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ANJA MESTNIK

IDENTIFIKACIJA SPOJIN V MEDU FRANCOSKEGA IZVORA IN NJIHOVE POTENCIALNE ANTIMIKROBNE LASTNOSTI

IDENTIFICATION OF COMPOUNDS IN HONEY OF FRENCH ORIGIN AND THEIR POTENTIAL ANTIMICROBIAL PROPERTIES

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The experimental work for this Master thesis had been in process from March 2016 until June 2016 at the Department of Analytical Chemistry and Bromatology at the Faculty of Pharmacy in Montpellier, France, under the supervision of prof. dr. Michel Larroque.

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STATEMENT

I hereby declare that this Master thesis was done independently by me under mentorship of assoc. prof. dr. Janez Ilaš, Ph. D. and co-mentorship of prof. dr. Michel Larroque.

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ABSTRACT

Honey has been appreciated for its therapeutic action from ancient time. Honey and its antimicrobial properties are getting more attention nowadays since they demonstrated activity against resistant bacteria. In the master thesis, we therefore wanted to research the antimicrobial compounds that can be found in different types of honey.

Practical work for the master thesis was conducted during Erasmus+ exchange in France, at the Faculty of Pharmacy, Université de Montpellier. Honey samples were obtained from the local beekeepers or bought in store, all of them originated from the south of France.

The aim of our research was to extract undefined compounds from honey, to test if they have any antimicrobial activity and further analyse the extracts. We wanted to qualify the components responsible for antimicrobial (antifungal) activity.

In the beginning, we used liquid-liquid extraction to extract interesting compounds. The main idea was to separate different compounds in honey with TLC, explore different modifications of the basic method and try to identify new antimicrobial compounds. Antimicotic activity was tested on four types of *Candidae: Candida albicans, Candida parapsilosis, Candida krusei, Candida glabrata.* Some types of honey proved more potent activity than others.

Later on, we wanted to identify the compounds on TLC plates with visualisation reagents. We used standards of phenolic acids to identify the specific ones in our honey samples. Since the identification proved difficult, we used HPLC to qualify and quantify the content of phenolic acid.

Key words:

Honey, antimicrobial compounds, antimicotic, TLC, HPLC

POVZETEK

Med se je uporabljal v tradicionalni, ponovno pa postaja priljubljen tudi v sodobni medicini, uporablja se pri zdravljenju kroničnih ran in gastrointestinalnih boleznih. Med sestavljajo predvsem sladkorji (od 80 do 85%; fruktoza, glukoza, saharoza in drugi), voda (15-17%), anorganske spojine (0,2%), proteini, amino kisline in v sledeh encimi, vitamini ter druge spojine. Na sestavo in lastnosti medu znatno vpliva vir, iz katerega je pridobljen. Narejenih je vrsta raziskav, ki preiskujejo vpliv medu na zaviranje rasti mikroorganizmov, pretežno bakterij. Z našo raziskavo smo želeli preučiti, kakšno je njegovo protiglivično delovanje.

Laboratorijski del magistrske naloge je bil opravljen v času izmenjave Erasmus+ na fakulteti v Franciji, Université de Montpellier. Med, ki smo ga uporabili za raziskave, je bil tako izdelan na jugu Francije, v regiji Occitanie, kjer se tudi nahaja fakulteta. Nekatere vzorce medu smo dobili od čebelarjev, nekatere pa kupili v lokalnih trgovinah.

Delo je bilo razdeljeno na dva dela, kemijske analize in antimikrobne analize. Namen dela je bil testirati različne komponente medu in njihovo protimikrobno delovanje. Glavna ideja je bila ločiti različne spojine v medu z ekstrakcijo. S tankoplastno kromatografijo smo preverili vsebnost različnih komponent pri različnih vrstah medov. Sledilo je analiziranje protiglivičnega delovanja z mikrodilucijsko metodo. Pri medovih, ki so se izkazali za učinkovite, smo nadalje želeli kvalificirati spojine s TLC, z izboljšavo metode TLC in nadaljevali s HPLC analizo, s katero smo želeli doseči boljšo kvalitativno in kvantitativno identifikacijo.

Iz pridobljenih vzorcev medu smo pripravili raztopine z destilirano vodo, ki smo jih ustrezno hranili in uporabili za raziskave. Z ekstrakcijo tekoče - tekoče smo ekstrahirali različne spojine iz več vrst medu. S TLC smo preverili, ali so bile ekstrakcije uspešne, določene spojine so fluorescirale. S TLC smo želeli doseči ločitev spojin, da bi bila možna kasnejša identifikacija.

Ekstrakte, ki so pod UV svetlobo fluorescirali, kjer so bile torej prisotne določene spojine, smo uporabili za antimikotične teste. Glivične okužbe ali mikoze povzročajo glive, ki so pogoste v našem okolju. Večinoma so nenevarne, nekatere pa povzročajo tudi hujše okužbe. *Candida* je najbolj pogosta kvasovka, ki povroča oportunistična obolenja. To pomeni, da so to glive, ki so del normalne človeške mikrobne flore, osebe z oslabljenim imunskim

sistemom pa se zaradi njihove prekomere razširjenosti razmeroma lahko okužijo z njmi. S testi na glivah *Candida albicans, Candida parapsilosis, Candida krusei* in *Candida glabrata* smo preverjali potencialno antimikrobno učinkovitost medu, določali smo MIK. MIK ali minimalna inhibitorna koncentracija, angl. MIC, je najnižja koncentracija določenega protimikrobnega sredstva, ki še zavira rast preiskovanega mikroorganizma. Na splošno so ekstrakti medu učinkoviteje zavirali rast *C. albicans* in *C. glabrata*, manj pa rast *C. parapsilosis* in še manj rast *C. krusei*. Enake vrste medu (npr. kostanjev med, cvetlični, hrastov) tudi niso pokazale enake stopnje inhibicije rasti gliv. Zato lahko zaključimo, da se protiglivično delovanje med medovi razlikuje, ne moremo ga predpisati določenemu viru ali geografski regiji nastanka medu. Za primerjavo smo ponovili analizo, že opravljeno v našem laboratoriju, kjer smo kot protimikrobno sredstvo uporabili raztopino medu, ne ekstrakt. Rezultati so bili pri preizkušanih glivah nasprotni, raztopine medu so zavirale rast *C. parapsilosis* in *C. krusei*. Podobno kot sladkor pa so po določenih redčitvah raztopine medu povečale rast gliv, rast je bila bolj intenzivna kot pri pozitivni kontroli. Zaključili smo, da ima pri raztopinah medu velik vpliv sladkor. Pri visokih koncentracijah zavira rast, ko pa med dovolj razredčimo z vodo, ta isti sladkor deluje kot hrana za glive. Ker so se ekstrakti medu izkazali za učinkovite pri zaviranju rasti gliv, smo nadaljevali s kemijskimi analizami.

Preizkusili smo različna organska topila za ekstrakcije, z ekstrakcijo smo želeli odstraniti sladkor in vodo, tako da je v vzorcu ostalo nekaj hidrofilnih in še manj hidrofobnih spojin. Na podlagi predhodnih raziskav je bilo izbrano topilo etil acetat. Preizkusili smo tudi ekstrakcijo z manj polarnimi topili, kloroformom in heksanom, a smo delo z njima prekinili, ker je bila ekstrakcija manj učinkovita.

Po ekstrakciji smo vzorce nanesli na TLC plošče. Prva mobilna faza, ki smo jo preizkusili, je bila mešanica ocetne kisline (22%), butanola (56%) in vode (22%), analizo pa smo izvedli na polarnih TLC ploščah. V nadaljevanju smo uporabili reverznofazne – nepolarne TLC plošče. Za najboljšo mobilno fazo se je izkazala mešanica vode (5%) in acetonitrila (95%).

Pri TLC smo uporabili orositvene reagente, s katerimi smo želeli detektirati: primarne amine (fluoreskamin), flavonoide (orositveni reagent s polietilenglikolom za dokazovanje naravnih pripravkov NP/PEG), polifenole (DPPH) in nedoločene spojine (splošen/nespecifičen orositveni reagent: vanilin žveplova kislina). Le pri slednjem smo opazili nove lise (opazovanje pri vidni svetlobi) z nižjim retencijskim faktorjem R_f kot preostale spojine.

Spojino smo popraskali s plošče, ekstrahirali iz silike in jo predali za nadaljne delo, za identifikacijo z masno spektrometrijo.

Po pregledu člankov smo v vzorcih predvideli prisotnost fenolnih kislin. Na TLC plošče smo tako skupaj z nekaterimi vzorci medu nanesli standardne reference fenolnih kislin: kofeinske, vanilinske, ferulične in p-kumarne kisline. Zaradi podobnosti med R_f spojin je bilo nemogoče potrditi ali zanikati prisotnost fenolnih kislin v medu. Za nadaljno identifikacijo kromatografskih markerjev in za primerjavo kromatografskih profilov različnih vrst medu smo nato izvedli HPLC analizo.

S HPLC analizo smo najprej določili odstotek ekstrakcije tako, da smo primerjali A (površina pod krivuljo) ekstraktov in A ekstraktov z dodatkom znanih količin fenolnih kislin. Povprečen odstotek ekstrakcije je bil 51%. Učinkovitost ekstrakcije bi lahko povečali z večkratnimi ekstrakcijami. Podatek o učinkovitosti smo uporabili pri izračunih koncentracij uporabljenih fenolnih kislin v vzorcih medu. Analizirali smo vse pripravljene ekstrakte, torej vseh 25 vrst medu in jim določili koncentracijo naslednjih fenolnih kislin: kofeinske, vanilinske, ferulične, galne, siringične in p-kumarne kisline. Pričakovali smo razlikovanje med vsebnostjo fenolnih kislin med vzorci medu, tudi med istimi vrstami. Uporabili pa smo dva vzorca medu iste vrste istega proizvajalca iz istega leta, ki sta se zelo razlikovala v vsebnosti fenolnih kislin. Zato smo zaključili, da metoda ni zanesljiva in rezultatov ne moremo uporabiti za nadaljne delo.

Tekom raziskave smo želeli ugotoviti, ali ima med potencial kot protiglivična učinkovina. Glivične okužbe so pogoste, pacientov z njmi je vedno več, glive pa razvijajo rezistenco na obstoječe antimikotike. Zato smo želeli preučiti možnosti iskanja novih učinkovin v medu. Med ima protiglivično delovanje, z mikrodilucijsko metodo testiranja občutljivosti gliv smo dokazali njegovo inhibitorno delovanje na rast gliv. Kot najbolj učinkovit se je izkazal kostanjev med. Opazili smo tudi, da so ekstrakti medu bolj učinkoviti pri inhibiciji rasti gliv kot sam med oziroma raztopine medu.

Ko smo potrdili antimikotično aktivnost medu, smo nadaljevali s TLC analizo. Cilj je bil ločiti in identificirati različne spojine, ki so odgovorne za protiglivično delovanje. TLC je dostopna, robustna metoda. Omogoča pogled na sestavo medu, omogoča preučevanje več vzorcev hkrati. Če bi na podlagi naših raziskav razvili metodo TLC za razlikovanje različnih vrst medu, bi bila metoda lahko uporabna za splošno javnost. Čebelarji in industrija bi to

lahko uporabili za določitev vira medu, ustreznost navedb na embalaži in pridobitev potrdil o kakovosti.

Nadaljevali smo s HPLC analizo za določanje koncentracije fenolnih kislin. Naša metoda ni bila zanesljiva, zato je za nadaljnje raziskave treba upoštevati drugačen pristop. Predlagamo uporabo več komplementarnih metod. Pri delu z naravnimi proizvodi kot je med običajno ne obstaja le ena najboljša tehnika. Upoštevati moramo tudi sinergijo komponent, ki je pogosto pomemben dejavnik za bioaktivnost.

Čeprav sta protiglivična analiza in TLC analiza dali ponovljive rezultate, niso dovolj natančni za raziskovalno uporabo ali rutinsko določanje antimikrobne učinkovitosti medu. Potrebna je optimizacija metode, s katero se zagotovi robustnost in ponovljivost.

Ključne besede:

Med, antimikrobne spojine, antimikotične spojine, TLC, HPLC

ABBREVIATIONS

AFST - Antifungal susceptibility testing

CLSI - The Clinical & Laboratory Standards Institute

EUCAST - European Committee on Antimicrobial Susceptibility Testing

HPLC - high performance liquid chromatography

MGO - methylglyoxal

MIC - minimum inhibitory concentrations

MP – mobile phase

R_f - retention factor

TLC - thin-layer chromatography

UV – ultraviolet light

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1. INTRODUCTION

1.1. Honey

Honey is a natural sweetener, often used as a healthier sugar substitute. It has been used for its functional properties as a therapeutic substance in gastrointestinal disorders and antimicrobial treatment in healing the wounds (1).

Honey can be produced from many sources, which influences its composition and properties. The physical properties depend on the water content, the components (the proportion of the specific sugars), the type of flora used to produce it (pasturage). It is typically made by bees (most common European honey bee *Apis mellifera*) foraging nectar from flowers. Bees convert it into honey by a process of regurgitation and evaporation, while the nectar is stored in wax honeycombs inside the beehive as a primary nutrition source. Honey contains approximately 200 distinct chemical compounds, besides sugars (80-85%), water (15-21%), inorganic compounds (0,2%), proteins and amino acids (0.1-0.4%), and trace amounts of vitamins, enzymes, phenolic compounds and others (2). Honey has about the same relative sweetness as granulated sugar. It is the result of the present monosaccharides, mainly fructose and glucose. Honey is classified by the floral source of the nectar from which it was collected. It can be made from specific types of flower nectar or blended after collection. Bees can use honeydew instead of nectar. Honeydew is the sweet secretion of aphids or other plant sap-sucking insects. Honeydew honey has darker brown colour, a rich fragrance, and is not as sweet as nectar honey (3) (4). In general, the density of honey is 1.42 kg/L at 20 °C. pH varies from 3.2 and 4.5. It is generally lower than 4 for honeys from nectar and higher than 5 for honeys from honeydew (3) (5).

Honey and its antimicrobial properties are getting more attention since they have also shown activity against resistant bacteria (1; 6). After the “golden era” of antibiotic discovery in the 1940s to 1960s, when all the important antibiotics (tetracyclines, cephalosporins, aminoglycosides and macrolides) were discovered, we are now facing an important global concern to public health: microbial resistance. This is the reason why discovery of new

antimicrobial compounds is an important issue. Natural products are a great source of complex and structurally diverse compounds (1).

