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Antibacterial effect of essential oils and supercritical extracts from Cambodian spices against food pathogens in vapour phase

BACHELOR'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Antibacterial effect of essential oils and supercritical extracts from Cambodian spices against food pathogens in vapour phase independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 16.04.2021

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Abstract

Consumption of food contaminated by pathogenic bacteria is still a cause of illnesses with severe or fatal outcomes worldwide. The elimination of chemical food additives by natural products has recently gained attention of researchers. The use of natural antibacterial agents in vapour phase is promising option for food preservation (e.g., as smart packaging).

The aim of this thesis is to evaluate of *in vitro* growth-inhibitory effect of essential oils and supercritical CO_2 extracts of Cambodian spices (*Citrus hystrix*, *Curcuma zedoaria*, Kampot pepper and *Limnophila aromatica*) against foodborne pathogens, namely *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella Typhimurium*. Essential oils and supercritical CO_2 extracts were obtained by hydrodistillation and supercritical fluid extraction using carbon dioxide, respectively. Subsequently, their antibacterial activity was assayed using broth microdilution volatilisation method and the minimum inhibitory concentrations were determined. It was found, that essential oil of *C. zedoaria* inhibited growth of Gram-positive bacteria (*B. cereus* and *L. monocytogenes*) with MIC $\geq$ 1024 μg/mL both in liquid and vapour phase.

Although the active concentrations determined for *C. zedoaria* are relatively high, the results suggest that application potential of this species in the area of food preservation could be further studied.

Key words: Essential oils, supercritical extracts, antimicrobial activity, food pathogens, Cambodian plants

Abstrakt

Konzumace potravin kontaminovaných patogenními bakteriemi je stále příčinou onemocnění s vážnými až fatálními následky po celém světě. Eliminace chemických potravinářských aditiv přírodními produkty se v nedávné době dočkala pozornosti ze strany vědců. Použití přírodních antibakteriálních látek v plynné fázi je slibnou možností konzervace potravin (např. jako inteligentní balení).

Cílem této práce je hodnocení *in vitro* růstově inhibičního účinku esenciálních olejů a superkritických CO_2 extraktů kambodžského koření (*Citrus hystrix, Curcuma zedoaria,* Kampot pepper a *Limnophila aromatica*) proti potravním patogenům, zejména *Bacillus cereus, Escherichia coli, Listeria monocytogenes* a *Salmonella Typhimurium*. Esenciální oleje a superkritické CO_2 extrakty byly získány pomocí hydrodestilace a superkritické extrakce využívající oxidu uhličitého. Následně byla jejich antibakteriální aktivita podrobena bujónové mikrodilační volatilizační metodě a byly stanoveny minimální inhibiční koncentrace. Bylo zjištěno, že esenciální olej *C. zedoaria* inhiboval růst grampozitivních bakterií (*B. cereus* a *L. monocytogenes*) s MIC ≥ 1024 μg/ml jak v kapalné, tak v plynné fázi.

I když aktivní koncentrace stanovené pro *C. zedoaria* jsou poměrně vysoké, výsledky naznačují, že potenciální aplikace koření tohoto druhu by mohla být v rámci konzervace potravin dále studována.

Klíčová slova: esenciální oleje, superkritické extrakty, antimikrobiální aktivita, potravní patogeny, kambodžské koření

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List of the abbreviations used in the thesis

ATCC	American Type Culture Collection
DEC	diarrheagenic *E. coli*
DMSO	Dimethyl sulfoxide
EAEC	enteroaggregative *E. coli*
EHEC	enterohaemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EOs	Essential oils
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
FDA	United States Food and Drug Administration
HD	hydrodistillation
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MTT	Thiazolyl Blue Tetrazolium Bromide
ND	not determined
SF	Supercritical fluids
SFE	Supercritical fluid extracts
WHO	World Health Organization

1. Introduction

Food contamination caused by bacteria pathogens are a worldwide threat that affect millions of people annually. Consuming contaminated food or water may evoke severe or fatal outcomes, thus, access to sufficient amounts of safe and nutritious food is key to sustaining life and promoting good health (WHO 2020). While 99.8 % of all food products and beverages need to be packaging and food security must be adhered, several methods using nature volatile compounds were explored (Otoni et al. 2016).

Essential oils epitomize volatile agents naturally produced as secondary metabolites of plants because of the protection from some pathogenic microorganisms and the reduction of the appetite of some herbivores. Therefore, essential oils obtained wide range of properties e.g., antibacterial, antifungal, antiparasitic, antitoxigenic, antiviral, and insecticidal (Burt 2004).

The antimicrobial activity of a volatile compound dependent on several conditions. One of the main factors is bacterial susceptibility. Generally, EOs are slightly more active against Gram-positive than Gram-negative bacteria due to reaction of chemical compounds of EOs to outer bacteria cell membrane. Another determining factor is the extraction method of EOs used. Distillation techniques have traditionally been applied for the removal of the essential oils from plant materials, however, recent years showed widely use of new environmentally friendly method, supercritical fluid extraction, which furthermore showed better yields of extracts from plant materials (Pourmortazavi & Hajimirsadeghi 2007; Singh et al. 2019).

In food preservation, direct contact between the packaging material and the food product is not required for EOs to exert their antimicrobial activity (Manso et al. 2015). This is making vapours of EOs promising option for food preservation (e.g., as smart packaging). The EOs from tropical species representing phytochemically less explored plants are promising antimicrobial agents of further studies. Evaluating antimicrobial activity of EOs and volatile compounds in the vapour phase is unfortunately still difficult because lack of standardized method.

2. Literature Review

2.1. Food pathogenic bacteria

Foodborne illnesses are usually caused by bacteria entering the body through contaminated food or water (WHO 2020). There is a significant increase in the occurrence of foodborne illnesses due to new nutritional trends that support consuming raw and fresh food, dry products, and exotic ingredients. Bacterial strains that contaminate food in different stages of production and cause foodborne diseases are referred as foodborne pathogens (Martinović et al. 2016). *Campylobacter* spp., enterohaemorrhagic *Escherichia coli* (EHEC*)*, and *Salmonella* spp. are among the most common foodborne pathogens that affect millions of people annually, sometimes with severe and fatal outcomes. Access to sufficient amounts of safe and nutritious food is key to sustaining life and promoting good health (WHO 2020).

Food protection against pathogen bacterial strains is controlled by several methods of food preservation such as pasteurization and sterilization, however, heat treatment is not desirable for all foods and cross-contamination cannot always be prevented (Burt & Reinders 2003). As for food industry, the first attempt to fight microbial contamination was based on the direct addition of antimicrobials (e.g., food preservatives) to food products. However, this strategy proved to be limited due to the rapid diffusion of the antimicrobial substance from the surface to the mass of the product (Radusin et al. 2013). According to 99.8 % of all food and beverages have to be encased in some sort of packaging during their existence, food industry represented innovative ways of antimicrobial substances implemented in the food packaging material (Otoni et al. 2016). These substances include chemicals such as organic acids, triclosan, antibiotics, chlorine dioxide, nitrites, and ammonium salt. In recent years, they are slowly being replaced by more natural such as bacteriocins, enzymes, phages, biopolymers, metal nanoparticles, natural extracts, and compounds, EOs and their components (Becerril et al. 2020).

2.1.1. *Bacillus cereus*

This aerobic, endospore-forming bacterium have been suspected as an agent of foodborne illness since the early days of microbiology. The cells of *B. cereus* are observed microscopically as large, Gram-positive rods that are motile by means of peritrichous flagella. The cells are typical 1,0 – 1,2 μm in diameter by 3,0 – 5,0 μm in length. Minimum growth range

is around 10 – 12 °C and a maximum of 48 – 50 °C. The organism grows rapidly in foods held in the 30 – 40 °C range. Growth has been demonstrated over the pH range 4,9 – 9,3. The spores posse a resistance to heat (Bennet 2001).

