillus anthracis, Bacillus subtilis, Bacillus pumilus, Staphylococcus aurelia, Salmonella enterides, E. typhi, Proteus mirabilis, Bordetella bronchiseptica, Pseudomonas aeruginosa*) proved the antimicrobial activity of the tested plants. Based on the results, they proved that both plants can be considered disinfectants or antiseptics, which confirms their use in folk medicine, because the extracts are in different concentrations (0,1, 0,2, 0,3 and 0,4 g mL-1) showed an inhibitory effect on Gram-positive and Gram-negative bacteria. The ethanol extract of prunus mahaleba showed antibacterial activity *against P. mirabilis, B. anthracis and S. aureus, B. licheniformis* while a satisfactory inhibitory effect was found with parsley extract and action on *Br. melitensis, E. coli, B. licheniformis* and *Salmonella enterides* in low concentrations (0,1 and 0,2 g mL^{-1}), which is in accordance with our research.

A gram-positive, polymorphic, rod-shaped bacterium, *Listeria monocytogenes*, was first described in 1926 by Murray, Webb and Swann at the University of Cambridge. It was identified as the causative agent of the disease in laboratory animals (rabbits and guinea pigs) in 1924. The disease was manifested by a high number of mononuclear leukocytes (monocytosis). Today, listeriosis is a rare but serious disease caused by the bacterium *Listeria monocytogenes*, which can survive and grow on certain high-risk foods. Its characteristic features, such as reproduction within a wide range of pH and temperature, and its strong resistance to high concentrations of sodium chloride allow listeria to survive various processes used in food processing. If we add to this the ability to multiply at storage temperatures (+4°C), it is not surprising that this bacterium represents a significant problem for the food industry, and a potential danger to human health. Our conducted research did not record the influence of the tested extracts on *Listeria monocytogenes*, the established inhibition zones are very low values (3,33 mm).

The research is in line with the research of [28] who examined the antimicrobial activity of other plants: Thymus capitatus (L.) *Origanum elongatum* (Bonet) and *Mentha suaveolens* Ehrh., and also achieved very low zones of inhibition in the value of 3,01 mm, which is in accordance with our results.

Similar research was carried out by [29] in order to evaluate the effectiveness of five plant essential oils: thyme, mint, bay, sage and orange oils as natural food preservatives. The effect of plant essential oils on *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus* and *Candida albicans* in concentrations of 5 to 20 micro L/disc (diameter 6 mm) and 0,5-3% (v/v) was studied in agar diffusion test medium . The essential oils of these extracts showed an extremely antibacterial and bacteriostatic effect, with thyme showing the highest inhibition, and orange the lowest, but still not high enough to characterize the bacteria as sensitive.

CONCLUSION

Significant statistical differences at the level of significance ($p<0.01$) with a medium zone of inhibition of 21,67 mm prove the antimicrobial activity of parsley, and the sensitivity of *Escherichia coli* to the tested plant. Turmeric extract with an average inhibition zone of 22,26 and garlic extract 19,63 mm showed a significant reaction of the examined extracts to *Escherichia coli*, and they were characterized as ***S - sensitive.

With an inhibition zone of 19,052 mm on average, Curcuma longa showed the strongest effect on the tested bacterium *Salmonella enteritidis*, which tells us that it is a very sensitive bacterium to the extract of the mentioned plant. Microorganism resistance is still a challenge for health and food production, and based on the demand for natural products to combat microorganisms, we conclude that the garlic extract has a statistically significant antimicrobial activity on the tested bacteria with an average zone of inhibition of 17,75 mm and parsley extract 16,22 mm. The research determined very low inhibition zones (> 8mm) in all three examined extracts: *Allium sativum* L., *Curcuma longa* L. *Petroselinum crispum* L. on *Listeria monocytogenes*.

The obtained results of the experimental research are the basis for further research on the topic of the influence of solvents on the antimicrobial activity of the extracts of the tested plants, because the presented

results of numerous scientists prove that the daily consumption of the aforementioned herbs improves the organoleptic properties of foods, but also reduces the population of tested pathogens in food, and this also improves people's health.

