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PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF MONOFLORAL HONEYS – EVALUATION OF SLOVENIAN LIME AND PORTUGUESE EUCALYPTUS SAMPLES

VREDNOTENJE FIZIKALNO-KEMIJSKIH IN BIOLOŠKIH LASTNOSTI GOZDNIH VRST MEDU – SLOVENSKEGA LIPOVEGA IN PORTUGALSKEGA EVKALIPTUSOVEGA MEDU

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Declaration

I declare that I have elaborated this bachelor thesis independetly, under the mentorship of prof. Mirjana Gašperlin, Ph.D., M.Pharm. and co-mentorship of assist. prof. Lídia Pinheiro, Ph.D..

Izjava

Izjavljam, da sem diplomsko nalogo izdelala samostojno, pod mentorstvom prof. dr. Mirjane Gašperlin, mag. farm. in somentorstvom asist. prof. dr. Lídie Pinheiro, mag. farm..

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ABSTRACT

Honey is a naturally occurring food that people have used as a sweetener and for other traditional uses, such as treating burns, wounds and other skin conditions throughout centuries. It consists of about 200 substances among which are proteins, amino acids, vitamins, minerals, flavonoids, phenolic acids and others.

The aim of this work was to determine the physicochemical composition of Portuguese eucalyptus and Slovenian lime honey and to assess their antimicrobial, antifungal and antioxidant capacity.

Considering the uses of honey that we have been familiar with, we have assumed both honeys will show antibacterial and antifungal properties. Our hypotheses were also that the total flavonoid and phenolic contents are in positive correlation with colour and that free radical scavenging capacity of both honeys is in positive correlation with total flavonoid content since those compounds are supposedly responsible for antioxidant properties of honey.

We have used different methods to determine physicochemical properties of both lime and eucalyptus honey. We have determined the colour of both samples and classified them according to Pfund scale. We have measured the electrical conductivity, moisture and water content. We have also measured the pH and determined the free acidity for evaluation of acids in their free form. Moreover, we determined the physicochemical composition of both honey samples by measuring the protein content using the Lowry method and sugar content based on the values for total soluble solids (TSS). We have also determined the content of hydroxymethylfurfural content using the White method and proline content using colorimetric method for both lime and eucalyptus honey.

Within antioxidant assays, we have determined total phenolic content using Folin-Ciocalteu method and total flavonoid content using Dowd method. The antioxidant properties of both honey samples were determined by performing three assays: ferric reducing antioxidant power (FRAP), free radical scavenging capacity (DPPH) and oxygen radical absorbance capacity (ORAC).

For determination of antimicrobial and antifungal activity we have tested honey samples against four bacteria - *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* a*nd Enterococcus faecalis* and two yeasts - *Saccharomyces cerevisie and Candida albicans.*

By performing those assays, we have determined that both Portuguese eucalyptus and Slovenian lime honey have antibacterial and antifungal activity, which corresponds to its traditional uses for treatment of colds, burns, wounds etc. We have also confirmed that both honeys have good antioxidant activity, that this activity is the result of flavonoids and other phenolic compounds. Moreover, we concluded that the darker the honey, the stronger are these capacities.

Keywords

honey
eucalyptus
lime
physicochemical composition
antibacterial activity

POVZETEK

Med je živilo, ki ga v našem vsakdanu pogosto uporabljamo. Po njem posežemo, ko nas boli grlo, ko se lažje opečemo ali pa takrat, ko želimo navaden bel sladkor nadomestiti z bolj zdravo alternativo.

Med je sestavljen iz preko 200 spojin. Največ je sladkorjev, predvsem monosaharidov glukoze in fruktoze, prisotne pa so tudi beljakovine, aminokisline, organske kisline, karotenoidi, mnogo vitaminov in mineralov, pigmenti, hlapne spojine, trdni delci, aromatične snovi in nenazadnje voda. Med je bogat s flavonoidi in drugimi fenolnimi spojinami, ki so odgovorne za njegove antioksidativne lastnosti (1).

Barva, vonj in okus medu so odvisni ne samo od vrst cvetja, na katerem čebele nabirajo med, ampak tudi od geografskega porekla, klimatskih razmer in vrst čebel, ki med nabirajo (1).

Med se je skozi človeško zgodovino uporabljal v številne kozmetične in zdravstvene namene. Že več tisoč let se med uporablja za zdravljenje ran in opeklin. Še danes je priljubljen kot vehikel pri izdelavi zeliščnih izvlečkov, včasih pa so ga uporabljali za vlaženje suhe kože, za barvanje las, mehčanje ustnic in za razne obrazne maske. Na Kitajskem verjamejo, da med preprečuje nastanek brazgotin, odstranjuje depigmentacije in pege ter pripomore k splošno boljšemu videzu kože. Rahlo kisel pH medu namreč blagodejno vpliva na zaščitni kislinski plašč kože, ki je pomemben pri vzdrževanju njene normalne funkcije. Arabska medicina med uporablja za zdravljene glivičnih infekcij kože, v Burkini Faso pa ga delavci uporabljajo za čiščenje kože zaradi njegovih baktericidnih lastnosti (5).

Pomembna skupina spojin, prisotnih v medu, so fenolne spojine. Delimo jih na fenolne kisline, flavonoide, stilbene in lignane. Analiza fenolnih spojin je uporabna za določitev cvetličnega in geografskega porekla medu. Na primer, hesperidin je tipičen označevalec citrusnih medov, kemferol je značilen za rožmarinov med, kvercetin za sončnični med, elagična kislina za jesenov med in hidroksicinamati za kostanjev med (7,8).

Flavonoidi so fenolne spojine, za katere so značilne protibakterijske, protivnetne, protialergijske in antitrombotične lastnosti. So kemijske strukture z dvema benzopiranskim skeletom. Antioksidativna aktivnost flavonoidov je posledica ujetja reaktivnih kisikovih spojin, zaviranja encimov, odgovornih za nastanek superoksidnih anionov, kelacije kovinskih ionov, vpletenih v nastanek radikalov in preprečevanja peroksidacije preko zmanjševanja nastajanja alkoksilnih in peroksilnih radikalov (7,8).

Visoka koncentracija sladkorja, prisotnost vodikovega peroksida, nizek pH in metilglioksal ter protimikrobni čebelji peptid defenzin-1 so odgovorni za protibakterijske lastnosti medu. Ena izmed glavnih protibakterijskih spojin v medu je vodikov peroksid H_2O_2. Nastane med proizvodnjo medu, ko čebele nabranemu medu dodajo glukoza oksidazo, encim, ki pod aerobnimi pogoji spremeni glukozo v vodikov peroksid in glukonsko kislino. Vodikov peroksid naj bi ščitil med pred mikrobiološko kontaminacijo, dokler ne doseže zadostne koncentracije sladkorjev. Ko med dozori, se glukoza oksidaza deaktivira, vendar se ob zadostni redčitvi medu ponovno aktivira (9).

Namen našega dela je bil, da preverimo, ali imata portugalski evkaliptusov in slovenski lipov med protibakterijsko delovanje. Predpostavili smo tudi, da je barva medu odvisna od koncentracije flavonoidov in drugih fenolnih spojin, ter da je zmožnost spojin v medu za lovljenje radikalov pozitivno odvisna od koncentracije flavonoidov.

Obema vrstama medu smo določili fizikalno-kemijske lastnosti. Izvedli smo analizo barve in s pomočjo Pfundove lestvice določili, da je lipov med barve svetlega jantarja, evkaliptusov pa barve temnega jantarja. Izmerili smo tudi električno prevodnost, ki je za bila lipov med 0,53 mS in za evkaliptusov med 0,71 mS. Izmerjena vsebnost vode z uporabo Abbejevega refraktometra je bila v lipovem medu 19 % in v evkaliptusovem 17,2 %. pH lipovega medu je znašal 3,92 in evkaliptusovega 3,98. Z uporabo Lowry metode smo določili vsebnost beljakovin in sicer 0,39 g/100 g za lipov in 0,51 g/100 g za evkaliptusov med. Na podlagi vrednosti, ki smo jih dobili pri določaju vsebnosti topnih trdnin v naših vzorcih, smo izračunali skupno vsebnost sladkorja. Vrednost za lipov med je znašala 79,39 g/100 g medu in za evkaliptusov med 81,25 g/100 g medu. Vsebnost prolina v lipovem medu je znašala 440,8 mg/kg in v evkaliptusovem 300,2 mg/kg. Z uporabo metode po White-u smo določili tudi vsebnost hidroksimetilfurfurala (HMF). Vsebnost HMF v evkaliptusovem medu je znašala 16,6 mg/kg in v lipovem 59,4 mg/kg.

Za določanje bioloških lastnosti medu smo z ekstrakcijo na trdni fazi in tekoče-tekoče pripravili izvlečke obeh vzorcev. To smo naredili zato, ker smo predvidevali, da so za antioksidativno ter protibakterijsko delovanje odgovorni flavonoidi in želeli dokazati, da je v obliki izvlečka med bolj učinkovit.

Vsebnost fenolnih spojin smo določili z metodo po Folin-Ciocalteu. Lipov med je imel več fenolnih spojin v obliki izvlečka (0,0132 mg GAE/mL) kot čisti med (0,0159 mg GAE/mL). Ravno nasprotno pa je bila vsebnost fenolnih spojin pri evkaliptusovem medu višja pri vzorcu čistega medu (0,0332 mg GAE/mL) kot v izvlečku (0,0145 mg GAE/mL).

Vsebnost flavonoidov smo določili z uporabo metode po Dowd-u. Vsebnost je bila nižja pri izvlečkih (0,0069 mg QE/mL za lipov in 0,0079 mg QE/mL za evaliptusov med), kot pri vzorcih čistega medu (0,0101 mg QE/mL za lipov in 0,0166 mg QE/mL za evaliptusov med).

