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Department of Crop Sciences and Agroforestry

Chemical composition and antimicrobial activity of essential oils and supercritical carbon dioxide extracts of Asian spices against food pathogens in liquid and vapour phase

DISSERTATION THESIS

Author: Ing. Kateřina Vihanová

Thesis supervisor: prof. Ing. Ladislav Kokoška, Ph.D.

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DECLARATION

I hereby declare that I have completed this thesis entitled “Chemical composition and antimicrobial activity of essential oils and supercritical carbon dioxide extracts of Asian spices against food pathogens in liquid and vapour phase” independently, except for the jointly authored publications that are included. In the case of such publications, my specific contributions have been clearly stated at the start of the relevant publication chapter. Furthermore, I confirm that proper acknowledgement has been provided within this thesis for any references made to the works of others, I also ensure that this work has not been, nor is it currently submitted, for any other degree, to this or any other university. All information sources have been quoted and acknowledged by means of complete references.

In Prague, 15/03/ 2024

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Ing. Kateřina Vihanová

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ABSTRACT

Foodborne microbial diseases present a significant public health concern necessitating the preservation of food products to extend their shelf life and ensure safety. An emerging method of food preservation involves the antimicrobial modified atmosphere packaging. Spices and condiments containing volatile oils hold promise for such applications. In this study, the *in vitro* growth-inhibitory activity of 14 essential oils (EOs) and 17 supercritical carbon dioxide extracts (CO_2 extracts) obtained from spices originating from various parts of Asia was assayed using broth microdilution volatilisation method against four bacterial food pathogens, namely *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* Typhimurium. Among the EOs, and CO_2 extracts tested, 6 EOs (*Armoracia rusticana*, *Cinnamomum cassia* bark and fruits, *Cinnamomum verum*, *Curcuma zedoaria*, and *Syzygium aromaticum*) and 5 extracts (*Alpinia officinarum, C. cassia* bark and fruits, *C. verum* and *S. aromaticum*) exhibited certain degree of antibacterial potential, whereas *Cinnamomum* EOs showed the strongest effect. Their MIC values were ranging from 256 to 1,024 μg/ml in both liquid and vapor phase. EO from fruits of *C. cassia*, known as cassia buds, demonstrated the highest activity against all bacteria tested with MIC values ranging from 256 to 512 μg/ml in both phases. Subsequently, chemical profiles of most potent samples were examined using gas chromatography-mass spectrometry (GC-MS) apparatus equipped with two columns of different polarity (HP-5/DB-HeavyWAX). In all *Cinnamomum* samples, (*E*)-cinnamaldehyde (values ranging from 89.36/88.91% to 49.79/49.19%) was as the most abundant component accompanied by sesquiterpenoids, esters and monoterpenoids. Subsequent time series of headspace sampling by solid phase microextraction and analysis of vapours above the mixture of growth medium and *C. cassia* fruits EO revealed a notable decrease in content of (*E*)-cinnamaldehyde in the headspace. Initially representing 23.11/16.35% of the headspace, the amount of this compound gradually diminished yielding to peaks of δ-cadinene (comprising 11.58/14.43% of the headspace after 12 hrs of incubation) and γ-muurolene (present in 12.74/17.54%). Although EOs and CO_2 extracts isolated from traditional Cambodian spices produced weak or no antibacterial effects, GC-MS analysis of *Amomum kravanh*, *Citrus hystrix* and *Piper nigrum* 'Kampot' showed new information about their phytochemical profiles. Variations in the chemistry of the EOs and CO_2 extracts were evident across all three species. Notably, the most substantial disparity was observed in the *A. kravanh* EO, characterised predominantly by eucalyptol (78.8/72.6%), while the CO_2 extract exhibited richness in fatty acids (13/55.92%) and long-chain alkanes (25.55/9.54%). Furthermore, distinct chemical

compositions were observed in the CO_2 extract of *A. kravanh*, with tricosane (14.74%) and oleic acid (29.26%) identified as the main compounds when utilising the HP-5 or DB-HeavyWAX columns, respectively. Additionally, the EO and CO_2 extract from *P. nigrum* 'Kampot' red peppercorns, along with the CO_2 extract from *C. hystrix* fruit peel, were isolated and analysed for the first time. The EO contained respective amounts of 34.84/39.55% β-caryophyllene and 30.2/28.9% β-pinene, while the CO_2 extract contained 54.21/55.86% β-caryophyllene and 28.9% β-pinene. The findings of the study suggest that EOs and CO_2 extracts derived from *Cinnamomum* spp. hold promise as natural food preservatives in the food industry. However, further experiments addressing organoleptic acceptance and effectiveness in appropriate food models are necessary to verify their potential commercial use. In addition, the results of experiments with *A. kravanh*, *C. hystrix*, and *P. nigrum* suggest the potential application of supercritical CO_2 for their extraction. Nevertheless, further research is necessary to determine the most efficient extraction parameters before considering commercial applications.

Key words: antimicrobial activity, essential oil, GC-MS analysis, spice, supercritical fluid extraction, vapour phase

TABLE OF CONTENTS

ABSTRACT **4**

LIST OF TABLES **8**

LIST OF FIGURES **8**

LIST OF ABBREVIATIONS USED IN THE THESIS **9**

1. INTRODUCTION **1**

2. LITERATURE REVIEW **4**

2.1. Microbial foodborne diseases 4

2.1.1. Food bacterial pathogens 5

2.1.2. Antimicrobial preservation of foods 8

2.2.3. Antimicrobial packaging 10

2.2.4. Antimicrobials in foods 11

2.3. Plant essential oils (EOs) 15

2.3.1. Chemistry 16

2.3.2. Methods of extraction 20

2.3.3. Methods of chemical analysis 24

2.3.4. Antimicrobial effects of EOs 27

2.4. Spices of Asia 33

2.4.1. History 34

2.4.2. Economic importance 35

2.4.3. Botany 36

2.4.4. Cambodian cuisine and spices 38

3. HYPOTHESES **42**

4. RESEARCH QESTIONS **42**

5. OBJECTIVES **43**

6. MATERIALS AND METHODS **44**

6.1. Plant materials and sample preparation 44

6.2. Hydrodistillation of EOs 45

6.3. Supercritical CO_2 extracts preparation 45

6.4. Bacterial strains and culture media 45

6.5. Antimicrobial assay 46

6.6. GC–MS analysis 49

6.7. Identification of constituents, quantification, and statistical analysis 50

6.8. HS-SPME 51

7. RESULTS 52

7.1. Physicochemical characteristics of EOs and CO_2 extracts 52

7.2. Antibacterial effect of spice EOs and supercritical CO_2 extracts in liquid and vapour phase 54

7.3. Chemical composition of EOs, supercritical CO_2 extracts and their vapours of *Cinnamomum* spp. barks and fruits 57

7.4. Chemical composition of EOs and supercritical CO_2 extracts from *Amomum kravanh*, *Citrus hystrix* and *Piper nigrum* 'Kampot' 80

8. DISCUSSION 98

8.1. Physico-chemical characteristics of EOs and CO_2 extracts 98

8.2. Screening of antimicrobial activity of EOs and CO_2 extracts 98

8.3. Chemical composition of EOs and supercritical CO_2 extracts from *Cinnamomum* spp. barks and fruits in liquid and vapour phase 102

8.4. Chemical composition of EOs and supercritical CO_2 extracts from *Amomum kravanh*, *Citrus hystrix* and *Piper nigrum* 'Kampot' 105

9. CONCLUSIONS 108

10. REFERENCES 110

11. APPENDICES I

List of the appendices I

Appendix 1: II

Appendix 2: IV

Appendix 3: V

Appendix 4: VIII

LIST OF TABLES

Table 1: Botanical characteristics and origin of tested spices 44

Table 2: Yields and physical properties of EOs and CO_2 extracts.....53

Table 3: *In vitro* growth inhibitory effect of spice essential oils (EOs) and CO_2 extracts against food pathogens in liquid and vapour phase 55

Table 4: Chemical composition of *C. cassia* bark essential oil and CO_2 extract.....60

Table 5: Chemical composition of *C. cassia* fruits essential oil and CO_2 extract.....64

Table 6: Chemical composition of *C. verum* bark essential oil and CO_2 extract.....68

Table 7: Chemical composition of a headspace above *C. cassia* fruit EO and MH broth at a concentration 256 μg/ml 73

Table 8: Chemical composition of *A. kravanh* EO and CO_2 extract.....82

Table 9: Chemical composition of *C. hystrix* EO and CO_2 extract.....86

Table 10: Chemical composition of *P. nigrum* 'Kampot' EO and CO_2 extract.....91

LIST OF FIGURES

Figure 1 Schematic layout of multiplate design for plate (a) and lid (b) of broth microdilution volatilisation method 48

Figure 2: Clamps and wooden pads for fastening lids and plates together prior to incubation 48

Figure 3: Visual evaluation of living colonies of *B. cereus* with MTT dye on (a) plate and (b) lid.....49

Figure 4: Preparation of samples and HS-SPME sampling of volatile constituents above the lowest MIC obtained in antimicrobial assay.....51

Figure 5: GC-MS chromatogram of *C. cassia* bark CO_2 extract when (a) analysed with HP-5 column and (b) analysed with DB-HeavyWAX column and the main volatile constituents detected 63
Figure 6: GC chromatograms of *C. cassia* fruits EO when analysed (a) with HP-5 column and (b) with DB-HeavyWAX column and main volatile compounds detected 67
Figure 7: GC-MS chromatograms of *C. verum* a) EO and b) CO_2 extract analysed by DB-HeavyWAX column and the main volatile compounds detected 72
Figure 8: GC-MS chromatograms of the headspace above *C. cassia* fruits EO after a) 0 hrs, b) 9 hrs 77
Figure 9: Contents of major components of EO from *C. cassia* fruits in the headspace detected by a) HP-5 column and b) DB-HeavyWAX at 3-hrs intervals 78
Figure 10: Heatmap of11 main constituents of C. cassia fruits EO headspace during overtime analysis 79
Figure 11: GC-MS chromatograms of EOs of a) *A. kravanh*, b) *C. hystrix* and c) *P. nigrum* 'Kampot' (analysed with HP-5 column).....96
Figure 12: GC-MS chromatograms of CO_2 extracts of a) *A. kravanh*, b) *C. hystrix* (analysed on DB-HeavyWAX column) and c) *P. nigrum* 'Kampot' (analysed with HP-5 column). 97
Figure 13: 1: Eugenol aceate and **4:** oleic acid 97

LIST OF ABBREVIATIONS USED IN THE THESIS

ADI	Acceptable daily intake
ATCC	American type culture collection
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
DMAPP	Dimethyl allyl pyrophosphate
DOXP	1-deoxy-D-xylulose 5-phosphate
DWP	Plant dry weight
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EO	Essential oil
EPEC	Enteropathogenic *E. coli*

ETEC	Enterotoxigenic *E. coli*
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FID	Flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GMP	Good Manufacturing Practice
GRAS	Generally Recognised as Safe
HACCP	Hazard Analysis Critical Control Points
HPLC	High performance liquid chromatography
HS-SPME	Headspace analysis using solid-phase microextraction
HUS	Haemolytic uremic syndrome
IPP	Isopentyl pyrophosphate
IS	Internal standard
ISO	International Standards Organization
LAB	Lactic acid bacteria
LP	Lactoperoxidase
MAE	Microwave assisted extraction
MAP	Modified atmosphere packaging
MEP	Methylerythritol 4-phosphate
MH broth	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MTT	Thiazolyl blue tetrazolium bromide
RI	Retention indices
RRF	Relative response factor
RT	Retention time
SFE	Supercritical fluid extraction
SPI	*Salmonella* pathogenicity islands
SPME	Solid-phase micro extraction

STEC	Shiga-like toxin *E. coli*
UAE	Ultrasound assisted extraction
USD	United States dollar
WHO	World Health Organization

1. INTRODUCTION

The term spice refers to dried plants, or their parts, that are used to enhance food flavour and taste rather than to contribute to the nutritional value (Ceylan & Fung, 2004; Peter & Shylaja, 2012). Nowadays, more than 400 spices and condiments are used worldwide, of which among 275 species have their origins in tropical regions of South-East Asia. *Cinnamomum cassia* and *verum*, *Curcuma longa*, *Elettaria cardamomum*, *Myristica fragrans*, *Piper nigrum*, *Syzygium aromaticum*, and *Zingiber officinale* are examples of commodities of global economic importance. In 2021, the total global spice market values accounted for 21.3 billion USD, and it is forecasted to reach 27.4 billion USD by the end of 2026 (Future market insights, 2021). Flavouring dishes to create smell, taste, and consistency is primary function of spices. Besides their seasoning properties, they are also used as natural colorants in the food industry due to the presence of pigments. Moreover, due to the numerous proven beneficial effects attributed to active biochemicals present in spices, they are utilized in aromatherapy, cosmetics, nutraceuticals, perfumes, and pharmaceuticals. Furthermore, introduction of spices into meals stimulates saliva excretion and promote digestion. Spices can be added to foods in various forms, such as whole, ground, or in the form of highly concentrated extracts (Gottardi et al., 2016; Shylaja & Peter, 2007).

In most spices, essential oils (EOs) are the main constituents responsible for their taste and olfactory properties. EOs are usually comprised of many individual constituents (up to 400) with one or two dominant compounds, mostly classified as terpenes and their oxygenated derivatives. Other chemicals present in EOs include phenylpropanoids, which contribute to the aromatic properties of spices. Significant representatives include phenols or phenolic ethers. A few spices also contain molecules with heteroatoms like nitrogen or sulphur in their EOs. From these, allicin from *Allium sativum* or allyl isothyocyanates from *Armoracia. rusticana* can be named (de Groot & Schmidt, 2016; Moghaddam & Mehdizadeh, 2017; Viuda-Martos et al., 2007). Furthermore, alkaloids also contribute to the olfactory characteristics of EOs, especially pungent principle of some spices (Ngo et al., 2018; Rivera-Perez et al., 2021). The extraction of EOs can be carried out using a wide range of techniques; however, distillation (steam, water, and steam-water) remains the most common method applied on an industrial scale (Embuscado, 2015; Westphal et al., 1989). However, the most important shortcomings of distillation are high

consumption of plant material, loss of thermos-sensitive compounds and long extraction times associated with higher energy consumption (Sovilj et al., 2011).

To overcome the above-mentioned drawbacks, various green extraction techniques have recently been developed. Supercritical fluid extraction (SFE) provides multiple advantages associated with the utilization of supercritical fluids as solvents possessing different physicochemical properties. Their lower viscosity and higher diffusivity result in higher extraction rates and an overall faster process. Moreover, their density, which influences the solvent capacity, can be modified by adjusting the extraction parameters (da Silva et al., 2016; Sovilj et al., 2011). Although several solvents can potentially be used during SFE, carbon dioxide (CO_2) is the most used supercritical fluid due to its wide availability at low cost, and non-toxic and non-flammable properties. Moreover, its temperate critical pressure and temperature can ensure the preservation of thermo-labile compounds in the final extract (Barbosa et al., 2014; Fornari et al., 2012). All the above-mentioned characteristics make supercritical CO_2 a highly attractive green solvent, which has led to multiple practical applications in different industries. Hop extract, decaffeinated coffee, nicotine-free tobacco, and specialty oils are a few examples of commercial products utilizing supercritical CO_2 extraction at an industrial level (Montalban & Villora, 2022; Uwineza & Waskiewicz, 2020). Extensive research has considered the possible alternative uses of SFE in the extraction of bioactive components from spices during recent decades and has led to the availability of a wide variety of products on the market, including CO_2 extracts from *Cinnamomum verum*, *Piper nigrum*, and *Zingiber officinale* (Fornari et al., 2012). Regarding the differences in chemical composition of EOs and supercritical CO_2 extracts from spices and aromatic plants, a plethora of studies have been conducted. Most commonly, results indicated significant differences in the quantities of individual compounds identified. EOs obtained through distillation contained higher amounts of low-molecular-weight components, like monoterpenoids and phenylpropanoids, while CO_2 extracts were richer in constituents of higher molecular weight, like sesquiterpenoids, diterpenoids, and fatty acid derivatives (Danh et al., 2013; Marongiu et al., 2004; Maxia et al., 2012; Mesomo et al., 2013; Pereira & Meireles, 2010).

Despite significant advancements in food production processes and hygiene, food contamination caused by bacteria still constitute a worldwide threat, affecting millions of people annually. Consumption of contaminated food or water may result in severe to fatal outcomes, especially in immunodeficient individuals like children, elderly, and pregnant women (WHO, 2020). Bacterial pathogens can contaminate food product in different stages of

production, transport, storage, and final preparation. In addition, some bacteria can also produce toxins, from which many of them are thermostable and cannot be neutralised by conventional methods of preparation such as drying, freezing, and cooking (Martinovic et al., 2016; van Seventer & Hamer, 2016). Common foodborne pathogens such as *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes,* and *Salmonella enterica* Typhimurium are responsible for a significant number of outbreaks and cases of food poisoning (Oussalah et al., 2007; Stenfors Arnesen et al., 2008).

Due to the increasing negative consumers perception of artificial food preservatives, the exploration of naturally occurring compounds as antimicrobial agents in food preservation has emerged as a promising approach to extend the shelf life and safety of processed food products (Burt, 2004; Oussalah et al., 2007; Smith-Palmer et al., 1998). Over the last century, various techniques have been developed in the food preservation industry, including modified atmosphere packaging (MAP). MAP enables easily perishable food products to be sealed in packaging systems while fresh, maintaining their nutritional value, desirable flavour, and appearance without compromising organoleptic properties. However, MAP used alone may foster bacterial growth, rendering the technique insufficient for ensuring food shelf life and safety. Combining MAP with natural antimicrobial agents such as EOs and plant extracts presents a viable option for enhancing the effectiveness of this method (Mastromatteo et al., 2010).

Antimicrobial effects of EOs and their potential application in food preservation has significantly been investigated and reviewed (Burt, 2004; Calo et al., 2015). A significant challenge remains, as EOs often achieve better results in *in vitro* conditions, than in actual food matrices. Achieving equivalent result in foods would require much higher concentrations. Moreover, the direct application of EOs can compromise the organoleptic properties of certain food products and pose potential health risks to susceptible individuals (Burt, 2004; Tongnuanchan & Benjakul, 2014). Therefore, application of EO vapours emerged as a promising approach (Laird & Phillips, 2012; Tyagi et al., 2012) in food preservation, especially in conjunction with MAP (Mastromatteo et al., 2010). However, assessing the potential antimicrobial effects of EOs in the vapour phase presents challenges. Unlike the well-established standardized methods for testing direct-contact antibacterial effects, no standardized techniques currently exist for evaluating vapour-phase antimicrobial activity. Consequently, comparing findings across the literature often requires recalculations of the reported inhibitory concentration values (Becerril et al., 2007; Houdkova & Kokoska, 2020; Kloucek et al., 2012).

With aim to evaluate in vitro potential of vapours of EOs and CO_2 extracts obtained from Asian spices to inhibit growth of food pathogenic bacteria, we assessed their antibacterial effects using broth microdilution volatilisation method in both liquid and vapour phase. Furthermore, we performed gas chromatography-mass spectrometry (GC-MS) analysis of chemical profiles of EOs and CO_2 extracts of the most effective and phytochemically less investigated spices.

2. LITERATURE REVIEW

2.1. Microbial foodborne diseases

Despite the current improvement in food production processes and hygiene, foodborne diseases still occur in unacceptable high frequencies. It has been estimated that 30% of the population in developed countries suffer from foodborne diseases every year and up to 2 million deaths are estimated per year in developing countries (Abebe et al., 2020; Burt, 2004). These diseases can be caused by various biological infectious agents (bacteria, fungi, parasites, and viruses), while bacteria belong to the most common food pathogens. Foodborne infectious bacteria exist in various shapes, types, and properties and some of them are also able to produce thermostable toxins (e.g., *Clostridium perfringens*, *B. cereus*) or spores (e.g., *C. botulinum*, *Staphylococcus aureus*) (Bintsis, 2017; Burt, 2004; Martinovic et al., 2016; van Seventer & Hamer, 2016).

Bacteria, viruses, fungi, and parasites can contaminate food in different stages of its production, storage, and delivery and some of them are also able to produce toxins. Intoxication, toxic and food infections are main types of food contamination. Intoxication is described as production of toxins after ingestion of harmful microorganisms in food. Although the microorganism excreting toxins into the food product might be killed, the associated food poisoning is caused by the toxins present in the product. Food infection is defined as disease caused by eating of food containing live pathogenic bacteria. Once the contaminated food is consumed, bacterial cells continue to grow, resulting in food poisoning symptoms. Food poisoning usually manifests itself with acute gastroenteritis like vomiting, diarrhoea, dehydration, and headaches. Usually, these infections can be cured within days, however, in some cases of immunodeficient individuals, they can become fatal (Martinovic et al., 2016; Serag et al., 2022).

Among the foodborne bacterial pathogens, some are particularly important in terms of frequency and severity of the caused disease. Although 31 bacteria have been identified as causative agents of food poisoning, *S. aureus*, *Salmonella* species, *Campylobacter* species, *L. monocytogenes* and *E. coli* are the leading cause of foodborne diseases and related deaths worldwide. Furthermore, it has been reported that Gram-negative bacterial strains were

responsible for 69% of bacterial foodborne diseases (Abebe et al., 2020). In frame of this thesis, *B. cereus*, *E. coli*, *L. monocytogenes,* and *S. enterica* Typhimurium bacterial strains will be described in more detail in next chapters with rough subdivision of general description followed by epidemiology and disease symptoms.

2.1.1. Food bacterial pathogens

B. cereus is a Gram-positive, motile, and spore-forming rod belonging to a family *Bacillaceae*. Most of the *Bacillus* spp. are ubiquitous in the environment and can be found in soils, sediments, fresh and marine waters, dust, and plants. Spores formed by this species are equipped with appendages and/or pilli and therefore are more adhesive and hydrophobic than spores produced by other representatives of the family. These spore properties make *B. cereus* spores very resistant to conventional methods of hygiene and sanitation. The vegetative cells growth temperature ranges from 4 to 55 °C with preferred temperature 30-40 °C. The pH for growth is ranging from 4.9 to 9.3, but presence of this bacterium has also been reported on meat at pH 4.35 (Bintsis, 2017). *B. cereus* is associated with two different food-poisoning syndromes, both caused by different toxins. These two syndromes are usually mild and self-limiting, however more severe, and lethal cases have also been reported (Stenfors Arnesen et al., 2008). The diarrheal syndrome, resembling the *C. perfringens* food poisoning, is caused by protein thermo-labile enterotoxin pre-formed already in the consumed food product or produced in the small intestine. Patients experience watery diarrhoea, abdominal cramps, and pain, rarely accompanied by vomiting and elevated temperature usually 6 to 16 hrs after ingestion of contaminated food products and symptoms generally dissipate after 12 to 24 hrs. This syndrome is associated with various food products; however, protein rich products like meat-based dishes are the most common (Bintsis, 2017; Drobniewski, 1993). The emetic syndrome is caused by thermostable emetic toxin cereulide (known also as vomiting factor) produced by bacteria during the exponential growth phase in the food product. This syndrome is characterised by short incubation period from 1 to 5 hrs after ingestion of contaminated food and accompanied with nausea and vomiting that persist for 6 to 24 hrs. Food products linked to this poisoning syndrome are rice-based dishes, pasta, noodles, and pastries (Bintsis, 2017; Stenfors Arnesen et al., 2008).

E. coli is a Gram-negative, non-spore forming rod belonging to the family *Enterobacteriaceae*. This bacterium might or may not be mobile and some of them might also be flagellated. *E. coli*

is a facultative anaerobe and grows at pH values ranging from 4.3 to 10 with optimum values ranging from 6 to 8. It belongs to one of the most prevalent commensals present in the gastrointestinal tract of humans and warm-blooded animals and mostly lives in mutually beneficial association with its host. Most of the strains are therefore harmless, however, some of them possess the ability to produce toxins. Strains with such ability are responsible for a broad spectrum of diseases of gastrointestinal, urinary, or central nervous system. Due to outbreaks occurring worldwide are considered major concern to public health. Moreover, their infection dose is low and can be transmitted *via* faecal-oral route through food and water. Transmission usually occurs in the case of consumption of food or water contaminated with faeces and cross contamination of animal products mostly takes place during slaughter and further processing. Based on the mechanism of their pathogenicity, strains have been divided into at least six major groups as follows: Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC, or STEC named after shiga-like toxin production resembling those produced by *Shigella dysenteriae*), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC) and attaching and effacing *E. coli* (A/EEC), from which EHEC, EPEC and ETEC are responsible for severe outbreaks worldwide (Allocati et al., 2013; Bintsis, 2017; Nataro & Kaper, 1998). Current research is predominantly focused on EHEC (STEC) strains of *E. coli* for its high virulence and continuous connection with severe food borne diseases contracted from contaminated fruits, vegetables, meats, and dairy products. Typical poisoning manifests itself as mild to severe diarrhoea, which might develop into Haemolytic Uremic Syndrome (HUS) in susceptible individuals (5 to 10%). HUS symptom is a protrusive bleeding which might lead to kidney failure and death. One of the EHEC *E. coli* representatives is strain O157:H7, which has been associated with many outbreaks in the past and hence, its presence needs to be reported to health authorities by food producers (Allocati et al., 2013; Bintsis, 2017). ETEC strains are the most common pathogens associated with traveller's diarrhoea in humans of all age groups manifesting as mild to severe watery diarrhoea, while EAggEC strains belong to the second most common origin. EAggEC *E. coli* manifests itself as persistent diarrhoea in humans and is often present in the intestine of asymptomatic individuals. These strains have also been recognized as a cause of multiple outbreaks worldwide. EIEC strains are closely related to *Shigella* spp. and have been investigated for possible linkage to Crohn's disease. EPEC strains are linked to diarrhoea in children mostly under poorer hygiene conditions and in animals (Nataro & Kaper, 1998).

L. monocytogenes is a Gram-positive, non-spore forming flagellated rod belonging to the family *Corynebacteriaceae*. The bacterium can be found in environments such as decaying vegetables, sewage, soil, and water and can survive in extreme temperatures (1-50°C, with optimal growth values 30-37 °C) and salinity up to 10%. Therefore, unlike many foodborne pathogens, can multiply in cold environments such as refrigerators and can only be killed by pasteurization and cooking. Therefore, ready-to-eat products or fresh produce represents the highest risk of *L. monocytogenes* contamination. It has 13 serotypes, namely 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7, whereas 1/2a, 1/2b, and 4b are linked to most food related infections. The organism can be found in dairy products (cheeses, curd, yoghurts, cultured buttermilks, and other fluid dairy products), meats (salami and ground beef/pork, ready to eat poultry products, fresh turkey parts and fish products), vegetables (pre-packed mixed salads and fresh produce, lettuce, and radishes), and potatoes (Bintsis, 2017; Farber & Peterkin, 1991). Infectious disease caused by *L. monocytogenes* is called listeriosis and although it's relatively sporadic incidence, the higher mortality rate (20-30%) marks it as one of the deadliest food-related diseases. This disease can occur in two forms, invasive and non-invasive listeriosis. The non-invasive form is mild and affects mainly adults, otherwise healthy individuals. It manifests itself as diarrhoea accompanied with fever and muscle pain with a short incubation period (usually a few days). Invasive listeriosis is more severe form of the disease and affects higher risk groups of the population include pregnant women, immunodeficient patients (undergoing treatment of AIDS, cancer, or organ transplants), elderly and children. Symptoms of this disease include fever, muscle pain, septicaemia, and meningitis and in case of pregnant women can result in miscarriage of a foetus or premature delivery. If left untreated, maternal listeriosis dissolves rapidly after delivery, however, the neonate remains critically ill (Schlech & Acheson, 2000; WHO, 2018a).

S. enterica **Typhimurium** is a Gram-negative, motile, flagellated, aerobic to facultative anaerobic, non-spore forming rod. According to current nomenclature, this bacterium is classified as a serotype (among approximately 2500 identified to date) within a species *S. enterica* subsp. *enterica* and belongs to the family *Enterobacteriaceae.* In general, *Salmonella* is a ubiquitous, hardy organism that can survive weeks in dry environments and months in

water. It can be found in the gastrointestinal tract of several domestic animals, ruminants, birds, reptiles, and insects. Some serotypes are host-specific and while transmitted to humans, cause invasive and life-threatening disease. However, most serotypes, including *S. enterica* Typhimurium can inhabit gastrointestinal tract of various hosts and typically cause gastroenteritis in humans. Together with serotype *S. enterica* Enteridis, this organism comprises two of the most important zoonotic serotypes of *Salmonella* (transmitted from animals to humans) (Bhunia, 2018; Crump & Wain, 2017; WHO, 2018b). The ability of *Salmonella* to cause infections is chromosomally encoded. The regions responsible for the virulence factors are called *Salmonella* pathogenicity islands (SPIs) and 14 of these have been identified (SPI 1-14). Not all serotypes contain all the islands and differential pathogenicity in various hosts can be related to presence or absence of a certain island. The most important are SPIs 1 and 2, associated with a systemic spread of a bacteria in the host organism (Bintsis, 2017; Crump & Wain, 2017). The disease is called salmonellosis and is usually linked to food products of animal origin, mainly eggs, poultry, other meats, and milk, but green vegetables contaminated by manure can also be associated with its transmission. Moreover, transmission from pets to humans can also occur, while these domestic animals might not show symptoms of the disease. Nontyphoidal salmonellosis manifests itself as with rapid onset of fever, abdominal pain and diarrhoea accompanied by nausea and sometimes vomiting. The incubation period varies from 6 to 72 hrs and symptoms are usually mild with no need for specific treatment. However, in vulnerable, immunodeficient individuals (infants, elderly) the associated dehydration might become severe (WHO, 2018b).

2.1.2. Antimicrobial preservation of foods

Food preservation involves action taken to maintain food products microbiologically safe and with preserved quality for as long as possible. It has been practiced for centuries with salting officially recognized as the earliest form of preservation, alongside with sugar use to preserve fruits. Together with salting and drying, pungent spices could have also been used with the aim to mask unpleasant odours of easily perishable foods products, such as meat. Though the exact origins of spice use as preservatives remain somewhat elusive, families employing spices were notably less susceptible to foodborne illnesses and symptoms of poisoning. Furthermore, they were able to store food for extended periods without spoilage, thus enduring through times of

food scarcity. The adoption of spice-based preservation practices by neighbouring families likely spread rapidly throughout communities through observation and imitation of culinary habits. Consequently, the antimicrobial properties inherent in spices have played a pivotal role in both food preservation and traditional medicinal systems. In modern times, food preservation requires a nuanced approach, considering complex factors. Today, food preservation methods can be broadly categorized into three main approaches (Gould, 1995; Sherman & Billing, 1999).:

- Procedures that prevent the access of microorganisms into the food product or avoiding recontamination during processing. Examples include packaging, aseptic packaging of heated products, hygienic storage, Hazard Analysis Critical Control Points (HACCP)-a systematic preventive approach during which critical control points in the production steps are identified and corrective actions established, and Good Manufacturing Practice (GMP) -actions applied to production of food, drugs, and medical equipment production. GMP is a prerequisite of HACCP programs implementation, as HACCP addresses specific critical control points, where the hazard of microbial contamination is higher. The critical limits, their measurement, corrective actions, and their validation is subsequently established (de Oliveira et al., 2016; Gould, 1995).
- Procedures that directly target and inactivate undesired microorganisms in food products. Pasteurization and sterilization, enzyme addition, ohmic heat, ionizing radiation, cooking, and blanching can serve as examples.
- Procedures that prevent or slow down growth of undesired microorganisms and deterioration by controlling the environment. Examples involve lower temperature storage like chilling and freezing, fermentation, reduction of water activity, addition of food preservatives, vacuum and modified atmosphere packaging and hurdle technology. Hurdle technology is based on combination of several factors (hurdles), that microorganism should not overcome (Gould, 1995; Leistner, 1994).