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INFLUENCE OF PROCESS PARAMETERS ON HAWTHORN (*CRATAEGUS MONOGYNA* JACK.) EXTRACTION

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ABSTRACT:

Given that many synthetic medications can induce a variety of negative reactions in patients, a search for natural substances with minimal side effects in patients has been conducted. Nowadays, researchers are focusing on plant medicines, which have been used to heal illnesses since ancient times. The plant *Crataegus monogyna* Jack. (hawthorn) is the most abuntant plant in the Rosaceae family that is also used in traditional medicine. *C. monogyna*'s pharmaceutical, phytochemical, functional, and therapeutic qualities are based on a wide range of useful secondary metabolites, which include phenolic compound (flavonoids, anthocyanins, tannins), vitamin C and antioxidants. Total (poly)phenols, flavonoids and anthocyanins contents in *C. monogyna* Jacq. extracts were measured using the Folin-Ciocalteu reagent, aluminium chloride and the pH differential methods, respectively. The extraction lasted 15 to 120 min, with a solid-to-solvent ratio of 1:15 w/v and 1:30 w/v and solvents of 30% and 60% ethanol. According to the results, the extraction process has the highest velocity within the first 15 min, when the majority of (poly)phenols and flavonoids are extracted, but it becomes slower as time passes. Higher yields are obtained by utilizing a solid-to-solvent ratio of 1:30 w/v rather than a solid-to-solvent ratio of 1:15 w/v, which indicates that when the amount of drug increases over a certain optimal value, the resistance to mass transfer from a solid material to liquid increases. Finally, the results about the impact of the ethanol content in the solvent demonstrate that a larger ethanol content greatly favors the extraction of flavonoids, but this is not as evident for the extraction of total (poly)phenols and anthocyanins.

KEYWORDS: anthocyanins, extraction, flavonoids, (poly)phenols

INTRODUCTION

It is well known that the usage of medicinal herbs is a significant factor that improves people's overall health, and the study of its positive influence is becoming an increasingly popular topic of contemporary research.

Medicinal plants are rich in (poly)phenolic compounds and as such represent a good antioxidant agent. One of a such medicinal plants is hawthorn. The Hawthorn species belongs to the Rosaceae family and the genus *Crataegus* [1]. The Rosaceae family's most significant genus, *Crataegus L.*, is thought to have between 150 and 1200 species, depending on the species concept used and the insertion of several plausible taxonomic hybrid origins [2]. Even though the taxonomy of the genus is quite complicated and, in the past, the species name *C. oxyacantha L.* (and *C. oxyacantha* Jacq. and other names) was used, the common hawthorn is primarily referred to as *C. monogyna* Jacq [3].

Hawthorn is a semi-evergreen shrub or small tree with thorns that may grow up to 5-15 m [4]. Leaf shape, seed characteristics, seed quantity, and fruit color are all very variable. The bark is smooth grey, with shallow longitudinal cracks and small ridges in the older phenological stage. Each flower has five to twenty-five anthers, five sepals, and five petals. The petals are white and pink, and they are frequently longer than the sepals. The Hawthorn fruit is normally fleshy and can be yellowish, reddish, or blackish-purple. Each fruit contains one to five solid seeds [3], [4].

Herbal drugs are made from the leaves, flowers, and fruits of the hawthorn. Flowers are harvested early in the spring when they are fully developed, leaves in the summer, and fruits in the middle of the fall when they are ready. Complexes of flavonoid heterosides in the amount of 1-2% are found in hawthorn flower and leaf. Fruits include fewer flavonoids (approximately 0.1%) but more sugars, organic acids, carotenoids, and vitamin C [5].

The high content of phenolic compounds such as flavonoids, proanthocyanidins, catechins, phenolic acids, essential oils, and terpenoids explain the use of hawthorn extract as natural therapy for a variety of pharmacological diseases [6]-[9]. Furthermore, some studies claim that carotenoids have an important antioxidant function in plants by deactivating singlet oxygen, which is formed during photosynthesis [10]. Hawthorn also has tonic effects on the heart [8], and multiple studies have shown that it can decrease a variety of cardiovascular risk factors such as hypertension, hypercholesterolaemia, and so on [11], [12].

Various solvents, including methanol, ethanol, acetone, and ethyl acetate, as well as their mixtures, have been employed to extract (poly)phenolic compounds from fresh, frozen, or dried plant material [13]. The type and amount of extracted (poly)phenolic compounds are influenced by the extraction solvent concentration, solid-to-solvent ratio, temperature and duration of extraction process. Methanol and methanol-water mixes are the most often used solvents for phenolics extraction, however ethanol and ethanol-water mixtures also yield good results. Some authors recommend using ethanol as a solvent for phenolic extraction since it is non-toxic and allows them to alter the ethanol to water ratio to modify the polarity of the mixture [14], [15].

MATERIAL AND METHODS

PLANT MATERIALS AND REAGENTS

Fresh hawthorn leaves and flowers, harvested in spring, was used for the extraction. Ethanol was used for sample extraction, while extract characterization was performed using the following reagents: Folin-Ciocalteu reagent (Carlo Erba, Germany), sodium carbonate (Lach:ner, Czech Republic), gallic acid (Sigma Aldrich, USA), aluminum chloride (Lach:ner, Czech Republic), sodium hydroxide (Lach:ner, Czech Republic), sodium nitrite (Zorka Šabac, Serbia), catechin hydrate (Sigma Aldrich, USA), and potassium chloride buffer pH=1.0 (Lach:ner, Czech Republic).