Za določitev antioksidativnih lastnostih naših vzorcev medu smo opravili štiri teste. Z njimi smo dobili rezultate tako o moči kot o mehanizmu antioksidativnega delovanja medu. Določili smo aktioksidativno moč redukcije železa, ki je bila višja pri vzorcih čistega medu (0,0761 mmol Fe(II)/L za lipov in 0,1671 mmol Fe(II)/L za evkaliptusov med), kot v izvlečkih (0,0461 mmol Fe(II)/L za lipov in 0,0662 mmol Fe(II)/L za evkaliptusov med). Preučili smo tudi zmožnost lovljenja radikalov naših vzorcev z analizo DPPH. Lipov med je bil bolj učinkovit kot vzorec čistega medu (19 % zaviranje radikala DPPH), kot pa kot izvleček (5,06 % zaviranje radikala DPPH). Nasprotno pa je bil evakliptusov med uspešnejši pri inhibiciji v obliki izvlečka (9,79 % inhibicija), kot pa v obliki čistega medu (1,4 %). Zmožnost absorpcije kisikovega radikala (ORAC) smo testirali z metodo mikroplošče. Dobljena vrednost za lipov med je znašala 10,32 μmol TE/g in za evkaliptusov 10,48 μmol TE/g.

Izvedeni protibakterijski in protiglivični testi so pokazali, da sta oba vzorca aktivna zgolj v obliki izvlečkov. Evkaliptusov med je uspešno zaviral rast *S. aureus* in *E. faecalis*, lipov med pa je bil uspešen proti *E.coli*. Evkaliptusov med je pokazal najmočnejšo baktericidno aktivnost proti *E. coli*, lipov med pa proti *P. aeruginosa.* Oba vzorca sta pokazala dobro protiglivično delovanje proti *Saccharomyces cerevisiae* in *Candidi albicans.* Evkaliptus je najmočneje inhibiral rast *C. albicans,* lipov med pa je proti obema glivama kazal približno enako močno inhibicijo.

Evkaliptusov med je imel višjo koncentracijo fenolnih spojin in flavonoidov kot lipov med, zato se je posledično tudi bolje obnesel pri antioksidativnih testih. Prav tako je imel evkaliptusov med večinoma višje vrednosti fizikalno-kemijskih parametrov (električna prevodnost, vsebnost beljakovin, pH), saj so vse te lastnosti povezane z barvo medu in evkaliptusov med je temnejši od lipovega. Oba vzorca pa sta zavirala rast glivic, ter bila uspešna pri zaviranju rasti bakterij.

Ključne besede

med

evkaliptus

lipa

fizikalno-kemijske lastnosti

protibakterijsko delovanje

LIST OF ABBREVIATIONS

SPE - solid-phase extraction

HPLC - high performance liquid chromatography

CE - capillary electrophoresis

GC - gas chromatography

UV - ultra-violet

LLE - liquid-liquid extraction

C_{18} - octadecyl

DAD - diode-array detection

UMF - unique manuka factor

MGO - methylglyoxal

DHA - dihydroxyacetone

AMPs - antimicrobial peptides

DMSO - dimethyl sulfoxide

UV-VIS - Ultraviolet–visible spectroscopy/Ultraviolet-visible spectrophotometry

IHC - International Honey Commission

BSA - bovine serum albumin

DNS - dinitrosalicylic acid

HMF - hydroxymethylfurfural

FRAP - ferric reducing antioxidant power

DPPH - 2,2-diphenyl-1-picrylhydrazyl

ORAC - oxygen radical absorbance capacity

MH - Mueller-Hinton

SAB - Sabouraud

NOR - Norfloxacine

MIC - minimum inhibitory concentration

MBC - minimum bactericidal concentration

MFC - minimum fungicidal concentration

TSS - total soluble solids

TPC - The total phenolic content

GAE - gallic acid equivalents

TFC - Total flavonoid content

QE - quercetin equivalents

FRAP - The ferric reducing antioxidant power

TPTZ - tripyridyl-s-triazine

EC_{50} - Half maximal effective concentration

IC_{50} - The half maximal inhibitory concentration

DPPH - 2,2-diphenyl-1-picrylhydrazyl

ORAC - The oxygen radical absorbance capacity

AAPH - 2,2'-azobis (2-methylpropionamidine) dihydrochloride

FL - fluorescein

AUC - area under the curve

TE - Trolox equivalents

Ef - *Enterococcus faecalis*

Ec - *Escherichia coli*

Pa - *Pseudomonas aeruginosa*

Sa - *Staphylococcus aureus*

Ca - *Candida albicans*

Sc - *Saccharomyces cerevisiae*

1 INTRODUCTION

1.1 HONEY

Honey is a food of natural source, that we most commonly use as a sweetener. With about 200 substances it has a very diverse composition. The most abundant compounds in honey are sugars, followed by proteins (enzymes), amino and carboxylic acids, many vitamins and minerals, carotenoids and pigments, a lot of different volatile compounds, solid particles, aromatic substances and of course, water. Honey is also a rich source of flavonoids and phenolic acids, which are responsible for honey's antioxidative activity.

The composition as well as the colour, aroma, and flavor of honeys differ as a result of the flower source and also geographical region, climate and honeybee species involved in their production. Other factors that may affect those properties are also the weather conditions, processing, handling, packaging of the final product, and at the end storage time (1).

1.2 COMPONENTS OF HONEY

Sugars

Almost 99% of all solids in honey are represented by sugars. They are the reason for honey's high viscosity, density, and energy value, and also its hygroscopicity and the tendency to granulate. Around ¾ of all sugars in honey are represented by monosaccharides. The remaining are disaccharides, followed by other sugars in smaller quantities. The most abundant sugars in honey are glucose, fructose, sucrose, maltose and dextrin, which can also be described as higher sugars.

Factors that affect the sugar composition of honey are of botanical and geographical origin, meaning it is important which flowers the bees visit and where they collect pollen, as well as the climate, processing, and storage. The amount of glucose and fructose present, and the fraction of each, is an important factor for classification of honey. In most cases, the fraction of fructose in honey is higher than that of glucose, but there are also exceptions in which the concentration of glucose is higher (1, 2).

Proteins

Sources of proteins and amino acids are either animal or vegetal origin. Some of them come in the form of fluids and nectar, which honeybees secrete through salivary glands and pharynx, but the most come from pollen.

The protein content of honey varies between different species of honeybees that produce it. Honeybee species called *Apis cerana* produces honey with protein content between 0,1 % and 3,3 %, and *Apis mellifera* from 0,2 % to 1,6 %.

Amino acids represent around 1% (w/w) of all the compounds found in honey. Depending on whether the origin of honey is nectar or honeydew, their proportion changes.

An amino acid that honey is most rich in, is proline and can be found in fractions from 50 to 85 %. However, proline is just one of them, as we can also find glutamic acid, alanine, phenylalanine, tyrosine, leucine, isoleucine, and many others but in much smaller fractions. The concentration of proline in honey is a very important marker for the maturity of honey and possible contamination with sugar.

Enzymes represent a small fraction of proteins in honey. Among them are invertase, the α- and β-glucosidase, catalase, acid phosphatase, diastase and glucose oxidase (1, 2).

Carboxylic acids

Carboxylic acids represent approx. 0.57 % of honey and are the reason for its slight acidity. Honeybees, when producing honey from nectar, secrete enzymes which transform sugars into carboxylic acids. Carboxylic acids play many important roles. Beside affecting the acidity, pH, and electrical conductivity, they also take part in determining the organoleptic properties (colour and flavor) of honey.

Carboxylic acids which can be found in honey are glutamic, aspartic, citric, acetic, formic, lactic, malic, pyruvic acid and many others.

The main acid in honey is gluconic acid, which originates from glucose oxidase provided by honeybees during ripening. Along with citric acid, they are used to separate floral honey from honeydew. These two acids, along with levulinic and formic, are responsible for higher concentrations of acids in free form (free acidity) in honey (1, 2).

Vitamins

Apart from other substances, honey is also a source of vitamins, primarily of the B complex: thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5),

pyridoxine (B6), biotin (B8 or H) and folic acid (B9). The source of named vitamins is pollen. Most types of honey also contain vitamin C that shows antioxidant effects. The low pH is the main reason for the stability of vitamins and, therefore, their presence in honey (1).

Minerals

Different types of honey, contain different groups of chemical compounds, among them being micro as well as macro element minerals. Most abundant are potassium, magnesium, calcium, iron, phosphorus, sodium, manganese, iodine, zinc, lithium, cobalt, nickel, cadmium, copper, barium, chromium, selenium, arsenic, and silver.

The content of minerals in honey varies. In light honeys, mineral content is approximately 0.4 %, and 0.2 % in dark honeys. Trace elements in honey are important for the determination of botanical origin since their content is dependent on the soil type which the flower source grew on. Potassium represents 33 % of all minerals and is, therefore, most abundant mineral in honey (1, 2).

Phenolic compounds

Phenolic compounds are, with approximately 10,000 compounds, a chemically heterogeneous group. They consist of non-flavonoids and flavonoids. Non-flavonoids are phenolic acids, while flavonoids are divided into 7 classes: flavones, flavonols, flavanones, flavanols, anthocyanidin, isoflavones and chalcones. Phenolic compounds are chemical structures with an aromatic ring and at least one hydroxyl group. They can either be simple molecules or very complex phenolic polymers.

Polyphenols possess antioxidative properties and are as such able to fight free radicals and prevent lipid oxidation.

According to their structure, they can be separated into two subgroups: the hydroxybenzoic and hydroxycinnamic acids.

Phenolic compounds in honey are used as floral markers and are studied for their antioxidant activity. Flavonoid's contribution to the total antioxidant activity is fairly significant, which makes them the main functional component of honey (1).

Volatile compounds

Complex mixtures of volatile compounds are responsible for the honey flavor. Their fractions vary based on honey's origin and collected nectar, as well as processing and storage conditions. When dealing with unifloral honey, certain volatile organic compounds can be the reason for honey's distinctive flavor of the plant.

