UNIVERZA V LJUBLJANI

FAKULTETA ZA FARMACIJO

NASTJA LUNAR

DIPLOMSKA NALOGA

UNIVERZITETNI ŠTUDIJSKI PROGRAM KOZMETOLOGIJA

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PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF MONOFLORAL HONEYS –EVALUATION OF SLOVENIAN ACACIA AND PORTUGUESE HEATHER SAMPLES

VREDNOTENJE FIZIKALNO-KEMIJSKIH IN BIOLOŠKIH LASTNOSTI GOZDNIH MEDOV –SLOVENSKEGA AKACIJEVEGA IN PORTUGALSKEGA JESENOVEGA MEDU

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Diplomsko nalogo in njen praktični del sem opravljala na Fakulteti za farmacijo v Lizboni pod somentorstvom asist. prof. Lídie Pinheiro, teoretični del pa na Fakulteti za farmacijo v Ljubljani, pod mentorstvom prof. dr. Mirjane Gašperlin.

Zahvala

Zahvaljujem se mentorici prof. dr. Mirjani Gašperlin za prijaznost, nasvete, usmeritve, spodbudo in strokovno pomoč pri izdelavi diplomskega dela. Iskrena hvala gre tudi somentorici asist. prof. Lídii Pinheiro in asist. prof. Célii Faustino za prijaznost in pomoč pri delu v laboratoriju v času študija v Lizboni.

Navsezadnje pa se zahvaljujem tudi svojim staršem, ki so me moralno in finančno podpirali tekom študija in izdelave diplomske naloge v tujini, me vzpodbujali in verjeli vame.

Izjavljam, da sem diplomsko delo izdelala samostojno pod vodstvom mentorice prof. dr. Mirjane Gašperlin in somentorice asist. prof. Lídie Pinheiro.

Nastja Lunar

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ABSTRACT

Honey is a natural product, consisting of many different compounds, which are the reason for its antiseptic, antibacterial and antioxidant properties. Among the most important compounds are phenolic compounds, especially flavonoids. In this thesis we focused on evaluation of physicochemical and biological properties of two different honey samples, Slovenian acacia honey and Portuguese heather honey. Further, we tested their inhibition of acetylcholinesterase.

We prepared honey sample solutions and performed different physicochemical and biological assays. We determined colour of honeys and its intensity, electrical conductivity, moisture, pH and free acidity, protein content, apparent reducing sugar content, proline content and hydroxymethylfurfural content. From obtained results of physicochemical assays we concluded that heather honey has higher values in almost all assays, except in measurements of water content. Further, biological properties were evaluated where we determined antioxidant, antibacterial and antifungal activity of honey samples. With performed antioxidant assays we determined total phenolic content, total flavonoid content, antioxidant power, free radicals scavenging capacity and oxygen radical absorbance capacity. Results from antioxidant assays showed that heather honey has better antioxidant properties than acacia honey. We continued evaluation with determining antibacterial and antifungal activities. We evaluated antibacterial and antifungal activities against four bacterial strains and two yeasts. Pure honeys did not show any activity, only extracts. We continued with performing assays, which tested anti-acetylcholinesterase activity. With the obtained results we concluded that our honey samples have no anti-acetylcholinesterase activity.

We concluded that both, heather and acacia honey, have good antibacterial and antifungal properties, but heather honey turned out to have better antioxidant properties. Further, we concluded that neither heather nor acacia honey inhibit acetylcholinesterase to prevent acetylcholine from degradation.

Keywords: honey, phenolic compounds, physicochemical properties, antioxidant activity, antibacterial activity

POVZETEK

Med je naravni produkt, sestavljen iz različnih komponent, ki so odgovorne za njegovo antiseptično, protibakterijsko in antioksidativno delovanje. Med najpomembnejšimi komponentami so fenolne spojine, še posebej flavonoidi. V diplomski nalogi smo se osredotočili na vrednotenje fizikalno-kemijskih in bioloških lastnosti dveh različnih vzorcev medu, slovenskega akacijevega in portugalskega jesenovega ter preverili njuno anti-acetilholinesterazno delovanje.

Pripravili smo raztopine vzorcev medu in izvedli različne fizikalno-kemijske in biološke teste. V obeh vzorcih smo določili barvo medu in njeno intenziteto, električno prevodnost, vsebnost vode, pH in proste kisline, vsebnost proteinov, reducirajočih sladkorjev, prolina in hidroksimetilfurfurala (HMF). Na osnovi dobljenih rezultatov fizikalno-kemijskih testov smo zaključili, da ima jesenov med višje vrednosti pri skoraj vseh testih, edino vsebnost vode je bila nižja. V nadaljevanju smo vrednotili biološke lastnosti, kjer smo določili antioksidativno, protibakterijsko in protiglivično delovanje obeh vzorcev medu. S testi antioksidativnega delovanja smo določili vsebnost fenolnih spojin in flavonoidov, antioksidativno moč, antioksidativni potencial in učinkovitost lovljenja kisikovih radikalov. Rezultati antioksidativnih testov so pokazali, da ima jesenov med v primerjavi z akacijevim boljše antioksidativne lastnosti. Protibakterijsko in protiglivično delovanje smo vrednotili proti štirim bakterijskim sevom in dvema kvasovkama. Čisti med ni pokazal nobenega učinka proti bakterijam in kvasovkam, medtem ko so ekstrakti pokazali protibakterijsko in protiglivično delovanje. S preverjanjem anti-acetilholinesteraznega delovanja smo ugotovili, da testirani vzorci medu niso zavirali acetilholinesteraze.

Zaključili smo, da imata oba, jesenov in akacijev med, dobre protibakterijske in protiglivične lastnosti, vendar pa ima jesenov med boljše antioksidatvne lastnosti. Noben od obeh vzorcev medu pa ne zavira acetilholinesteraze, s čimer bi preprečili razgradnjo acetilholina.

Ključne beside: med, fenolne spojine, fizikalno-kemijske lastnosti, antioksidativno delovanje, antibakterijsko delovanje

LIST OF ABBREVIATIONS

AAPH	2,2’-azobis(2-methylpropionamidine)dihydrochloride
ACh	acetylcholine
AChE	acetylcholinesterase
AUC	area under the curve
BuChE	butyrylcholinesterase
BSA	bovine serum albumin
BSAE	bovine serum albumin equivalents
DMSO	dimethylsulfoxide
DNS	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5’-dithiobis(2-nitrobenzoic acid)
FA	free acidity
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalents
GE	glucose equivalents
HMF	hydroxymethylfurfural
HPLC	high-performance liquid chromatography
HPLC-MS	high-performance liquid chromatography-mass spectrometry
LPE	L-protein equivalents
MBC	minimum bactericidal concentration
MFC	minimum fungicidal concentration
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
NOR	Norfloxacine
ORAC	oxygen radical absorbance capacity
Ph	potential of hydrogen
SAB	Sabouraud
TE	Trolox equivalents
TFC	total flavonoid content
TPC	total phenolic content
TPTZ	2,4,6-tripyridyl-*s*-triazine

TSS	total soluble solids
UV	ultraviolet
QE	quercetin equivalents

1 INTRODUCTION

1.1 The formation of honey

Honey is a brown-gold viscid substance that is made from flower nectar with the help of bees (1). Bees have their body shaped in a way, which enables them to extract the nectar from blossoms. They keep the nectar inside of their stomach, where the enzymes change the pH and chemical composition, so that they can store it for a longer time. (2).

Honey is made, when nectar is converted by bees, which regurgitate it and then evaporate the water out (1). Inside the hive bees are passing the nectar to each other's mouth. When nectar is almost digested they drop it off into a honeycomb, where nectar is still in a liquid form (2).

Constant fanning by the bee's wings causes evaporation, where all the extra water gets out and honey creates (3). When there is almost no water left in the honeycomb, bees excrete a liquid from its abdomen and with time the liquid turns into beeswax (2). This whole process is shown in figure 1. Honey is stored in wax honeycombs, protected in a beehive, where bees use it as source of food. People then harvest honey from beehives for human consumptions. The colour and flavour of honey varies, depending on the type of flower nectar (1).

Honey is harvested by beekeepers, who collect the honeycomb frames and scrap off the wax to get honey out of the comb. Then they remove the frames and place them in an extractor. Inside of the extractor the frames are spun and honey is forced to come out. Spinning pushes honey to the sides of an extractor and because of the gravity it is pulled down to the bottom, where it can be collected. (3).

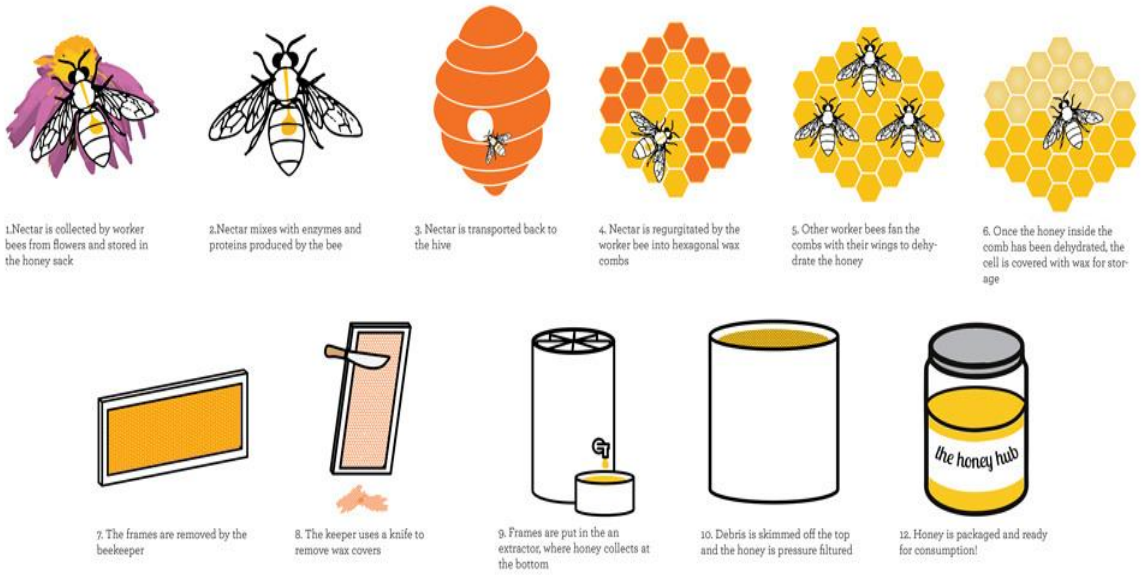

Figure 1: Honey production (4)

1.2 The composition of honey

Honey is naturally made substance, which consists mostly from sugars, but also contains water and other compounds, such as proteins, flavonoids, minerals, vitamins, enzymes, organic acids, phenolic acids and volatile compounds (figure 2). It depends on the floral and geographical origin, what is the amount of these compounds in honey and they determine the differences between various types of honey (colour, aroma, taste). Processing, handling and storage time has also impact on the structure of honey. Flavonoids, phenolic acids, ascorbic acid, catalase, peroxidase, carotenoids and products of Maillard reactions are components in honey that are known to be the main reason for its antioxidant activity (5).

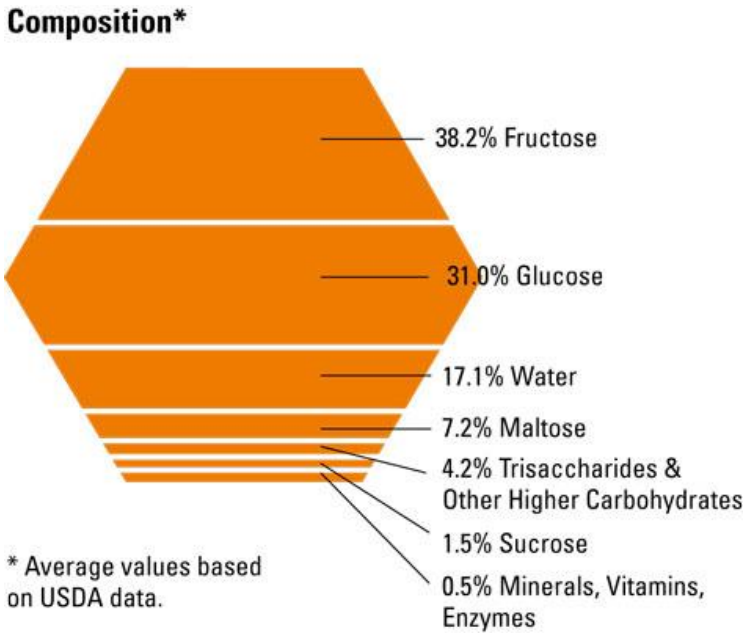

Figure 2: The composition of honey (6)

1.2.1 Sugars

There are three kinds of sugar in honey: fruit sugar (fructose), grape sugar (glucose) and ordinary sugar (sucrose) (7). Sugars have impact on physical properties of honey – high viscosity and density, "stickness", tendency of creating granules, tendency to absorb moisture from the air and they protect honey from spoilage. The ratio of sugars depends on the flower source and enzyme invertase, which is the reason for breaking down regular sugar in grape and fruit sugar (8). This enzyme can be found in a flower, but is also present in the bee's body (7).