EXTRACTION

The traditional maceration technique was utilized in this study to extract (poly)phenolic compounds from the drug material. The experiment was carried out at room temperature in laboratory beakers containing the plant and the solvent. Occasional mixing ensures that the plant is in contact with the solvent, which promotes extraction.

The maceration procedure was performed under the following process conditions:

- Extraction time [min] – 15, 30, 45, 60 and 120,
- Percentage of ethanol in the extraction solvent [vol. %] – 30 and 60 and
- Solid-to-solvent ratio [w/v] – 1:15 and 1:30.

The influence of previously mentioned process parameters on the yield of total (poly)phenols, flavonoids, and anthocyanins in extracts was examined in this paper.

METHODS

Determination of total (poly)phenols content is based on oxidation-reduction reactions involving hydroxyl groups of phenol and the Folin-Ciocalteu reagent, as well as polymer complex ions of molybdenum and tungsten. The reaction requires a basic environment, which is created by adding sodium carbonate to the reaction mixture. The measurement is spectrophotometric, at wavelength of 765 nm, and gallic acid is utilized as the standard [16]. A Shimadzu 1800 spectrophotometer was utilized for spectrophotometric determination, with the calibration curve ranging from 50 to 500 mg/l of gallic acid. The results are given in milligrams of gallic acid equivalent per gram of plant material (mg GAE/g).

The flavonoids content of the sample is determined using the colorimetric technique with aluminum chloride. In an acidic solution, aluminum chloride forms stable complexes with the C-4 keto group or the C-3 and C-5 hydroxyl groups of the present flavones and flavonols, and unstable complexes with orthodihydroxyl groups in the A or B ring of flavonoids. Measurement is spectrophotometric at wavelenght of 510 nm, with catechin hydrate as the standard [17]. For determination of flavonoids the calibration curve was in range 20 to 200 mg/l of catechin hydrate. The results are given in milligrams of catechin hydrate equivalents per gram of plant material (mg CTH/g).

The quantitative determination of total anthocyanins (non-degraded monomers and products of their degradation) is based on the property of anthocyanins to reversibly change their structure when the pH of the environment changes, which also changes the absorption spectrum. The content of total anthocyanins is determined by the "single" method, described in the paper [18], which is based on measuring the absorbance of the anthocyanin solution at pH=1.

The total anthocyanins concentration in the sample is determined as cyanidin-3-glucoside equivalent (mg Cy3G/g) using the formula:

$$C_{tot} = (A \cdot M \cdot F \cdot 10^3)/(\varepsilon \cdot l \cdot R) \quad (1)$$

where are:
C_{tot} – total anthocyanins content,
$A = (A_{520nm} - A_{700nm})_{pH=1,0}$,
M = molar mass (for Cy3G it is 449,2 g/mol),
F = dilution factor,
10^3 = factor for converting grams to miligrams,
ε = molar absorption extinction coefficient (for Cy3G it is 26900 $Lmol^{-1}$ cm^{-1})
l = cuvette thickness (1 cm) and
R – factor for recalculating the value of anthocyanins per gram of drug.
A Shimadzu 1800 spectrophotometer was used to determine anthocyanins, same as it was for total (poly)phenols and flavonoids.

STATISTICAL ANALYSIS

Statistical analysis was performed using MINITAB 21. Given that a high rate of extraction is anticipated at first, followed by a gradual slowing [19], a concave function was employed to approximate the experimental data. The formula for that function is as follows:

$$Y = a \cdot X/(b + X) \quad (2)$$

where are:
Y – (Poly)phenols (flavonoids or anthocyanins) content [mg/g],
X – Time [min] and
a, b – coefficients.

By choosing a confidence level of 95%, the coefficients a and b in the previous concave function were determined. The algorithm used is Gauss-Newton, the maximum number of iterations is 200, while the tolerance is 0.00001.

RESULTS AND DISCUSSION

Figure 1 depicts the time dependence of extracted (poly)phenols under constant other process parameters (solid-to-solvent ratio and percentage of ethanol in the extraction solvent). Since the parameters solid-to-solvent ratio and percentage of ethanol in the extraction solvent have two levels per experimental design setup, a total of 2^2=4 combinations will be utilized to illustrate the time dependency of total (poly)phenolic yield.