These chemical compounds come from various origins. They can be transferred from the floral source, or produced by honeybees.

Some of the 400 identified volatile compounds are common indicators of commercial honey. For example, 3,9-epoxy-1-p-mentadiene, t-8-p-mentan-oxide-1,2-diol, and cis-rose have been suggested as indicators of lemon honey, main indicators of eucalyptus honey are diketones, sulfur compounds and alkanes, while the lavender honey's aroma is mainly formed of hexanal and heptanal.

Flavors of honey can be either spicy or rancid. This is determined by the lengths of the carboxylic acids carbon chains. In case the chains are short, for example, acetic acid, the aroma and flavor are spicy, but if the chain is longer (butanoic or hexanoic acid), the aroma is rancid. Alcohols are yet another important chemical structure in honey because they are responsible for its freshness (1).

1.3 MAIN TYPES OF HONEY

There are hundreds of types of honey around the world and each one is different in taste, depending on flowers honeybees visit during harvesting. If the honeybees collect honey only from one type of flowers, we are talking about single varietal honeys or uniflower/monoflower honeys. This process is monitored and aided by beekeepers who strategically place beehives in orchards or near one single type of flower.

Different types of honey based on the flower the pollen is collected from:

Alfalfa honey is made out of a plant with purple blossoms and found throughout North America (Canada and USA). This honey has a mildly flavored honey with an aroma similar to beeswax.

Avocado honey is a fully developed honey with a rich caramelized molasses flavor that leaves a flowery aftertaste.

Basswood honey tastes fresh, reminding of green, ripening fruit and is often differentiated by its distinctive lingering flavor.

Blueberry honey has an aroma similar to green leaves with a hint of lemon, and a gentle fruity flavor with a delicious aftertaste.

Buckwheat honey is a highly-flavored honey with molasses and malty flavors, and a lingering aftertaste.

Clover honey has a sweet, flowery flavor and a pleasing malt taste. It is the most important plant in production of honey.

Eucalyptus honey has a moderately sweet honey, whose flavor reminisces of herbs followed by a fruity aftertaste. In some, mild menthol flavor can be tasted.

Fireweed honey is a gentle, sweet honey with slight, tea-like notes.

Orange blossom honey has a sweet and fruity aroma that brings out the taste of citrus blossoms.

Sage honey is rich and light with a strong clover-like sweet flavor and a subtle floral aftertaste.

Sourwood honey is anise aromatized honey with a sweet, spicy flavor and a delightful, continuing aftertaste.

Tupelo honey is a honey of a complex aroma and taste. It is smooth and combines several flavors and aftertastes.

Chestnut honey is dark and spicy with a hint of smoke and leather. Suitable for those who do not like too sweet, because of its slightly bitter taste.

Meadowfoam honey is produced from various meadow flowers. The colour is golden-yellow, sometimes yellow-brown. This type of honey has a very sweet aroma and pleasant sweet taste. It has strong anti-bacterial, anti-inflammatory and analgesic characteristics (3, 4).

Manuka honey is mostly used for medical applications. It is made from nectar collected from the Manuka tree (*Leptospermum scoparium*), found in New Zealand and Southeastern Australia (6).

Lime honey has a typical light yellow to light amber colour. Fast crystallization is typical for this type of honey. It is of mild flavor and aroma. Lime honey is either of honeydew origin or it comes from a flower source.

Acacia honey is the most popular and widely used honey in Slovenia. It is known for its mild flavor, aroma, and colour. The colour is very light, almost white and its consistency is, opposed to other types of honey, very liquid (5).

Other honey floral sources include Black Locust, Blackberry, Brazilian pepper, Chinese Tallow, Cotton, Gallberry, Goldenrod, Mesquite, Mint Raspberry, Safflower, Saw Palmetto, Snowberry, Soybean, Star Thistle, Sunflower, Thyme, Tulip Polar.

There are also different forms of honey: liquid, whipped and comb. Centrifugal force, gravity or straining are used to extract liquid honey from the comb and that is why it is free of any crystals or wax. Whipped honey is thoroughly crystallized, resulting in a creamy and spreadable product. Comb honey is honey in its completely natural form– in the honeybee's wax comb (3, 4).

1.4 USE OF HONEY

In addition to being used as food, honey also has properties that have made it suitable for cosmetic and medical applications throughout human history.

In ancient cultures, it has been used as a binder or vehicle for herbal extracts and remedies, as skin moisturizer, hair dye, lip softener, face mask. Honeybee products have also been used for embalming, therefore preservation of corpses.

Honey has a wide spectrum of use. It has been used to treat wounds and burns for thousands of years. It is one of the oldest skin care ingredients and is still used to soothe skin infections and aging today. Chinese believe honey helps to prevent scars, clears away discoloration and freckles, and helps to improve the general skin appearance. Arab medicine uses honey for fungal infections of the skin, while in Burkina Faso laborers use it as a skin cleanser, probably due to its bacterial properties.

Honey is primarily used by dermatologists for its antimicrobial qualities, which are the result of hydrogen peroxide, released by enzymes from the honey. Hyperosmolarity of honey suppresses the growth of bacteria, while the inhibines (hydrogen peroxide, flavonoids, and phenolic acids) have a direct antibacterial effect.

There have been studies to confirm the ability of honey to help heal the skin and to impede and eliminate bacteria. One study showed honey treats superficial and partial-thickness burns more efficiently than sulphadiazine, an antibiotic used to treat burns in patients.

Medi-honey, a FDA-approved, irradiated with gamma rays and available in commercial stores, active manuka honey- has also been studied for its aid in treating chronic pressure ulcers in patients that had a spinal cord injury. It showed major improvement in wounds and antibacterial properties, which are, according to *in vitro* studies, due to methylglyoxal and osmotic effect, extracting moisture out of the environment and dehydrating bacteria.

Acidic environment (pH of honey ranges from 3,2 to 4,5) is as well an important factor for inhibition of microorganism growth. Besides Manuka honey, Ulmo honey also showed efficiency against methicillin-resistant *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa*. It is even considered to be potentially more effective than Manuka (6).

Honey's acidic nature is beneficial to regulating the pH of the upper protective skin layer, which is mildly acid. In addition, honey also nurtures the skin, has humectant and cleansing properties, shows soothing and anti-irritant characteristics, and is, therefore, suitable for a wide range of products, even for sensitive skin and babies. Various cosmetic products most commonly contain between 1 and 10 % of honey, because it's sticky, and difficult to dissolve. By combining honey with oily agents and emulsifiers, concentrations up to 70 % can be reached, while maintaining a satisfactory performance for the application.

It has been suggested, that honey could be used instead of emulsifiers in body-care products for bathing and shampooing. Even small addition of honey to a shampoo (3-20 %) helps to make hair fuller, preserves wave and lubricates, making it easier to comb. It has been suggested, that honey also possesses keratolytic properties, which makes it suitable for facial radiance-enhancing and anti-wrinkle preparations (7).

1.5 ANTIBACTERIAL ACTIVITY OF HONEY

The high sugar concentrations, hydrogen peroxide, low pH and also methylglyoxal and the antimicrobial peptide bee defensin-1 are responsible for honeys antibacterial properties.

Honey has been known for its antibacterial activity since the 19th century when it was used to treat and prevent infections of wounds. Its use has been reduced with the arrival of the antibiotics, but since the resistance is growing and development of new antibiotics is not increasing, the traditional use of honey is again becoming interesting. Even though the knowledge of honey's antibacterial activity is still incomplete, several honeys have been approved for clinical applications. Sterilization is a necessary step when eliminating potential bacterial presence in honey used for medical applications. This is usually done with gamma-radiation. Most commonly used medical-grade honeys are Manuka honey from New Zealand and Revamill®, which source is grown in greenhouses.

Unique manuka factor (UMF) is an industry standard phenol-equivalent scale used to express the antibacterial activity of manuka honey. This factor is important when

conducting radial diffusion assay with *Staphylococcus aureus* as targeted bacteria. It gives us the concentration of phenol solution needed for achieving a similar zone of growth inhibition as tested honey.

Because the antibacterial activity differs between batches, they are individually tested.

The assay using UMF has its advantages and disadvantages. While it gives us information about the antibacterial ability, it does not provide any information about the compounds responsible for it. Revamill® honey is approved for treatments of wounds but does not possess certified antibacterial abilities (8, 9).

1.5.1 ANTIBACTERIAL COMPONENTS IN HONEY

Honey, when ripened, contains 80 % sugars and less than 18 % of water. This combination of high sugar concentration and low moisture results in osmotic stress, preventing the honey to spoil. When honey is less than 40 % diluted, the antibacterial activity is due to high sugar content. Above this level, the antibacterial effects are caused by other components of honey.

A major antibacterial component in honey is hydrogen peroxide (H_2O_2). During the production of honey, honey bees add glucose oxidase to the collected honey, which is an enzyme that converts glucose to H_2O_2 and gluconic acid under aerobic conditions. H_2O_2 is supposed to protect the honey from microbial growth when the sugar levels are not yet high enough. Glucose oxidase stops working when the honey is ripe, but after dilution, it activates again. Highest concentrations of H_2O_2 are found in 30-50 % honey solutions, while its quantity decreases fast once the concentration of honey is below 30 %. This is caused because glucose oxidase, that comes from honeybees, has low affinity for glucose.

The accumulation of H_2O_2 reduces when it's degraded by honey or can be affected by inactivation of glucose oxidase when exposed to heat or light.

Various honeys, for example, manuka honey, possess significant antibacterial activity that is caused by components that are not H_2O_2. One of them is methylglyoxal (MGO), which is built from sugars while exposed to heat or lengthy storing of foods and beverages containing carbohydrates. In Manuka honey, the source of MGO is converted dihydroxyacetone (DHA), which can be found in the nectar of *Leptospermumm scoparium* flowers in extremely high concentrations. Because this conversion is not aided by enzymes, it occurs slowly during honey storage.