1.2.2 Minerals

Mineral content are small remains of ash, which are obtained after drying and burning honey (8). There is not a huge amount of minerals in the honey, which makes honeys that are richer in minerals more valued in human consumption. Minerals that are most commonly found in the honey are: potassium, chloride, sulfur, calcium, sodium, phosphorus, magnesium, silicon, iron, manganese and copper. The content of minerals is usually higher in darker kinds of honeys (7).

1.2.3 Amino acids and proteins

Proteins come in honey from nectar and pollen. They can be present as complex structures or as simple structures like amino acids (7). Proteins cause honey to have a lower surface tension, which results in foaming and encouraging the formation of air bubbles. Chemical or digestive processes cause the decomposition of proteins, where amino acids are obtained. There is small or no nutritional significance to free amino acids present in honey. Previous research showed that 11 to 21 different free amino acids can be present in honey. Amino acids are reacting slowly with sugars, but in the presence of heat they react rapidly and yellow or brown materials are produced. Due to this, honey gets darker with age or heating (8).

1.2.4 Vitamins

Vitamin C is one of the most common vitamins, along with the B complex vitamins, such as riboflavin, pantothenic acid, pyridoxine, biotin, nicotinic acid (7). Vitamin C improves immune system and protects the body from infections. It has good impact on skin as face cleanser and it has the ability to ease symptoms that can appear on skin (burns, rashes, dry skin), because it can be topically absorbed (9).

1.2.5 Other components

Carboxylicic acids (acetic, butanoic, formic, citric etc.) are also present in honey, but they represent less than 0.5 percent of solid compounds. They contribute to the flavour and are the reason for honey's stability against microorganisms (10, 8). When glucose is oxidized by glucose oxidase, gluconic acid is obtained, which is the major organic acid present in honey (10).

1.3 The therapeutic uses of honey

Honey is often used as a natural antiseptic. It has antibacterial effects and a role in removing the damaged tissue for its better and faster healing (debridement). It stimulates wound tissue, which results in faster healing and it removes the unpleasant smell in malodorous wounds. It helps in the healing process of dormant wounds and it maintains moisture in the wound, which speeds up the healing process. In honey we can also find nutraceuticals, which play

an important role in removing free radicals from the body. This helps in improving immunity of our body against many conditions, even cancer or heart disease. Honey is also very good for skin care; often it is served with milk, because together these ingredients can create smooth and nice skin (11).

1.4 Main types of honey

It is known that there are two different kinds of honey: floral honey and honeydew. It depends on the source from which it originates. The source of floral honey are mainly flowers from different kinds of plants, while honeydew is obtained from sweet liquid, which is excreted from some insects after they eat the juice of the plants (12).

1.4.1 Floral honey

There are many different kinds of floral honey: acacia, linden, chestnut, sage, etc. It is important from which plant the nectar is collected from, because this is the main factor, which determines the type of honey. Each of the species has different colour and flavour. (12).

1.4.2 Honeydew honey

Honeydew honey comes from the beech forests of New Zealand (13). It is an excretion from insects, which is known for its sweetness and "stickiness". Insects eat the juice from the plant, which goes into their food canal and forces the previously absorbed juice to go out on the other end (14). They secrete this sugar-like substance on the trees, where bees feed on it and develop organic end product (13). Honeydew is highly valued in human consumption, because it contains high amount of minerals (12).

1.5 Phenolic compounds

Phenolic compounds are present in plants, where they represent one of the most important components. They are known to be secondary metabolites and have a role to protect against stress and oxidative damage. Previous research showed that phenolic compounds can reduce inflammation and formation of blood clots, they have anticarcinogenic effects, they improve the immune system and they can reduce the pain (15, 16). It is reported that some

degenerative diseases (cardiovascular diseases, diabetes, obesity and cancer) can be prevented or the risk of getting them can be reduced by longer intake of phenolic compounds (17). Phenolic compounds are transferred via the nectar to the honey. They are divided into two groups: phenolic acids with their derivatives and flavonoids (18). Analysis of phenolic compounds is useful for studying floral and geographical origins of honey (15).

1.5.1 Phenolic acids

Phenolic acids are known to be secondary metabolites in plants and are placed amongst phenolic compounds (19). Most common phenolic acids are gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids. Lignins and hydrolysable tannins usually contain phenolic acids, which are normally in the bound form (17). In the structure of phenolic acids there are two carbon frameworks: the hydroxycinnamic (Xa) and hydroxybenzoic (Xb) structures. Often there can be aldehyde analogues (Xc) in the structure too (figure 3). The elementary skeleton remains the same, only numbers and positions of the hydroxyl groups on the aromatic ring change. Phenolic acids are vital for the synthesis of proteins (they can inhibit transcription of proteins that are responsible for control of inflammation, cell proliferation and differentiation), important for nutrient uptake, activity of enzymes, photosynthesis, structural components and allelopathy. They also have impact on food, regarding colour, sensory qualities, nutritional and antioxidant properties. We ingest phenolic acids through fruits and vegetables and they protect us against oxidative damage disease, such as coronary heart disease, stroke and different types of cancers (20). They can be found in berries, pomegranate, cereals and herbs (17).

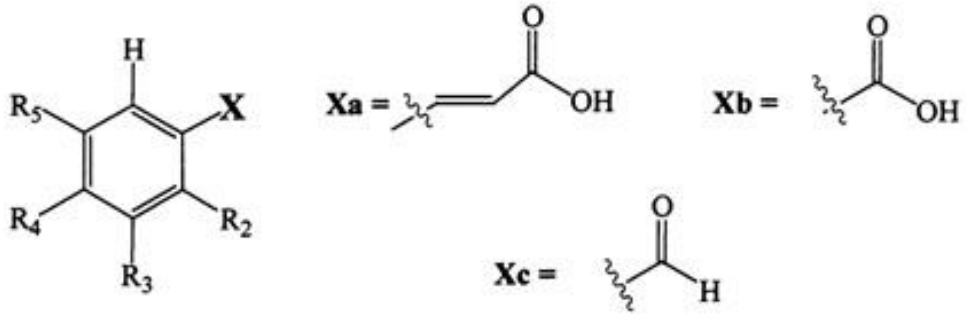

Figure 3: Structures that can be present in phenolic acids (21)

1.5.2 Flavonoids

Flavonoids are considered to be plant metabolites called C6-C3-C6 phenolics. They have antioxidant properties and play an important role in cell signalling (22, 23). Many fruits and vegetables consist of flavonoids and they are also present in honey. Flavonoids are molecules that are placed amongst polyphenols. Their chemical structure contains 15 carbon atoms and their basic structure is called flavon or 2-fenil-1,4-benzopyrone (22). According to the nature of the C3 fragment flavonoids can be classified into 6 structural groups: flavonols, flavones, flavan-3-ols, flavanones, isoflavones and anthocyanidins (17) (figure 4). They are soluble in water, because of a sugar moiety, which is usually present on C3, but sugar can be also attached on C5 or C7 (22). Usually the UV spectroscopy is used to characterize the flavonoids, because they absorb the UV radiation (23). They perform several functions: essential pigments to attract insects, chemical messengers, nitrogen fixation, UV filtration, cell cycle inhibition. Most important function of flavonoids in honey is antioxidant activity, but they also have other valuable effects; they reduce the inflammation, treat viral infections, prevent the development of cancer and they treat or prevent allergic reactions. (22).

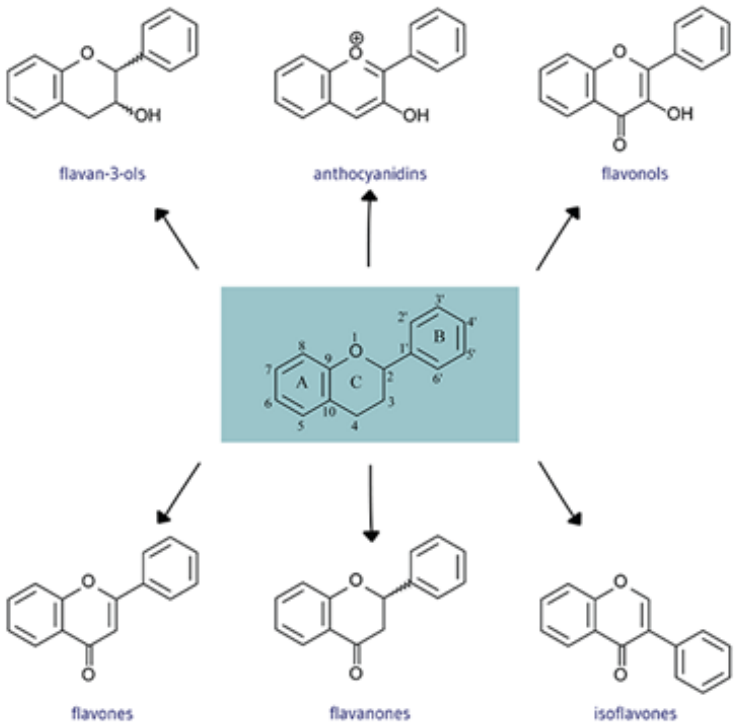

Figure 4: Basic structures of flavonoid subclasses (24)

Flavonols

Most common flavonoids that occur in the plant food are flavonols. Their colour varies from white to yellow and their structure is very similar to flavones. They are mostly represented by quercetin, kaempferol, myricetin and also quite common is isorhamnetin. Most of the fruits and vegetables contain quercetin in high concentrations (17).

Flavones

The only structural difference between flavonols and flavones is the absence of the hydroxyl group at the 3-position on the C-ring. Apigenin and luteolin are most commonly known flavones and can be found in significant concentrations only in celery, parsley and artichoke, so their dietary intake is very low. Citrus species contain nobiletin, scutellarein, sinensetin and tangeretin, which are placed amongst polymethoxylated flavones. Observations revealed that methoxylated flavones increase membrane transport in the intestine/liver and metabolic stability and also the oral bioavailability can be improved (17).

Flavan-3-ols

Different beverages, food and herbals contain flavan-3-ols, where they represent functional ingredients. Regarding food they have impact on quality parameters such as astringency, bitterness, sourness, sweetness, aroma and colour. Structurally, they are the most complex subclass of flavonoids, because they can exist as simple monomers (catechin) and its isomer (epicatechin) and more complex structures such as oligomeric and polymeric proanthocyanidins. Esterification of flavan-3-ols with gallic acid can cause a formation of catechin gallates and hydroxylation can cause a formation of gallocatechins. Some fruits (apricots, sour cherries, grapes and blackberries) can contain flavan-3-ols and they can also be found in nuts, mint, basil, rosemary, sage, dill, red wine, beer and green tea (17).

Flavanones

As regards the flavanones structure a double bond is absent and a chiral centre is present at the carbon-2. Flavanones have very reactive structure, which is the reason for hydroxylation, glycosylation and O-methylation reactions. Most common flavanones are naringenin,

hesperetin and eriodictyol, but sakuranetin and isosakuranetin also occur. Citrus fruits contain flavanones in a form of glycosides. They are also present in tomatoes, kiwi and bananas (3, 2, 17).

Anthocyanidins

Some fruits and flowers contain anthocyanidins, which are water-soluble plant pigments that cause different shades of red, blue and purple colour. They are present as glycosides and their sugar parts are attached at the 3-position on the C-ring or 5-position on the A-ring. They protect plants from excessive light and attract pollinating insects. Unlike other subgroups of flavonoids anthocyanidins's charge is positive at acidic pH. There are around 17 anthocyanidins present in the nature, but most commonly present are cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin. Anthocyanidins can be found in grapes, berries, cherries, plums, peaches, red onions and pomegranate (17).

Isoflavones

As regards the structure isoflavones have the B-ring attached at C3 rather than the C2 position. They are not widely distributed among the plants and can be only found in leguminous species. Isoflavones reduce blood cholesterol, blood pressure, dimensions of arteries and oxidative stress, which results in reducing the risk of coronary heart disease. Isoflavones have estrogenic activity, because they are capable of binding to estrogen receptor, which is the reason for higher attention in preventing breast cancer and osteoporosis. One of the rarest sources of isoflavones is soybeans. Genistein, daidzein and glycitein can also be found in black beans and green peas and they represent most frequent isoflavones. Isoflavones can exist as simple or more complex isoflavonoids, which is obtained with methylation, hydroxylation or polymerization (17).