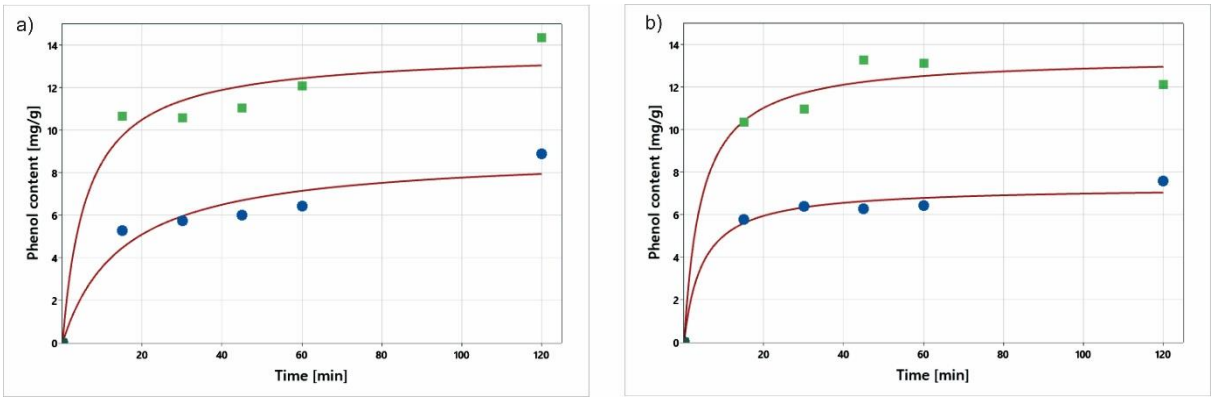

Figure 1. Effect of extraction time on the content of extracted total (poly)phenols with a) 30% ethanol and b) 60% ethanol (• - solid-to-solvent ratio of 1:15 w/v and ▪ - solid-to-solvent ratio of 1:30 w/v)

According to the findings, time has a substantial influence on the extraction of (poly)phenolic compounds from the hawthorn. There are two extraction periods: a fast extraction period (during the first 15 min of extraction) in which (poly)phenolic compounds are extracted intensely from the drug, and a slow extraction period in which the rate of extraction is significantly slower. These findings are consistent with previous research on the extraction of phenols from medicinal plants [20].

However, when the extraction periods are examined independently, it is discovered that parameters solid-to-solvent ratio and percentage of ethanol in the extraction solvent have different effects on the extraction rate. When the solid-to-solvent ratio is 1:15 w/v and 30% ethanol is used (lower extraction conditions), the extraction is slower in the beginning, and after 15 min, only 5.27 mg GAE/g of (poly)phenols are extracted. However, there is a further rise in (poly)phenol in the extract during the second period, with the amount present increasing by 40.7% (to a value of 8.9 mg GAE/g). The application of a solid-to-solvent ratio of 1:30 w/v and 60% ethanol (higher extraction parameters) have the opposite effect. During a fast extraction period, 10.37 mg GAE/g of (poly)phenols was extracted from hawthorn, but with further prolongation of the extraction time, the (poly)phenols content increased to 12.15 mg GAE/g, representing a 14.7% increase. So, when lower extraction parameters are used, the extraction

velocity is low at first but steadily increases during the second extraction period, but when higher extraction parameters are used, the extraction velocity is high at first but relatively unchanged during the second extraction period.

There are a similar increase in the second extraction period with the solid-to-solvent of 1:30 w/v and the use of 30% ethanol and with the solid-to-solvent ratio of 1:15 w/v and the use of 60% ethanol; in the first case, there is an increase from 10.68 mg GAE/g to 14.35 mg GAE/g (which represents a 25.5% increase), while in the second case, there is an increase from 5.79 mg GAE/g to 7.58 mg GAE/g (which represents an increase in the amount of 23.6%).

Observing the parameter percentage of ethanol in the extraction solvent, for example, after 120 min of extraction and with a solid-to-solvent ratio of 1:30 w/v, it is observed that the content of (poly)phenols (12.15 mg GAE/g) with 60% ethanol is slightly lower than the content of (poly)phenols with 30% ethanol (14.35 mg GAE/g). Therefore, increasing the ethanol content in the solvent above 30% reduces the yield of (poly)phenols. Such behavior was noticed by other authors [21].

