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In vitro growth-inhibitory activity of spice essential oils and supercritical CO_2 extracts against food pathogenic bacteria in vapor phase

MASTER'S THESIS

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I hereby declare that I have written this diploma thesis called “*In vitro* growth-inhibitory activity of spice essential oils and supercritical CO_2 extracts against food pathogenic bacteria in vapor phase“ independently and used solely the sources which I quote in the enclosed list of literature.

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Ondřej Horák

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ABSTRACT

In recent years, the importance of antimicrobials in foods has rapidly increased. However, the current awareness of consumers about chemical additives leads to demand for their natural alternatives. Non-direct contact of antimicrobials with the surfaces of the food products is a new emerging alternative approach in food preservation. Recently, the antimicrobial activity of food ingredients in vapor phase attracted attention of many researches.

In this diploma thesis, *in vitro* growth-inhibitory activity of spice essential oils and supercritical CO_2 extracts were tested against food pathogenic bacteria in the liquid and in the vapor phase. Firstly, essential oils were isolated by hydrodistillation and simultaneously, the supercritical CO_2 extracts were obtained by supercritical fluid extraction using carbon dioxide as a solvent. Subsequently, the antimicrobial activity was assayed using the novel broth microdilution volatilization method against 4 foodborne pathogenic bacteria, namely *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica typhimurium*. As a result of antibacterial susceptibility testing, *C. cassia* (bark and fruits) and *C. verum* (bark) exhibited significant growth-inhibitory effects against all tested foodborne pathogens in the liquid and in the vapor phase. Their effectiveness varied from 256 to 1,024 µg/ml in broth and agar media.

These results suggest that both *Cinnamomum* spp. could be potentially used in food industries as natural food preservatives, for example, in modified atmosphere packaging. However, further studies focused on their effectiveness in various food models will be necessary prior their practical use in foods and food industry.

Key words: antimicrobial activity, essential oils, supercritical CO_2 extracts, food preservation, vapor phase

ABSTRAKT

Přítomnost antimikrobiálních látek v potravinách nabývá v poslední době na důležitosti, a zároveň povědomí spotřebitelů o chemických aditivech vede k poptávce po jejich přírodních alternativách. Použití bezkontaktních antimikrobiálních látek je novým alternativním přístupem v konzervaci potravin. V nedávné době si konzervace potravin v plynné fázi získala pozornost výzkumu.

V této diplomové práci byl zkoumán *in vitro* inhibiční účinek esenciálních olejů a superkritických CO_2 extraktů vůči potravinovým patogenním bakteriím v kapalné a plynné fázi. Nejprve byly získány esenciální oleje pomocí hydrodestilace a extrakty za použití superkritické fluidní extrakce využívající oxidu uhličitého. Následně proběhlo testování jejich antimikrobiální aktivity pomocí bujónové mikrodiluční volatilizační metody a byly stanoveny minimální inhibiční koncentrace každého testovaného vzorku. Bylo zjištěno, že esenciální oleje spolu s extrakty všech tří testovaných vzorků získaných z rodu *Cinnamomum* vykázaly určitou antibakteriální aktivitu v kapalné, ale především v plynné fázi. Nejúčinnější esenciální olej i superkritický extrakt byl shledán u *Cinnamomum cassia*, který dosáhl nejnižší koncentrace, tj. 256 µg/ml, a to v obou fázích. Ostatní vzorky koření skořice ukázaly rovněž příznivé výsledky a jejich efektivita se pohybovala v rozmezí od 256 do 1,024 µg/ml v bujónu i v agaru.

Tyto výsledky naznačují, že oba testované druhy koření skořice by mohly být použity v potravinářském průmyslu jako alternativní přírodní konzervanty, například při konzervaci v modifikované atmosféře. Nicméně před použitím těchto látek v praxi, je nezbytně nutné provést další studie zaměřené na jejich efektivitu v různých potravinových modelech.

Klíčová slova: antimikrobiální aktivita, silice, superkritické CO_2 extrakty, konzervace potravin, plynná fáze

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CAS	Chemical Abstracts Service
DMSO	Dimethyl sulfoxide
EO	Essential oil
FAO	Food and Agriculture Organization of the United Nations
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MTT	Thiazolyl Blue Tetrazolium Bromide
SFE	Supercritical fluid extraction
WHO	World Health Organization

1 INTRODUCTION

Nowadays, almost all foods are somehow processed or treated before the final consumption. Foods that come directly from land lose their freshness usually in a few hours and therefore, we are forced to preserve our foods immediately. The main reason for food preservation is to keep food stable, with no foodborne pathogens. Traditional preservation methods such as drying, salting, sugaring, acidulation, pickling, use of caustic components, curing, smoking, and fermentation have been used for centuries. Although heating, cooling, freezing and new emerging technologies such as use of electrical impulses and high-pressure are currently used in the food preservation, the use of antimicrobial agents is still important approach in food preservation and processing.

In recent years, the importance of antimicrobials has rapidly increased. However, the current awareness of consumers about chemical additives leads to demand for their natural alternatives. For example, EOs (essential oils) have been discussed in many research papers as potential agents for food conservation. Similarly, supercritical extracts obtained from spice plants are considered as prospective materials for development of new safer alternatives to current preservatives, especially because of their high efficiency and ecological nature. Due to the specific physico-chemical properties such as high volatility, the potential of EOs and supercritical extracts can be seen especially in the area of modified atmosphere packaging. Nevertheless, one of the main limitations for the previous research was the lack of methods suitable for testing of these agents in vapor phase.

Recently, Houdkova et al. (2017) have developed a new method for evaluation of natural volatile agents that has been proven for identification of new antimicrobial agents effective in vapor phase. Therefore, the aim of this study is determination of in vitro antibacterial activity of EOs and supercritical CO_2 extracts obtained from commonly used species of spices against food pathogenic microorganisms in vapor phase. It is possible to suppose that the most effective agent identified in this study can potentially be used for development new food preservatives for modified atmosphere packaging.

2 LITERATURE REVIEW

2.1 Food spoilage bacteria

Together with viruses, parasites and chemical substances, bacteria are the main causes of foodborne illnesses that are the worldwide problems and threats to food safety with a high prevalence in developing countries. Except chemical substances, it is possible to collectively label bacteria, viruses and parasites as so-called foodborne pathogens which enter the body through contaminated food or water. Food can be contaminated at every stage of its process beginning in the primary production via processing and it ends during distribution to the final consumers (WHO 2017).

2.1.1 Main pathogens

Bacterium as an organism is usually source of major foodborne illnesses. In this particular thesis, the focus is given to four bacterial strains, which are examined in the experimental part and therefore, following chapters are about *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica typhimurium*. Every chapter is roughly structured into the following order: general description, favorable conditions, occurrence, link to diseases and consequences.

Bacillus cereus

B. cereus is a Gram-positive, aerobic, usually motile, and spore-forming rod. Its growth temperature varies between 4 °C and 50 °C, optimum temperature ranges from 25 °C to 37 °C. *B. cereus* does not thrive below pH 4.5. It is widely distributed in natural environment including soils, water, dust, plants, animals, and humans (Bhunia 2008). Due to its ability to adhere to surfaces and produce toxins, it can easily contaminate any of the food production or processing systems. By spore formation it may survive gamma-ray irradiation, pasteurization process and higher temperatures. Infection of *B. cereus* cause approximately 63,400 illnesses annually in the United States (Kotirantaa et al. 2000). This bacterium can cause diarrheal and emetic food poisoning by diarrheagenic and emetic toxins, respectively. Emetic syndrome is associated with rice, pasta, poultry, beef, infant formulas, milk pudding and vanilla sauce, while diarrheal syndrome is associated mainly with fish, soups, vegetables, corn potatoes and dairy products. Generally, food which is contaminated by two major previously mentioned enterotoxins can cause vomiting, nausea, diarrhea and gastrointestinal disorders (Bhunia 2008).

Escherichia coli

E. coli is a Gram-negative, aerobic, 1-2 μm long, motile bacterium. It belongs to the family Enterobacteriaceae. A broad range of *E. coli* strains is harmless and non-pathogenic and their presence is common in intestinal microflora of humans and animals. Nevertheless, some existing strains are pathogenic and they can cause a variety of diseases such as dysentery, gastroenteritis, septicemia, pneumonia, meningitis, hemolytic uremic syndrome and urinary tract infection. Major pathogenic groups of *E. coli* are, namely: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli*, Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli*, Adherent-invasive *E. coli*, from which EHEC, EPEC and ETEC are well known sources of severe diseases worldwide. Current research is focused predominantly on EHEC group because of their continuous association with serious foodborne diseases from various foods, for instance fruits, vegetables, meats and dairy products (Bhunia 2008). *E. coli* infection causes approximately 73,480 illnesses each year in the United States, leading to 61 deaths and 2,168 hospitalizations annually, and it is an important cause of acute renal failure in children (Rangel et al. 2005). Foodborne diseases caused by pathogenic *E. coli* can result in abdominal cramps and diarrhea which can progress in bloody diarrhea. Vomiting and fever may also occur. The incubation time can vary from 3 to 8 days and the most patients are recovered within 10 days. Nevertheless, children and elderly are the risk groups and this particular infection may lead to the life-threatening disease in their case. The prevention is necessary and it requires control measures in all stages of food chain. It starts from the agricultural production, via processing, preparation of foods in commercial establishments or in household kitchens (WHO 2018).