Nowadays, numbers of new techniques of food preservation are being developed to satisfy consumer demands in nutritional and sensory aspects, safety, convenience, absence of artificial preservatives and environmental safety. Therefore, food preservation starts with a complete analysis of the whole food chain (including growing, harvesting, processing, packaging, and distribution) and integrated approach needs to be applied with aim to identify desired food characteristics that need to be preserved. Moreover, causes of possible deterioration of food

product also need to be addressed, because food deterioration can facilitate potential microbial contamination and growth in food. Food deterioration, whether chemical or biological, often stems from mishandling during production, storage, and distribution. To counteract chemical deterioration, methods like antioxidants, anti-browning compounds, and anti-stalling agents are employed (Davidson, 2005; Gould, 1995; Rahman, 2007). In frame of this thesis, an emphasis is given to antimicrobial packaging and antimicrobials of natural origin and their possible combination. Therefore, these methods of preservation will be described in more details in subsequent chapters.

2.2.3. Antimicrobial packaging

Food distribution has undergone two major changes over the last century, canning and freezing, which gave consumers the availability of most food products. However, the shift towards green consumerism and the need for healthy and fresh food have created the need for a technology that allows distribution of fresh produce all year long. One technique fulfilling the criteria is modified atmosphere packaging (MAP). This packaging concept improves the quality of fresh produce and easily perishable food products (fresh fruits and vegetables and minimally processed meat and fish products) and allows them to be packed when they are fresh and maintain them in that condition. MAP has been used as a preservation technique mainly to extend shelf-life of fresh fruits and vegetables, because they respire after the harvest and produce CO_2. During these biological processes, deterioration happens and in adverse climatic conditions can get even accelerated. The use of MAP can slow this process down by changing the atmosphere around the product (increasing CO_2 and decreasing O_2). The MAP system can either be passive (only sealed in polymeric packaging hermetically, where gas permeability influences the headspace composition around the product), or active (intentionally introducing a gas mixture into the packaging). The use of MAP therefore results in decreased physiological and biochemical processes of ripening, senescence and ethylene production and maintains quality, freshness, and microbial safety of fresh produce and ready to eat products (Jiang et al., 2022; Mastromatteo et al., 2010; Tajkarimi et al., 2010; Van Haute et al., 2016).

N_2, O_2, CO_2, and water vapor are the most employed gases in the MAP, used individually or in combination. Each of them has unique properties and their use needs to be carefully evaluated for specific food product types, which can be a challenging task requiring expensive technologies. Furthermore, MAP might lead to physicochemical processes that enhance growth

of certain pathogenic bacteria due to increased water accumulation in enclosed environment. Therefore, this technique is used complementary to cooled storage temperatures and extensive research is being conducted to add another hurdle to these two preservations techniques. Moreover, there is the above-mentioned tension in consumer view on artificial and "dangerous" chemical preservatives (Calo et al., 2015; Mastromatteo et al., 2010). Therefore, the most studied option is adding of naturally occurring compounds with well-established antimicrobial effect, including plant EOs, organic acids, enzymes, and bacteriocins (Jiang et al., 2022; Mastromatteo et al., 2010; Tajkarimi et al., 2010; Van Haute et al., 2016).

2.2.4. Antimicrobials in foods

Antimicrobials can be defined as compounds added or present in foods, food packaging, food contact surfaces or food processing environment that prevents or inhibits growth of pathogenic and spoilage microorganisms. Based on their origin, antimicrobials can be divided into traditional (or synthetic, chemical) and naturally occurring (microbial, plant and animal sources). The first group of antimicrobials is approved by many international regulatory agencies, while the second group is mostly only suggested to be used in foods (Davidson, 2005). According to the US Food and Drug Administration (FDA) antimicrobial agents can be classified into three categories, namely processing aids, secondary direct food additives and direct food additives. To be classified as a processing aid, the additive should be added to food during processing and either removed or converted into regular food constituent. Secondary direct food additives are added to the food during manufacture for functionality but removed from the final product. Both categories do not provide technical effects and thus no labelling is necessary. Agents considered direct food additives provide technical effect and therefore should be listed on the food label. The use of an antimicrobial depends on several factors such as antimicrobial spectrum, legal limits of use and effect on food (Crozier-Dodson et al., 2005). In the European Union (EU), use of food additives is strictly regulated, and additive must be authorized before its use in foods. Prior to the authorization, additives and their safety are vigorously assessed by European Food Safety Authority (EFSA). As part of the safety assessment, EFSA seeks to establish acceptable daily intake (ADI), which is defined as the amount of a substance that people can consume daily during their whole life without any appreciable health risk. ADIs are usually expressed in mg per kg of body weight per day (mg/kg bw/day). Based on the safety assessment provided by EFSA, European Commission then decides whether to approve a new additive. All authorised additives are identified by an E number, which consists of an E followed by 3 or 4 digits and must be mentioned on the food

product label in each member state. EU approved additive can be classified into four major categories, namely food protection (antimicrobials and antioxidants), dyes, sugar substitutions (intense sweeteners) and structure and technology (thickeners, gelling agents, and stabilizers) (Lagana et al., 2017).

With the aim to subdivide large and heterogenous group of food antimicrobials, division into functional categories based on an overview provided by Crozier-Dodson et al. (2005) will be used. Functional groups will be discussed in the following order: Acid antimicrobials, chemical antimicrobials, lacto-antimicrobials, ovo-antimicrobials, bacto-antimicrobials, and plant secondary metabolites (phyto-antimicrobials).

Acid antimicrobials include various organic acids and their salts, which have a long history of use as food preservatives. This category primarily includes saturated straight chain monocarboxylic acids and their derivatives (unsaturated, hydroxylic, phenolic, polycarboxylic and salts) (Ricke, 2003). In addition to their antimicrobial effect, these acids can also enhance or contribute to the flavour of fermented foods like sausages, cheeses, pickles, and sauerkraut. The efficacy of acid antimicrobial depends on its dissociation constant (pKa), or pH at which 50% of total acid is dissociated. Because the undissociated form is believed to be an active form, antimicrobial effect of acid preservative is improved when accompanied by lower pH. Furthermore, acid antimicrobials have also exhibited synergism with other compounds like antioxidants (Crozier-Dodson et al., 2005; Ricke, 2003).

The most common representatives of this functional group include one of the most common preservatives like benzoic acid (E 210), naturally occurring in *Styrax benzoin* (Gum Benjamin tree) bark and various berries. Together with its salt sodium benzoate (E 211) are commonly employed in fruit juices and soft drinks with naturally acidic pH range. Both benzoic acid and sodium benzoate are also used in edible coatings, however, their toxicological properties have led to recent efforts to replace these compounds with other preservatives (Davidson, 2005; Qi et al., 2009). Other extensively used preservatives in multiple industries are sorbic acid (E 200), first isolated from unripe *Sorbus acuparia* (rowanberry) oil and its salt potassium sorbate (E 202). Their cross-disciplinary use is based on the proven ability to inhibit numerous microorganisms, mostly yeasts and moulds, but also bacteria (Luck, 1990). Lactic acid (E 270) has been extensively used for its sensory qualities in the past, but recently this acid is utilized in the meat industry as a rinse for beef, pork, and chicken carcasses. Its antimicrobial capacity is associated with reduced growth of *L. monocytogenes* and *S.e. Typhimurium* (Davidson, 2005).

Inorganic chemical antimicrobials include traditionally used sulphur dioxide and sulphites, that to some extents are naturally occurring in wines. Sulphur dioxide (E 220) and sulphites are used traditionally in winemaking to control malolactic fermentation. They are also added to dried fruits and vegetables, vinegars, malt and beer beverages, fruit juices and sports drinks (Davidson, 2005; Mani-Lopez et al., 2016). Other compounds with extensive use in meat industry are nitrites (NO_2^-) and nitrates (NO_3^-) with the main aim to inhibit growth of *Clostridium botulinum*, causing life-threatening botulism. However, their effective preservation characteristics are limited by their possible adverse health effects, causing life threatening methemoglobinemia or formation of carcinogenic nitrosamines. Therefore, ADIs have been established (EFSA, 2017a; Mani-Lopez et al., 2016).

Lacto-antimicrobials is a category of naturally occurring preservatives in milk. Milk is the first functional food devised by nature to develop the immune system of a newborn mammal and therefore contains a plethora of anti-microbial substances. The isolated antimicrobials are usually broad-spectrum agents protecting the neonate against bacteria, fungi, viruses, and parasites. Moreover, protein like structure of these milk or hyper immunized colostrum constituents provides an additional nutraceutical benefit (Davidson, 2005; Naidu, 2000).

Representatives of this functional group include lactoperoxidase (LP) and lactoferrin, both naturally occurring in milk, tears, saliva. LP is the most abundant enzyme in bovine milk and catalyses the oxidation of thiocyanate, a compound in milk, yielding bacteriostatic and even bactericidal products with such effects on various bacteria, including *E. coli* and *Pseudomona*s spp. Industrial applications of LP as a bio preservative include raw milk preservation and oral care products and a potential use in ground beef (Davidson, 2005; FAO/WHO, 2006). Lactoferrin, an iron-binding glycoprotein, exhibits bacteriostatic effects on Gram-negative bacteria and inhibits the growth of pathogens like *S. aureus*, *L. monocytogenes*, and *Bacillus* spp. Its current uses include dietary iron supplements, infant formulas, chewing gums, fermented milk, and other milk products, with ongoing research indicating its promising role as a bio preservative in food (Naidu, 2000; Niaz et al., 2019).

Ovo-antimicrobials are antimicrobials of animal origin found in hen eggs. Opposed to a mammal immune system that produces proteins when needed, the main function of ovo-antimicrobials is to keep undesired microorganisms away from the yolk, which provides nutrients for the developing embryo (Naidu, 2000).

Lysozyme, a bacteriolytic enzyme occurring in animal tissues, organs, serum, tears, and saliva, represents this functional group. Commercially, lysozyme (E 1105) is isolated from hens' egg white and demonstrates antimicrobial activity against vegetative cells of Gram-positive bacteria but is ineffective against Gram-negative bacteria and dormant spores. In the food industry, lysozyme is utilized in the ripening process of hard and semi-hard cheeses (such as Edam, Gouda, Provolone, and Emmentaler) to prevent late blowing caused by the contaminant *Clostridium tyrobutyricum*. Additionally, several Japanese patents explore lysozyme as a preservative in various food products, including fresh vegetables, meat, seafood products, mustards, and beverages like sake and infant milk formulas (Gaare et al., 2013; Mani-Lopez et al., 2016).

Bacto-antimicrobials, originating from microbial sources, manifest as protective fermentative cultures or bacteriocins, small peptides synthesized by bacteria to outcompete closely related microbial species in polymicrobial environments. The majority of bacteriocins are produced by Lactic Acid Bacteria (LAB), some of which exhibit probiotic properties recognized as safe (GRAS). Bacteriocins, easily degraded by proteolytic enzymes in the gastrointestinal tract, pose no harm to humans (Negash & Tsehai, 2020; Simons et al., 2020).

Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis via* fermentation, represents this group. This polypeptide bacteriocin is particularly effective against Gram-positive bacteria (including spores) associated with food poisoning including *B. cereus*, *L. monocytogenes,* and *Clostridium* spp. Nisin (E 234) is currently approved by EFSA as a preservative added to processed cheeses, pasteurized dairy products, canned vegetables, pasteurized liquid egg products and semolina and tapioca puddings (Delves-Broughton et al., 1996; EFSA, 2017b; Shin et al., 2016). While no other bacteriocin has attained approval as a food preservative, ongoing research explores the potential utilization of pediocin from several *Pediococcus* spp., reuterin from *Lactobacillus reuteri*, and sakacin from *Lactobacillus sakei*, with pediocin and sakacin showing efficacy against *L. monocytogenes* (Naidu, 2000).

Plant-derived products (phyto-antimicrobials) form an extensive class of compounds that could potentially be used as food preservatives. Plants contain an enormous variety of constituents belonging to the category called secondary metabolites. As opposed to primary metabolites, secondary metabolites do not contribute to plant primary growth and development but provide an important ecological function. They play an important role in plant defence against pests, herbivores, and pathogens, as an attractant to pollinators and seed-dispersing animals and in allelopathy (emitting chemicals influencing competition among plant species).

Moreover, secondary metabolites can also play role as plant signalling molecules. Secondary metabolites are usually distributed very specifically among plant taxa and therefore can be used as taxonomic markers and contribute to specific odours, tastes, and colours of plants (Bennett & Wallsgrove, 1994; Hyldgaard et al., 2012; Makkar et al., 2007). Plant secondary metabolite secretion is influenced by biotic and abiotic stress and therefore, aromatic plants are more abundant in areas with higher biodiversity and climatically favourable conditions. Biotic factors like plenteous competitive flora and more pollinators and pathogens as well as higher humidity and favourable temperature affects the secondary metabolite production. For these reasons, occurrence of aromatic plants is associated more with tropical and subtropical areas (Akula & Ravishankar, 2011). There are several classes of compounds that belong to this vast category including alkaloids, glycosides, phenolics and polyphenols with distinct role in the plant life and distinct pharmacological activity (Bennett & Wallsgrove, 1994; Cowan, 1999). In frame of this thesis, EOs, forming only a small proportion of this extensive group, will be discussed in next chapters.

2.3. Plant essential oils (EOs)

According to International Standards Organization (ISO) EOs are defined by their production method as products obtained from vegetable raw material either by distillation with water or steam, or in case of epicarp from *Citrus* fruits by mechanical process or dry distillation. Furthermore, EOs can undergo physical treatments that do not influence the chemical composition (ISO 9235, 2021). Alternative definitions include also physicochemical perspective and EOs can be described as complex mixtures of volatile compounds in liquid state isolated from several plant organs that are often poorly soluble in water (Burt, 2004; Calo et al., 2015). They have been used since antiquity in perfumery, cosmetics, medicine and have been added to food as spices and herbs. Nowadays, approximately 3000 EOs are known, of which around 300 have economic importance in flavours and fragrance market (Baser & Buchbauer, 2010; Westphal et al., 1989).

Plants can produce EOs by organs where biosynthesis and storage of EOs occurs, or by specialized anatomical structures, where the yield of EO is higher. Individual cells producing EOs are called secretory idioblasts and can be found in aromatic flowers in epidermal layer of their petals (*Jasminum* spp., *Rosa* spp.), aromatic woods (*Lauraceae*) and roots (*Vetiveria zizanioides*). Many species have specialized anatomical structures where secretion and accumulation of volatiles takes place and therefore the EO yield is higher. These specialized anatomical structures include secretory cavities, oil ducts, glandular trichomes, and oil cells.

Secretory cavities consist of large extracellular storage space, where the produced oil is contained between the cell wall and cuticula surrounding the cell nucleus. Such cavities are typical for *Myrtaceae* and *Rutaceae,* where these aggregated cells form large subepidermal glands. Oil ducts are typical for *Apiaceae*, *e.g.*, *F. vulgare*, *C. carvi*, etc. Glandular trichomes are epidermal outgrowths characterized by the presence of a head made of cells that possess the ability to secrete and store large amounts of oil. They can be found in *Lamiaceae* or *Pelargonium* spp. Furthermore, there are several plants with more than one secretory structure, *e.g.*, trichomes and canals. The ability to produce and accumulate EOs is scattered throughout the plant kingdom and there are 108 families of higher plants known for such capability. Most of them are represented by one species only, however some families are represented by several aromatic plants. Economically, the most important families yielding EOs are among others *Myrtaceae* (*Eucalyptus* spp.), *Lamiaceae* (*M.* x *piperita*), *Lauraceae* (*Litsea* spp., *Cinnamomum* spp.), *Rutaceae* (*Citrus* spp.), *Apiaceae*, *Asteraceae*, *Poaceae* (*Vetiver* spp., *Cymbopogon* spp.), *Rosaceae,* and *Santalaceae.* Some cryptogams also yield EOs, *e.g.*, lichen *Evernia prunastri* (Oakmoss) and seaweed *Fucus vesiculosus* (Bladder wreck) (Baser & Buchbauer, 2010; Figueiredo et al., 2008; Huchelmann et al., 2017).

2.3.1. Chemistry

EOs are multicomponent mixtures of hundreds of chemical constituents and some of them are still unknown. The number of individual chemicals comprising the oil usually differs from 100 to 250, but in some oils the number of compounds is ever higher. Usually, an EO contains one or a few dominant compounds, which together constitute from 50 to 90% of the oil and are called character-impact components. Most of the compounds can be grouped into a few major functional groups, however some of them are difficult to classify. In this text, chemical constituents will be discussed in the following order: aliphatic compounds, terpenes and terpene derivatives, benzene derivatives and miscellaneous compounds (de Groot & Schmidt, 2016; Westphal et al., 1989).

2.3.1.1. Aliphatic hydrocarbons

Aliphatic compounds are defined as acyclic organic compounds with straight or branched carbon chain, but not containing an enclosed aromatic ring. This chain can be saturated or unsaturated, depending on the number of double (alkenes) and triple bounds (alkynes) between

carbon atoms. These compounds can be derived from fats or amino acids undergoing their metabolic conversion. Examples of these constituents are among many other aldehydes, (*E*)-4-decenal in *C. carvi* and *E. cardamomum* EOs, esters like hexyl propionate found in *Lavender* spp. oil and simple aliphatic alcohols and ethers like 1-octanol and methyl hexyl ether. In most EOs, aliphatic molecules are usually present only in trace amounts, but when they have oxygenated functional groups attached, their odours are usually noticeable (Moghaddam & Mehdizadeh, 2017; Westphal et al., 1989).

2.3.1.2. Terpenes and terpene derivatives

Terpenes and their derivatives terpenoids comprise most volatile substances. They are defined as substances consisting of isoprene (2-methylbutadiene) units (C_5H_8) and their classification is also based on the number of isoprene units connected head to tail. Terpenes in EOs are mostly built from 2 units of isoprene (monoterpenes), 3 isoprene units (sesquiterpenes) and rarely 4 units (diterpenes). These compounds are either biosynthesized from precursor acetyl-CoA *via* mevalonate pathway, or from pyruvate and glyceraldehyde phosphate *via* non-mevalonate (methylerythritol) pathway. Mevalonate pathway appears in the cell cytoplasm and generally provides precursors for synthesis of sesquiterpenes. The partial steps of this metabolic pathway include a) creation of mevalonic acid with six atoms of carbon, b) the rearrangement of this acid by several enzymatic reactions leading to isopentyl pyrophosphate (IPP) molecule and its isomer dimethyl allyl pyrophosphate (DMAPP) and c) IPP molecule is condensed with DMAPP to generate isoprenoid precursors of different chain lengths, with geranyl diphosphate being the precursor for monoterpenes (C10) and farnesyl diphosphate being the precursor for sesquiterpenes (C15). Methylerythritol pathway appears in plastids and generally supplies precursors for diterpenoids, carotenoids, gibberellins, and chlorophylls. In this pathway, 2-C-methylerythritol 4-phosphate (MEP) and 1-deoxy-D-xylulose 5-phosphate (DOXP) are involved resulting from the condensation of glyceraldehyde phosphate and pyruvate. Subsequently, the condensation of MEP and DOXP yields IPP and DMAPP. Finally, geranyl diphosphate or geranylgeranyl diphosphate as precursor for diterpenoids (C20) (Moghaddam & Mehdizadeh, 2017; D. Yang et al., 2012). Mevalonate pathway is the only biosynthetic pathway for isoprenoid production in animals and fungi, while methylerythritol pathway is utilized in bacteria. In plants, however, both pathways are involved and yet, the linkage between these two processes is still unclear. It has been suggested by Pu et al. (2021), that emergence of mevalonate pathway could have been involved during the plant transition from aquatic to

subterrestrial and terrestrial environment, allowing them to colonize the land and provide evolutionary advantage.

When a terpene molecule has a functional group attached to it, this molecule is described as terpenoid. The "oid" ending means "like" or "derived from". Functional group can be defined as an atom or a group of atoms that significantly determine the characteristic chemical properties of the molecule containing it. Most of the functional groups in EOs contain heteroatoms (atoms different than carbon or hydrogen), particularly oxygen. The major category of terpenoids are oxygenated derivatives. In alphabetical order they can be classified as follows: acids, alcohols, aldehydes, esters, ethers, ketones, lactones, oxides, peroxides, and phenols (Moghaddam & Mehdizadeh, 2017).

Monoterpenes Monoterpene hydrocarbons comply with the molecular formula $C_{10}H_{16}$. Their name is derived from a Latin word *mono* meaning one (two isoprene units forming one terpene). Their main carbon chain can be acyclic (myrcene), cyclic (limonene), bicyclic (pinene, carene) or even tricyclic (cyclofenchene, tricyclene). Monocyclic monoterpenes form the largest group of monoterpenes occurring in EOs. An important monocyclic monoterpene is limonene, which comes in two isomers. Dextrorotatory D-limonene can comprise up to 90% of *Citrus* fruits peel EO and its isomer L-limonene is much less prevalent in nature and occurs in *Pinus* EO. Furthermore, α-, β- and γ-terpinenes, terpinolene, and β-phellandrene occur widely in spice EOs. In general, monoterpenes have harsh, turpentine-like odour. For the organoleptic properties of EOs, the oxygenated monoterpene derivatives are the most important. The aliphatic monoterpene alcohol linalool has also two isomers, D-linalool typical for *Coriandrum* EO and its floral aroma and L-linalool occurring in *Lavandula* EO. Other important alcohols include menthol, isopulegol, and borneol. Significant monoterpene ketone representative is carvone in both isomers. D-carvone occurs in *C. carvi* EO and L-isomer can be found in *M. spicata* EO. Other significant monoterpene ketones are piperitone and pulegone (piperitenone) occurring in *Piper* EO. An example of monoterpene aldehyde can be citral, present in *Cymbopogon citratus* EO (Baser & Buchbauer, 2010; Moghaddam & Mehdizadeh, 2017; Westphal et al., 1989).

Sesquiterpenes Sesquiterpenes form the most diverse chemical group of spice derived volatiles. Their molecular formula is $C_{15}H_{24,}$ and they have lower volatility and higher boiling point than monoterpenes. The name comes from the Latin word *sesqui* meaning one and a half

in connection to three isoprene units forming one and a half terpene. These compounds are biosynthesized through numerous reactions from farnesyl pyrophosphate and their structure, irregularities and possible variations differ substantially. Significant sesquiterpene representative in spice industry is caryophyllene, first isolated from the *S. aromaticum* EO. β-Caryophyllene is the most usual isomeric form and occurs among others in *P. nigrum* EOs. An important isomeric structure is α-humulene, which was first discovered in *Humulus lupulus* EO. This structure is widely present numerous EOs. Other important constituents are zingiberene, present in *Z. officinale* rhizome EO, or sesquisabinene from *P. nigrum*. Sesquiterpene hydrocarbons are easily subjected to oxidation and therefore a huge variety of oxygenated derivatives can be encountered. For example, citronellal as aldehyde in *C. citratus* EO, nerolidol and bisabolol as alcohols in EOs of several plants. Occasionally, diterpenes can be also found in EOs. They have the molecular formula of $C_{20}H_{32}$ and are arranged of four isoprene units. These compounds are generally found in resins, but a few representatives can be also part of EOs. Diterpenic alcohol phytol and geranyl citronellol can serve as examples as well as diterpenes found in *Z. officinale* rhizome EO (Baser & Buchbauer, 2010; R. B. Berger, 2007).

2.3.1.3. Benzene derivatives

Many compounds with spicy and pungent odour belong to benzene derivatives and therefore, they can be called also aromatic compounds. All these compounds contain a characteristic benzene nucleus to which one or more functional groups are attached (*e.g.*, allyl, hydroxyl, methoxy, aldehyde, and many others). Most benzene derivatives belong to phenylpropanoids and are biosynthesized *via* shikimate pathway. Shikimate pathway is a sequence of seven metabolic steps starting with condensation of phosphoenolpyruvate and erythrose-4-phosphate. Such steps are gradually leading *via* shikimate as an intermediate product to synthesis of chorismate, which subsequently serves as a precursor to other metabolic pathways or for synthesis of aromatic amino acids and other aromatic secondary metabolites (Herrmann & Weaver, 1999; Westphal et al., 1989).

These constituents contain one or more C_6-C_3 units with C_6 being a benzene ring and the most important representatives are oxygenated derivatives. Anethole from *P. anisum*, eugenol from *S. aromaticum* and safrole from *Sassafras albidum* EOs are important representatives in the group of alcohols. The aldehydes are other important oxygenated derivatives and

cinnamaldehyde, responsible for the organoleptic properties of *Cinnamomum*, is one of the most important representatives. Its methoxy derivative (methoxycinnamaldehyde) can be found in *C. cassia* EO and is responsible for the characteristic aroma of this spice. Cuminaldehyde also serves as an example and this compound is responsible for the aroma of *C. cyminum*. Another significant phenylpropanoid is elemicin, which can be found in both seeds and arils of *M. fragrans*. Aromatic lactone coumarin is also important representative, which can be found in several EOs and is responsible for typical aroma of *C. cassia* (Moghaddam & Mehdizadeh, 2017; Sadgrove et al., 2022; Westphal et al., 1989).

2.3.1.5. Miscellaneous compounds

This category includes compounds also containing heteroatoms of nitrogen and sulphur. Nitrogen containing compounds in EOs provide an interesting aroma, with a faecal smell at high concentrations, but floral scent in dilution. In spices, they often bring pungent and bitter sensation. Capsaicinoids from *C. annuum* can be mentioned as an example. These constituents can be classified as vanillyl-amides of saturated or unsaturated monocarboxylic acids with straight chains (C_8-C_{11}). Amide piperine, responsible for a pungent principle of *P. nigrum*, can also serve as an example. Sulphur containing compounds are very characteristic and can be found in *A. cepa*, *A. rusticana*, *A. sativum* and Brassicaceae family. Examples include di-allyl disulphide, allyl isothiocyanathes and 4-hydroxybenzyl isothyocyanate. Isocyanates are usually bound to the sugar moiety by the sulphur end in the plant tissue and are released when the tissue is damaged by chewing or cutting (Sadgrove et al., 2022; Westphal et al., 1989).

2.3.2. Methods of extraction

The method of extraction is one of the key factors, that can significantly impact the chemical composition of volatile compounds present in the EOs. EOs can be obtained through variety of extraction methods, each developed for specific purposes. The selection of the technique typically depends on raw plant material, its condition, and the intended application. The methods can be subdivided into two major categories: conventional (classical) and advanced (innovative, green) techniques (Asbahani et al., 2015; Stratakos & Koidis, 2016).

Expression or cold pressing is one of the oldest methods of obtaining EOs and is used almost exclusively to isolate oil from *Citrus* fruit peels. During the process, the oil sacs in the pericarp are broken to release the EO localized in external part of the mesocarp (in secretory cavities). This results in yielding a watery emulsion, from which the oil is subsequently centrifugated and

separated. Until the beginning of the 20th century, cold pressing of *Citrus* oils was carried out manually, but nowadays special machines are utilized industrially. The reason for employment of such conventional method is thermolability of constituents of the *Citrus* fruit EOs, especially the aldehydes (Asbahani et al., 2015; Stratakos & Koidis, 2016).

Enfleurage, an ancient method originating from France, has been conventionally utilized primarily for capturing fragrant compounds from delicate flowers or petals, such as *Jasminum officinale*. The process involves spreading colourless purified cold fat onto the plant material, allowing the flowers to release their aromatic compounds, which dissolve into the layer of grease. Subsequently, old flowers are replaced with new ones, and the process is repeated until the fat reaches saturation. The saturated fat is then collected and extracted with alcohol. Although considered labour and time-intensive, and seemingly obsolete for EOs used in the food industry, enfleurage remains widely employed in the fragrance industry. It has been noted to produce EOs with characteristics closely resembling the natural scents of flowers (Paibon et al., 2011; Stratakos & Koidis, 2016).

Distillation is the most employed technique for EOs extraction, which can be carried out by boiling water (hydrodistillation), by steam (steam distillation), or by combination of boiling water and steam, where direct contact of boiling water and plant material is avoided (steam-water distillation) (Asbahani et al., 2015; Oktavianawati, 2020). During hydrodistillation, raw plant material is immersed in water in a vat and subsequently brought to a boiling point, when the EO is released *via* evaporation. Subsequently, vapours are condensed and the EO and water are then easily separated by decantation. On a laboratory scale, hydrodistillation by Clevenger-type apparatus is recommended by the European Pharmacopoeia (EDQM, 2013). Hydrodistillation is suitable for recovering EOs from flowers and petals, but has several drawbacks, including long extraction time (from 3 to 6 hrs) and chemical degradation of thermolabile constituents due to the long exposure to boiling water. Therefore, formation of artifacts and loss of some polar molecules due to overheating can occur. Steam distillation works on the same principle, but the raw plant material is not in direct contact with boiling water. It is placed on a grid and exposed to vapours above boiling water. Steam distillation is suitable for isolation of EOs stored in plant glandular trichomes and the extraction time and formation of artifacts is lower than in case of hydrodistillation. There are a few subtypes of steam distillation, namely vapour-hydrodistillation, vapour-distillation and hydrodiffusion, where vapor flow occurs downwards with gravity (Asbahani et al., 2015; Stratakos & Koidis, 2016).

Solvent extraction is employed for extracting EOs from plant material that yields low amounts of EO or for highly resinous or delicate EOs unsuitable for distillation. In this method, plant material is immersed in a solvent bath, which dissolves it, and the solvent is then evaporated. This process yields a liquid mixture termed "concrete," containing EO, waxes, and pigments. Treating the concrete with alcohol and subjecting it to distillation at low temperatures releases the EO. While this method offers advantages such as lower temperatures and shorter extraction times compared to distillation, EOs obtained via solvent extraction invariably retain residual amounts of solvent. Therefore, their use in the food industry is not feasible (Asbahani et al., 2015; Stratakos & Koidis, 2016; Tongnuanchan & Benjakul, 2014).

Research into new potential technologies for EO extraction over recent decades has yielded a plethora of innovative, often termed "green" extraction techniques. These advancements are aimed at mitigating the limitations of conventional methods, which are characterized by high consumption of plant material and time (Stratakos & Koidis, 2016; Tongnuanchan & Benjakul, 2014). Moreover, the adoption of green extraction techniques contributes to a reduction in environmental impact attributed to the accumulation of toxic solvents and energy consumption. This reduction can be achieved through two fundamental approaches: the utilization of alternative solvents such as supercritical or subcritical fluids, or the implementation of innovative heating technologies or pretreatment methods (Chemat et al., 2012).