The solid-to-liquid ratio, on the other hand, has major effects on the yield of (poly)phenols. For example, after 120 min of extraction and 30% alcohol, the (poly)phenols content is 8.9 mg GAE/g with a solid-to-solvent ratio of 1:15 w/v, and 14.35 mg GAE/g with a solid-to-solvent ratio of 1:30 w/v. Similarly, after 120 min of extraction and the use of 60% ethanol, the level of (poly)phenols is 7.58 mg GAE/g with a solid-to-solvent ratio of 1:15, and 12.15 mg GAE/g with a solid-to-solvent ratio of 1:30 w/v. Based on the previous results, better yields of (poly)phenols per unit mass are obtained at larger solid-to-solvent ratios. These results were consistent with mass transfer principles where the driving force for mass transfer is considered to be the concentration gradient between the solid and the solvent [15], [22]. Higher solid-to-solvent ratio increases the concentration gradient, leading to an increased diffusion rate of the compounds from the extracted solid material into the solvent, but also determines the increasing of the necessary period of time to achieve equilibrium. Solid-to-solvent ratio could significantly affect the equilibrium constant and characterize the relationship between yield and solvent use as a steep exponential increase followed by a steady state to give the maximum yield [15], [23].

The statistical program MINITAB 21 was used to determine the regression equation for the dependence of the (poly)phenols yield in the extract on the extraction time. The results of the statistical analysis are shown in Table 1.

Table 1. Determination of coefficients a and b in regression equations of (poly)phenols content dependency on time

Constant values	Summary		Parameter	Estimate	SE[a] Estimate	95% Cl[b]
Solid-to-solvent ratio of 1:15 and 30% ethanol	Iterations	14	a	8.9463	1.11727	(6.29654, 14.3942)
	Final SSE[c]	2.57136	b	14.9691	7.18346	(0.43926, 58.4699)
	DFE[d]	4				
	MSE[e]	0.642840				
	S[f]	0.801773				
Solid-to-solvent ratio of 1:30 and 30% ethanol	Iterations	10	a	13.7233	1.06688	(10.9998, 17.6454)
	Final SSE	4.33319	b	6.1009	3.18328	(-1.0474, 20.1872)
	DFE	4				
	MSE	1.08330				
	S	1.04082				
Solid-to-solvent ratio of 1:15 and 60% ethanol	Iterations	9	a	7.31804	0.37160	(6.34958, 8.5092)
	Final SSE	0.594733	b	4.56873	1.92009	(0.00023, 11.5076)
	DFE	4				
	MSE	0.148683				
	S	0.385595				
Solid-to-solvent ratio of 1:30 and 60% ethanol	Iterations	8	a	13.4421	0.78474	(11.4795, 15.8545)
	Final SSE	2.70162	b	4.3462	2.18051	(-0.5118, 11.8669)
	DFE	4				
	MSE	0.675406				
	S	0.821831				

where are:
[a]SE – standard error
[b]95% Cl - The 95% Confidence Interval
[c]SSE – Sum of Squares for Error
[d]DFE - Degrees of Freedom for Error
[e]MSE – Mean Squared Error
[f]S – Standard Deviation

The coefficients of the regression equation and the divergence of the actual from the theoretical values were estimated based on data processing in MINITAB 21.

The regression equations of the dependence of the total (poly)phenols content in the extract on the extraction time (0-120 min) have the following form:

1. Solid-to-solvent ratio of 1:15 w/v and 30% ethanol
$Y = 8.94628 \cdot X / (14.9691 + X)$
2. Solid-to-solvent ratio of 1:30 w/v and 30% ethanol
$Y = 13.7233 \cdot X / (6.10089 + X)$
3. Solid-to-solvent ratio of 1:15 w/v and 60% ethanol
$Y = 7.31804 \cdot X / (4.56873 + X)$
4. Solid-to-solvent ratio of 1:30 w/v and 60% ethanol
$Y = 13.4421 \cdot X / (4.34617 + X)$

where are:
Y – (Poly)phenols content [mgGAE/g] and
X – Time [min].

Figure 2 depicts the time dependence of extracted flavonoids under constant other process parameters (solid-to-solvent ratio and percentage of ethanol in the extraction solvent). As with content of (poly)phenols, a total of 2^2=4 combinations will be utilized to illustrate the time dependency of flavonoids yield.