Listeria monocytogenes

L. monocytogenes is a Gram-positive, nonspore-forming, 1-2 μm long bacterium. Its presence is common in the soil, water, plants, sewage. It survives in inhospitable extreme environmental conditions including a wide range of pH (4.1 - 9.6), high salinity up to 10 % and low temperatures which are usually found in the refrigerators. Foods are the main vehicles of infection that is transmitted often by meat, dairy products, vegetables or fish

(Bhunia 2008). Infectious disease caused by the bacterium *L. monocytogenes* is called listeriosis. There are two forms, namely: invasive form and non-invasive form. The invasive form of listeriosis is more severe which affects high risk groups of population including infants, children, pregnant women, patients undergoing treatment for cancer. Symptoms of invasive form are fever, meningitis and muscle pain and the incubation period is usually from 1 to 2 weeks. The non-invasive form of listeriosis is not as severe as the invasive form. It affects healthy people and it results in diarrhea, fever, headache and in muscle pain. The incubation period is relatively short and it usually takes a few days. The antibiotics are often used to treat listeriosis symptoms (WHO 2018). The mortality rate is the highest from all mentioned foodborne pathogens, i.e. 20 - 30 % and, in fact, the mortality rate may be even higher in the risk groups of population (Bhunia 2008; Ranjbar & Halaji 2018; WHO 2018).

Salmonella enterica typhimurium

S. e. typhimurium is a Gram-negative, facultatively anaerobic, motile bacterium. It belongs to the family Enterobacteriaceae. They grow between 5 – 45 °C and they thrive around 35 – 37 °C. Despite the fact, *S. e. typhimurium* is discussed here as one of the main pathogen it is present in intestinal tract of humans, domestic animals, reptiles, birds and even insects. In terms of foodborne diseases, poultry and eggs represent a major source for human foodborne salmonellosis. Nevertheless, meat, milk, vegetables and fruits can also be a common sources of transmission. *S. e. typhimurium* can cause gastroenteritis, bacteremia and typhoid fever (Bhunia 2008).

2.2 Antimicrobials in food

According to Davidson et al. (2005), food antimicrobials can be defined as chemical compounds present in or added to foods, food packaging, food contact surfaces, or food processing environments that inhibit the growth of, or inactivate, pathogenic or spoilage microorganisms. Furthermore, it is possible to classify food antimicrobials as preservatives which are used to prevent biological deterioration. Generally, there are two types of deterioration of foods, namely chemical and biological. In order to prevent and/or slow down chemical and biological deterioration, suitable preservation method must be used. Preservatives used to prevent chemical deterioration include antioxidants, antibrowning compounds and antistaling compounds. On the other hand, preservatives used to prevent biological deterioration are already mentioned antimicrobials.

Crozier-Dodson et al. (2005) provides an overview of the antimicrobials that are used in the food industry. Following division is made according to desired effect, legal limits of use and the effects on particular food. It is predominantly focused on some of the most effective and/or promising agents used today as part of process control in food manufacturing operations. The list of the antimicrobials merged into the specific groups is as follows: acid antimicrobials, chemical antimicrobials, ovo-antimicrobials, lacto-antimicrobials, bacto-antimicrobials and phyto-antimicrobials.

Acid-antimicrobials including organic acids, such as lactic, acetic and citric acids, can enhance or contribute to the flavor of acidified or fermented foods, for instance, pickles, cheeses, sausages or sauerkraut. These acids in combination with other compounds have exhibited synergism. Lactic acid and citric acid with potassium sorbate have been reported to inhibit *S. e. typhimurium*, *Pseudomonas fluorescens* and *Yersinia enterocolitica* (Crozier-Dodson et al. 2005). Citric acid is present in the numerous plants and animals but the commercial production is driven by fermentation of sugar by *Aspergillus niger*. Since it is a natural ingredient of citrus fruits, it adapts itself well to beverages with such a flavor, i.e. juices made out of citric fruits. Sorbic acid, potassium sorbate, and calcium sorbate are highly efficient and safe food preservatives and they are often used as standard products in many branches of the food industry. The organic acids, potassium sorbate and sorbic acid, are used in cheeses, bakery products and salad dressing (Jorge 2003). Benzoic acid and sodium benzoate, which is the salt of benzoic acid, are used to prevent microbial growth in fruit juices, fruit products, sauces, condiments etc. The upper allowed concentration is 0.015 – 0.5 % in the EU while in the US it is 0.1 %. A major market for sodium benzoate as preservative is in the soft drink industry, as a result of the demand for high-fructose corn syrup in carbonated beverages. However, it can be also used as a preservative in pharmaceuticals, anticorrosive agents or newly as a strengthening substance in plastic production (WHO 2000; Encyclopedia Britannica. 2019). Salts of lactic acid, or so-called lactates, such as sodium lactate and potassium lactate, are able to inhibit gram-positive organisms more effectively than gram-negative organisms. They can effectively lower pH, decrease water content and the combination of sodium lactate and sodium diacetate has shown synergistic effect against *L.*

monocytogenes. Therefore, the application in ready-to-eat meat products such as hot dogs has been widely accepted. Buffered sodium citrate is also effective antimicrobial agent against *L. monocytogenes*, as well as acidified sodium chlorite that has shown clear results against *E. coli* and *S. typhimurium* (Crozier-Dodson et al. 2005).

Chemical antimicrobials are for example trisodium phosphate, chlorine dioxide, peracetic acid (peroxyacetic acid), sodium nitrite and nitrate while ovo-antimicrobials include lysozyme that naturally occurs in in both the animal and plant kingdoms. Lysozyme plays an important role in defend mechanism and is effective against both Gram-negative and Gram-positive bacteria. It is used as a natural food preservative due to its inhibiting properties against bacterial cells and on the other hand, it is harmless to human cells. Commercially used lysozyme is made from hen egg albumin (Crozier-Dodson et al. 2005).

Lacto-antimicrobials include lactoferrin that is characterized as glycoprotein present in milk. It is able to kill Gram-negative and Gram-positive bacteria and its effectiveness is associated with tightly bounded and adherent pathogens in raw meat that are hard to remove using traditional intervention processes. Bacto-antimicrobials are for instance nisin, pediocin, lacticin, macedocin, reuterin, sakacin and colicin. Nisin is an antimicrobial peptide and bacteriocin produced by certain strains of *Lactococcus lactis* that possesses the widest antimicrobial spectrum out of the previously mentioned bacteriocins. It disrupts and penetrates the cell membrane of Gram-positive bacteria while Gram-negative bacteria, molds and yeasts remain undisrupted. The only difference is in the complexity and the structure of the cell membranes (Crozier-Dodson et al. 2005).

Phyto-antimicrobials includes e.g. spices that are added to food based on the flavor or aroma profiles. However, a different kinds of spices possess a different properties and the level of antimicrobial activity that is based on their inherent bioactive compounds. Nowadays, a lot of efforts is made to discover additional natural agents with antimicrobial properties (Crozier-Dodson et al. 2005). Phyto-antimicrobials in foodstuffs are the main subjects of this diploma thesis and therefore, deeper explanation and characterization of this topic is provided in the chapters below.

Generally, the effectiveness of the antimicrobials which have been used is predominantly dependent upon the concentration used and the food product in question. Although there have been many innovations in the use of antimicrobials in and on food products, sequential research is definitely needed (Crozier-Dodson et al. 2005).

In addition, antimicrobials, e.g. antibiotics, are significant tools to treat infections caused by bacteria. However, their overuse in veterinary and human medicine caused the emergence and dissemination of resistant bacteria. Antimicrobial resistance became s one of the main threats and major global health concern in modern medicine. When pathogens become resistant to antimicrobial agents, they can pose a greater human health risk as a result of potential treatment failure and loss of treatment options and thus contribute to severity of disease (FAO 2015; WHO 2017). The resistance of microorganisms is a problem which is related not only to human and veterinary medicine but also in food industry. The adaptation is obvious act of living organisms and the previous paragraph emphasizes this particular issue that is currently discussed and therefore, there is a space for new options and alternatives, such as naturally-based products (Ghosh 2019).

Nowadays consumers are demanding foods without artificial chemicals. They perceive them as a harmful and unnecessary chemicals present in their foods, including those which are used as antimicrobials and preservatives in foods. Therefore, interest in the natural occurring compounds is rapidly increasing (Calo et al. 2015). Naturally occurring bioactive compounds are abundantly present in our living environment. Some of them are already used in food preservation but the majority is currently studied to be used in food industry. The main source are plants with their derived EOs and isolated compounds which can inhibit or kill bacteria, yeast, molds etc. and they may stop the production of their metabolites. Nevertheless, the most of plant-derived compounds have not been explored and tested yet (Davidson et al. 2005). There is a special classification group called aromatic plants that represents a great reservoir of bioactive secondary metabolites. They confer protection against predators, code for signaling molecules and increase the plant resistance against stresses. Compounds present in their structures have been examined as potential inhibitors of bacterial growth and these antimicrobial properties have been directly linked to EOs and plant extracts (Calo et al. 2015, Corocho et al. 2015). Some of them have achieved the Generally Recognized As Safe (GRAS) label. Food and Drug Administration (FDA, USA) provides comprehensive overview of substances that are intentionally added to food as food additives that are safe with no harmful effects and all these additives are mentioned in the GRAS list. Within this document is possible to find the so-called “green chemicals” present in the plants that are used as flavor ingredients. Oregano oil, thymol, carvacrol, cinnamon oil and clove oil are some of the most important. There is a vast number of foodstuffs where EOs are applied, for instance, meat, dairy products, fish, fruits and vegetables (Burt 2004). In addition, recent developments have also been done in the packaging management, with some films surfaces being impregnated with EOs, namely with carvacrol and thymol (Corocho et al. 2015).

The presence of natural products in foodstuffs is undoubtedly prosperous, beneficial and even promising tool how to address customers and to some extent general public. But of course, the question is emerging: What people perceive as added artificial chemicals and what do they know about them? The answer for this particular question is perfectly summarized in the investigation called “Consumers’ ratings of the natural and unnatural qualities of foods” made by Evans et al. (2010). In this work, several hypotheses have been set up and discussed, for example, the hypothesis related to E-numbers denoting food additives numbered in accordance with EU directives. E-numbers are perceived as artificial chemicals regardless their origin. Even the use of chemical names has been perceived more natural than the use of E-numbers. Surprisingly to respondents, this particular classification system contains not only artificial substances but also compounds derived from natural sources.