Food-poisoning strains of *B. cereus* and other species of this genus produce a number of toxins and other defined extracellular products. The metabolic components most commonly associated with such strains include lecithinase, proteases, hemolysin, ß-lactamase, mouse lethal toxin, cereloysins, and food-poisoning toxins. The enterotoxins are responsible for two food-poisoning syndromes caused by this organism. Diarrheal Syndrome was the first recognized as the illness caused by the consumption of foods contaminated with large numbers of enterotoxigenic *B. cereus*. This syndrome is primarily characterized by abdominal cramps with profuse watery diarrhoea, rectal tenesmus, and occasionally nausea that seldom results in vomiting. It has an incubation period within the range of 8 – 16 h. The symptoms generally resolve in 12 – 24 h. Emetic Syndrome is the second type of illness caused by B. cereus characterized by an acute attack of vomiting that occurs 1 – 5 h after consumption of contaminated food. The presence of the diarrheal factor is usually associated with proteinaceous foods, vegetables, sauces, and puddings. In contrast, the emetic form of the illness is associated with farinaceous foods, particularly cooked rice and other starchy foods (Bennet 2001; Rasko et al. 2005; Vidic et al. 2020).

The ubiquity of *B. cereus* in the general environment, the stability and resistance of their spores, and their presence on raw agricultural products provide justifiable concern for *Bacillus* spp. as actual or opportunistic foodborne pathogens. Their presence on raw agricultural products ensures possible contamination of the food-processing environment and equipment. As a consequence, effective prevention and control measures would include the control of *Bacillus* spore germination and prevention of proliferation of the vegetative cells in foods. Effective heat or irradiation treatment may be necessary where complete destruction of the organism is desired. The creation of unfavourable conditions such as low temperatures, low Aw, or low pH in foods may greatly reduce the spore germination of enterotoxigenix *Bacillus* spp., thus preventing toxin formation in foods. A number of cooking methods such as steaming under pressure, through roasting, frying, and grilling are likely to destroy both vegetative cells and spores, although cooking at temperatures below 100 °C might allow survival of *Bacillus* spores. Of major concern to the consumer is the multiplication of the organism during inadequate cooling or the holding of moist foods in a nonrefrigerated state over periods that would allow cell proliferation. Favourable conditions for enterotoxigenic *Bacillus* are sometimes provided by cooking procedures that activate spores and then by slow cooling and

mass storage is necessary, it should be cooled rapidly to a temperature (8 °C) that prevents growth of the organism. If food must be held in warm state, such as might be necessary in institutional settings, the temperature should be maintained above 60 °C. Of major concern to the food processor and retailer in the prevention of food-poisoning outbreaks should be effective utilization of hazard analysis critical control point (HACCP) systems by all who are involved in the harvest, manufacture, distribution, storage, and serving of food (Bennet 2001).

2.1.2. *Escherichia coli*

The genus *Escherichia* consists of Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium that belong to the family Enterobacteriaceae. *E. coli* strains live harmlessly in the colon and seldom cause disease in healthy individuals, although several pathogenic strains can cause intestinal and extraintestinal diseases both in healthy and immunocompromised individuals (Gomes et al. 2016). Diarrheal illnesses are a severe public health problem. Moreover, this bacterium continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries (Sarantuya et al. 2004; Gomes et al. 2016). Enteroaggregative *E. coli* (EAEC), EHEC, enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) are five distinct classes of diarrheagenic *E. coli* (DEC) recognized as being associated with diarrheal disease (Sarantuya et al. 2004).

As for the DEC categories, EAEC appears to have been increasingly recognized as an emerging pathogen causing low fever watery diarrhoea, presenting mucus, with or without blooding and abdominal pain, and vomiting in both developing and industrialized countries (Sarantuya et al. 2004; Gomes et al. 2016). Contamination by EAEC were detected in milk samples from infant feeding bottles that were handled by mothers with low socioeconomic status and were also isolated in tabletop sauces from Mexican restaurant. In the case of animals, it is presumed, that they did not represent a reservoir of human pathogenic EAEC (Gomes et al. 2016).

EHEC strains produce Shiga toxins, which are the major virulence factor, and a defining characteristic of EHEC. They also present leadership to death and many other symptoms in patients such as crampy abdominal pain and a short-lived fever usually accompanied by nonblood diarrhoea (Nataro & Kaper 1998). Vehicles of transmission of EHEC are widespread since they can survive in the soil, manure, pastures, and water. Generally, they can be transmitted by food, water and from person to person. Because Stx-producing *E. coli* can be

found in the faecal flora of a wide variety of animals including cattle, most cases are caused by ingestion of contaminated foods of bovine origin. Animal reservoir represents not only cattle but also cats, dogs, goats, gulls, chickens, pigs, and sheep (Nataro & Kaper 1998; Gomes et al. 2016). In the United States, ingestion of undercooked hamburgers has been a particularly important cause of outbreaks. North America in 1992 reported a large outbreak due to contaminated hamburgers from a fast-food restaurant chain (Griffin 1995). Contamination of the hamburgers implicated in these outbreaks was the result in part of modern food-processing technology. Beef from thousands of cattle raised on hundreds of farms is ground together in a single hamburger plant, which then distributes frozen patties to thousands of restaurants in several states (Nataro & Kaper 1998).

EIEC is responsible for diarrhoea illnesses. Water, cheese (milk products), and beef were described as potential sources, as well as the direct transmission through person-to-person contact (Gomes et al. 2016).

EPEC is an important category of diarrheagenic *E. coli*, which has been linked to infant diarrhoea in developing countries where it is hosting primarily children, especially new-borns (0 – 6-month age group), who may die in a result of watery diarrhoea due to dehydration. Transmission of enteropathogenic *E. coli* happens face-oral through contaminated surfaces, weaning fluids, and human carriers however particular types of food were not identified. Transmission was also determined from dust and aerosols (Nataro & Kaper 1998).

ETEC strains are associated with two major clinical syndromes: weanling diarrhoea among babies and young children in tropical areas with poor sanitary conditions, and diarrhoea linked with travelling into these countries (Nataro & Kaper 1998; Gomes et al. 2016). Epidemiologic investigations have implicated contaminated food and water as the most common vehicles for ETEC infection. ETEC is also an economic burden to farmers and industry, where it is an important pathogen for broilers, swine, cattle, and other farm animals (Gomes et al. 2016).

2.1.3. *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, facultative anaerobic, 1 – 2 μm long rod-shaped bacterium that is responsible for the disease, listeriosis (Roberts et al. 2020). Due to having the ability to cross three key barriers - the intestinal barrier, the blood – brain barrier and the fetoplacental barrier, it can infect organs such as the brain or uterus, and cause severe life-threatening infections such as meningitis, encephalitis, spontaneous abortion, or miscarriage.

L. monocytogenes is ubiquitous in the environment (soil, water, sewage, green plant material, decaying vegetation, and numerous species of birds and mammals, including humans); therefore, contamination of the food processing environment is inevitable unless stringent efforts are in place to prevent such contamination. *L. monocytogenes* can survive for long periods of time in a seemingly hostile environment such as a food processing facility partially due to its ability to endure various stresses, such as sanitizers, pH and temperature (Jordan et al. 2018). Meats, spreads, and soft cheeses, as well as raw milk and milk products are examples of contaminated foods by *L. monocytogenes* (Roberts et al. 2020). In 2000–2001, consumption of Mexican-style soft cheese (Queso Fresco) made with unpasteurized milk resulted in 12 cases of listeriosis and 5 miscarriages in a Hispanic community in North Carolina. In 2002, ready-to-eat turkey deli meat was implicated in a multistate outbreak involving nine states resulting in 54 illnesses, 8 deaths, and 3 stillbirths. In 2003, raw milk cheese was responsible for an outbreak in Texas, and in 2005, a multistate outbreak involving consumption of turkey deli meat affected nine states and caused 12 illnesses (Bhunia 2008).

2.1.4. *Salmonella Typhimurium*

S. Typhimurium is a serovar of *Salmonella enterica* characterized as Gram-negative, facultatively anaerobic, motile bacterium, and express peritrichous flagella. They can grow in a temperature range of 5–45 °C with optimum temperature of 35–37 °C. They are able to grow at low pH and are generally sensitive to increased concentrations of salt (Bhunia 2008). Is classified as an invasive enteric pathogen associated with diarrhoea, acute intestinal inflammation, and the presence of neutrophils in stool samples (Winter et al. 2010).