Studies have recently identified peptide bee defensin-1 as a potential antimicrobial component of honey. This peptide, also known as royalsine, can be found in honeybee hemolymph, honeybee head, and thoracic glands, and in royal jelly, that is primary food of queen bee larvae. Bee defensin-1 has potential activity against Gram-positive bacteria, including *B. subtilis, S. aureus*, and *Paenibacillus larvae*. Antimicrobial peptides (AMPs) are an important part of honeybee's innate immune system. When infected with *E. coli* the hemolymph produces four types of AMPs: hemenoptecin, bee defensin-1, apidaccin and the group of abaccin peptides. Together they work against all main groups of microorganisms since each one has a specific range of antimicrobial activity. But so far bee defensin-1 has only been identified in Revamill® and not in other honeys. Honeybees secrete bee defensin-1from their hypopharyngeal glands, which they also produce honey and royal jelly with (therein referred to as "royalisin").

Various phenolic compounds with antibacterial properties have been found in honeys, but it is not yet completely clear to what extent they contribute to the overall antimicrobial activity of honey (8).

1.5.2 METHODS TO ASSESS THE ANTIBACTERIAL ACTIVITY OF HONEY

The method of choice for estimation of antibacterial activity of honey used for medical applications is the agar diffusion assay with *S. aureus,* but it has some disadvantages. For example, because not all species of bacteria react in the same way to honey and its components, measuring antibacterial activity against *S. aureus* is not very representative.

This assay estimates the activity of honey according to the size of the growth inhibition zone, but this size is not only dependent on the antimicrobial activity but on the movement of antibacterial components through the agar as well. And since high molecular antibacterial compounds move difficultly through the matrix, honey with these molecules could be characterized as having low antibacterial activity. This method also does not differentiate between growth inhibiting and bactericidal activity and does not offer a possibility for quantification of bactericidal activity or the motion of killing. By using a quantitative liquid bactericidal assay and a wider range of bacterial species, all these disadvantages could be overcome (8).

1.6 ABOUT PHENOLIC COMPOUNDS

Phenolic compounds or polyphenols are molecules with a polyphenol structure, meaning they have several hydroxyl groups on an aromatic ring. Several thousands of these molecules have been identified in higher plants, and several hundred in edible plants, which means they are one of the biggest classes of plant occurring molecules.
Polyphenols are also secondary metabolites of plants and help in the defense against ultraviolet radiation or aggression by pathogens. Analysis of polyphenols in honey is believed to be a suitable method for determining floral and geographical origins of honey.
As a function of the number of phenol rings and of the structural elements that bind rings together, these compounds can be categorized in different groups: phenolic acids, flavonoids, stilbenes, and lignans. The most known among stilbenes is resveratrol, which can be found in red wine, and has shown anticarcinogenic effects. The richest dietary source of lignans is linseed, but small amounts can also be found in algae, legumes, gluten containing cereals, some fruits and vegetables. Phenolic acids and flavonoids can be, among other sources, found in honey (10).

1.6.1 PHENOLIC ACIDS

Phenolic acids are classified into two groups: derivatives of benzoic acid and derivatives of cinnamic acid. Hydroxybenzoic acids are not very common in the human diet and have therefore not been extensively studied as opposed to hydroxycinnamic acids. These are mostly represented by *p*-coumaric, caffeic, ferulic, and sinapic acids, which are rarely found in free form, except in processed food. The richest sources of hydroxycinnamic acid are blueberries, kiwis, plums, cherries and apples, especially outer parts of ripe fruits. The phenolic acid that is most common is caffeic acid, which represents 75-100% of hydroxycinnamic acids found in fruits, followed by ferulic acid, most abundant in cereals and that exists mostly in *trans* form.
Analysis of phenolic compounds can be used for the research of honey origin, floral as well as geographical. For instance, hesperidin is a known indicator of citrus honey, kaempferol of rosemary honey, quercetin of sunflower honey, ellagic acid of heather honey and hydroxycinnamates of chestnut honey.
Based on the concentration of flavonoids derived from propolis, we can differentiate between Australian and European Eucalyptus honey. This is important because the

botanical origin of honey is a basic quality indicator, which also commonly affects the price (10,11).

1.6.2 FLAVONOIDS

Flavonoids are chemical structures with the basic structure of benzopyran. They possess antibacterial, anti-inflammatory, anti-allergic and anti-thrombotic properties (10,11).

The reason for antioxidant activity of flavonoids is their capability of trapping the reactive oxygen species and suppression of superoxide anions producing enzymes. They are also able to chelate transition metals, that contribute to the formation of radicals and to reduce alcoxyl and peroxyl radicals, therefore keeping peroxidation process from happening (11).

Flavonoids can be very good for our health, but the effects are dependent on how much of them we consume and on their bioavailability. There are six subclasses of flavonoids, depending on the type of heterocycle involved: flavonols, flavanones, flavones, anthocyanidins, isoflavones and flavanols (catechins and proanthocyanidins).

1.6.3 ISOLATION AND IDENTIFICATION

To determine individual phenolic compounds, we must follow some basic steps. First, we must isolate the phenolic compounds from a simple matrix, which is usually done by solid-phase extraction (SPE) or by using different solvents, then follows analytical separation, usually achieved with high performance liquid chromatography (HPLC), capillary electrophoresis (CE) or gas chromatography (GC). After completing these two steps, we can identify and quantify the compounds.

To achieve detection of phenolic compounds ultraviolet (UV) absorption, in association with a photodiode detector and various mass-spectral methods is used.

The sample of honey we are determining polyphenols in must be representative, in order to avoid inconsistency in results. This means it has to possess the same properties as the whole batch. To achieve that, we must thoroughly stir the sample, either by hand or using a machine, to homogenize it. In case of crystallization, we can use a stove or a thermostatic bath to heat it, but the temperature must not exceed 40-50°C.

For a successful analysis of phenolic compounds in honey, we must first remove the sugars. This step will remove matrix components and also isolate and concentrate analytes. When using liquid-liquid extraction (LLE), ethyl acetate or ethanol are usually used. LLE

is used to isolate aglycones, while some other methods can be directed at isolating both aglycones and conjugates.

When using solid phase extraction (SPE) for the removal of the matrix, we pack the cartridges with different solvents. C18 silica is used most commonly, but it has been found, that polymer sorbents (polymeric Strata-X) are a better choice, because they offer, due to their aromatic structure, a better performance. Studies showed that Amberlite XAD2 is able to completely sorb kaempferol, *p*-coumaric acid and syringic acid, but using methanol for the recovery of quercetin is bad (only 54% recovery).

Separation of phenolic compounds and flavonoids is usually achieved using HPLC equipped with RP columns. An alternative is using HPLC with monolithic columns which is faster. When we are trying the recognize how complex the phenolic profile of our honey sample is, we can use gradient elution.

The most common mobile phase used is a binary system of an aqueous component and a less polar organic solvent. Acids are added for maintenance of pH.

The usual elution pattern is benzoic acid, cinnamic acid, flavanone glycoside, followed by flavonols and flavone glycosides and then free aglycones in the same order.

The most popular technique, for separation of polyphenolic compounds, is HPLC, but capillary electrophoresis, as an alternative, is also gaining in popularity. Because CE is fairly new as a separation technique, it is still in the process of evolution. The detection of analytes using HPLC and CE, is usually performed by measuring UV absorption, frequently done by diode-array detection (DAD), which appears to be very successful (11).

2 THE AIM OF THE WORK AND WORKING HYPOTHESES

Honey has been used for treatment of burns, colds, sore throat, cough, etc. for a long time. Therefore, we have decided to investigate the physicochemical and biological properties of two different honeys, to find out which compounds are responsible for the believed effects of honey, or whether the supposed medical applications are merely a myth.

We will determine the antibacterial and antifungal activity of Slovenian lime and Portuguese eucalyptus honey, by investigating the content of sugars, proteins, proline, phenolic compounds, flavonoids and hydroxymethylfurfural. Also, the ferric reducing antioxidant capacity, free radical scavenging capacity and oxygen radical absorbance capacity will be determined.

In addition, we will determine the colour, pH, free acidity, electrical conductivity and water content to try and find connections between different parameters.

Based on other studies that have already been carried out and the traditional uses, we hypothesize that:

- honey has antibacterial and antifungal properties,
- the total flavonoid and phenolic content are in positive correlation with colour,
- free radical scavenging capacity of honey is in positive correlation with total flavonoid content.

3 MATERIALS AND METHODS

3.1 HONEY SAMPLES AND PREPARATION OF EXTRACTS

For evaluation of physicochemical and biological properties we have used Slovenian lime honey from Vajda Maksimilijan and Portuguese SerraMel eucalyptus honey.

During this research, all pure honey samples and extracts prepared by liquid-liquid extraction were stored in the dark at room temperature, and the extracts prepared by solid phase extraction were stored in the dark at 80°C.

Eucalyptus honey extract was prepared by SPE using a column of Amberlite XAD-2. Honey sample was prepared by dissolving 10 g of eucalyptus honey in 50 mL of 0,01 M HCl (pH 2). After the elution of sugars with acid aqueous solution and polar compounds with deionized water, phenolic compounds remained in the column and were eluted with 100 mL of methanol. Methanol extract was then concentrated in a rotary evaporator at 40 °C. The residue was dissolved in 15 mL of deionized water and extracted 3 times with 10 mL of diethyl ether for further purification of the flavonoids. Diethyl ether was then removed in a rotary evaporator at 40 °C and dissolved the residue in 5 mL of methanol. There was quite a bit of oily compounds from honey visible in the sample and we can assume they are probably the reason for higher mass of extracted phenolic compounds (1,4 g).

Liquid-liquid extraction was used for preparation of both eucalyptus and lime honey extracts. 10 g of eucalyptus honey was dissolved in 50 mL of 0,01 M HCl solution (pH) and 2 g of lime honey in 10 mL of 0,01 M HCl solution (pH 2). Eucalyptus honey sample was extracted 3 times with 25 mL of ethyl acetate and lime honey sample 3 times with 10 mL of ethyl acetate. Acetate from organic extract phases was evaporated with rotary evaporator at 40 °C.