1.1.1. ANTIMICROBIAL ACTIVITY OF HONEY

Honey has the ability to inhibit the growth of microorganisms, namely it has antibacterial and antimicotic effect. It contains components like: proteins, minerals, free amino acids, vitamins and enzymes. Minerals that have been found in honeys include: potassium, sulphur, calcium, chlorine, magnesium, phosphorus, iron, sodium, manganese and copper. There are free amino acids present in honey, proline being the main one. Honey also contains small quantities of vitamins: riboflavin, pantothenic acid, thiamine, niacin, pyridoxine and ascorbic acid (5). In general, honeys contain similar phenolic acids, such as syringic acid, gallic, caffeic, *p*-coumaric, vanillic, ferulic acid; flavonoids, including apigenin, chrysin, hesperetin, galangin, pinocembrin, kaempferol, and quercetin; antioxidants, like tocopherols, ascorbic acid; enzymes superoxide dismutase (SOD) and catalase (CAT), and reduced glutathione (GSH). Each component has different medicinal and nutritional properties. They act synergistically, leading to a variety of beneficial effects (2).

In most honeys, the antimicrobial activity is due to the enzymatic production of H_2O_2. Its mechanism is based on chemical oxidation of cellular components (4). The enzyme glucose oxidase is added to the collected nectar by honey bees during production of honey. Glucose oxidase is activated at dilution of honey and it converts glucose into H_2O_2 and gluconic acid. Glucose oxidase protects the ripening honey (diluted) against pathogenic microorganisms, whereas it is present but not active in the mature honey. Mature honey is protected by other mechanisms, high osmotic pressure and low pH. The factors that lower the accumulation of H_2O_2 are exposure to heat or light, which inactivates glucose oxidase; and degradation of H_2O_2 by honey itself. Neutralization of H_2O_2 decreases the antimicrobial activity of the majority of honeys analysed, demonstrating the important role of H_2O_2. On the other hand, a large number of honey samples keep their antimicrobial activity even after H_2O_2-neutralization, indicating there are other active ingredients present (1). Kralj Kunčič et al. conducted a research with Slovenian honeys and their antimicrobial activity. They tested non-peroxide antibacterial activity by degrading hydrogen peroxide with catalase. The

reduction in hydrogen peroxide was tested with glucose oxidase assay kit and later tested for antibacterial activity against *E. coli*. The results showed decreased activity, and they came to the conclusion that: »the antibacterial action of this Slovenian honey is mainly due to its peroxide properties« (7).

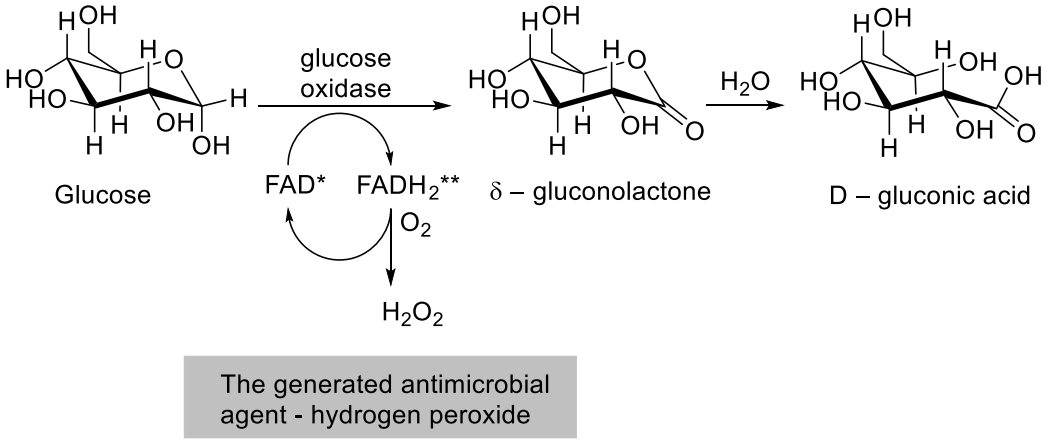


Figure 1: Generation of hydrogen peroxide, reaction catalysed by glucose oxidase

Honey is a saturated solution of sugars, 80-85% of it represent a mixture of the monosaccharides fructose and glucose, at a distant third place is saccharose. Other disaccharides (maltose, isomaltose, turanose, nigerose, and maltulose) are present in honey in very small amounts. The water content is generally 15-21%. A small quantity of higher sugars, oligosaccharides, and dextrins are present in honey at about 1% or less of the total sugars (5). The high concentration of sugars disables growth of bacteria and fungi, especially the ones that are sensitive to high osmotic pressure. Therefore, microorganisms do not grow in fully matured honey. The more diluted the honey is, the more organisms can grow in it (8).

Honey is characteristically acidic with pH between 3.2 and 4.5, which is low enough to inhibit the growth of numerous microorganisms (4). The acidity is primarily due to the content of gluconolactone/gluconic acid (average values of 0.23-0.98%) present as the result of enzymatic action in the maturing nectar. Acidity does contribute to the overall antimicrobial activity of honey, but is not the most important. Crousilles (9) found out during her research that honeys with almost identical pH values differ in antibacterial activity a lot, some have remarkable activity (Balsam spurge - *Euphorbia balsamifera*) whereas others (flower honey) have none. On the other hand, she discovered antibacterial activity in chestnut honeys with high pH (pH 5-6). The effect of pH may be neutralized if honey is

diluted with buffering solutions (e.g. body fluids) (8). The dominant acid in honey is gluconic acid, produced by glucose oxidase from bee secretions acting on glucose. Other acids have been identified, such as acetic, citric, butyric, lactic, formic, maleic, malic, oxalic, and succinic acid (5).

Manuka honey is one of the most potent and well-investigated honeys for its antimicrobial and wound healing activities. Its effectiveness is mainly attributed to methylglyoxal (MGO). MGO is a by-product of a number of metabolic reactions, it is formed during heat treatment or prolonged storage from food and beverages containing carbohydrates. It is found in most types of honey, but generally in small amounts. In manuka honey MGO is present in high amounts. MGO is the consequence of the conversion of dihydroxyacetone (DHA), which is present in very high concentrations in the nectar of manuka myrtle flowers (*Leptospermum scoparium)*. This nonenzymatical conversion happens at a slow rate during the storage of honey. Kwakman et al. (10) proved that neutralization of MGO eliminated the activity of manuka honey against *S. aureus* and significantly reduced the activity against *B. subtilis.* However, it did not affect the activity against *E. coli* and *P. aeruginosa*. Thus, MGO is not the only nonperoxide antimicrobial ingredient in manuka honey (1).

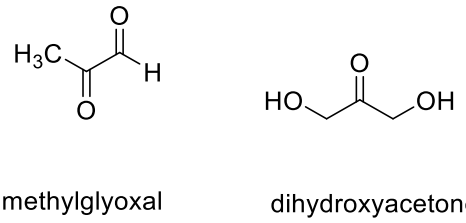


Figure 2: Structure of methylglyoxal and dihydroxyacetone

Another antimicrobial compound can be found in honey, peptide bee defensin-1 (also called royalisin) which was found in honeybee's haemolymph, the insect's equivalent of blood, in honeybee thoracic glands and head, and in royal jelly. Kwakman et al. (10) demonstrated that bee defensin-1 contributes to the bactericidal activity of honey, besides other factors (high concentration of sugars, low pH, H_2O_2). They proved its effectiveness but only against Gram-positive bacteria including *S. aureus, B. subtilis,* and *Paenibacillus larvae* (1; 10).

Phenolic compounds are found in small quantities and contribute to antimicrobial activity of honey. Phenols are aromatic hydrocarbon systems directly bonded with one or more hydroxyl groups (-OH). Phenolic compounds also include functional derivates (esters,

methyl ethers, glycosides etc.). Because of their hydroxyl group, phenols are similar to alcohols, yet in water they are reacting as weak acids. Some authors have studied the phenolic and flavonoid contents of honey to determine if there is a correlation with the floral source and to determine potential antimicrobial activity.

- *Phenolic acids*

Phenolic acids contain phenolic ring and an organic carboxylic acid function (C6-C1 skeleton). Furthermore, three subgroups can be differentiated: benzoic derivates, cinnamic acid derivates and other phenolic acids and derivates. The benzoic acids have the basic chemical structure of C6-C1, whereas the cinnamic acids are based on C6-C3.

Different phenolic acids were found in honeys: caffeic acid, ferulic, *p*-coumaric acid, syringic, vanillic, gallic, dihydroxybenzoic, 4-hydroxybenzoic, 3-hydroxybenzoic, *m*-coumaric, *o*-coumaric, cinnamic, chlorogenic, homogentisic acid.

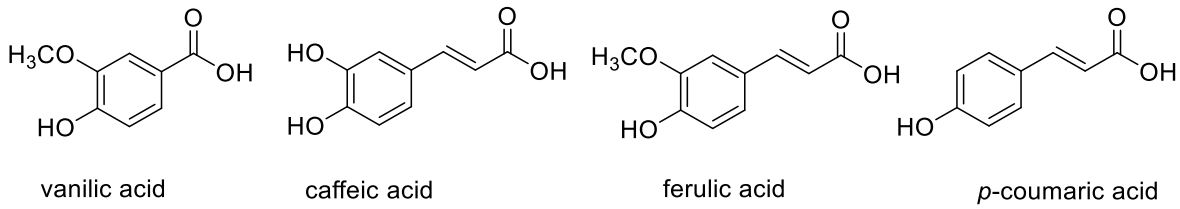
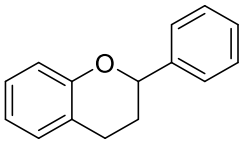


Figure 3: Structures of some phenolic acids: vanillic, caffeic, ferulic, p-coumaric acid

- *Flavonoids*

Flavonoids are pigments of plants, they consist of two phenyl rings and heterocyclic ring and have the general structure of a 15-carbon skeleton. They affect honey's colour, taste and flavour. There have been several activities attributed to them, i.e., antioxidant, antimutagenic, anti-inflammatory, anticarcinogen, antidepressant properties. They also proved antibacterial activity and synergistic activity with antibiotics, and they suppressed bacterial virulence factors in numerous *in vitro* and in a limited number of *in vivo* studies.

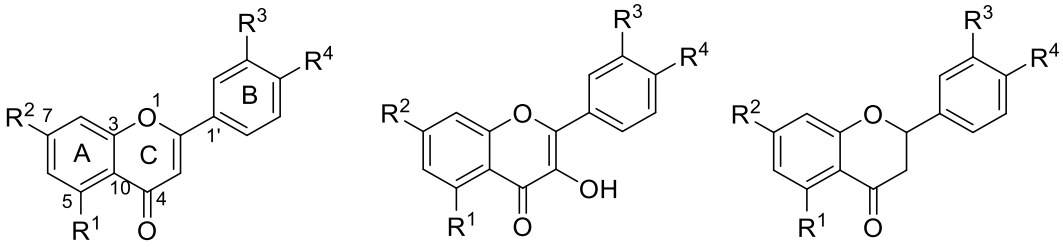


flavan

Figure 4: Flavan - basic structure of most flavonoids

Flavonoids found in honey:

- Flavone type: apigenin, luteolin, chrysin, pinocembrin, tricetin;
- Flavonol type: quercetin, kaempferol, galangin, myricetin, pinobanksin, rhamnetin, rutin;
- Flavanone type: hesperetin, naringenin;
- Isoflavonoids: genistein.



flavone type flavonol type flavanone type

Figure 5: Structures of flavonoids: flavone type, flavonol type, flavanone type

Even the removal of the identified effective compounds (e.g. neutralisation of H_2O_2 by the addition of catalase, neutralisation of osmotic pressure by diluting the honey), honey still proves antimicrobial. Antimicrobial activity in honeys varies depending on their type, and the complex nature of honey is an attractive challenge for further research.

1.2. Fungal infections

Fungal diseases or mycoses are often caused by fungi that are common in human environment. They live outdoors, in the soil, on plants, on trees, and also on the indoor surface and on human skin. Most fungi are not dangerous, but some cause harm to people's health. *Candida* is a yeast that is the most common cause of fungal infections worldwide.

Candida species are detected in healthy individuals, we are exposed to them repeatedly in food and other sources (11). *C. albicans* forms normal flora of the mucus membranes of the respiratory, gastrointestinal and female genital tracts. When mucosal barriers are disrupted or the immune system is compromised *Candida* species can attack and cause superficial, as well as life-threatening diseases (12; 11). Occurrence of fungal disease in the modern world is increasing rapidly, not only with high-risk patients (e.g. bone marrow transplant recipients or HIV-infected patients) but also amongst non-trauma emergency surgery patients (11).

Candida albicans is an opportunistic fungal pathogen that is responsible for candidiasis in human hosts. It is the most common dimorphic pathogen in humans, it causes oral infections, over 50% of candidaemia cases and more than 90% of vaginal candidiasis (13).

Candida glabrata is another organism commonly connected to fungal infection. Until recently, *C. glabrata* was assumed to be mostly non-pathogenic organism. But with the increasing number of immunocompromised patients, *C. glabrata* turned out to be a highly opportunistic pathogen of the bloodstream (Candidemia) and the urogenital tract. In fact, it became the dominant non-*C. albicans Candida* species involved in these infections. The infections can be mucosal or systemic and difficult to treat since they are often resistant to many azole antifungal agents (12).

Candida parapsilosis is another fungal species that has become an important cause of sepsis, and wound and tissue infections in immunocompromised individuals. The incidence of infections caused by *C. parapsilosis* may continue to rise since the pathogen has a high affinity for parenteral nutrition, it colonizes the hands of healthcare workers, and forms biofilm on prosthetic surfaces and central venous catheters (14).

Candida krusei is an evolving nosocomial pathogen (hospital-acquired infection (HAI)) mainly found in the immunocompromised patients and patients with haematological malignancies. It has been recognized as a potentially multidrug-resistant (MDR) fungal pathogen, due ti its natural resistance to fluconazole, on top of the recent reports of decreased susceptibility to both flucytosine and amphotericin B (15).

1.3. Methods for antifungal testing

There are several methods for antifungal susceptibility testing. Most used ones are disk diffusion, well diffusion and broth or agar dilution method. There are also others such as flow cytofluorometric and bioluminescent methods, but they are not as common because they entail more complex evaluation.

Dilution methods (broth or agar) are the most useful ones for determination of MIC (minimum inhibitory concentration) values since it is possible to estimate the concentration of the used antimicrobial agent in the agar or broth medium. These methods can be used to quantitatively measure the *in vitro* antimicrobial activity against fungi. MIC is defined as the lowest concentration of the tested antimicrobial agent that inhibits the visible growth of the microorganism, and it is expressed in μg/mL or mg/L. Antifungal susceptibility tests (AFST) are developed and validated by EUCAST (European Committee on Antimicrobial Susceptibility Testing) and the CLSI (The Clinical & Laboratory Standards Institute). They provide standards and guidelines for uniformed clinical and laboratory testing (16).