Supercritical fluid extraction (SFE) belongs to innovative techniques based on the first approach using alternative solvents. For fluids, supercritical state can be achieved by subjecting them to a certain pressure (critical P) and temperature (critical T). After achieving supercritical state, fluids then exhibit very promising properties for extraction, like low viscosity, high diffusivity, and density close to liquids. All these properties would qualify as an excellent solvent, therefore SFE techniques based on this state have been developed. The principle of this technique is based on the use and recycling of the fluid in repeated steps of compression and decompression. By compressing and heating, the fluid reaches its supercritical state and passes through the loaded plant material. Taking away the volatile fraction and other extractable compounds, the fluid then continues to the separator or collecting flask and is subsequently decompressed, which leads to evaporation and yield of a solvent-free extract. Although several solvents (butane, ethane, pentane, etc.) can potentially be used during the SFE, CO_2 remains the most employed. Its moderate and easily achievable critical parameters (P = 74 bar, T = 32 °C) ensure the preservation of thermolabile compounds in the extract. In addition, CO_2 is ubiquitous in the environment, non-toxic to human health, non-flammable and widely available

at low cost (Asbahani et al., 2015; Barbosa et al., 2014; Fornari et al., 2012). All above-mentioned characteristics make supercritical CO_2 highly attractive to be used as "green solvent" and have led to multiple practical applications in different industries. Decaffeinated tea and coffee and later isolation of hop extract were the first commercially used processes of this innovative method and they became successful, large-scale industrial applications of CO_2 based SFE. Nowadays, several products including specialty oils, nicotine-free tobacco, and spice extracts (*Cinnamomum*, *P. nigrum* black and white and *Z. officinale*) are examples of commercial utilization of SFE (Fornari et al., 2012; Montalban & Villora, 2022). The option of using SFE for obtaining EOs has been a subject of multiple studies of various research teams worldwide. The most common conclusion regarding to the differences in chemical profiles of EOs and supercritical CO_2 extracts was higher abundance of monoterpenoids and phenylpropanoids in the hydrodistilled EOs opposed to higher recovery of sesquiterpenoids, diterpenoids and fatty acid derivatives and waxes in CO_2 extracts (Danh et al., 2013; Marongiu et al., 2013; Maxia et al., 2012; Mesomo et al., 2013). SFE has been concluded as an efficient and suitable method for extraction of volatile substances from plants with the final extract resembling the natural plant aroma more than distilled EOs. However, the most important shortcomings are the high initial cost of the high-pressure equipment, its installation and maintenance requirements. Moreover, processing a plant sample with higher moisture content might lower the extraction yield (Pereira & Meireles, 2010; Perrut, 2000; Sovilj et al., 2011).

Microwave assisted extraction (MAE) represents the second approach implementing the innovative heating technology. MAE utilizes microwave energy to rapidly heat the solvent and plant material, enhancing the extraction process by promoting the breakdown of cell walls and facilitating the release of bioactive compounds. Compared to conventional extraction methods, MAE typically requires shorter extraction times and lower energy input, making it more environmentally friendly. Its efficiency in extracting target compounds while using reduced temperatures and shorter processing times has led to its widespread adoption in various industries, including pharmaceuticals, food, and cosmetics (Asbahani et al., 2015; Chemat et al., 2012; Stratakos & Koidis, 2016).

Ultrasound assisted extraction (UAE) is an example of utilization of innovative pre-treatment technology. Ultrasound waves are employed to disrupt cell structures and improve the mass transfer of compounds from the plant material into the solvent. This method offers several advantages, including enhanced extraction efficiency, shorter extraction times, and lower energy consumption compared to conventional techniques (Asbahani et al., 2015; Stratakos &

Koidis, 2016). UAE has found applications in diverse fields such as natural product extraction, food processing, and pharmaceutical production. Its ability to extract target compounds efficiently under mild conditions makes it a valuable tool for sustainable extraction processes, for instance in combination with distillation (Chemat et al., 2012) or SFE (Riera et al., 2004).

2.3.3. Methods of chemical analysis

Since EOs and natural volatiles are widely used in commercial applications across various industries, their chemical analysis has a long tradition. Moreover, higher prices of natural products opposed to synthetic materials often led producers to adulterate products by adding a lower value ingredient or by recreating commercially valuable oils. Therefore, authentication is an important tool for consumers (Do et al., 2015; Sadgrove et al., 2022). At the same time, the scientific interest and curiosity regarding atmospheric chemistry, ecology, chemistry, and biochemistry of plant volatiles have led to the development of numerous analytical methods for their investigation. However, the study of chemical profile of EOs and their individual constituents is a challenging task due to their physicochemical properties. Many constituents are only present in trace amounts and therefore are difficult to detect. Another complication is presented by similarity of compounds (*e.g.*, isomers or enantiomers), that are difficult to distinguish (Fokou et al., 2020; Sadgrove et al., 2022). Furthermore, the volatile profiles are highly method-dependent, and no single technique can provide a complete analysis of volatiles present in the sample. Therefore, a combination of broad spectral profiling methods, together with targeted methods for analysis of key volatiles probably occurring in the sample, will continue to be utilized in chemical analysis of EOs. While a myriad of analytical methods can be used in the chemical analysis, two basic approaches can be distinguished: a) direct sampling of the air (headspace) above the sample and b) traditional solvent-based extraction methods (Rowan, 2011). Due to their volatile nature, gas chromatography (GC) is the most preferred technique to analyse the constituents of EOs. However, GC alone does not provide enough data for sufficient chemical profiling. Therefore, other analytical tools like mass spectrometry (MS), infrared spectroscopy a nuclear magnetic resonance have also been utilized. Moreover, other techniques can also be used as an enhancement to GC to improve its chemical profiling. These include chiral selective GC to detect isomers or multidimensional GC, mostly GC-MS. In addition, high performance liquid chromatography (HPLC) with many of its advances can also be a method of choice in EO analysis, especially in combination with MS as multidimensional technique (Fokou et al., 2020; Jalali-Heravi & Parastar, 2011).

2.3.3.1. GC in EO analysis

Gas chromatography (GC) has a rich history in chemical profiling of essential oils (EOs). Like other chromatographic methods, GC relies on separating individual compounds from a complex matrix using two phases: stationary (typically a column) and mobile (the carrier gas in GC). Volatile constituents are separated based on their affinity to surfaces, shape, and mass. During GC analysis, the EO is mixed and diluted with a solvent (typically pentane or hexane) and injected into the injector using a syringe. The mixture is vapourised and carried by an inert carrier gas (such as He, H_2, or N_2) to the stationary phase (column), where compounds are retained based on their interactions with the column. Compounds with stronger interactions elute later, while those with weaker interactions elute earlier. The quality of separation depends on factors such as molecular weight, polarity of volatiles, column properties (polarity, length, diameter), and mobile phase flow rate. The final step is detection, typically using a flame ionization detector (FID) in EO analysis. The FID burns the compound, and the resulting increase in flame temperature, proportional to the compound amount, is detected and displayed as a peak on a computer screen. The chromatogram, the final output, represents the pattern of peaks and summarizes all detected constituents in the sample.

To enhance the analytical power of GC, mass spectrometer is commonly integrated as a detector. Since analytical columns usually separate compounds solely by their boiling points, different constituents with the same boiling point can end up with the same retention time (RT). When a mass spectrometer is coupled to the GC, neutral molecules leave the column and are subsequently ionized in the ion source. Molecular ions are than produced and can degrade to individual fragment ions, which are subsequently separated in the mass analyser and detected by their mass: charge (*m/z*) ratio. Nowadays, the GC-MS is one of the most employed techniques for analysis of volatile compounds in many scientific fields. It provides an advantage in the ability to analyse large number of samples, high sensitivity, low detection limits and additional separation power based on mass spectra (Fokou et al., 2020; Jalali-Heravi & Parastar, 2011; Rowan, 2011). To achieve higher accuracy and quality in volatile compounds separation and avoid co-elusion and overlapping of peaks, analysis can be carried out using two columns of different polarities. Non-polar columns separate constituents largely based on boiling points and those might be highly similar in case of oxygenated and purely hydrocarbon constituents. Therefore, EO containing high number of oxygenated components might result in chromatogram with overlapping peaks in short elution range with similar retention times. Adding a polar column will therefore provide the benefit of extending the elution range by

retaining polar compounds (oxygenated constituents) longer. In this way, the various component classes are pulled apart and greater separation space is available in the sample. Therefore, identification and subsequent quantification of individual constituents is ameliorated (Marriott et al., 2001).

2.3.3.2. Headspace analysis using GC-MS

Headspace sampling (HS) involves collection of the volatile compounds directly from the air surrounding the sample (headspace). This non-destructive extraction of airborne molecules from plant samples corresponds more with authentic volatile profile of plant samples compared to traditional, solvent based extraction techniques or hydrodistillation. HS can be static or dynamic (purge and trap). In static headspace analysis, no air circulation appears and volatiles in the sample are equilibrated with the air in the airtight container. After reaching the equilibrium, the known amount of the headspace above the sample is collected and injected directly to the GC-MS apparatus. Sample of the headspace is usually collected using a gas-tight syringe or solid-phase micro extraction (SPME) fibre. During the dynamic headspace, a known volume of air is passed over the sample and entrained airborne compounds are concentrated onto an adsorbent trapping material, capturing the volatile constituents in substantial amounts enabling multiple analyses.

HS-SPME is currently the most employed technique for static HS and has a wide range of applications for its simplicity and cost effectiveness. This robust and sensitive method is based on the absorption of volatiles on a fibre coated with polymeric adsorbent, from which the compounds can be thermally desorbed directly into a GC inlet. This fibre is protected by a needle, which protects the fibre through the passage septa and GC injector. For analysis of airborne compounds, the adsorbent-coated fibre is extended from a needle and exposed to the HS above the sample (or solution) for a pre-defined amount of time. Volatiles are absorbed by the fibre and after some time, the fibre is removed and injected into a GC inlet. SPME is used for qualitative or semi-quantitative analysis, although quantification of compounds might present a challenge. Analytes might compete for binding sites on the SPME fibre and bias the results. An alternative to SPME would be a direct injection of a headspace using a gas-tight syringe directly into a GC chromatogram. Such simple technique requires no sophisticated instrumentation and is readily instrumented, however, lacks sensitivity. Therefore, SPME is the method of choice for sampling of small amounts of volatiles for its remarkable sensitivity and flexibility (Qualley & Dudareva, 2009; Rowan, 2011).

2.3.4. Antimicrobial effects of EOs

It has long been recognized that plant EOs possess antibacterial, antifungal, antiviral, antimycotic, antioxidative, antiparasitic, insecticidal and other significant pharmacological properties, which are probably related to their function in plants (Burt, 2004; Guenther, 1948). One of the first scientific studies regarding the antimicrobial activity of EOs derived from spices was conducted in 1880 and reported *Cinnamomum* to be active against spores of *Bacillus anthracis* (Ceylan & Fung, 2004; Tajkarimi et al., 2010). Ever since, there is an extensive amount of scientific literature dealing with the potential utilization of spices and their EOs as novel food preservatives (Calo et al., 2015; Dussault et al., 2014; Hyldgaard et al., 2012; Reyes-Jurado et al., 2020; Tajkarimi et al., 2010).

Although the exact mechanism of the antimicrobial effect has not been fully elucidated and more processes can be involved, it is probably related to the hydrophobic nature of EOs, which allows them to destabilize bacterial cell membranes integrity (Cowan, 1999). After the partition of lipids on bacterial cell membrane, it becomes more permeable. Consequently, leakage of ions and other cell contents occurs, gradually leading to the cell death (Burt, 2004; Hyldgaard et al., 2012). Gram-negative bacteria are generally less susceptible to EOs, because their outer cell membrane contains more hydrophilic lipopolysaccharides than Gram-positive bacteria. Those structures create a barrier against hydrophobic compounds and therefore can tolerate more such compounds as those present in EOs (Reyes-Jurado et al., 2020). EOs rich in phenolic compounds are mostly associated with the strongest antimicrobial effects. Thymol and carvacrol, occurring in *O. vulgare*, *Satureja* spp. and *T. vulgaris* can serve as examples of the most investigated compounds and species. Furthermore, alcohols and aldehydes are also associated with antimicrobial effect and many studies have concluded that the higher content of these oxygenated compounds in EOs, the greater the antimicrobial effect (Burt, 2004; Reyes-Jurado et al., 2020). In addition, there is evidence that minor compounds of EOs can also contribute to the overall antimicrobial efficacy, probably by exhibiting synergistic effects with major components of EOs. Synergistic effect of carvacrol and *p*-cymene against *Vibrio cholerae* in foods can serve as an example (Falleh et al., 2020; Rattanachaikunsopon & Phumkhachorn, 2010).

Microbial contamination is one of the most important concerns at the different stages of food processing and reason for the deterioration of food products. Application of synthetic

antimicrobial compounds can prevent the microbial growth and reduce the related complications with foodborne diseases. However, some synthetic preservatives can have harmful effect on human health, including contribution to development of cancer, multiple sclerosis, etc. Therefore, scientific teams worldwide are focusing on developing an alternative of natural origin with no or negligible adverse side effects. In this context, applications of EOs as food preservatives is a novel growing interest (Falleh et al., 2020; Serag et al., 2022; Singh et al., 2022). Accordingly, many EOs or their components were approved by the EC as well as by the FDA as GRAS to be utilized as flavourings and/or preservatives in food products (Falleh et al., 2020; Hyldgaard et al., 2012). On the FDA website, EOs derived from *C. verum*, *C. sativum*, *L. burnati*i or *L. intermedia, M. fragrans*, *O. basilicum*, *O. vulgare*, *S. officinalis* and *S. rosmarinus*, *S. aromaticum*, *T. vulgaris,* and *Z. officinalis* are labelled as GRAS. Furthermore, some EO derived compounds like vanillin, carvacrol, citral, cinnamaldehyde, linalool, carvone, eugenol, thymol and limonene are also listed there (Burt, 2004; Singh et al., 2022).

EOs can be applied to food matrices in various ways. The first option is inclusion of EOs directly in the food system, however, this method comes with several shortcomings. The strong odour of these volatiles can hamper the organoleptic properties of food products, resulting in low acceptance on the consumer's side. Furthermore, the higher volatility makes EOs easily degradable under the treatment of heat, light, and other environmental factors. In addition, food matrix characteristics (pH, protein, fat content, etc.) can also interfere with the antimicrobial properties of EOs. To overcome these restrictions with the direct use of EOs, several alternative technologies have been suggested. Encapsulation of EOs with biodegradable polymeric complexes (e.g., chitosan, alginate, zein, lecithin, whey protein and inulin) is one technique used to enhance their efficacy and stability (Asbahani et al., 2015; Singh et al., 2022). Several researchers have demonstrated better sensory acceptance of food containing encapsulated EOs. At low concentrations, encapsulated EOs can control microbial growth without altering the organoleptic properties of food products despite their strong flavour (Reis et al., 2022). A specific type of encapsulation is nano emulsion, which is defined by the size of particles smaller than 100 nm. In these particles EO, surfactant and water are used together to facilitate dispersibility and controlled release in food systems. Compared to other encapsulation techniques, nano emulsion has been shown to be one of the most effectives, due to the high surface area-volume ratio and better stability of encapsulated compounds (Maurya et al., 2021). Edible coating as part of active packaging system is also an alternative for addition of EOs into food systems. EOs are incorporated into biodegradable films of polysaccharides, proteins or

lipid-based matrices. These edible films promote the antimicrobial efficacy of EOs as well as help in the control of their release. The most promising results have been obtained when using bioactive coatings in combination with other non-thermal preservation methods, especially MAP (Sanchez-Gonzalez et al., 2011; Singh et al., 2022).

2.3.4.1. Methods for evaluation of antimicrobial potential of EOs

Various laboratory methods can assess the in vitro growth-inhibitory potential of extracts or pure compounds. Among the most recognized are the disc-diffusion and broth or agar dilution methods, which involve direct contact of microorganisms with antimicrobial substances. These methods have been thoroughly established and standardized by organizations like the Clinical and Laboratory Standards Institute (CLSI) and are commonly employed in routine investigations related to potential antibiotic agents (Balouiri et al., 2016).

However, the high volatility and hydrophobic nature of essential oils (EOs) present challenges for direct-contact assays. In diffusion assays, individual components of EOs may partition through the agar based on their affinity to water. Additionally, dilution methods necessitate the use of emulsifiers or solvents (such as Tween 80, DMSO, or ethanol) due to the poor water solubility of EOs, which can potentially impact their antimicrobial activity (Nedorostova et al., 2009). Moreover, volatility may result in the loss of active substances during sample handling, experimental preparation, and incubation (Houdkova & Kokoska, 2020).

In contrast to the established screening methods for testing antimicrobial susceptibility on solid or liquid matrices, standardized procedures for assessing the antimicrobial potential of volatile compounds in the vapor phase, as set by organizations like CLSI, NCCLS, or EU-CAST, are lacking. Consequently, comparing results from various research teams in recent decades proves challenging. While several methods have been developed to investigate the potential antimicrobial effects of volatile agents, they largely represent modifications of agar disc diffusion assays (Houdkova & Kokoska, 2020; Nedorostova et al., 2009). Although these modifications lead to creation of relative values, they are expressed in different units of different values and their comparison mostly requires several recalculations. These units include diameter of inhibition zones, inhibition ratios, minimum inhibitory dose per colony forming unit and several definitions of minimum inhibitory concentrations (MIC) (Burt, 2004; Houdkova & Kokoska, 2020; Tyagi et al., 2012). Principles of some methods for testing volatile antimicrobial agents will be briefly described in next chapters, newly categorized according to

review by Houdkova & Kokoska (2020), with special emphasis on broth microdilution volatilisation method.

Solid matrix volatilisation methods are laboratory methods, where tested volatile agents are applied onto solid matrices, *e.g.*, paper disc.

- **Disc volatilisation assay and modifications** are based on disc volatilisation assay, one of the simplest methods for antimicrobial evaluation of volatiles in vapour phase, ideal for preliminary screening of the most promising EOs (Bueno, 2015). The method involves inoculating Petri dishes containing solidified media with a solution of the microorganism to be tested. Sterile filter paper discs impregnated with the volatile constituent at the desired concentration are then placed on the lid of the dish, which is free of medium. The plates are sealed with parafilm or adhesive tape and subjected to incubation. Antimicrobial potential is measured by the diameter of inhibition zones, providing qualitative assessment. However, this method is associated with high material consumption and workload (Houdkova & Kokoska, 2020). Approximate minimum inhibitory concentration (MIC) values can be calculated from inhibition zone diameters, as demonstrated by Lopez et al. (2007) evaluating growth inhibitory effect of cinnamon, thyme, and oregano EO vapours. Nevertheless, results may vary depending on factors such as Petri dish diameter, media composition, and the amount of compound on the disc. Kloucek et al. (2012) modified this assay by dividing Petri dishes into four sections, allowing for simultaneous testing of more bacteria and enabling higher throughput screening.
- **Airtight apparatus disc volatilisation methods** represent an improvement of disc volatilisation assay. In this method, Petri dishes are inserted into an airtight box of known volume, as demonstrated by Inouye et al. (2001). Authors of the study introduced aluminium foil into the box to protect its walls from direct contamination by EOs and plastic absorption. A paper disc impregnated with EO at a known concentration (or a glass vessel containing pure EO) is placed atop the airtight box, away from the Petri dish inoculated with bacteria. The boxes are then incubated under specified conditions, and the minimum inhibitory dose (MID), preventing bacterial growth, is recorded. This method offers the flexibility to use various inoculated materials and larger objects inside the box. However, the main drawback is the space required for storing larger number of experiments (Bueno, 2015; Houdkova & Kokoska, 2020).

- **Experimental airtight apparatus** designed for simultaneous screening of different concentrations of EO has been developed by Seo et al. (2015). This apparatus consists of two separate chambers divided by an O-ring. The upper chamber contains seven wells with a special nutrient agar medium containing D-glucose and bromocresol purple for pH determination. In the lower chamber, seven wells with sterile paper discs and two-fold serially diluted concentrations of EO are placed. The inhibitory concentration is assessed based on the colour change in the nutrient agar from purple to yellow, indicating pH alteration due to bacterial fermentation of glucose. Subsequently, the MIC is determined. The main advantage of this method is the ability to test multiple concentrations simultaneously, though it requires specialized apparatus not commonly available and is only suitable for glucose-fermenting bacteria (Houdkova & Kokoska, 2020; Seo et al., 2015).

Liquid phase volatilisation methods are *in vitro* testing methods, where the volatile substances are tested in liquid form, either as pure compounds or dissolved in solvents or growth media.

- **Broth microdilution volatilisation method** has recently been developed by Houdkova et al. (2017) and combines principles of standard broth microdilution method and disc volatilisation assays. The method is performed on standard 96-well immune plates with tight fitting lids, specifically designed to reduce growth media evaporation. With aim to prepare vapor phase of the method, melted agar is pipetted into every flange of the lid and subsequently inoculated with bacterial suspension on agar surface. For evaluation of liquid phase, seven two-fold serial dilutions of six different volatile substances are prepared on a microtiter plate. The plate is then inoculated with bacterial suspension as well and finally, the lid and plate are connected and fastened by clamps with handmade wooden pads. After the incubation under specific conditions, MIC values are determined by visual evaluation after colouring of metabolically active bacterial colonies with thiazolyl blue tetrazolium bromide dye (MTT). When the interface of colour changes from yellow to purple (containing metabolically active bacteria), value is recorded. The lowest concentrations inhibiting the microbial growth are assessed as the MICs. This method is suitable for high-throughput screening for its simplicity and determination of both liquid and vapor phase simultaneously. Several volatile compounds can be tested at the same time in different concentrations utilizing only one 96-well plate without the

need for special apparatus and the assay is based on CLSI standardized method. However, it does not solve the drawbacks of previously described methods which is the physicochemical characteristics of volatiles and their distribution in throughout the well/flange. Therefore, the MIC values for vapours can be taken as indicative values only. Moreover, wooden pads and clamps are necessary for better sealing the plate and its lid.

- **Broth macrodilution volatilisation method** was designed to overcome some drawbacks of the above-mentioned screening technique (Houdkova et al., 2021). This assay combines the principles of broth microdilution volatilisation method and standard broth macrodilution method. On the contrary to the above-mentioned method, 2 mL standard Eppendorf tubes with snap caps are used. The vapour phase determination takes places on the caps, where melted agar is pipetted, and bacterial suspension is subsequently inoculated on its surface. The liquid phase takes place inside the microtubes as during standard microdilution method. After inoculation, the microtubes containing liquid medium with serially diluted tested volatiles are finally closed together to prevent losses of active volatiles. After the incubation, MIC values are determined by visual evaluation as described in the previous method. The main advantage of this technique is no need for special apparatus and supporting material like clamps and wooden pads. Furthermore, method is also more suitable for slower growing microorganisms like fungi, that require longer incubation time for MIC assessment.
- **Airtight apparatus liquid volatilisation method** developed by Sekiyama et al. (1996) is conducted in a sealed container, where inoculated agar plate and a Petri dish containing the tested volatile are located. After incubation, the inhibitory effect is determined by measuring colony diameter, with the MID defined as the lowest concentration inhibiting colony formation by 50% (Nakahara et al., 2013).
- **Agar plug-based vapour phase assay** designed by Amat et al. (2017) uses two agar plates for qualitative and quantitative assessment of vapour phase antimicrobial effects. Agar plugs obtained from an inoculated plate are placed on sterile caps embedded in the second plate. Known volumes of EO are added to inner compartments of the caps. After incubation, bacterial growth is visually assessed, and quantification involves recovery and enumeration of bacteria cells from plugs compared to controls. The method allows quantitative assessment and easy recovery of bacterial cells from the plug for further analysis.

2.4. Spices of Asia

According to the ISO, spices and condiments are defined as plant products or their mixtures that are used for flavouring, seasoning and imparting aroma to foods (ISO 676, 1995). Another definition from the FDA describes spices as any aromatic vegetable substances in the whole, broken or ground form, whose significant function is seasoning rather than nutrition, that is true to name and from which no portion of any volatile oil or other flavouring principle has been removed. With aim to distinguish spices and herbs from aromatic plants also being utilized for their volatile oils, following division has been defined: the spices are species in which direct use of the whole, dried, or ground plant parts in food flavouring is predominant to its EO extraction and subsequent use in perfumery as in case of aromatic plants. It is estimated that 400-500 species of spices have been used worldwide, while roughly 275 of them belong to species originating from Southeast Asia region. The tropical Asian spices include *Cinnamomum cassia*, *C. verum*, *Curcuma longa*, *Elettaria cardamomum*, *Myristica fragrans*, *P. nigrum*, *Syzygium aromaticum,* and *Zingiber officinalis*, while *Capsicum annuum*, *Pimenta dioica,* and *Vanilla planifolia* are native to tropical regions of America. The temperate spices (*Anethum graveolens, Brasica* spp, *Coriandrum sativum*, *Cuminum cyminum*, *Foeniculum vulgare*, *Laurus nobilis*, *Ocimum basilicum*, *Origanum vulgare*, *Salvia officinalis*, *Salvia rosmarinus*, *Thymus vulgaris,* and *Trigonella foenum-graecum*) are mostly native to the Mediterranean region. Only a few species (*Armoracia rusticana, Artemisia dracunculus,* and *Carum carvi*) are native to cold temperate regions (Future market insights, 2021; Westphal et al., 1989).

Southeast Asian region consists of 11 countries (Brunei, Cambodia, East Timor, Indonesia, Laos, Malaysia, Myanmar, Singapore, Philippines, Thailand, and Vietnam. Often referred to as "food bowl of Asia" for its favourable climate conditions, fertile soil, and monsoon rains ideal for cultivating a wide variety of crops. The cuisine of Southeast Asia is dominated by rice and noodles as two staples, complemented with fish as a primary source of protein, and an extensive array of fruits and vegetables and abundant use of spices and herbs. Many of sought-after spices have their origins in countries of Southeast Asia and play pervasive role in their culinary traditions. It should be noted that the use of spices over the centuries has been primarily to enhance the flavour and palatability of the food. However, the unexpected added nutritional and health promoting value of these plant materials have elevated the status of these ingredients to more prominent level and attracts the attention of wider culinary audience (Ooraikul et al., 2008).

2.4.1. History

Although the beginning of spice use is not fully elucidated, no commodity has ever played such a crucial role in the development of modern civilizations, explorations, and commerce. The history of spices dates to at least 2600 BC, when antient Egyptians used spices for embalming practices, body ointments and fumigations of rooms. In ancient Mesopotamia, spices were used as remedies as encouraged in the code of Hammurabi in 1700 BC. Oriental spices have added flavour and spiciness to otherwise bland and staple diet full of rice already before 1000 BC in India and Far East. Ancient civilizations considered spices as luxury goods because of their scarcity. Spices are also mentioned in three "Holly books", the Old and New testaments of the Bible and the Koran (Mrad, 2005; Sherman & Billing, 1999; Westphal et al., 1989). Oriental spices were imported from China, India and "Spice Islands" (Moluccas in Indonesia nowadays) by Arab traders, who sold their spices in Babylon, Egypt, and Nineveh. By 980 BC, they became the "middlemen" and acted as intermediaries between Orient and Middle East and the West. By 1st century BC, Arabs were monopolizing the spice market, because they knew the spice routes and kept them a secret. Ancient Greeks and Romans also highly appreciated spices and consequently, Alexandria became the main trading centre between Mediterranean region and the East. Moreover, knowledge of spices flourished that time and spices were mentioned also in texts from Hippocrates. In the 9th century, cultivation, and use of Mediterranean spices expanded throughout Europe (Mrad, 2005).

During the Middle Ages, the trading centre and control point moved to Venice, Genoa, and Pisa and spices became extremely expensive and in great demand. In those times, *P. nigrum* was used as a currency to pay taxes, rents and interestingly, also dowries for brides. Furthermore, the crusades also contributed to spreading spices across Europe and changed the eating and cooking habits of European population. To gain the independence from the monopoly of Mediterranean traders, spice merchants of Western Europe gradually organized themselves into guilds to gain direct access to the East. During the age of discoveries, Marco Polo was sent to an exploratory journey in 1271 to China and India. The main purpose of his travels was to bypass the Arab middlemen with their 300% markup prices. After his return, he wrote a travelogue, where among others, great abundance of spices and other luxury goods in India is described. During the renaissance, many European nations were motivated to find the sea route to the Far East and in 1492, Christopher Columbus set up a sail to discover the West Indies but ended up in the American continent instead. From his voyage, he brought back *C. annuum*,

Solanum tuberosum, *V. planifolia,* and *Theobroma cacao* leaves, used by native American population (Mrad, 2005).

In 15th and 16th centuries, the Portuguese were the first Europeans to become the market leaders. In 1498, Vasco Da Gama discovered a sea route to East India around Africa and after several more exploratory voyages by 1515, Portugal had well established spice trades between East Indies and Western Europe and by the end of 16th century, also a monopoly. However, their position was short lived and was soon taken over by the Dutch. Between 1605 and 1621, the Dutch managed to drive Portuguese out of the Spice Islands, which lead to their virtual monopoly in *M. fragrans* and *S. aromaticum* trade. Netherlands found a powerful trading company called Dutch East India company and it has been successful over 200 years in securing its monopoly by implementing aggressive policy in Spice Islands and destroying three fourths of *M. fragrans* and *S. aromaticum* trees, leading to prices increase in Europe (Dhont, 2023; Mrad, 2005).

Alongside the Netherlands, England also established route to Far East and found a powerful company called British East India Company. During several exploratory voyages, the English also visited Moluccas and Java and often fought their Dutch counterparts. The tension between these two countries rose into several Anglo-Dutch wars in the 17th and 18th centuries, when the Dutch suffered severe losses. The ruthless era of the Dutch domination in the spice Islands ultimately led to smuggling of seedlings of nutmeg and cloves plants into areas out of the reach of the Dutch. The smuggling led to Zanzibar and ever since it became the country specialized in *S. aromaticum* tree production. The *M. fragrans* seedlings were transported to Grenada and other areas in the Caribbean. These events have led to the beginning of era of free commerce since then no country has obtained the monopoly to produce any kind of spice. Nowadays, some countries have become specialists at growing specific kinds of spices (*e.g.*, Zanzibar in Tanzania for *S. aromaticum*, Madagascar for *V. planifolia,* and Vietnam for growing *P. nigrum*). The spices industry has gradually become as competitive as the food market and is continually growing (Mrad, 2005; Westphal et al., 1989).

2.4.2. Economic importance

In 2021, the global spice market's total value amounted to $21.3 billion USD, with a forecasted increase to $27.4 billion USD by the end of 2026. The surge in demand for spices is primarily driven by several factors, including the rising preference for diverse and flavourful cuisines, the

expansion of the fast-food and packaged food sectors, and consumers' growing inclination towards healthier and more natural products. Additionally, there's a notable global trend towards clean-label products, necessitating transparency in ingredients, which presents new opportunities for food manufacturers to innovate in the spice and seasoning market. Geographically, the Asia-Pacific region held the largest revenue share in 2021, accounting for 35% of the market, and is expected to maintain its dominance due to the region's rich history and tradition of spice usage in culinary practices (Market reports, 2021).