Salmonella spp. are present in the intestinal tract of birds, reptiles, turtles, insects, farm animals, and humans. Poultry are a major source for human foodborne salmonellosis, in part due to high-density farming operations which allow colonized birds to quickly spread salmonellae to other birds within a flock. Intestinal colonization by salmonellae increases the risk for contamination during slaughter. Human salmonellosis is generally foodborne and is contracted through consumption of contaminated food of animal origin such as meat, milk, poultry, eggs. as well as food products such as chocolate, ice cream, cheese, and peanut butter. Furthermore, fruits and vegetables such as lettuce, tomatoes, cilantro, alfalfa-sprouts, and almonds have also been implicated in outbreaks. Animal-to-human or human-to-human transmission can also occur (Bhunia 2008; Eng et al. 2015). *S. Typhimurium* is one of the leading serovars responsible for human and animal salmonellosis, globally (Wang et al. 2019).

2.2. EOs and supercritical extracts

EOs are liquid volatile agents naturally produced as secondary metabolites of plant families such as the Alliaceae, Apiaceae, Asteraceae, Chenopodiaceae, Cyperaceae, Lamiaceae, Lauraceae, Myrtaceae, Poaceae, Piperaceae, Rutaceae, Verbenaceae, and Zingiberaceae (Pandey et al. 2017). They can be synthesized in all plant organs such as bark, buds, flowers, fruits, leaves, stems, seeds, roots, twigs, or wood. According to cellular level, EOs are found in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali et al. 2008). As for other secondary metabolites, the role of EOs is the protection of the plant organism against some pathogenic microorganisms and the reduction of the appetite of some herbivores. On the other hand, they also may attract some insects to promote the dispersion of pollens and seeds (Nazzaro et al. 2017).

EOs are known for their range of properties e.g., antibacterial, antifungal, antiparasitic, antitoxigenic, antiviral, and insecticidal (Burt 2004). Currently a range of synthetic protection against foodborne pathogens used for conservation food items accumulate evidence of possibility that they could be toxic and carcinogenic. Therefore, EOs as a natural antimicrobials and antioxidants seem to be the most promising answer to many of the increasing concerns and could yield better results than synthetic food preservatives (Purkait et al. 2018). Pleasant odour and distinctive taste are also among EOs properties thus used by flavouring and cosmetic industries. Although incorporation of EOs into food products may cause quality defects such as undesirable colour changes or off-flavour (Bagheri et al. 2020).

2.2.1. Antibacterial properties

The antimicrobial activity of EOs is strictly connected to their chemical composition (Kalemba & Kunicka 2003). EOs contain primarily terpenoids, especially monoterpenes and sesquiterpenes, although diterpenes may also be present. A variety of other molecules also occur, such as acids, alcohols, aldehydes, aliphatic hydrocarbons, acyclic esters or lactones, rare nitrogen- and sulphur-containing compounds, coumarins, and homologues of phenylpropanoids (Nazzaro et al. 2013).

The chemical composition and yield of EOs depend on provenance of the plant, the part of plant used, the stage of plant development, climatic and growth condition (temperature, soil, fertilizers, etc.) as well as distillation and storage conditions (Kalemba & Kunicka 2003). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that

there are several targets in the cell. An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable.

Most studies investigating the action of EOs against food spoilage organisms and food borne pathogens agree that, generally, EOs are slightly more active against Gram-positive than Gram-negative bacteria (Burt 2004). The outer membrane of Gram-negative bacteria contains hydrophilic lipopolysaccharides that acts as a barrier to macromolecules and hydrophobic compounds such as those found in EOs (Pandey et al. 2017).

The antimicrobial activity of EOs can be determined by its composition which is strongly dependent on extraction method used (Burt 2004). EOs are generally extracted from various aromatic plants localized in temperate to warm countries like Mediterranean and tropical countries (Bakkali et al. 2008).

2.2.2. Extraction methods

Distillation techniques such as steam and hydrodistillation (HD) have traditionally been applied for the removal of the essential oil from plant materials. These techniques have some disadvantages e.g., losses of volatile compounds, low extraction efficiency, and long extraction time. In addition, elevated temperatures and water could cause the possibility of degradation or chemical modifications of essential oil component. Thus, past several years presented alternative methods for extraction of EOs using solvents at high pressure, or supercritical fluids (SF) which were used especially in food, pharmaceutical and cosmetic industries (Pourmortazavi et al. 2007).

SF appear as a compressed gas; therefore, SF are similar to a liquid with elevated density and low compressibility and at the same time they are similar to a gas with elevated diffusivity and low viscosity. Owing to their high penetration power inside plant materials, supercritical fluids became a good solvent. Supercritical fluid extraction (SFE) has become the most widely used method for extracting and isolating EOs from aromatic plants. This technique provides effective and quick extraction, requires only moderate temperatures, eliminates clean-up steps, and avoids the use of harmful organic solvents. Currently, more than 90 % of SFE processes are performed using CO_2. In addition to having a relatively low critical temperature and pressure, CO_2 as a solvent in supercritical fluid extraction offer several properties such as moderately non-flammable, non-explosive, non-toxic, available at low cost and high purity, recyclable. CO_2 performs an excellent medium for extraction of nonpolar species from plant

matrices such as alkenes and terpenes (Yousefi et al. 2013). However, some investigations showed higher number of compounds extracted by HD method, in that case HD method is being more effective in antimicrobial activity (Singh et al. 2019).

Today, SFE is mainly used for decaffeination of coffee and tea as well as production of hop extracts on a large scale. Also, growing interest in this extraction method caused applications in other industrials. The use of SFE was demonstrated with a variety of samples including spices, chewing gum, orange peel, spruce needles, and cedar wood. For example, SFE of oil from *Piper nigrum* L., using CO_2 as a solvent, showed a significant increase of extraction rate thus caused higher levels of contained sequiterpene hydrocarbons, leading to higher sesquiterpene to monoterpene ratios as compared to that obtained from hydrodistillation (Capuzzo et al. 2013).

2.2.3. EOs in food systems

Substances that are added to food to maintain or improve the safety, freshness, taste, texture, or appearance of food are known as food additives (WHO 2018). They can be derived from plants, animals, or minerals, or they can be synthetic. With the company's growing interest in preserving food other than synthetic additives, EOs regarded as a promising replacement (Purkait et al. 2018). According to this affirmation and subsequently research, several EOs and their components have been approved as Generally Recognized as Safe by United States Food and Drug Administration (FDA), to be used for food flavourings or preservatives (Pandey et al. 2017). For example, the application of thyme and cinnamon EOs in ham has been shown to significantly decrease the *L. monocytogenes* population (Dussault et al. 2014). Furthermore, the shelf-life of mortadella has been extended with the use of rosemary/thyme EOs (Viuda-Martos et al. 2010[2]), while the shelf-life of bologna sausages was extended using oregano EO (Viuda-Martos et al. 2010[1]). Application of EOs to food products is although still uncommon and mainly limited to cooked meat products (Laranjo et al. 2017).

Several studies declare internal food factors such as: proteins, fats, water, carbohydrates, antioxidants, salt, and food pH have an impact on EOs effect. For example, meat products and their higher fat content caused a reduction in the antibacterial activity of EOs, such as fish products also showed. For fruit, the main indicator was pH. The lower the pH of the fruit, the more effective the effect of EOs (Burt 2004). On the other hand, there are also some potential synergists used in the food industry in combination with EOs, such as various additives: sodium

chloride, sodium nitrate and nisin, or various processing techniques such as: heat treatment, high hydrostatic pressure and anaerobic packaging (Calo et al. 2015).

Usually, the antimicrobial effect of EOs is higher *in vitro* than in food products. Their major advantage when compared to other phytochemicals and natural extracts used as antimicrobials, is their volatility. This advantage is mostly mentioned in active packaging when the meaning is that no direct contact between the packaging material and the food product is required for EOs to exert their antimicrobial activity (Manso et al. 2015). Echegoyen & Nerin (2015) determined the effect of active packaging with cinnamon oil on postharvest deterioration in mushrooms. They found that the active packaging prevented weight loss and browning when compared to nonactive paraffin-based packaging.

Nano- or microencapsulation of EOs could offer possible solutions to solve challenges facing their applications in food. Pan et al. (2014) studied thymol encapsulated in sodium caseinate and found that the encapsulated thymol was more effective than un-encapsulated compound in inhibiting foodborne pathogens in milk, due to the enhanced distribution and solubility. Another possible usage of EOs are food coatings and films. Kafirin and zein are alcohol-soluble prolamin-type proteins that can be used to make biodegradable, environmental-friendly bioplastic films and coatings. Especially kafirin, protein extracted from sorghum, is widely used for bioactive films incorporating plant essential oils (Taylor et al. 2005). For example, kafirin films containing citral can cause strong antimicrobial activity against *C. jejuni, L. monocytogenes* and *Pseudomonas fluorescens*. Peretto et al. (2014) prepared edible films of strawberry puree with carvacrol and methyl cinnamate and used them in clamshells to provide controlled release of vapours without direct contact with the fruit. It was a significant delay in the severity of visible decay. Fruits remained firmer and brighter in colour as compared to untreated strawberries.