1.6 Antibacterial activity of honey

Honey is known for its antibacterial activity and antiseptic properties for many years (8). Antibacterial properties are due to its high osmolarity, acidic properties and presence of hydrogen peroxide and non-peroxide antibacterial components (25). So it is not appropriate medium for survival of bacteria, because it is acidic (its pH is between 3.2 and 4.5, which is

low enough to inhibit the growth of several bacteria) and sugar content is too high for their growth (8, 25). Osmotic effect is when bacteria are killed by sugar, which causes the drought of bacteria (8). In many honeys antibacterial activity is due to hydrogen peroxide, which occurs in enzymatic production and it is produced by inhibine. Enzyme glucose oxidase is responsible for the oxidation of glucose to gluconic acid and hydrogen peroxide (figure 5). In honey peroxide has the ability to prevent the growth of certain bacteria. For the synthesis of glucose oxidase are responsible bees and enzyme catalase, which originates from flower pollen. The amount of glucose oxidase determines, how much of hydrogen peroxide will be obtained. Heating and catalase can block the activity of hydrogen peroxide. Floral type, age and heating are responsible for the quantity of inhibine, which has an impact on peroxide accumulation (25, 8). The other type of honey is labelled as non-peroxide honey. In this honey the effect of hydrogen peroxide is prevented. The antibacterial activity is due to presence of non-peroxide components such as methylglyoxal and methyl syringate (25).

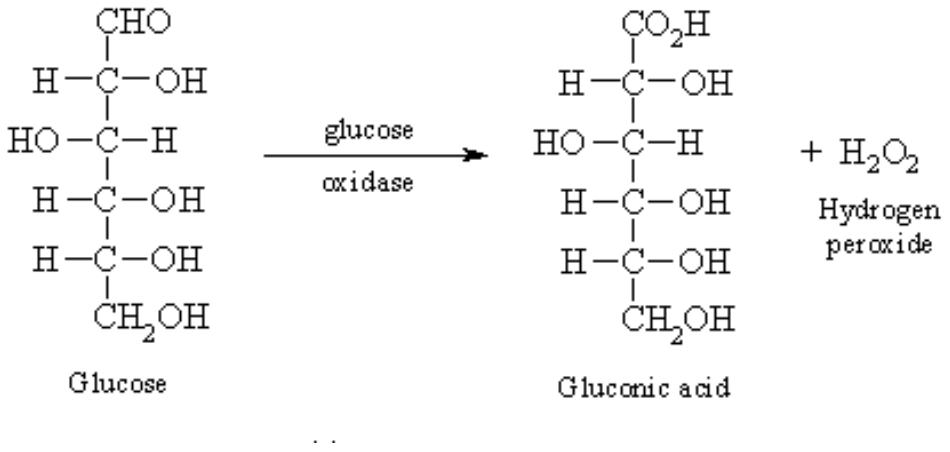

Figure 5: Glucose oxidation (26)

Honey can help in healing skin infections which are a consequence from burns and wounds and it can also help in treating ulcers and bed sores. Honey improves wound healing, because it maintains moisture in the wound and because its high viscosity ensures a barricade that blocks an infection. Faster healing and new tissue growth is due to honey's antibacterial effects. (25).

1.7 Anti-acetylcholinesterase activity

1.7.1 Cholinesterases

Cholinesterases are placed amongst the enzymes, which are responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh). They cause the decomposition of acetylcholine into choline and acetic acid. This reaction enables the return of cholinergic neuron to its resting state after activation. There are two classes of those enzymes:

- Acetylcholinesterase (AChE) can be present in many different tissues: nerve and muscle, central and peripheral tissues, motor and sensory fibres and cholinergic and noncholinergic fibres. The enzyme can be present in various molecular forms, which have catalytic features that are alike, but there is a difference in oligomeric structure and the way of attaching to the cell surface (27).
- Pseudocholinesterase (BuChE) can also be named butyrylcholinesterase and they can primarly be present in the liver. The difference between AChE and BuChE is that BuChE is more susceptible to hydrolysis of butyrylcholine than acetylcholine (27).

1.7.2 Acetylcholinesterase inhibitors

Flavonoids have been reported to be the inhibitors of the enzyme acetylcholinesterase (28). AChE inhibitors can block the acetylcholinesterase, which prevents the decomposition of acetylcholine. This results in increased level and duration of the neurotransmitter action (figure 6). Considering the way of action inhibitors are classified as irreversible and reversible. Reversible inhibitors can be used for therapeutic purposes, while irreversible inhibitors are considered to cause toxic effects (27).

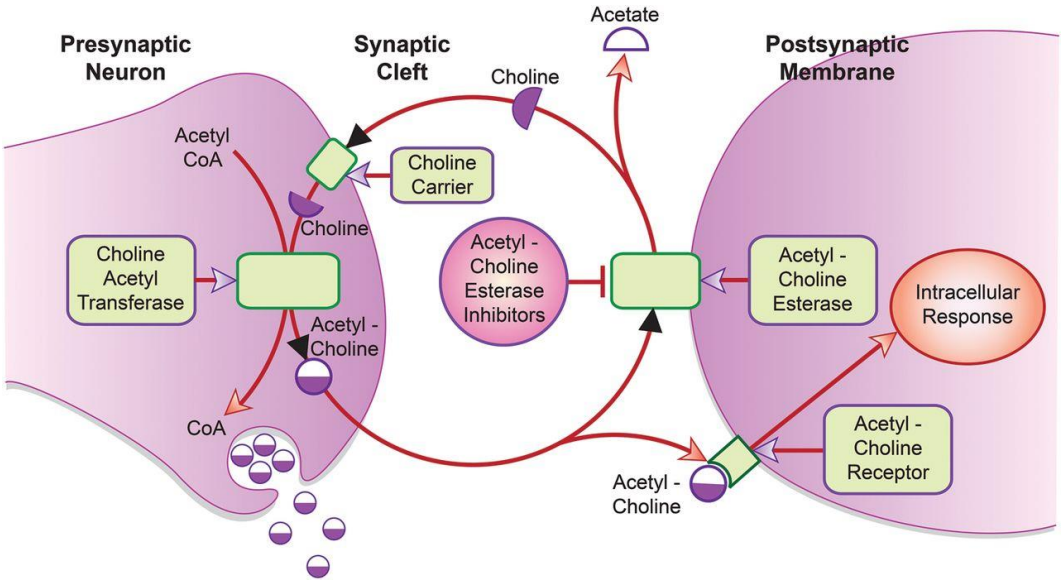

Figure 6: Acetylcholinesterase inhibitors in cholinergic nerve transmission (29)

Reversible acetylcholinesterase inhibitors

Reversible acetylcholinesterase inhibitors have been used for treating various diseases (myasthenia gravis, Alzheimer's disease, post-operative ileus, bladder distention, glaucoma) (27).

The use of acetylcholinesterase inhibitors has caused a lot of attention in treatment of Alzheimer's disease (type dementia). Alzheimer's disease is a chronic neurodegenerative disorder which results in a loss of cognitive ability (memory loss), behavioural abnormalities and ultimately death (30). People with Alzheimer's disease suffer the loss of cholinergic neurons in the brain and the concentrations of ACh in CNS are decreased (27). One treatment strategy to improve cholinergic functions is the use of acetylcholinesterase inhibitors, which prevent degradation of ACh, so the amount of acetylcholine increases in the synapses between cholinergic neurons and more information is delivered to other cells (30, 27). Reversible AChE inhibitors can only ease the symptoms related to memory, thinking, language, judgement and similar thought processes, but there is no cure for Alzheimer's disease (27).

Irreversible acetylcholinesterase inhibitors – organophosphorus compounds

Irreversible acetylcholinesterase inhibitors are also known as organophosphorus compounds and are represented by esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid. They cause phosphorylation of esterases in the central nervous system, which is non-reversible and causes toxicological effects. Organophosphorus compounds phosphorylate enzyme AChE to obstruct the hydrolysis of neurotransmitter (ACh) and acetylcholine starts to accumulate in the synaptic cleft. Symptoms that indicate acute poisoning are agitation, muscle weakness, hypersalivation, sweating etc. (27).

The phosphorylated enzyme can remain inhibited or in some cases nucleophilic reagents (fluoride or oximes) can be used to force the recovery and reactivate the enzyme. Also, the phosphoryl enzyme can undergo a dealkylating reaction called aging. This results in non-reactivity of the enzyme, because the negative charge of the aged phosphoryl group enables its better stability (figure 7) (31).

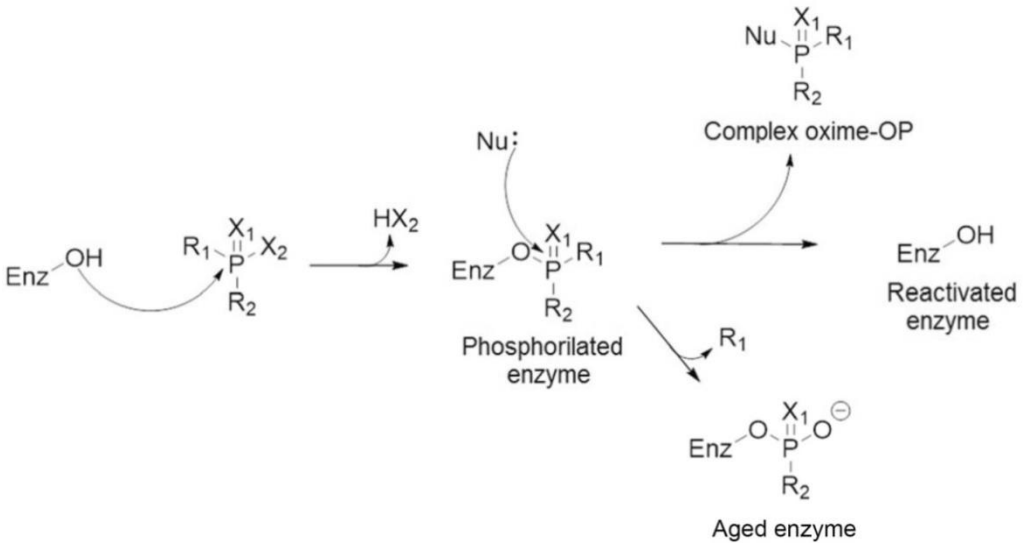

Figure 7: Inhibition of esterases and cholinesterases caused by organophosphorus compounds and its reactivation by the presence of nucleophilic compounds (32)

Organophosphorus compounds have been commonly used as nonspecific insecticides, which are used for controlling insects in agronomics (27).

2 AIM OF WORK

The aim of diploma thesis is to study the composition of two different honey samples and their physicochemical and biological properties. With that obtained knowledge we will be able to develop products, consisting honey components, which have antibacterial, antifungal or antioxidant activity. In this experimental work we will study the physicochemical properties such as water content, protein and proline content, apparent reducing sugar content, pH and free acidity, honey freshness, colour analysis and electrical conductivity and biological properties such as antibacterial, antifungal and antioxidant activity of one Slovenian and one Portuguese honey. The antioxidant assays will include determination of total flavonoid content, total phenolic content, ferric reducing antioxidant power assay, determination of free radicals scavenging capacity and oxygen radical absorbance capacity. We will also check the anti-acetylcholinesterase activity. In all assays we will use Slovenian acacia honey (Belokranjski hram) and Portuguese heather honey (Serra mel) samples.

3 METHODS

3.1 Physicochemical assays

3.1.1 Colour analysis

We determined honey colour according to the colour based on the Pfund scale (mm) concerning the absorption of pure honey at 560 nm (in practice the absorbance at 635 nm is read and converted to mm).

Colour intensity (in mAU) of the 50 % (w/v) honey solutions was determined from the difference in the absorbances at 450 nm and at 720 nm (ABS_{450}).

For the preparation of samples we weighed 2.5 g of honey and dissolved it with deionized water in a 5 mL volumetric flask (honey sample solution 50 % (w/v), 0.50 g/mL). The absorbance of the honey sample solution was measured at the wavelengths of 720 nm, 635 nm and 450 nm against a blank of deionized water. Triplicate assays were performed.

3.1.2 Determination of electrical conductivity

Electrical conductivity of honey was determined as that of a 20 % (w/v) aqueous honey solution corresponding to 20 g of anhydrous honey in 100 mL of deionized water at the reference temperature of 20 °C.

For sample preparation we accurately weighed a honey amount equivalent to 1.2 g of dry honey (calculated according to the water content previously determined by refractometry) and dissolved it with deionized water in a 5 mL volumetric flask (honey sample solution 24 %, (w/v), 0.24 g/mL). First we did the blank measurement with electrodes immersed in deionized water and then we determined the cell constant by immersing the electrode in 0.01 mol/L KCl standard solution and read the conductance. We immersed the electrode in our samples and read the conductance. After each measurement the electrode was rinsed with deionized water. Triplicate assays were performed.

3.1.3 Determination of moisture (water content) in honey – refractometric method

We determined the moisture in honey by refractometric method. We placed a drop of honey at the centre of the refractometer prism and read the refractive index in the refractometer scale. We determined water content from the refractive index of honey by reference to a standard table.