Economically, the most important spice in international trade is black pepper, known as "king," of the spices. In 2023, the black pepper market size has reached 4.3 billion USD and is likely to nearly double almost 8 million USD by 2032. According to the latest black pepper market report, food and beverage is the dominant segment holding most of the market share. Following industries are pharmaceuticals and personal care, owing to the presence of bioactive compounds, particularly piperine. (Future market insights; Hammouti et al., 2019; Imargroup, 2023). Beyond their role as flavour enhancers, certain spices also serve as source of natural colorants in food industry, owing to the presence of pigments within them. Crocin and crocetin from *Crocus sativus*, curcumin from *C. longa* rhizome and capsanthin and capsorubin from *C. annuum* can serve as examples (Peter & Shylaja, 2012).

2.4.3. Botany

Spices form botanically heterogenous commodity and occur throughout the plant kingdom, approximately in 35 distinct plant families. Nevertheless, in following families the occurrence of spices is more significant, containing a high number of economically important spices.

- **Apiaceae** is one of the largest taxa of higher plants, consisting of 3780 species and 434 genera. Plants from this family have traditionally been used in food and fragrance as well as a natural remedies in households since antiquity. It is mostly distributed in northern temperate regions or higher altitudes in the tropical areas. The most significant representatives of spices cultivated worldwide are *A. graveolens*, *Carum carvi*, *C. sativum*, *C. cyminum*, *Foeniculuim vulgare, Petroselinum crispum,* and *Pimpinella anisum* (Sayed-Ahmad et al., 2017).

- **Lamiaceae** family contains many valuable aromatic plants and spices. It consists of 241 genera and contains approximately 7530 species. Significant spices include *Mentha* x *piperita*, *Ocimum basilicum*, *Origanum vulgare*, *Salvia officinalis*, *Salvia rosmarinus*,

and *Thymus vulgaris*. Most of plants from this family are native to the Mediterranean region, where production of their EOs is profitable. Their EOs and their potential utilization as food preservatives has extensively been investigated (Karpinski, 2020; Liaqat et al., 2018; Nieto, 2017).

- **Lauraceae** is an important family of higher plants containing 55 genera and approximately 2500 species. Plants of this family are extensively used in traditional medicine and as spices and are native to the tropical areas of east Asia, south and north America. Most species possess aromatic roots, stems, and leaves. Many popular spices originate from this family, including *C. cassia, C. verum,* and *L. nobilis* (Simic et al., 2004).

- **Myrtaceae** family contains 132 genera and roughly 5950 species of shrubs or trees occurring mostly in tropical forests worldwide. Trees from this family are known for the high yield of EOs (*Eucalyptus globulus*) and are used in folk medicine. The major spice and potential nutraceuticals representatives include *P. dioica* and *S. aromaticum* (Christeneusz & Byng, 2016; Stefanello et al., 2011).

- **Piperaceae** is a small family with 5 genera and approximately 3700 species from which many are used in folk medicine. Plants from this family might be small trees, shrubs, or herbs. The most well-known representative is *P. nigrum* for its economic importance. Pepper or peppercorn refers to dried fruits of *P. nigrum*, a perennial vine native to Western Ghats in India. Its relatives also include many other important pepper spices, while the most known are *Piper longum* and *Piper cubeba* (Benitez et al., 2009; Kato & Furlan, 2007).

- **Rutaceae** is a family generally known as a citrus family and contains 154 genera and roughly 2070 species. Species from this family range in size from plants to shrubs or small trees and have very fragrant flowers or fruits, from which EOs are commercially obtained. Citrus fruits are highly cultivated and used as food or condiments worldwide and their smell and taste are pleasant. Citrus EOs are GRAS and are extensively used for food, beverage liquors, flavouring agents but also aromatic agents in cosmetics (Christeneusz & Byng, 2016; Giovanelli et al., 2020; Liaqat et al., 2018).

- **Zingiberaceae** is well known as the ginger family and contains 50 genera with roughly 1600 species. Plants from this family are aromatic and widely distributed in tropical areas worldwide and are especially abundant in South-East Asia. Many species from the

ginger family have been used as spices for their intensive aroma and well-known examples include *Alpinia galanga*, *Curcuma longa*, *Elettaria cardamomum,* and *Z. officinale.* Other representatives include *Aframomum melegueta, Amomum subulatum*, *Boesenbergia rotunda, Etlingera* spp., *Kaempferia galanga,* and many others (Christeneusz & Byng, 2016; Zahara et al., 2018).

Moreover, spices emanate from distinct botanical components such as flower buds (*C. cassia*, *S. aromaticum*), flowers (*C. sativus*), subterranean rhizomes (*A. galanga*, *C. longa,* and *Z. officinale*), roots (*A. rusticana*), leaves (*L. nobilis*), reproductive organs such as fruits (*P. longum* and *P. nigrum*), seeds (*C. carvi*, *C. cyminum*, *Myristica fragrans* nutmeg) and their protective covers as arils (*M. fragrans* mace) and inner stem bark (*C. cassia* and *C. verum*). They also include various herbs in whole form such as *C. sativum*, *M.* x *piperita*, *Origanum majorana*, *Origanum sativum*, *P. crispum*, *Salvia officinalis,* and *T. vulgaris* (Sulieman et al., 2023).

2.4.4. Cambodian cuisine and spices

Cambodia, along with other Southeast Asian countries, has a cuisine that is generally considered to be healthy and delicious due to the abundant use of fish and the incorporation of many vegetables, fruits, herbs, and spices into each meal. The national main staple is rice, but traces of French colonial influence are evident in the use of bread and French-style baguettes. Fish and seafood sourced from the Gulf of Thailand are very popular and served at almost every dining table. Some examples include sour fish soup (Somlar Machou Banle) and spicy prawn soup (Bangkang). Other national Cambodian popular dishes include rice noodles in a coconut-based sauce (Khao Poun) and fish with coconut milk steamed in a banana leaf (Hamok Trei). Herbs and spices are integral components in traditional Cambodian dishes, with an average consumption of 42 g of condiments and spices per person daily (In et al., 2015; Ooraikul et al., 2008). In the following paragraphs, examples of less phytochemically explored Cambodian spices alongside with as spices with international recognition originating in various parts of Asia used in this thesis will briefly be described.

- ***Alpinia officinarum*** **Hance** commonly referred as galangal is a major spice in Southeast Asian cuisine. Belonging to the Zingiberaceae family, its rhizome is recognized for pungent aroma and is daily used in Khmer cuisine in curries, sauces, meat and fish marinades or in fruit or vegetable smoothies. Traditionally, the rhizome has been used

against rheumatism, bronchial cataract and to control incontinence (La plantation, 2024; Srividya et al., 2010).

- ***Amomum kravanh* Pierre ex Gagnep** is a plant belonging to the Zingiberaceae family, widely cultivated in Cambodia, Thailand, Vietnam, and South China. In Thailand it is commonly known as "Krevanh", and its fruits and leaves are used to flavor curries, white meats, rice, and vegetables. Furthermore, fruits, known as "Bai Dou Kou" are used in traditional Chinese medicine to treat digestive disorders and stomach diseases (Diao et al., 2014; Zhang et al., 2020).
- ***Amomum subulatum* Roxb** commonly known as black cardamom, is another plant from the Amomum genus, which belongs to one of the major crops distributed in Eastern Himalayas in Bhutan, India, and Nepal. Although it is native to temperate to subtropic regions, it is widely utilized in Southeast Asia as a stimulant and carminative. Fruits are also used as condiments to flavour food, confections, and beverages, and as a food preservative (Satyal et al., 2012).
- ***Boesenbergia rotunda* (L.) Mansf** commonly known as fingerroot or Chinese keys for its finger look-like rhizome is another representative from the Zingiberaceae family, which is a common edible ingredient throughout Southeast Asia. It is commonly cultivated in small home ranches and used as a condiment in soups and curries for its aromatic flavour, which promotes appetite. The rhizome has also a wide range of applications in traditional medicine (Eng-Chong et al., 2012; Ongwisespaiboon & Jiraungkoorskul, 2017).
- ***Cinnamomum cassia* L. J. Presl** commonly referred as Chinese cinnamon is an evergreen tree indigenous to Southern China, which is widely cultivated in various parts of Southeast Asia. Several parts of this tree are utilized in food flavouring for a long time and besides the inner stem bark, immature fruits called cassia buds are another commodity used as a flavouring food additive. *C. cassia* fruits are added to pickles, curries, beverages and confectioneries and possess unique and subtle flavour (Guoruoluo et al., 2017; Sandner et al., 2018).
- ***Cinnamomum verum* J. Presl** commonly known as Ceylon or "true" cinnamon is another evergreen tree, native to Sri Lanka. Its inner bark has been used as a spice for thousands of years. Together with *C. cassia*, their inner bark are sought-after spices in European and US markets. Furthermore, *C. verum* bark has a long tradition in Ayurvedic medicine (Jayaprakasha & Rao, 2011).

- ***Citrus hystrix* DC**, commonly known as kaffir lime, is one of the examples from Rutaceae family used as regional condiment in Cambodia. Leaves and fruit juice from this citrus are used for various flavouring purposes in Khmer cuisine, while EO from the fruit pericarp finds its applications in cosmetics and beauty products. Previous research has demonstrated that kaffir lime EO exhibits diverse biological activities, including antimicrobial, antioxidant, insect-repellent, and antiviral properties (Suresh et al., 2021; Waikedre et al., 2010).
- ***Curcuma zedoaria* (Christm.) Roscoe** commonly called zedoary or white turmeric is a member of *Curcuma* genus within Zingiberaceae family, which is known as essential source of colouring and flavouring agents in Asian cuisines. *C. zedoaria* is widely cultivated across Southeast Asia and used both as a spice and medicinally. For its unique smell, it is widely used in curries or to flavour meat or fish (La plantation, 2024; Thin et al., 2022).
- ***Elettaria cardamomum* (L.) Maton** commonly known as green cardamon, or often referred as "queen of all spices" is a well-known spice. Its seeds have been used in Indian culinary practice since antiquity to bring flavour to wide array of products like curries, coffee, cakes, breads, sweet dishes, and drinks. This plant is widely cultivated in India, Sri Lanka, but also Morocco, where it has an abundant use in cooking (Singh et al., 2008).
- ***Etlingera littoralis* (J.Koenig) Giseke** is a tropical plant belonging to *Etlingera* genus within Zingiberaceae family, which is commonly known as "golden star torch ginger" for its remarkable orange to red blooms with yellow margins. In Malaysia, the hearts of young shoots, inflorescences and fruits are consumed by indigenous communities as condiments, consumed raw or cooked as a vegetable. In Thailand, fruits are edible, and the young stems are eaten raw or cooked after removing the outer parts (Chan et al., 2013)
- ***Kaempferia galanga* L.** commonly known as black galangal or "kenjur" is another plant from the Zingiberaceae family. The aromatic rhizome of this plant has traditionally been used as spice and as a part of herbal mixture that ensures body vitality. Furthermore, this plant has a wide application in traditional medicine, including cough treatment, arthritis alleviation, body detoxication and many others (Sudatri et al., 2019).
- ***Piper nigrum* L. 'Kampot'** is one of Cambodia's most renowned spices, garnering international recognition for its exceptional quality. It is a cultivar of *P. nigrum* L. grown

in the Kampot province, which has unique climatic and soil conditions, giving the pepper its distinctive aroma and flavour compared to other types of peppercorns. Its exportation to Europe originated in 1870, during the French Protectorate, highlighting its long-standing reputation for outstanding sensory properties. Currently, four different types of Kampot pepper can be found on the market: green, black, red, and white. Although all Kampot peppers have an excellent reputation regarding their sensory properties, red peppercorns are particularly rare due to the intricate process involved in their production. Harvested at full maturity, the red berries undergo blanching, sun-drying, and meticulous manual sorting (Morm et al., 2020; Verner et al., 2022).

- ***Syzygium aromaticum* (L.) Merr. & L.M.Perry** is an evergreen tree native to Moluccas islands in Indonesia, belonging to Myrtaceae family. Its buds, commonly known as cloves, serves as a popular spice and condiment worldwide. Furthermore, it is utilized to bring aroma and flavour to "Kretek" cigarettes. In addition, its essential oil is used for many health benefits and addresses various ailments, including dental carries (Das et al., 2021).
- ***Zingiber zerumbet* (L.) Sm** commonly referred as "shampoo ginger" also belongs to the Zingiberaceae family. Its rhizome is used as spice, food flavouring and appetizer in various Southeast Asian cuisines. Furthermore, the rhizome has a wide array of use in traditional medicine for treatment of various ailments. The flower head is used in production of shampoos and conditioners (Yob et al., 2011).

Traditional use as spices as both culinary ingredients and in traditional medicine systems hints at their potential antimicrobial properties due to the presence of volatile compounds. A detailed examination of the *in vitro* growth inhibitory effects of EOs and CO_2 extracts isolated from these spices, both in liquid and vapour phases, has been undertaken. Moreover, the chemical profiles of EOs and CO_2 extracts from phytochemically less explored spice species have been analysed. Uncovering the antimicrobial potential of these natural extracts against foodborne bacterial pathogens would shed light on their potential applications in food preservation and safety. Additionally, investigating the chemical composition of the EOs and CO_2 extracts from various spice species could provide insights to the diverse array of chemical constituents present and facilitate a comparison of two extraction methods.

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3. HYPOTHESES

1. Volatile agents isolated by hydrodistillation and supercritical CO_2 extraction from Southeast Asian spices will differ in their physico-chemical properties;
2. Volatile agents present in plant parts used in Southeast Asia as spices and food condiments will produce antibacterial effects against food pathogens in liquid and/or vapour phase;
3. Antibacterial efficacy of spice EOs, CO_2 extracts and their vapours will be related to the differences in their chemical compositions;
4. Phytochemical analysis of less explored species of Cambodian spices will reveal new chemical aspects of their volatile agents.

4. RESEARCH QESTIONS

1. What are the main differences in physico-chemical properties of EOs and supercritical CO_2 extracts isolated from spice species originated in Southeast Asia?

2. Which EOs and CO_2 extracts obtained from spices traditionally used in South Asian foods produce antibacterial effects of in liquid and vapour phase?

3. What are the dominant compounds of the most effective spice EOs, CO_2 extracts and their vapours?

4. What are the chemical profiles of phytochemically less explored Cambodian spices?

5. OBJECTIVES

The main aim of the study is determination of chemical composition of EOs and supercritical CO_2 extracts isolated from spices of South-East Asian origin and evaluation of their *in vitro* growth-inhibitory effects against food pathogenic bacteria in liquid and vapour phase.

The specific objectives are:

1. comparison of physico-chemical properties of EOs and supercritical CO_2 extracts isolated from spices originated in Southeast Asia;
2. determination of antibacterial effects of isolated EOs and CO_2 extracts in liquid and vapour phase using broth microdilution volatilisation method;
3. GC-MS analysis of the most effective antibacterial EOs and CO_2 extracts and their vapours using two columns of different polarity in liquid and vapour phase;
4. GC-MS analysis of chemical composition of EOs and supercritical CO_2 extracts isolated from phytochemically less explored Cambodian spices using two columns of different polarity.

6. MATERIALS AND METHODS

6.1. Plant materials and sample preparation

Samples of *A. subulatum*, *C. cassia*, *C verum*, *E. cardamomum* and *S. aromaticum*, internationally sought-after spices with origin in various parts of Asia have been purchased from different suppliers in the Czech Republic in local spice stores (Ex Herbis, Prague, CZ, U Salvatora, Prague, CZ). Samples of *Alpinia officinarum*, *Amomum kravanh*, *B. rotunda*, *Citrus. hystrix*, *Curcuma zedoaria*, *Etlingera littoralis*, *K. galanga*, *Piper nigrum* 'Kampot' and *Zingiber zerumbet*, less phytochemically explored Cambodian spices, were obtained in Cambodia during the expedition in 2019 either in local markets or collected from the wild populations as described in Table 1. Samples of *A. sativum* and *A. rusticana* were used as a positive control for their distinct origin and previously known antimicrobial effect, even in vapour phase. Dried plant material was ground and homogenised using a Grindomix apparatus (GM 100, Retsch, Haan, DE). Subsequently, residual moisture content was evaluated gravimetrically at 130 °C for 1 h using a Scaltec SMO 01 analyser (Scaltec Instruments, Gottingen, DE) according to the AOAC (2002).

Table 1: Botanical characteristics and origin of tested spices

Plant species	**Family**	**Plant part**	**Source (voucher specimen No. when collected)**
Allium sativum L. **(PC)**	Alliaceae	Bulbs	Billa, spol. s r.o. (Prague, CZ)
Alpinia officinarum Hance	Zingiberaceae	Rhizome	La Plantation, (Kampot, KH)
Amomum kravanh Pierre ex. Gagnep.	Zingiberaceae	Fruits	Orussey market (Phnom-Penh, KH)
Amomum subulatum Roxb.	Zingiberaceae	Seeds	Prodejna u Salvatora (Prague, CZ)
Armoracia rusticana P.Gaertn., B.Mey.& Scherb **(PC)**	Brassicaceae	Root	Prodejna u Salvatora (Prague, CZ)
Boesenbergia rotunda (L.) Mansf.	Zingiberaceae	Rhizome	Orussey market (Phnom-Penh, KH)
Cinnamomum cassia (L.) J.Presl bark	Lauraceae	Inner bark	Prodejna u Salvatora (Prague, CZ)
Cinnamomum cassia (L.) J.Presl fruits	Lauraceae	Fruits	Ex Herbis (Prague, CZ)
Cinnamomum verum J.Presl	Lauraceae	Inner bark	Prodejna u Salvatora (Prague, CZ)
Citrus hystrix DC	Rutaceae	Fruit pericarp	Stung Treng market
Curcuma zedoaria (Christm.) Roscoe	Zingiberaceae	Rhizome	Orussey market (02642KBFR5)
Elettaria cardamomum (L.) Maton	Zingiberaceae	Seeds	Prodejna u Salvatora (Prague, CZ)
Etlingera littoralis (J.Koenig) Giseke	Zingiberaceae	Flower	Sen Monorom (02644KBFR4)
Kaempferia galanga L.	Zingiberaceae	Rhizome	Orussey market

Piper nigrum L. 'Kampot'	Piperaceae	Seeds	La Plantation, (Kampot, KH)
Syzygium aromaticum (L.) Merr. & L.M.Perry	Zingiberaceae	Flower buds	Prodejna u Salvatora (Prague, CZ)
Zingiber zerumbet (L.) Sm.	Zingiberaceae	Rhizome	Sen Monorom (02640KBFR3)

PC: positive control

6.2. Hydrodistillation of EOs

EOs were extracted by hydrodistillation of 100 g of pulverized plant materials in one litre of distilled water for 3 hrs using Clevenger-type apparatus (Merci, Brno, CZ). The distillation rate was 2-3 ml of liquid/ml as described in European Pharmacopoeia (EDQM, 2013). Since hydrodistillation belongs to the most utilized methods for the commercial production of EOs, the properties of those prepared in this investigation should be alike to those commercially available. All EOs were stored in 2 ml sealed glass vials at 4 °C until further use.

6.3. Supercritical CO_2 extracts preparation

Supercritical CO_2 extraction was carried out using a Spe-ed SFE helix system (Applied Separations, Allentown, PA, USA). Initially, 10 g of ground material was placed into a 100 ml stainless steel extraction vessel between a glass wool bilayer. Subsequently, the filled vessel was installed into the extraction module and the extraction process was conducted using following parameters: isocratic pressure 200 bar, temperature 40 °C and flow rate of 5 litres per minute. The extraction continued until no more CO_2 extracts were obtained (assessed by visual confirmation) whereas total extraction time ranged from 5 to 12 mins. Since this study was a first screening of potential use of CO_2 extraction for spices, parameters recommended in the literature have been chosen. Following the extraction, extracts were captured in 60 ml glass collection vials (Applied Separations, Allentown, PA, USA) and stored in 2 ml sealed glass vials at 4 °C until further utilisation.

6.4. Bacterial strains and culture media

Following standard strains of the American Type Culture Collection (ATCC) were used: *B. cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644 and *Salmonella enterica* Typhimurium ATCC 14028. Both cultivation and assay media (broth/agar) were Mueller-Hinton complemented by defibrinated horse blood in case of *L. monocytogenes*

agar. The pH of broths was equilibrated to a final value of 7.6 using Trizma base (Sigma-Aldrich, Prague, CZ). All microbial strains, growth media and their additions were purchased from Oxoid (Basingstoke, Hampshire, UK).

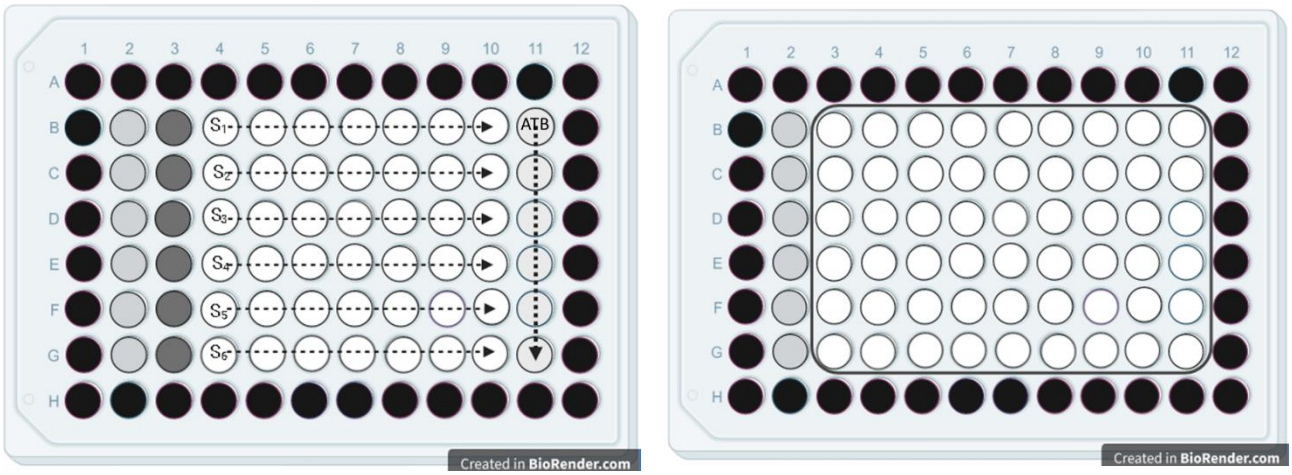
Stock cultures of bacterial strains were cultivated in appropriate medium at 25 °C for 24 hrs prior to testing and then the turbidity of bacterial suspension was adjusted to 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, CZ) to reach the final concentration of 10^7 CFU/ml. Since the conventional antibiotics are not volatile, susceptibility of tested bacteria to amoxicillin (90%, CAS: 26787-78-0) x *E. coli*, ampicillin (84.5%, CAS: 69-52-3) x *L. monocytogenes*, chloramphenicol (98%, CAS: 56-75-7) x *B. cereus* and tetracycline (98%, CAS: 60-54-8) x *S. e.* Typhimurium (Sigma-Aldrich, Prague, CZ) were checked as a positive antibiotic control in liquid phase only.

6.5. Antimicrobial assay

The *in vitro* growth inhibitory effect of spice EOs and supercritical CO_2 extracts in liquid and vapour phase was assessed using broth microdilution volatilisation method (Houdkova et al., 2017) with slight modifications for testing complex EOs (Houdkova et al., 2018a). Experiments were performed on standard 96-well microtiter plates (well volume = 400 µl) covered by tight-fitting lids with flanges designed to reduce growth media evaporation (SPL Life Sciences, Naechon-Myeon, KR). With aim to test vapour phase of this method, 30 µl of melted agar was transferred using electronic pipette (Xplorer, Eppendorf, DE) into every flange of the lid except the outermost flanges and inoculated with 5 µl of bacterial suspension after solidification of agar on its surface. In one column, flanges with agar were not inoculated (sterility controls). Subsequently, inoculated lids were left until further handling while liquid phase preparations were carried out. For evaluation of liquid phase, microtiter plate was prepared in the same way as in case of standard broth microdilution method (CLSI, 2015). Each sample of EOs and supercritical CO_2 extracts was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Prague, CZ), which was used at maximum concentration of 1%, and diluted in an appropriate broth medium. The multiplate design was used for testing of samples, while seven two-fold serially diluted concentrations starting from 1024 µg/ml were prepared for all EOs and supercritical CO_2 extracts. Since this concentration can be taken as an indicative value only (antimicrobial agent is distributed between liquid and solid matrix, and therefore, its particular concentrations in agar and broth media are always lower than 1024 µg/ml), this approach is in

consonance with generally accepted rules for evaluation of antimicrobial potential of plant extracts and EOs suggesting that agents with MIC values higher than 1000 μg/ml should be considered as ineffective and avoided from process of further evaluation (Cos et al., 2006; Kokoska et al., 2019; Rios & Recio, 2005). The final volume of liquid in each well was 100 μl. The plates were subsequently inoculated with bacterial suspension using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, CZ). Wells containing inoculated and non-inoculated broth were prepared as growth and sterility controls simultaneously. As a positive control, appropriate antibiotic was arranged in a separate column. All outermost wells were left empty to prevent edge effect. Finally, clamps (Lux Tool, Prague, CZ) were used to fasten lids and plates together, with the handmade wooden pads (size 8.5 × 13 × 2 mm) for better fixing and the microtiter plates were incubated at 25°C for 24 hrs. Following day, MICs were evaluated by visual assessment of bacterial growth after colouring of metabolically active bacterial colonies with MTT dye at a concentration of 600 μg/ml (Sigma-Aldrich, Prague, CZ) using electronic pipette (Xplorer, Eppendorf, DE) with 20 μl for the lid and 25 μl for the plate. When the interface of colour changed from yellow to purple (relative to that of colours in control wells and flanges), it was recorded both in agar and broth rows. DMSO used as negative control at a concentration of 1% did not inhibit any of the strains tested either in broth or agar growth media. All experiments were set in triplicates as three independent measurements. Schematic layouts of plate and lid can be seen in Figure 1a and 1b.

For liquid phase, MIC vas determined as the lowest value that inhibited bacterial growth compared to the compound free growth control and expressed in μg/ml (1024, 512, 256, 128, 64, 32, 16 and 8). Results of vapour phase measurements were expressed in the same way, however, due to the psychochemical properties of volatile compounds, should be taken as indicative values only. Theoretically, if volatile compounds are distributed evenly throughout the well between liquid and gaseous phase, concentrations could be expressed as weight of volatile agent per volume unit of the well. In that case, their real values would be 256, 128, 64, 32, 16, 8, 4, and 2 μg/cm^3 for 1,024; 512; 256; 128; 64; 32; 16, and 8 μg/ml, respectively. The mode and median were used for the final value calculation when the triplicate endpoints were within the two- and three-dilution range, respectively.



(a) **(b)**

Figure 1 Schematic layout of multiplate design for plate (a) and lid (b) of broth microdilution volatilisation method

Plate (a): Black wells: empty wells, Grey wells (column 2): sterility control, Dark grey wells: growth control, white wells: samples 1-6 two-fold serial dilutions starting from 1024 µg/ml to 8 µg/ml, Light grey wells (column 11): Positive antibiotic control two-fold serial dilutions

Lid (b): Black flanges: Empty flanges, Grey flanges: purity control (containing agar only), White flanges: inoculated flanges (containing both agar and bacterial suspension)

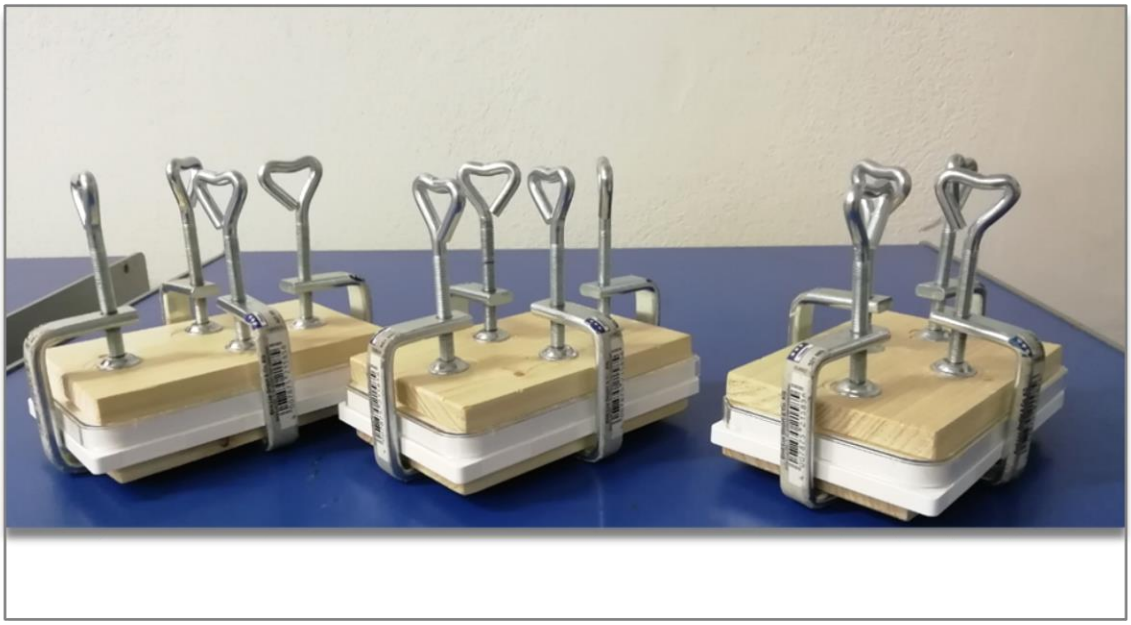
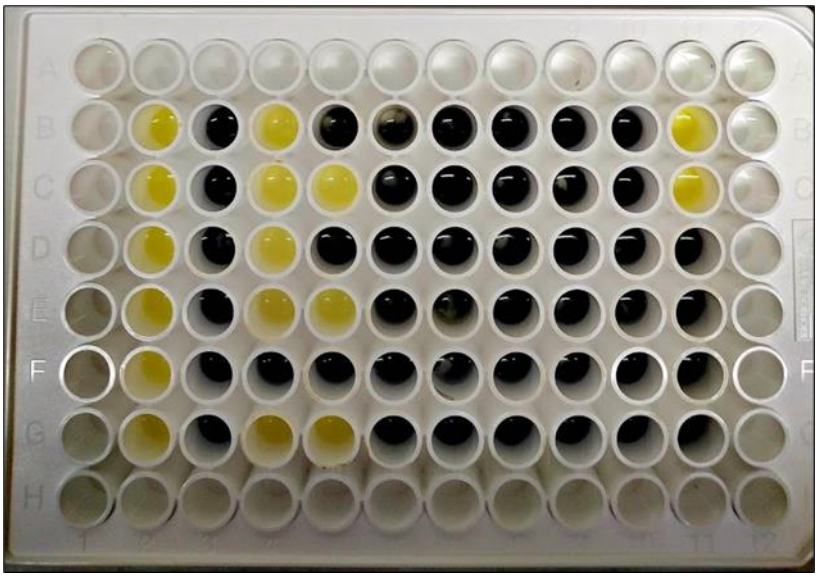
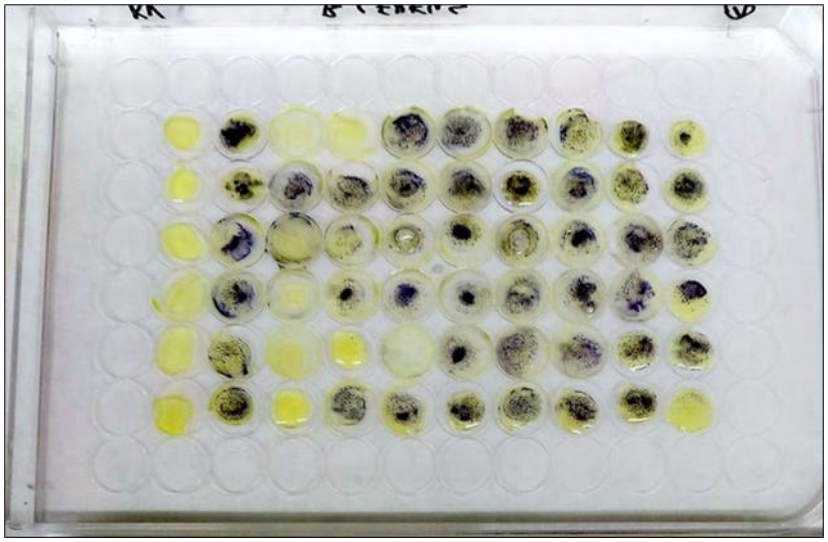


Figure 2: Clamps and wooden pads for fastening lids and plates together prior to incubation



(a)



(b)

Figure 3: Visual evaluation of living colonies of *B. cereus* with MTT dye on (a) plate and (b) lid

Purple wells (flanges): infected medium, yellow wells (flanges): non-infected medium, white wells (flanges): unused

6.6. GC–MS analysis

For the determination of chemical composition of the EOs and supercritical CO_2 extracts, GC-MS analysis has been performed using a non-polar HP-5 column and a polar DB Heavy WAX column. An Agilent GC-7890B system was utilized, which is equipped with auto sampler Agilent 7,693, two columns, a fused-silica HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 19091s-433) and a DB-Heavy WAX (30 m × 0.25 mm, film thickness

0.25 µm, Agilent 122–7132), and a FID coupled with single quadrupole mass selective detector Agilent MSD-5977B (Agilent Technologies, Santa Clara, CA, USA). Helium was used as a carrier gas at a flow rate of 1 ml/min and the injector temperature was set to 250 °C for both columns. The oven temperature was raised for both columns after 3 min, from 50 to 280 °C. Initially, the heating velocity was 3 °C/min, until the system reached 120 °C. Subsequently, the velocity increased to 5 °C/min until a temperature of 250 °C was reached, and after 5 min holding time, the heating speed reached 15 °C/min until 280 °C was finally obtained. Heating was followed by a 20 min isothermal period. Samples of EOs and supercritical CO_2 extracts were diluted in *n*-hexane for GC–MS (Merck KGaA, Darmstadt, DE) at a concentration of 20 µl/ml. An amount of 1 µl of the solution was injected in split mode in a split ratio of 1:30. The mass detector was set to the following conditions: ionization energy 70 eV, ion source temperature 230 °C, scan time 1 s, and mass range 40–600 *m*/*z*.