One of the most basic antifungal susceptibility methods is broth dilution method. It is performed using an agent of which the antifungal effect we want to test, and its appropriate dilutions. Broth dilution methods, depending on the used material, can be macrodilutions (the medium is dispensed in tubes of minimum volume of 2 mL) or microdilutions (the medium is dispensed in smaller wells in microtitration plate). Each tube or well is inoculated with a fungi inoculum. The antifungal effect of the agent can be determined visually or spectrophotometrically (16).

1.4. Chemical analysis

1.4.1. Liquid-liquid extraction

Liquid-liquid extraction is an analytical technique used for separation of compounds based on their relative solubilities in two different immiscible liquids, two solvents, most commonly water and an organic solvent. Extraction can be used to separate or “partition”

ionic or polar low-molecular-weight substances into an aqueous phase and less polar water-insoluble substances into an immiscible liquid organic phase. This phenomenon is governed by the partition (P) or distribution coefficient (D). It is the ratio, K, of solubility of the analyte dissolved in the organic layer to the solubility of analyte dissolved in the aqueous layer:

K = solubility of organic (g/100 mL) / solubility of water (g/100 mL)

Depending on the system, the distribution ratio can be a function of temperature, the concentration of chemical species in the system, and a large number of other parameters.

Different apparatus can be used for extraction, most common for small scale chemical labs are separatory funnels. Sample solution and another immiscible solvent are mixed, two-phased system is produced so that the small droplets of less polar phase mix with more polar phase. After mixing the system is left to separate again into two phases. This way an analyte can be separated from a sample with more analytes, the sample can also be separated in fractions with different polarities (17).

1.4.2. Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is a planar chromatography technique used to separate non-volatile mixtures. It consists of three steps: spotting, development, and visualization. TLC is performed on a plate made of glass or aluminium foil, which has a thin layer of adsorbent material on the surface, usually silica gel, aluminium oxide, or cellulose. The sample is applied on this layer of adsorbent, which is called the stationary phase. The samples are prepared with appropriate solvents and applied on the plate, Chromatograph is developed by putting the plate into a shallow tank of development solvent. This solvent or solvent mixture, called the mobile phase, is drawn up the plate via capillary action. Different interactions happen between molecules of the sample, mobile phase and stationary phase. Different analytes migrate up the TLC plate at different rates, therefore their separation is achieved. TLC plate is then removed from the pool and the solvent is evaporated. Separated spots could be observed with visible or UV light. If fluorescent plates are used, a number of compounds can be seen by illuminating the plate with Short-wave UV light is used for fluorescent plates, where compounds that illuminate can be seen. Compounds can also quench the fluorescence which causes dark spots appear on the surface of the plate. Spots

that do not fluoresce can be derivatised with staining reagents. Each spot has a retention factor (R_f) which is equal to the distance travelled over the total distance covered by the solvent. The R_f value can be used to identify and distinguish compounds. R_f is characteristic for a compound, but can change depending on the condition of the method (mobile and stationary phase).

There are two options when it comes to chromatographic techniques: normal phase and revered phase chromatography. Normal phase is with polar stationary phase, most common silica gel with binders, while mobile phase is nonpolar. Reversed phase or nonpolar chromatography is when silanol groups are esterified with long chained carbon hydrogens, making it nonpolar (this is useful with samples dissolved in water).

There could be different enhancements made to the original TLC method. The improved method is named HPTLC or "high-performance TLC". It allows to automate the different steps in the process, to increase the resolution achieved, and to achieve more accurate quantitative analysis. For this method, thinner layers of stationary phase and smaller sample volumes are used which results in better resolution.

TLC is very simple to use and an inexpensive technique. When the best mobile phase is found, it can be applied to other separation methods, like high-performance liquid chromatography (HPLC). TLC is also used to ensure purity (of a compound), separation and identification of compound(s), especially if using standard reference, and quantitative analysis of compound(s). The disadvantages are high detection limit and short stationary phase, which makes the length of separation limited (17).

1.4.3. High-performance liquid chromatography (HPLC)

HPLC is a technique used to separate, identify and quantify components in a mixture. In HPLC, a pump is used to force a liquid mobile phase through a column filled with adsorbent, called the stationary phase. The result is the separation of the input sample components.

The column is the main part of HPLC, containing the adsorbent, typically a granular material made of solid particles (e.g. silica, polymers). The analytes in the sample are separated from each other based on their interaction with the adsorbent particles. The interactions can be

hydrophobic (dispersive), dipole-dipole and ionic, or a combination. The interactions also depend on the mobile phase, its composition (usually a mixture of solvents, such as water, acetonitrile and/or methanol) and temperature. HPLC instrument consists of a sampler, pumps and a detector. After the sampler brings the sample in the column, where the pump dictates the desired flow, the detector detects eluted substances and generates a signal. The signal is proportional to the amount of the sample that is emerging, therefore a quantitative analysis of the sample component is possible. There are several types of detectors used, like UV/VIS, photodiode array (PDA) or based on mass spectrometry.

There are two variations of use of HPLC depending on the relative polarity of the solvent and the stationary phase, normal phase and reversed phase HPLC.

Normal phase HPLC is essentially the same as thin layer chromatography or column chromatography. The column is filled with small silica particles, and the solvent is nonpolar (e.g. hexane). The polar compounds in the sample therefore interact longer with the polar silica particles than nonpolar compounds, which pass through the column quicker.

On the contrary, reversed phase HPLC consists of nonpolar stationary phase. The silica is modified, long hydrocarbon chains (typically with 8 or 18 carbon atoms) are attached to its surface making it nonpolar. A polar solvent is used, and therefore there are stronger interactions between the solvent and polar molecules in the mixture being forced through the column. That means that now the polar molecules travel through the column quicker. Reversed phase HPLC is the most commonly used chromatographic technique (17).

2. RESEARCH AIM AND OBJECTIVES

The aim of our research was to extract undefined compounds from honey, test them if they have any antimicrobial (antifungal) activity and further analyse the active extracts.

It is commonly known that honey has therapeutic action. Most of the studies on potential therapeutic activity have been done by testing raw honey. By neutralizing different compounds and their effect, they discovered possible active ingredients.

Our method was a little different; we wanted to directly analyse the unidentified antimicrobial compounds. We tested the extracts to see if they have any potential for antifungal activity. We wanted to qualify the components responsible for antifungal activity. The main idea was to separate different compounds in honey, explore different methods for doing that and try to identify new antimicrobial compounds, test their efficiency. Later on, we carried out modification of our method of qualification.

During our work, we performed the following experiments:

- Liquid-liquid extractions with different solvents,
- TLC chromatography with different mobile phases,
- Identifying compounds separated with TLC with visualisation reagents,
- Identifying polyphenols (phenolic acids) present in honey with TLC using standard references of phenolic acids,
- HPLC for quantifying the content of phenolic acid, potentially use the information for identification of type of honey,
- Determination of antimicrobial and antifungal activity of extracts.

3. MATERIALS AND METHODS

3.1. MATERIALS

We analysed different honeys that were harvested in a southern region of France, previously known as Languedoc-Roussillon region, now belonging to Occitanie. Honeys were obtained from beekeepers or bought in local stores. As a control sample sugar analogue was used, consisting of fructose, glucose and saccharose, and treated the same way as honey samples. Honeys were stored in the original airtight packaging at room temperature in the dark. For further tests, honey solutions with distilled water were prepared, containing 1:1 w/w ratio (30 g of honey, 30 g of water). Honey solutions were used for liquid-liquid extractions. Solutions of honey and water as well as honey extracts were stored in the refrigerator at 4 °C.

Table 1: List of honey samples

N°	**Type of honey (*Latin name*)**	**Name/brand of honey**
1	Forest honey	Rayon d'Or, Miel et Miellat de forêt
2	Chestnut (*Castanea sativa*)	Rayon d'Or, Miel de châtaignier
3	Organic chestnut (*Castanea sativa*)	Rayon d'Or, Miel biologique de châtaignier
4	Chestnut (*Castanea sativa*)	Châtaignier Miel
5	Chestnut (*Castanea sativa*)	Châtaignier des Pyrénées catalanes
6	Chestnut (*Castanea sativa*)	Miel de Châtaignier des Cévennes
7	Chestnut (*Castanea sativa*)	Miel d'Ardèche
8	Chestnut (*Castanea sativa*)	Miel soleil du roussillon
9	Chestnut (*Castanea sativa*)	Miel de Châtaignier LLUCH
10	Honeydew	Le Rucher de marina
11	Chestnut (*Castanea sativa*)	Chataignier Escano
12	Chestnut (*Castanea sativa*)	Maître rucher, miel de châtaignier
13	Chestnut (*Castanea sativa*)	Les Bons Miels du Soleil
14	Chestnut (*Castanea sativa*)	Miel de châtaignier, NYER
15	Sugar analogue	Mélange sucre (35g fructose + 35g glucose + 10g saccharose)

16	Oak	Miel de chêne
20	Euphorbe/spurge (*Euphorbia mellifera*)	Matéro, Miel d'Euphorbe Bio
21	Oak	Les Butineuses catalanes
22	Chestnut (*Castanea sativa*)	Miel Rico
23	Chestnut (*Castanea sativa*)	Nyer 2014, châtaignier N°2
24	Chestnut (*Castanea sativa*)	Châtaignier Escano 10/2015
25	Forest honey	Les Bons Miels du Soleil
26	Chestnut (*Castanea sativa*)	Miel Thiebaurd
27	Mountain wildflowers	Miel Véro récolte 2012, Nyer
28	Chestnut (*Castanea sativa*)	Miel châtaignier Fuillat
29	Local wildflowers	Miel de garrigue

3.2. ANTIFUNGAL ANALYSIS

3.2.1. Materials

For antifungal susceptibility testing we used broth microdilution method. The fungal strains used were reference strain for antimicrobial studies and were obtained from the laboratory Laboratoire de Parasitologie et Mycologie Médicale, UFR de Pharmacie, where the analysis was performed.

Table 2: List of Candidae strains

Fungal strain	*Candida albicans:* ATCC 90028 *Candida parapsilosis:*ATCC 22019 *Candida krusei:*ATCC 6258 *Candida glabrata:* ATCC 90030

A suspension with saline of each strain was prepared for further use. The medium used was RPMI (*Roswell Park Memorial institute medium*) 1640 + MOPS. In 1 L of distilled water we prepared: 10.04 g of RPMI 1640 medium (R6504 Sigma RPMI-1640 Medium With L-

glutamine, without sodium bicarbonate, powder, suitable for cell culture) and 34.53 g of MOPS (Sigma M1254). The suspension was thoroughly diluted, adjusted to pH 7 and filtered.

Honey solutions

Solutions of honey and distilled water were prepared in concentration of 1 g/mL.

Honey extracts

Concentrated honey extracts were prepared as follows: the solution of honey and distilled water was extracted with EtOAc, dried and redissolved in water, corresponding to concentration of 3.67 g/mL (3667.5 mg/mL).

3.2.2. Microdilution method

The working solutions of honey extracts were prepared out of dry EtOAc extracts that were dissolved in water. The stock solution that was the initial solution used for additional dilutions was concentrated to an estimated concentration of 3.67 g/mL of initial honey in each extract. This means that MIC 100% represents concentration 3.67 g/mL. (100 µL of the stock solution therefore contains 0.367 g of honey). We prepared further dilutions of stock solution with RPMI 1640, which is the liquid medium used for the *in vitro* growth of the fungi.

Table 3: Preparation of the range of dilutions

Tube	Concentration stock solution % (sample)	Volumes : stock solution (ml) + RPMI (ml)	Concentration	Estimated concentration of honey (g/ml)
1	Solution stock		100%	3.67
2	Stock (100%)	1 + 1	50%	1.835
3	Stock (100%)	0.5 + 1.5	25%	0.918

4	Stock (100%)	0.5 + 3.5	12.5%	0.459
5	Tube 4 (12. 5%)	1 + 1	6.25%	0.230
6	Tube 4 (12. 5%)	0.5 + 1.5	3.125%	0.115
7	Tube 4 (12. 5%)	0.5 + 3.5	1.563%	0.0575
8	Tube 7 (1.653%)	1 + 1	0.781%	0.02875
9	Tube 7 (1.653%)	0.5 + 1.5	0.391%	0.01438
10	Tube 7 (1.653%)	0.5 + 3.5	0.195%	0.007188

Dilution method involved preparing two-fold dilutions of antifungal sample (in our case, honey extract or honey solution) in a liquid medium dispensed in 96 well microdilution plate with flat-bottom wells, with a nominal capacity of approximately 300 μL. Wells 1 to 10 of each column of the microdilution plate were filled with 100 μL from corresponding tube. Column 11 served as negative control (without inoculum – sterility control) and was filled only with RPMI 1640 (200 μL in each well). Column 12 served as positive control (growth control wells) and we dispensed 100 μL of RPMI 1640 in each well (no honey extract).

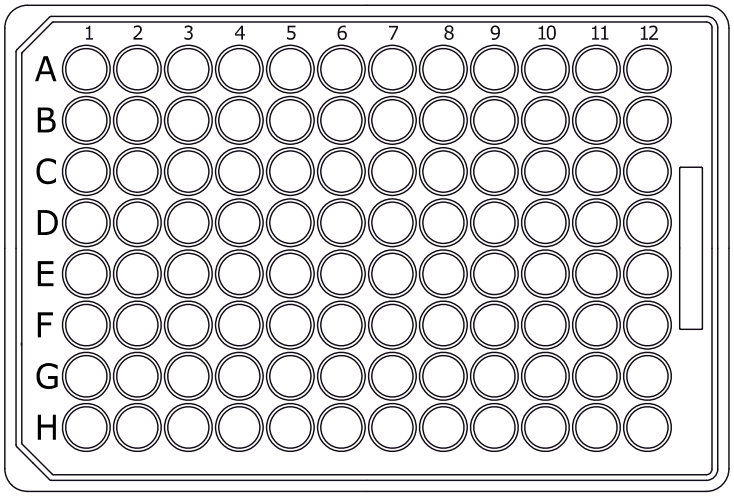


Figure 6: Plate 96 well

We included the results from analysis previously done in our laboratory using the same method, where solutions of honey and distilled water were used as working solutions. We repeated the test with our raw honey samples (honey solutions) using the same microdilution method as for honey extracts.

Preparation of inoculum

An inoculum is the number of yeasts (colony forming units) suspended in a certain volume. The inoculum is expressed as colony forming units per millilitre (CFU/mL).

We used 4 different colonies of fungi: *Candida albicans, Candida parapsilosis, Candida glabrata, Candida krusei* for the preparation of the fungi dilutions. A suspension of strain with saline was prepared using fresh cultures 24-48 hours old. The opacity of suspensions had to be adjusted to the standard 0, 5 McFarland standard which contains 1-5 × 10^6 CFU/mL. Opacity was verified at a wavelength of 530 nm, standard's opacity was 0.179 and allowed deviation was ± 0.020. The adjusted opacity – the cell density gave a suspension of 1-5 x 10^6 CFU/mL. Working suspensions were prepared by diluting 10 times with RPMI to yield 1-5 × 10^5 CFU/mL.