For the determination of water content we used homogeneous samples of pure non-crystallised honeys.

3.1.4 Determination of pH and free acidity – titrimetric method

We determined the pH with pH meter and free acidity by titrimetric method. Honey free acidity corresponds to the content of acids in their free form, expressed in miliequivalents/kg of honey, determined by titration with sodium hydroxide (NaOH).

For sample preparation we accurately weighed 5 g of honey and dissolved it with deionized water in a 50 mL volumetric flask (honey sample solution 10 % (w/v), 0.10 g/mL).

3.1.5 Determination of protein content – Lowry method

We determined the protein content in honey samples with colorimetric method of Lowry, by pretreatment of the honey sample with copper(II) alkaline solution (stabilized with sodium and potassium tartrate) to form a metal-protein complex. The complex reduces the phosphomolibdate and the phosphotungstate of the Folin-Ciocalteu reagent (yellow) to heteropolymolibdate and tungsten (both blue), which absorb between 550-750 nm.
For the preparation of samples we accurately weighed 0.10 g of honey and dissolved it with 10 mL of deionized water (honey sample solution 1.0 % (w/v), 10 mg/mL).
We measured the absorbance of the solutions at 660 nm against water blank and performed triplicate assays. We determined the protein content from the calibration curve obtained by plotting the absorbance of the BSA standards solutions against BSA concentration according to sample dilution and mass of honey in the sample.

3.1.6 Determination of apparent reducing sugars – DNS method

We determined the apparent reducing sugars and apparent sucrose with DNS method (colorimetric method). The content of apparent reducing sugars (sugars that contain aldehyde (glucose) or ketone (fructose) groups) is defined as the amount of sugar able to reduce an oxidant agent (Fehlin's reagent) at specified conditions. The method of 3,5-dinitrosalicylic acid (DNS) is based on the oxidation of aldehyde or ketone groups of reducing sugars to carboxylic acid by DNS (yellow), which is reduced to 3-amino-5-nitrosalicylic acid (red) in alkaline media.
For sample preparation we accurately weighed 1 g of honey and dissolved it with deionized water in a 100 mL volumetric flask (honey solution 1 % (w/v), 10 mg/mL). For the determination of apparent reducing sugars content, we added 1 mL of honey solution to a 10 mL volumetric flask and completed the volume with deionized water (honey diluted solution 0.1 % (w/v), 1 mg/mL).
We measured the absorbance of the solutions at 540 nm against water blank. We determined the amount of reducing sugars in the sample, based on the amount of DNS formed, which was quantified from the calibration curve obtained by plotting the absorbance of the glucose standard solutions against glucose concentration. We determined total sugar content from total soluble solids (° Bx), where 1° Bx corresponds to 1 g of sugar per 100 g of honey.

3.1.7 Determination of proline content – colorimetric method

We determined the proline content in honey by colorimetric method. The proline content is defined as the colour developed with ninhydrin compared to a proline standard, after addition of 2-propanol. Proline and ninhydrin form a coloured complex that absorbs at 510 nm. The method can be used for determination of proline, because proline is the only amino acid that does not react with ninhydrine like all the other amino acids, where the coloured complex is purple. The complex created between ninhydrine and proline gives the yellow colour. The reason is that proline is a secondary amino acid, where nitrogen is in the ring structure and cannot react with ninhydrine.

For the sample preparation we accurately weighed 0.5 g of honey and dissolved it with 10 mL of deionized water (honey sample solution 5 % (w/v), 0.05 g/mL).

We measured the absorbance of the solutions at 510 nm against water blank and performed triplicate assays. We determined the proline content from a calibration curve obtained by plotting the absorption of the L-proline standard solutions against L-proline concentration according to the sample dilution and the mass of honey in the sample.

3.1.8 Determination of hydroxymethylfurfural (HMF) content – White method

We determined the HMF content by White method. This method is based on the absorbance of HMF at 284 nm. To eliminate interferences from honey colour, we determined the difference between the absorbances of a clean aqueous honey solution and the same solution after reduction of HMF by addition of bisulfite. The HMF content is calculated after subtraction of the absorbance background at 336 nm. The HMF forms, because of degradation of sugars by the normal honey acidity at room temperature. This degradation is accelerated during heat processing or storage at high temperature (36). HMF content depends on honey pH and storage temperature. HMF content is an indicator of honey freshness and purity.

The HMF content (in mg/kg) was given by:

HMF (in mg/kg) = $(A_{284} - A_{336})$ x 149.7 x 5 x (D/m)

where A_{284} and A_{336} correspond to the mean absorbance values at 284 nm and 336 nm, 149.7 is a constant (149.7 = (126 x 1000 x 1000)/(16830 x 10 x 5), D is the dilution factor if A_{284} > 0.6 and m is the mass in g of the honey sample.

For sample preparation we prepared 10 % (w/v) honey sample solution or 0.10 g/mL.

We determined the sample absorbance against the reference solution at 284 nm and at 336 nm. We performed triplicate assays.

3.2 Antioxidant assays

3.2.1 Total phenolic content (TPC) – Folin - Ciocalteu method

We determined the total phenolic content (TPC) by the colorimetric method of Folin-Ciocalteu reagent. This method is based on the reduction of a yellow solution of molybdotungstophosphoric acid (Folin-Ciocalteu reagent) by the phenolic compounds in honey. They form a blue complex in alkaline media, which absorbs at 760 nm.

For sample preparation we accurately weighed 0.5 g of honey and dissolved it with 10 mL of deionized water (honey sample solution 5 % (w/v), 0.05 g/mL) and we weighed 1 g of artificial honey and dissolved it in 2 mL of deionized water (0.5 g/mL).

We determined TPC from the calibration curve obtained by plotting the absorption of gallic acid standard solutions against gallic acid concentration according to sample dilution and mass of honey in the sample.

3.2.2 Total flavonoid content (TFC) – Dowd method

We determined the total flavonoid content (TFC) in honey by Dowd method (aluminium chloride method). This method uses 1 % aluminium chloride solution in methanol and is based on the formation of a yellow complex between Al^{3+} ion and the carbonyl and hydroxyl groups of flavonoids (flavones and flavonols).

For the sample preparation we accurately weighed 0.5 g of honey and dissolved it with 10 mL of methanol (honey sample methanolic solution 5 % (w/v), 0.05 g/mL). We weighed 0.5 g of artificial honey and dissolved it with 10 mL of deionized water (0.05 g/mL)

We determined the TFC from the calibration curve obtained by plotting the absorption of the quercetin standard solutions against quercetin concentration according to the sample dilution and mass of honey sample.

3.2.3 Ferric reducing antioxidant power (FRAP)

We determined the reducing power of the antioxidant agents in the sample by the ferric reducing antioxidant power assay (FRAP). The method is based on the reduction of ferric

2,4,6-tripyridyl-*s*-triazine (Fe^{3+}-TPTZ) to the blue ferrous complex (Fe^{2+}-TPTZ) in acidic media to maintain iron solubility.

For the sample preparation we accurately weighed 0.5 g of honey (and artificial honey) and dissolved it with 10 mL of deionized water (honey sample solution 5 % (w/v), 0.05 g/mL).

We determined the FRAP from the calibration curve obtained by plotting the absorption of the ferrous sulphate standards solutions against ferrous sulphate concentration according to the sample dilution and mass of honey sample.

3.2.4 Determination of free radical scavenging capacity – DPPH

The method is based on the evaluation of the capacity of the antioxidant agents to reduce DPPH (stable nitrogen organic radical with an intense purple colour and absorption maximum at 515 nm) to the corresponding pale yellow hydrazine. The remaining DPPH is quantified by measuring the absorbance at 515 nm whose intensity decreases proportionally to the DPPH consumption and therefore to the amount of antioxidant agent in the solution.

For the sample preparation we accurately weighed 1 g of honey and dissolved it with 10 mL of methanol (honey sample solution 10 % (w/v), 0.10 g/mL) and we weighed 1 g of artificial honey and dissolved it with 10 mL of deionized water (0.1 g/mL).

We calculated the radical scavenging ability, expressed as percentage of inhibition of DPPH radical. We expressed the result based on the calibration curve obtained by plotting of the inhibition percentage of the Trolox standard solutions against Trolox concentration according to the sample dilution and mass of honey sample.

3.2.5 Oxygen radical absorbance capacity (ORAC) (microplate method)

The oxygen radical absorbance capacity (ORAC) assay is based on the ability of the antioxidant agents present in the sample to inhibit oxidation reactions induced by peroxyl radicals ($ROO^{\cdot}$) generated by thermal decomposition of 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), through interruption of the radical chain via hydrogen atom transfer. The method uses fluoresceine as fluorescent probe, which reacts with peroxyl radical forming a nonfluorescent product and thus the fluorescence of the probe decays with time.

For sample preparation we accurately weighed 0.10 g of honey and dissolved it with 10 mL of phosphate buffer (honey sample solution 1 %, 0.01 g/mL).

We determined the concentration of the samples from the corresponding corrected AUC based in the calibration curve constructed with the Trolox standard solutions, and expressed the results in μmol of Trolox equivalents per mg of honey (μmol TE/mg).

We determined the fluorescence means and corresponding standard deviations for each cycle. We normalized the fluorescence (*f*) curves as a function of time (*t*) obtained for the blank, the samples and the standard solutions, according to:

$f_N = f_i/f_0$

where f_N is normalized fluorescence, f_0 is initial fluorescence (for t = 0) and f_i is fluorescence for t = i. We plotted f_N against time for the blank, standard solutions and honey samples.

From the normalized curves, we calculated the area under the curve (AUC) of the fluorescence decay, according to:

$AUC = 1 + \Sigma (f_i/f_0) = \Sigma f_N$

We corrected the obtained AUC by subtracting the AUC of the blank to the AUC of the samples and to the AUC of the standard solutions. We plotted the corrected AUC for the Trolox standard solutions against Trolox concentration and calculated the regression equation of the calibration curve. We determined the concentration of the samples from the corrected AUC based in the calibration curve constructed with the Trolox standard solutions, and expressed the results in μmol of Trolox equivalents per mg (or per g) of honey (μmol TE/mg).

3.3 Antimicrobial and antifungal activity of honey samples

To determine the antibacterial and antifungal activity of honey samples, we did the microplate broth microdilution method (MIC) and the well diffusion assay (MBC). We determined MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values. This showed us if our honey samples are bacteriostatic (stop bacteria/yeast from growing) or bactericidal (kill bacteria/yeast). We were testing four bacterial strains: *Staphylococcus aureus*, *Enterococcus faecalis* (both Gram positive bacteria), *Pseudomonas aeruginosa*, *Escherichia coli* (both Gram negative bacteria) and two yeasts: *Candida albicans* and *Saccharomyces cerevisiae*.

The reagents and solvents used were obtained from commercial suppliers and used without further purification. For the antibacterial assays Mueller-Hinton (MH) broth was used, and Sabouraud (SAB) for antifungal assay. Positive control for Gram positive bacteria was vancomycin (1 mg/mL) and for Gram negative bacteria norfloxacine (NOR) (1 mg/mL) was

used. Nystatin was used as positive control for yeasts. Negative control for both bacteria and yeasts was distilled water (for samples diluted in water) and dimethyl sulfoxide (DMSO) (for samples diluted in DMSO).

For pure honey we dissolved 1 g of honey in 2 mL of deionized water in order to obtain the 500 mg/mL solutions. For honey extracts we dissolved 10 mg of extract in 10 mL of DMSO in order to obtain the concentration of 1 mg/mL. We also prepared honey extracts with concentration 10 mg/mL by dilution in DMSO (2 mL of extract in 200 μL of DMSO). To check if the inhibition of bacteria is caused by sugars with osmotic effect (which causes the drought of bacteria), we prepared an artificial solution with concentration of 500 mg/mL. For the preparation of the artificial honey solution we dissolved 77 g of glucose and 3 g of sucrose in 20 mL of deionized water. But to reach the concentration 500 mg/mL we weighed 1 g of artificial honey solution and dissolved it in 2 mL of deionized water.

MIC was determined using the microplate broth microdilution method. To every well of 96 wells microtiter plate were added 100 μL of broth: MH for bacteria and SAB for fungus. To the first well were added 100 μL of sample. Then using the multichannel electronic pipette all samples were diluted by taking 100 μL of broth-sample solution from the first well to the next well, taking the solution in and out of the pipette tip to ensure homogenous mixture, and continued to do so until reaching the second last well. Afterwards each well was inoculated with pre-prepared 10 μL of bacteria/fungus suspension. The last well, containing only broth and bacteria/fungus, was used for growth control. Each microtiter plate also included positive and negative control. All together were assayed 6 microtiter plates, 4 for antibacterial assay and 2 for antifungal assay. After inoculation of samples the microtiter plates were incubated in incubator at 37 °C for 24 hours. After 24 hours turbidity measurements were taken to determine, which concentrations prevented the growth of bacteria/fungus, comparing each well to the last one in the row, containing only broth and inoculant. The last well not containing any growth determined the MIC of each sample, which was defined as the lowest concentration of honey that prevented the growth of the tested microorganisms. Assays were done in triplicate for each microorganism tested.