Generally, consumers appear to have become more aware of what is in the foods they buy and they always tend to choose the additives of natural origin than their synthetic analogues. Nevertheless, the lack of knowledge about food additives and poor recognition are still not negligible. The significant factors which influence perception and knowledge about food additives in general are mainly age, gender and educational level (Evans et al. 2010).

2.3 Essential Oils

EOs are defined as a volatile aromatic oily liquids which are obtained from various plant materials e.g. leaves, twigs, wood, bark, flowers, buds, fruits and seeds (Burt 2004). To complete precise definition, EOs may be characterized as a complex mixtures of different aromatic compounds. Generally, herbs and spices that are commonly used in food flavoring consist the most of the EOs (Calo et al. 2015). From the raw plant materials, it is possible to obtain EOs by several methods, for instance, distillation, extraction, expression, fermentation. Nevertheless, distillation is used the most for commercial production of EOs (Burt 2004). From the historical perspective, the process of distillation of EOs has begun in the Orient i.e. India, Persia and Egypt. Furthermore, the written sources from ancient Greece and Rome reveal the oil of turpentine. However, Catalan physician Arnaldus de Villa Nova had made the first record of proper distillation of EOs in the 13th century. Since that time, EOs became used in medicine for their favorable pharmacological effects. Nevertheless, in the 19th century, the importance of EOs as medicinal drugs decreased and simultaneously, the usage of EOs in the foods, beverages and perfumes rapidly increased (Guenther 1948). EOs are produced by angiospermic families, predominantly by Rutaceae, Lamiaceae, Myrtaceae and Zingiberaceae. In total, it signifies 17,000 aromatic plant species. Different kinds of the EOs have been used as flavoring, condiments, seasoning and sometimes as preservative, throughout the world especially in the countries with high biodiversity (Prakash et al. 2015). High temperature coupled with high humidity create richness of biodiversity and therefore, the tropics are abundant in the various plant species which serves as a reservoir of EOs. Accumulation of secondary metabolites is caused by the environmental stresses such as drought, high and low temperature, salinity, alkalinity, UV stress and pathogen infection. The plant as a living organism is adapting and overcoming those environmental stresses and it results in higher amount of secondary metabolites in the tissues (Akula & Ravishankar 2011). Among these secondary metabolites, it is estimated that approximately 3,000 EOs are known, of which about 300 are commercially significant and used in the flavoring and fragrance industries (Bassolé & Juliani 2012). The antimicrobial activity of EOs obtained from plants depends on the extraction method but also on the initial quantity of EO present in the plant. Within the plant, the levels of constituents and extract composition can substantially vary due to several factors, for instance, geographical zone of cultivation or local growing conditions (Davidson et al. 2005). According to Mann and Markham (1998), the antimicrobial effectiveness of particular compound is usually described in its minimum inhibitory concentration (MIC), i.e. the lowest concentration of the compound that is capable of inhibiting the growth of the pathogens.

Spices have become the subject for isolation of EOs and supercritical CO_2 extracts in this diploma thesis. Spices are defined as plant materials which are used in flavoring foods and beverages. Each different condiment possesses a unique aroma and flavor and thus it enhances the sensory perception and palatability of the foodstuffs in general. Nevertheless, spices are used not only for flavoring and coloring the foods but also for its beneficial properties e.g. antimicrobial and antiviral properties. The main principle is that plants use their secondary metabolites as a protective compounds and the use of spices takes the advantage of them (Sherman & Billing 1999).

The antimicrobial substances in the plant materials are commonly contained in the EOs extracted from leaves (e.g. *Salvia officinalis*, *Rosmarinus officinalis*), flowers or flower buds (e.g. *Syzygium aromaticum*), bulbs (e.g. *Allium cepa*, *Allium sativum*), rhizomes (e.g. *Ferula assa-foetida*), fruits (e.g. *Elettaria cardamomum*, *Piper* spp.), and other plant parts. Major components with antimicrobial activity found in plants, and spices made out of the plants, are phenolic compounds, terpenes, ketones, aliphatic alcohols, acids, aldehydes, and isoflavonoids. It has been reported that antimicrobial activity of EOs is dependent on the chemical structure of their components and their actual concentration (Davidson et al. 2005). EOs such as aniseed (*Pimpinella anisum*), camphor (*Cinnamomum camphora*), cinnamon (*Cinnamomum cassia* or *Cinnamomum verum*), citronella (*Cymbopogon citratus*), clove (*Syzygium aromaticum*), eucalyptus (*Eucalyptus* spp.), lavender (*Lavandula angustifolia*), mint (*Mentha piperita*) nutmeg (*Myristica fragrans*), rosemary (*Rosmarinus officinalis*), basil (*Ocimum basilicum*) and vetiver (*Chrysopogon zizanioides*) have been traditionally used by people for various purposes in different parts of the world. Representatives of the family Rutaceae are also widely used as a source of EOs, for example, lemon (*Citrus limon*), orange (*Citrus sinensis*) and lime (*Citrus aurantifolia*) that has shown immunomodulatory effect in humans. Lavender oil has displayed antibacterial activity and positive effective to treat burns and insect bites. EO extracted from *Cinnamomum* spp. possesses antibacterial, antifungal and even antidiabetic properties. Anti-inflammatory activity has been found in basil (Prabuseenivasan et al. 2006).

Table 1 summarizes the list of some of the more highly recognized plant species that have been reported as sources of natural antimicrobials.

Table 1: Plant species and their major antimicrobial compounds (taken and edited from Davidson et al. 2005).

Plant species	Spice	Family	Major antimicrobial compounds
Allium cepa	Onion	Amaryllidaceae	d-n-propyl disulfide, methyl-n-propyl disulfide
Allium sativum	Garlic	Amaryllidaceae	Diallyl disulfide, diethyl sulfide, diallyl trisulfide, allicin
Apium graveolens	Celery seed	Apiaceae	d-limonene
Artemisia dracunculus	Tarragon	Compositae	Methyl chavicol, anethole
Brassica hirta	Mustard	Brassicaceae	Allyl-isothiocyanate
Carum carvi	Caraway seed	Apiaceae	Carvone, limonene
Cinnamomum zeylanicum	Cinnamon	Lauraceae	Cinnamic aldehyde, l-linalool, p-cymene, eugenol
Coriandrum sativum	Coriander	Apiaceae	d-linalool, d-α-pinene, β-pinene
Cuminum cyminum	Cumin	Apiaceae	Cuminaldehyde, p-cymene
Cymbopogon citratus	Lemongrass	Graminae	Citral, geraniol
Foeniculum vulgare	Fennel	Apiaceae	Anethole
Laurus nobilis	Bay	Lauraceae	Cineol, l-linalool, eugenol, geraniol
Ocimum basilicum	Basil	Lamiaceae	d-linalool, methyl chavicol, eugenol, cineol, geraniol
Origanum majorana	Marjoram	Lamiaceae	Linalool, cineol, methyl chavicol, eugenol, terpinineol
Origanum vulgare	Oregano	Lamiaceae	Thymol, carvacrol, α-pinene, p-cymene
Petroselinum crispum	Parsley	Apiaceae	α-pinene, fenol-eter-apiol
Pimenta dioica	Allspice	Myrtaceae	Eugenol, methyl ether cineol
Piper nigrum	Black pepper	Piperaceae	Monoterpenes, sesquiterpenes
Rosmarinus officinalis	Rosemary	Lamiaceae	Borneol, cineol, camphor, α-pinene, bornyl acetate
Salvia officinalis	Sage	Lamiaceae	Thujone, cineol, borneol, thymol, eugenol
Syzygium aromaticum	Clove	Myrtaceae	Eugenol, cariofilene
Thymus vulgaris	Thyme	Lamiaceae	Thymol, carvacrol, l-linalool, geraniol, p-cymene
Vanilla planifolia	Vanilla	Orchideaceae	Vanillin, vanillic, p-hydroxbenzoic, p-coumaric acids

2.4 Supercritical CO_2 Extracts

Supercritical CO_2 extracts are produced by relatively new and highly efficient extraction process – supercritical fluid extraction (SFE). It is an efficient technique for solid materials that is widely used for the separation of bioactive compounds from the plants. The importance of this technology is rapidly increasing and due to low critical parameters, non-toxicity, nonflammability and its availability in high purity at low cost, the usage of CO_2 remains the most common among all other gases and liquid solvents. Using carbon dioxide as a solvent for extracting compounds from raw material results in the production of superior extracts which can be used in food and medicine industries. The CO_2 extracts are similar to EOs traditionally derived by hydrodistillation or extracted using certain chemical solvents such as hexane, heptane or ethanol, however, the supercritical carbon dioxide extracts and their creation process possess several benefits. The usage of large amounts of solvents is not environmentally friendly and it is accompanied with a high costs and in the case of hydrodistillation, the quality of obtained EO is dependent upon the temperature and time used for distillation. Thus, SFE represents alternative extraction technique with high efficiency where the extracts are not altered by the high temperature, and therefore, the higher number of chemical substances present in the sample has been undamaged. To summarize, when compared to other extraction methods, SFE has many advantages such as low operating temperatures to reduce energy consumption, preservation of heat-sensitive compounds and avoidance of residual toxic solvent. Furthermore, it maintains the organoleptic features of the natural plants, and it is surely better than the conventional extraction approaches (Vági et al. 2005, Eden Botanicals 2019). Due to its proven benefits, supercritical extracts became quickly popular in e.g. pharmaceutical and food industry. The main commercial food applications using SFE are: extraction of hops for beer production, decaffeination of the coffee and the tea, separation of free fatty acids from vegetable oils, extraction of oleoresins from spices, deoiling of snack-foods, flavors and fragrances extraction, extraction of natural food color agents, extraction of natural food preservatives, extraction of herbs for natural medicines, extraction of natural pesticides, production of cholesterol-free food products etc. (Applied Separations 2019).