6.7. Identification of constituents, quantification, and statistical analysis

Identification of constituents was based on a comparison of their retention indices (RI), retention time (RT) and mass spectra with those to the National Institute of Standards and Technology Library ver 2.0.f (NIST) as well as in the literature. The certain identified compounds were confirmed by co-injection of authentic standards, namely benzaldehyde (99%, CAS: 100-52-7), borneol (97%, CAS: 464-45-9), bornyl acetate (95%, CAS: 5655-61-8), camphene (97.5%, CAS: 79-92-5), β-caryophyllene (80%, CAS: 87-44-5), carvone (99%, CAS: 6485-40-1), cinnamaldehyde (95%, CAS: 104-55-2), α-humulene (96%, CAS: 6753-98-6), linalool (97%, CAS: 78-70-6), α-phellandrene (95%, CAS: 4221-98-1), α-pinene (99%, CAS: 7785-70-8), β-pinene (99.0%, CAS: 18172-67-3), and γ-terpinene (97%, CAS: 99-85-4) (Sigma-Aldrich, Prague, CZ). The RI were calculated for the compounds separated by the HP-5 column using the RT of the *n*-alkanes series ranging from C_8 to C_{40} (Sigma-Aldrich, Prague, CZ). For each analysed EO and CO_2 extract, the final number of individual constituents was computed as the sum of components simultaneously identified using both columns and the remaining compounds detected by individual columns only. Quantitative data are expressed as the relative percentage content of the constituents determined by FID. Chemical analysis of Cambodian EOs and CO_2 extracts was performed in triplicates and the relative peak area percentages were expressed as the mean average of these three independent measurements ± standard deviation.

6.8. HS-SPME

Chemical analysis of the headspace above the mixture of MH broth and *C. cassia* fruits EO at a concentration 256 µg/ml (the lowest MIC value obtained during antimicrobial assay) was conducted using HS-SPME sampling technique. Sampling was performed using fibre coated with 50/30 µm layer of divinylbenzene/carboxen/polydimehylsiloxane (SUPELCO, Bellefonte, PA). For time series HS sampling, a set of 5 samples was prepared, intended for 0, 3, 6, 9 and 12 hrs of incubation. Volume of 2 ml of the mixture was introduced into a 4 ml glass vial and except for the first sample (t = 0), all vials with samples were placed in an oven and incubated at 25 °C until they were analysed after 3, 6, 9, and 12 hrs of incubation. The needle of HS-SPME assembly was inserted into the vial and fibre was exposed for adsorption of volatile compounds at 25 °C for 15 min. Grey fibre was subsequently removed from the vial and inserted into a GC injector where the desorption of analytes was carried out. The injector temperature was 250 °C and fibre was retained in the injector for the whole analysis duration until the next measurement. Measurements have been repeated each 3 hrs in 12 hrs total period of incubation. Identification of constituents was carried out as described above in chapter 6.7. and quantification is expressed as a relative percentage content of compounds determined by the FID.

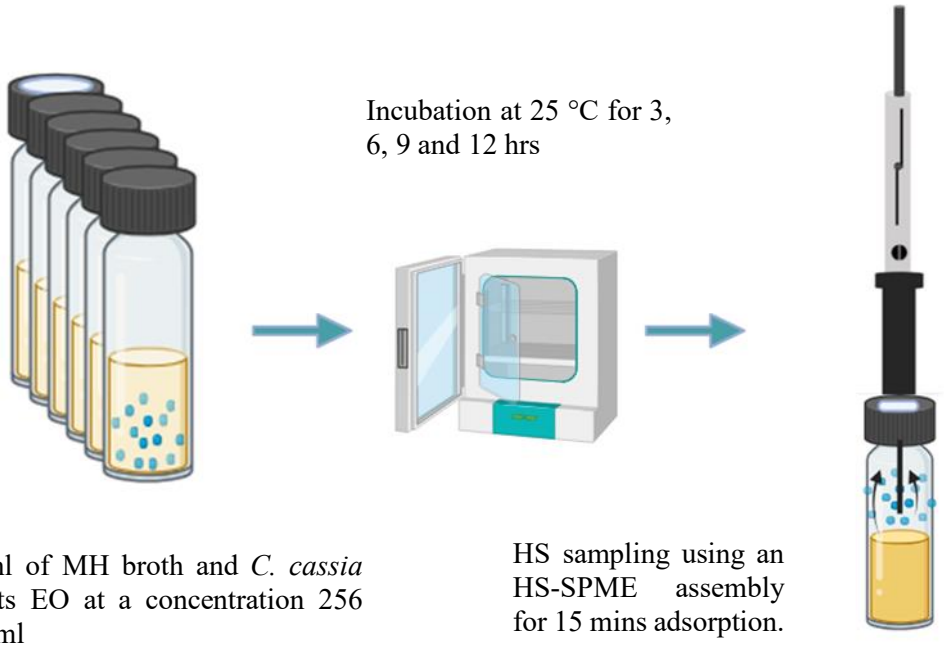


Figure 4: Preparation of samples and HS-SPME sampling of volatile constituents above the lowest MIC obtained in antimicrobial assay

7. RESULTS

7.1. Physicochemical characteristics of EOs and CO_2 extracts

In this investigation, dried plant material from 16 spice species originating in Asia was obtained either commercially (local spice stores, traditional markets or farms) or collected from the wild. In total, 13 EOs were isolated from 12 species by hydrodistilaltion and the yields exhibited significant variations between species, as seen in Table 2. The highest yielding plants were representatives from the Zingiberaceae family, namely *A. kravanh* and *E. cardamomum* with respective yields 5.22 and 4.7%. On the other hand, the lowest yielding species were *A. rusticana* and *A. sativum* with their respective yields 0.18 and 0.21%. In case of *B. rotunda*, *E. littoralis*, *K. galanga,* and *Z. zerumbet*, EOs were not obtained as there was insufficient material available due to various reasons. In case of *B. rotunda* and *Z. zerumbet*, rhizome got contaminated by mould during the transportation. For *E. littoralis* and *K. galanga*, material available on location was limited. Using the SFE, 17 extracts were obtained from 16 species and their yields also presented substantial variations. The highest yields were obtained by SFE from *S. aromaticum* and *E. littoralis* with respective yields 11.96 and 10.12%, followed by *C. hystrix* (8.35%). Species with the lowest yields were *A. sativum* (0.36%) and *A. rusticana* (0.42%). In general, higher yields were obtained by SFE than by hydrodistillation, with exception of *A. kravanh*, *C. cassia* fruits, *E. cardamomum,* and *P. nigrum* 'Kampot'.

Variations were also observed in terms of physical properties and appearances of EOs and CO_2 extracts. For instance, *S. aromaticum* yielded translucent EO, but its CO_2 extract was of vivid yellow colour. Similarly, *C. zedoaria* produced intense yellow EO and dark lime-green extract. Generally, EOs colour warried between translucent and intense yellow, but extracts displayed more distinctive colours. Broad spectrum colours ranging from white and intense yellow to orange and green have been observed. Interestingly, CO_2 extract from *Z. zerumbet* displayed expressive, blood red colour. In terms of density of EOs, all isolated EOs exhibited a lower density than water, with exception of EO from *C. cassia* bark and *S. aromaticum*. Furthermore, the consistency of extracts from *A. kravanh*, *A. sativum, A. rusticana*, *C. cassia* fruits, *C. verum*, *K. galanga* and *Z. zerumbet* has been classified as semi-solid to solid.

Table 2: Yields and physical properties of EOs and CO_2 extracts

Plant species	Dry matter (%)	Yield		Description	
		EO	CO_2 extract	EO	CO_2 extract
Allium sativum	70.46	0.21	0.36	pale yellow	intense yellow
Alpinia officinarum	80.61	1.24	3.72	translucent	yellow orange
Amomum kravanh	84.21	5.22	1.20	translucent	white semi solid
Amomum subulatum	75.53	1.77	4.25	translucent	intense yellow
Armoracia rusticana	80.91	0.18	0.42	translucent	pale yellow
Boesenbergia rotunda	66.20	n.d.	2.82	n.d.	pale yellow semi solid
Cinnamomum cassia bark	84.06	0.90	1.66	pale yellow	intense yellow
Cinnamomum cassia fruits	81.42	2.25	1.08	pale yellow	intense yellow
Cinnamomum verum	85.13	0.47	0.60	pale yellow	yellow orange
Citrus hystrix	77.49	3.89	8.35	translucent	greenish yellow
Curcuma zedoaria	79.06	2.64	4.03	pale yellow	intense yellow
Elettaria cardamomum	76.55	4.70	2.86	pale yellow	greenish yellow
Etlingera littoralis	80.63	n.d.	10.12	n.d.	pale grey
Kaempferia galanga	77.26	n.d.	5.87	n.d.	yellow orange semi solid
Piper nigrum L. 'Kampot'	85.61	2.57	1.88	pale yellow	intense orange
Syzygium aromaticum	72.78	3.30	11.96	translucent	intense yellow
Zingiber zerumbet.	88.17	n.d.	0.94	n.d.	bloody red

Footnotes: n.d.: not determined

7.2. Antibacterial effect of spice EOs and supercritical CO_2 extracts in liquid and vapour phase

In the following stage of this investigation, *in vitro* growth-inhibitory effect against four food pathogenic bacterial strains has been assessed. The tested strains encompassed two Gram-positive strains *B. cereus* and *L. monocytogenes* and two Gram-negative species *E. coli* and *S. enterica* serovar Typhimurium. The MIC values acquired during broth microdilution volatilisation assay for liquid and vapour phase are presented in the Table 3. Results of this screening revealed variable degrees of antibacterial effects against tested food bacterial pathogens.

Among all EOs tested, 6 EOs produced certain degree of antimicrobial effect, *C. cassia* bark and fruits and *C. verum* showed the strongest growth-inhibitory effects with MICs ranging from 256 to 512 μg/ml in liquid and from 256 to 1024 μg/ml in vapour phase. The lowest MIC values (256 μg/ml) in broth were observed in case of *C. cassia* bark EO against *E. coli*, *L. monocytogenes,* and *S. e*. Typhimurium. In agar, *C. cassia* fruits EO was the most effective against *B. cereus* and *L. monocytogenes* and displayed MIC 256 μg/ml. Weak antibacterial effect was observed in EOs acquired from *A. rusticana* (tested as a positive spice control) and *S. aromaticum*, displaying MIC 1024 μg/ml against most tested bacteria. *C. zedoaria* EO also showed negligible growth inhibitory effect against *B. cereus* and *L. monocytogenes*. Among all supercritical CO_2 extracts tested, 3 exhibited considerable degree of antibacterial activity. *C. cassia* bark, *C. cassia* fruits and *C. verum* had the greatest inhibitory effects against all tested bacteria from all extracts and their MIC values ranged from 256 to 1024 μg/ml in both liquid and vapour phase. The most effective extract was obtained from *C. cassia* bark, and its effect was even slightly stronger than in case of EO. In case of *C. verum*, the antimicrobial effect of CO_2 extract was also higher in agar (512 μg/ml) than in case of the EO (1024 μg/ml) against most tested bacteria. A negligible antimicrobial activity was also observed in *S. aromaticum* extract, that inhibited all tested bacterial strains at a concentration of 1024 μg/ml only in liquid phase. Extract acquired from *A. officinarum* inhibited only *B. cereus* at the same concentration in liquid phase only. In broth, *B. cereus*, *E. coli* and *L. monocytogenes* (MICs 256-512 μg/ml) were slightly more susceptible than *S. e.* Typhimurium (MICs 256-1024 μg/ml) when exposed to the most effective EOs and extracts. In agar, similar pattern of bacterial susceptibility was observed, while MICs for *B. cereus*, *E. coli,* and *L. monocytogenes* ranged from 256 to 1024 μg/ml, while MICs for *S. e.* Typhimurium were ranging from 512 to 1024μg/ml.

Table 3: *In vitro* growth inhibitory effect of spice essential oils (EOs) and CO_2 extracts against food pathogens in liquid and vapour phase

Species	Extraction method/Bacteria/Growth medium/MIC (µg/ml)															
	EOs								CO_2 extracts							
	B. cereus		*E. coli*		*L. monocytogenes*		*S. e.* Typhimurium		*B. cereus*		*E. coli*		*L. monocytogenes*		*S. e.* Typhimurium	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
Alpinia officinarum	-	-	-	-	-	-	-	-	1024	-	-	-	-	-	-	-
Amomum kravan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amomum subulatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Boesenbergia rotunda	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-
Cinnamomum cassia bark	512	512	256	256	256	1024	256	512	256	512	512	512	256	256	512	512
Cinnamomum cassia fruits	256	256	512	512	256	256	512	512	512	512	512	1024	512	512	1024	1024
Cinnamomum verum	512	1024	512	512	512	1024	512	1024	256	512	512	1024	512	512	512	512
Citrus hystrix	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Curcuma zedoaria	1024	1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Elettaria cardamomum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Etlingera littoralis	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-
Kaempferia galanga	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-
Piper nigrum L. 'Kampot'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Syzygium aromaticum	1024	1024	1024	1024	1024	1024	1024	-	1024	-	1024	-	1024	-	1024	-
Zingiber zerumbet	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-
Positive antibiotic control																
Amoxicilin	NT	NT	8	>8	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Ampicilin	NT	NT	NT	NT	0.5	>1	NT	NT	NT	NT	NT	NT	0.5	>1	NT	NT
Chloramphenicol	4	>8	NT	NT	NT	NT	NT	NT	4	>8	NT	NT	NT	NT	NT	NT
Tetracycline	NT	NT	NT	NT	NT	NT	4	>32	NT	NT	1	>4	NT	NT	2	>32
Positive spice control																
Allium sativum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Armoracia rusticana	1024	1024	1024	1024	-	-	1024	1024	-	-	-	-	-	-	-	-

Footnotes: NT: not tested (in main table body: this type of extract was not obtained for lack of material reason and thus not tested, in positive ATB control: this ATB is not used as a control for such bacterial strain), -: Not determined =>1024 µg/ml with no further MIC specification, B: Broth-liquid phase, A: Agar-vapour phase

7.3. Chemical composition of EOs, supercritical CO_2 extracts and their vapours of *Cinnamomum* spp. barks and fruits

In this step of the investigation, chemical composition of three EOs and three CO_2 extracts isolated from *Cinnamomum* species has been determined together with the vapours of most effective antimicrobial agent. Complete chemical analyses in liquid phase of all samples are provided in Table 4-6 and the time-series headspace analysis of *C. cassia* fruits EO at a concentration 256 μg/ml is provided in the Table 7.

In EOs obtained from *C. cassia* bark and fruits and *C. verum* bark, a total of 23, 42, and 51 constituents have been identified during an H5-5 column analysis, representing 99.05, 99.20, and 99.33% of their total respective contents. When using a DB-HeavyWAX column, 29, 47, and 53 compounds have been determined amounting to 99.07, 99.26, and 98.97% of the total oils, respectively. Total respective numbers of identified constituents in EOs were 30, 56, and 61 in *C. cassia* bark and fruits and *C. verum* bark. In CO_2 extracts isolated from *C. cassia* bark and fruits and *C. verum* bark, a total number of 23, 30, and 27 compounds have been discovered using HP-5 column accounting for 99.59, 99.75, and 98.26% of their total respective content. During a DB-HeavyWAX column analysis, a total number of 24, 46, and 43 compounds have been discovered that amounted to 99.83, 99.73, and 99.77 of the total extracts. Total numbers of constituents were 34 for *C. cassia* bark and 52 for both *C. cassia* fruits and *C. verum* bark. Aldehydes, primarily (*E*)-cinnamaldehyde, emerged as the predominant chemical group in all analysed samples, followed by sesquiterpenoids and esters. Additionally, various other compounds such as monoterpenoids, ketones, carboxylic acids, fatty acid derivatives, diterpenoids, and sterols were identified, although no derivatisation step has been performed prior to analyses. A key disparity between EOs and supercritical extracts lay in the higher concentration of monoterpenoids in EOs, along with the detection of minor quantities of fatty acid derivatives across all CO_2 extracts.

In EO extracted from the bark of *C. cassia*, (*E*)-cinnamaldehyde has been detected as the most abundant compound constituting 86.15/85.19% of the total sample when measured with HP-5/DB-HeavyWAX columns, respectively. Following (*E*)-cinnamaldehyde, cinnamyl and bornyl acetates ranked as second and third most abundant constituents with percentage values of 5.61/4.5 and 2.19/1.59% when measured by HP-5/DB-HeavyWAX, respectively. In CO_2 extract, (*E*)-cinnamaldehyde comprises 69.26/63.88% of the total sample. Notably, while

coumarin is only detected in EO by a DB-WAX column at 0.4%, the supercritical CO_2 extract contains significantly higher amount, ranking as the second most abundant constituent at 16.57% when identified by an HP-5 column, followed by cinnamyl acetate (3.99%). Contrastingly, cinnamyl acetate was the second most abundant component (10.46%) of the extract, followed by coumarin (4.15%) when analysed by a DB-WAX column.

Comparatively, both EO (89.36/88.91%) and CO_2 (87.15/89.17%) extract acquired from fruits of *C. cassia* contained the highest amounts of (*E*)-cinnamaldehyde among all samples. The contents of the following two compounds, 2-methoxycinnamaldehyde and δ-cadinene, are similar for both EO and CO_2 extract. In the EO, 2-methoxycinnamaldehyde is present at 1.03/1.28%, followed by δ-cadinene with percentage values of 0.82/1.01%. Slightly different values are obtained for 2-methoxycinnamaldehyde (3.96/1.3%) and for δ-cadinene (1.36/0.96%) in CO_2 extract. γ-Muurolene emerged as the last compound with a percentage value higher than one (1.01%) when determined by an HP-5 column.

In EO obtained from *C. verum*, the percentage of (*E*)-cinnamaldehyde was significantly lower than in samples isolated from *C. cassia* (49.79/49.19). Nonetheless, (*E*)-cinnamaldehyde still represents 73.39/58.58% of the CO_2 extract, values not far from those of *C. cassia* samples. Other components present in both EO and CO_2 extract in larger amounts include cinnamyl acetate with percentage values of 22.68/23.1% and benzyl benzoate (4.8/5.5%). Values for CO_2 extracts are slightly lower, with 8.15/10.56% for cinnamyl acetate and 2.63/2.74% for benzyl benzoate.

In case of EO isolated from fruits of *C. cassia*, the composition of a headspace above the mixture of *C. cassia* fruit EO at a concentration 256 μg/ml and MH broth has been measured in a time series every 3 hrs over a 12-hour period. Using the HP-5 column, the total number of components ranged from 49 to 59, representing 90.86% to 96.73% of the total samples, while with the DB-HeavyWAX column, the number of constituents varied from 45 to 52, representing 86.62% to 96.3% of their contents. The predominant chemical group in the headspace comprised sesquiterpene hydrocarbons, primarily δ-cadinene, γ-muurolene, and δ-cadinene, along with aldehydes, represented by (*E*)-cinnamaldehyde and benzaldehyde. Other chemical categories present in minor amounts in the headspace included monoterpenoids, esters, and aliphatic or cyclic hydrocarbons. Similar to its presence in the EO, (*E*)-cinnamaldehyde was the major component of the headspace at time 0 (23.11%/16.35% using HP-5/DB-WAX columns). However, during the sample incubation, the amount of this aldehyde gradually decreased to 6.39%/3.74% after 12 hrs. Contrastingly, δ-cadinene exhibited the opposite trend, being present

at 8.18%/14.06% in freshly prepared samples and becoming the most abundant after 3 hrs of incubation, with its amount continuously increasing until it reached the highest percentage value of 12.94%/17.78% at 9 hrs. A similar pattern was observed for γ-muurolene, which was present at 5.77%/10.93% in freshly mixed samples, becoming the second predominant component after 6 hrs, with its amount progressively rising to 11.58%/14.43% of the sample after 12 hrs of incubation. The gradual changes in the contents of these three main compounds are depicted in

Table 4: Chemical composition of *C. cassia* bark essential oil and CO_2 extract

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
929	939	α-Pinene	MH	0.200	0.142	-	-	RI, GC-MS, Std	GC-MS, Std
943	945	Camphene	MH	0.323	0.241	-	-	RI, GC-MS, Std	GC-MS, Std
956	961	Benzaldehyde	A	0.790	0.947	-	0.370	RI, GC-MS, Std	GC-MS, Std
972	980	β-Pinene	MH	0.126	0.092	-	-	RI, GC-MS, Std	GC-MS, Std
1020	1024	*p*-Cymene	MH	0.092	0.118	-	-	RI, GC-MS	GC-MS
1025	1031	Limonene	MH	0.088	0.108	-	-	RI, GC-MS	GC-MS
1029	1033	Eucalyptol	MO	0.893	0.765	0.222	-	RI, GC-MS	GC-MS
1042	1036	Benzene acetaldehyde	A	0.097	0.180	-	-	RI, GC-MS	GC-MS
1164	1165	Endoborneol	MO	0.331	0.476	0.180	-	RI, GC-MS, Std	GC-MS, Std
1174	NA	2-Methylcumarone	K	-	-	0.245	-	GC-MS	-
1177	1177	Terpinen-4-ol	MO	-	-	0.306	0.241	RI, GC-MS	GC-MS
1189	1189	α-Terpineol	MO	0.190	-	0.640	0.700	RI, GC-MS	GC-MS
1219	1214	(*Z*)-Cinnamaldehyde	A	0.102	0.562	-	3.259	RI, GC-MS, Std	GC-MS, Std
1222	1228[a]	2-Hydroxycineole	MO	0.064	0.112	-	-	RI, GC-MS	GC-MS
1227	1227[b]	Bornyl formate	E	0.095	0.092	-	-	RI, GC-MS	GC-MS
1243	1242	Carvone	SH	0.324	0.365	-	0.284	RI, GC-MS, Std	GC-MS, Std
1277	**1270**	**(*E*)-Cinnamaldehyde**	**A**	**86.150**	**85.198**	**69.264**	**63.884**	**RI, GC-MS, Std**	**GC-MS, Std**
1286	**1287**	**Bornyl acetate**	**E**	**2.194**	**1.589**	**-**	**0.623**	**RI, GC-MS, Std**	**GC-MS, Std**
1311	1305	Cinnamyl alcohol	SO	-	-	0.733	0.690	RI, GC-MS	GC-MS
1354	1351	α-Cubebene	SH	-	-	0.260	-	RI, GC-MS	-
1374	NA	Cinnamic acid ethyl ester	E	-	-	0.525	-	GC-MS	-
1377	1376	α-Copaene	SH	0.226	0.242	1.713	0.642	RI, GC-MS	GC-MS
1419	1411	α-Bergamotene	SH	-	-	0.592	0.537	RI, GC-MS	GC-MS
1425	1418	β-Caryophyllene	SH	-	-	1.547	-	RI, GC-MS, Std	-
1450	**1429**	**Coumarin**	**K**	**-**	**0.403**	**16.573**	**4.150**	**RI, GC-MS**	**GC-MS**

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
1451	**1454**	**Cinnamyl acetate**	**E**	**5.611**	**4.505**	**3.993**	**10.459**	**RI, GC-MS**	**GC-MS**
1460	1440	α-Humulene	SH	-	-	0.166	-	RI, GC-MS, Std	-
1505	1500	α-Muurolene	SH	-	-	0.291	0.818	RI, GC-MS	GC-MS
1526	1528	Calamenene	SH	0.159	0.242	-	0.352	RI, GC-MS	GC-MS
1529	1524	δ-Cadinene	SH	-	-	0.627	0.766	RI, GC-MS	GC-MS
1589	1581	Caryophyllene oxide	SO	0.546	0.486	0.322	0.499	RI, GC-MS	GC-MS
1637	NA	Di-epi-1,10-cubenol	SO	-	-	0.262	0.107	GC-MS	GC-MS
1647	1653	α-Cadinol	SO	0.148	0.042	-	-	RI, GC-MS	GC-MS
1652	1636	δ-Cadinol	SO	0.099	0.179	-	10.459	RI, GC-MS	GC-MS
1668	NA	Cyclocopacamphenol	SO	-	-	0.207	-	GC-MS	-
1672	NA	α-Ylangene	SH	-	-	0.020	0.136	GC-MS	GC-MS
2134	NA	Equilin	S	-	-	0.647	-	GC-MS	-
2144	NA	Retinol acetate	DH	-	-	0.263	-	GC-MS	-
2228	NA	Bornyl cinnamate	E	0.207	0.143	-	-	GC-MS	GC-MS
-	1145	Camphene hydrate	E	-	0.050	-	-	-	GC-MS
-	1162	Benzenepropanal	A	-	0.236	-	-	-	GC-MS
-	1478	γ-Muurolene	SH	-	-	-	0.207	-	GC-MS
-	1641	τ -Muurolol	SO	-	0.126	-	0.221	-	GC-MS
-	1356	Eugenol	PP	-	-	-	0.302	-	GC-MS
-	NA	Limonene glycol	MO	-	0.173	-	-	-	GC-MS
-	1347	Hydrocinnamic acid	CA	-	0.148	-	-	-	GC-MS
-	1452	Cinnamic acid	CA	-	1.106	-	0.002	-	GC-MS
-	NA	Isopropyl palmitate	FAD	-	-	-	0.118	-	GC-MS
		Total identified [%]		**99.053**	**99.068**	**99.599**	**99.828**		

[a] RI = retention indices; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 2007), [a] (Apel et al., 2004), [b] (Priestap et al., 2003), NA = RI values not available in the literature, [c)] C = Class; A - Aldehydes, CA - Carboxylic acids, DH - Diterpene hydrocarbons, E - Esters, FAD - Fatty acid and fatty acid derivatives, K - Ketones, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, PP-Phenylpropanoids, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes, S – Sterols, [d)] Identification method: GC-MS = Mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching literature database; Std = constituent identity confirmed by co-injection of authentic standards [e)] Retention indices were not calculated for compounds detected only by DB-HeavyWAX column, - = not detected. **In bold** = main volatile compounds detected and total percentage of identified constituents

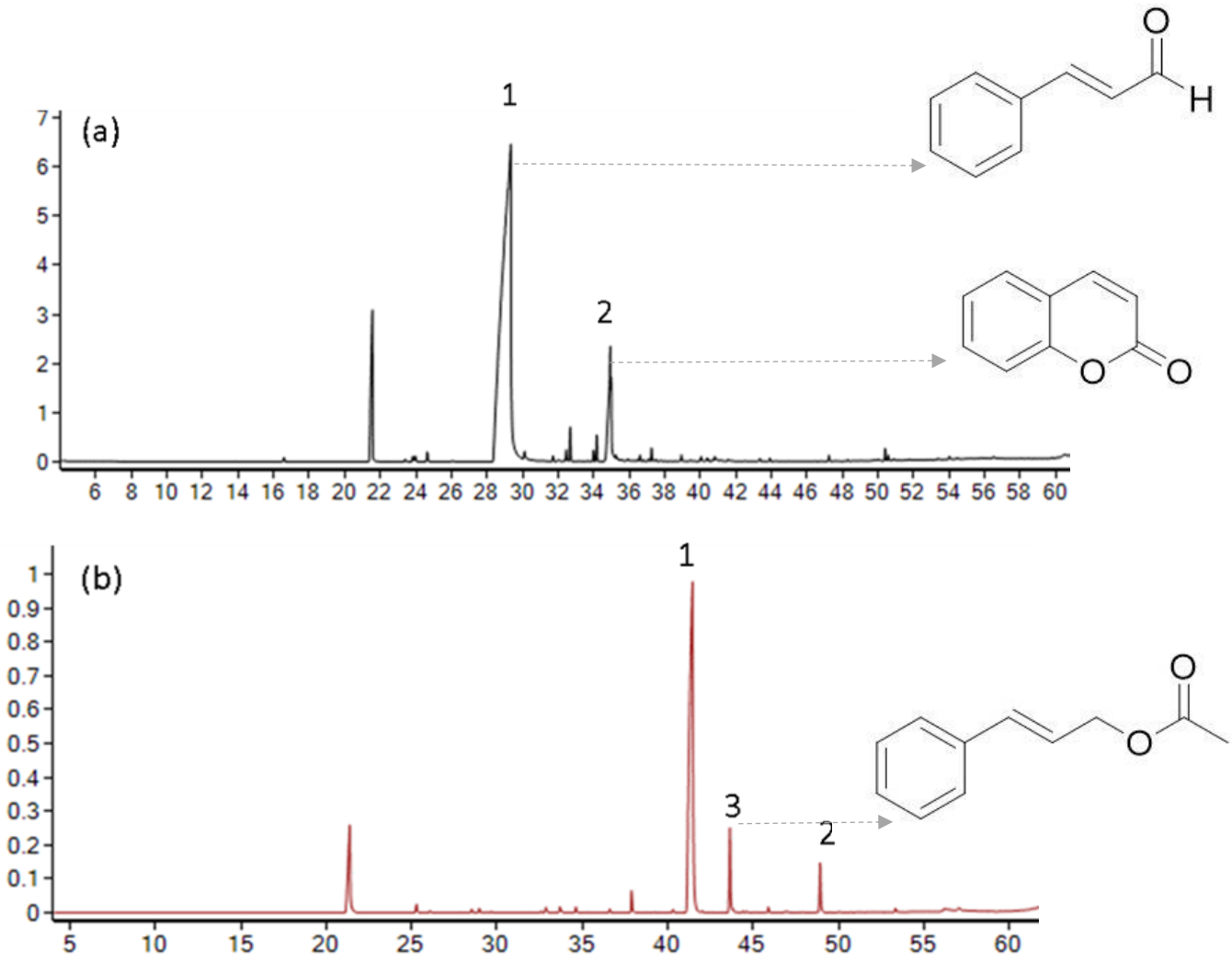


Figure 5: GC-MS chromatogram of *C. cassia* bark CO_2 extract when (a) analysed with HP-5 column and (b) analysed with DB-HeavyWAX column and the main volatile constituents detected **1**-(*E*)-cinnamaldehyde, **2**-coumarin, **3**-cinnamyl acetate