Some essential oils against *E. coli* were aims of many of studies. *Allium sativum, Armoracia rusticana, Brassica nigra, Cinnamomum cassia, Cinnamomum verum, Cistus ladaniferus, Coriandrum sativum, Cupressus sempervirens, Cymbopogon citratus, Cymbopogon nardus, Lippia berlandieri, Melaleuca alternifolia, Mentha × piperita, Mentha spicata, Monarda didyma, Monarda fistulosa, Origanum majorana, Origanum vulgare, Pelargonium graveolens, Pinus sylvestris, Piper nigrum, Salvia lavandulifolia, Salvia sclarea, Satureja hortensis, Satureja montana, Syzygium aromaticum, Thymus capitatus, Thymus mastichina, Thymus pulegioides, Thymus serpyllum, Thymus vulgaris, Thymus zygis, Trachyspermum ammi*, and *Zataria multiflora* proved their antibacterial activity against *E. coli*

in vapour phase (Houdkova & Kokoska 2020). These vapours could be used in food industry. For example, films containing halloysite nanotubes loaded with thyme oil showed antimicrobial activity against *E. coli* (Lee et al. 2017).

Anti *B. cereus* agents which may co-operate with food security and may lead to potential usage in food preservation are EOs from plant species such as *A. sativum*, *C. verum*, *Citrus bergamia*, *C. citratus*, *O. vulgare*, *P. graveolens*, *S. aromaticum*, *T. capitatus*, *T. vulgaris*, and *Z. multiflora* (Houdkova & Kokoska 2020). Lin et al. (2017) demonstrated that the cinnamon essential oil/β-cyclodextrin proteoliposomes incorporated into poly(ethylene oxide) nanofibers can significantly extend the shelf life of beef, prevent from contamination of *B. cereus*, and have potential application in active food packaging.

As for *S. Typhimurium* strains, there was explored a great sensitivity to EOs from *A. sativum*, *B. nigra*, *Cinnamomum* sp., *C. cassia*, *C. verum*, *M. didyma*, *O. vulgare*, *S. hortensis*, *S. aromaticum*, *T. vulgaris*, *T. zygis*, *T. ammi*, and *Z. multiflora* plant species (Houdkova & Kokoska 2020). These EOs especially from *T. vulgaris* are potential antimicrobials in food industry against *S. Typhimurium*. For example, Lin et al. (2019[2]) indicated that silk fibroin nanofibers encapsulating thyme EOs treated with cold plasma inhibit *S. Typhimurium* in poultry meat.

Due to sensitiveness to EOs it was found, that *A. sativum*, *A. rusticana*, *C. cassia*, *C. verum*, *C. bergamia*, *Citrus limon*, *C. citratus*, *Cymbopogon martini*, *Ledum groenlandicum*, *Melaleuca quinquenervia*, *Melissa officinalis*, *Mentha* × *piperita*, *M. didyma*, *M. fistulosa*, *O. vulgare*, *S. hortensis*, *S. montana*, *Solidago canadensis*, *S. aromaticum*, *T. pulegioides*, *T. serpyllum*, *T. vulgaris*, *T. zygis*, *T. ammi*, and *Z. multiflora* plant species EOs vapours are proving antibacterial activity against *L. monocytogenes* strains and could be further useful in exploring anti agents in food industry developing new nature technology such as active packaging (Houdkova & Kokoska 2020). For example, gelatin nanofibers including moringa essential oil in cheese packaging (Lin et al. 2019[1]).

2.3. Antibacterial activity in vapour phase

Since EOs belong to the volatile agent group, their vapours have bioactive potential. Their physicochemical feature, volatility, was not fully developed yet in industrial antimicrobial applications. However, there exists a few fumigants that provide greenhouse application, e.g., Eco-Oil (Organic Crop Protectans), Pre-AM (Orgo Agri International), Requiem (Bayer AG),

and inhaler nasal sticks (Vicks). These examples were commercially presented by vapours of plant volatiles. Ingredients include camphor, limonene, menthol, and the EOs of *Abies sibirica*, *Citrus* sp., and *Melaleuca alternifolia* (Isman & Tak 2017; Procter & Gamble 2020).

Before we can practically use the vapours of volatile substances, we need to get detailed information about their efficiency and sapphire. *In vitro* screening is commonly the first step in this process. Disc-diffusion, drop-agar-diffusion, broth microdilution, and direct-contact technique in agar represent the most common methods utilized for screening (Tyagi & Malik 2010). The pitfalls of volatility are within the lost active substance via evaporation during sample handling, experiment preparation, and time, temperature associated with incubation (Kalemba & Kunicka 2003; Rondevaldova et al. 2017). Kind of matrix onto which they are applied (e.g., paper disc, cultivation broth) is one of the reasons at what speed and with what intensity the vapours will evaporate into the atmosphere. For example, it was found that little evaporation was provided when the compound was mixed into the broth (Orchars & van Vuuren 2017).

There is no standardized method for evaluating antimicrobial activity of volatile compounds in the vapour phase resulting in lots of results in different values (e.g., minimum inhibitory dose per colony-forming units (Amat et al. 2017), the percentage inhibition of radial microbial growth (Perumal et al. 2016), various definitions of minimum inhibitory concentration (Nedorostova et al. 2009; Kloucek et al. 2012; Matusiak et al. 2018)), resulting in a complicating comparison of results.

2.4. Cambodian Spices

According to the FDA, Spices are aromatic plant substances in the whole, broken, or ground form, whose significant function in food is seasoning rather than nutrition (FDA 1980). For centuries, spices have been used for food preparation and preservation, as well as for embalming, in areas where plants are native.

Spice is not a botanical category, but it is a culinary term. However, it comes from different parts of herbaceous plants such as roots, rhizomes, bark, leaves, aerial parts, flowers, fruits, or seeds. Herbs represent fresh state, on the other hand, spices are generally dried (Sherman & Billing 1999).

The function of spices is different. We can divide its basic functions into primary and secondary. Flavouring of dishes, which serve to create smells, tastes, consistency belong to the

primary functions. Secondary properties include their preservative, antimicrobial, nutritional and health activity (Raghavan 2006). The introduction of spices through the meals has other beneficial effects such as stimulating saliva excretion, promoting digestion, preventing from cold, flu and reducing nausea and vomiting (Gottardi et al. 2016).

The role of spices has always been associated with valuable commodities, not only with culinary delight, but also worked as a symbol of wealth (Raghavan 2006). The history of the first spice application dates back to the Sumerian empire (3000 BC), which records the use *Foeniculum vulgare*, *Sesamum indicum*, *Coriandrum sativum*, and others (Kopecka 2016). The spice trade had its most important centre in Asia, especially in China, Southeast Asia, and India, that's where it comes from e.g., turmeric, nutmeg, cloves, anaesthema. Another major metropolis in the spice trade was the North African or Mediterranean region, where spices such as cumin, coriander, rosemary came from (Raghavan 2006). In Europe, we became acquainted with exotic raw materials during the period of maritime discoveries in the 15th century. Spices were then used for enhancing the taste of dish, preserving meat and fish, neutralizing odours, and furthermore in cosmetics and perfumes production (Gottardi et al. 2016). Their important role was mainly in the field of medicine. It was used for the prevention or treatment of certain diseases. In its time it has become, thanks to its healing abilities, a special part of religious ceremonies. Certain cultures believed in their miraculous and magical qualities (Raghavan 2006).

As for Cambodia itself, there was a significant historical influence of the French colony, which was reflected in the availability of baguettes, which are very popular in Cambodia. The surrounding countries also had an influence. It was drawn from the recipes of Thailand, Vietnam, but also China. Khmer cuisine, or Cambodian traditional cuisine, is mainly based on rice, freshwater and sea fish due to the summer monsoon season, which brings good conditions for rice growth and the water source of the Mekong River, Lake Tonle Sap for the source of freshwater fish and the seaside area for the source of sea fish.