A supercritical fluid is defined as the phase of a material at critical temperature and critical pressure. It combines useful properties of gas and liquid phases and this is what creates perfect solvent for the extraction process. Figure 1 displays the basic principle of SFE on the simple phase diagram. Solid, liquid, and gas region are defined areas with a boundaries that are connected in the common equilibrium; the triple point. Critical temperature and critical pressure create together a critical point (Tc = 31.04 °C, Pc = 73.8 bar) where the phase curve between liquid and gas phases disappears and supercritical material is formed (Vági et al. 2005; Raja & Barron 2019).

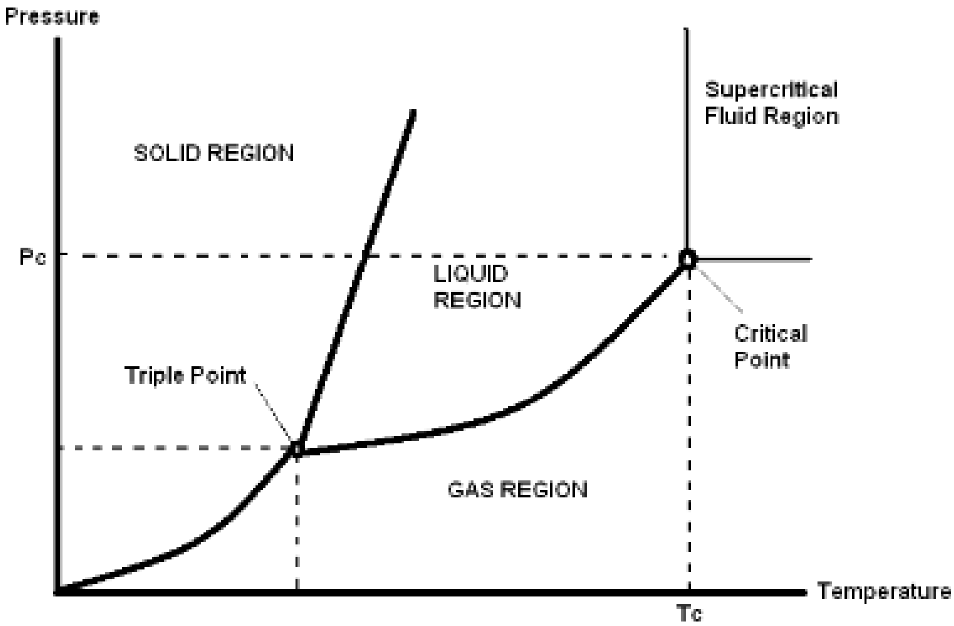

Figure 1: The scheme of the formation of a supercritical fluid (taken from Raja & Barron 2019).

2.5 Plant species used in this diploma thesis

Following chapters focus on eight plant species which has been used during experimental part of this diploma thesis. The information structure of each chapter is roughly organized into the following order: taxonomy, origin and distribution, botanical description, plant part which is used, food and medicinal uses, chemical composition and bioactive compounds with their antimicrobial properties.

Aframomum melegueta

A. melegueta known under the English name grains of paradise belongs to the family Zingiberaceae. It is widely distributed across tropical Africa including Nigeria, Ghana, Sierra Leone, Liberia, Cameroon, Ivory Coast and Togo. The plant can be characterized as an herbaceous, perennial plant from the tropical rainforest. *A. melegueta* is valued for both edible and medicinal uses. The seeds that have pungent peppery taste are used in Moroccan and Tunisia spice mixtures. In the West, grains of paradise are now hard to obtain, but still valuable for people following old recipes (e.g. for sausages or spiced wine). The seeds are used either for flavoring foods or preparation of liquors and alcoholic beverages or either against dysentery and gastrointestinal troubles in the traditional medicine. The taste is bitter, spicy and hot due to a volatile oil (Okwu 2005). *A. melegueta* can be used in phytomedicine and furthermore, it can be included in health care systems due to its high amounts of phytochemicals coupled with the presence of minerals and vitamins (Doherty 2010; Okwu 2005). Moreover, the seeds contain various classes of compounds, such as flavonoids, tannins, saponins, terpenoids, alkaloids and glycosides. Presence of these phytochemical constituents inside of each seed creates the effective antimicrobial agent (Doherty 2010). The EO from *A. melegueta* is dominated by the sesqui-terpene hydro-carbons humulene, α- and β-caryo-phyllene (83 %) and their oxides (9 %). The volatile oil of this species was tested for antimicrobial effects on five pathogenic bacteria which include *B. cereus, Staphylococcus aureus, E. coli, S. typhimurium* and *Klebsiella pneumonia*. The results have shown the inhibition effect to the growth of *Klebsiella pneumonia* and *S. typhimurium* (Doherty 2010).

Allium sativum

A. sativum, also called garlic, belongs to the family Amaryllidaceae. It originates from central Asia and in ancient times, garlic spread to the Mediterranean region, India and China. Nowadays, it is cultivated almost all over the world at latitudes from 5° to 50° in both hemispheres. From botanical perspective, it is erect herb which is grown as an annual and reach up to 0.6 m in height. The bulb is composed of several lateral bulbs, also called cloves (van der Meer 1993). Garlic is predominantly used as a condiment of foods, especially meat, fish and salads. Both form fresh and dried are used. Garlic is also valuable medicinal crop and its medicinal properties have been scientifically proved. It is used to lower cholesterol level and blood sugar level, cure headache, toothache and rheumatism (Sulistriarini 1999). Garlic cloves contain the water-soluble nutrients, e.g. amino acids, sugars, vitamins, enzymes, and fat-soluble nutrients, e.g. organosulfur compounds (alliicin, methiin, allylmethyl, dimethyl, -mono to -hexa sulfides) (Putnik 2018). According to Martins et al. (2016), the amount and the quality of present bioactive compounds are determined not only by pre-harvest factors but also by post-harvest conditions. Factors that affect the chemical composition of garlic before harvesting are predominantly genotype, growing conditions as well as irrigation and soil fertility. However, processing and storage also influence the bioactive compounds content. Last studies have shown considerable variations in the final chemical composition according to the type of garlic processing and alterations. Moreover, in terms of maintaining of instable organosulfur compounds, proper storage conditions are crucial to retain the high quality of garlic bulbs and its by-products. Alliicin has already been mentioned above among organosulfur compounds. This compound is converted from odorless alliin during damaging the tissue and therefore, the highest amounts of alliicin has been detected in freshly crushed garlic. Almost 70 – 80 % garlic smell comes from this particular compound. It has been found that alliicin has certain antibiotic activity against Gram-positive and Gram-negative bacteria (Putnik 2018).

Amomum subulatum

A. subulatum is a Latin name of black (large) cardamom which belongs to family Zingiberaceae. It is also known under the vernacular name Nepal cardamom due to its origin (Jansen 1999). *A. subulatum* is a large perennial, herbaceous, evergreen plant that consists of subterranean rhizomes and several leafy aerial shoots, co-called tillers, and spikes which grow up to 1.5 to 2.5 m in height. The leaves are green, simple, linear and glabrous on both sides. The fruit is red-brown capsule which can reach up to 2.5 cm and it contains several aromatic seeds held together by a viscous sugary pulp. Seeds and fruits of *A. subulatum* are used as spices because of its pleasant aromatic odor (Bisht et al. 2011). However, the specific flavoring aroma is not the only important feature of black cardamom. There are proven positive medicinal effects which have been used since ancient times. From ethnobotanical perspective, black cardamom has been used by indigenous people as preventive as well as a curative for inflammation of eyelids, digestive disorders, respiratory problems, pulmonary tuberculosis and migraine. The EO of black cardamom is reported to have hepatoprotective and anti-inflammatory activity and the major components are: 1,8-cineole (65 to 80 %), limonene (10.3 %)., α-terpineol, α-pinene, β-pinene and allo-aromadendrene (Bisht et al. 2011; Joshi et al. 2012). The volatile oil content of the seeds varies between 1.95 to 3.32 % in different varieties of the black cardamom. *A. subulatum* has a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids having antimicrobial activities, nevertheless, it is not the only biological property of this species. Analgesic activity, anti-inflammatory properties, antioxidant activity, antiulcer activity and hypolipidemic activity have been identified as well. The antimicrobial activity has been reported against two bacteria *Streptococcus mutan*s and *Streptococcus aureus* and two fungi *Candida albicans* and *Saccharomyces cerevisiae* (Bisht et al. 2011).

Armoracia rusticana

A. rusticana, commonly known as a horseradish, belongs to family Brassicaceae. It originates in south-east Europe and western Asia. Nowadays, it is distributed in many temperate regions, mostly in Europe and North America. However, cultivation of horseradish is also possible in tropical regions but in higher elevations only. The plant can be described as erect, up to 1.5 in height, perennial herb which is grown as an annual crop. The valuable plant part is the root which can be consumed raw, dried and in various of mixtures of seasonings, vinegar etc. Due to its specific pungent taste, horseradish is used as a condiment of foods, for instance meats, fish, sauces and salads. Horseradish has shown certain medicinal properties and antimicrobial activity against bacteria (de Guzman 1999; Nichols and Jansen 1999). According to Nguyen et al. (2013), *A. rusticana* is rich in glucosinolates which are the secondary products that play an important role in the plant protection. From the chemical perspective, glucosinolate is organic anion that is stable and soluble in water and their amount is strongly affected by the age of the certain plant and its living environment, for example the soil fertility. In the roots of horseradish, eight different glucosinolates have been detected. Sinigrin (2-propenyl or allyl glucosinolate) and gluconasturtiin (phenethylglucosinolate) have been found in the highest quantities compared with the others. However, the active substances are the isothiocyanates which are formed by hydrolyzed glucosinolates. The most common is allyl isothiocyanate which is responsible for the pungent taste of the roots. Based on observations in animal and human cell studies, isothiocyanates inhibit the development of cancer cells and even eliminate established cancer cells, and therefore, they can be classified as a promising anticarcinogenic agents. Antimicrobial activity has been reported against several microorganisms, i.e. *Helicobacter pylori*, *E. coli*, *S. e. typhimurium*, *Staphylococcus aureus*, *Streptococcus mutans*, *Penicillium notatum*, *B. cereus* and *Vibrio parahaemolyticus*.