Table 5: Chemical composition of *C. cassia* fruits essential oil and CO_2 extract

RI[a]		Compound [b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
930	939	α-Pinene	MH	0.158	0.152	-	0.130	RI, GC-MS, Std	GC-MS, Std
945	953	Camphene	MH	0.087	0.098	-	0.087	RI, GC-MS, Std	GC-MS, Std
957	961	Benzaldehyde	A	0.403	0.567	-	0.510	RI, GC-MS, Std	GC-MS, Std
1114	1116	Phenylethyl alcohol	SO	-	0.051	0.045	0.049	RI, GC-MS	GC-MS
1163	1165	Benzenepropanal	A	0.708	0.657	-	0.640	RI, GC-MS	GC-MS
1166	1165	Borneol	MO	0.161	0.145	-	0.109	RI, GC-MS, Std	GC-MS, Std
1220	1219	(*Z*)-Cinnamaldehyde	A	0.322	0.465	-	0.472	RI, GC-MS, Std	GC-MS, Std
1274	**1270**	**(*E*)-Cinnamaldehyde**	**A**	**89.360**	**88.911**	**87.153**	**89.170**	**RI, GC-MS, Std**	**GC-MS, Std**
1375	1372	Ylangene	SH	0.110	0.050	0.071	0.038	RI, GC-MS	GC-MS
1380	1376	α-Copaene	SH	0.260	0.238	0.305	0.224	RI, GC-MS	GC-MS
1424	1418	β-Caryophyllene	SH	0.357	0.312	0.419	0.305	RI, GC-MS, Std	GC-MS, Std
1441	1428	Coumarin	K	-	0.061	0.246	0.066	RI, GC-MS	GC-MS
1448	1454	Cinnamyl acetate	E	0.473	0.651	0.441	0.656	RI, GC-MS	GC-MS
1460	1440	α-Humulene	SH	0.051	0.036	-	0.074	RI, GC-MS, Std	GC-MS, Std
1467	1461	Alloaromadendrene	SH	0.015	0.114	0.196	0.101	RI, GC-MS	GC-MS
1481	**1477**	**γ-Muurolene**	**SH**	**0.616**	**0.546**	**1.006**	**0.533**	**RI, GC-MS**	**GC-MS**
1485	1485	α-Amorphene	SH	0.235	-	0.298	-	RI, GC-MS	-
1492	1485	β-Selinene	SH	0.120	0.218	0.219	0.208	RI, GC-MS	GC-MS
1499	1491	Valencene	SH	-	0.016	0.327	-	RI, GC-MS	GC-MS
1500	1499	Eremophilene	SH	0.240	-	-	-	RI, GC-MS	-
1504	1499	α-Muurolene	SH	0.299	0.333	0.531	0.326	RI, GC-MS	GC-MS
1519	1513	γ-Cadinene	SH	0.398	-	0.745	-	RI, GC-MS	-

RI[a]		Compound [b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
1529	**1524**	**δ-Cadinene**	**SH**	**0.815**	**1.007**	**1.356**	**0.980**	**RI, GC-MS**	**GC-MS**
1536	**NA**	**2-Methoxycinnamaldehyde**	**A**	**1.031**	**1.283**	**3.955**	**1.307**	**GC-MS**	**GC-MS**
1544	1538	α-Cadinene	SH	0.132	0.107	0.166	0.107	RI, GC-MS	GC-MS
1550	1544	α-Calacorene	SH	0.118	0.170	0.114	0.168	RI, GC-MS	GC-MS
1579	1568	Caryophyllenyl alcohol	SO	0.150	0.123	0.103	0.112	RI, GC-MS	GC-MS
1586	1576	Spathulenol	SO	0.378	0.013	0.264	0.356	RI, GC-MS	GC-MS
1592	1581	Caryophyllene oxide	SO	0.486	0.325	0.461	0.470	RI, GC-MS	GC-MS
1601	1590	Viridiflorol	SO	0.059	-	-	-	RI, GC-MS	-
1610	NA	Epiglobulol	SO	0.079	0.231	-	0.233	GC-MS	GC-MS
1619	1606	Humulene epoxide II	SO	0.104	0.025	0.073	0.026	RI, GC-MS	GC-MS
1623	NA	Junenol	SO	0.062	-	-	-	GC-MS	-
1634	1642	Cubenol	SO	0.073	0.084	0.118	-	RI, GC-MS	GC-MS
1642	1630	γ-Eudesmol	SO	0.088	-	-	-	RI, GC-MS	-
1649	1640	tau-Cadinol	SO	0.306	0.420	0.186	0.438	RI, GC-MS	GC-MS
1655	NA	δ-Cadinol	SO	0.059	0.075	-	0.072	GC-MS	GC-MS
1661	NA	β-Selinenol	SO	0.047	-	-	-	GC-MS	-
1663	1653	α-Cadinol	SO	0.375	0.369	0.237	0.364	RI, GC-MS	GC-MS
1681	NA	Aromadendrene oxide-(2)	SO	0.088	0.095	0.093	-	GC-MS	GC-MS
1684	1674	Cadalene	SH	0.128	-	0.159	0.053	RI, GC-MS	GC-MS
1713	NA	9,10-dehydroisolongifolene	O	0.074	0.058	-	-	GC-MS	GC-MS
1773	1762	Benzyl benzoate	SO	0.084	0.128	0.107	0.130	RI, GC-MS	GC-MS
1798	NA	8-Oxo-9H-cycloisolongipholene	O	0.087	0.070	0.084	0.079	GC-MS	GC-MS
2161	2173[c]	Linoleic acid	FAD	-	-	0.277	-	RI, GC-MS	-
-	1071	β-Terpinene	MH	-	0.050	-	0.053	-	GC-MS
-	1024	Limonene	MH	-	0.064	-	0.057	-	GC-MS

RI[a]		Compound [b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
-	1502	γ-Patchoulene	SH	-	0.090	-	0.021	-	GC-MS
-	1479	Curcumene	SH	-	0.096	-	0.090	-	GC-MS
-	1528	Calamenene	SH	-	0.202	-	0.193	-	GC-MS
-	NA	Di-epi-1,10-cubenol	SO	-	0.050	-	0.048	-	GC-MS
-	1590	Globulol	SO	-	0.117	-	0.118	-	GC-MS
-	1356	Eugenol	PP	-	0.077	-	0.076	-	GC-MS
-	1521	Eugenol acetate	E	-	0.245	-	0.237	-	GC-MS
-	NA	Phenethyl benzoate	E	-	0.061	-	0.130	-	GC-MS
-	1434	γ-Elemene	SH	-	-	-	0.047	-	GC-MS
-	1613	Epicubenol	SO	-	-	-	0.075	-	GC-MS
		Total identified [%]		**99.196**	**99.257**	**99.755**	**99.737**		

[a)] RI = retention indices; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 2007),[c] (Alves et al., 2005) NA=RI values not available in the literature, [b)] C = Class; A - Aldehydes, CA- Carboxylic acids, DH - Diteprene hydrocarbons , E-Esters, FAD - Fatty acid and fatty acid derivatives, K - Ketones, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, PP-Phenylpropanoids, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes, S – Sterols, [d)] Identification method: GC-MS = Mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching literature database; Std = constituent identity confirmed by co-injection of authentic standards, [e)] Retention indices were not calculated for compounds detected only by DB-WAX column, - = not detected, **In bold** = main volatile compounds detected and total percentage of identified constituents

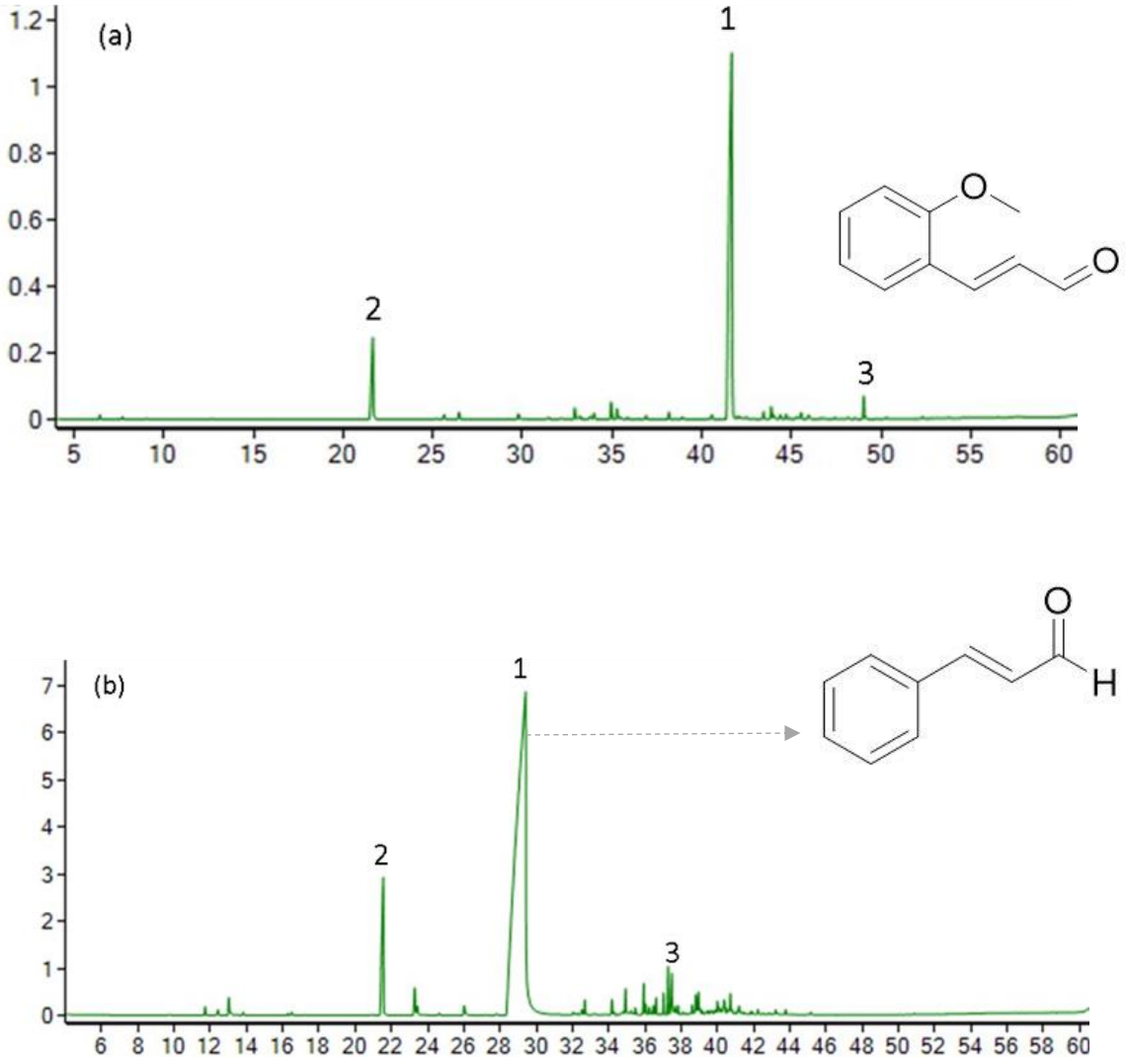


Figure 6: GC chromatograms of *C. cassia* fruits EO when analysed (a) with HP-5 column and (b) with DB-HeavyWAX column and main volatile compounds detected **1**- (*E*)-cinnamaldehyde, **2**- methyl octanoate (internal standard), **3**- 2-methoxycinnamaldehyde

Table 6: Chemical composition of *C. verum* bark essential oil and CO_2 extract

RI [a]		Compound [b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
930	939	α-Pinene	MH	0.274	0.191	-	-	RI, GC-MS, Std	GC-MS, Std
945	953	Camphene	MH	0.113	0.084	-	-	RI, GC-MS, Std	GC-MS, Std
957	961	Benzaldehyde	A	0.042	0.086	-	-	RI, GC-MS, Std	GC-MS, Std
973	980	β-Pinene	MH	0.091	0.069	-	-	RI, GC-MS, Std	GC-MS, Std
1003	1005	α-Phellandrene	MH	0.392	0.312	-	-	RI, GC-MS, Std	GC-MS, Std
1008	1011	3-Carene	MH	0.034	0.030	-	0.093	RI, GC-MS	GC-MS
1015	1018	α-Terpinene	MH	0.187	-	-	-	RI, GC-MS	-
1024	1024	*p*-Cymene	MH	0.646	0.530	0.068	0.164	RI, GC-MS	GC-MS
1027	NA	β-Thujene	MH	-	-	0.067	0.133	RI, GC-MS	GC-MS
1028	1031	β-Phellandrene	MH	1.136	0.743	-	-	RI, GC-MS	GC-MS
1029	1033	Eucalyptol	MO	0.123	-	-	-	RI, GC-MS	-
1058	1062	γ-Terpinene	MH	0.044	0.039	-	-	RI, GC-MS	GC-MS
1088	1088	Terpinolene	MH	0.078	0.148	-	-	RI, GC-MS	GC-MS
1100	1098	Linalool	MO	2.564	2.315	0.653	2.091	RI, GC-MS, Std	GC-MS, Std
1122	NA	(*Z*)-2-p-Menthen-1-ol	MO	0.061	0.053	-	-	GC-MS	GC-MS
1152	NA	1-Methyl-1-indanol	O	0.067	-	-	-	GC-MS	-
1163	1165	Benzenepropanal	A	0.354	0.284	0.086	0.161	RI, GC-MS	GC-MS
1166	1165	L-Borneol	MO	0.034	-	-	-	RI, GC-MS, Std	-
1174	NA	2-Methylcumarone	K	0.047	0.102	-	-	GC-MS	GC-MS
1177	1177	Terpinen-4-ol	MO	0.540	0.542	0.303	1.017	RI, GC-MS	GC-MS
1186	1183	p-Cymen-8-ol	MO	0.060	0.011	-	-	RI, GC-MS	GC-MS
1186	1183	Cryptone	MO	-	-	0.117	0.168	RI, GC-MS	GC-MS
1191	1186	α-Terpineol	MO	0.709	0.478	0.286	0.893	RI, GC-MS	GC-MS

RI [a]		Compound [b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
1199	1195	Estragole	MO	0.104	0.098	-	-	RI, GC-MS	GC-MS
1220	1214	(*Z*)-Cinnamaldehyde	A	0.327	0.365	-	0.187	RI, GC-MS, Std	GC-MS, Std
1241	NA	Cumaldehyde	A	0.091	0.150	-	-	GC-MS	GC-MS
1245	1242	Carvone	MO	0.692	0.667	-	-	RI, GC-MS, Std	GC-MS, Std
1274	**1270**	**(*E*)-Cinnamaldehyde**	**A**	**49.795**	**49.188**	**73.390**	**58.581**	**RI, GC-MS, Std**	**GC-MS, Std**
1292	1282	Anethole	MO	0.322	0.373	-	-	RI, GC-MS	GC-MS
1297	NA	Benzylidenemalonaldehyde	A	-	-	0.093	-	GC-MS	-
1306	1298	Carvacrol	MO	0.063	0.111	-	-	RI, GC-MS	GC-MS
1310	1303	Cinnamyl alcohol	SO	-	0.165	0.512	2.179	RI, GC-MS	GC-MS
1354	1346	α-Terpinyl acetate	MO	0.069	-	-	-	RI, GC-MS	-
1364	1356	Eugenol	PP	3.276	3.413	2.931	6.935	RI, GC-MS	GC-MS
1374	1373[d]	Benzenepropyl acetate	E	-	-	0.096	0.299	RI, GC-MS	GC-MS
1375	NA	Hydrocinnamyl acetate	E	0.398	0.462	-	-	GC-MS	GC-MS
1380	1376	α-Copaene	SH	0.293	0.264	0.152	0.373	RI, GC-MS	GC-MS
1410	1413[e]	Isocaryophyllene	SH	-	-	0.113	-	RI, GC-MS	-
1425	1418	β-Caryophyllene	SH	4.959	4.284	2.195	2.318	RI, GC-MS, Std	GC-MS, Std
1453	**1454**	**Cinnamyl acetate**	**E**	**22.680**	**23.095**	**8.154**	**10.555**	**RI, GC-MS**	**GC-MS**
1460	1440	α-Humulene	SH	0.968	0.837	0.437	0.503	RI, GC-MS, Std	GC-MS, Std
1487	1483	Curcumene	SH	0.074	-	-	-	RI, GC-MS	-
1530	1524	δ-Cadinene	SH	0.113	0.066	-	-	RI, GC-MS	GC-MS
1536	NA	2-Methoxycinnamaldehyde	A	0.447	0.555	2.402	5.520	RI, GC-MS	GC-MS
1568	1581	Caryophyllene oxide	SO	0.046	0.087	0.398	0.710	RI, GC-MS	GC-MS
1580	1568	Caryophyllenyl alcohol	SO	0.207	0.220	0.092	0.108	RI, GC-MS	GC-MS
1587	1576	Spathulenol	SO	0.025	0.074	-	-	RI, GC-MS	GC-MS
1593	1581	Caryophyllene oxide 2nd isomer	SO	0.706	0.755	-	0.527	RI, GC-MS	GC-MS
1607	NA	Methoxyeugenol	O	-	-	0.198	0.392	GC-MS	GC-MS

RI [a]		Compound [b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
1610	NA	Epiglobulol	SO	0.180	-	-	-	GC-MS	-
1614	1611	Tetradecanal	A	0.270	0.229	-	-	RI, GC-MS	GC-MS
1619	1606	Humulene epoxide II	SO	0.171	0.165	0.079	-	RI, GC-MS	GC-MS
1651	1641[f]	τ-Muurolol	SO	0.024	-	-	-	RI, GC-MS	-
1674	NA	6,7-Dimethoxy-2-tetralone	SO	-	-	0.240	0.455	GC-MS	GC-MS
1681	1682[g]	Caryophylladienol II	SO	0.167	0.228	0.027	0.115	RI, GC-MS	GC-MS
1775	**1762**	**Benzyl benzoate**	**SO**	**4.805**	**5.496**	**2.623**	**2.741**	**RI, GC-MS**	**GC-MS**
1821	NA	3-Isolongifolol	SO	-	-	0.118	0.083	GC-MS	GC-MS
1865	NA	Benzoic acid phenethyl ester	E	0.072	0.080	-	-	GC-MS	GC-MS
1889	1885	Clovanediol	SO	-	-	0.146	0.074	RI, GC-MS	GC-MS
2011	NA	α-Phellandrene dimer	O	0.165	0.206	0.381	0.309	GC-MS	GC-MS
2022	NA	Methyl abietate	DH	-	-	0.078	-	GC-MS	-
2085	2099	*E*-Cinnamyl benzoate	E	-	-	0.181	0.094	RI, GC-MS	GC-MS
2112	NA	(*Z*)-1, 2-Cyclododecanediol	FAD	-	-	0.066	-	GC-MS	-
2135	NA	Equilin	S	0.224	0.082	0.641	-	GC-MS	-
2144	NA	Gibberellic acid	O	-	-	0.685	0.179	GC-MS	GC-MS
2162	NA	Linalyl cinnamate	E	-	-	0.156	-	GC-MS	-
2254	NA	8,11-Octadecadiynoic acid, methyl ester	FAD	-	-	0.051	-	GC-MS	-
2265	NA	Methyl 9,11-octadecadiynoate	FAD	-	-	0.047	-	GC-MS	-
-	988	β-Myrcene	MH	-	0.033	-	-	-	GC-MS
-	1024	Limonene	MH	-	0.244	-	-	-	GC-MS
-	1001	4-Carene	MH	-	0.047	-	-	-	GC-MS
-	1137	(*Z*)-Sabinol	MO	-	0.066	-	0.158	-	GC-MS
-	1285	Safrole	MO	-	0.049	-	-	-	GC-MS
-	1409	β-(*Z*)-Caryophyllene	SH	-	-	-	0.277	-	GC-MS

RI [a]		Compound [b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract		HP-5MS	DB-WAX
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX		
-	NA	10,12-Octadecadiynoic acid	FAD	-	0.107	-	-	-	GC-MS
-	1652	α-Cadinol	SO	-	0.008	-	-	-	GC-MS
-	1521	Eugenol acetate	PP	-	0.165	-	-	-	GC-MS
-	NA	α-Kessyl acetate	SO	-	-	-	0.161	-	GC-MS
-	NA	Z-Hexadec-7-enal	FAD	-	-	-	0.113	-	GC-MS
-	1234	Ascaridole	MO	-	-	-	0.107	-	GC-MS
-	1984[b)]	Palmitic acid	FAD	-	0.525	-	-	-	GC-MS
-	1866	Pentadecanoic acid	FAD	-	-	-	0.167	-	GC-MS
-	NA	Retinol acetate	DH	-	-	-	0.246	-	GC-MS
-	NA	Isopropyl palmitate	FAD	-	-	-	0.252	-	GC-MS
-	1428	α-Ionone	K	-	-	-	0.222	-	GC-MS
-	NA	Panaxydol	FAD	-	-	-	0.067	-	GC-MS
-	NA	6,9,12-Octadecatrienoic acid, methyl ester	FAD	-	-	-	0.020	-	GC-MS
-	3408	β-Sitosterol	S	-	-	-	0.027	-	GC-MS
		Total identified [%]		**99.332**	**98.972**	**98.264**	**99.774**		

[a]RI = retention indices; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 2007), [d] (Beaulieu & Grimm, 2001), [e] (Elias et al., 1997), [f](Karioti et al., 2003), [g] (Smelcerovic et al., 2007) NA=RI values not available in the literature, [b)] C = Class; A - Aldehydes, CA- Carboxylic acids, DH - Diteprene hydrocarbons, E-Esters, FAD - Fatty acid and fatty acid derivatives, K - Ketones, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, PP-Phenylpropanoids, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes, S – Sterols, [d)] Identification method: GC-MS = Mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching literature database; Std = constituent identity confirmed by co-injection of authentic standards, [-)] Retention indices were not calculated for compounds detected only by DB-WAX column, - = not detected, . **In bold** = main volatile compounds detected and total percentage of identified constituent

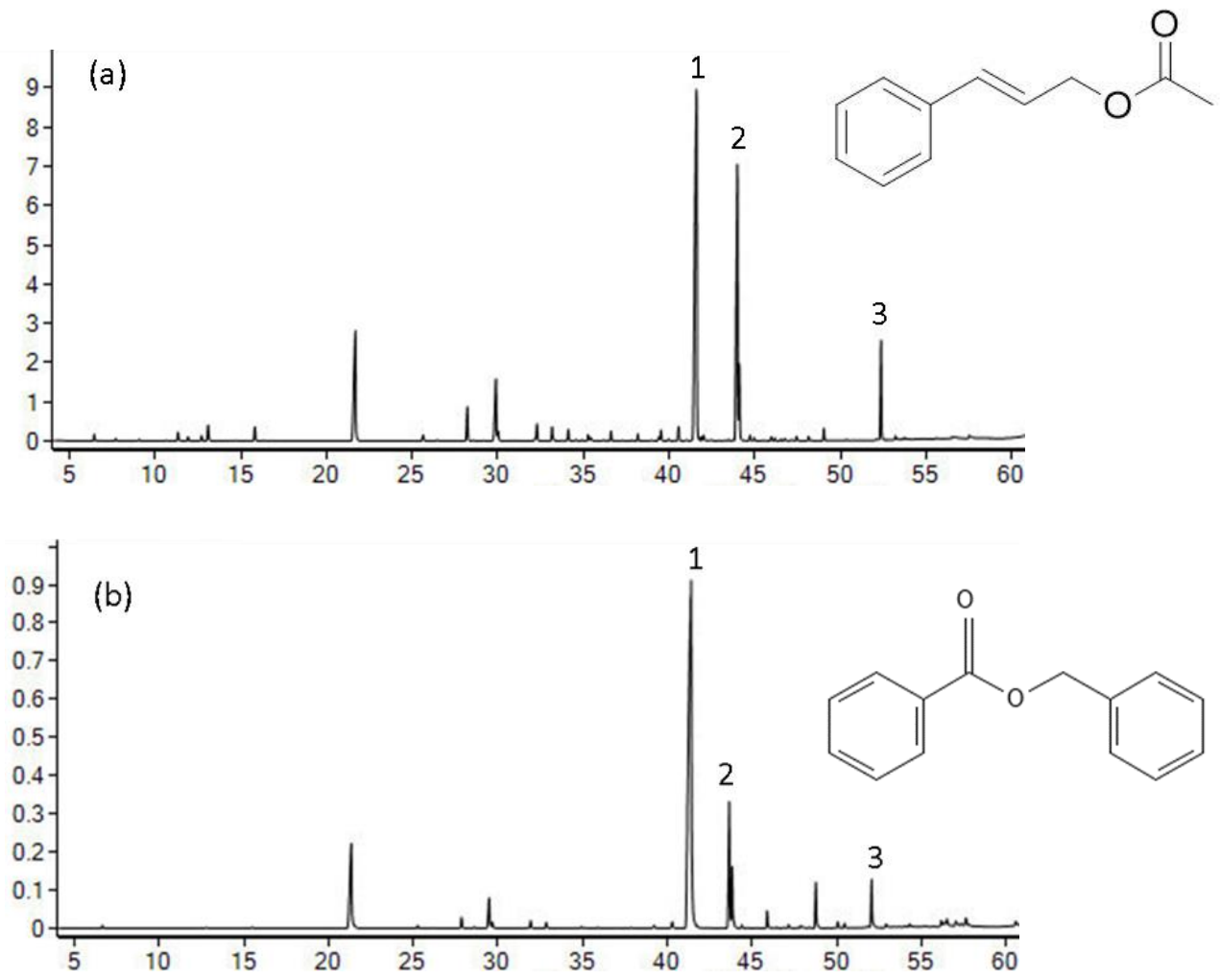


Figure 7: GC-MS chromatograms of *C. verum* a) EO and b) CO_2 extract analysed by DB-HeavyWAX column and the main volatile compounds detected **1**- (*E*)-cinnamaldehyde, **2**-cinnamyl acetate, **3**- benzyl benzoate

Table 7: Chemical composition of a headspace above *C. cassia* fruit EO and MH broth at a concentration 256 µg/ml

RI[a]		Compound[b]	C[c]	Time (h)/Column/Content (%)									
				0		3		6		9		12	
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX
-	654	Isovaleral	A	-	0.42	-	0.51	-	0.36	-	-	-	-
824	NA	Dimethyl sulfoxide	O	0.09	1.46	0.19	2.54	0.25	1.70	0.32	1.15	0.27	1.45
854	863	4-Methyloctane	AH	0.47	-	0.15	-	-	-	-	-	-	-
885	890	Styrene	CH	0.58	0.81	0.23	0.62	0.26	0.48	0.20	0.39	0.23	0.32
-	911	2,5-Dimethylpyrazine	O	-	0.36	-	0.37	-	0.30	-	-	-	0.26
929	930	α-Pinene	MH	0.23	-	0.26	-	0.40	0.34	0.24	0.32	0.14	0.38
943	953	Camphene	MH	0.46	0.57	0.43	0.81	0.63	0.89	0.33	0.78	0.15	0.68
956	961	Benzaldehyde	A	5.25	3.90	2.74	3.70	2.08	3.45	2.27	2.19	2.07	2.19
971	974	β-Pinene	MH	0.44	0.54	0.43	0.60	0.60	0.70	0.32	0.76	0.18	0.56
-	1024	Limonene	MH	-	0.28	-	0.65	-	0.62	-	0.79	-	1.01
1022	1024	*p*-Cymene	MH	1.15	1.73	0.71	0.73	0.82	0.07	0.32	0.25	0.19	0.81
1026	1031	Limonene	MH	1.62	1.18	1.34	1.34	1.76	1.47	0.82	1.43	0.42	0.61
-	1082	m-Cymene	MH				0.76	-	0.20	-	-	-	-
1028	1033	Eucalyptol	MO	0.33	-	0.30	0.07	-	-	0.13	0.04	0.23	0.05
1101	1100	Undecane	AH	0.23	-	-	-	-	-	-	-	-	-
1124	1123	Methyl octanoate	E	0.42	0.38	0.22	0.28	0.17	0.21	0.09	0.15	0.09	-
1164	1165	Endoborneol	MO	1.18	-	0.60	-	0.55	-	0.54	-	0.40	-
1219	1214	(*Z*)-Cinnamaldehyde	A	0.18	-	-	-	-	-	-	-	-	-
1257	NA	Benzylcarbinyl acetate	E	0.17	-	-	-	-	-	-	-	-	-
1275	**1270**	**(*E*)-Cinnamaldehyde**	**A**	**23.11**	**16.35**	**10.80**	**11.36**	**8.86**	**8.60**	**9.21**	**4.33**	**6.69**	**3.74**
1287	1287	Bornyl acetate	E	1.46	1.44	0.70	1.61	0.57	0.22	0.35	1.08	0.41	0.88
-	1335	δ-Elemene	SH	-	0.19	-	-	-	0.27	-	-	-	-
1368	1369	Cyclosativene	SH	0.30	-	0.65	-	0.49	-	0.86	-	0.83	0.93