The well diffusion assay was used to determine MBC of the compounds. All wells with no apparent growth in the MIC assay were subcultured onto fresh nutrient broth (Mueller Hinton for bacteria and Sabouraud medium for yeasts) and the microplates were incubated at 37 °C for 24 h (bacteria) or 25 °C for 72 h (yeasts) to determine whether viable bacteria or yeasts had persisted. The minimum bactericidal concentration (MBC) and the minimum

fungicidal concentration (MFC) were determined as the lowest concentration of honey to prevent the survival of viable bacteria or yeasts, respectively (34, 35). Each assay was performed in triplicate.

3.4 Anti-acetylcholinesterase activity of honey samples

To check if our honey samples inhibit the enzyme acetylcholinesterase, we used microplate and put the components and reagents in wells in special order and then measured the absorbance at 405 nm with spectrophotometric multiwell plate reader. First we had to check the enzyme activity (without inhibitor), which was good (around 850). Then we had to check the inhibition with the inhibitor tacrine, for which we know that inhibits the acetylcholinesterase (the concentration of tacrine solution was 3 μM). The inhibition was good (around 85 %). After we made sure that the enzyme was suitable for the assay, we started with our tests. First we prepared the Ellman's reagent (DTNB), because it oxidizes with time, so it always has to be prepared fresh. Before every test, we always had to do the blank test. We had to dilute the DMSO samples 1:100, so we diluted 10 μL of sample with 990 μL of deionized water. When we were testing the DMSO samples (honey extracts) we had to test the DMSO to see how much the DMSO inhibited the enzyme.

4 RESULTS AND DISCUSSION

4.1 Physicochemical assays

4.1.1 Colour analysis

In tables I, II and III are shown the absorbances of honey samples at different wavelengths, colour classification and colour intensity of honey samples.

Table I: Absorbances of honey samples at 720 nm, 635 nm and 450 nm

SAMPLES	A_{720} (average)	A_{635} (average)	A_{450} (average)
ACACIA HONEY	0.063	0.073	0.142
HEATHER HONEY	0.364	0.479	1.349

Table II: Colour classification of honey

SAMPLES	mm Pfund scale	COLOUR
ACACIA HONEY	24.3	**White**
HEATHER HONEY	159.5	**Dark amber**

Legend: mm Pfund scale

COLOUR NAME	mm Pfund scale
Water white	< 9
Extra white	9 – 17
White	18 – 34
Extra light amber	35 – 50
Light amber	51 – 85
Amber	86 – 114
Dark amber	>114

Table III: Colour intensity

SAMPLES	ABS_{450} (mAU)
ACACIA HONEY	79
HEATHER HONEY	985

According to the mm Pfund scale the colour of acacia honey is classified as white and heather honey as dark amber. The results were similar to the ones in previous reports, which classify the colour of acacia honey as water or extra white and heather honey as amber (36, 37). The colour intensity of heather honey was a lot higher than intensity of acacia honey, which was expected, because heather honey has a lot darker colour than acacia.

4.1.2 Determination of electrical conductivity

Table IV represents experimental conductance values, electrical conductivity and total ash content in honey samples.

Table IV: Experimental conductance value (G), electrical conductivity (K) and total ash content of honey samples (A)

SAMPLES	G (mS)	K honey (mS/cm)	A (g/100 g)
ACACIA HONEY	0.080	0.092	-0.029
HEATHER HONEY	0.760	0.881	0.423

The measured electrical conductivity of heather honey was a lot higher than conductivity of acacia honey (table IV). The results meet standards and correspond to the results in previous reports, where it was said that the conductivity of heather honey is relatively high and can reach the values up to 0.97 mS/cm (46). Heather honey had a high amount of total ash content, which is typical for darker honeys, but for acacia honey we got the negative value, because the method is not valid for honeys with k < 0.143 mS/cm (table IV). Regarding the quality of the honey it means that heather honey has better quality than acacia, because it has higher amount of the ash. The amount of total ash content in heather honey was higher than amount in previous reports (38), but it was still within the limitations, which are usually below 0.6 g/100 g for blossom honeys.

4.1.3 Determination of moisture (water content) in honey

Honey water content is usually between 15-20 % (corresponding to TSS content of 80-86 ° Bx) and the maximum limit allowed is 21 %, except for heather and clover honeys, which can be up to 23 %. The water content increases with total soluble solids (TSS), which is expressed in degrees Brix (° Bx). 1 ° Bx corresponds to 1 g of sugar per 100 g of solution. The refractive index, water content, relative density and total soluble solids content of honey samples are shown in table V.

Table V: Refractive index (n_D), water content (W), relative density (d) and total soluble solids (° Bx) content of honey samples

SAMPLES	n_D (average)	W (g/100 g honey)	d	° Bx
ACACIA HONEY	1.4950	16.6	1.4267	81.9
HEATHER HONEY	1.4975	15.6	1.4338	82.9

Our results showed that the water content in acacia honey was higher than in heather honey. Compared to the results, obtained in previous reports, the water content for acacia honey is

very similar, but for heather honey our results showed lower values. (39). The results corresponded to standards.

The amount of TSS was higher in heather honey than in acacia, which tells us that heather honey has higher amount of sugar and this confirms the fact that its viscosity is higher than in acacia honey.

4.1.4 Determination of pH and free acidity

Honey free acidity corresponds to the content of acids in their free form. The total acidity of honey is usually between 40-50 miliequivalents (meq) per kg of honey and the maximum limit allowed is 50 meq/kg. In table VI are shown the results of measured pH and free acidity of honey samples.

Table VI: pH and free acidity of honey samples

SAMPLES	pH	FA (meq/kg)
ACACIA HONEY	3.48	15.99
HEATHER HONEY	4.24	38.34

The results showed that acacia honey has lower pH than heather honey, which leads to a conclusion that it contains higher amount of organic acids. Consequently, because of lower pH value is less suitable medium for bacteria. Our results are similar to the ones from the reports obtained previously (38, 40).

The free acidity of heather honey was higher than the one in acacia honey. According to this results heather honey contains higher amount of acids in their free form. The values that we obtained are higher than the ones reported in previous reports (38, 41). Inconsistency between pH and free acidity is probably due to mistakes done, while doing measurements.

4.1.5 Determination of protein content

The amount of protein content might refer to the origin of honey and the type of pollens (42). In table VII are shown the absorbances of water, standard solutions and honey samples at 660 nm and figure 8 represents the calibration curve, which we used to determine the protein content.

Table VII: Absorbances of water, standard solutions and honey samples at 660 nm

SAMPLES	A (average)	A (average-corrected)
DEIONIZED WATER	0.263	0
0.050 mg/mL BSA STANDARD SOLUTION	0.295	0.032
0.100 mg/mL BSA STANDARD SOLUTION	0.298	0.035
0.200 mg/mL BSA STANDARD SOLUTION	0.346	0.083
0.300 mg/mL BSA STANDARD SOLUTION	0.408	0.145
0.500 mg/mL BSA STANDARD SOLUTION	0.655	0.392
ACACIA HONEY (PURE)	0.352	0.089
HEATHER HONEY (PURE)	0.563	0.300

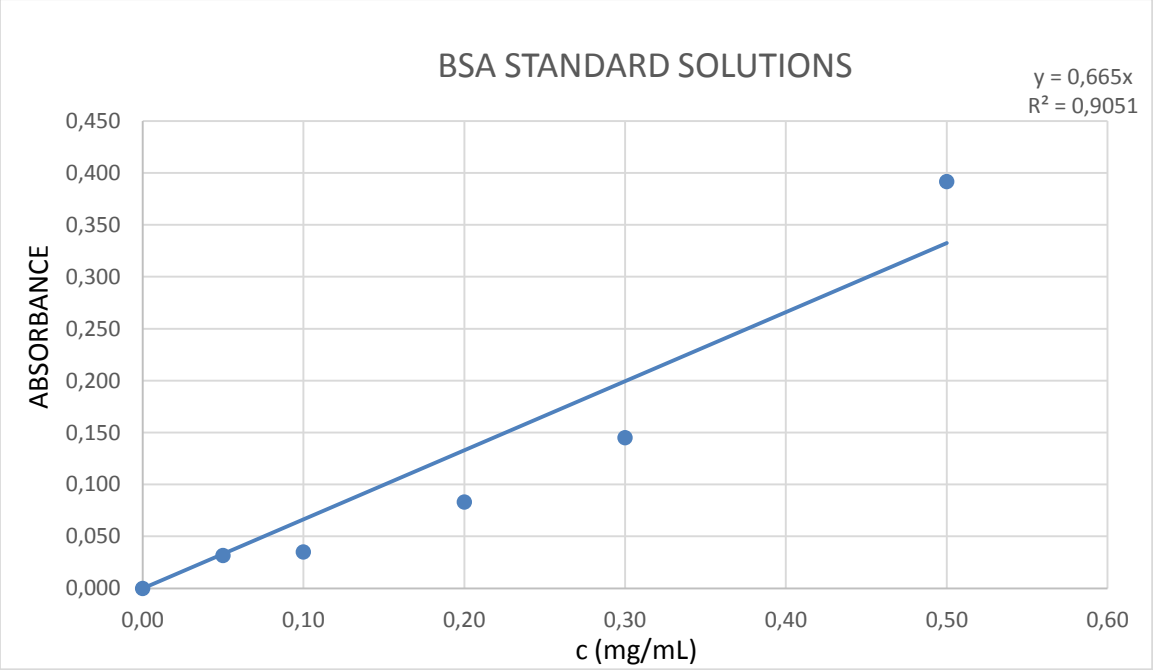

Figure 8: Calibration curve of BSA standard solutions

Table VIII: Protein content of honey samples

SAMPLES	PROTEIN CONTENT (mg BSAE/100 g honey)	PROTEIN CONTENT (mg BSAE/mL sample)
ACACIA HONEY	0.13	0.134
HEATHER HONEY	0.45	0.451

Heather honey was richer with proteins than acacia honey, which leads to conclusion that heather honey has lower surface tension and higher tendency to foam and form air bubbles (table VIII). The measured content of proteins was lower than usually, where protein concentrations are between 1-100 mg/mL.

4.1.6 Determination of apparent reducing sugars – DNS method

The apparent reducing sugar content should not be below 60 g/100 g honey (new proposal suggests a minimum of 65 g/100 g honey) for blossom honeys or 45 g/100 g for honeydew honeys and mixture of blossom and honeydew honeys. Absorbances of water, standard solutions and honey samples at 540 nm are shown in table IX and figure 9 represents the calibration curve, which was used to determine the apparent reducing sugars.

Table IX: Absorbances of water, standard solutions and honey samples at 540 nm

SAMPLES	A (average)	A (average-corrected)
DEIONIZED WATER	0.035	0
0.25 mg/mL GLUCOSE STANDARD SOLUTION	0.157	0.122
0.50 mg/mL GLUCOSE STANDARD SOLUTION	0.365	0.330
0.75 mg/mL GLUCOSE STANDARD SOLUTION	0.538	0.503
1.00 mg/mL GLUCOSE STANDARD SOLUTION	0.710	0.675
1.25 mg/mL GLUCOSE STANDARD SOLUTION	0.868	0.833
ACACIA HONEY (PURE)	0.714	0.679
HEATHER HONEY (PURE)	0.855	0.820

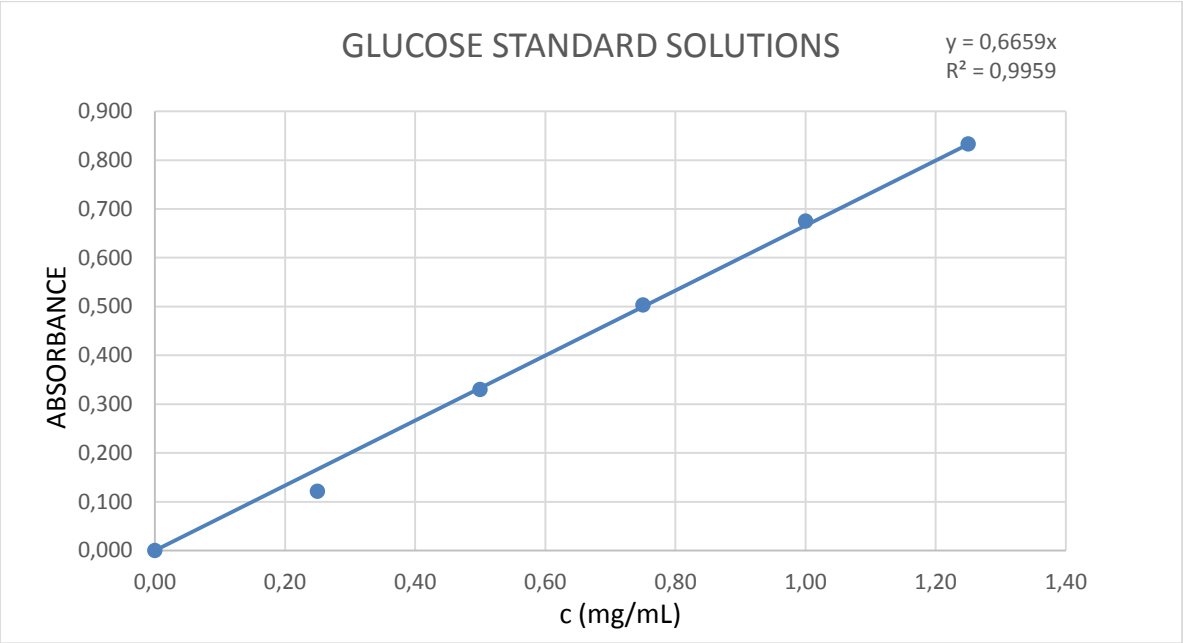

Figure 9: Calibration curve of glucose standards solutions

Table X: Apparent reducing sugar and total sugar content in honey samples

SAMPLES	APPARENT REDUCING SUGAR CONTENT (g GE/100g honey)	TOTAL SUGAR CONTENT(g/100 g honey)
ACACIA HONEY	40.8	81.9
HEATHER HONEY	49.2	82.9

The apparent reducing sugar content was higher in heather honey than in acacia (table X). Both results differed from the ones in previous report, where values for the apparent reducing sugar were higher. Acacia honey had 71.5 % of apparent reducing sugars and heather honey 72.16 % (43, 44). Total sugar content was higher in heather honey than in acacia, which corresponds to a higher viscosity of heather honey (table X). Both results for total sugar content were within the limitations.