Cinnamomum cassia

C. cassia, also called Chinese cinnamon or Chinese cassia, belongs to the family Lauraceae. In the scientific literature, *Laurus cassia* L. (1753) and *Cinnamomum aromaticum* C. Nees (1831) are also often used as synonyms. It is distributed in South China, Myanmar, Laos and also Vietnam. Furthermore, it has been introduced into Indonesia, Sri Lanka, South America and also Hawaii. Nowadays, commercial cultivation occurs in China and Vietnam only. *C. cassia* is evergreen tree up to 18 m high with thick bark which is smooth during juvenile stage but it is becoming rough in mature stage. The leaves are alternate, leathery, shiny, dark green which are about 10 cm long with extended acuminate tips. It can be defined as a multipurpose tree with several benefits that are used by humans. The bark, twigs, buds and also the leaves can be somehow used in the food industry, medicine and perfumery. For example, flavoring of foods by cassia bark is widely used either in households or either in food industry in a large scale. Cassia bark oil is used for instance in perfumes and soaps. Cassia leaf oil, simply referred as a cassia oil, acquired by distilling twigs and leaves is used in flavoring of foods, perfumery but it is specifically used in cola-type drinks. Cassia fruits, so-called cassia buds, also possess important features which are widely used mainly in flavoring of foods (Dao et al. 1999). The protection of the plant itself is assured by particular secondary metabolites present in the plant, namely cinnamic aldehyde, cinnamic acid, coumarin. A wide range of these secondary metabolites are unconsciously used in the traditional medicine. For the medicinal purposes, *C. cassia* is usually used for treating blood circulation disturbances, inflammatory diseases, dyspepsia and gastritis in traditional medicine. The bark possesses anti-inflammatory activity and antibacterial effects against antibiotic-resistant isolates of *Bacillus megaterium* and *Enterococcus faecalis*. However, other plant parts contain certain biological activity as well. The leaves and buds have displayed antioxidant activity and even certain inhibition activity against the human breast cancer cell (Sharma et al. 2018). In the scientific article of Guoruoluo et al. (2018), the chemical composition of *C. cassia* fruits has been studied. In total, 15 active compounds have been isolated including phenylethyl glycosides, phenylmethanol glycosides, butanol glycosides, a megastigmane sesquiterpenoid, monoterpenoid glycoside, isomeric monoterpenoids and their glycosides, geraniol type monoterpenoid, phenolic glycosides and cinnamic acid. According to the other relevant article Liu et al. (2018), the phytochemical structure of

C. cassia twigs has been studied. It resulted in identification of 39 active compounds. 32 out of these identified substances are reported for the first time from this plant. Among identified compounds are flavonoid glycosides, lignans, sesquiterpenoids, phenolic amides, cinnamaldehyde derivatives, phenols and indole derivatives.

Cinnamomum verum

C. verum, or *C. zeylanicum*, belongs to the family Lauraceae. Vernacular names such as Ceylon cinnamon or true cinnamon are commonly used. It is distributed in the wild of south-west India, western Sri Lanka and some regions in Myanmar. The habitat of *C. verum* resembles *C. cassia* that is described in the previous chapter. It is evergreen tree up to 18 m high approximately 10 mm thick, strongly aromatic bark. The leaves are opposite, glabrous, shiny and dark green. The major uses of *C. verum* are in foods either in households for domestic culinary purposes or in processed foods such as delights, sauces, beverages, puddings, bakery products. The part which is commonly used is the inner part of the bark in a whole or ground from and its EO is dominated by two phenylpropanoids cinnamaldehyde (65 – 75 %) and eugenol (5 – 10 %). From the medicinal point of view, *C. verum* is used as a stimulant, carminative or as astringent. Same as *C. cassia*, leaf oil can be extracted from the leaves by distillation and afterwards used e.g. cosmetics. Additionally to this, *C. verum* can be used in perfumery products or detergents (Flach & Siemonsma 1999; Gernot Katzer 1999). According to Kamran et al. (2018) *C. verum* bark extracts are effective antimicrobial agents. In his experimental trial, manganese nanoparticles synthesized from C. *verum* bark extract showed promising antimicrobial activity against *Staphylococcus aureus* and *E. coli* bacterial strains.

Elettaria cardamomum

E. cardamomum, or so-called true cardamom or small cardamom, belongs to the family Zingiberaceae. In the scientific literature it can be found under the following synonyms *Amomum cardamomum* L. (1753), *Amomum repens* Sonnerat (1782), *Alpinia cardamomum* (L.) Roxb. (1819). From the botanical point of view, *E. cardamomum* is a robust, perennial plant which grows up to 5 m. The leaves are dark green, glabrous above and light green, glabrous occasionally pubescent beneath (Wardini & Thomas 1999). The seeds are used as a source of EOs and they have a great economical value. Normally, the whole fruits (pods) are sold on the markets due to quick loss of their typical pleasant fragrance. The content of EOs in the seeds depends on storage conditions, but it can reach almost 8 %. The oil is composed of oxygenated monoterpene derivatives: α- terpineol 45 %, myrcene 27 %, limonene 8 %, menthone 6%, β-phellandrene 3 %, 1,8-cineol 2 %, heptane 2 % and sabinene 2 % (Gernot Katzer 2015).

Syzygium aromaticum

S. aromaticum belongs to family Myrtaceae. In the literature, it can be found under the other valid synonyms such as *Caryophyllus aromaticus* L. (1753) or *Eugenia aromatica* (L.) Baill. (1876), however, the general public recognizes this plant species under the name clove. It originates on the islands of the Moluccas, Indonesia, but nowadays, it grows also outside of Asia, in Zanzibar or Madagascar. It possess tree habitat that is up to 20 m tall, branched from the base with opposite, simple and glabrous leaves. The fruit, so-called mother of cloves, is a dark red berry that is 2 - 2.5 cm long and it usually contains only 1 oblong seed. *S aromaticum* is widely used for food flavoring (the flower bud) but also for medicinal purposes (the flower bud and the fruit). A vast portion of a total production is associated with tobacco to produce kretek cigarettes, which are smoked mainly in Indonesia. The flower buds are the valuable plant part that is used as a condiment in dried form as such or after grinding (Verheij & Snijders 1999). They are harvested shortly before the opening of the flowers and the EO can be produced from the flower buds (content 15 - 17 %), from the flower stems (content 6 %) and also from the leaves (content 2 - 3 %). The oil can be characterized as a clear, colorless to yellow liquid (changing into brown with increasing age) with a strong characteristic sweet and spicy clove odor and spicy flavor. It is composed from eugenol (from 70 to 85 %), eugenol acetate (15 %), β-caryophyllene (from 5 to 12 %) and it contains about 2 % of the triterpene oleanolic acid (Gernot Katzer 2008).

2.6 Antimicrobial activity in vapor phase

Nowadays, the new preservation approach is emerging - preservation in vapor phase. Instead of incorporating antimicrobials directly to the foods or using the active films in the direct contact packaging-food system, one of the new alternative approach is using preservation in vapor phase. This method is suitable for bigger volume and large areas and its main advantage lies in non-direct contact with the surfaces of the products.

From the historical point of view, usage of EOs vapors is known from 4^{th} century BC but Europeans have not used them until 16^{th} century. The first serious report of antimicrobial activity from EOs vapors was in 1960. Since that time, the importance of EOs as antimicrobial agents obviously increased. Their promising properties have been repeatedly proven in liquid phase and even in vapor phase. However, there are no specific observations which EO in vapor phase will be effective against which type of microorganism, and therefore, a broad spectrum of them have to be tested against particular microorganism experimentally (Laird & Phillips 2011).

The antimicrobial effectivity of vapors is usually assessed by an initial *in vitro* screening methods and basically, there are two main types. The first is an adapted disc diffusion method where the impregnated filter disc by EO is placed on the lid of the petri dish and afterwards, zones of inhibition are measured. In the second method, the EO and microorganisms are placed into closed environment separately, for example, jars. It results in the individual testing of EO with certain type of pathogen. Recently, Kloucek et al. (2012) proposed another adaptation of the disc diffusion method using a four-sectioned petri dish that reduces the costs both in terms of materials and labor. It is a relatively new screening method, which is faster and more precise, developed by several modifications of already existing methods. In this particular article, 69 EOs have been successfully tested and their MICs against 6 microorganisms have been obtained. Nevertheless, there is newly developed screening method proposed by Houdkova et al. (2017) that is suitable for evaluation of antimicrobial activity in both liquid and vapor phase simultaneously, using microtiter plates covered by tight-fitting lids which reduce evaporation and thus, the results are even more precise. It is a new concept that aims on the determination of antimicrobial potential of plant volatile compounds against foodborne pathogens. Broth microdilution volatilization method allows cost and labor effective high-throughput screening of volatile bioactive compounds with no need of special device. Due to its advantages and certain benefits, it is suitable for the topic of this diploma thesis that focuses on antimicrobial activity of spices against foodborne pathogens in the liquid and in the vapor phase.

Hence, there is a space for further research not only in the EOs but also in the supercritical carbon dioxide extracts. In the future, it would be valuable to develop active packaging for extending the shelf life of food by means of the antimicrobial properties of volatile compounds of EOs and supercritical CO_2 extracts in the atmosphere around the food in non-direct contact packaging-food system.