Cambodians have perfected the art of blending spices into pastes using ingredients including cloves, cinnamon, star anise, nutmeg, cardamom, ginger, and turmeric. They also blend other native ingredients with these spices including galangal, garlic, shallots, lemongrass, cilantro, kaffir lime leaves, and turmeric to make a distinctive and complex spice blend known as kroeung samlar m'chou. Dried bell peppers, sweet peppers, and chilis are also used according to taste and to add colour. The characteristics of a Cambodian cook's kroeung are distinctive and considered a measure of respect in Khmer culture. Kroeung and prohok (the fermented fish

product as an essential ingredient in a wide variety of dishes) blended together become a unique aromatic combination that is the most distinctive signature ingredient in the Khmer kitchen. These and other essential ingredients including tamarind, lemon grass, basil, sugar, and lime (commonly designated in Cambodia as lemon) flavour sauces, soups, stir-fries, and many other foods (LeGrand et al. 2020).

2.4.1. *Citrus hystrix* DC

C. hystrix or Kaffir limet is a tropical plant of the species *Citrus*, family Rutaceae. Its fruits, leaves and peel have a multi-purpose use. Due to its nutritional and functional properties, it interferes with the aromatherapy, food industry, aromatization and preservation, pharmaceutical and cosmetic industries (Suresh et al. 2021). In tropical areas, it is mainly used for culinary purposes as a flavouring agent in Asian cuisine (Ulhaq et al. 2020).

It is a shrub or low-tree plant, 0.15 – 0.9 meters tall, which has numerous branches with barbs. The leaves are alternately built, the blade of the leaf is oblong-ovate to orbicular-ovate, dark green, and glossy in upper parts, which is aromatic after bruised. The flowers are fragrant, yellowish with violet edges, with calyx 4 - lobed, 4 - 5 petals. The fruit is wound, rough on the surface, yellow when ripened. The peel is thick and yellowish green, the pulp is yellowish, sour, and gently bitter. *C. hystrix* is cultivated on a wide scale in Indonesia, Malaysia, the Philippines, Myanmar, Southeast Asia (Vietnam, Thailand, Cambodia), Sri Lanka and in some parts of India. At local markets we come across both fresh and dried leaves, which are added to soups, fish patties, curries, and peels, which both candied and dried, are used as ingredients in curry pastes (Staples & Kristiansen 1999).

By chemical analysis of *C. Hystrix* DC we obtain bioactive molecules such as mainly phenolic compounds, limonoids. flavanones, which are important in preventing diseases and improving an individual's well-being in terms of their antibacterial, antioxidant, antitussive and antileukemic activities (Laohavechvanich et al. 2010; Norkaew et al. 2013). The essential oil obtained from pericarp contains limonene, monoterpene hydrocarbon, and sabinene with antibacterial effects to *S. Typhi* strains (MIC 100 μg/ml), while essential oil from fresh leaves showed compounds such as citronellal, citronellol , and limonene with antibacterial efficiency to *B. cereus* (MIC 25 μg/ml) and *S. Typhi* strains (MIC 50 μg/ml) (Phanthong et al. 2013; Agouillal et al. 2017).

2.4.2. *Curcuma zedoaria* (Christm.) Roscoe

It is a plant belonging to the genus *Curcuma* and the family Zingiberaceae known also as zedoary or white turmeric. Its origin extends to South Asia and Southeast Asia, today it is an extended plant of tropical and subtropical regions (Ullah et al. 2014). The dried rhizome of *C. zedoaria* is used for beverage preparation and as pharmaceutical preparation. It is producing anti-diarrheal, diuretic, antimicrobial, antiviral, and antioxidant effects (Mau et al. 2003). Substances responsible for antimicrobial and antioxidant activity are EOs containing terpenes, alcohols, ketones (Sharifi-Rad et al. 2020). The rhizome of white turmeric is rich in curcuminoids, compounds of yellow-orange colour, that used as dyeing materials for industrial applications, cosmetic products, and culinary spices.

2.4.3. *Limnophila aromatica* (Lam.) Merr.

Limnophila aromatica (Lam.) Merr. or rice-paddy herb, belongs to the genus Scrophulariaceae and its origin is in Southeast Asia. It is a widely used plant species in the system of traditional medicine due to its content of flavonoids, terpenoids, etc. Its biological activity is reflected in the antimicrobial and antioxidant properties (Gorai et al. 2014). In previous study published by Houdkova et al. (2018), *L. aromatica* EOs produced *in vitro* growth-inhibitory effect against *Haemophilus influenzae* (MIC 256 μg/mL) when assayed by broth microdilution volatilization method .

This water perennial is characterized by its branched glabrous, glandural stem. Sessile leaves grow opposite or in whorls of three. Their blade is shaped lanceolate to ovate-lanceolate with toothed margins. Flowers have a corolla that is trumpet shaped and from pale or dark blue, or purple coloured. Fruit rarely produced in cultivation as well as flowers take the form of compressed capsule brown coloration (Staples & Kristiansen 1999).

The expansion extends to the tropical countries of India, Sri Lanka, Southeast Asia, China, Southern and Central Japan, Malaysia, Indonesia, New Guinea, and Northern Australia. It grows in an area that has a good water source such as wet prairies, rice paddies, pools, and ponds etc. Cambodians use it as an ingredient in soups. (Gorai et al. 2014).

2.4.4. *Piper nigrum* 'Kampot'

Kampot pepper is a cultivar of *Piper nigrum* L., belonging to the family Piperaceae, which is grown in the province of Kampot in Cambodia. It is used for strengthening food flavour, but also for food preservation and in the pharmaceutical industry due to its chemical

compounds such as phenolic compounds, vitamin C, and minerals. Piperine, an alkaloid responsible for the pungency of black pepper, is known for its anti-diarrheal, anti-inflammatory, and anti-hypertensive properties (Ahmad et al. 2012; Nwofia et al. 2013). *P. nigrum* showed antibacterial effect against *E. coli* strains with MIC 2.50 µL/mL (Seo et al. 2015).

In Cambodia, we come across four types of Kampot pepper on the local markets: green, black, red, and white pepper. The difference between these products comes from the harvest period and the post harvesting operations. Green pepper is the fresh grains, harvested before maturity but when the fruit kernel is well formed. It is served in fresh (i.e., undried) form or preserved in saline or oil. It is characterized by its delicateness and is prepared most often for grilled octopus. The three other types of pepper are dried. Black pepper is the result of the drying of the grains harvested before maturity (yellow to green colour). It has a strong and at the same time gentle aroma and is used in the preparation of fish. Red pepper is the result of the drying of mature grains, which present a red colour before and after the drying characterized by a strong and fruity aroma and is used from wild meat seasoning to vanilla desserts. Finally, white pepper is the product that is obtained by removing the pericarp of mature grains prior to their drying. White pepper is an important product mainly used in food items where the dark particles are undesirable, such as salad dressings, soups, mayonnaise, light-coloured sauces, etc. The grains have a spherical shape with a diameter around 5 - 7 mm. They shrink during their drying, to reach a diameter around 4 - 5 mm. The mass of a single grain is about 0.1 g (Morm et al. 2020).

Most fresh vegetables and fruit have a short shelf-life because they present a relatively high moisture content, promoting microbial spoilage and the development of detrimental enzymatic reactions. Consequently, these food products cannot be stored for a long period, and drying is one of the traditional techniques used to improve their shelf-life. Traditionally, the drying of Kampot pepper is performed during three to four days, depending on the weather conditions, by spreading the grains on the ground, outdoor, exposed to direct sunlight. Usually, before the drying, the grains are soaked in boiling water for a few minutes. In this paper, this operation is referred to as the pre-treatment. It is thought to play an important role in reducing the drying time, ensuring the microbiological quality, and inactivating the polyphenol oxidases, enzymes causing the browning of the pepper during the drying. Achieving the desired colour of the product plays indeed an important role towards its acceptability by the consumers (Varzakas & Tzia 2015).

3. Aims of the Thesis

The main aim of the thesis was to evaluate *in vitro* growth-inhibitory activity of essential oils and supercritical extracts from plants traditionally used in Cambodia as spices and food condiments against foodborne pathogenic bacteria in vapour and liquid phase using broth microdilution volatilization method.