For antioxidant assays honey extracts (1 mg/mL) were prepared by dissolving 10 mg of lime honey and 10 mg eucalyptus honey extract in 1 mL of DMSO.

Artificial honey was prepared with thoroughly mixing 77 g of glucose and 3 g of sucrose in 20 mL of distilled water. Artificial honey was used in the assays for determination of sugar interference.

3.2 PHYSICOCHEMICAL ASSAYS

3.2.1 COLOUR ANALYSIS – SPECTROPHOTOMETRIC METHOD

For colour determination of honey samples, we dissolved 2,5 g of each honey in 5 mL of deionised water (honey sample solution 50% (w/v), 0,50 g/mL). We then read the absorbance of each honey sample solution using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer at the wavelengths of 720 nm, 635 nm and 450 nm against a blank of deionised water, performing triplicate assays.

3.2.2 DETERMINATION OF ELECTRICAL CONDUCTIVITY

Samples for determination of electrical conductivity were prepared by dissolving 1,2 g of lime and 1,2 g of eucalyptus honey in 5 mL of deionised water (honey sample solution 20 % (w/v), 0,20 g/mL). Electrical conductivity was measured at 22°C with conductivity meter using 0,01 mol/L potassium chloride solution (κ = 1,413 mS/cm at 25°C) as calibration standard.

3.2.3 DETERMINATION OF MOISTURE (WATER CONTENT) IN HONEY

The water content was determined from the refractive index of honey by reference to a standard table. Refractive index was measured using Digital Abbe refractometer, by placing 2 drops of each honey at the centre of the prism and reading the value on the refractometer scale for each honey, at 22°C. After performing triplicate assays, we calculated mean refractive index (n) and obtained the corresponding water content (W, in g/100 g honey), relative density (d, adimensional) and total soluble solids content (°Bx) from the Conversion Table available from the International Honey Commission (IHC) for honey.

3.2.4 DETERMINATION OF pH AND FREE ACIDITY

For determination of pH and free acidity 5 g of each honey was dissolved in deionized water in a 50 mL volumetric flask (honey sample solution 10 % (w/v), 0,10 g/mL). The initial pH was measured using pH meter.

For determination of free acidity titrimetric method was used and titration was carried out using 0,10 M sodium hydroxide solution, which was standardised with 0,010 M HCl. To each of honey sample solutions 2 drops of indicator were added and then titrated with 0,10

M NaOH solution until the formation of stable pink colour. Duplicate assays were performed and the volume of 0,10 M NaOH used was registered.

3.2.5 DETERMINATION OF PROTEIN CONTENT

For estimation of protein content in honey colorimetric method of Lowry was used (12). Bovine serum albumin (BSA) was used as standard for calibration curve. Honey samples were prepared by dissolving 0,10 g of each honey in 10 mL of deionised water (honey sample solution 1,0 % (w/v), 10 mg/mL).

The absorbance of solutions was read at 660 nm using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer against water blank. We performed triplicate assays.

3.2.6 DETERMINATION OF APPARENT REDUCING SUGARS

For determination of apparent reducing sugars, the colorimetric method of 3,5-dinitrosalicylic acid (DNS) was used. This method 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 (13). Anhydrous D-glucose (M = 180,16 g/mol) was used as standard for calibration curve.

Samples were prepared by dissolving 1g of both lime and eucalyptus honey in deionised water in 100 mL volumetric flasks (honey solution 1% (w/v), 10 mg/mL).

The absorbances were read at 540 nm using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer against water blank.

3.2.7 DETERMINATION OF PROLINE CONTENT

For determination of proline content colorimetric method was used. Samples were prepared by dissolving 0,5 g of both lime and eucalyptus honey in 10 mL of deionised water (honey sample solution 5 % (w/v), 0,05 g/mL). L-proline standard solutions were used as standard for calibration curve. The reagents used in this assay were 1,5 % (w/v) ninhydrin solution in glacial acetic acid and 50 % (v/v) 2-propanol aqueous solution. We measured the absorbance of the solutions at 510 nm against a water blank. We performed triplicate assays.

3.2.8 DETERMINATION OF HYDROXYMETHYLFURFURAL (HMF) CONTENT

For determination of 5-(hydroxymethyl)furan-2-carbaldehyde (5-hydroxymethyl-2-furfuraldehyde or hydroxymethylfurfural, HMF) content in honey samples the White method (White, 1979) was used (14).

Samples were prepared by dissolving 5 g of honey in deionised water in a 50 mL volumetric flask, after adding 0.5 mL of Carrez I solution (1.5 g of potassium hexacyanoferrate(II) ($K_4Fe(CN)_6.3H_2O$, M = 422.39 g/mol) dissolved in 10 mL of deionised water) and 0.5 mL of Carrez II solution (3.0 g of zinc acetate ($Zn(CH_3COO)_2.2H_2O$, M = 219.51 g/mol) dissolved in 10 mL of deionised water) (honey sample solution 10% (w/v), 0.10 g/mL). The solutions were filtered and first 10 mL of filtrate was rejected.

We determined the sample absorbance against the reference solution at 284 nm and at 336 nm using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer.

3.3 ANTIOXIDANT ASSAYS

3.3.1 TOTAL PHENOLIC CONTENT (TPC) ASSAY

For determination of total phenolic content Folin-Ciocalteu method was used. Pure honey samples (honey sample solution 5% (w/v) 0,05 g/mL) were prepared by dissolving 0,50 g of eucalyptus honey and 0,5 g of lime honey in 10 mL of deionized water.

Artificial honey sample was prepared by dissolving 1 g of artificial honey in 2 mL of deionized water (artificial honey solution 50% (w/v) 0,5 g/mL).

Gallic acid was used as standard solution for the calibration curve. To all samples we added 3,5 mL of Folin-Ciocalteu reagent and vortexed the solutions. After 5 minutes, we added 2 mL of 7,5% sodium carbonate aqueous solution to each test tube. We incubated prepared solutions for 2 hours in the dark at room temperature and read the absorbance at 760 nm against water blank using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer. We performed triplicate assays.

3.3.2 TOTAL FLAVONOID CONTENT (TFC)

For determination of total flavonoid content Dowd method was used. Pure honey samples were prepared by dissolving 0,50 g of eucalyptus honey and 0,5 g of lime honey in 10 mL of methanol (honey sample solution 5 % (w/v) 0,05 g/mL).

Artificial honey sample was prepared by dissolving 0,5 g of artificial honey in 10 mL of

Quercetin was used as standard solution for the calibration curve. We added 2,5 mL of aluminium chloride solution to all samples. We covered the test tubes with aluminium foil to avoid methanol evaporation and incubated for 10 minutes in the dark at room temperature. We measured the absorbance using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer at 415 nm against methanol blank. We performed triplicate assays.

3.3.3 FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

By performing ferric reducing antioxidant power assay, we have measured the reducing power of the antioxidant agents in the sample.

Pure honey samples were prepared by dissolving 0,50 g of eucalyptus honey and 0,50 g of lime honey in 10 mL of deionised water (honey sample solution 5 % (w/v) 0,05 g/mL).

Artificial honey sample was prepared by dissolving 0,50 g of artificial honey in 10 mL of deionized water (artificial honey solution 5 % (w/v) 0,05 g/mL).

Ferrous sulphate was used as standard solution for the calibration curve. We added 4,5 mL of FRAP reagent to all samples, covered them with aluminium foil and incubated for 10 minutes in the dark at 37°C. We measured the absorbance of solutions at 593 nm against a water blank using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer. We performed triplicate assays.

3.3.4 DETERMINATION OF FREE RADICAL SCAVENGING CAPACITY

For determination of free radical scavenging capacity DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used.

Pure honey samples were prepared by dissolving 1,0 g of eucalyptus honey and 1,0 g of lime honey in 10 mL of methanol (honey sample solution 10 % (w/v) 0,10 g/mL).

Artificial honey sample was prepared by dissolving 1,0 g of artificial honey in 10 mL of deionized water (artificial honey solution 10 % (w/v) 0,10 g/mL).

Trolox was used as standard solution for the calibration curve. We added 4,5 mL of 0,06 DPPH solution to all samples and shook them with vortex. We covered the test tubes with aluminium foil to avoid methanol evaporation and incubated for 30 minutes in the dark at room temperature. We measured the absorbance of solutions at 517 nm against methanol blank using UV-VIS spectrophotometer HITACHI U-2000 spectrophotometer. We performed triplicate assays.

3.3.5 OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC) ASSAY

For determination of oxygen radical absorbance capacity, microplate method was used. Pure honey samples were prepared by dissolving 0,10 g of eucalyptus honey and 0,10 g of lime honey in 10 mL of phosphate buffer (honey sample solution 1 % (w/v) 0,010 g/mL).
Trolox was used as standard solution for the calibration curve. We added 150 μL of 0,08 μM fluorescein solution to all samples, covered the microplate and incubated it at 37°C for 10 minutes. We then added 25 μL of AAPH solution to all wells and shook the microplate for 3 seconds. We read the fluorescence at 37°C every 5 minutes for 4 h, at the excitation wavelength of 485 nm and at the emission wavelength of 535 nm using a microplate reader.

3.4 ANTIMICROBIAL AND ANTIFUNGAL ACTIVITY

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).

3.4.1 SAMPLE PREPARATION

Pure honey samples were prepared by dissolving 1 g of each honey in 2 mL of deionized water (honey sample solution 50 % (w/v) 0,5 g/mL).
Honey extracts were prepared by dissolving 10 mg of extract and dissolving in 1 mL of DMSO (honey extract solution 0,1 % (w/v) 1 mg/mL).