The microdilution plates had to be inoculated within 30 min of preparing the inoculum suspension, in order to maintain the viable cell concentration. Each well was inoculated with 100 µL of the 1-5 × 10^5 CFU/mL fungi suspension, one fungi inoculum was tested in duplicate, per two rows. We did not inoculate the column 11, which served as a negative control. Each *Candida* was tested in duplicate.

The microdilution plates were incubated at 37 °C. First reading of the results was done after 24 hours and then the plates were re-incubated for another 24 hours and then re-read. We tested all the samples in duplicate.

3.3. CHEMICAL ANALYSIS

3.3.1. Materials

For liquid-liquid extraction we used:

Solvents	Ethyl acetate, CARLO ERBA Reagents
	Chloroform, Acros Organics
	Hexane, VWR International
Other chemicals	Sodium sulphate anhydrous, CARLO ERBA Reagents

Apparatus	Separatory funnel
	Orbital shaker, Stuart
	Centrifuge, Selecta

For TLC we used:

Mobile phase	**1. Acetic acid (22%), butanol (56%) and water (22%)** Acetic acid, Sigma Aldrich Butanol, Merck Distilled water, produced at the faculty
	2. water 95%, acetonitrile 5% Acetonitrile, CARLO ERBA Reagents Distilled water, produced at the faculty
	3. water 5%, acetonitrile 95% Acetonitrile, CARLO ERBA Reagents Distilled water, produced at the faculty
	4. buffer pH 3 5%, acetonitrile 95% Buffer solution pH 3.776 (25 °C); Sigma Aldrich
	5. water 10%, acetonitrile 90% Acetonitrile, CARLO ERBA Reagents Distilled water, produced at the faculty
Reference standards	Vanillic acid, Sigma Aldrich Ferulic acid, Sigma Aldrich Coumaric acid, Sigma Aldrich Caffeic acid, Sigma Aldrich
Staining reagents	Fluorescamine, Alfa Aesar Acetonitrile, CARLO ERBA Reagents
	2-aminoethyl diphenylborinate, Sigma Aldrich Methanol, Sigma-Aldrich Polyethylenglycol-4000 (PEG), Sigma Aldrich Ethanol
	2,2-Diphenyl-1-picrylhydrazyl – Sigma Aldrich Hexane, VWR International

	Vanillin, Prolabo Methanol, Sigma Aldrich Sulphuric acid, Sigma Aldrich
TLC plates	**Polar (standard) TLC plates**: HPTLC silica gel 60 F254, glass plates 20 × 10 cm, Merck **Nonpolar TLC plates (reverse phase)**: HPTLC silica gel 60 RP-18 F254s, glass plates 20 × 10 cm, Merck
Device	Automatic TLC sampler 4, CAMAG, works with the program win CATS TLC scanner, CAMAG

For HPLC we used:

Device	HPLC and UV detector, Shimadzu
Column	ZORBAX SB-Phenyl Agilent 4.6 × 250 mm, 5 μm
Mobile phase	**A: Methanol formic acid 0.01%** Methanol, Sigma Aldrich Formic acid, Acros Organics **B: Ammonium formate/Formic acid buffer** HPLC water, Sigma Aldrich Ammonium formiate, Sigma Aldrich
Reference standards	Syringic acid, Sigma Aldrich Gallic acid, Sigma Aldrich Vanillic acid, Sigma Aldrich Ferulic acid, Sigma Aldrich Coumaric acid, Sigma Aldrich Caffeic acid, Sigma Aldrich

3.3.2. Liquid-liquid extraction

For separating and identifying the compounds in honey we chose the method of liquid-liquid extraction. A part of the proceedings was chosen based on previous work in the same laboratory at the faculty (9); some were based on experimental work. Extraction is a good method for handling the honeys since it eliminates some previously identified antimicrobial compounds. The goal was to extract the moderately lipophilic compounds; among solvents which can be used for extraction, we chose more polar ones. Another consideration for further work was the evaporation of the solvent that is why we chose more volatile solvents. The solvents were chosen to avoid the presence of sugars in the extract samples. We also tested solvents with different polarities to extract the maximum of compounds of interest. When sugars (fructose, glucose, saccharose, maltose, other sugars) and water were eliminated from the composition of honey, mostly hydrophilic compounds and a minority of hydrophobic compounds remained.

Solvent: ethyl acetate (EtOAc)

The first choice of solvent for extraction was EtOAc. EtOAc was chosen based on the previous work in the same laboratory. It is a polar solvent with boiling point of 77.1 °C. It is relatively non-toxic and a common choice of solvent.

Since honey is a viscous liquid we first prepared honey solutions with distilled water in the ratio 1:1 w/w (e.g. 15 g of honey, 15 g of water). Considering that the density of honey is 1.42 g/mL, the starting concentration of honey was 0.587 g/mL (586.8 mg/mL). For the extraction, we used the same amount of honey solution and EtOAc (e.g. 25 mL of honey solution, 25 mL of solvent). The separatory funnel was mixed and then put on the orbital shaker for 10 minutes at 200 rpm. The two layers needed to be mixed together vigorously to provide maximum surface contact between the two immiscible layers so that substances could be pulled or extracted from one into the other. Afterwards we separated organic and water phases. If the phases were not clearly separated, we used centrifuge (5 minutes on 3000 rpm). We collected the organic phase and evaporated the solvent, using heater at 80-85 °C. For our further chemical part of analysis, we used concentrated EtOAc extracts. Dry extracts were re-dissolved in smaller amount of EtOAc (2 mL) which corresponds to a

concentration of honey of 7.34 g/mL (7335 mg/mL), and stored in refrigerator for further work.

Solvents: hexane, chloroform

The aim of our further work was to verify if we can extract other remaining compounds with potential antimicrobial activity. The extraction was carried out using honey solutions and solvent in ratio 1:1. The extracts were not concentrated in the end; we used the organic phase for further chemical analysis. Since we were working with smaller volumes (2 mL of each phase) the extraction was executed in tubes. They were mixed on rotator for 20 min on 40 rpm. The resulting solution was then centrifuged (5 minutes on 3000 rpm) to separate the organic and aqueous layers. After that the aqueous phase was removed with Pasteur pipet. When working with chloroform the aqueous phase stays on top because chloroform has higher density than water (1.49 g/mL). Since the extracts were not concentrated and assuming the extraction was complete (100%), we were working with concentration corresponding to 0.587 g/mL (586.8 mg/mL) of honey.

3.3.3. TLC

TLC chromatography was the first technique that we used to examine the extracts. In the beginning, we compared TLC chromatographs of extracts prepared with different extraction solvents, to see how a choice of solvent influences the extractions. Next, we examined how different mobile phases influence the results, if we can achieve better separation of chromatographic spots. We also examined the influence of polar and nonpolar plates. We used staining reagents to examine if there were other compounds present that we had not observed yet. In the end, we used reference standards of phenolic acids to confirm or disconfirm their presence in the extracts.

Primary analysis

TLC chromatography was executed using polar silica plates. The mobile phase was a mixture of acetic acid (22%), butanol (56%) and water (22%). We applied 10 μL of each sample onto a point, on the plate. Between each drop we waited for the solvent to evaporate, we used blow dryer to speed up the drying process to prevent any interfering. The TLC plates

were then put into the developing chamber with mobile phase where they were submerged into the mobile phase for about 0.5 cm. The mobile phase needed a few hours to move up the plate by capillary action, meet the sample mixture and carry it up the plate (elute the sample). The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase, and dried.

The results of TLC were observed under UV light. We used wavelength of 366 nm for observing the compounds that fluoresce. Wavelength of 254 nm was used to observe the compounds that quench the UV light, and dark spots appeared.

Modifications of primary analysis

- Nonpolar TLC plates - reversed phase

TLC was performed using nonpolar plates – reversed phase. We used HPTLC silica gel plates 60 RP-18 F 254. For the application of the samples in bands we used Automatic TLC sampler. In the beginning, we deposited 40 µL of the samples. After the first plate was developed we figured that we could improve the resolution by depositing less product. Therefore, 10 µL of each sample was used for further analyses instead.

For the next nonpolar plates, we used MP 2, mixture of water 5%, acetonitrile 95%.

Later we tested the mobile phase MP 3 buffer pH 3 5%, acetonitrile 95%. Since we suspected the presence of acidic compounds (phenolic acids), we lowered the pH of mobile phase with buffer to reduce the potential streaking.

Visualisation reagents

After the plate developed it was dried to remove the mobile phase. Usually the separated compounds are detected visually, in daylight or under UV light. Substances that cannot be seen this way have to be visualized with suitable detection reagents to form coloured, fluorescent, or UV-absorbing compounds by means of derivatisation reactions (post chromatographic derivatisation). We used different visualisation reagents, their preparation is described below. We prepared one TLC plate with 8 depos of the same honey (chestnut

honey 11) and sprayed visualisation reagents from number 1 to 4 in order, two depos for each reagent. For the last visualisation reagent, number 5, we used another plate.

1. **Primary amines**

Fluorescamine was used for detection of primary and secondary amines, peptides, sulphonamides. Plate was sprayed with a solution of 0.5 mg/mL 4-phenylspiro [furan-2(3*H*), -1'-phthalan]-3, 3'-dione in acetonitrile that needs to be prepared daily and used fresh.

2. **Flavonoids**

For detection of flavonoids NP/PEG natural products-polyethylenglycol reagent was used. The plate was sprayed with 1% methanolic diphenyboricacid-β-ethylamine ester (diphenylboryloxyethylamine) (NP), followed by 5% ethanolic polyethylenglycol-4000 (PEG) (10 mL and 8 mL, respectively).

3. **Polyphenols**

DPPH (2, 2-diphenylpicrylhydrazyl) was used to detect polyphenols. 100 mM solution in hexane was prepared and used fresh.

4. **Non-specific staining reagent**

Vanillin/sulfuric acid is a universal staining reagent that can be used for detection of higher alcohols, phenols, steroids, essential oils, and esters. Solution of 3 g of vanillin, 100 mL of methanol, and 3 mL sulphuric acid was sprayed on the plate which was then heated on 120 °C for 10 min.

Identification of phenolic acids using standard references of caffeic acid, vanillic acid, ferulic acid and *p*-coumaric acid

Honey extracts contain different compounds. One group that could also be responsible for the antibacterial activity are phenolic compounds. We performed qualitative comparison of our extracts with TLC standards of phenolic acids: caffeic acid, vanillic acid, ferulic acid and *p*-coumaric acid. HPTLC was performed to identify chromatographic markers and to compare chromatographic fingerprints of different honeys.

We used honey extracts:

- Forest honey (Rayon d'Or, Miel et Miellat de forêt) (1),
- Honeydew honey (Le Rucher de marina) (10),
- Chestnut honey (Miel de châtaignier, NYER) (14),
- Spurge honey (Matéro, Miel d'Euphorbe Bio) (20),
- Chestnut honey (Miel Rico) (22),
- Mountain wildflowers' honey (Miel Véro récolte 2012, Nyer) (27).

Honey extracts were prepared with liquid-liquid extraction with solvent EtOAc.

The standard references were caffeic acid, vanillic acid, ferulic acid and *p*-coumaric acid. Standard references were prepared in concentration 0.1 mg/mL, the solvent system was formic acid buffer (60%) and methanol with formic acid (40%).

We used standard phase plate (polar) (HPTLC silica gel 60 F_{254}, glass plates 20 × 10 cm, Merck) and MP 1: acetic acid 22%, butanol 56%, and water 22%.

3.3.4. HPLC

Identification and qualification of phenolic acids (syringic acid, gallic acid, caffeic acid, *p*-coumaric, vanillic acid, ferulic acid) in honey by the use of HPLC-UV

First, we wanted to determine the percentage of extraction. A dilution of honey with distilled water was made, by weighing 10 g of honey and 10 g of water. 20 mL of the honey solution (approx. 8.4 mL of pure honey) were extracted with 20 mL of EtOAc. A second extraction was done the same way using the same honey but by adding 4 mg of each phenolic acid (syringic acid, gallic acid, caffeic acid, *p*-coumaric, vanillic acid, ferulic acid). After 10 minutes of rotation and 5 minutes of centrifugation the water phase was detached and the organic phase was evaporated. Afterwards, the residues were diluted in 2 mL of 40% methanol, formic acid 0.01% and 60% ammonium formate/formic acid buffer. The concentration of added phenolic acids correlates to a concentration of 2 mg/mL. This concentration was too high to be directly analysed by HPLC, therefore the sample was diluted to a concentration of 0.01 mg/mL.

With the information about the percentage of extraction we could determine the concentration of the phenolic acids in honey. All the honey extracts were prepared in the same manner as previously described and analysed by the following HPLC method.

Column:

ZORBAX SB-Phenyl RRHT, Agilent

ID 4, 6mm × 250 mm, (5 μm) threaded column

Mobile phase:

A: MeOH formic acid 0.01%

B: Ammonium formate/Formic acid buffer (pH 3.3, ionic strength 7.4 mM)

Flow rate:

0.8 mL/min

Table 4: Gradient of mobile phase

Gradient	A (Me-OH)	B (Buffer)
0 min	40%	60%
13 min	40%	60%
15 min	100%	0%
17 min	100%	0%
17.1 min	40%	60%
20 min	40%	60%

Detection UV: 200-360 nm

The honey's phenolic compounds analysis was performed using HPLC equipped with UV detector. The sample was injected in the HPLC system with a high-performance microparticulate phenyl column (ZORBAX SB-Phenyl RRHT, Agilent). The injection volume for all samples was 10 μL. The elution was with a flow rate of 0.8 mL/min and the

gradient program was as stated in the table above. The identification was carried out using the peak retention time and spectrum compared with phenolic acids commercial standards.

4. RESULTS AND DISCUSSION OF RESEARCH

4.1. ANTIFUNGAL ANALYSIS

Our method of antifungal analysis was a combination of *in vitro* antifungal susceptibility tests by institutes CLSI and EUCAST. Both institutes provide a standardized method which results in credible results (in the dynamic field of mycology). The newest, third edition of CLSI's method (»Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved standard M27-A3«) was published in 2008. It includes a description for testing susceptiblity to antifungal agents of yeast that cause invasive infections (*Candida* spp. and *C. neoformans*). Other methods can be developed from it, using it as a reference method..

According to the definition in CLSI »Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved standard M27-A3« the definition of MIC is: »MIC is the lowest concentration of an antimicrobial agent that causes a specified reduction in visible growth in an agar or broth dilution susceptibility test. The magnitude of reduction in visible growth is assessed using the following numerical scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease (~50%) in visible growth; 3, slight reduction in visible growth; and 4, no reduction in visible growth« (18).