Specific objectives were:

a) Isolation EOs and CO_2 extracts from *C. hystrix*, *C. zedoaria*, *L. aromatica*, and *P. nigrum* 'Kampot', determination of their yields and characterization of their physical properties (colour, fragrance).
b) Susceptibility determination of *B. cereus*, *E. coli*, *L. monocytogenes*, and *S. Typhimurium* (MIC values) to EOs and CO_2 extracts.

4. Materials and Methods

4.1. Plant material

Four local plant species *C. hystrix, C. zedoaria, L. aromatica*, and *P. nigrum* 'Kampot', were selected in Cambodia as phytochemically less explored representatives of taxa containing essential oils. The plant material was collected from various districts of Cambodia. In case of *P. nigrum* 'Kampot', seeds were collected from La Plantation farm, one of Cambodia's premier *P. nigrum* 'Kampot' plantations, in Kampot province. *L. aromatica* aerial parts were purchased in the local market of Sen Monorom, *C. Hystrix* and *C. zedoaria* were collected from wild populations of plants in the rainforests near the Stung Treng town and Banlung town in Ratanakiri province, respectively. Identification of species was performed by ethnobotany expert Prof Ladislav Kokoska. Detailed description of plant material is summarised in Table 1.

4.2. Preparation of essential oils

EOs were obtained by hydrodistillation of dried crushed plant material in 1 L of distilled water for 3 h using a Clevenger-type apparatus (Merci, Brno, CZ) according to the procedures described in the European pharmacopoeia (2013). The EOs were collected in sealed glass vials and stored at 4 °C. The data on yields of obtained essential oils are shown in Table 1. As for *L. aromatica*, due to complications with hydrodistilled apparatus, stock vial sample of EOs past used in the study Houdkova et al. (2018) were further studied.

4.3. Preparation of SFE

Supercritical extracts from plant species were obtained by fluid extractor Helix SFE System, Basic Model (Applied Separations, Allentown, Pennsylvania, USA). In first step the dried and crushed plant material was weighted and filled into the extraction vessel. Pressure and temperature were adjusted to 200 Bars and 40 °C, respectively. The flow rate of CO_2 was kept at approximately 5 litters per minute (LPM) and it did not exceed 10 LPM. SFE was than collected in 60 ml collection vial and taken out by microliter syringe (100 µl; Hamilton Company, Reno, Nevada, USA) into sealed glass vial and stored at 4 °C. The data of SFE yields are shown in Table 1.

4.4. Moisture determination

Percentage of moisture of 1 g each grinded plant samples were determined using moisture analyzer (SMO 01, Scaltec Instruments, Germany). The assessment was repeated three times and average values were then used for restatement on dry matter content.

4.5. Chemicals

During the research following chemicals were used: dimethyl sulfoxide – DMSO (Penta, Prague, Czech Republic), ethanol 90 % pharmacological grade (Penta, Prague, Czech Republic), distilled water, tetracycline CAS number 60-54-8, chloramphenicol CAS number 56-75-7, thiazolyl blue tetrazolium bromide – MTT (Sigma-Aldrich, Prague, Czech Republic), Mueller-Hinton (MH) agar (Oxoid, Basingstoke, Hampshire, UK), MH broth (Oxoid, Basingstoke, Hampshire, UK), Trizma base (Sigma-Aldrich, Prague, Czech Republic), sodium chloride - NaCl (Sigma-Aldrich, Prague, Czech Republic) and potassium chloride - KCl (Sigma-Aldrich, Prague, Czech Republic).

4.6. Bacteria strains and culture media

The following standard strains of the American Type Culture Collection (ATCC) were used: *B. cereus* ATCC 1177, *E. coli* ATCC 25322, *L. monocytogenes* ATCC 29213 and *S. Typhimurium* ATCC 14028. The cultivation and assay media (broth/agar) for bacteria growth were MuellerHinton (MH). The pH of broths was equilibrated to a final value of 7.6 using Trizma base (Sigma-Aldrich, Prague, CZ). All microbial strains and cultivation media were purchased from Oxoid (Basingstoke, UK).

Stock cultures of bacterial strains were cultivated at 37 °C for 24 h. After that bacterial suspension was adjusted to 0.50 McFarland standard using Densi-La-Meter II (Lachema, Brno, CZ) to get the final concentration of 107 CFU/mL. The susceptibilities of *B. cereus*, *E. coli*, *L. monocytogenes*, and *S. Typhimurium* to tetracycline (88 %, CAS 60-54-8) and chloramphenicol (98 %, CAS56-75-7), respectively, purchased from Sigma-Aldrich (Prague, CZ), were checked as positive antibiotic controls (CLSI 2015).

Table 1. Botanical and physical characteristics of plant-derived products.

Scientific name	Family	Area of collection	Plant part used	Extraction method	Weight of sample (g)	Yield % (v/w)		Colour	
						EOs	CO_2 extracts	EOs	CO_2 extracts
Citrus Hystrix DC	Rutaceae	Stung Treng	Pericarp	HD	53.01	3.89	ND	Colourless	ND
				SFE	40.20	ND	8.35	ND	Pale yellow
			Leaves	HD	12.85	0.65	ND	Colourless	ND
Curcuma zedoaria (Christm.) Roscoe	Zingiberaceae	Banlung	Rhizomes	HD	27.84	2.64	ND	Slightly yellow	ND
Limnophila aromatica (Lam.) Merr.	Plantaginaceae	Sen Monorom	Aerial parts	HD	13.80	1.20	ND	Slightly yellow	ND
Piper nigrum L.	Piperaceae	Kampot	Seeds	HD	54.39	3.01	ND	Colourless	ND
				SFE	46.24	ND	2.20	ND	Bright yellow

Footnotes: ND – not determined, HD – hydrodistillation , SFE – Supercritical fluid extraction

4.7. Antimicrobial assay

The antibacterial potential of plant essential oils in liquid and vapour phase was determined using a broth microdilution volatilisation method (Houdkova et al. 2017). The experiments were performed in standard Nunclon 96-well microtiter plates (well volume = 400 μL), covered by tight-fitting lids with flanges designed to reduce evaporation (Thermo Scientific, Roskilde, DK). In the first part 100 μL of MH buffer was pipetted into every part of microtiter plate except wells for maximum concentration of samples. In the second part each sample of EOs and SFE was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Prague, CZ) at maximum concentration of 1 %, and diluted in 792 μL MH buffer broth medium. Seven two-fold serially diluted concentrations of samples starting from 1024 μg/mL were prepared. The final volume in each well was 100 μL. In the third part, 30 μL of agar was pipetted into every flange on the lid except the outermost flanges and inoculated with 5 μL of bacterial suspension after agar solidification and also the plates were then inoculated with bacterial suspension using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, CZ). The wells containing inoculated and non-inoculated broth were prepared as growth and purity controls simultaneously. The outermost wells were left empty to prevent edge effect. Finally, clamps (Lux Tool, Prague, CZ) were used for fastening the plate and lid together, with the handmade wooden pads (size 8.5 × 13 × 2 mm) for better fixing and the microtiter plates were incubated at 37 °C for 24 h.

The minimum inhibitory concentrations (MICs) were evaluated by eye control of bacterial growth after colouring of a metabolically active bacterial colony with thiazolyl blue tetrazolium bromide dye (MTT) in a concentration of 600 μg/mL (Sigma-Aldrich, Prague, CZ). When the interface of colour change from yellow to purple (relative to that of colours in control wells) was recorded in broth and agar the meaning was about bacterial activity, when the colour stayed yellow (30 minutes minimal) the antibacterial activity was proven and MIC was determined. The MIC values as the lowest concentrations that inhibited bacterial growth were compared with the compound-free control and expressed in μg/mL. 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. All experiments were carried out in triplicate in three independent experiments and results were expressed as median/modal MICs values.

5. Results and Discussion

In this study, five EOs and two CO_2 extracts derived from different parts of four Cambodian medicinal and edible plant species were obtained in yields ranging from 0.65 to 8.35 % (v/w). The highest yields showed supercritical CO_2 extracts (8.35 %) followed by EOs (3.89 %) of *C. hystrix* pericarp. In comparison of volatile oils obtained from *C. hystrix* pericarp, it was found that CO_2 extracts afforded higher yields than hydrodistilled EOs. This result could be supported by study of Piggott et al. (1997), who used steam distillation, solvent extraction, supercritical fluid extraction and liquid CO_2 extraction to obtain the volatile oil from *Santalum spicatum* and found, that SFE afforded the highest yields of extractable material and total volatile. On the other hand, in this study, *P. nigrum* 'Kampot' showed higher yield obtained by hydrodistillation. This result is in correspondence with study of Vilegas et al. (1994), who found that supercritical carbon dioxide extraction provided lower yields than steam distillation. In contrast to colourless or slightly yellow EOs, CO_2 extracts were of yellow colour. Further details of plant material are summarised in Table 1.