Artificial honey was prepared by thoroughly mixing 77 g of glucose and 3 g of sucrose in 20 mL of deionized water. 1 g of this solution was then dissolved in 2 mL of deionized water (artificial honey solution 50 % (w/v) 0,5 g/mL). Artificial honey was used as negative control in antibacterial/antifungal assay to exclude the possibility of high sugar content (and therefore high osmolarity) being the reason for antibacterial/antifungal activity of honey.

3.4.2 MICROORGANISMS AND GROWTH CONDITIONS

The *in vitro* antimicrobial assays were performed using both Gram positive and Gram negative bacteria. Gram positive bacteria used were *Staphylococcus aureus* and *Enterococcus faecalis*, Gram negative *Escherichia coli* and *Pseudomonas aeruginosa*, and yeasts *Saccharomyces cerevisiae* and *Candida albicans*.

3.4.3 MICRODILUTION METHOD

The minimum inhibitory concentration (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.

3.4.4 WELL DIFFUSION ASSAY

The well diffusion assay was used to determine the minimum bactericidal concentration (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. Each assay was performed in triplicate.

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 (15, 16).

4 RESULTS AND DISCUSSION

4.1 PHYSICOCHEMICAL ASSAYS

Physicochemical assays were perfomed due to importance of organoleptic properties and composition of honey for determination of floral and geographical origin of honey. They can also be an important factor in determining the quality and pureness of honey samples.

4.1.1 COLOUR ANALYSIS

According to colour, based on the Pfund scale (mm), honey is divided in 7 classes.

If the value is lower than 9 mmPfund, the honey is classified as water white. When the value is between 9 and 17 mmPfund, the colour of honey is extra white and between 18 and 34 mmPfund white. Honeys with values from 35 to 50 mmPfund are extra light amber. The following class is light amber for honeys with values between 51 and 85 mmPfund. Honeys with Pfund values from 86 to 114 mm are classified as amber and those with values over 114 mm as dark amber (17).

Literature classifies lime honey, according to colour, from white to light amber, with mm Pfund values from 19 to 78 (18). Our obtained value, 72,75 mm Pfund, as shown in the table 1, puts lime honey in the darkest possible class for lime honeys.

Obtained value for colour classification of eucalyptus honey was 118,77 mmPfund (Table 1), which is higher than the values in literature, which vary from 90,54-93,88 mm Pfund (19). Our value classifies eucalyptus honey as dark amber, and literature classifies it as amber.

Table 1: Concentration, mean absorbance at 720 nm, 635 nm and 450 nm, mm Pfund, colour (class) and colour intensity of honey samples.

	c (g/mL)	**A_{720}**	**A_{635}**	**A_{450}**	**mm Pfund**	**Colour classification of honey**	**ABS450 (mAU)**
LIME HONEY	0,5	0,18	0,22	0,5	72,75	Light amber	320
EUCALYPTUS HONEY	0,5	0,29	0,36	0,87	118,77	Dark amber	581

4.1.2 ELECTRICAL CONDUCTIVITY

As shown in Table 2, electrical conductivity of lime honey is 0,53 mS and electrical conductivity of eucalyptus honey is 0,71 mS. Electrical conductivity values for lime honey

in literature vary from 0,2 to 0,8 mS (18, 20) and the values for eucalyptus honey are around 0,5 mS (21, 22). That means the electrical conductivity value for lime honey matches the reference values and the obtained value for eucalyptus honey is a bit high. But since the value doesn't exceed 0,8 mS, the results are according to The European Union rules (23).

Our obtained total ash content for eucalyptus honey (0,33 g/100 g) matches the values found in literature which vary from 0,32 to 0,46 g/100 g (22).

Literature shows total ash content in lime honey varies from 0,23 to 0,30 g/100 g (18, 20), meaning our obtained value 0,27 g/100 g is in agreement.

Table 2: Concentration, mean conductance, electrical conductivity and total ash content of honey samples

	c (g/mL)	**G (mS)**	***K* honey (mS/cm)**	**Total ash content (A) (g/100 g)**
LIME HONEY	0,2	0,53	0,62	0,27
EUCALYPTUS HONEY	0,2	0,62	0,71	0,33

4.1.3 MOISTURE (WATER CONTENT)

As shown in Table 3 the obtained water content of lime honey sample was 19 %, which matches the literature data that show water content in lime honey varies from 15-19 % (18).

Literature data for eucalyptus honey shows water contents between 14 and 19 %. Our obtained value, 17,2 %, therefor matches the expected criteria. The corresponding TSS value 81,25°Bx is also in agreement with the literature values which vary between 80 and 82°Bx (21, 22).

Table 3: Mean refractive index, water content, relative density and total soluble solids (TSS) content of honey samples.

	nD (average)	**W (g/100 g HONEY)**	***d***	**°Bx**
LIME HONEY	1,489	19	1,41	79,39
EUCALYPTUS HONEY	1,494	17,2	1,42	81,25

4.1.4 pH AND FREE ACIDITY

The initial pH of lime honey measured was 3,92 (Table 4), which matches the values found in literature where they vary from 3,8 to 4,6 (18, 20). Free acidity of lime honey varies from 12,8 to 45,6 meq/kg, according to the literature, meaning our obtained value 24,24 meq/kg is in expected range (18, 20).

According to the literature pH of eucalyptus honey is between 3,4 and 4,7 (22), which matches our initial pH measured (3,98).

The free acidity we obtained for eucalyptus honey is a bit high, since most of the literature data shows values between 17-27 meq/kg (21, 22). But since there are cases of free acidity exceeding 40 meq/kg, our value 36,14 meq/kg is not that unusual (21).

Table 4: Initial pH, mean volume of NaOH used and free acidity of honey samples.

	pHi	**V NaOH (mL)**	**Free acidity - FA (meq/kg)**
LIME HONEY	3,92	0,6	24,24
EUCALYPTUS HONEY	3,98	0,9	36,14

1.1.1 PROTEIN CONTENT

Protein content was measured in honey samples pre-treated with copper(II) alkaline solution (stabilized with sodium and potassium tartrate) to form a metal-protein complex and quantification was made based on the absorbance of the solution at 660 nm from a calibration curve using bovine serum albumin (BSA) as standard (model protein).

Table 5: Concentration, mean absorbance and protein content of honey samples

	c (mg/mL)	**A**	**PROTEIN CONTENT (mg BASE/mL SAMPLE)**	**PROTEIN CONTENT (g BASE/100 g HONEY)**
LIME HONEY	10	0,261	0,392	0,39
EUCALYPTUS HONEY	10	0,339	0,510	0,51

The obtained protein content for lime honey was 0,39 g/100 g and for eucalyptus honey 0,51 g/100 g (Table 5). The literature data for protein content of lime honey is between 0,15 and 0,51 g/100 g honey (20), and for eucalyptus between 0,23 and 0,49 % (24), which means our values are more or less in the expected range.

4.1.5 APPARENT REDUCING SUGARS

The content in apparent reducing sugars, i.e. sugars containing aldehyde (e.g., glucose) or ketone (e.g., fructose) groups, is defined as the amount of sugar able to reduce an oxidant agent (e.g., Fehling's reagent) at specific conditions. The fructose/glucose ratio and the sucrose content are quality criteria of honey which allow differentiation between monofloral honeys.

We have calculated the total sugar content based on the values for total soluble solids (TSS) obtained when measuring the water content of our samples. The calculations were based on the fact that 1 °Bx equals 1 g of sugar per 100 g of honey.

Table 6: Concentration, mean absorbance, reducing sugars and total sugar content of honey samples.

	c (mg/ml)	**A**	**REDUCING SUGARS CONTENT (mg GE/mL)**	**APPARENT REDUCING SUGARS CONTENT (g GE/100 g HONEY)**	**TOTAL SUGAR CONTENT (g/100 g HONEY)**
LIME HONEY	2,5	0,122	0,14	13,85	79,39
EUCALYPTUS HONEY	2,5	0,089	0,106	10,47	81,25

Literature data shows total sugar content for eucalyptus honey varying between 67,80 and 88,30 (24), and our obtained values (Table 6) are in agreement with that. But the values for apparent reducing sugars in literature are a lot higher (72,34 ± 2.46 %) than our obtained one (10,47 %) (24).

Literature show total sugar content values for lime honey around 72 % (25), therefore our obtained value is in agreement. But the apparent reducing sugars content was again too low, because the rules suggest it should be above 65 %. A possible reason for this result could be that the analysis was not long enough for the sugars to completely react with Fehling's reagent.

4.1.6 PROLINE CONTENT

The proline content, expressed in mg per kg, 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 proline content is used as

a criterion of honey ripeness and eventual sugar adulteration when the value is below a certain limit.

Table 7: Concentration, absorbance and proline content of honey samples.

	c (mg/mL)	**A**	**PROLINE CONTENT (mg LPE/kg HONEY)**	**PROLINE CONTENT (mg LPE/ 100 g HONEY)**
LIME HONEY	50	0,086	440,8	44,08
EUCALYPTUS HONEY	50	0,058	300,2	30,02

The proline content was determined from a calibration curve obtained by plotting the absorption of the L-proline standard solutions against L-proline concentration. Obtained proline content for lime honey, as shown in Table 7, was 440,8 mg/kg. The literature data shows, that proline content in lime honey varies all the way from 250 to 790 mg/kg (18, 20), meaning our samples' proline content is around the mean value.

Proline content for eucalyptus honey was 300,2 mg/kg. Literature values vary from 112 to 987 mg/kg, the mean value being 429,5 mg/kg (21). That means, our obtained value is in expected range.

Since the values for both lime and eucalyptus honey are above 180 mg/kg, we can say that both honeys are ripe.

4.1.7 HMF CONTENT

The HMF, formed by heating of hexoses in acidic media or by Maillard reactions, is an indicator of honey freshness, being almost inexistent in fresh honey. The concentration of HMF increases during storage and HMF content depends on honey pH and storage temperature.

For elimination of honey colour interference, the difference between the absorbances of a clean aqueous honey solution (using Carrez reagent as clarifying agent) and the same solution after reduction of HMF by addition of bisulphite was be determined. The HMF content is calculated after subtraction of the absorbance background at 336 nm.