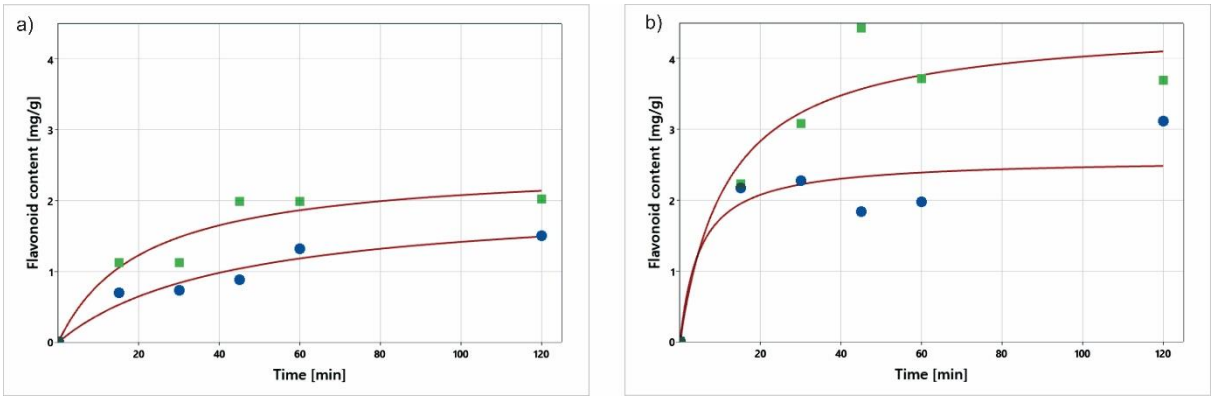

Figure 2. Effect of extraction time on the content of extracted flavonoids with a) 30% ethanol and b) 60% ethanol (• - solid-to-solvent ratio of 1:15 w/v and ▪ - solid-to-solvent ratio of 1:30 w/v)

Based on Figure 2, it can be concluded that the content of flavonoids is aproximetely 3-6 times smaller then the content of (poly)phenols, depends on other process condition.

As with extraction of total (poly)phenols, we also here differentiate two extraction periods: initial (fast extraction period) extraction and steady (slow extraction period).

The increase in the yield of flavonoids in the extract during the period of slow extraction is similar for all four cases: with a solid-to-solvent ratio of 1:15 w/v and 30% ethanol there is an increase in the content of flavonoids from 0.7 mg CTH/g to 1.5 mg CTH/g, which represents an increase of 53.3%, with a solid-to-solvent ratio of 1:30 w/v and 30% ethanol there is an increase of 44.6% (from 1.12 mg CTH/g to 2.02 mg CTH/g), with a solid-to-solvent ratio of 1:15 w/v and 60% ethanol there is an increase in the amount of 30.1% (from 2.18 mg CTH/g to 3.12 mg CTH/g), while with a solid-to-solvent ratio of 1:30 w/v and 60% ethanol there is an increase of 39.5% (from 2.24 mg CTH/g to 3.7 mg CTH/g). Therefore, the extraction of flavonoids is somewhat slower in the initial extraction period, but the extraction velocity does not decrease drastically in the second period.

When the solid-to-solvent ratio is examined in relation to flavonoids yield, it is discovered that as this ratio grows, so does the yield. As an example, after 120 min of extraction with 30% ethanol, the extracted flavonoids content is 1.5 mg CTH/g at a solid:liquid ratio of 1:15 w/v, and 2.02 mg CTH/g at a solid-to-solvent ratio of 1:30 w/v. When 60% ethanol is used, the same response is observed. As a result, as with (poly)phenols extraction, using a smaller amounts of the drug results in a better yield per unit mass of the drug.

On the other hand, observing the influence of the ethanol content in the solvent on the yield of flavonoids, an interesting effect is observed. For example, after 120 min of extraction and a solid-to-solvent ratio of 1:15 w/v, using 30% ethanol the yield of flavonoids is 1.5 mg CTH/g, while using 60% ethanol the yield is 3.12 mg CTH/g. Likewise, at the same time of extraction but with a solid-to-solvent ration of 1:30 w/v, using 30% ethanol, the flavonoids content is 2.02 mg CTH/g, and using 60% ethanol, this content is 3.7 mg CTH/g. Thus, the yield of flavonoids increases twice with the use of a solvent containing 60% ethanol compared to a solvent containing 30% ethanol.

The regression equation for the dependency of the yield of flavonoids in the extract on the extraction time was determined using the statistical application MINITAB 21. Table 2 displays the statistical analysis findings.

Table 2: Determination of coefficients a and b in regression equations of flavonoid content depending on time

Constant values	Summary		Parameter	Estimate	SE Estimate	95% CI
Solid-to-solvent ratio of 1:15 and 30% ethanol	Iterations	8	a	2.0195	0.3853	(1.24213, 4.355)
	Final SSE	0.0853752	b	42.3903	18.9276	(8.89972, 175.697)
	DFE	4				
	MSE	0.0213438				
	S	0.146095				
Solid-to-solvent ratio of 1:30 and 30% ethanol	Iterations	7	a	2.5127	0.4036	(1.67868, 4.2524)
	Final SSE	0.240507	b	20.8372	10.8189	(2.21770, 77.0574)
	DFE	4				
	MSE	0.0601268				
	S	0.245208				
Solid-to-solvent ratio of 1:15 and 60% ethanol	Iterations	10	a	2.58846	0.45598	(1.54698, 5.8812)
	Final SSE	0.871403	b	4.90151	6.78238	(-6.65384, 83.2469)
	DFE	4				
	MSE	0.217851				
	S	0.466745				
Solid-to-solvent ratio of 1:30 and 60% ethanol	Iterations	11	a	4.5078	0.63293	(3.17555, 6.7978)
	Final SSE	1.01243	b	11.7599	7.27309	(-0.57881, 43.8438)
	DFE	4				
	MSE	0.253108				
	S	0.503098				