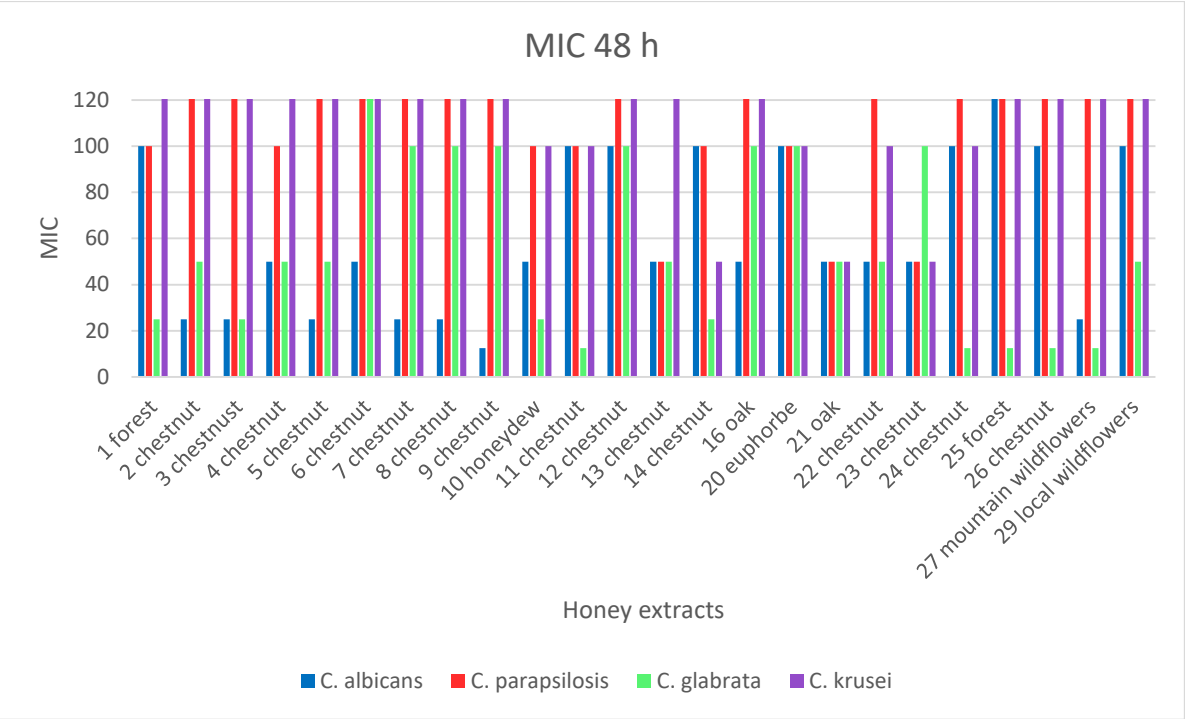
EUCAST also developed methods to establish MIC. They could be used as reference methods to establish the activity of new antifungal agents, as in our case. EUCAST defines MIC as: »the lowest concentration, recorded in mg/L, of an agent that inhibits the growth of a fungus to a predefined degree (e.g. 50%, 90%, or complete growth inhibition)« (19). The EUCAST method is principally similar to the CLSI M27-A3 method with modifications of some test parameters such as inoculum preparation, inoculum size, and the MIC reading method which is spectrophotometric in EUCAST guideline and visual in CLSI guideline (16).

The differences in test parameters of CLSI M27-A2 11 and EUCAST E.Dis 7.1 47 broth dilution methods are written in the Table 5 below (20).

Table 5: Test parameters in CLSI M27-A1 and EUCAST E. Dis 7.1.

Test parameter	**CLSI M27-A2**	**EUCAST E.Dis 7.1**
Test medium	RPMI 1640 with glutamine, without bicarbonate glucose concentration: 0.2%	RPMI 1640 with glutamine, without bicarbonate glucose concentration: 2%
Microdilution plates	96 U-shaped wells	96 flat-bottom wells
Inoculum density	0.5–2.5×10^3 CFU/mL	1–5×10^5 CFU/mL
MIC reading method	Visual	Spectrophotometric (530 nm)
MIC reading time point	48 h	24 h

In our study, MIC was the lowest concentration of tested agent that inhibited the growth of a microbe for 50%, compared to the positive control, with an unaided eye. MIC was evaluated visually, measuring the growth of the fungi and comparing it on a scale 0-6. The positive control's growth (column 12 in 96 well) was marked with 4, which is an optimum growth of fungi. If the growth in other wells was estimated to be smaller, the mark was lower. Where the growth was seen to be reduced by 50% it was marked with 2 and this counted as MIC. Mark 5 and 6 were given if the growth exceeded the growth of positive control. The reading was done at 24h and 48h, but the final MIC was determined at 48h. The results are shown below.



Graph 1: MIC 48h of honey extracts

Table 6: MIC and corresponding estimated concentration of honey

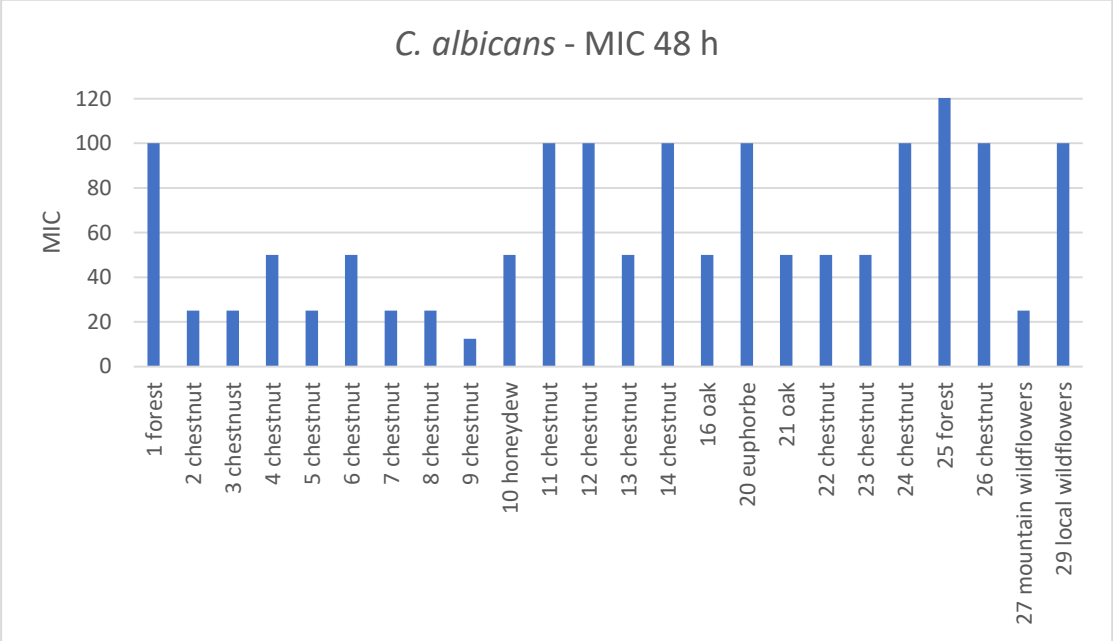
MIC > 100	no visible inhibition (>100% needed)
MIC 100	inhibiton of growth at 375 mg/ 100 µL of honey
MIC 50	inhibiton of growth at 188 mg/ 100 µL of honey
MIC 25	inhibiton of growth at 93.8/ 100 µL mg of honey
MIC 12.5	inhibiton of growth at 46.9 mg/ 100 µL of honey
MIC 6.25	inhibiton of growth at 23.4 mg/ 100 µL of honey
MIC 3.1	inhibiton of growth at 11.7 mg/ 100 µL of honey
MIC 1.6	inhibiton of growth at 5.86 mg/ 100 µL of honey
MIC 0.8	inhibiton of growth at 2.93 mg/ 100 µL of honey
MIC 0.4	inhibiton of growth at 1.47 mg/ 100 µL of honey
MIC 0.2	inhibiton of growth at 0.73 mg/ 100 µL of honey

The results of MIC value of all tested honeys are shown in the Graph 1. For clearer view, the results for each *Candida* strain are shown in Graph 2 (C. albicans), Graph 3 (*C. parapsilosis*), Graph 4 (*C. glabrata*) and Graph 5 (*C. krusei*). The more potent the antifungal

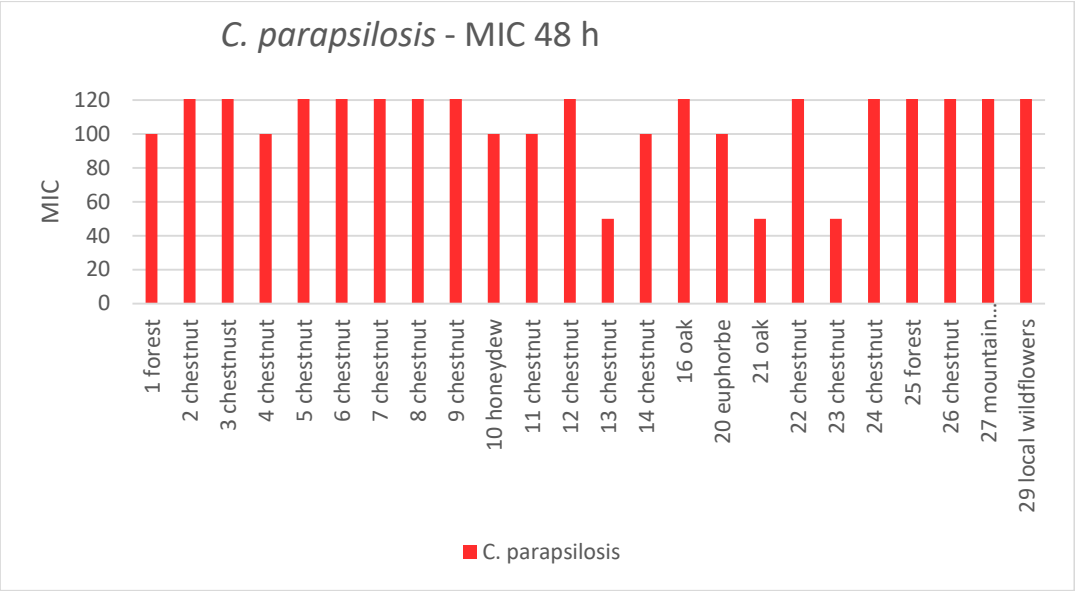
activity of honey extract is, the lower the column. There was no inhibition of growth of fungi where MIC is over 100.

In general, all the honey extracts were more efficient in the inhibition of growth of *Candida albicans* and *Candida glabrata*, less in inhibition of growth of *Candida parapsilosis* and even less for *Candida krusei*.

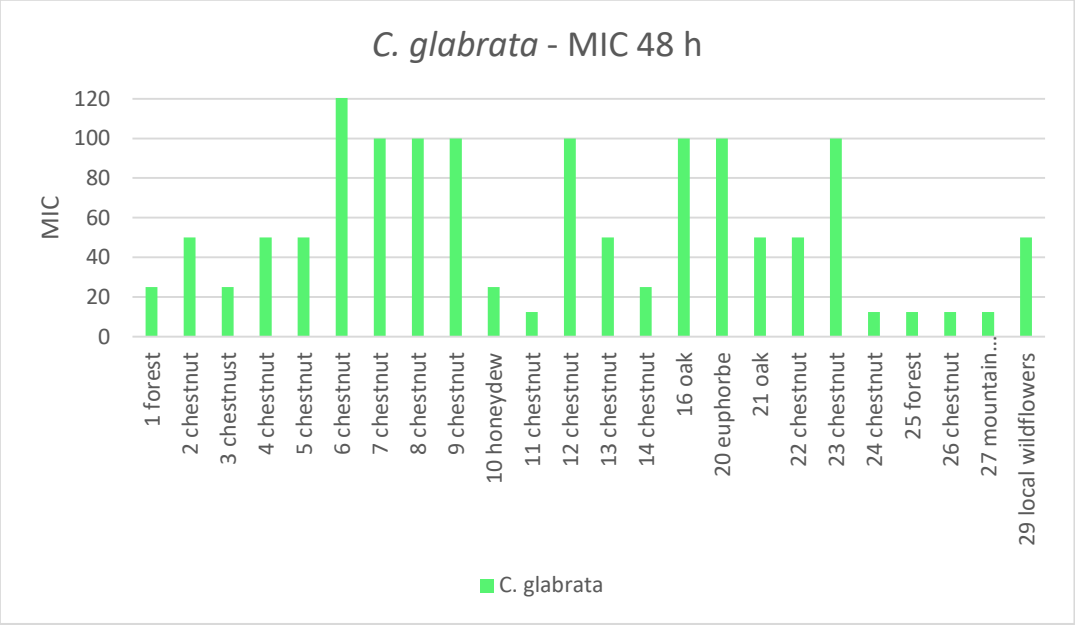
Our results showed that not all honeys from the same source demonstrate the same antifungal activity. Therefore, we cannot combine the results from all chestnut honeys. Furthermore, the fungi were not uniformly affected by honey. We can conclude that the antifungal ability of honeys varies widely and cannot be attributed to a specific source or geographic region (south of France).