Table 8: Concentration, absorbance at 284 nm, absorbance at 336 nm and HMF content of honey samples.

	c (g/mL)	A_{284}	A_{336}	HMF (mg/kg)
LIME HONEY	0,1	0,251	0,052	59,4
EUCALYPTUS HONEY	0,1	0,083	0,027	16,6

Literature data for eucalyptus honey shows HMF content from around 3 to 33 mg/kg (21, 22), but most of the values are around 15 mg/kg, which matches our obtained value of 16,6 mg/kg (Table 8).

However, the obtained value for lime honey is a lot higher than in literature, with the value being 59,4 mg/kg, and reference numbers varying from 0,5 to 14,7 mg/kg (18), which can be due to the transportation of honey from Slovenia to Portugal. The Codex Alimentarius standards stipulate 80 mg/kg of HMF as maximum limit while the European Unit has a limit of 40 mg/kg and a value ≤ 60 mg/kg is recommended after processing and/or mixture (26).

4.2 ANTIOXIDANT ASSAYS

With the use of different methods, we have assessed the antioxidant capacity of both lime and eucalyptus honey. We have determined the content of polyphenols and also just flavonoids in our samples, since these compounds are most likely responsible for antioxidant properties of honey.

4.2.1 TOTAL PHENOLIC CONTENT (TPC)

The total phenolic content (TPC), expressed in mg of gallic acid equivalents (GAE) per 100 g and kg of honey, was determined from the absorbance of honey sample at 760 nm based on the calibration curve (plot of absorbance of gallic acid standard solutions against gallic acid concentration) and according to sample dilution of honey in the sample.

Table 9: Concentration, absorbance at 760 nm and total phenolic content of honey extracts.

	c (mg/ml)	A_{760}	TPC (mg GAE/mL) SAMPLE (per mg of extract)	TPC (mg GAE) per g of extract
LIME EXTRACT	1	0,127	0,0132	13,21
EUCALYPTUS EXTRACT	1	0,139	0,0145	14,53

Table 10: Concentration, absorbance at 760 nm, total phenolic content for honey samples and artificial honey, and corrected TPC values.

	c (mg/ml)	**A_{760}**	**TPC (mg GAE/mL) SAMPLE**	**TPC (mg GAE/kg honey) corrected values**
ARTIFICIAL HONEY	0,5	0,036	0,0035	**(for sugar interference)**
LIME HONEY	0,05	0,185	0,0194	381,88
EUCALYPTUS HONEY	0,05	0,346	0,0367	726,10

Total phenolic content in lime honey was higher in the extract, 0,0132 mg GAE/mL (Table 9), than in pure honey sample, 0,0159 mg GAE/mL (Table 10, corrected value).

In eucalyptus honey, total phenolic content was higher in pure honey sample, 0,0332 mg GAE/mL (corrected value), than in the extract, 0,0145 mg GAE/mL.

Literature data shows TPC values for eucalyptus honey around 12 mg GAE/kg (27) and for lime honey around 9 mg GAE/kg (28, 29). Our obtained values were a lot higher, 381,88 mg GAE/kg for lime honey and 726,10 mg GAE/kg for eucalyptus honey. These high values could mean that Folin-Coicalteu reagent not only reacted with polyphenols, but with other compounds as well.

4.2.2 TOTAL FLAVONOID CONTENT (TFC) – DOWD METHOD

Total flavonoid content (TFC) was determined by use of aluminium chloride method, also known as Dowd method. It is based on the formation of a yellow complex between Al^{3+} ion, from 1 % aluminium chloride solution in methanol, and the carbonyl and hydroxyl groups of flavonoids (flavones and flavonols), which absorbs at 415 nm.

The total flavonoid content (TFC), expressed in mg of quercetin equivalents (QE) per 100 g of honey was determined from the absorbance of the sample solution at 415 nm based on the calibration curve (plot of the absorbance of the quercetin standard solutions against quercetin concentration) and according to the sample dilution and mass of honey in sample.

Table 11: Concentration, absorbance at 415 nm and total flavonoid content of honey extracts.

	c (mg/ml)	A_{415}	TFC (mg QE/mL SAMPLE)	TFC (mg QE/g of extract)
LIME EXTRACT	1	0,068	0,0069	6,90
EUCALYPTUS EXTRACT	1	0,078	0,0079	7,92

Table 12: Concentration, absorbance at 415 nm and total flavonoid content of honey samples.

	c (mg/ml)	A_{415}	TFC (mg QE/mL SAMPLE)	TFC (mg QE/100 g honey)
ARTIFICIAL HONEY	0,05	0,020	0,0020	3,992
LIME HONEY	0,05	0,100	0,0101	20,30
EUCALYPTUS HONEY	0,05	0,163	0,0166	33,15

Total flavonoid content was lower in honey extracts, 0,0069 mg QE/mL for lime and 0,0079 mg QE/mL for eucalyptus extract (Table 11), than in pure honey samples, where the values were 0,0101 mg QE/mL for lime and 0,0166 mg QE/mL for eucalyptus honey (Table 12).

Total average flavonoid content for eucalyptus honey in literature varies from 3,85 to 7,61 mg QE/100 g of honey (27) and for lime honey 0,63 mg QE/100 g of honey (30). That means are obtained values were much higher than expected.

4.2.3 FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

The ferric reducing antioxidant power (FRAP) assay measures the reducing power of the antioxidant agents in the sample. The method is based in the reduction of ferric 2,4,6-tripyridyl-s-triazine (Fe^{3+} - TPTZ) to the blue ferrous complex (Fe^{2+}-TPTZ) in acidic media (pH 3,6) to maintain iron solubility.

The ferric reducing antioxidant power (FRAP), expressed in milimols of Fe^{2+} equivalents per 100 mg of honey (mmol Fe(II)/100 mg), was determined from the absorbance of the honey sample based on the calibration curve (plot of the absorbance of the ferrous sulphate standard solutions against ferrous sulphate concentration) according to the sample dilution

and mass of honey in the sample. The EC_{50} value (sample concentration able to reduce 50 % of Fe^{3+} ions to Fe^{2+} ions) was determined from the plot of solution absorbance against concentration (of samples or standards).

Table 13: Concentration, absorbance at 593 nm and ferric reducing antioxidant power of honey extracts.

	c (mg/ml)	**A_{593}**	**FRAP (mmol Fe(II)/L SAMPLE)**	**FRAP (μmol Fe(II)/g extract)**
LIME EXTRACT	1	0,115	0,0461	46,06
EUCALYPTUS EXTRACT	1	0,165	0,0662	66,21

Table 14: Concentration, absorbance at 593 nm, ferric reducing antioxidant power and corrected FRAP values of honey samples.

	c (mg/ml)	**A_{593}**	**FRAP (mmol Fe(II)/L SAMPLE)**	**FRAP (μmol Fe (II)/L) corrected values**	**FRAP (μmol Fe (II)/100 g) corrected values**
ARTIFICIAL HONEY	0,5	0,014	0,0056	(for sugar interference)	(for sugar interference)
LIME HONEY	0,5	0,204	0,0817	76,1	152,2
EUCALYPTUS HONEY	0,5	0,431	0,1727	167,1	334,3

Ferric reducing antioxidant power is higher in pure honey samples, 0,0761 mmol Fe(II)/L for lime honey and 0,1671 mmol Fe(II)/L for eucalyptus honey (Table 14), than in the extracts, where the values were 0,0461 mmol Fe(II)/L for lime and 0,0662 mmol Fe(II)/L for eucalyptus extract (Table 13).

Ferric reducing antioxidant power of lime honey is 76,1 (μmol Fe (II)/L), which is a bit lower than the values found in literature where they vary from 98,5 to 139,1 (μmol Fe (II)/L) (29). FRAP of eucalyptus honey was 167,1 (μmol Fe (II)/L), which is in agreement with values found in literature, where they vary from 130,95 to 373,09 (μmol Fe (II)/L) (27).

4.2.4 FREE RADICAL SCAVENGING CAPACITY – DPPH

The method is based on the evaluation of the capacity of the antioxidant agents to reduce 2,2-diphenyl-1-picrylhydrazyl (DPPH) to the corresponding pale-yellow hydrazine. DPPH

is a stable nitrogen organic radical commercially available with an intense purple colour and an absorption maximum at 515 nm.

The remaining DPPH was quantified by measuring absorbance of samples at 517 nm. The antioxidant concentration necessary to reduce 50 % of the initial DPPH concentration, which is called EC_{50} or IC_{50}, was obtained from the plot of inhibition percentage against antioxidant concentration (of sample or standard).

The results were expressed in milimols of Trolox equivalents per 100 g of honey, based on the calibration curve (plot of inhibition of the Trolox standard solutions against Trolox concentration) according to honey dilution and honey mass in the sample.

Table 15: Concentration, absorbance at 517 nm and inhibition (expressed in %, mmol TE/L and μmol TE/g) for honey extracts

	c (mg/ml)	**A_{517}**	**% INHIBITION**	**mmol TE/L SAMPLE**	**μmol TE/g extract**
LIME EXTRACT	1	0,475	5,06	0,0117	11,65
EUCALYPTUS EXTRACT	1	0,451	9,79	0,0225	22,53

Table 16: Concentration, absorbance at 517 and inhibition (expressed in %, mmol TE/L and μmol TE/100 g (corrected values) for honey samples

	c (mg/ml)	**A_{517}**	**% INHIBITION**	**mmol TE/L SAMPLE**	**μmol TE/100 g HONEY Corrected values**
ARTIFICIAL HONEY	0,1	0,500	0,07	0,0002	(for sugar interference)
LIME HONEY	0,1	0,405	19,1	0,0438	43,69
EUCALYPTUS HONEY	0,1	0,493	1,47	0,0034	3,219

As shown in Table 16 lime honey showed higher free radical scavenging capacity as pure honey (19,0 % inhibition), than as extract (5,06 %), shown in Table 15. But eucalyptus honey showed higher percentage of inhibition as extract (9,79 %) than as pure honey (1,4 %).