EOs and supercritical CO_2 extracts can perfectly fit into this new approach due to their antimicrobial effects and presence of volatile compounds (Chen et al. 2018). Several studies have been reported to deal with this topic e.g. Lopez et al. 2005, Goñi et al. 2009, Kloucek et al. 2012, Reyes-Jurado et al. 2019. However, to our best knowledge, none of available research has been focused especially on the supercritical CO_2 extracts in vapor phase. Although, the EOs showed antimicrobial activity in vapor phase with promising results in several studies, the supercritical carbon dioxide extracts are still not deeply and precisely tested.

3 OBJECTIVES OF THE THESIS

The main aim of this study is determination of *in vitro* antibacterial activity of essential oils and supercritical CO_2 extracts obtained from commonly used species of spices against food pathogenic microorganisms in vapor phase that could be potentially used in food preservation (e.g. in modified atmosphere packaging).

The specific objectives are:

a) Isolation of essential oils, extraction of supercritical CO_2 extracts, determination of physical characteristics of both antimicrobial agents;

b) Determination of MICs of isolated essential oils and supercritical CO_2 extracts against food pathogenic microorganisms in liquid and vapor phase.

4 METHODOLOGY

4.1 Plant material and sample preparation

Following plant species have been selected based on their common use as food condiments and literature data on their antimicrobial properties as well as on the content of EOs, namely: *A. melegueta*, *A. sativum*, *A. subulatum*, *A. rusticana*, *C. cassia*, *C. verum*, *E. cardamomum*, *S. aromaticum*. In total, nine samples have been purchased from commercial suppliers in the Prague, Czech Republic (see the Table 2).

The form of spices has been purposely chosen according to usage in our daily diet and thus, spices have been predominantly used in the dried form except *A. sativum* and *A. rusticana* that have been fresh. Seeds (*A. melegueta*, *A. subulatum*, *E. cardamomum*), bark (*C. cassia*, *C. verum*), buds (*C. cassia*, *S. aromaticum*), lateral bulbs, so-called cloves (*A. sativum*) and root (*A. rusticana*) have been chosen for the testing

Initially, all spice samples have been finely ground into powder using an electric mill Grindomix (GM100 Retsch, Haan, Germany). Required amount of powdered sample has been determined according to further processing, either hydrodistillation or SFE.

4.2 Chemicals

Following chemicals were used: amoxicillin CAS number 26787-78-0, ampicillin CAS number. 69-53-4, chloramphenicol CAS number 56-75-7, dimethyl sulfoxide - DMSO (Penta, Prague, Czech Republic), ethanol 70 % and 96 % pharmacological grade (Penta, Prague, Czech Republic), sterile distilled water, tetracycline CAS number 60-54-8, thiazolyl blue tetrazolium bromide – MTT (Sigma-Aldrich, Prague, CZ).

4.3 Bacterial strains and culture media

Bacteria were grown in Mueller-Hinton (MH) broth (Oxoid, Basingstoke, Hampshire, UK) and MH agar (Oxoid, Basingstoke, Hampshire, UK). The pH of broth was equilibrated to final value of 7.6 using buffer containing Trizma base (Sigma-Aldrich, Prague, Czech Republic), sodium chloride - NaCl (Sigma-Aldrich, Prague, Czech Republic) and potassium chloride - KCl (Sigma-Aldrich, Prague, Czech Republic). Subsequently, the required pH has been adjusted by hydrochloric acid 0.1 M (Lach-Ner, Neratovice, Czech Republic) using Cyberscan PC 510 Meter (Eutech Instruments, Singapore).

Following standard strains of the American Type Culture Collection (ATCC) were used: *B. cereus* ATCC 11778, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, *S. e. typhimurium* ATCC 14028. All bacterial strains were purchased from Oxoid (Basingstoke, Hampshire, UK).

4.4 Moisture determination

The moisture and dry matter have been determined in all nine samples. Using moisture analyzer (SMO 01, Scaltec Instruments, Germany), total percentage of moisture and dry matter present in 1 g of each grinded sample have been established. The assessment has been repeated three times for each sample and the average value has been taken into account.

4.5 Preparation of essential oils

EOs have been obtained by hydrodistillation of dried material in 1 liter of distilled water using hydrodistillation equipment including Clevenger apparatus (Merci, Brno, Czech Republic) according to the procedures described in the European Pharmacopoeia (European pharmacopoeia 2013). The process itself begins at the boiling point and it takes three hours. Afterwards, the EOs are collected and stored at 4 °C in airtight glass vials. The data on yields of obtained essential oils are shown in the Table 3.

4.6 Supercritical fluid extraction

Supercritical CO_2 extracts have been prepared by supercritical fluid extractor Helix SFE System, Basic Model (Applied Separations, Allentown, Pennsylvania, USA). The whole process of extraction has been done according to given instructions in the original operations manual, version R3.2U. It begins with finely grinded material (10 g) which is filled into the extraction vessel that is subsequently installed on the extraction module. The extracts have been obtained at constant pressure 200 bars and temperature 40 °C. The flow rate of CO_2 was kept at approximately 5 LPM (liters per minute) and it did not exceed 10 LPM. The obtained saturated solution of extract in supercritical carbon dioxide was expanded to atmospheric pressure, and the extract was collected in 60 ml collection vial (Applied Separations, Allentown, Pennsylvania, USA). Collected supercritical CO_2

extract was taken out by microliter syringe (100 µl; Hamilton Company, Reno, Nevada, USA) and stored at 4 °C in airtight glass vials. The data on yields of obtained supercritical CO_2 extracts are shown in the Table 3.

4.7 Antimicrobial assay

Testing of antimicrobial properties of EOs and supercritical CO_2 extracts in liquid and vapor phase was determined by recently developed broth-microdilution volatilization method by Houdkova et al. (2017).

Whole assay is composed from specifically determined steps and it takes three days. It is necessary to prepare bacteria one day in advance. Bacterial strains have been cultivated in appropriate conditions for 24 h at 37 °C. Prepared sample with particular bacteria contains 5 ml of MH pure broth with 1 ml of chosen bacteria. Afterwards, immediately before testing, prepared bacterial strain was suspended in 10 ml of pure broth and turbidity of suspension was increased to 0.5 McFarland standard, using Densi-La-Meter II (Lachema, Brno, Czech Republic).

According to Houdkova et al. (2017), broth microdilution volatilization assay is suitable for determination of antimicrobial potential of plant volatile compounds in the liquid and the vapor phase at different concentrations. The experiments have been performed in SPL Life Sciences 96-well Immuno Plates (well volume: 400 µl; Pocheon, Gyeonggi, South Korea), covered by tight-fitting lids with flanges which reduce evaporation. At the beginning, the lid has been inoculated by chosen bacteria and then covered by 30 µl of MH agar. EOs and supercritical CO_2 extracts have been dissolved in DMSO at maximum concentration 1 % and then diluted in 792 µl of MH buffer broth. Seven two-fold serially diluted concentrations of samples starting from 1,024 µg/ml have been prepared for all EOs and supercritical CO_2 extracts. The outer wells and flanges have been omitted to prevent edge effect that could influence the results of an assay. The wells containing inoculated and non-inoculated MH buffer broth have been prepared as growth and purity controls. The DMSO assayed as the negative control at concentration of 1 % did not inhibit any of the strains tested either in broth or agar media. Finally, the plate and the lid have been fastened by handmade wooden pads (lower: 120 × 75 ×10 mm; upper: 120 × 80 × 10 mm) and clamps to enhance fixation. In this way fixed plate and lid have been incubated for 24 h at 37 °C.

Thiazolyl blue tetrazolium bromide (MTT) has been used as coloring agent in evaluation of antimicrobial assay. The solution has been prepared according to ratio 600 µg of MTT diluted in 1,000 µl of distilled water. Thereafter, it has been added to each well (25 µl) on the plate and each flange (20 µl) on the lid. Due to metabolization of MTT, which changes color from green to purple, is possible to clearly distinguish effectiveness of the EOs or supercritical CO_2 extracts in liquid and even in vapor phase. As a result, wells/flanges are obtained with yellow (absence of bacteria) or purple (presence of bacteria) color and therefore, MIC of each EO can be determined.

All experiments have been done in triplicate in three independent experiments and results have been expressed as average MICs values.

Table 2: Botanical data on spices tested.

Plant species	Family	Plant part used	Form	Origin
Aframomum melegueta K.Schum.	Zingiberaceae	seeds	dried	Koření od Samuela (Chomutov, CZ)
Allium sativum L.	Alliaceae	lateral bulbs	fresh	Billa, spol. s r.o. (Prague, CZ)
Amomum subulatum Roxb.	Zingiberaceae	seeds	dried	Prodejna u Salvátora (Prague, CZ)
Armoracia rusticana P.Gaertn., B.Mey. & Scherb.	Brassicaceae	root	fresh	Prodejna u Salvátora (Prague, CZ)
Cinnamomum cassia (L.) J.Presl bark	Lauraceae	bark	dried	Prodejna u Salvátora (Prague, CZ)
Cinnamomum cassia (L.) J.Presl fruits	Lauraceae	buds	dried	Ex Herbis (Prague, CZ)
Cinnamomum verum J.Presl	Lauraceae	bark	dried	Prodejna u Salvátora (Prague, CZ)
Elettaria cardamomum (L.) Maton	Zingiberaceae	seeds	dried	Prodejna u Salvátora (Prague, CZ)
Syzygium aromaticum (L.) Merr. & L.M.Perry	Myrtaceae	buds	dried	Prodejna u Salvátora (Prague, CZ)

5 RESULTS

5.1 Physical characteristics of EOs and supercritical CO_2 extracts

The assessment of moisture and dry matter has been done in all nine samples. The highest dry matter content has been found in *C. verum* (85.13 %) and *C. cassia* bark (84.06 %) whereas the lowest dry matter content has been found in *A. sativum* (70.46 %) and *S. aromaticum* (72.78 %). The dry matter content of remaining spices is in the order: *C. cassia* fruits > *A. rusticana* > *A. melegueta* > *E. cardamomum* > *A. subulatum*. For exact values of dry matter in each sample see the Table 3.