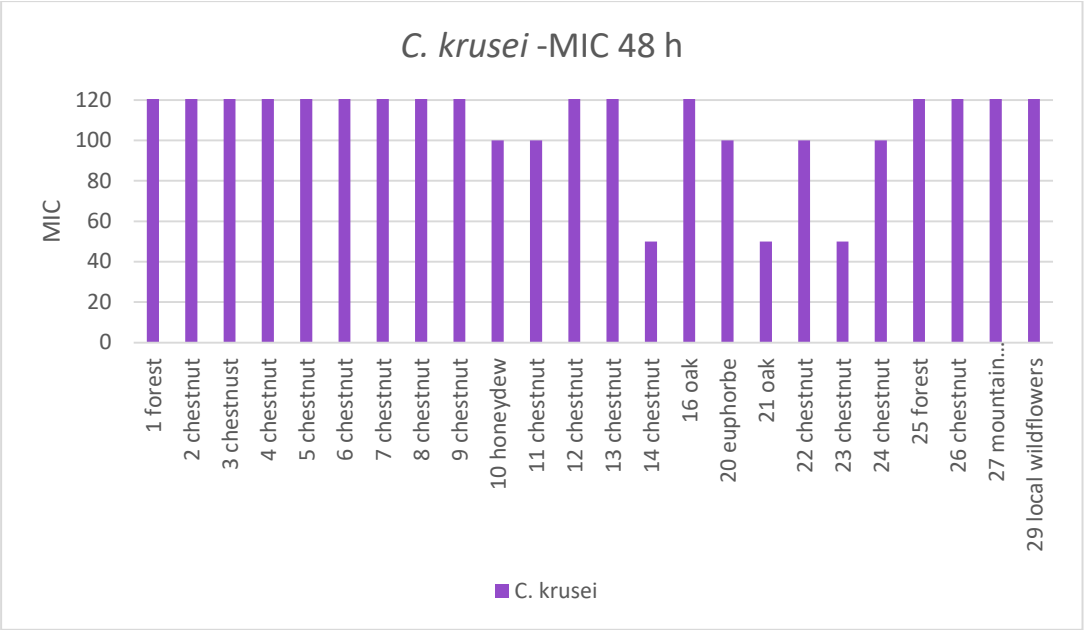
Graph 2: MIC 48 h for C. albicans



Graph 3: MIC 48 h for C. parapsilosis



Graph 4: MIC 48 h for C. glabrata



Graph 5: MIC 48 h for C. krusei

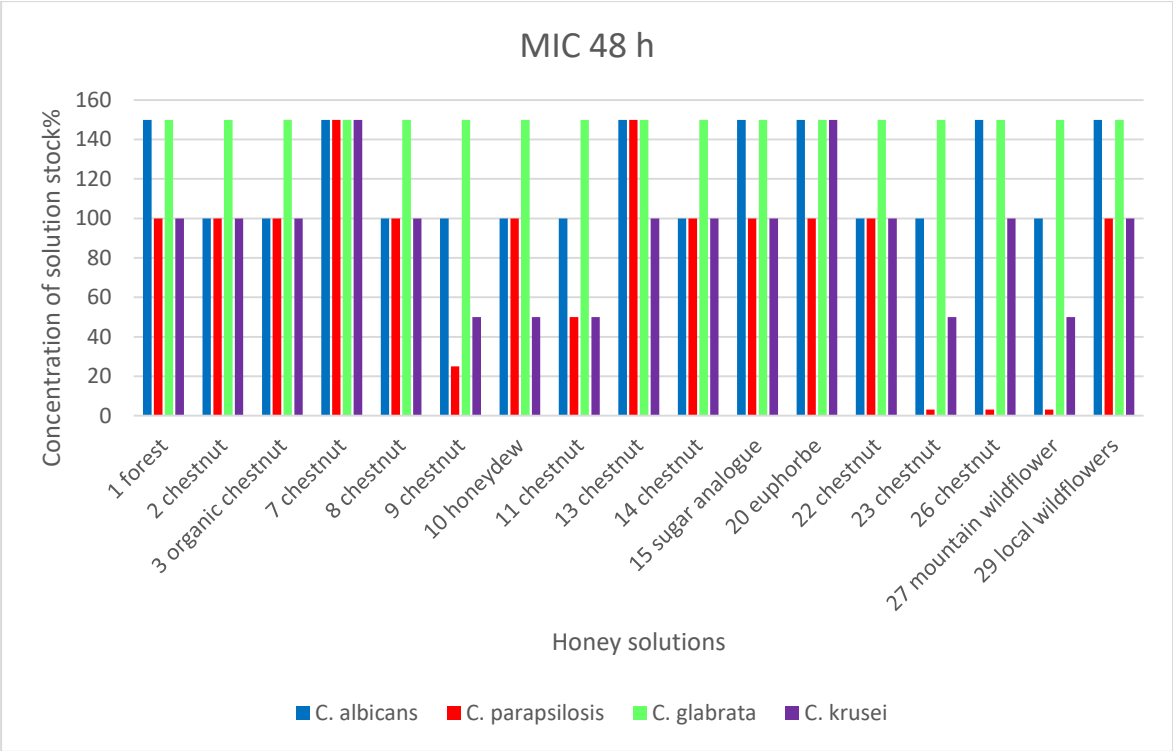
There are studies regarding honey and its potential therapeutic action against different pathogens. Whether the studies were focused on honey's antifungal or antibacterial activity, most of the researchers were using raw honey and/or honey solutions. Methods usually included different concentrations of honey that were used on agar diffusion methods or dilution methods in wells.

To determine the rate of inhibition caused by sugar, hydrogen peroxide, or other recognised antimicrobial components of honey, the components are tested alongside honey samples. The usual four types of inhibition against pathogens as noted by M.A. Mundo et al. and tested in studies are: inhibition due to high sugar concentration (reduced water activity), hydrogen peroxide formation, presence of proteinaceous antimicrobial compounds, or other unidentified components (21).

A similar method for antifungal activity of honey was performed in our laboratory beforehand, using raw honey against different strains of *Candida* (9). The researchers concluded that fungus is not that sensible to honey solution and that there are not many differences between honey's and sugar's antimicotic activity. We repeated the same method of antifungal test as the researchers in our laboratory beforehand with our samples of honey, the results are shown in Graph 6.

As already mentioned, honey extracts were more efficient in the inhibition of growth of *Candida albicans* and *Candida glabra*ta, less in inhibition of growth of *Candida parapsilosis* and even less for *Candida krusei*. The results of raw honey samples show opposite outcome; honey (honey solution with water) was more effective in inhibiting *Candida parapsilosis* and *Candida krusei*. The results also show similarities with sugar analogue and honey samples in their fungi growth inhibition. None of the honeys inhibited the growth of *Candida glabrata*. Although this is not visible from the table, we should note that in most cases honey solution increased the growth of fungi. In the first column of wells where the concentration of honey was 1 g/mL we noticed inhibition of growth, but with further dilutions we could see an increase in growth. The results were the same with sugar analogue. There was an inhibition of growth of *Candida parapsilosi*s and *Candida krusei* at 1 g/mL, and with further dilutions the growth was comparable to positive control. With *Candida albicans* and *Candida glabrata* the increase in growth was higher than in the positive control.

We can establish that the effect of honey solutions is at least partly sugar-dependent; i.e. its concentration is not high enough for hyperosmolar effect but it is high enough to represent extra nutrition for fungus, it helps them grow. But since this effect is not constant, there are other compounds that are antimicrobial.



Graph 6: MIC 48h of honey solutions

4.2. CHEMICAL ANALYSIS

4.2.1. Liquid-liquid extraction

Honey is in essence a highly concentrated water solution of sugars. Many other substances also occur in honey and they make up about 6%, but sugars are by far the most prevalent components (76%). As already mentioned, sugar is one of the main reasons for antimicrobial activity of honey. To eliminate its effect, we decided to work with extracts and not pure honey. Moreover, sugar could also influence the results of TLC, not just antimicrobial testing, since it prevents other components to rise on TLC plate. If sugar and water are eliminated from honey there is a small percentage of hydrophilic compounds left and even fewer hydrophobic compounds. With a proper choice of solvent, we could extract components in the hydrophilic part of honey. Besides polarity, the volatility of the organic solvent is also important. Solvents with low boiling points were chosen since the material does not need to be exposed to too high temperatures during the evaporation of the solvent. There are also practical concerns regarding chosen extraction solvent. Accessibility, cost and

toxicity of the used material should be considered. Researchers in our laboratory ruled out diethyl ether and dichloromethane as possible solvents (too volatile) and they chose EtOAc as their choice of solvent.

Solvent: EtOAc

Extraction with EtOAc produced extracts that were used for further TLC analysis and are described in chapter 4.2.2. With some honeys, we noticed the occurrence of emulsion between the phases so the samples were centrifugated. EtOAc extracts were the samples we used for antifungal analysis.

Solvents: hexane

When using hexane as a solvent there were no visible spots under UV lamp. Hexane is a very nonpolar solvent, with it we could extract more nonpolar components but we concluded that there are almost none in honeys. We ended our work with hexane afterwards.

Solvent: chloroform

When chloroform was used as a solvent it extracted some compounds from honey, but very few. Hence, work with chloroform was terminated after visually observing the TLC plates.

Extraction efficiency

At the beginning, liquid-liquid extraction was used as a step during qualification of the components of honey. Later, we also ran HPLC analysis for quantification purposes and we could assess the effectiveness of extractions. The results of HPLC are described in chapter 4.2.3. We can see that the average percentage of extraction of 6 phenolic acids was 51%. We could improve the effectiveness with multiple extractions. Since the distribution coefficient is a ratio, unless K is very large, not the entire solute would reside in the organic layer after one extraction. Usually two or more extractions of the aqueous layer with an organic solvent are needed in sequence in order to take away as much of the desired product from the aqueous layer as possible.

$$\text{fraction extracted into B} = \left(\frac{1}{1 + \frac{V_B}{V_A n K}} \right)^n$$

Equation 1: Fraction of extracted material

The equation provides the fraction of material extracted by solvent B where *n* is the number of extractions performed, K is the distribution coefficient, V_A is the volume of solvent A and V_B is the volume of solvent B.

4.2.2. Thin-layer chromatography

Primary analysis

The first analysis was performed using all honeys. EtOAc was used as an extraction solvent and mobile phase was a mixture of acetic acid 22%, butanol 56%, and water 22%. We noticed differences between the composition of honeys, especially between chestnut honeys and the rest. We could notice that not all chestnut honeys have the same composition. Based on the results we chose the extracts for further TLC analysis.

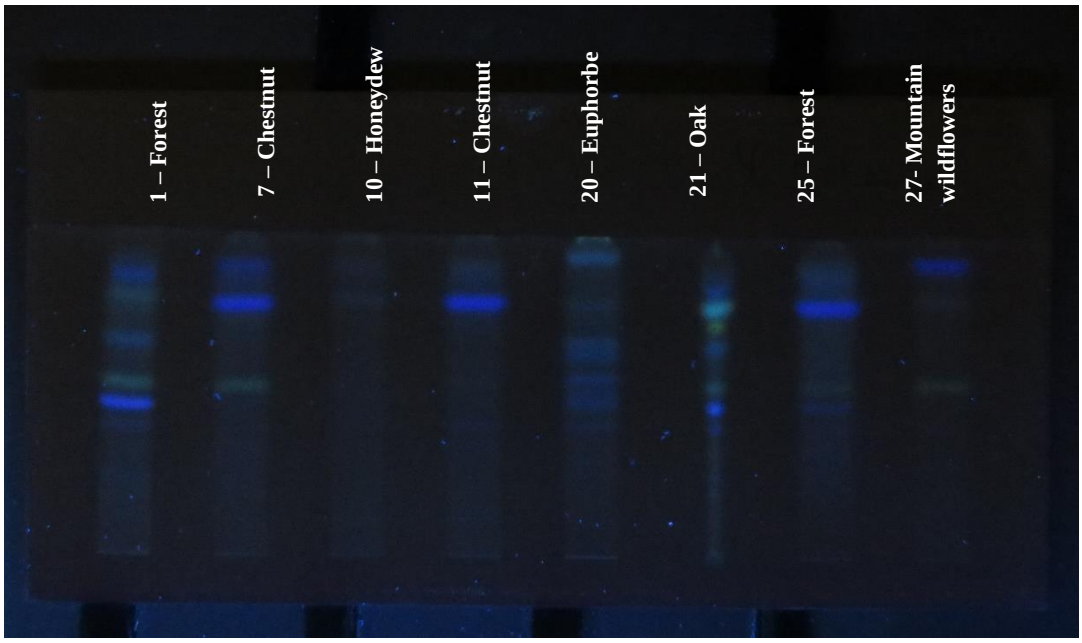


Figure 7: TLC standard phase (polar) plate, UV 366 nm

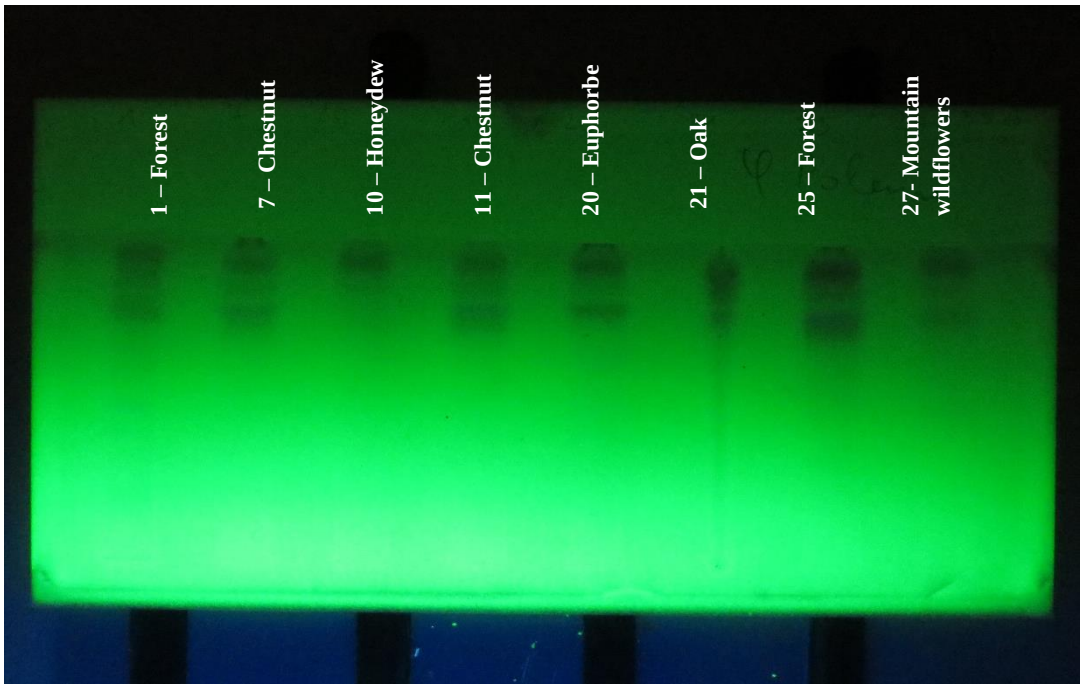


Figure 8: TLC standard phase plate (polar), UV 254 nm

Modifications of primary analysis

- Nonpolar TLC plates - reversed phase

The best mobile phase for the nonpolar plates was a mixture of water 5%, acetonitrile 95%. We used 40 µL and 10 µL samples to deposit on the plate. The results were better, less blurred, with 10 µL samples, which were used for further work.