Literature shows values for eucalyptus honey's % of inhibition being around 70 %, which is not in agreement with our obtained value (27).

Lime honey also showed lower % of inhibition (19,0 %) than in literature, where the values are around 65 % (28). Perhaps the reason for these results is not sufficient enough time of analysis and therefore the honeys did not react with DPPH completely.

4.2.5 OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC)

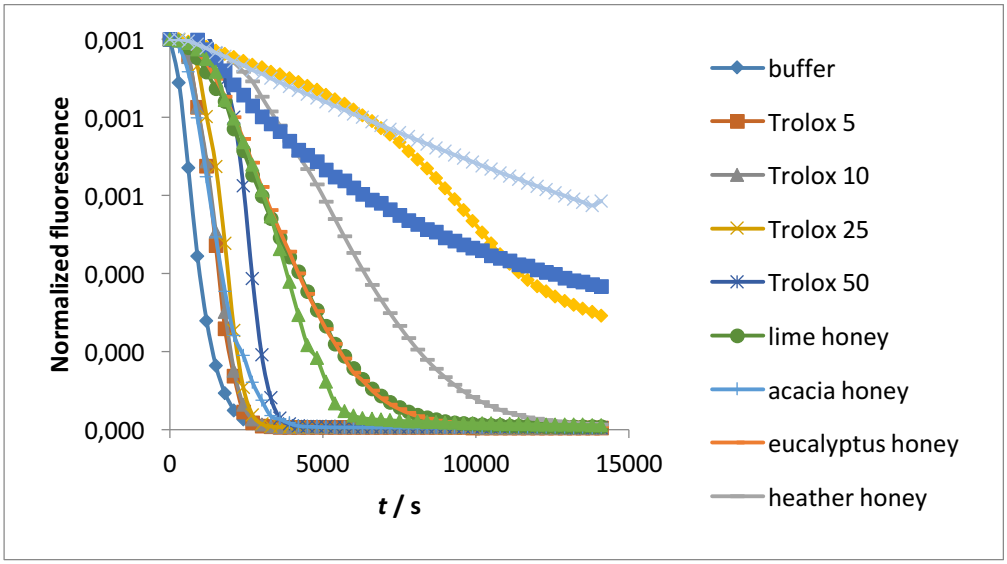
The oxygen radical absorbance capacity (ORAC) assay is based on the ability of the antioxidant agents present in the sample to inhibit oxidation reactions induced by peroxyl radicals ($ROO^{\cdot}$) generated by thermal decomposition of 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), through interruption of the radical chain via hydrogen atom transfer. The method uses fluorescein (FL) as fluorescent probe, which reacts with peroxyl radical forming a nonfluorescent product and thus the fluorescence of the probe decays with time. The peroxyl radical scavenging ability by the antioxidant agents in the sample is also a measure of their antilipoperixidant activity since these radical species are involved in lipid peroxidation (31).

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

Table 17: Normal and corrected values for AUC, concentration of samples expressed in μmol of Trolox equivalence per g and litre of honey. Values marked with "*" are too high, meaning the reaction did not finish in time of the assay.

	AUC_{normal}	$AUC_{corrected}$	*μ*mol TE/L	*μ*mol TE/g
LIME HONEY	12,46	8,57	103,2	10,32
EUCALYPTUS HONEY	12,59	8,71	104,8	10,48
LIME EXTRACT	32,52	28,63	344,5*	344,54*
EUCALYPTUS EXTRACT	11,32	7,43	89,4	89,43

Figure 1: Normalized fluorescences of honey samples and Trolox standard solutions against time



Literature data shows ORAC values for lime honey are around 9,5 μmol TE/g (32) and around 3,6 μmol TE/g for eucalyptus honey (33). The value we obtained, shown in Table 17, for lime honey sample is therefore in agreement with literature (10,32 μmol TE/g), but the obtained value for eucalyptus honey is much higher (10,48 μmol TE/g).

4.3 ANTIMICROBIAL AND ANTIFUNGAL ACTIVITY

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. As shown in Table 18, eucalyptus extract was most effective against *S. aureus* (36.46 µg/ml), *E. faecalis* (104.17 µg/ml), which is in agreement with other studies (34) and *C. albicans* (62.5 µg/ ml) (35). Lime extract mostly inhibited the growth of *S. aureus* (52.08 µg/ml) and *E. coli* (104.17 µg/ml) and it showed the same inhibitory growth properties against both yeasts (93.75 µg/ml).

Eucalyptus extract showed the strongest bactericidal activity against *E. coli* and lime extract against *P. aeruginosa.*

Table 17: MIC and MBC values for honey and extract samples, and artificial honey against different bacteria.

MIC/MBC (µg/mL)

	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 Eucalyptus Honey	250000 / x > 250000 / x > 2500000 / x	250000 / x > 250000/x > 2500000 / x	250000 / x > 250000/x > 2500000 / x	250000 / x > 250000/x > 2500000 / x
Pure Lime Honey	> 250000 / x > 250000 / x > 250000 / x	> 250000 / x > 250000 / x > 250000 / x	> 250000 / x > 250000 / x > 250000 / x	125000 / > 250000 > 250000 / x > 250000 / x
Eucalyptus extract	62.5 / > 500 125 / > 500 125 / > 500	125 / 500 125 / 500 125 / 500	125 / 500 125 / > 500 125 / 500	156.25 / > 5000 62.5 / > 500 31.25 / > 500
Lime extract	125 / > 500 250 / > 500 125 / > 500	125 / 500 62.5 / > 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

Legend:
Ef - *Enterococcus faecalis*
Ec - *Escherichia coli*
Pa - *Pseudomonas aeruginosa*
Sa - *Staphylococcus aureus*
Values in black are from day 1 of testing, values in red from day 2 and values in green from day 3, as we have performed triplicate assays.

Table 18: MIC and MBC values for honey and extract samples, and artificial honey against different yeast.

MIC/MBC (µg/mL)

SAMPLES	Ca	Sc
Artificial honey	> 250000 / x > 250000 / x	> 250000 / x > 250000 / x
Pure Eucalyptus Honey	> 250000 / x > 250000 / x	> 250000 / x > 250000 / x
Pure Lime Honey	> 250000 / x > 250000 / x	125000 / > 250000 250000 / >250000
Eucalyptus extract	62.5 / 125 62.5 / 125	62.5 / 62.5 125 / 125
Lime extract	62.5 / 125 125 / 125	62.5 / 125 125 / 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:
Ca - *Candida albicans*
Sc - *Saccharomyces cerevisie*
Values in black are from day 1 of testing, values in red from day 2 and values in green from day 3, as we have performed triplicate assays.

Eucalyptus honey extract showed the strongest antibacterial activity against *E. faecalis* and *S. aureus,* respectively. Both extracts showed the same antibacterial activity against *P. aeruginosa*. The eucalyptus extract showed weaker activity against *E. coli*. Both extracts showed strong antibacterial activity against *S. aureus*. Eucalyptus extract showed the strongest antifungal activity against *C. albicans*, while lime extract had almost the same activity against both yeasts.

4.4 CORRELATIONS BETWEEN PARAMETERS

Before starting the research, we have assumed the colour of honey depends on its flavonoid in phenolic content. The results (Table 19) have shown fairly good positive correlation between colour and total phenolic content (r = 0,979) and colour and total flavonoid content (r = 0,946). That means the darker the honey, the higher the total flavonoid and phenolic content, and vice versa. Therefore, we can confirm our hypothesis that the colour of honey is in positive correlation with total flavonoid in phenolic content.

We have also assumed that free radical scavenging capacity of honey is in positive correlation with total flavonoid content, but the results we have obtained showed very poor (r = 0,474) correlation between those two parameters, which means, the content of flavonoids in honey does not contribute much to its radical scavenging capacity. However, this does not coincide with the fact that one of the main activities of flavonoids is exactly the capacity of trapping radicals, therefore, the reason for this result must be an error in one of the assays.

In addition, the results have also shown good positive correlation (r = 0,999) between ferric reducing antioxidant power and total flavonoid content and between ferric reducing antioxidant power and total phenolic content (r = 0,990).

Table 19: Pearson correlations (r) between different physicochemical and antioxidant parameters.

	PEARSON
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
MOISTURE/TSS	-1,000
EC/TSS	0,090
FRAP/TFC	0,999
FRAP/ORAC	0,912
FRAP/DPPH	0,506
FRAP/TPC	0,990
DPPH/TPC	0,379
DPPH/TFC	0,474
DPPH/ORAC	0,321
ORAC/TPC	0,936
ORAC/TFC	0,903

5 CONCLUSION

Honey is a food that we commonly reach out to, whether we have a sore throat or want to use a healthier alternative to granulated sugar.

With our assays, we have confirmed that colour of honey is in positive correlation with phenolic content, but free radical scavenging capacity is not in correlation with total flavonoid content.

The antibacterial assays showed that pure honey samples have no antibacterial activity, but the honey extracts do. Eucalyptus honey was able to successfully inhibit growth of *S. aureus* and *E. faecalis*, and lime honey was successful against *E.coli*. Eucalyptus extract showed the strongest bactericidal activity against *E. coli* and lime extract against *P. aeruginosa.* In addition to that, both honeys showed significant antifungal activity against *Saccharomyces cerevisiae* and *Candida albicans.* Eucalyptus extract showed the strongest antifungal activity against *C. albicans*, while lime extract had almost the same activity against both yeasts.

To conclude, both Portuguese eucalyptus and Slovenian lime honey have antibacterial and antifungal activity, which corresponds to its traditional uses for treatment of colds, burns, wounds etc. We have also confirmed that both honeys have good antioxidant activity, that this activity is the result of flavonoids and other phenolic compounds, and that the darker the honey, the stronger are these capacities.

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