In total, nine EOs out of eight various plant species have been distilled and also extracted. The yields have been calculated according to following formula: mL/g (weight of dry sample) x 100 %. The highest yielding EO became *E. cardamomum* (4.70 %) followed by *S. aromaticum* (3.30 %), *C. cassia* fruits (2.25 %) and *A. subulatum* (1.77 %). The rest of the tested compounds yielded less than 1 % in the order: *C. cassia* bark > *C. verum* > *A. melegueta* > *A. sativum* > *A. rusticana*. In terms of yield, SFE seems to be more effective method than hydrodistillation. In general, carbon dioxide extracts possess higher yield except of *C. cassia* fruits and *E. cardamomum*. The highest yielding extract became sovereignly *S. aromaticum* (11.96 %) while the lowest yielding became *A. sativum* (0.36 %) and *A. rusticana* (0.42 %).

EOs and extracts vary in smell, color and density. In general, the EOs and extracts are colorless and translucent or yellow. However, certain differences have been observed, for example, *S. aromaticum* displayed pure colorless oil but the extract has been vivid yellow. For exact colors of EOs and carbon dioxide extracts see the Table 3. Predominantly, the color of EOs varies between limpid and pale yellow and in the case of extracts, the color has been detected from pale yellow and intense yellow via green and orange nuances of yellow. *C. cassia*, either bark or buds, and *C. verum* possess a well-known typical pleasant smell. On the other hand, *A. sativum* and *A. rusticana* have specifically pungent smell which is unique and unmistakable for those species. In terms of smell, no differences between EOs and supercritical extracts have been identified. Interestingly, the consistency of extracts from *A. sativum*, *A. rusticana*, *C. cassia* fruits and *C. verum* has been classified as a solid.

Table 3: Determination of physical characteristics.

Plant species	Dry matter (%)	Yield (%)		Color	
		EOs	CO_2 extracts	EOs	CO_2 extracts
Aframomum melegueta K.Schum.	80.23	0.44	3.43	colorless	intense yellow
Allium sativum L.	70.46	0.21	0.36	pale yellow	intense yellow
Amomum subulatum Roxb.	75.53	1.77	4.25	colorless	intense yellow
Armoracia rusticana P.Gaertn., B.Mey. & Scherb.	80.91	0.18	0.42	colorless	pale yellow
Cinnamomum cassia (L.) J.Presl bark	84.06	0.90	1.66	pale yellow	intense yellow
Cinnamomum cassia (L.) J.Presl fruits	81.42	2.25	1.08	pale yellow	intense yellow
Cinnamomum verum J.Presl	85.13	0.47	0.60	pale yellow	yellow-orange
Elettaria cardamomum (L.) Maton	76.55	4.70	2.86	pale yellow	greenish yellow
Syzygium aromaticum (L.) Merr. & L.M.Perry	72.78	3.30	11.96	colorless	intense yellow

5.2 Antibacterial activity of EOs in liquid and vapor phase

The results show positive antimicrobial effect in five tested EOs. Nevertheless, the most effective EOs became *C. cassia* bark, *C. cassia* fruits and *C. verum* in both liquid and in vapor phase. Their effectiveness varied substantially ranging from 256 to 1,024 µg/ml in broth and agar media. The bark of *C. cassia* was the most effective against *E. coli* in broth and agar at the same concentration 256 µg/ml. Against *L. monocytogenes* it reached the same concentration but only in the liquid phase. In the case of *C. cassia* fruits, the lowest concentration, i.e. 256 µg/ml, was observed against *B. cereus* in broth and agar as well. The mild antimicrobial effects could be observed in the case of *A. rusticana* and *S. aromaticum*. Spices from *A. melegueta*, *A. sativum*, *A. subulatum* and *E. cardamomum* possess no inhibitory effect against all tested foodborne pathogens. In case of EOs, the most sensitive bacterium was *E. coli*. Detailed overview of results of *in vitro* growth-inhibitory activity of spice EOs against *B. cereus*, *E. coli*, *L. monocytogenes*, *S. e. typhimurium* in liquid and vapor phase using the broth microdilution volatilization method, see the Table 4.

5.3 Antibacterial activity of CO_2 extracts in liquid and vapor phase

From all tested supercritical CO_2 extracts, only three possessed decent degree of antibacterial activity in both media, broth and agar. *C. cassia* bark, *C. cassia* fruits and *C. verum* had the greatest positive inhibitory effect against all bacterial strains from all tested extracts. Their effectiveness varied substantially ranging from 256 to 1,024 µg/ml in broth and agar media. *A. melegueta* and *S. aromaticum* displayed certain antimicrobial activity as well, however, in comparison with the *Cinnamomum* spp. it is almost negligible. *A. melegueta* has been active only against *L. monocytogenes* at concentration 1,024 µg/ml. in liquid phase. In the case of *S. aromaticum*, the mild inhibitory activity has been proven only in the liquid phase at concentration 1,024 µg/ml while in the vapor phase no antimicrobial activity has been observed. In case of supercritical CO_2 extracts, the most sensitive bacterium was *L. monocytogenes*. The final results of *in vitro* growth-inhibitory activity of supercritical CO_2 extracts against *B. cereus, E. coli, L. monocytogenes, S. e. typhimurium* in liquid and vapor phase using the broth microdilution volatilization method are displayed in the Table 5.

Table 4: The antibacterial activity of EOs against foodborne pathogens in liquid and vapor phase.

Essential oils	*Bacillus cereus*		*Escherichia coli*		*Listeria monocytogenes*		*Salmonella enterica typhimurium*	
	Broth	**Agar**	**Broth**	**Agar**	**Broth**	**Agar**	**Broth**	**Agar**
Aframomum melegueta K.Schum.	-	-	-	-	-	-	-	-
Allium sativum L.	-	-	-	-	-	-	-	-
Amomum subulatum Roxb.	-	-	-	-	-	-	-	-
Armoracia rusticana P.Gaertn., B.Mey. & Scherb.	1024	1024	1024	1024	-	-	1024	1024
Cinnamomum cassia (L.) J.Presl bark	512	512	256	256	256	1024	256	512
Cinnamomum cassia (L.) J.Presl fruits	256	256	512	512	256	256	512	512
Cinnamomum verum J.Presl	512	1024	512	512	512	1024	512	1024
Elettaria cardamomum (L.) Maton	-	-	-	-	-	-	-	-
Syzygium aromaticum (L.) Merr. & L.M.Perry	1024	1024	1024	1024	1024	1024	1024	-
Positive Antibiotic control								
Amoxicillin	NT	NT	8	>8	NT	NT	NT	NT
Ampicillin	NT	NT	NT	NT	0.5	1	NT	NT
Chloramphenicol	2	>8	NT	NT	NT	NT	NT	NT
Tetracycline	NT	NT	NT	NT	NT	NT	4	>32

Explanatory notes: NT: not tested; -: not determined (>1024 μg/ml)

Table 5: The antibacterial activity of supercritical CO_2 extracts against foodborne pathogens in liquid and vapor phase.

Supercritical CO_2 extracts	***Bacillus cereus***		***Escherichia coli***		***Listeria monocytogenes***		***Salmonella enterica typhimurium***	
	Broth	**Agar**	**Broth**	**Agar**	**Broth**	**Agar**	**Broth**	**Agar**
Aframomum melegueta K.Schum.	-	-	-	-	1024	-	-	-
Allium sativum L.	-	-	-	-	-	-	-	-
Amomum subulatum Roxb.	-	-	-	-	-	-	-	-
Armoracia rusticana P.Gaertn., B.Mey. & Scherb.	-	-	-	-	-	-	-	-
Cinnamomum cassia (L.) J.Presl bark	256	512	512	512	256	256	512	512
Cinnamomum cassia (L.) J.Presl fruit	512	512	512	1024	512	512	1024	1024
Cinnamomum verum J.Presl	256	512	512	1024	512	512	512	512
Elettaria cardamomum (L.) Maton	-	-	-	-	-	-	-	-
Syzygium aromaticum (L.) Merr. & L.M.Perry	1024	-	1024	-	1024	-	1024	-
Positive Antibiotic control								
Amoxicillin	NT	NT	1	>4	NT	NT	NT	NT
Ampicillin	NT	NT	NT	NT	0.5	>1	NT	NT
Chloramphenicol	4	>8	NT	NT	NT	NT	NT	NT
Tetracycline	NT	NT	NT	NT	NT	NT	2	>32

Explanatory notes: NT: not tested; -: not determined (>1024 μg/ml)

6 DISCUSSION

In this study, EOs of *C. cassia* and *C. verum* exhibited the strongest antibacterial activity against *B. cereus, E. coli, L. monocytogenes* and *S. e. typhimurium*. Previously, several studies using conventional susceptibility testing methods such as broth dilution and disk diffusion assays have shown similar results. For instance, Chaudhry and Tariq (2006) who examined the EOs of *C. cassia* bark against *E. coli* and *Salmonella* spp. In another study, Prabuseenivasan et al. (2006) confirmed the antimicrobial activity of EO obtained from *C. cassia* against *E. coli* by disc diffusion method as well. The results of our study agree with the findings of Keloth et al. (2018) who reported remarkable antimicrobial potential of crude extracts of cinnamon species (including *C. cassia* and *C. verum*). The extracts of bark and the leaves have been tested with the positive growth-inhibitory effects against *E. coli* and *S. e. typhimurium*. The bark extracts possess better results than leaves and the author also recommends further purification of the extracts from their crude form. It would definitely enhances their antimicrobial efficiency against foodborne pathogens. Based on this fact, the SFE could be the suitable extraction method due to its benefits and the results directly support this theory. Nevertheless, the scientific sources about supercritical CO_2 extracts of *C. cassia* and *C. verum* and their antimicrobial activity are limited. Santos (2018) prove the antibacterial activity of *C. cassia* extract by satisfactory results in inhibition of *E. coli*. The positive results and promising properties of *Cinnamomum* spp. are obviously caused by the presence of antimicrobial compounds such as cinnamic aldehyde and eugenol (Davidson et al. 2005, Lopez et al. 2007, Guoruoluo et al. 2018, Liu et al. 2018). Moreover, there are also studies that confirm their growth-inhibitory effects in vapor phase due to the volatile substances. The antimicrobial activity of *C. verum* has previously been determined in vapor phase against *B. cereus*, *E. coli*. *L. monocytogenes* and *Salmonella* sp. (Lopez et al., 2007). However, according to our best knowledge, this is the first report on *C. cassia* EO and supercritical CO_2 extracts of bark and fruits and supercritical CO_2 extract of *C. verum* bark in vapor phase.