RI[a]		Compound[b]	C[c]	Time (h)/Column/Content (%)									
				0		3		6		9		12	
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX
1374	1372	Ylangene	SH	0.72	1.38	1.20	1.42	0.14	1.85	1.57	2.15	1.52	2.25
1378	1376	α-Copaene	SH	1.99	3.75	3.73	3.76	1.14	4.70	5.09	5.62	4.96	5.32
1387	1387	β-Bourbonene	SH	-	-	-	-	-	-	0.15	-	0.15	-
1393	1389	β-Elemene	SH	0.18	0.45	0.36	0.51	0.83	-	0.51	-	0.53	-
-	1390	Sativene	SH	-	0.25	0.33	0.19	3.79	0.39	0.66	0.14	0.66	0.28
1408	1418	β-Caryophyllene	SH	3.81	3.46	4.88	3.72	5.05	4.33	5.25	5.05	5.21	5.66
1409	1400	β-Longipinene	SH	-	-	0.28	-	0.18	-	0.30	-	0.28	-
-	1409	α-Gurjunene	SH	-	0.98	0.22	0.51	0.57	0.23	0.34	1.10	0.27	0.74
-	1409	Isocaryophyllene	SH	-	0.31	-	-	-	-	-	-	-	-
1416	NA	6-epi-Shyobunol	SO	0.31	-	-	-	0.33	-	-	-	-	-
1426	NA	Guaia-1(10),11-diene	SH	0.47	-	1.11	-	-	-	-	1.40	-	-
1432	1430	β-Copaene	SH	0.11	-	0.29	-	0.20	-	0.45	-	0.46	-
1442	1439	Aromandendrene	SH	0.43	1.02	0.72	0.89	0.77	0.83	1.12	1.25	1.16	2.85
1447	NA	Guaia-6,9-diene	SH	0.24	-	0.36	-	0.39	-	0.41	-	0.38	-
1458	1452	α-Humulene	SH	0.85	0.26	1.31	0.56	1.94	0.49	-	1.10	1.35	0.99
1460	NA	Valerena-4,7(11)-diene	SH	0.15	0.68	0.42	-	0.47	0.45	-	0.45	0.54	1.23
1465	1461	Alloaromadendrene	SH	1.20	2.53	2.50	1.45	2.10	3.02	2.38	3.29	0.55	3.06
-	1475	γ-Gurjunene	SH	-	0.39	-	0.70	-	-	-	-	-	-
-	1475	β-Chamigrene	SH	-	-	-	-	-	0.06	-	-	-	0.61
1481	**1478**	**γ-Muurolene**	**SH**	**5.77**	**10.93**	**9.33**	**10.59**	**9.60**	**15.15**	**10.75**	**13.33**	**11.58**	**14.43**
-	1479	α-Curcumene	SH	-	0.75	-	0.47	-	0.36	-	0.73	-	1.20
1484	1483	α-Amorphene	SH	2.75	-	3.85	-	4.02	-	-	-	-	-
1491	1489	β-Selinene	SH	1.79	2.08	2.70	1.91	3.05	2.22	3.32	2.66	3.17	2.60
1498	1486	Eremophilene	SH	2.80	-	4.07	-	3.96	-	4.20	-	4.03	-
-	1493	Epicubebol	SO	-	0.25	-	0.29	-	-	-	-	-	-
1503	1500	α-Muurolene	SH	2.98	7.04	4.83	6.49	4.87	7.50	9.36	8.88	10.33	8.61

RI[a]		Compound[b]	C[c]	Time (h)/Column/Content (%)									
				0		3		6		9		12	
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX
1519	1513	γ-Cadinene	SH	3.77	-	5.59	-	5.84	-	6.28	-	7.10	-
1528	**1524**	**δ-Cadinene**	**SH**	**8.18**	**14.06**	**12.30**	**13.09**	**12.98**	**15.56**	**12.94**	**17.78**	**12.74**	**17.54**
[c)]	1528	Calamenene	SH	-	3.97	-	4.02	-	4.61	-	-	-	4.60
1543	1538	α-Cadinene	SH	1.62	1.63	2.12	1.46	2.32	1.65	2.47	2.09	2.64	2.36
1549	1548	α-Calacorene	SH	1.38	1.22	1.55	0.85	1.96	1.33	1.59	1.34	1.57	1.37
1566	NA	Aromadendrene oxide	SO	0.48	0.53	0.41	-	0.38	-	0.22	0.37	0.25	0.52
1573	NA	1,5-Epoxysalvial-4(14)-ene	SO	0.59	-	-	-	-	-	-	-	-	-
1577	1570	Caryophyllenyl alcohol	SO	0.80	-	0.61	-	0.65	-	0.42	-	0.47	-
1581	1566	Maaliol	SO	0.29	-	0.20	-	-	-	-	-	-	-
1585	1577	Spathulenol	SO	1.37	0.83	0.93	0.80	0.98	0.72	0.67	0.38	0.78	0.48
1587	NA	Longifolenaldehyde	A	0.59	-	0.41	-	-	-	-	-	-	-
1590	1582	Caryophyllene oxide	SO	2.67	1.19	2.15	1.72	1.92	0.86	1.33	0.80	1.55	0.79
1607	1590	Globulol	SO	0.17	0.34	0.26	0.52	-	0.16	0.22	0.20	0.13	0.21
1611	NA	Isoaromadendrene epoxide	SO	2.06	1.30	1.78	0.57	0.48	0.97	0.27	0.62	0.30	0.65
1618	1612	Calarene epoxide	SO	1.04	0.16	1.10	-	0.59	-	0.19	-	0.37	-
1621	1627	Epicubenol	SO	0.43	0.20	0.32	-	0.18	0.23	0.13	-	0.24	0.10
1627	1618	Junenol	SO	0.94	-	0.74	-	-	0.40	-	-	-	-
1633	NA	Epiglobulol	SO	0.43	0.35	0.34	0.17	0.31	-	0.11	0.16	-	-
1635	NA	Di-epi-1,10-cubenol	SO	0.44	-	-	-	-	-	-	-	-	-
1640	1645	Cubenol	SO	0.59	-	0.69	0.92	0.35	0.35	0.36	0.18	0.50	0.24
1644	1639	Caryophylladienol II	SO	0.92	0.01	0.60	-	0.66	-	0.93	0.46	0.44	0.49
1648	1640	tau-Cadinol	SO	1.30	0.56	0.95	0.65	1.01	0.50	0.72	0.30	0.79	0.35
1653	1636	δ-Cadinol	SO	0.35	-	0.20	-	-	-	0.05	-	0.16	-
1661	1653	α-Cadinol	SO	0.49	0.50	0.36	-	0.44	0.42	0.33	0.27	0.37	0.30
1682	1675	Cadalene	SH	1.01	1.04	0.91	0.81	1.05	0.82	0.87	0.70	1.03	0.79
[-)]	NA	Selin-6-en-4α-ol	SO	-	0.42	-	0.41	-	0.33	-	0.33	-	-
[-)]	NA	Selina-3,7(11)-diene	SH	-	0.21	-	0.22	-	0.33	-	0.53	-	-

RI[a]		Compound[b]	C[c]	Time (h)/Column/Content (%)									
				0		3		6		9		12	
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX
[-)]	NA	4,5,9,10-Dehydro-isolongifolene	O	-	0.21	-	0.17	-	0.21	-	0.23	-	0.24
[-)]	NA	Diepicedrene-1-oxide	SO	-	0.36	-	0.12	-	0.49	-	0.88	-	0.86
[-)]	NA	α-Elemene	SH	-	1.11	-	0.72	-	0.90	-	0.57	-	-
		Total identified [%]		**96.16**	**96.30**	**96.73**	**86.62**	**92.93**	**92.28**	**91.96**	**89.00**	**90.86**	**95.60**

[a]RI = retention indices; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 2007), NA=RI values not available in the literature, [c)] C = Class; A - Aldehydes, CH-Hydrocarbons, E-Esters, K - Ketones, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes, [-)] Retention indices were not calculated for compounds calculated only by DB-WAX column, . **In bold** = main volatile compounds detected and total percentage of identified constituents

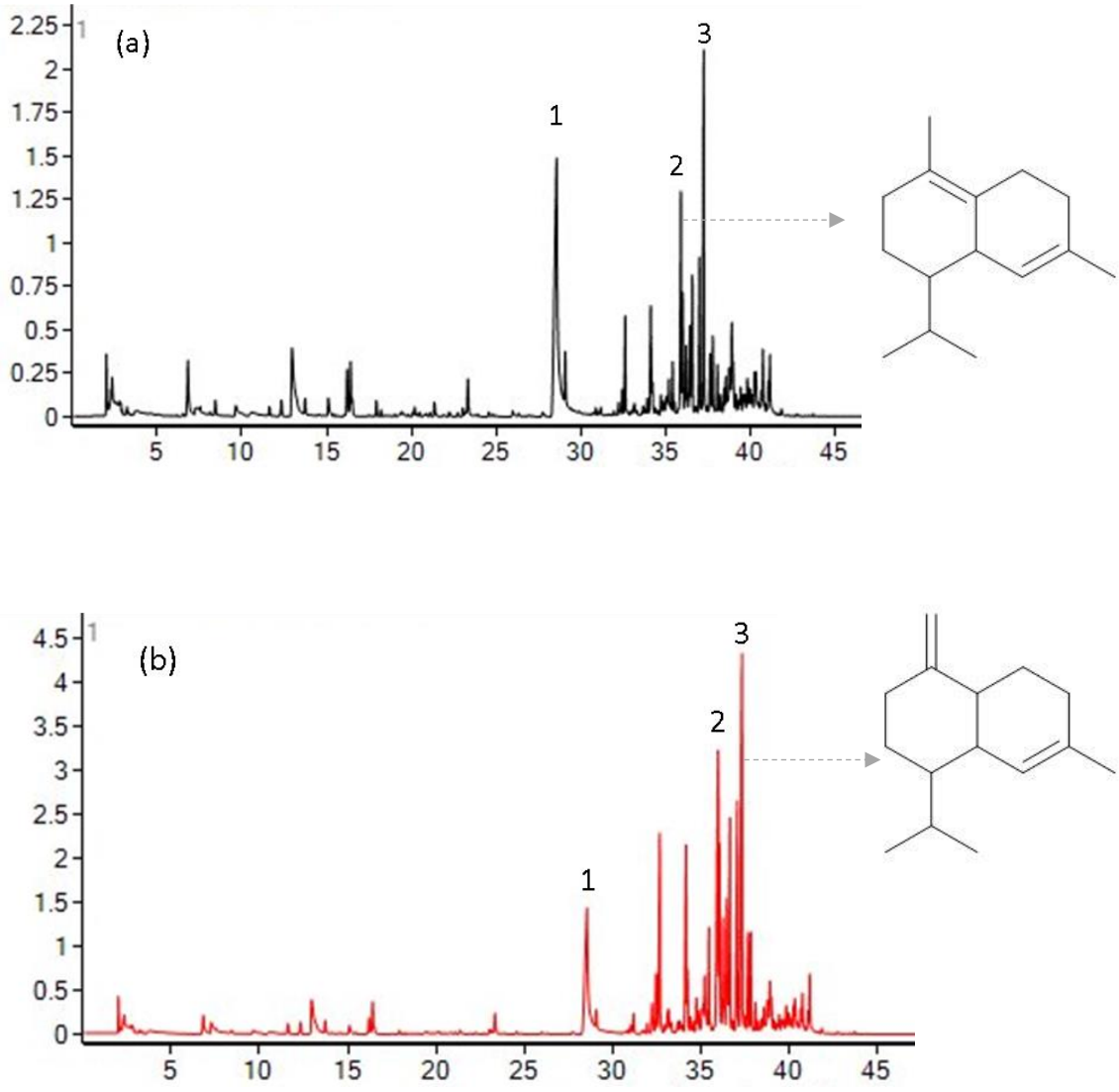


Figure 8: GC-MS chromatograms of the headspace above *C. cassia* fruits EO after a) 0 hrs, b) 9 hrs and Main volatile compounds detected **1**- (*E*)-cinnamaldehyde, **2**- γ-muurolene, **3**- δ-cadinene

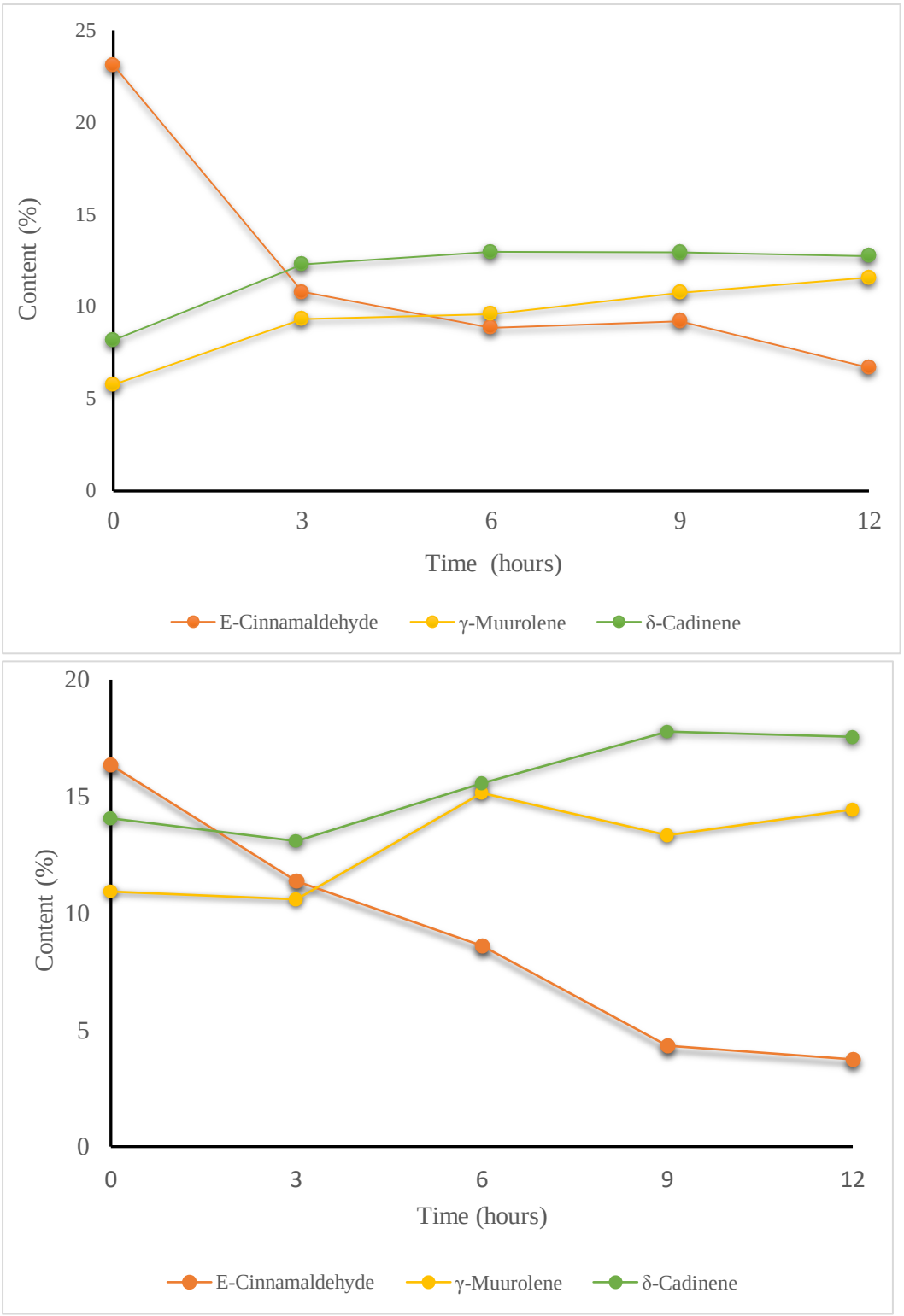


Figure 9: Contents of major components of EO from *C. cassia* fruits in the headspace detected by a) HP-5 column and b) DB-HeavyWAX at 3-hrs intervals

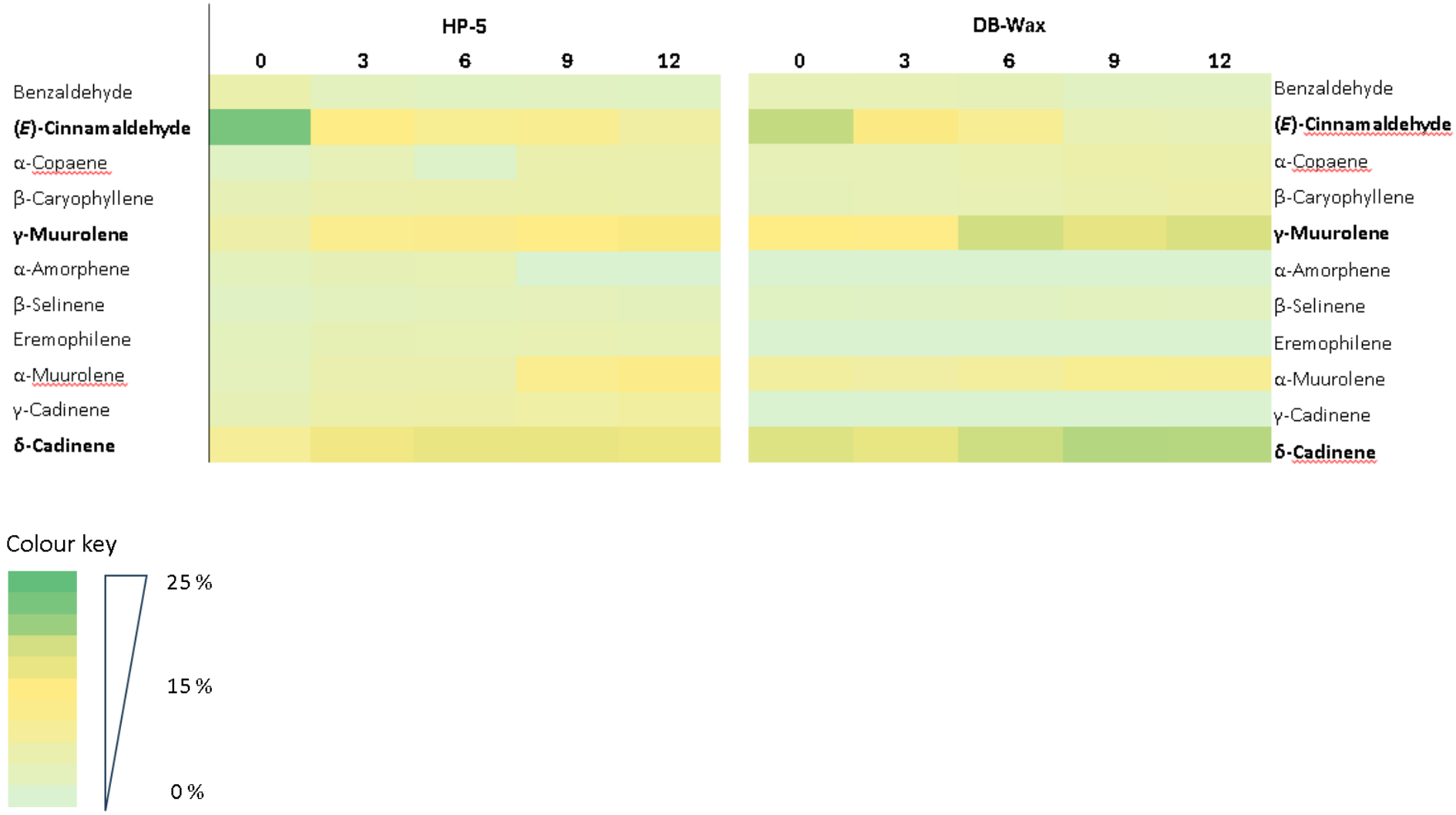


Figure 10: Heatmap of11 main constituents of *C. cassia* fruits EO headspace during overtime analysis

7.4. Chemical composition of EOs and supercritical CO_2 extracts from *Amomum kravanh*, *Citrus hystrix,* and *Piper nigrum* 'Kampot'

In following stage of this investigation, chemical profiles of three EOs and three CO_2 extracts isolated from traditional Cambodian spices have been analysed. Fruits of *A. kravanh*, *C. hystrix* and red peppercorns of *P. nigrum* 'Kampot', which are valuable commodities with a long tradition of culinary use, have been subjected to hydrodistillation and SFE and subsequently to analysis of both HP-5 and DB-HeavyWAX columns. Complete chemical analyses are provided in Table 8-10.

In EOs isolated from *A. kravanh*, *C. hystrix,* and *P. nigrum* 'Kampot', a total of 21, 38 and 35 individual constituents have been identified representing 99.88, 98.68, and 99.38% of their total respective contents when analysed with HP-5 column. Using the DB-HeavyWAX column, a total of 24, 50, and 41 compounds were detected, constituting 99.26, 98.06, and 99.15% of the total volatile oils, respectively. In CO_2 extracts from these same spices, a total number of 31, 32 and 36 compounds were determined during an HP-5 column analysis, amounting to 92.69, 99.36 and 98.65% of the total extracts. When utilising the DB-HeavyWAX column, 40, 54 and 40 components were identified, which accounted for 95.57, 98.06 and 96.74% of their total respective contents. Sesquiterpenes, monoterpenes, and their oxygenated derivatives were the most predominant chemical groups in almost all tested EOs and CO_2 extracts from these three spices, apart from the *A. kravanh* extract, where the most abundant chemicals were higher fatty acids and long-chained alkanes.

In *A. kravanh* EO, oxygenated monoterpene eucalyptol was the prevailing compound, comprising 78.8/72.6% of the total sample. Significant amounts of other compounds included the monoterpenes β-pinene (7.68/7.49%), α-pinene (2.3/2.2%), and the oxygenated derivative α-terpineol (4.31/4.67%). When analysed with the HP-5 column, L-terpinene-4-ol constituted 1.19% of the EO, but it wasn't detected by the DB-HeavyWAX column. Instead, monoterpene limonene emerged as the third most abundant component (5.12%). On the contrary, the chemical composition of the CO_2 extract differed substantially from the EO. When utilizing the HP-5 column, the long-chain alkane tricosane accounted for 14.74% of the total extract, followed by eugenol acetate which accounted for 14.02% of the extract. Oleic acid was the third-most prevailing constituent, comprising 12.21% of the extract accompanied by phenylpropanoid eugenol (7.91%) and long-chain alkane pentacosane (5.19%). In contrast with

these findings, analyses with the DB-HeavyWAX column differed considerably. Most of the extract consisted of oleic and palmitic acids, constituting 29.26 and 17.07% of the total respective content, followed by tricosane (5.26%), eugenol acetate (5.24%), and linoleic acid (5.17%).

Analysis of *C. hystrix* EO revealed that monoterpenes constituted the most prevalent class of chemical compounds. The primary constituents in the EO were monoterpenes, with β-pinene (29.95/29.45%), limonene (24.54/23.24%), and sabinene (9.94/10.23%), along with alcohols L-terpinene-4-ol (9.71/9.07%) and α-terpineol (3.7/3.62%). Similarly, the CO_2 extract displayed a dominance of monoterpenes including β-pinene (30.2/28.9%), limonene (23.99/23.74%), and sabinene (13.36/19.55%), with the aldehyde citronellal (5.21/4.28%) also being a major component. Upon analysis with the HP-5 column, furanocoumarin oxypeucedanin accounted for 2.96% of the total extract, although it remained undetected by the DB-HeavyWAX column.

In the EO of *P. nigrum* 'Kampot', the dominant compound was sesquiterpene β-caryophyllene constituting 34.84/39.55% of the total oil. This constituent was followed by monoterpenes 3-carene (18.72/18.48%), limonene (11.18/10.93%), and β-pinene (5.42/5.32%) when analysed using the HP-5/DB-HeavyWAX columns, respectively. Similarly, analysis of the CO_2 extract revealed an even higher content of β-caryophyllene (54.21/55.86%), along with 3-carene, limonene, and β-selinene, comprising 7.4/7.18%, 6.26/6.03%, and 5.24/4.76% of the total extract, respectively. Chromatograms of the EOs and CO_2 extracts can be observed in Figure 11 and

Figure 12.

Table 8: Chemical composition of *A. kravanh* EO and CO_2 extract

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
923	931	α-Thujene	MH	0.09 ± 0.01	- - -	- - -	- - -	RI, GC-MS	-
929	**932**	**α-Pinene**	**MH**	**2.3 ± 0.05**	**2.20 ± 0.03**	- - -	- - -	RI, GC-MS, Std	GC-MS, Std
-	945	α-Fenchene	MH	- - -	- - -	- - -	0.01 ± 0.01	-	GC-MS
944	946	Camphene	MH	0.07 ± 0.01	0.08 ± 0.00	- - -	- - -	RI, GC-MS, Std	GC-MS, Std
970	969	Sabinene	MH	0.2 ± 0.04	0.23 ± 0.00	- - -	- - -	RI, GC-MS	GC-MS
973	**974**	**β-Pinene**	**MH**	**7.68 ± 0.08**	**7.49 ± 0.09**	- - -	- - -	RI, GC-MS, Std	GC-MS, Std
989	988	β-Myrcene	MH	0.78 ± 0.03	0.86 ± 0.08	- - -	- - -	RI, GC-MS	GC-MS
1003	1002	α-Phellandrene	MH	0.08 ± 0.01	- - -	- - -	- - -	RI, GC-MS, Std	GC-MS, Std
1015	1009	4-Carene	MH	0.24 ± 0.03	0.17 ± 0.12	- - -	- - -	RI, GC-MS	GC-MS
-	1014	α-Terpinene	MH	- - -	0.22 ± 0.01	- - -	- - -	-	GC-MS
1025	1024	*p*-Cymene	MH	0.69 ± 0.04	0.87 ± 0.02	- - -	- - -	RI, GC-MS	GC-MS
1031	**1026**	**Eucalyptol**	**MO**	**78.89 ± 0.42**	**72.60 ± 0.89**	- - -	0.08 ± 0.01	RI, GC-MS	GC-MS
-	1031	Limonene	MH	- - -	5.12 ± 0.09	- - -	0.01 ± 0.01	-	GC-MS
1058	1054	γ-Terpinene	MH	1.05 ± 0.06	1.06 ± 0.02	- - -	- - -	RI, GC-MS	GC-MS
-	1083	Fenchone	MO	- - -	0.18 ± 0.02	- - -	- - -	-	GC-MS
1087	1086	Isoterpinolene	MH	0.43 ± 0.02	- - -	- - -	- - -	GC-MS	-
1105	1095	Linalool	MO	0.45 ± 0.01	0.50 ± 0.01	- - -	- - -	RI, GC-MS, Std	GC-MS, Std
1174	1162	δ-Terpineol	MO	0.39 ± 0.05	0.43 ± 0.01	- - -	- - -	RI, GC-MS	GC-MS
1182	1174	L-terpinen-4-ol	MO	1.19 ± 0.12	1.32 ± 0.02	- - -	- - -	RI, GC-MS	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]												Column type/Identification method[d]	
				Essential oil						CO_2 extract							
Obs.	Lit.			HP-5 MS			DB-Wax			HP-5 MS			DB-Wax			HP-5 MS	DB-Wax
1196	**1186**	**α-Terpineol**	**MO**	**4.31**	**±**	**0.17**	**4.67**	**±**	**0.08**	3.68	±	0.16	1.77	±	0.15	RI, GC-MS	GC-MS
1350	1346	α-Terpinyl acetate	MO	-	-	-	-	-	-	0.17	±	0.01	-	-	-	RI, GC-MS	-
1368	1356	Eugenol	PP	-	-	-	-	-	-	7.91	±	0.18	5.06	±	0.18	RI, GC-MS	GC-MS
-	1416	α-Santalene	SH	-	-	-	-	-	-	-		-	0.08	±	0.01	-	GC-MS
1421	1419	β-Caryophyllene	SH	-	-	-	-	-	-	1.37	±	0.25	0.66	±	0.02	RI, GC-MS, Std	GC-MS, Std
1457	1452	α Humulene	SH	-	-	-	-	-	-	0.41	±	0.03	0.12	±	0.10	RI, GC-MS, Std	GC-MS, Std
1486	1465	(*Z*)-muurola-4(14),5-diene	SH	0.16	±	0.01	-	-	-	-	-	-	-	-	-	RI, GC-MS	-
-	1478	γ-Muurolene	SH	-	-	-	0.16	±	0.02	-	-	-	-	-	-	-	GC-MS
1484	1484	Germacrene D	SH	-	-	-	-	-	-	1.38	±	0.02	-	-	-	RI, GC-MS	-
1490	1489	β-Selinene	SH	0.35	±	0.03	0.25	±	0.01	2.06	±	0.04	0.88	±	0.16	RI, GC-MS	GC-MS
1497	1496	Valencene	SH	-	-	-	-	-	-	0.69	±	0.10	0.09	±	0.01	RI, GC-MS	GC-MS
1508	1505	β-Bisabolene	SH	0.36	±	0.03	0.23	±	0.01	3.59	±	0.06	1.12	±	0.10	RI, GC-MS	GC-MS
1518	1513	γ-Cadinene	SH	-	-	-	-	-	-	1.34	±	0.09	0.73	±	0.11	RI, GC-MS	GC-MS
-	1514	Cubebol	SO	-	-	-	-	-	-	-	-	-	0.39	±	0.01	-	GC-MS
1525	1521	β-Sesquiphellandrene	SH	0.12	±	0.02	0.15	±	0.00	1.60	±	0.62	0.65	±	0.02	RI, GC-MS	GC-MS
1531	**1521**	**Eugenol acetate**	**PP**	**-**	**-**	**-**	**-**	**-**	**-**	**14.02**	**±**	**0.74**	**5.23**	**±**	**0.11**	RI, GC-MS	GC-MS
1558	1542	(*Z*)-Sesquisabinene hydrate	SO	0.12	±	0.02	0.15	±	0.00	0.51	±	0.11	0.20	±	0.01	RI, GC-MS	GC-MS
1566	1561	(*E*)-Nerolidol	SO	-	-	-	0.12	±	0.00	1.71	±	0.03	0.61	±	0.04	RI, GC-MS	GC-MS
1595	1577	(*E*)-Sesquisabinene hydrate	SO	-	-	-	-	-	-	1.11	±	0.30	0.44	±	0.01	RI, GC-MS	GC-MS
-	1577	Spathulenol	SO	-	-	-	-	-	-	-	-	-	0.15	±	0.00	-	GC-MS
1591	1582	Caryophyllene oxide	SO	-	-	-	-	-	-	0.39	±	0.10	0.25	±	0.02	RI, GC-MS	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
1675	1674	β-Bisabolol	SO	0.04 ± 0.04	0.12 ± 0.04	0.21 ± 0.00	0.07 ± 0.00	RI, GC-MS	GC-MS
1691	1685	α-Bisabolol	SO	- - -	- - -	0.21 ± 0.00	0.07 ± 0.00	RI, GC-MS	GC-MS
1714	1715	β-Santalol	SO	- - -	- - -	0.56 ± 0.04	0.90 ± 0.01	RI, GC-MS	GC-MS
-	**1959**	**Palmitic acid**	**FAD**	**- - -**	**- - -**	**- - -**	**17.07 ± 0.23**	-	GC-MS
2086	2100	Heneicosane	AH	- - -	- - -	2.12 ± 0.03	0.76 ± 0.00	RI, GC-MS	GC-MS
-	2113	Linoleic acid	FAD	- - -	- - -	- - -	5.17 ± 0.36	-	GC-MS
2166	**2141**	**Oleic Acid**	**FAD**	**- - -**	**- - -**	**12.21 ± 0.25**	**29.26 ± 0.42**	RI, GC-MS	GC-MS
-	2172	Stearic acid	FAD	- - -	- - -	- - -	2.16 ± 0.09	-	GC-MS
2186	2200	Docosane	AH	- - -	- - -	1.57 ± 0.09	0.51 ± 0.10	RI, GC-MS	GC-MS
2286	**2300**	**Tricosane**	**AH**	**- - -**	**- - -**	**14.74 ± 0.60**	**5.24 ± 0.09**	RI, GC-MS	GC-MS
2383	2400	Tetracosane	AH	- - -	- - -	1.94 ± 0.03	0.69 ± 0.05	RI, GC-MS	GC-MS
2482	2500	Pentacosane	AH	- - -	- - -	5.19 ± 0.39	1.88 ± 0.08	RI, GC-MS	GC-MS
-	2700	Heptacosane	AH	- - -	- - -	- - -	0.46 ± 0.02	-	GC-MS
2096	NA	Nonadecan-2-one	K	- - -	- - -	0.54 ± 0.02	- - -	GC-MS	-
2261	NA	Tetradec-9-enal	AL	- - -	- - -	0.24 ± 0.01	- - -	GC-MS	-
2267	NA	Palmitoleic acid	FAD	- - -	- - -	0.80 ± 0.06	1.94 ± 0.03	GC-MS	GC-MS
2464	NA	Hexadec-7-enal	AL	- - -	- - -	1.50 ± 0.13	0.11 ± 0.02	GC-MS	GC-MS
2691	2669[a]	Azelaic acid bis (2-ethylhexyl) ester	E	- - -	- - -	4.99 ± 0.12	1.96 ± 0.20	GC-MS	GC-MS
2810	NA	β-Monoolein	E	- - -	- - -	2.88 ± 0.23	- - -	GC-MS	-
-	2478[b]	Tricosanol	A	- - -	- - -	- - -	0.38 ± 0.01	-	GC-MS
-	NA	Cyclopentadecanone	K	- - -	- - -	- - -	0.46 ± 0.01	-	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
-	2483[c]	Pentacos-1-ene	AH	- - -	- - -	- - -	0.48 ± 0.04	-	GC-MS
-	2684[c]	Heptacos-1-ene	AH	- - -	- - -	- - -	1.31 ± 0.04	-	GC-MS
-	1942[d]	Hexadec-9-enoic acid	FAD	- - -	- - -	- - -	0.30 ± 0.04	-	GC-MS
-	3110[c]	Octacosanol	A	- - -	- - -	- - -	3.16 ± 0.06	-	GC-MS
-	NA	Squalene	TH	- - -	- - -	- - -	1.17 ± 0.05	-	GC-MS
-	NA	Glyceryl linolenate	E	- - -	- - -	- - -	1.43 ± 0.06	-	GC-MS
-	NA	β-Sitosterol	O	- - -	0.17 ± 0.14	- - -	- - -	-	GC-MS
		Total identified [%]		**99.88 ± 0.05**	**99.29 ± 0.16**	**91.64 ± 0.88**	**95.41 ± 0.46**		