4.1.7 Determination of proline content

The proline content is used as a criterion of honey ripeness and eventual sugar adulteration when the value is below a certain limit (honey with proline content less than 180 mg/kg is considered either non-ripe honey or adulterated honey). In table XI are represented

absorbances of water, standard solutions and honey samples at 510 nm and figure 10 shows a calibration curve, which was used to determine the proline content.

Table XI: Absorbances of water, standard solutions and honey samples at 510 nm

SAMPLES	A (average)	A (average-corrected)
DEIONIZED WATER	0.010	0
0.015 mg/mL L-PROLINE STANDARD SOLUTION	0.047	0.037
0.030 mg/mL L-PROLINE STANDARD SOLUTION	0.112	0.102
0.045 mg/mL L-PROLINE STANDARD SOLUTION	0.167	0.157
0.060 mg/mL L-PROLINE STANDARD SOLUTION	0.240	0.230
0.075 mg/mL L-PROLINE STANDARD SOLUTION	0.325	0.315
ACACIA HONEY	0.018	0.008
HEATHER HONEY	0.163	0.153

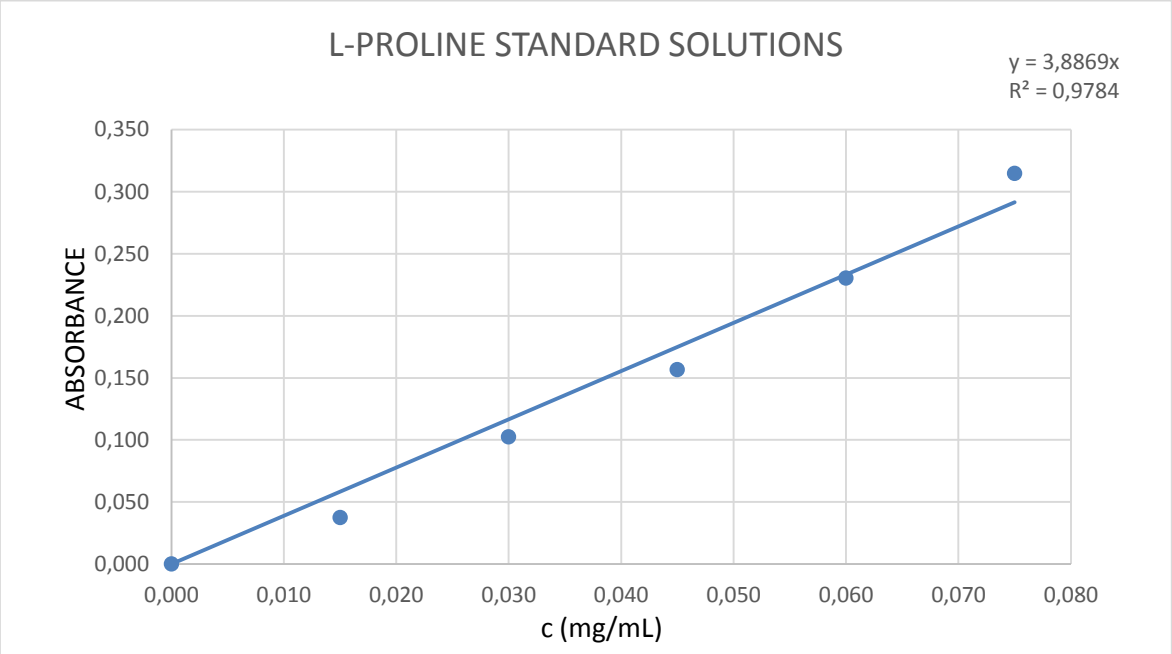

Figure 10: Calibration curve of L-proline standard solutions

Table XII: Proline content of honey samples

SAMPLES	PROLINE CONTENT (mg LPE/kg honey)	PROLINE CONTENT (mg LPE/mL sample)
ACACIA HONEY	41.2	0.0021
HEATHER HONEY	787.3	0.0394

The obtained experimental results showed that heather honey had much higher amount of proline content than acacia honey (table XII). According to the limits and the measured proline content, acacia honey is considered as non-ripe honey. Both results differed from the ones in previous reports. Heather honey had a lot higher amount of proline and acacia lower amount of proline compared to other reports (45).

4.1.8 Determination of hydroxymethylfurfural (HMF) content

HMF is an indicator of honey freshness and purity. In tables XIII and XIV are shown absorbances of honey samples at 284 and 336 nm.

Table XIII: Absorbances of honey samples at 284 nm

SAMPLES	A (average)
ACACIA HONEY	0.021
HEATHER HONEY	0.075

Table XIV: Absorbances of honey samples at 336 nm

SAMPLES	A (average)
ACACIA HONEY	-0.002
HEATHER HONEY	0.021

Table XV: HMF in honey samples

SAMPLES	HMF (mg/kg)
ACACIA HONEY	7.0
HEATHER HONEY	16.0

The measured HMF content was lower in acacia honey than in heather honey, which leads to a fact that acacia honey was fresher in comparison with heather honey (table XV). They were both stored at the same temperature, but considering the pH, we can conclude from our results that lower the pH of honey, lower the HMF content and fresher the honey. This result

contradicts to a fact that HMF content is higher in more acidic medium. The results obtained from the assay, were within the recommended range, where 80 mg/kg of HMF is the maximum limit, while the European Union has a limit of 40 mg/kg and a value ≤ 60 mg/kg is recommended after processing and/or mixture. The values were lower compared to other reports (46, 47).

4.2 Antioxidant assays

4.2.1 Total phenolic content (TPC)

Absorbances of water, standard solutions and honey samples at 760 nm are shown in table XVI and figure 11 represents the calibration curve, which was used to determine TPC.

Table XVI: Absorbances of water, standard solutions and honey samples at 760 nm

SAMPLES	A (average)
DEIONIZED WATER	0
0.010 mg/mL GALLIC ACID STANDARD SOLUTION	0.097
0.020 mg/mL GALLIC ACID STANDARD SOLUTION	0.193
0.030 mg/mL GALLIC ACID STANDARD SOLUTION	0.287
0.040 mg/mL GALLIC ACID STANDARD SOLUTION	0.377
0.050 mg/mL GALLIC ACID STANDARD SOLUTION	0.468
ARTIFICIAL HONEY	0.036
ACACIA HONEY (PURE)	0.075
HEATHER HONEY (PURE)	0.583
ACACIA HONEY (EXTRACT)	0.088
HEATHER HONEY (EXTRACT)	0.147

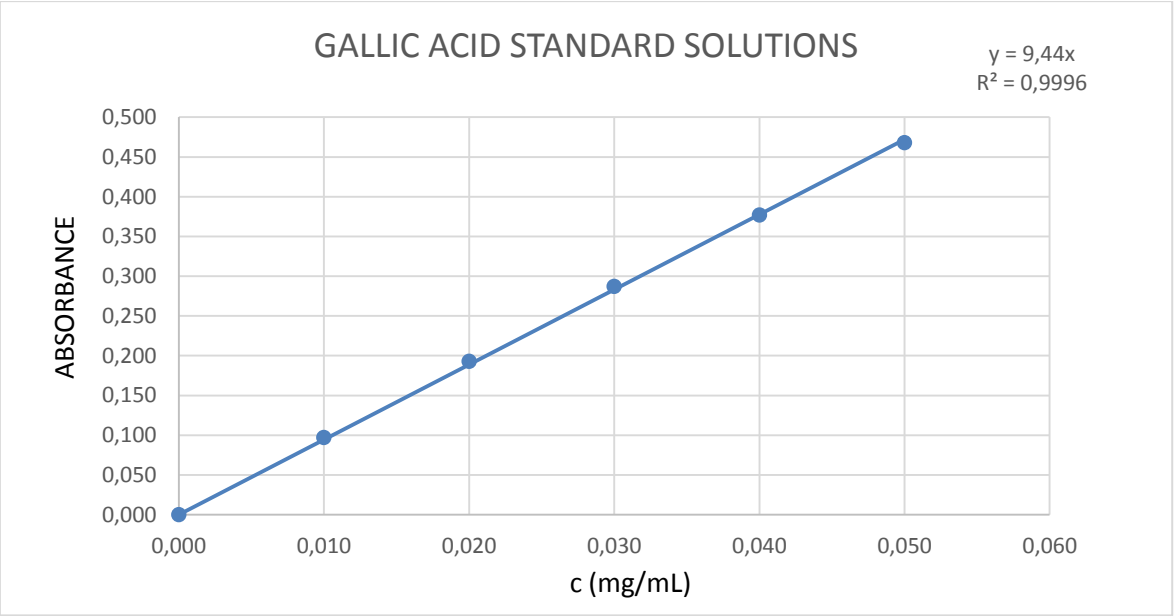

Figure 11: Calibration curve of gallic acid standard solutions

Table XVII: TPC in honey extracts

SAMPLES	TPC (mg GAE/mL sample)	TPC (mg GAE/ g of extract
ACACIA HONEY (EXTRACT)	0.0091	9.08
HEATHER HONEY (EXTRACT)	0.0154	15.42

Table XVIII: TPC in artificial and pure honey

SAMPLES	TPC (mg GAE/mL sample)	TPC (mg GAE/100 g honey)
ARTIFICIAL HONEY	0.0035	0.703
ACACIA HONEY (PURE)	0.0077	15.37
HEATHER HONEY (PURE)	0.0620	123.91

The total phenolic content in pure heather honey was higher than in pure acacia honey and it was the same with the extracts (table XVII, table XVIII). The measured results for heather honey were similar to the ones from previous reports, but for acacia honey the amount of total phenolics was lower (47, 48).

4.2.2 Total flavonoid content (TFC)

Flavonoids are very important for their antioxidant properties. Absorbances of methanol, standard solutions and honey samples at 415 nm are shown in table XIX and figure 12 show the calibration curve, which we used to determine TFC.