As far as antibacterial activity of plant species tested in this study is considered, all of EOs and SFE were not previously tested for their antimicrobial effect on foodborne pathogens both in liquid and vapour phase using broth microdilution volatilization method. In this study, in liquid phase, the lowest MIC value was observed of *C. zedoaria* rhizomes essential oil (1024) μg/mL against *B. cereus* and *L. monocytogenes*. No antibacterial activity in broth medium was determined for *C. hystrix* pericarp, *L. aromatica* aerial parts, and *P. nigrum* 'Kampot' seeds. As well as in broth, *C. zedoaria* rhizomes essential oil was the most effective antibacterial agent against *B. cereus* in vapour phase with MIC = 1024 μg/mL. Other essential oils and supercritical extracts did not show any antibacterial activity in vapour phase. The results are summarized in Table 2.

Although the antibacterial effect of *C. zedoaria* rhizomes it is quite week with MIC 1024 μg/mL, this is the first described MIC in broth and agar medium of *C. zedoaria* rhizomes tested against *B. cereus* and *L. monocytogenes*. In comparison with other studies, no one tested rhizomes of *C. zedoaria* in vapour phase, only few studies examined MIC in vapour phase of plants from Zingiberaceae family, such as *Alpinia oymitra* rhizomes using broth microdilution method brought results of MIC 128 μg/mL against *Haemophilus influenzae* (Houdkova et al. 2018). From another point of view, the antibacterial effect of *C. zedoaria* could be supported by a study of Islam et al. (2017) who observed MIC 128 μg/mL of ethanol extract of *C. zedoaria*

rhizome against *B. cereus* and *E. coli* with MIC 64 μg/mL. These difference between MIC values of EOs could be caused by different extraction method used.

As for *L. aromatica* essential oil, in this study, there was no significant result related to antibacterial activity against foodborne pathogens. But according to Nanasombat & Teckchuen (2009), *L. aromatica* crude methanolic extracts using microbroth dilution test demonstrated susceptibility of *B. cereus* (MIC = 2.6 μg/mL), *L. monocytogenes* (MIC = 20.8 μg/mL), *S. typhimurium* (MIC = 10.4 μg/mL), and as for *E. coli*, related to this study, no susceptibility was indicated. Antimicrobial activity of *L. aromatica* EOs in vapour phase was previously demonstrated against *Haemophilus influenzae* (MIC = 256 μg/mL) by broth microdilution volatilization method (Houdkova et al. 2018). Different results obtained for these bacterial species can be caused by their specific susceptibility to tested EO.

EOs and supercritical CO_2 extracts of *C. hystrix* MIC did not produced any bacterial activity when assayed using broth microdilution volatilization method. However, Phanthong et al. (2013) discovered antibacterial effect of *C. hystrix* DC pericarp EO on *S. Typhi* (MIC = 100 μg/mL) and also fresh leaves EO against to *B. cereus* (MIC = 25 μg/mL) and *S. Typhi* (MIC = 50 μg/mL). using broth microdilution. Although other species belonging to Rutaceae family, such as *Citrus bergamina* and *Citrus limon* exhibited effect on *B. cereus* and *L. monocytogenes* in vapour phase (Fisher & Phillips 2006). (Fancello et al. 2020), *C. hystrix* did not produced antibacterial efficiency in vapour phase in this study.

Similar to *C. hystrix* DC, *P. nigrum* 'Kampot' did not show *in vitro* growth-inhibitory effect against foodborne pathogens. In the study of Seo et al. (2015), *Piper nigrum* L. showed antibacterial effect on *E. coli* strains with MIC 2.50 μL/mL in vapour phase using airtight apparatus disc volatilization method. Other study Karsha & Lakshmi (2010) also determined the MICs of *Piper nigrum* L. against *Bacillus*, *E. coli* and *Salmonella* (250, 62.5, 125 μg/mL MIC, respectively).

Higher resistance of bacteria strains *E.coli* and *S. Typhimurium* to EOs and CO_2 extracts could be explained on their hydrophilic lipopolysaccharides outer cell membrane, that acts as a barrier to EOs and CO_2 extracts and therefore, according to many of studies e.g., Burt (2004) and Pandey at al. (2017) it was confirmed that in comparison with Gram-positive bacteria (*B. cereus* and *L. monocytogenes*), Gram-negatives are slightly more resistant to volatile samples.

Table 2. Antibacterial effects of EOs and CO_2 extracts from Cambodian spice plants.

EOs	Part used	Minimum inhibitory concentrations (MICs)							
		B. cereus		***E. coli***		***L. monocytogenes***		***S. Typhimurium***	
		Broth (µg/mL)	Agar (µg/mL)	Broth (µg/mL)	Agar (µg/mL)	Broth (µg/mL)	Agar (µg/mL)	Broth (µg/mL)	Agar (µg/mL)
C. hystrix	Pericarp	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
C. zedoaria	Rhizomes	**≥1024**	**≥1024**	>1024	>1024	**≥1024**	>1024	>1024	>1024
P. nigrum 'Kampot'	Seeds	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
L. aromatica	Aerial parts	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
CO_2 extracts									
C. hystrix	Pericarp	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
P. nigrum 'Kampot'	Seeds	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024

6. Conclusions

In this study, *in vitro* growth-inhibitory activity of Cambodian spices EOs and supercritical CO_2 extracts have been tested against 4 foodborne pathogenic bacteria, namely *B. cereus*, *E. coli*, *L. monocytogenes*, and *S. typhimurium*, using the broth microdilution volatilization method in liquid and vapour phase. Among 4 essential oils and 2 supercritical CO_2 extracts tested, only *C. zedoaria* rhizomes EO has shown the significant growth-inhibitory effect against *B. cereus* both in liquid and vapour phase with MIC 1024 µg/mL and *L. monocytogenes* in liquid phase with MIC 1024 µg/mL. This study is one of the first providing the information about its significant *in vitro* antibacterial efficiency of EO from *C. zedoaria* against foodborne pathogens. Thus, it could be further studied, and future used in food industry as suitable preservative agent in smart packaging of food.

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Appendices

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Appendix 1: Photographic illustrations of plant samples

Figure 1: *Citrus hystrix* DC (Jay 2009)

Figure 2. *Curcuma zedoaria* (Christm.) Roscoe (Epharmacognosy 2012)

Figure 3. *Limnophila aromatica* (Lam.) Merr. (Fern 2014)

Figure 4. Piper nigrum 'Kampot' (Sullivan 2020)

Appendix 2: Photographic illustrations of plant extraction

Figure 5. Clevenger apparatus (Merci, Brno, Czech Republic) (Tůmová 2021)

Figure 6. Crushed material of *C. hystrix* pericarp (Tůmová 2021)

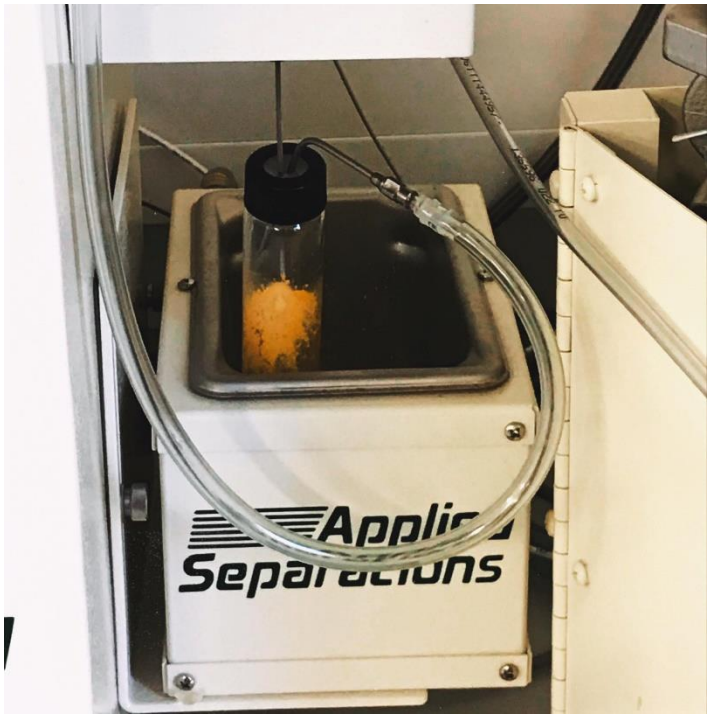

Figure 7. Obtained supercritical CO_2 extract of Kampot pepper collected in 60 ml collection vial (Tůmová 2021)