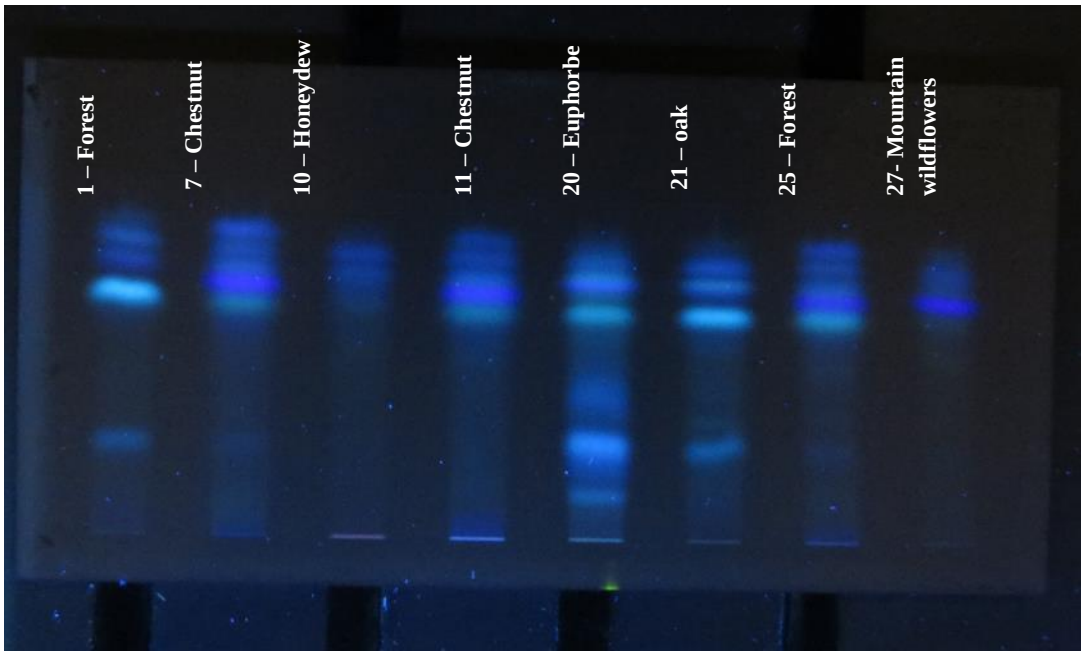


Figure 9: TLC reverse phase (nonpolar) plate, mobile phase water 5%, acetonitrile 95%, 10 µL of samples, UV 366 nm

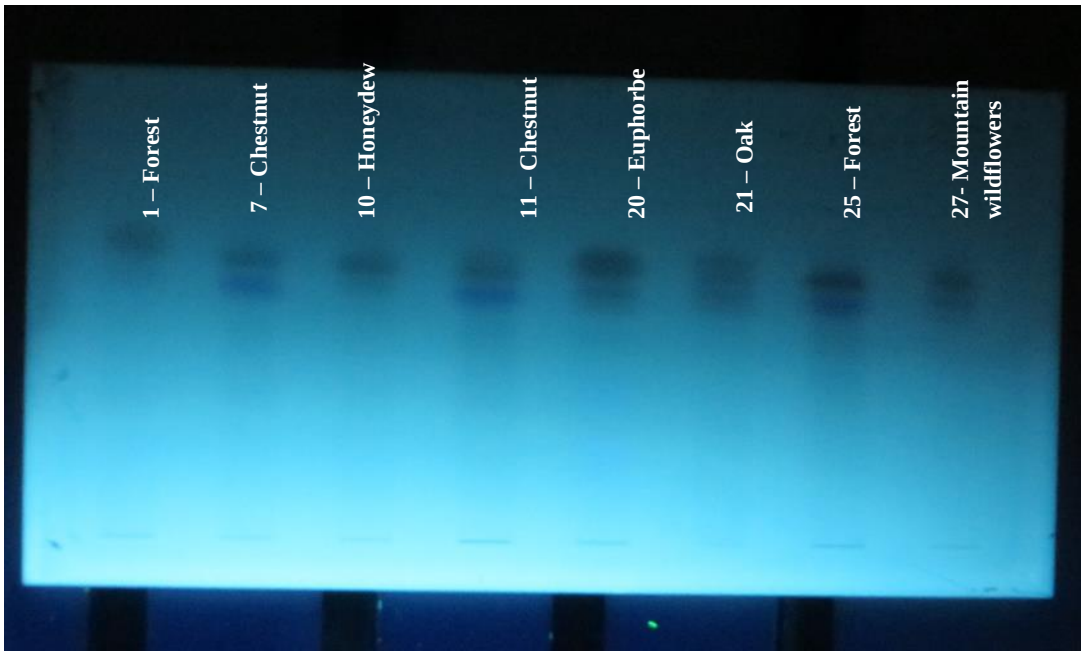


Figure 10: TLC reversed phase plate (nonpolar), mobile phase water 5%, acetonitrile 95%, 10 µL of samples, UV 254 nm

We tested mobile phase buffer pH 3 5%, acetonitrile 95%. Since we suspected the presence of acidic components we lowered the pH value of mobile phase to reduce streaking. This

mobile phase moved up quickly and as the lines are not that blurred, the streaking is reduced. But because not all compounds are clearly seen we chose the first mobile phase for further work with nonpolar plates.

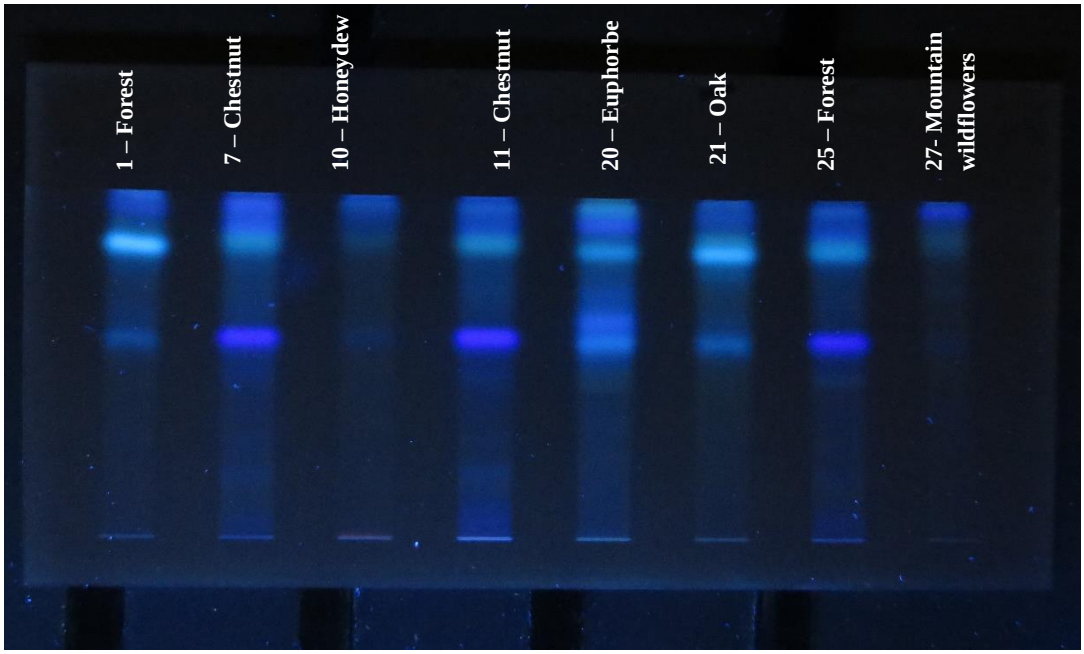


Figure 11: TLC reversed phase plate (nonpolar), mobile phase buffer pH3 5%, acetonitrile 95%, 10 µL of samples, UV 366 nm

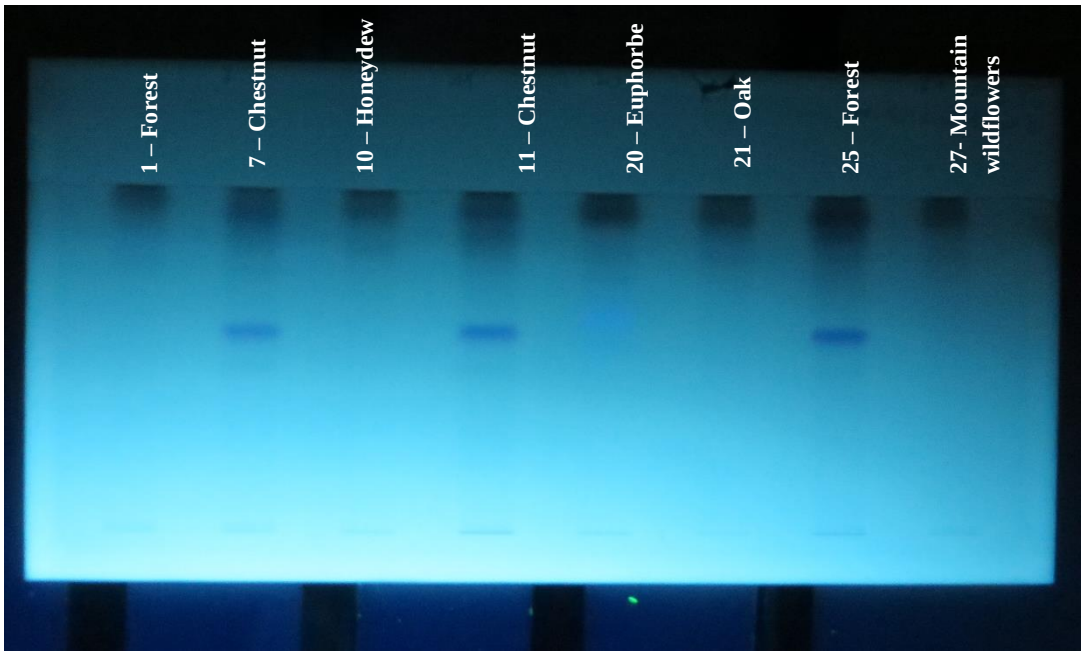


Figure 12: TLC reversed phase plate (nonpolar), mobile phase buffer pH3 5%, acetonitrile 95%, 10 µL of samples, UV 254 nm

Visualisation reagents

Four visualisation reagents were used for detection of: primary amines (fluorescamine), flavonoids (NP/PEG reagent), polyphenols (DPPH), general non-specific staining reagent (vanillin/sulfuric acid).

We only noticed difference with general non-specific staining reagent (vanillin/sulfuric acid). We noticed another compound, with lower R_f than the rest of them. The compound was scraped from the plate, extracted from the silica with solvent EtOAc and will be identified in the future work with mass spectrometry (MS).

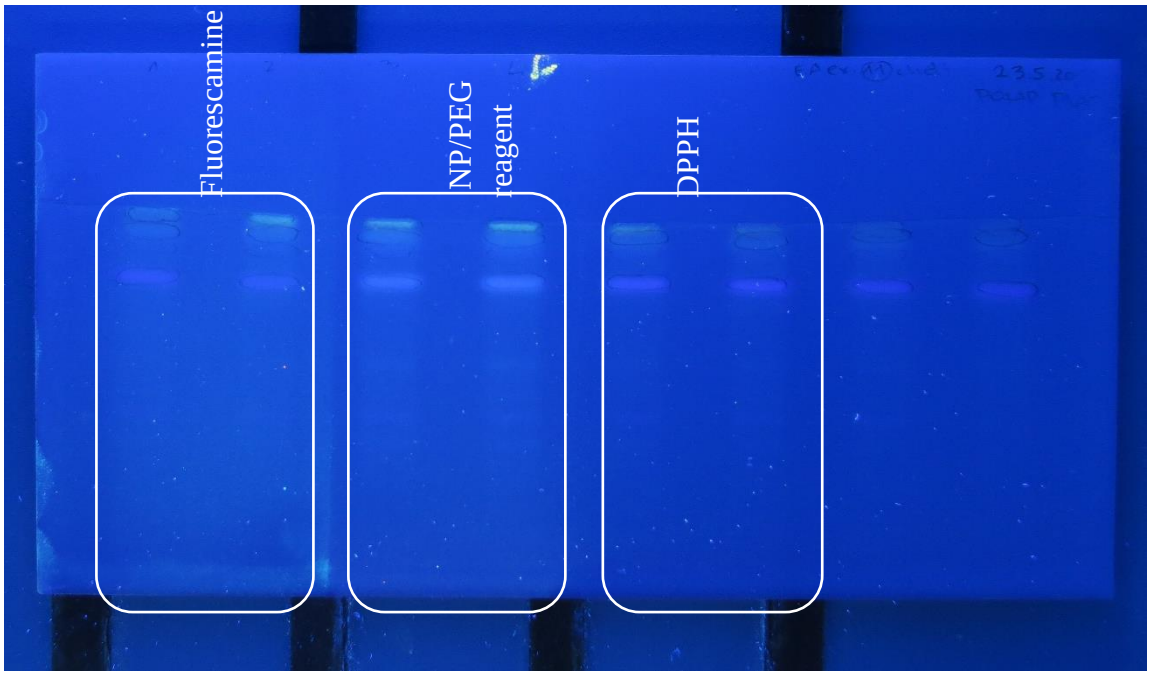


Figure 13: Staining of TLC plate with visualisation reagents- fluorescamine; NP/PEG reagent; DPPH

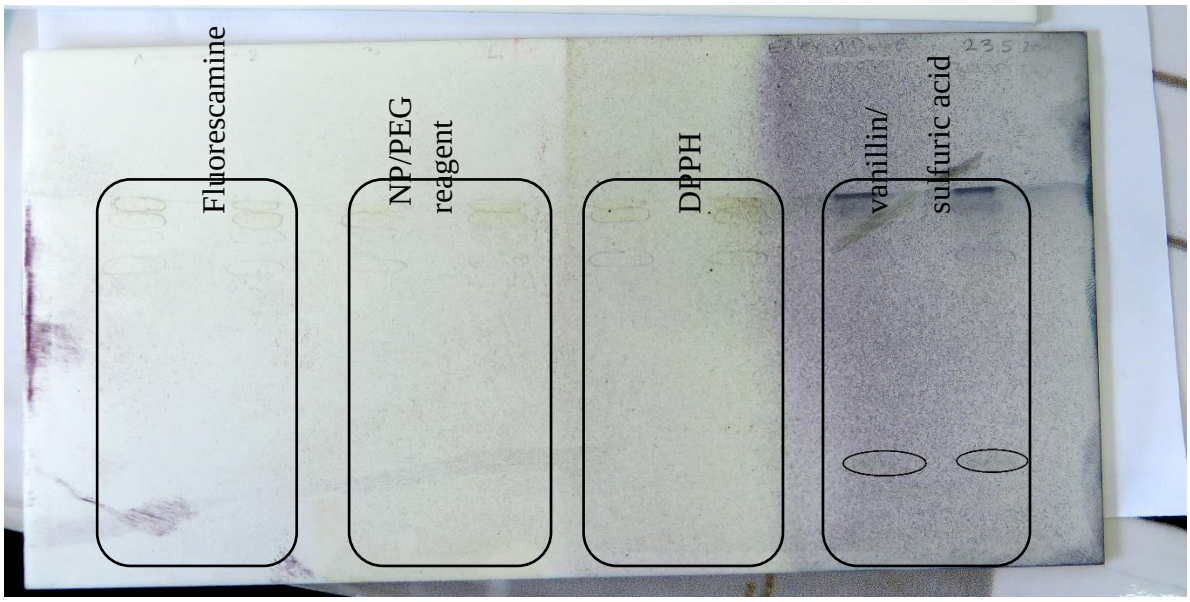


Figure 14: Staining of TLC plate with visualisation reagents - vanillin/sulfuric acid

Identification of phenolic acids using standard references of caffeic acid, vanillic acid, ferulic acid and *p*-coumaric acid

For identification of phenolic acids, we made a comparison of standard references (caffeic acid, vanillic acid, ferulic acid and *p*-coumaric acid) with honey extracts (forest honey, honeydew honey, chestnut honey, spurge honey, mountain wildflower honey) on a TLC plate. All 4 phenolic acids quench the fluorescence (UV 254 nm) but only caffeic and ferulic acid produce fluorescence (UV 366 nm). It is difficult to confirm or disconfirm the identity of compounds in honey based on TLC results because the R_f of phenolic acids are quite similar. The components of honeys in the similar area of R_f as the phenolic acids are not well separated. For further qualitative and quantitative evaluation of the compounds we performed HPLC analysis.