Table XIX: Absorbances of methanol, standard solutions and honey samples at 415 nm

SAMPLES	**A (average)**
METHANOL	0
0.010 mg/mL QUERCETIN STANDARD SOLUTION	0.182
0.020 mg/mL QUERCETIN STANDARD SOLUTION	0.281
0.030 mg/mL QUERCETIN STANDARD SOLUTION	0.320
0.040 mg/mL QUERCETIN STANDARD SOLUTION	0.334
0.050 mg/mL QUERCETIN STANDARD SOLUTION	0.476
ARTIFICIAL HONEY	0.020
ACACIA HONEY (PURE)	0.078
HEATHER HONEY (PURE)	0.272
ACACIA HONEY (EXTRACT)	3.728
HEATHER HONEY (EXTRACT)	0.125

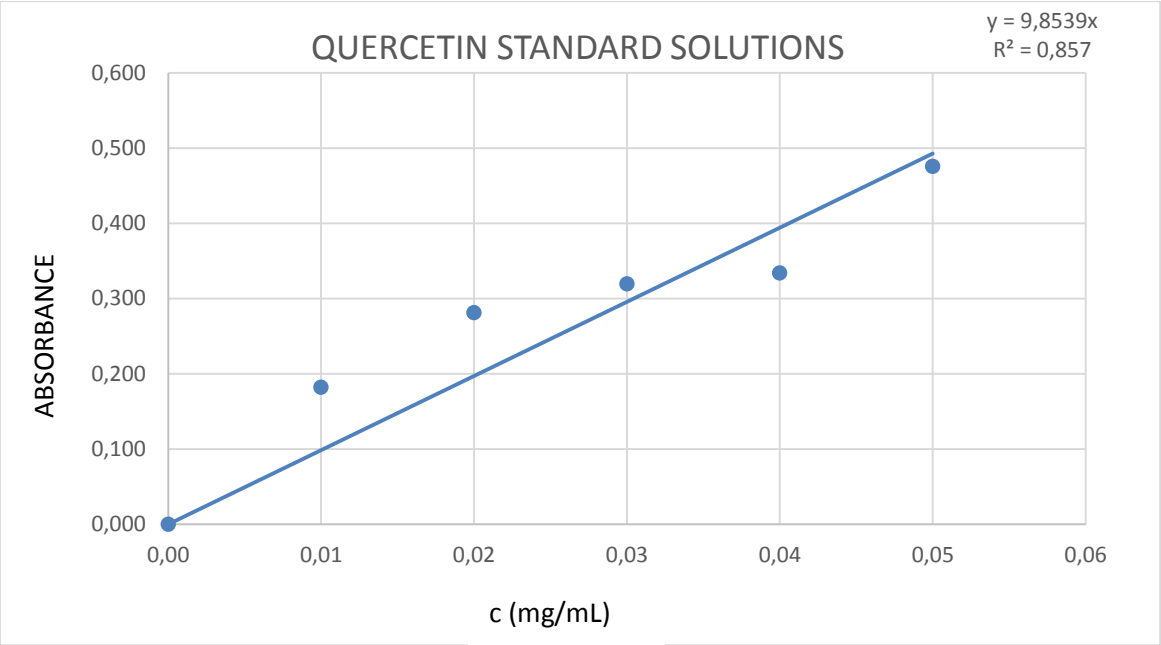

Figure 12: Calibration curve of quercetin standars solutions

Table XX: TFC in honey extracts

SAMPLES	TFC (mg QE/mL sample)	TFC (mg QE/ g of extract
ACACIA HONEY (EXTRACT)	0.3783	378.29
HEATHER HONEY (EXTRACT)	0.0127	12.69

The result for acacia extract was too high, because there was something wrong with the prepared sample, probably it was contaminated, which caused too high absorbance and consequently the total flavonoid content was too high. The amount of total flavonoids from heather extract was lower than the amount of flavonoids in pure heather honey (table XX).

Table XXI: TFC in artificial and pure honey

SAMPLES	TFC (mg QE/mL sample)	TFC (mg QE/100 g honey)
ARTIFICIAL HONEY	0.0020	3.992
ACACIA HONEY (PURE)	0.079	15.76
HEATHER HONEY (PURE)	0.0276	55.21

The amount of total flavonoids in pure heather honey was higher than in acacia, which is the reason for heather honey's better antioxidant properties (table XXI). The results, obtained in

this assay, corresponded to the results from previous reports, where the total flavonoid content in acacia honey varied from 6.61 to 18.05 mg QE/100 g and for the heather honey the value was 59.11 mg QE/100 g (46, 50).

4.2.3 Ferric reducing antioxidant power (FRAP)

In table XXII are shown the absorbances of water, standard solutions and honey samples at 593 nm and figure 13 represents the calibration curve, which was used to determine FRAP.

Table XXII: Absorbances of water, standard solutions and honey samples at 593 nm

SAMPLES	A (average)
DEIONIZED WATER	0
0.050 mmol/L FERROUS SULPHATE STANDARD SOLUTION	0.130
0.075 mmol/L FERROUS SULPHATE STANDARD SOLUTION	0.217
0.100 mmol/L FERROUS SULPHATE STANDARD SOLUTION	0.321
0.200 mmol/L FERROUS SULPHATE STANDARD SOLUTION	0.510
0.300 mmol/L FERROUS SULPHATE STANDARD SOLUTION	0.710
ARTIFICIAL HONEY	0.014
ACACIA HONEY (PURE)	0.089
HEATHER HONEY (PURE)	0.467
ACACIA HONEY (EXTRACT)	3.516
HEATHER HONEY (EXTRACT)	0.201

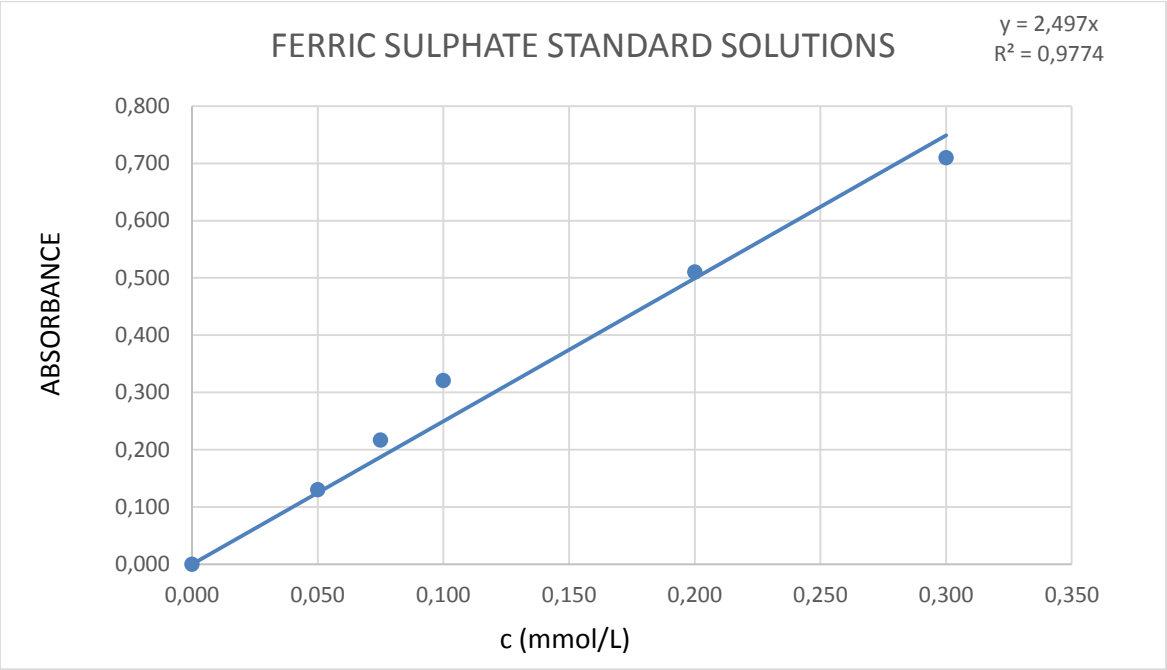

Figure 13: Calibration curve of ferric sulphate standard solutions

Table XXIII: FRAP of honey extracts

SAMPLES	FRAP (mmol Fe (II)/L sample)	FRAP (µmol Fe (II)/g extract)
ACACIA HONEY (EXTRACT)	1.4080	1407.96
HEATHER HONEY (EXTRACT)	0.0805	80.50

The result for acacia extract was too high, because there was something wrong with the prepared sample, probably it was contaminated, which caused too high absorbance and consequently the total antioxidant power was too high. The total antioxidant power of heather extract was lower than the total antioxidant power of pure heather honey (table XXIII).

Table XXIV: FRAP of artificial and pure honey

SAMPLES	FRAP (mmol Fe (II)/L sample)	FRAP (µmol Fe (II)/100 g)
ARTIFICIAL HONEY	0.0056	11.21
ACACIA HONEY (PURE)	0.0356	60.07
HEATHER HONEY (PURE)	0.1872	737.4

According to the results, obtained from the assay, the total antioxidant power of pure heather honey was higher than total antioxidant power of pure acacia honey (table XXIV). This assay also confirms that heather honey has better antioxidant properties than acacia. Both results differed from the results gathered in previous reports, where the value for acacia honey was 0.71 mmol Fe (II)/L and for heather honey 1092 μmol Fe (II)/100 g (51, 52).

4.2.4 Determination of free radical scavenging capacity (DPPH)

The table XXV represents absorbances of methanol, standard solutions and honey samples and % of inhibition of DPPH. Calibration curve is shown in figure 14 and it was used to determine free radical scavenging capacity.

Table XXV: Absorbances of methanol, standard solutions and honey samples at 517 nm and % of inhibition of DPPH

SAMPLES	**A (average)**	**% INHIBITION**
METHANOL	0.500	0.0
0.025 mg/mL TROLOX STANDARD SOLUTION	0.476	4.9
0.050 mg/mL TROLOX STANDARD SOLUTION	0.401	19.9
0.100 mg/mL TROLOX STANDARD SOLUTION	0.337	32.6
0.200 mg/mL TROLOX STANDARD SOLUTION	0.031	93.8
ARTIFICIAL HONEY	0.500	0.07
ACACIA HONEY (PURE)	0.334	33.2
HEATHER HONEY (PURE)	0.241	51.9
ACACIA HONEY (EXTRACT)	0.054	89.2
HEATHER HONEY (EXTRACT)	0.453	9.39

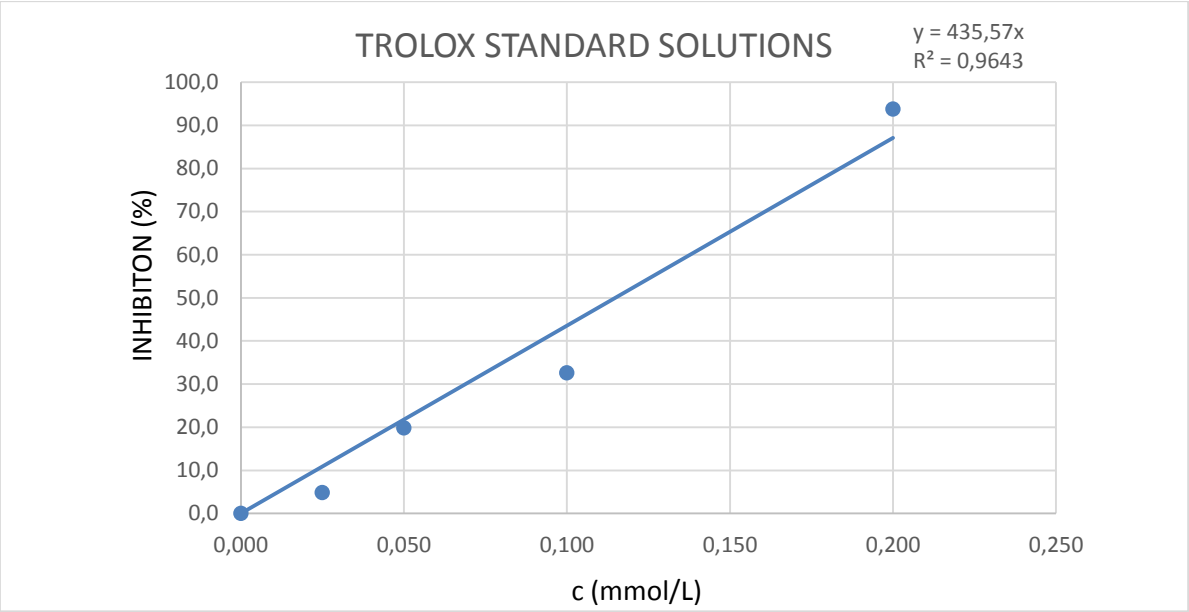

Figure 14: Calibration curve of Trolox standard solutions

Table XXVI: Free radical scavenging capacity of honey extracts

SAMPLES	DPPH (mmol TE/L sample)	DPPH (μmol TE/g extract)
ACACIA HONEY (EXTRACT)	0.2053	205.3
HEATHER HONEY (EXTRACT)	0.0216	21.61

The acacia extract had very high percentage of inhibition unlike the heather extract, which had very low percentage of inhibition (table XXVI).

Table XXVII: Free radical scavenging capacity of artificial and pure honey

SAMPLES	DPPH (mmol TE/L sample)	DPPH (μmol TE/100 g honey)
ARTIFICIAL HONEY	0.0002	0.153
ACACIA HONEY (PURE)	0.0765	76.34
HEATHER HONEY (PURE)	0.1194	119.3

The results showed that pure heather honey had higher percentage of inhibition of DPPH radical than pure acacia honey and again confirmed that heather's compounds are better antioxidants (table XXVII). The results corresponded to values, obtained in previous

research, where the radical scavenging activity, calculated as percent of inhibition, was similar for both samples of pure honey (46, 49).

4.2.5 Oxygen radical absorbance capacity (ORAC)

In table XXVIII is shown the area under the curve of Trolox standard solutions with different concentrations and in table XXIX is shown the area under the curve of honey samples. Figure 15 represents the calibration curve, which was used to determine ORAC.