Figure 8. Plant extracted material (Tůmová 2021)

Appendix 3: Photographic illustrations of antimicrobial assay

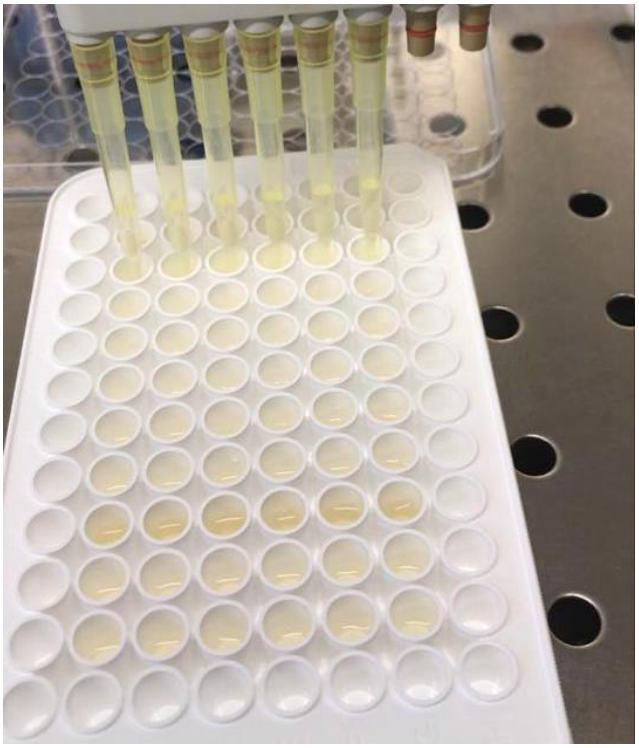

Figure 9. Dilution (Tůmová 2021)

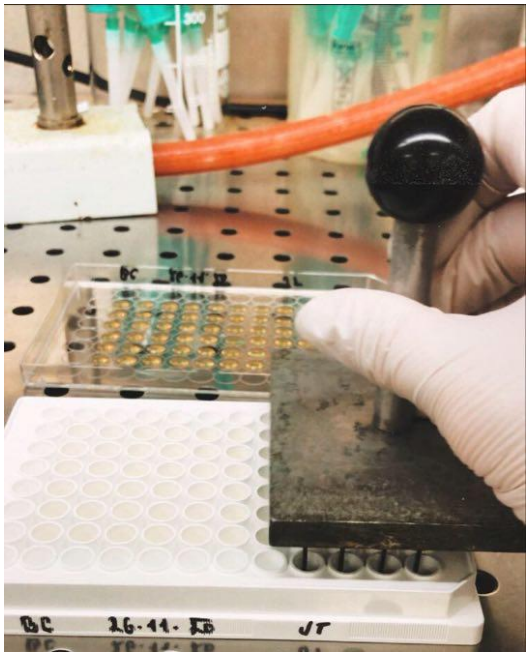

Figure 10. Inoculation of bacterial suspension (plate) (Tůmová 2021)

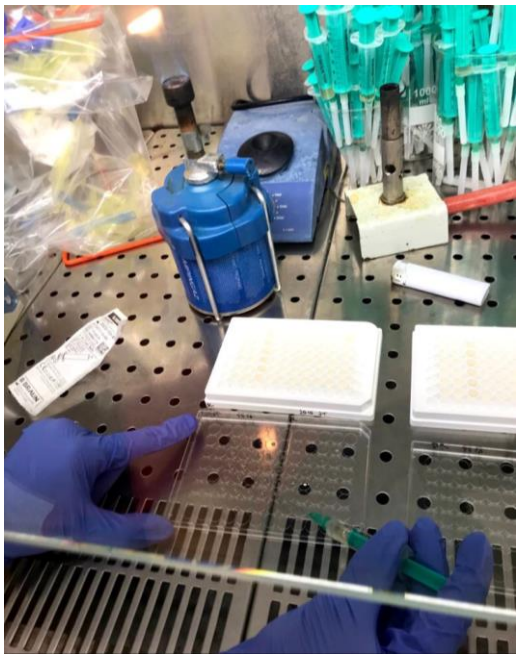

Figure 11. Inoculation of bacterial suspension (lid) (Tůmová 2021)

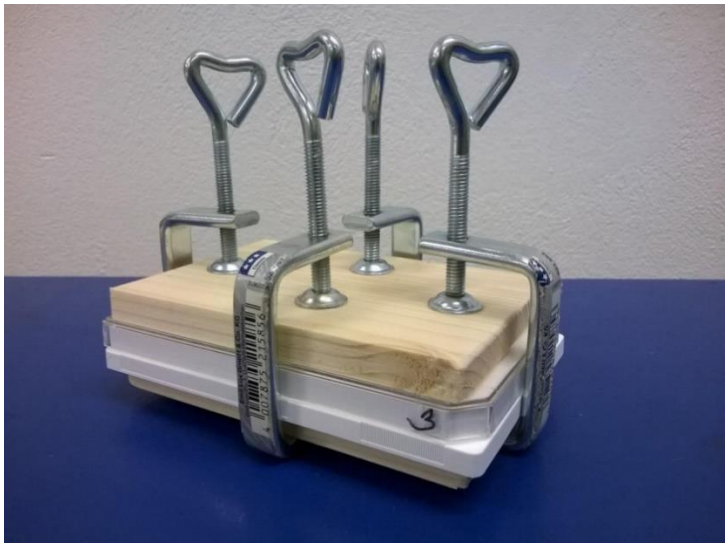

Figure 11. The plates and the lids fastened by handmade wooden pads and clamps to enhance fixation (Houdkova 2017)

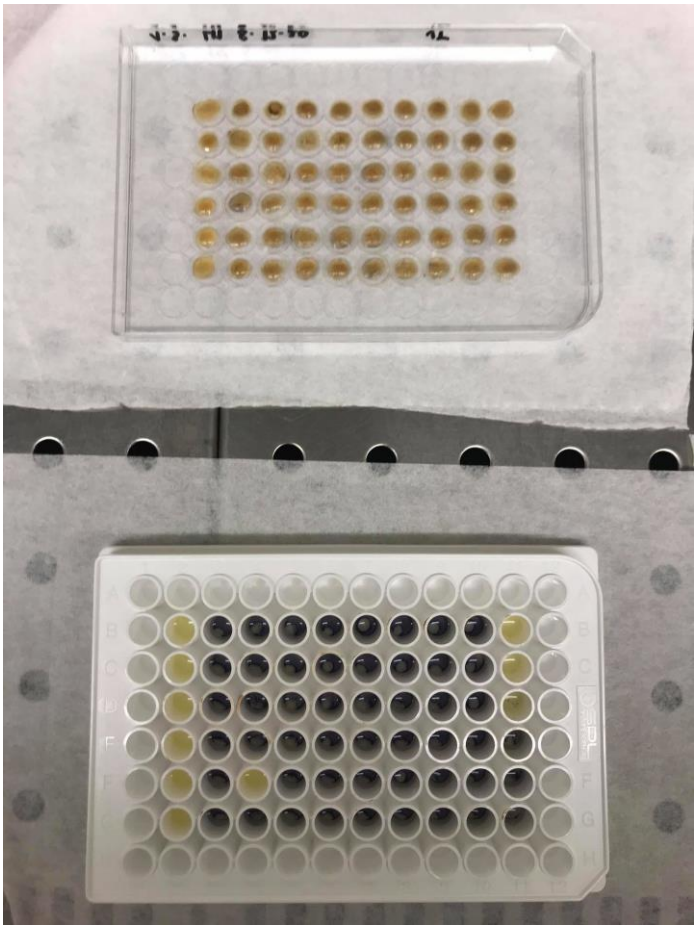

Figure 12. Determination of MICs on the plate and the lid (above). *L. monocytogenes* (Tůmová 2021)