The coefficients of the regression equation and the divergence of the actual from the theoretical values were estimated based on data processing in MINITAB 21.

The regression equations of the dependence of the flavonoids content in the extract on the extraction time (0-120 min) have the following form:

1. Solid-to-solvent ratio of 1:15 w/v and 30% ethanol
$Y = 2.0195 \cdot X / (42.3903 + X)$
2. Solid-to-solvent ratio of 1:30 w/v and 30% ethanol
$Y = 2.5127 \cdot X / (20.8372 + X)$
3. Solid-to-solvent ratio of 1:15 w/v and 60% ethanol
$Y = 2.58846 \cdot X / (4.90151 + X)$
4. Solid-to-solvent ratio of 1:30 w/v and 60% ethanol
$Y = 4.5078 \cdot X / (11.7599 + X)$

where are:
Y - Flavonoids content [mg CTH/g] and
X – Time [min].

The dependence of anthocyanins content in the extract on time under constant other process parameters (solid-to-solvent ratio and percentage of ethanol in the extraction solvent) is shown in Figure 3. As in previous cases, a total of 2^2=4 combinations will be utilized to illustrate the time dependency of anthocyanins yield.

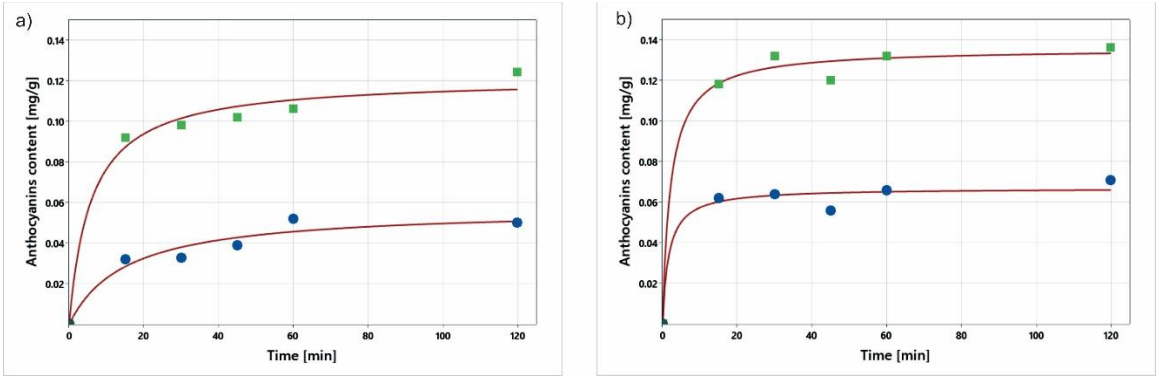

Figure 3. Effect of extraction time on the content of extracted anthocyanins with a) 30% ethanol and b) 60% ethanol (• - solid-to-solvent ratio of 1:15 w/v and ▪ - solid-to-solvent ratio of 1:30 w/v)

The findings and conclusions are similar to those for (poly)phenols and flavonoids: there is a period of fast extraction (the first 15 min) followed by a period of slow extraction. Examining through each of the diagrams individually, it can be concluded that the other process parameters have different effects on these extraction periods; in the period of slow extraction with lower extraction parameters (solid-to-solvent ratio of 1:15 w/v and usage of 30% ethanol), there are a 36% increase in anthocyanins content (from 0.032 mg Cy3G/g to 0.050 mg Cy3G/g), while at higher extraction parameters (solid-to-solvent ratio of 1:30 w/v and usage of 60% ethanol), there are a 13.2% increase from 0.118 mg Cy3G/g to 0.136 mg Cy3G/g).

Observing the effect of the solid-to-solvent ratio on anthocyanins content in the extract, it is discovered that increasing the ratio from 1:15 w/v to 1:30 w/v improves the anthocyanins content by 2-3 times, depending on other process parameters. For example, using 30% ethanol and after 15 min of extraction, at a solid-to-solvent ratio of 1:15 w/v the anthocyanins content is only 0.032 mg Cy3G/g, while at a solid-to-solvent ratio of 1:30 w/v the anthocyanins content is even three times higher and amounts to 0.092 mg Cy3G/g). If the amount of drug is more than optimal, it can be concluded that resistance to mass transfer exists in this situation as well.