Table XXVIII: Area under the curve of Trolox standard solutions with different concentrations

SAMPLES	AUC (corrected)
0 µmol/L TROLOX STANDARD SOLUTION	0
5 µmol/L TROLOX STANDARD SOLUTION	0.73
10 µmol/L TROLOX STANDARD SOLUTION	0.85
25 µmol/L TROLOX STANDARD SOLUTION	1.61
50 µmol/L TROLOX STANDARD SOLUTION	4.35

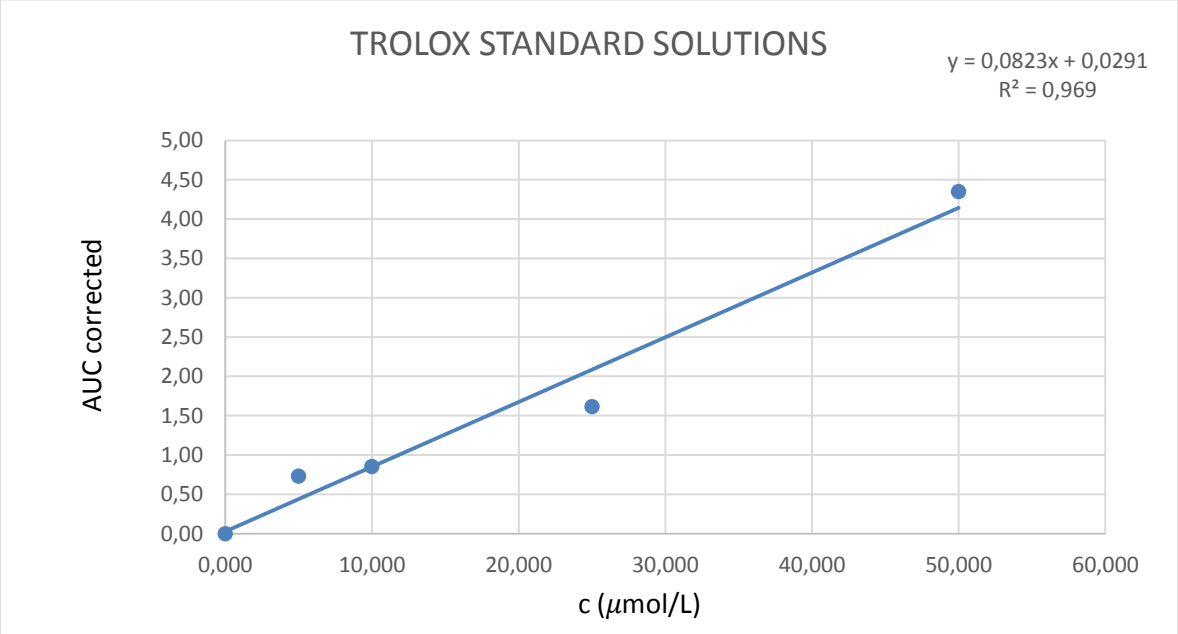

Figure 15: Calibration curve of Trolox standard solutions

Table XXIX: Area under the curve of honey samples

SAMPLES	AUC
ACACIA HONEY (PURE)	5.106
HEATHER HONEY (PURE)	19.224
ACACIA HONEY (EXTRACT)	28.788
HEATHER HONEY (EXTRACT)	36.464

Table XXX: ORAC of honey samples

SAMPLES	ORAC (μmol TE/g honey)
ACACIA HONEY (PURE)	0.062
HEATHER HONEY (PURE)	0.233
ACACIA HONEY (EXTRACT)	0.349
HEATHER HONEY (EXTRACT)	0.443

The obtained results showed higher ORAC values for pure heather honey and heather extract, which corresponds to the other antioxidant assays (table XXX). The results were lower compared to results from previous reports, where it was reported that the ORAC value for acacia honey was around 3 μmol TE/g honey and the ORAC value for heather honey was around 22.58 μmol TE/g honey (53, 54).

4.3 Antimicrobial and antifungal activity of honey samples

Table XXXI: MIC and MBC values of honey samples for different bacterial strains

	MIC/MBC (μg/ml)			
SAMPLES	**Ef**	**Ec**	**Pa**	**Sa**
Artificial honey	> 250000 / x > 250000 / x > 250000 / x	> 250000 / x > 250000 / x > 250000 / x	> 250000 / x > 250000 / x > 250000 / x	> 250000 / x > 250000 / x > 250000 / x
Pure acacia honey	250000 / x > 250000 / x > 2500000 / x	> 250000 / x > 250000 / x > 2500000 / x	> 250000 / x > 250000 / x > 2500000 / x	125000 / > 250000 > 250000 / x > 250000 / x
Pure heather honey	250000 / x > 250000 / x 250000 / > 250000	> 250000 / x > 250000 / x > 250000 / x	> 250000 / x 250000 / 250000 250000 / 250000	250000 / > 250000 250000 / > 250000 125000 / >250000
Acacia extract	125 / > 500 125 / > 500 125 / > 500	62.5 / 250 125 / 500 125 / 500	125 / 500 125 / 500 125 / 500	625 / > 5000 62.5 / > 500 31.25 / > 500

Heather extract	62.5 / 500 62.5 / > 500 125 / 500	62.5 / 500 125 / 500 125 / 500	125 / 500 125 / 500 125 / 500	312.5 / > 5000 62.5 / 500 62.5 / 500
Positive control	> 7.8125 / > 500	< 7.8125 / > 500 < 7.8125 / > 500	< 7.8125 / 31.25 < 7.8125 / 31.25	< 7.8125 / > 500
Negative control (DMSO)	62.5 / > 500 > 500 / x	62.5 / 500 > 500 / x	62.5 / 500 > 500 / x	31.25 / > 500 > 500 / x

Table XXXII: MIC and MFC values of honey samples for different yeasts

MIC/MFC (µg/ml)		
SAMPLES	**Ca**	**Sc**
Artificial honey	> 250000 / x > 250000 / x	> 250000 / x > 250000 / x
Pure acacia honey	> 25000 / x > 250000 / x	250000 / > 250000 > 250000 / x
Pure heather honey	> 250000 / x > 250000 / x	62500 / > 250000 125000 / > 250000
Acacia extract	62.5 / 125 125 / 125	62.5 / 125 125 / 125
Heather extract	62.5 / 125 125 / 125	62.5 / 62.5 125 / 125
Positive control	125 / 125 125 / 125	62.5 / 62.5 < 7.8125 / 62.5
Negative control (DMSO)	125 / 125 > 500 / x	62.5 / 125 > 500 / x

Legend (regards to table XXXI and table XXXII):

Date: 15. 3. 2017

Date: 22. 3. 2017

Date: 23. 3. 2017

Ef = Enterococcus faecalis

Ec = Escherichia coli

Pa = Pseudomonas aeruginosa

Sa = Staphylococcus aureus

Ca = Candida albicans

Sc = Saccharomyces cerevisiae

Artificial honey showed no antibacterial or antifungal activity, which means that sugars do not interfere with antibacterial or antifungal activity.

Pure honeys also showed no antibacterial (> 250000 μg/mL) or antifungal activity (> 25000 μg/mL) for each of the microorganisms tested, but honey extracts showed some activity. Acacia honey extract showed the best inhibitory growth properties against *S. aureus* (52.1 μg/mL) and *E. faecalis* (104.2 μg/mL) and it showed the same inhibitory growth properties against both yeasts (93.8 μg/mL). Heather honey extract showed the strongest activity against *S. aureus* (52.1 μg/mL) and *E. faecalis* (83.3 μg/mL) and the same inhibitory growth properties against both yeasts (93.8 μg/mL) (table XXXI, table XXXII).

Acacia honey extract showed the strongest bactericidal activity against *E. coli*, which added up to 416.7 μg/mL. Heather honey extract had the strongest ability to kill *E. coli* and *P. aeruginosa* both with the same MBC values (500 μg/mL) (table XXXI).

Previous findings reported stated that acacia honey has strongest antibacterial activity against *S. aureus* and the lowest antibacterial activity against *E. coli* and heather honey has the best inhibitory growth properties against *S. aureus* and the lowest inhibitory growth properties against *E. coli* (51, 55).

4.4 Anti-acetylcholinesterase activity of honey samples

None of the tested honey samples inhibited acetylcholinesterase, because through the reaction, the colour changed to yellow, which means that the enzyme reacted with the DTNB. If there would still be no colour, it would mean that the enzyme was inhibited and did not react with DTNB. We concluded that heather and acacia honeys have no anti-acetylcholinesterase activity.

4.5 Comparison of acacia and heather honey samples

In tables XXXIII, XXXIV are shown results of physicochemical and antioxidant assays and in table XXXV is represented correlation matrix.

Table XXXIII: Results of physicochemical assays for honey samples

	ACACIA HONEY	HEATHER HONEY
COLOUR CLASSIFICATION (mm)	24.3 (White)	159.5 (Dark amber)
COLOUR INTENSITY - ABS_{450} (mAU)	79	985
k (mS/cm)	0.092	0.88
A (g/100 g honey)	-0.029	0.423
W (g/100 g honey)	16.6	15.6
TSS (° Bx)	81.9	82.9
pH	3.48	4.24
FA (meq/kg)	15.99	38.34
PROTEIN CONTENT (mg BSAE/mL sample)	0.134	0.451
PROLINE CONTENT(mg LPE/kg honey)	41.2	787.3
APPARENT REDUCING SUGAR CONTENT (g GE/100g honey)	40.8	49.2
HMF (mg/kg)	7.0	16.0

From the obtained results we classified the colour of acacia honey as white and the colour of heather honey as dark amber. Heather honey is a lot darker, which confirms the measured colour intensity, which was higher than in acacia honey. The dark colour also implicates that heather honey has high value of minerals, which was confirmed by the results of ash content. The electrical conductivity was higher in heather honey, but the water content was lower than usually. Higher viscosity of heather honey implicates on higher value of sugar, which we confirmed with the value of total soluble solids, which was higher in heather honey. Free acidity was higher in heather honey, so was the amount of protein and proline content and apparent reducing sugars. Acacia honey was fresher and stored less time than heather honey, which was confirmed by the hydroxymethylfurfural content, which was lower in acacia honey.

Table XXXIV: Results of antioxidants assays for honey samples

	ACACIA HONEY (PURE)	HEATHER HONEY (PURE)	ACACIA HONEY (EXTRACT)	HEATHER HONEY (EXTRACT)
TPC (mg GAE/mL sample)	0.0077	0.0620	0.0091	0.0154
TFC (mg QE/mL sample)	0.0079	0.0276	0.3783	0.0127
FRAP (mmol Fe (II)/L sample)	0.0356	0.1872	1.4080	0.0805
DPPH (mmol TE/L sample)	0.0765	0.1194	0.2053	0.0216
ORAC (μmol TE/g honey)	0.062	0.233	0.349	0.443

Total phenolic content and total flavonoid content was higher in heather honey, so we concluded that heather honey has better antioxidant properties than acacia, which was confirmed by measuring antioxidant power, antioxidant potential and ability to remove oxygen radicals.

Table XXXV: Correlation matrix

CORRELATIONS	PERSON'S CORRELATION COEFFICIENT – R
COLOUR/TPC	0.979
COLOUR/TFC	0.946
COLOUR/FRAP	0.940
COLOUR/DPPH	0.195
COLOUR/ORAC	0.950
COLOUR/pH	0.961
COLOUR/MOISTURE	-0.405
FRAP/TPC	0.990
FRAP/TFC	0.999
FRAP/DPPH	0.506
FRAP/ORAC	0.912
DPPH/TPC	0.379
DPPH/TFC	0.474
DPPH/ORAC	0.321
ORAC/TPC	0.936
ORAC/TFC	0.903

TSS/ELECTRICAL CONDUCTIVITY	0.090
TSS/MOISTURE	-1.000

High correlation coefficients obtained by correlating FRAP values with total phenolic and total flavonoid content in honey samples show that phenolic compounds and flavonoids are responsible for the antioxidant activity of honey. We also obtained high correlations by correlating ORAC values with total phenolic and total flavonoid content. High correlation coefficients were obtained by correlating colour with total phenolic and flavonoid content, ORAC, FRAP and pH. We concluded that total phenolic and flavonoid content, pH and antioxidant properties have impact on honey colour. Perfect negative correlation was calculated between total soluble solids and moisture, which means higher the value of total soluble solids, lower the moisture.

5 CONCLUSIONS

The colour of heather honey was darker and more intense and heather honey also had better conductivity, higher amount of ash content, TSS amount, higher pH and free acidity and higher amount of protein and proline content. Acacia honey only had higher amount of water content, regarding physicochemical assays. Heather honey turned out to have better antioxidant properties, but none of the honey samples inhibited acetylcholinesterase. We concluded that heather honey and acacia honey have no anti-acetylcholinesterase activity.
We can conclude that both, heather and acacia honey have good antibacterial and antifungal properties.

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