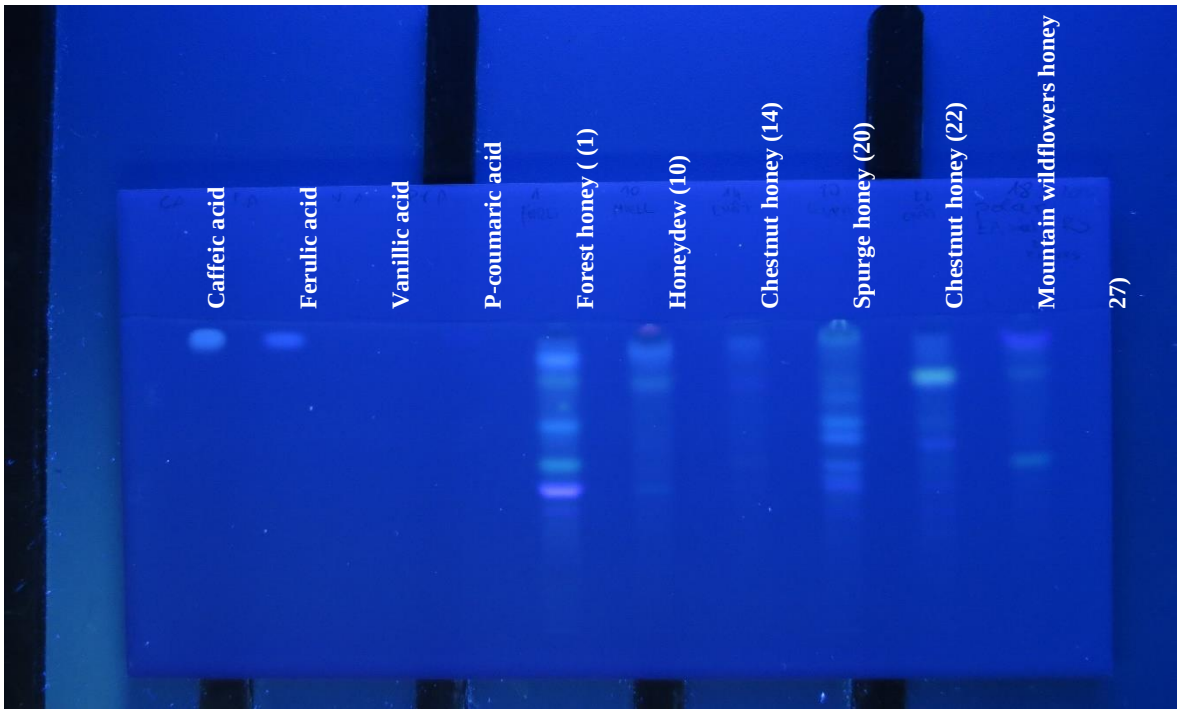


Figure 15: TLC of standard references and honey extracts, standard phase plate (polar), UV 366 nm

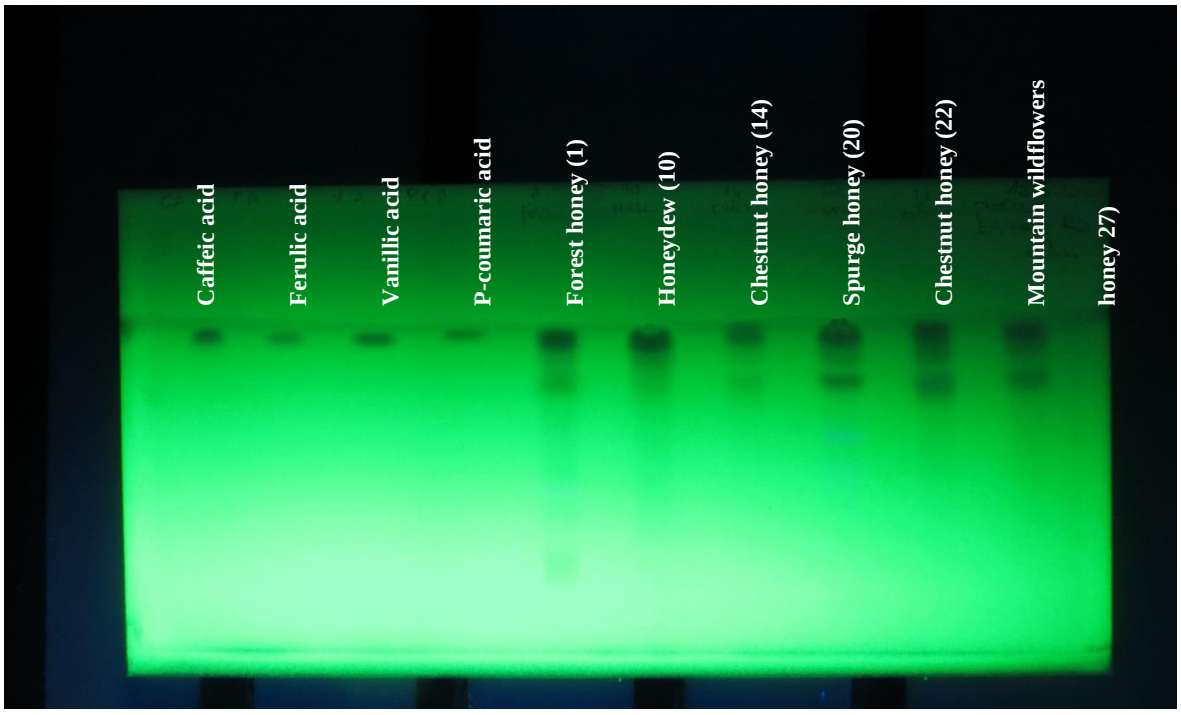


Figure 16: TLC of standard references and honey extracts, standard phase plate (polar), UV 254 nm

4.2.3. High-performance liquid chromatography

Determination of the extraction factor

Mix of the 6 phenolic acids at a concentration of 5 mg/L each

Solvent: 60% Ammonium formate/formic acid buffer, 40% MeOH formic acid 0.01%

Table 7: $Area_{5mg/L}$ and $Area_{10mg/L}$ of phenolic acids

Name	**Absorption wavelength (nm)**	**Retention time (min)**	**$A_{5mg/L}$**	**$A_{10mg/L}$** $A_{10mg/L}=2\times A_{5mg/L}$
p-Coumaric acid	310	12.29	424773	849546
Caffeic acid	325	7.61	136279	272558
Ferulic acid	325	14.12	170035	340070
Vanillic Acid	260	8.21	224494	448988
Gallic acid	271	4.03	206864	413728
Syringic acid	220	9.41	460215	920430

Extract diluted by the factor 200 with an **addition of 10 mg/L of each phenolic acid**

Table 8: Percentage of extraction of phenolic acids

Name	**Retention time (min)**	**A_{ex+ad}**	**A_{ad}** Corresponding to extracted addition of the acids (exclusion of area showed by the acids in the honey) $A_{ad}=A_{ex+ad}-(A_{ex}/200)$	**Percentage of extraction (ex%)** $ex\%=A_{ad}/A_{10mgL}\times100$
p-Coumaric acid	12.32	433938	433390	51.0%
Caffeic acid	7.62	143074	142590	52.3%
Ferulic acid	14.15	175503	175440	51.6%
Vanillic Acid	8.22	192767	192699	42.9%
Gallic acid	4.02	122075	11882	28.7%
Syringic acid	9.42	722486	722320	78.5%

Determination of the phenolic acids concentration in honey

Extract of honey number 14: Chestnut honey

Table 9: Phenolic acid concentration in honey

Name	**Retention time (min)**	**A_{ex}**	**Concentration in the extract (c_e) (mg/L)** $c_e=A_{ex}\times 5$ mg/L/$A_{5mg/L}$	**Concentration of the extract, for an extraction of 100% (c_{100}) (mg/L)** $c_{100}= c_e\times 100/ex\%$	**Concentration in honey (c_h in mg/kg honey)** $c_h=c_{100}$ mg/L×0,002L× 1000g/11.76g
***p*-Coumaric acid**	12.39	109531	1.29	2.53	0.43
Caffeic acid	7.59	96758	3.55	6.79	1.15
Ferulic acid	14.10	12643	0.4	0.72	0.12
Vanillic Acid	8.20	13515	0.3	0.70	0.12
Gallic acid	4.02	64930	1.6	5.47	0.93
Syringic acid	9.29	33161	0.4	0.46	0.08

Concentration of phenolic acids in each honey

Table 10: Phenolic acid concentration in each honey

Concentration in mg/kg						
Honey	**Caffeic acid**	**Ferulic acid**	**Vanillic acid**	**Gallic acid**	**Syringic acid**	***p*-coumaric acid**
(1) Forest	2.69	0.40	0.43	4.84	1.18	0.12

(2) Chestnut	1.70	0.68	0.64	0.01	0.48	0.19
(3) Biologic chestnut	3.09	0.47	0.45	0.02	0.32	0.17
(7) Chestnut	3.62	1.19	0.88	0.13	0.94	0.34
(8) Chestnut	3.14	0.95	0.34	0.08	0.83	0.31
(9) Chestnut	0.70	0.18	0.25	0.03	0.37	0.10
(10) Honeydew	1.33	0.35	0.32	0.78	0.18	0.08
(11) Chestnut	2.86	0.36	0.38	0.03	4.54	0.44
(13) Chestnut	3.67	1.36	0.64	0.06	0.59	0.42
(14) Chestnut	1.15	0.12	0.69	0.93	0.08	0.43
(20) spurge	0.09	0	0.05	0.03	0.07	0.01
(22) Chestnut	1.93	1.10	0.25	0.08	0.18	0.21
(23) Chestnut	6.30	0.73	0.93	5.70	0.35	0.15
(25) Forest	1.99	0.55	0.28	0.32	0.24	0.21
(26) Chestnut	4.68	0.69	0.34	0.09	0.05	0.11
(27) Mountain wild flower	3.68	0.56	0.91	0.10	0.67	0.12
(29) Wild flower	3.39	0.38	0.16	0.05	1.09	0.08

After examination of the results we noticed inconsistency that implied chosen method is not reliable. Honeys 14 and 23 are both chestnut honeys, from the same producer from the same year (Nyer 2014). But the amounts of phenolic acids they contain based on HPLC analysis differ a lot. One reason could be the method of preparations of the extracts. For further analysis, a validation of all preliminary procedures before HPLC needs to be done. This

includes adequate planning (defining the research process, creating the concept of research, and sampling) and conduct of the analysis (preparation, extraction, data processing).

For comparison, we would like to present data that other researchers conducted regarding the content of phenolic acid. As mentioned, majority of studies was done using raw honey. Therefore, also the results of total phenolic content that they got was from different samples then ours.

Bertoncelj et al. used modified Folin–Ciocalteu method for total phenolic content of seven most common honey types in Slovenia. The results showed that total phenolic content differs widely among different honey types. Phenolic content expressed as gallic acid equivalent ranged from 44.8 mg/kg in acacia honey to 241.4 mg/kg in fir honey. Chestnut honey contained 199.9 $mg_{gallic\ acid}$/kg (22).

Amiot et al. studied French honey and the total phenolic compounds they assayed by colorimetry using the Folin-Ciocalteu reagent, ranged from 5.8 mg/100 mg for acacia honey to 96 mg/100 g for the strawberry tree honey (23).

Estevinho et al. compared dark and clear honeys from Trás-os-Montes of Portugal. About 14 phenolic compounds have been identified (five flavonoids and nine phenolic acids) and the phenolic pattern of honey contains protocatequic acid, p-hydroxibenzoic acid, caffeic acid, chlorogenic acid, vanillic acid, p-coumaric acid, benzoic acid, ellagic acid, and cinnamic acid as well as the flavonoids naringenin, kaempferol, apigenin, pinocembrin and chrysin. The total phenolic content extracted of the two honey samples used was in average 4.1 mg/100 g for the clear honey and 13.0 mg/100 g of honey for the dark honey (24)

5. CONCLUSION

We wanted to research whether there is a potential in the use of honey as an antimicotic agent. Fungi diseases are common, so patient-wise it is worth researching. Since honey is one of the natural sources of antimicrobial agents, we looked into the possibilities of finding new active ingredients in it.

Honey has antibacterial, namely antimicotic activity. We have proven this with our experiment and came to a few conclusions. Firstly, chestnut honey has more potent antimicotic activity than honeydew honey. Therefore, it is worth further research.

We noticed that honey extracts are better at reducing fungi growth than raw honey (honey solutions). After we have proven antimicotic activity of honeys, we continued with TLC analysis. The aim was to separate, identify different compounds that are responsible for the activity. TLC is an affordable, robust method. It gives a glance into the composition of honey. If we developed a TLC method for differentiating different types of honeys based on our research, the method could be useful for general public. Beekeepers and the industry could use it to define the source of honey, the adequacy of the indications on the packaging, and acquiring quality certificates.

With TLC method, we also tried to identify certain phenolic acids. We did not get proper results with this analysis, as it was hard to identify certain acid. We continued with HPLC analysis for defining concentrations of certain phenolic acids. Our method was not reliable, so different approach needs to be considered for further research.

For further research work we suggest the use of complimentary techniques. When working with natural products like honey, there is usually not just one best technique that should be used. We need to keep in mind the synergy of ingredients, which is often an important factor for bioactivity.

Although the antimicotic analysis and TLC tests gave reasonably reproducible results, they are not accurate enough for research use or routine determination of antimicotic activity of honey. Optimization of the method is needed, where the robustness and repeatability is guaranteed. During the research process, we are acquainted with the weaknesses of our method, which should be taken into account.

For further work, the antimicotic tests and TLC could be joined in a technique called TLC direct bioautography. Using a properly developed TLC protocol, the compounds in honey could be physically separated on TLC plates. *In vitro* antifungal susceptibility test is then performed directly on the TLC plate.

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