Concerning the influence of percentage of ethanol in the extraction solvent, an increase in its content results in a rise in yield of anthocyanins, but it doesn't have as much impact as a solid-to-solvent ratio. For example, after 120 min of extraction and at a solid-to-solvent ratio of 1:30 w/v, using the 30% ethanol the anthocyanins content is 0.124 mg Cy3G/g, while using the 60% ethanol the anthocyanins content is slightly larger and amounts 0.136 mg Cy3G/g)

Observing some absolute values of anthocyanins content in the extract, e.g. after 120 min, it is observed that they range from 0.05 to 0.136 mg Cy3G/g; therefore, the content of anthocyanins in ethanole extracts of hawthorn is much smaller in comparison to the overall quantity of (poly)phenols in the ethanol extracts.

The results of the statistical analysis determined in the statistical program MINITAB 21 are shown in Table 3.

Table 3. Determination of coefficients a and b in the regression equations of the dependence of anthocyanins content in the extract on time

Constant values	Summary		Parameter	Estimate	SE[a] Estimate	95% CI[b]
Solid-to-solvent ratio of 1:15 and 30% ethanol	Iterations	6	a	0.0573	0.00675	(0.04177, 0.0844)
	Final SSE[c]	0.0000926	b	15.1665	6.81291	(1.71307, 47.8126)
	DFE[d]	4				
	MSE[e]	0.0000232				
	S[f]	0.0048122				
Solid-to-solvent ratio of 1:30 and 30% ethanol	Iterations	9	a	0.12149	0.00630	(0.105001, 0.1421)
	Final SSE	0.0001538	b	5.87788	2.10012	(0.838620, 13.6339)
	DFE	4				
	MSE	0.0000385				
	S	0.0062010				
Solid-to-solvent ratio of 1:15 and 60% ethanol	Iterations	8	a	0.06701	0.00431	(0.05591, 0.08110)
	Final SSE	0.0001024	b	1.67812	2.03425	(-2.95444, 9.54932)
	DFE	4				
	MSE	0.0000256				
	S	0.0050596				
Solid-to-solvent ratio of 1:30 and 60% ethanol	Iterations	7	a	0.13588	0.00493	(0.122916, 0.15056)
	Final SSE	0.0001283	b	2.19390	1.18955	(-0.706813, 6.04411)
	DFE	4				
	MSE	0.0000321				
	S	0.0056625				

Based on the data processing in MINITAB 21, the coefficients of the regression equation were obtained and the deviation of the actual from the theoretical values was also calculated.

The regression equations of the dependence of the anthocyanins content in the extract on the extraction time (0-120 min) have the following form:

1. Solid-to-solvent ratio of 1:15 w/v and 30% ethanol

$Y = 0.0573 \cdot X / (15.1665 + X)$

2. Solid-to-solvent ratio of 1:30 w/v and 30% ethanol

$Y = 0.12149 \cdot X / (5.87788 + X)$

3. Solid-to-solvent ratio of 1:15 w/v and 60% ethanol

$Y = 0.06701 \cdot X / (1.67812 + X)$

4. Solid-to-solvent ratio of 1:30 w/v and 60% ethanol

$Y = 0.13588 \cdot X / (2.19390 + X)$

where are:

Y - Anthocyanins content [mg Cy3G/g] and

X – Time [min].

CONCLUSION

The effect of process parameters (time, solid-to-solvent ratio, and percentage of ethanol in the extraction solvent) on the yield of (poly)phenolic chemicals in howthorn (*Crataegus Monogyna* Jack.) extract was investigated in this paper. In terms of time as a process parameter, it was established that there are two separate extraction periods: a fast extraction period, which occurs during the first 15 min, and a slow extraction period (after 15 min). By comparing the effect of different ethanol content in the solvent on the yield, it was determined that a higher ethanol content in the solvent enables better extraction only for flavonoids and anthocyanins, but not for total (poly)phenols. Higher solid-to-solvent ratios contribute to more effective extraction of all (poly)phenolic components due to easier diffusion into the liquid in the presence of a smaller amount of drug relative to the liquid.

When the content of anthocyanins in the extract is compared to the overall polyphenol content, it may be determined that just a small amount of the polyphenols is anthocyanins. By comparing extraction with different percentage of ethanol in the extraction solvent and the solid-to-solvent ratio, it was discovered that a higher percentage of ethanol in the solvent and a higher solid-to-solvent ratio promote anthocyanins extraction.

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