In this thesis, *A. rusticana* produced only week antimicrobial effect in both liquid an vapor phase. This is in contrast to study of Kloucek et al. (2012), who found *A. rusticana* as one of the most active among 69 EOs in vapor phase. The positive effect has been assured due to its major bioactive compounds, such as allyl isothiocyanate (63.7 %) and βphenylethyl isothiocyanate (23.9 %). The different results can be caused by different method and bacterial strains used in both studies.

Although EOs neither extracts of *A. melegueta*, *A. sativum*, *A. subulatum* and *E. cardamomum* did not show any significant antimicrobial properties, there are some studies and scientific articles about their growth-inhibitory effects. According to Nedorostova et al. (2009), EO of *A. sativum* is highly effective in the vapor phase and it could be potentially used against *E. coli* or *L. monocytogenes*. Doherty et al. (2010) reported certain antimicrobial activity of EOs, isolated from *A. melegueta* by ethanol extraction, against *E.coli* and *Salmonella* spp. Due to a different extraction technique, the results are not corresponding with this study. *A. sativum* did not display any antibacterial properties against all tested bacterial strains in this thesis, however, Kloucek et al. (2012) has observed certain growth-inhibitory effect due to its phytochemical composition of EOs. Major constituents are diallyl disulfide (43.8 %) and diallyl trisulfide (27.1 %). The reason for distinct results is probably in the selection of different bacterial strains.

The interesting related topic is synergism between the EOs and/or the extracts. It supports several scientific articles, for example, Goñi et al. (2009). The combination of EOs from *C. verum* and *E. aromaticum* at 50 % in volume against a wide range of bacteria in vapor phase showed that a synergistic effect could be achieved for some of the tested microorganisms. However, in this particular thesis, the compounds either EOs or extracts have been tested separately. Based on these facts, the EOs and likewise supercritical CO_2 extracts could be tested in combination and therefore, the promising synergistic effect that utilize the benefits of both antimicrobial agents could be achieved.

The comparison of yields obtained by SFE and hydrodistillation is provided above in the Table 3. The yields from hydrodistillation are predominantly lower than from SFE except the samples *C. cassia* fruits and *E. cardamomum*. Based on these facts, the SFE can be classified as a suitable extraction method with high efficiency. The advantages are e.g. low operating temperatures to reduce energy consumption, preservation of heat-sensitive compounds, avoidance of residual toxic solvent, and moreover, extracts are not altered by the high temperature. The main disadvantages include higher initial financial inputs and the necessity of skilled operating staff.

EOs as food preservatives may have a great potential use. Spices, which are commonly used as food additives in cuisine, are present in insufficient quantities for their antimicrobial properties to be significant (Dorman & Deans 2000). Furthermore, it is well-known, that in foodstuffs the concentrations needed for microorganism inhibition are usually higher than that obtained by *in vitro* methods (Burt 2004). Based on this two theses, the future practical use of EOs as natural food preservatives is becoming even more complicated.

To our best knowledge, this is the first study using broth microdilution volatilization method for testing *in vitro* antimicrobial activity of EOs simultaneously with supercritical CO_2 extracts in the liquid and in the vapor phase and provide the comparison between them. Generally, the research regarding antimicrobial activity of EOs is relatively available and accessible, however, the studies related to supercritical CO_2 extracts and their growth-inhibitory effects are still limited. Hence, the main tasks for the near future are to create and evaluate functional antimicrobial packaging systems using these natural antimicrobial substances. According to our results, this method was confirmed to be suitable for rapid determination of antibacterial potential of EOs and supercritical CO_2 extracts in the liquid and the vapor phase at different concentrations. However, in order to achieve comprehensive and definite results, further research is highly needed and recommended.

7 CONCLUSION

In this study, *in vitro* growth-inhibitory activity of spice EOs and supercritical CO_2 extracts have been tested against 4 foodborne pathogenic bacteria, namely *B. cereus*, *E. coli*, *L. monocytogenes*, and *S. e. typhimurium*, using the broth microdilution volatilization method in liquid and vapor phase. In total, 9 EOs have been successfully isolated from 8 plants species by hydrodistillation as well as extracted by SFE. As a result of antibacterial susceptibility testing, *C. cassia* (bark and fruits) and *C. verum* (bark) exhibited significant growth-inhibitory effects against all tested foodborne pathogens in the liquid and in the vapor phase. Their effectiveness varied from 256 to 1,024 μg/ml in broth and agar media. These results suggest that both *Cinnamomum* spp. could be potentially used in food industries as natural food preservatives, for example, in modified atmosphere packaging. However, further studies focused on their effectiveness in various food models will be necessary prior their practical use in foods and food industry.

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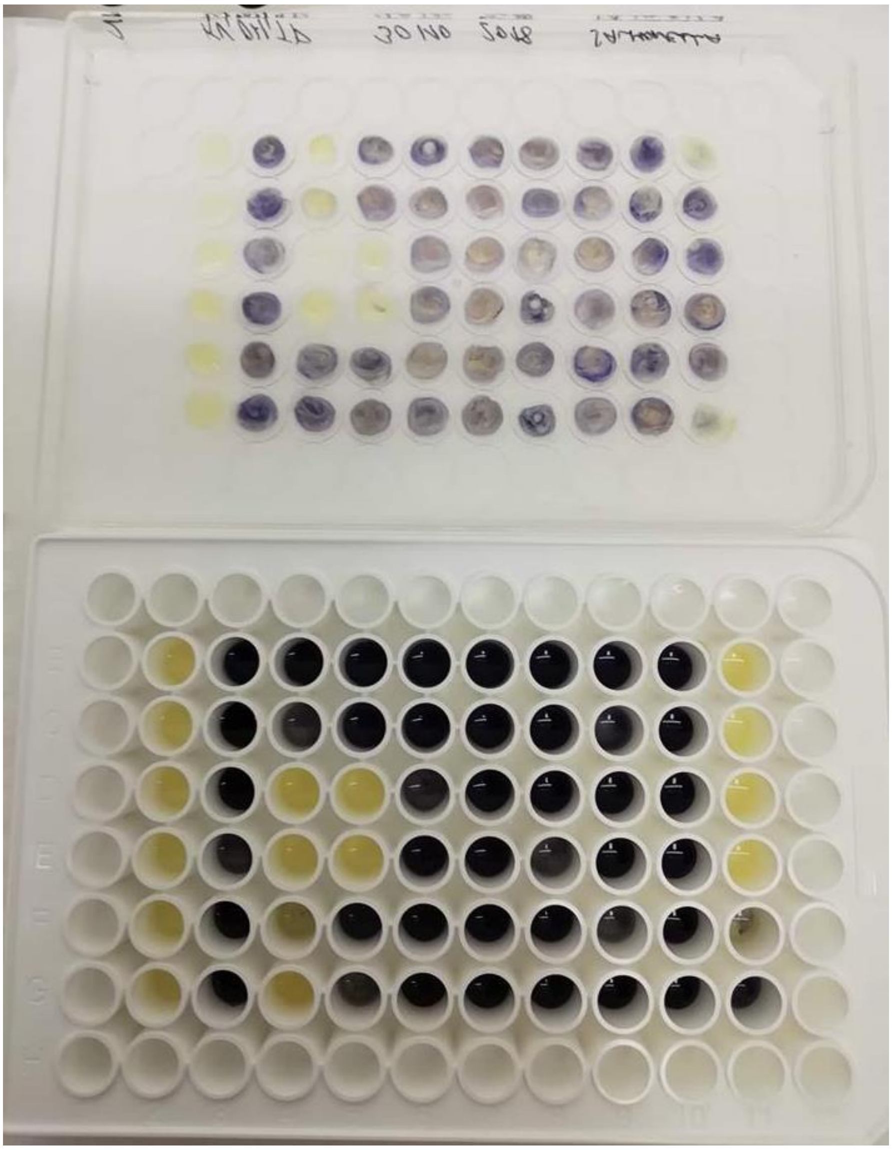
APPENDICES

APPENDIX 1: Hydrodistillation device with Clevenger apparatus (Merci, Brno, Czech Republic) (Ondřej Horák 2019).

APPENDIX 2: Supercritical fluid extractor (Helix SFE System, Basic Model, Applied Separations, Allentown, Pennsylvania, USA) (Ondřej Horák 2019).

APPENDIX 3: Broth-microdilution volatilization method. The plates and the lids fastened by handmade wooden pads and clamps to enhance fixation (Houdkova 2017).

APPENDIX 4: Evaluation of broth-microdilution volatilization method. Determination of MICs on the plate (below) and the lid (above and reversed). *S. e. typhimurium* (Ondřej Horák 2019).



APPENDIX 5: Filling the extraction vessel by grinded material. *A. sativum* (Ondřej Horák 2019).

APPENDIX 6: Obtained supercritical CO_2 extract collected in 60 ml collection vial (Applied Separations, Allentown, Pennsylvania, USA) (Ondřej Horák 2019).

APPENDIX 7: The comparison of the EO (on the left) and the supercritical CO_2 extract. *S. aromaticum* both (Ondřej Horák 2019).