[a)] RI = retention indices for HP-5 column; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 2007), [a] (Okumura, 1991), [b] (Nibret & Wink, 2010), [c] (Andriamaharavo, 2014), [d] (Zhao et al., 2008), [e] (Xu et al., 2009), NA= RI values were not available in the literature, [b)] C = Class; A - Aldehydes, AH-Aliphatic hydrocarbons, AL-Alcohols, DH - Diteprene hydrocarbons, E - Esters, FAD - Fatty acid and fatty acid derivatives, K-Ketones, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes, TH= Triterpene hydrocarbons, [d)]Identification method: GC-MS = Mass spectrum was identical to that of the National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching the literature database; Std = constituent identity confirmed by co-injection of authentic standard, [e)] Retention indices were not calculated for compounds calculated only by the DB-HeavyWAX column, **In bold** = main volatile compounds detected and total percentage of identified constituents

Table 9: Chemical composition of *C. hystrix* EO and CO_2 extract

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
923	931	α-Thujene	MH	0.31 ± 0.01	0.34 ± 0.02	0.32 ± 0.02	0.31 ± 0	RI, GC-MS	GC-MS
930	937	α-Pinene	MH	2.93 ± 0.16	2.96 ± 0.09	2.54 ± 0.25	2.52 ± 0.09	RI, GC-MS, Std	GC-MS, Std
-	945	Fenchene	MH	- - -	- - -	- - -	0.02 ± 0.02	-	GC-MS
945	946	Camphene	MH	0.16 ± 0.01	0.19 ± 0.01	0.14 ± 0.01	0.15 ± 0	RI, GC-MS, Std	GC-MS, Std
971	**976**	**Sabinene**	**MH**	**9.94 ± 0.22**	**10.2 ± 0.15**	**19.36 ± 0.97**	**19.55 ± 0.33**	RI, GC-MS	GC-MS
974	**980**	**β-Pinene**	**MH**	**29.95 ± 0.54**	**29.5 ± 0.45**	**30.2 ± 1.84**	**28.9 ± 0.55**	RI, GC-MS, Std	GC-MS, Std
990	991	β-Myrcene	MH	1.20 ± 0.05	1.38 ± 0.02	1.43 ± 0.06	1.58 ± 0.02	RI, GC-MS	GC-MS
-	1004	Pseudolimonene	MH	- - -	0.97 ± 0.07	- - -	0.97 ± 0.04	-	GC-MS
1003	1005	α-Phellandrene	MH	0.07 ± 0.01	0.06 ± 0	- - -	- - -	RI, GC-MS, Std	GC-MS, Std
-	1008	3-Carene	MH	- - -	0.01 ± 0.02	- - -	- - -	-	GC-MS
1015	1009	4-Carene	MH	- - -	- - -	0.02 ± 0.02	- ± -	RI, GC-MS	-
1015	1009	α-Terpinene	MH	0.51 ± 0.04	0.61 ± 0.01	- - -	- - -	RI, GC-MS	GC-MS
1025	1022	*p*-Cymene	MH	1.91 ± 0.01	2.34 ± 0.01	0.1 ± 0.01	0.21 ± 0.02	RI, GC-MS	GC-MS
1028	**1031**	**Limonene**	**MH**	**24.54 ± 0.16**	**23.2 ± 0.1**	**23.99 ± 0.45**	**23.74 ± 0.12**	RI, GC-MS	GC-MS
1058	1062	γ-Terpinene	MH	1.67 ± 0.01	1.63 ± 0.02	0.08 ± 0	0.1 ± 0.01	RI, GC-MS	GC-MS
1072	1065	(*Z*)-Sabinene hydrate	MO	- - -	- - -	0.78 ± 0.06	1.06 ± 0.02	RI, GC-MS	GC-MS
-	1071	β-Terpinene	MH	- - -	- - -	- - -	0.03 ± 0.01	-	GC-MS
1074	1074	Linalool oxide	MO	1.55 ± 0.02	1.57 ± 0.03	- - -	0.05 ± 0.01	RI, GC-MS	GC-MS
1087	1086	Terpinolene	MH	0.54 ± 0.03	0.52 ± 0.01	0.02 ± 0.01	0.05 ± 0	RI, GC-MS	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
1105	1095	Linalool	MO	0.72 ± 0.09	0.98 ± 0.02	0.52 ± 0.27	0.81 ± 0.07	RI, GC-MS, Std	GC-MS, Std
-	1098	(*E*)-Sabinene hydrate	MO	- - -	- - -	- - -	0.35 ± 0.03	-	GC-MS
-	1114	Fenchol	MO	- - -	0.02 ± 0.03	- - -	- - -	-	GC-MS
1145	1137	Sabinol	MO	- - -	- - -	0.08 ± 0.02	- ± -	RI, GC-MS	-
1150	1145	L-isopulegol	MO	0.22 ± 0.04	0.28 ± 0.03	0.07 ± 0.01	0.11 ± 0	RI, GC-MS	GC-MS
1154	1148	Citronellal	MO	1.38 ± 0.07	1.05 ± 0.04	5.21 ± 0.31	4.28 ± 0.25	RI, GC-MS	GC-MS
1174	1165	Borneol	MO	0.09 ± 0.02	- - -	- - -	- - -	RI, GC-MS	-
1161	1167	D,L-Isopulegol	MO	0.12 ± 0.02	- - -	- - -	- - -	RI, GC-MS	-
1184	**1174**	**terpinen-4-ol**	**MO**	**9.71 ± 0.16**	**9.07 ± 0.22**	**0.36 ± 0.04**	**0.42 ± 0.02**	RI, GC-MS	GC-MS
-	1176	*p*-Cymen-8-ol	MO	- - -	0.09 ± 0	- - -	- - -	-	GC-MS
-	1182	Pinocarveol	MO	- - -	0.03 ± 0.03	- - -	0.06 ± 0	-	GC-MS
1199	1189	α-Terpineol	MO	3.70 ± 0.09	3.62 ± 0.07	0.98 ± 0.17	1 ± 0.04	RI, GC-MS	GC-MS
-	1194	Myrtenol	MO	- - -	0.05 ± 0	- - -	- - -	-	GC-MS
1217	1205	(*E*)-Piperitol	MO	0.02 ± 0.04	- - -	- - -	- - -	RI, GC-MS	-
1237	1228	Citronellol	MO	0.75 ± 0.15	0.94 ± 0.03	0.43 ± 0.28	0.92 ± 0.04	RI, GC-MS	GC-MS
-	1249	Geraniol	MO	- - -	0.1 ± 0	- - -	- - -	-	GC-MS
1291	1273	(*Z*)-Ascaridole glycol	O	0.40 ± 0.10	0.69 ± 0.02	- - -	- - -	RI, GC-MS	GC-MS
-	1312	Citronellic acid	MO	- - -	0.3 ± 0.01	- - -	0.25 ± 0.01	-	GC-MS
1351	1345	α-Cubebene	SH	- - -	- - -	0.05 ± 0.02	0.07 ± 0	RI, GC-MS	GC-MS
1355	1354	Citronellyl acetate	MO	0.27 ± 0.03	0.32 ± 0.01	0.33 ± 0.11	0.44 ± 0.03	RI, GC-MS	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
1384	1365	Neryl acetate	MO	0.24 ± 0.04	- - -	0.22 ± 0.14	0.01 ± 0.02	RI, GC-MS	GC-MS
1379	1374	α-Copaene	SH	0.95 ± 0.03	0.75 ± 0.04	1.43 ± 0.13	1.21 ± 0.12	RI, GC-MS	GC-MS
-	1379	Geranyl acetate	MO	- - -	0.36 ± 0.02	- - -	0.51 ± 0.03	-	GC-MS
1391	1390	β-Cubebene	SH	0.37 ± 0.01	- - -	1.04 ± 0.15	- ± -	RI, GC-MS	-
1395	1391	β-Elemene	SH	0.05 ± 0.00	- - -	- - -	- - -	RI, GC-MS	-
1424	1419	β-Caryophyllene	SH	0.72 ± 0.02	0.57 ± 0.01	1.12 ± 0.09	1.05 ± 0.09	RI, GC-MS, Std	GC-MS, Std
-	1430	β-Copaene	SH	- - -	0.31 ± 0.01	- - -	0.96 ± 0.09	-	GC-MS
1461	1454	α Humulene	SH	0.23 ± 0.01	0.2 ± 0.01	0.35 ± 0.03	0.32 ± 0.02	RI, GC-MS, Std	GC-MS, Std
1488	1484	Germacrene D	SH	0.24 ± 0.01	0.07 ± 0	0.69 ± 0.05	0.49 ± 0.06	RI, GC-MS	GC-MS
1502	1495	Bicyclogermacrene	SH	- - -	- - -	0.14 ± 0.07	0.13 ± 0.01	RI, GC-MS	GC-MS
1505	1499	α-Muurolene	SH	0.10 ± 0.01	0.05 ± 0.02	0.1 ± 0.05	0.09 ± 0.01	RI, GC-MS	GC-MS
-	1514	Cubebol	SO	- - -	- - -	- - -	0.24 ± 0.02	-	GC-MS
1528	1524	β-Cadinene	SH	1.49 ± 0.04	1.06 ± 0.1	1.75 ± 0.11	1.57 ± 0.07	RI, GC-MS	GC-MS
-	1528	Calamenene	SH	- - -	0.01 ± 0.01	- - -	- - -	-	GC-MS
-	1548	Elemol	SO	- - -	0.09 ± 0.02	- - -	0.01 ± 0.01	-	GC-MS
1588	1574	Germacrene D-4-ol	SO	- - -	- - -	0.07 ± 0.01	0.17 ± 0.01	RI, GC-MS	GC-MS
-	1577	Spathulenol	SO	- - -	0.09 ± 0	- - -	0.09 ± 0.01	-	GC-MS
-	1582	Caryophyllene oxide	SO	- - -	- - -	- - -	0.05 ± 0	-	GC-MS
-	1608	Humulene epoxide	SO	- - -	0.01 ± 0.01	- - -	- - -	-	GC-MS
-	1619	Humulane-16-dien-3-ol	SO	- - -	- - -	- - -	0.04 ± 0	-	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
1641	1627	Epicubenol	SO	0.08 ± 0.01	0.08 ± 0.02	- - -	- - -	RI, GC-MS	GC-MS
1647	1631	γ-Eudesmol	SO	0.26 ± 0.01	0.41 ± 0.03	- - -	- - -	RI, GC-MS	GC-MS
-	1645	Cubenol	SO	- - -	0.07 ± 0.02	- - -	- - -	-	GC-MS
1656	1645	δ-Cadinol	SO	0.10 ± 0.00	0 ± 0.01	- - -	0.02 ± 0.02	RI, GC-MS	GC-MS
-	1649	β-Selinenol	SO	- - -	0.18 ± 0.02	- - -	0.04 ± 0	-	GC-MS
1671	1652	α-Eudesmol	SO	0.41 ± 0.01	0.06 ± 0.01	- - -	- - -	RI, GC-MS	GC-MS
-	1656	Patchouli alcohol	SO	- - -	- - -	- - -	0.04 ± 0.01	-	GC-MS
-	1949[d]	Isophytol	DO	- - -	- - -	- - -	0.06 ± 0	-	GC-MS
-	1984	Palmitic acid	FAD	- - -	- - -	- - -	0.62 ± 0.01	-	GC-MS
-	2132	Linoleic acid	FAD	- - -	- - -	- - -	0.28 ± 0.03	-	GC-MS
2521	2501	Oxypeucedanin	O	- - -	- - -	2.96 ± 0.64	- ± -	RI, GC-MS	-
2707	2707	β-Monolinolein	E	- - -	- - -	0.18 ± 0.01	- ± -	RI, GC-MS	-
1562	NA	Hedycaryol	SO	- - -	- - -	0.17 ± 0.01	0.17 ± 0.02	GC-MS	GC-MS
2009	NA	Thunbergol	DO	- - -	- - -	1.79 ± 0.12	1.4 ± 0	GC-MS	GC-MS
2010	NA	(*E*)-Geranylgeraniol	DO	0.79 ± 0.02	0.64 ± 0.01	0.31 ± 0.03	0.19 ± 0	GC-MS	GC-MS
-	1126[a]	2-p-Menthen-1-ol	MO	- - -	0.15 ± 0.01	- - -	- - -	-	GC-MS
-	1640	τ-Muurolol	SO	- - -	0.05 ± 0.01	- - -	- - -	-	GC-MS
-	1765[d]	Tetradecanoic acid	FAD	- - -	- - -	- - -	0.16 ± 0.01	-	GC-MS
-	2199	17-Octadecynoic acid	FAD	- - -	- - -	- - -	0.12 ± 0.08	-	GC-MS
-	2351[a]	Ricinoleic acid	FAD	- - -	- - -	- - -	0.06 ± 0.01	-	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Column type/Identification method[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
		Total identified [%]		**98.69 ± 0.22**	**98 ± 0.39**	**99.36 ± 0.07**	**98.06 ± 0.32**		

[a)] RI = retention indices for HP-5 column; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 1995), [a] (Andriamaharavo, 2014)[b] (Liu et al., 2006),[c] (Roussis et al., 2000), [d] (Palic et al., 2002), [e] (Tretyakov, 2007), NA = RI values were not available in the literature[b)] C = Class; A - Aldehydes, DO -Oxygenated diterpenes, E - Esters, FAD - Fatty acid and fatty acid derivatives, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes,, [d)]Identification method: GC-MS = Mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching the literature database; Std = constituent identity confirmed by co-injection of authentic standards [e)] Retention indices were not calculated for compounds calculated only by the DB-HeavyWAX column, **In bold** = main volatile compounds detected and total percentage of identified constituents

Table 10: Chemical composition of *P. nigrum* 'Kampot' EO and CO_2 extract.

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]												Identification[d]	
				Essential oil						CO_2 extract							
Obs.	Lit.			HP-5 MS			DB-Wax			HP-5 MS			DB-Wax			HP-5 MS	DB-Wax
923	924	α-Thujene	MH	0.059	±	0	-	-	-	-	-	-	-	-	-	RI, GC-MS	-
929	937	α-Pinene	MH	2.806	±	0.25	2.568	±	0.09	0.649	±	0.02	0.574	±	0.04	RI, GC-MS, Std	GC-MS, Std
944	946	Camphene	MH	0.04	±	0.01	0.05	±	0	-	-	-	-	-	-	RI, GC-MS, Std	GC-MS, Std
970	976	Sabinene	MH	0.048	±	0.03	0.091	±	0	-	-	-	-	-	-	RI, GC-MS	GC-MS
973	980	β-Pinene	MH	5.424	±	0.45	5.322	±	0.14	2.039	±	0.04	1.996	±	0.14	RI, GC-MS, Std	GC-MS, Std
989	991	β-Myrcene	MH	1.477	±	0.14	1.682	±	0.12	0.681	±	0.05	-	-	-	RI, GC-MS	GC-MS
-	1001	2-Carene	MH	-	-	-	-	-	-	-	-	-	0.076	±	0.02	-	GC-MS
1003	1005	α-Phellandrene	MH	1.803	±	0.14	1.481	±	0.08	0.762	±	0.03	0.681	±	0.03	RI, GC-MS, Std	GC-MS, Std
1008	**1008**	**3-Carene**	**MH**	**18.72**	**±**	**1.46**	**18.49**	**±**	**0.42**	**7.395**	**±**	**0.17**	**7.181**	**±**	**0.4**	RI, GC-MS	GC-MS
1025	1024	*p*-Cymene	MH	1.399	±	0.12	1.495	±	0.04	0.636	±	0.01	0.771	±	0.05	RI, GC-MS	GC-MS
1028	**1031**	**Limonene**	**MH**	**11.18**	**±**	**0.79**	**10.93**	**±**	**0.15**	**6.265**	**±**	**0.11**	**6.034**	**±**	**0.39**	RI, GC-MS	GC-MS
1058	1062	γ-Terpinene	MH	0.056	±	0.01	0.045	±	0	-	-	-	-	-	-	RI, GC-MS	GC-MS
1084	1086	Isoterpinolene	MH	0.194	±	0.04	0.4	±	0.02	0.09	±	0	-	-	-	RI, GC-MS	GC-MS
1087	1086	Terpinolene	MH	0.428	±	0.08	0.169	±	0	0.156	±	0.01	0.191	±	0.01	RI, GC-MS	GC-MS
1104	1095	Linalool	MO	0.354	±	0.03	0.453	±	0	0.238	±	0.05	0.386	±	0.01	RI, GC-MS, Std	GC-MS, Std
-	1140	Verbenol	MO	-	-	-	0.185	±	0.02	-	-	-	-	-	-	-	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]				Identification[d]	
				Essential oil		CO_2 extract			
Obs.	Lit.			HP-5 MS	DB-Wax	HP-5 MS	DB-Wax	HP-5 MS	DB-Wax
-	1179	p-Cymen-8-ol	MO	- - -	0.05 ± 0.05	- - -	- - -	-	GC-MS
-	1318	2,3-Pinanediol	MO	- - -	0.254 ± 0.01	- - -	- - -	-	GC-MS
-	1329	Piperonal	O	- - -	- - -	- - -	0.04 ± 0.01	-	GC-MS
1339	1339	δ-EIemene	SH	0.559 ± 0.02	0.588 ± 0.01	0.491 ± 0.01	0.516 ± 0.01	RI, GC-MS	GC-MS
-	1340	Piperitenone	MO	- - -	0.063 ± 0.05	- - -	- - -	-	GC-MS
1351	1351	α-Cubebene	SH	0.097 ± 0	0.086 ± 0.02	0.144 ± 0.01	0.117 ± 0.01	RI, GC-MS	GC-MS
-	1357	Octadecanal	A	- - -	0.57 ± 0.1	- - -	- - -	-	GC-MS
1378	1374	α-Copaene	SH	0.194 ± 0.01	0.17 ± 0.01	0.275 ± 0.01	0.247 ± 0.02	RI, GC-MS	GC-MS
1394	1391	β-Elemene	SH	1.483 ± 0.08	- - -	1.887 ± 0.03	1.303 ± 0.03	RI, GC-MS	-
1410	1409	α-Gurjunene	SH	0.164 ± 0.01	0.131 ± 0	0.257 ± 0.01	0.239 ± 0.03	RI, GC-MS	GC-MS
1416	1411	α-Bergamotene	SH	0.093 ± 0	0.01 ± 0.01	0.154 ± 0.01	0.018 ± 0	RI, GC-MS	GC-MS
1425	**1419**	**β-Caryophyllene**	**SH**	**37.84 ± 2.05**	**39.55 ± 1.12**	**54.21 ± 0.85**	**55.86 ± 1.37**	RI, GC-MS, Std	GC-MS, Std
-	1434	γ-Elemene	SH	- - -	0.057 ± 0	- - -	0.12 ± 0.01	-	GC-MS,
1440	1437	α-Guaiene	SH	0.983 ± 0.07	- - -	1.363 ± 0.02	- - -	RI, GC-MS	-
1456	1454	β-Farnesene	SH	0.101 ± 0.03	0.058 ± 0.05	0.143 ± 0	0.167 ± 0	RI, GC-MS	GC-MS
1459	1454	α-Humulene	SH	2.572 ± 0.22	2.52 ± 0.07	3.7 ± 0.02	3.465 ± 0.08	RI, GC-MS, Std	GC-MS, Std
-	1475	γ-Gurjunene	SH	- - -	0.9 ± 0.03	- - -	- - -	-	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]												Identification[d]	
				Essential oil						CO_2 extract							
Obs.	Lit.			HP-5 MS			DB-Wax			HP-5 MS			DB-Wax			HP-5 MS	DB-Wax
1493	1485	β-Selinene	SH	3.653	±	0.33	3.358	±	0.15	5.242	±	0.14	4.757	±	0.11	RI, GC-MS	GC-MS
1486	1492	Valencene	SH	0.136	±	0.01	-	-	-	0.224	±	0.01	-	-	-	RI, GC-MS	-
1501	1494	α-Selinene	SH	2.409	±	0.23	1.972	±	0.29	3.493	±	0.08	3.009	±	0.09	RI, GC-MS	GC-MS
1510	1506	β-Bisabolene	SH	1.131	±	0.1	0.887	±	0.1	1.711	±	0.06	1.284	±	0.05	RI, GC-MS	GC-MS
1524	1520	7-epi-α-Selinene	SH	0.114	±	0.01	-	-	-	0.168	±	0.03	-	-	-	RI, GC-MS	-
-	1528	Calamenene	SH	-	-	-	-	-	-	-	-	-	0.013	±	0	-	GC-MS
1533	1529	γ-Bisabolene	SH	0.069	±	0.01	-	-	-	-	-	-	-	-	-	RI, GC-MS	-
-	1561	Nerolidol	SO	-	-	-	-	-	-	-	-	-	0.076	±	0	-	GC-MS
-	1577	Spathulenol	SO	-	-	-	0.184	±	0	-	-	-	0.118	±	0.09	-	GC-MS
-	1579	Isoaromadendrene epoxide	SO	-	-	-	0.086	±	0	-	-	-	-	-	-	-	GC-MS
1593	1582	Caryophylene oxide	SO	2.941	±	0.24	3.295	±	0.26	2.036	±	1.01	3.013	±	0.2	RI, GC-MS	GC-MS
1621	1608	Humulene epoxide II	SO	0.154	±	0.02	0.158	±	0	0.128	±	0.01	0.139	±	0	RI, GC-MS	GC-MS
1643	1638	Isospathulenol	SO	0.492	±	0.05	0.421	±	0.32	0.463	±	0.02	0.526	±	0.08	RI, GC-MS	GC-MS
1668	1651	Pogostole	SO	0.154	±	0.02	0.215	±	0.1	-	-	-	0.09	±	0	-	GC-MS
-	1658	Neointermedeol	SO	-	-	-	0.054	±	0.01	-	-	-	-	-	-	-	GC-MS
1675	1665	Intermedeol	SO	0.05	±	0.02	0.07	±	0.01	-	-	-	-	-	-	RI, GC-MS	GC-MS
1950	1938	Pellitorine	O	-	-	-	0.006	±	0.01	1.191	±	0.09	1.669	±	0.06	RI, GC-MS	GC-MS

RI[a]		Compound[b]	C[c]	Extraction type/Column type/Peak area [%]												Identification[d]	
				Essential oil						CO_2 extract							
Obs.	Lit.			HP-5 MS			DB-Wax			HP-5 MS			DB-Wax			HP-5 MS	DB-Wax
-	1953	Hexadec-9-enoic acid	FAD	-	-	-	-	-	-	-	-	-	0.103	±	0	-	GC-MS
-	1959	Palmitic acid	FAD	-	-	-	-	-	-	-	-	-	0.423	±	0.04	-	GC-MS
-	2141	Oleic Acid	FAD	-	-	-	-	-	-	-	-	-	0.267	±	0.03	-	GC-MS
2707	2707	β-Monolinolein	E	-	-	-	-	-	-	1.84	±	0.98	-	-	-	RI, GC-MS	-
2018	NA	Heptadec-14-enal	A	-	-	-	-	-	-	0.122	±	0.04	-	-	-	RI, GC-MS	-
2815	NA	β-Monoolein	E	-	-	-	-	-	-	0.503	±	0.6	0.52	±	0.06	RI, GC-MS	GC-MS
-	NA	Hexadec-9-en-1-ol	O	-	-	-	-	-	-	-	-	-	0.238	±	0.01	-	GC-MS
-	2119[a]	17-Octadecynoic acid	FAD	-	-	-	-	-	-	-	-	-	0.025	±	0.02	-	GC-MS
-	NA	9-Tetradecenal	A	-	-	-	-	-	-	-	-	-	0.073	±	0.01	-	GC-MS
-	2153[b]	Kalecide	O	-	-	-	-	-	-	-	-	-	0.181	±	0	-	GC-MS
-	2351[b]	Ricinoleic acid	FAD	-	-	-	-	-	-	-	-	-	0.231	±	0.01	-	GC-MS
-	1143[b]	Sabinol	SO	-	-	-	0.072	±	0.01	-	-	-	-	-	-	-	GC-MS
		Total identified [%]		**99.4**	±	**0.2**	**99.2**	±	**0.1**	**98.7**	±	**0.7**	**96.7**	±	**0.1**		

[a]RI = retention indices for HP-5 column; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 1995), [a] (Tretyakov, 2007), [b] (Andriamaharavo, 2014), NA = RI values were not available in the literature [b)] C = Class; A - Aldehydes, DH - Diteprene hydrocarbons, E - Esters, FAD - Fatty acid and fatty acid derivatives, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes,, [d)]Identification method: GC-MS = Mass spectrum was identical to that of the National Institute of Standards and Technology

Library (ver. 2.0.f), RI = the retention index was matching the literature database; Std = constituent identity confirmed by co-injection of authentic standards, [e)] Retention indices were not calculated for compounds calculated only by the DB-HeavyWAX column, **In bold** = main volatile compounds detected and total percentage of identified constituents

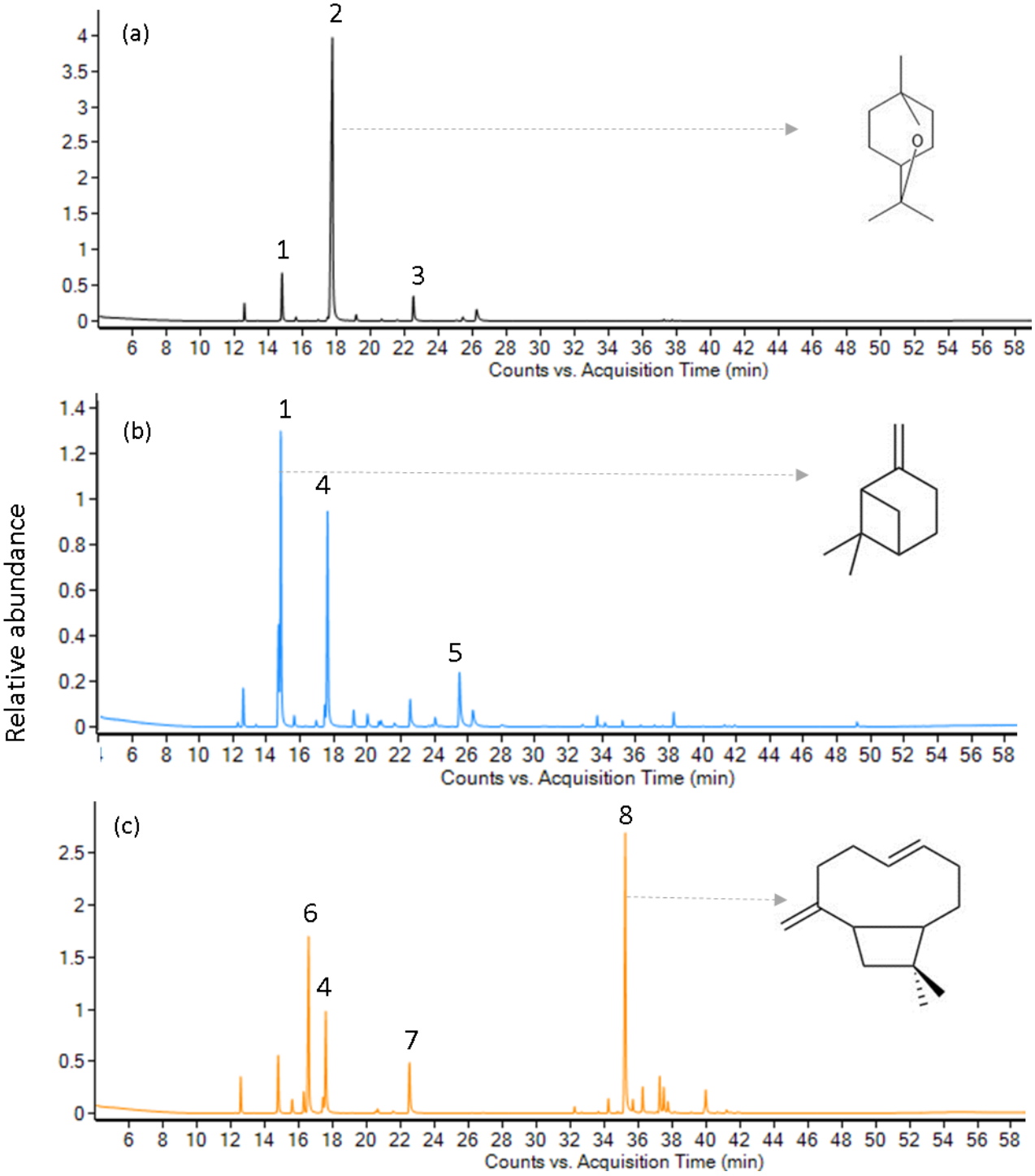


Figure 11: GC-MS chromatograms of EOs of a) *A. kravanh*, b) *C. hystrix* and c) *P. nigrum* 'Kampot' (analysed with HP-5 column). Peak numbers and constituents' names: **1**. β-pinene**, 2**. eucalyptol, **3**. α-terpineol, **4**. limonene, **5**. terpinene-4-ol, **6**. 3-carene, **7**-β-caryophyllene and **8**. β-selinene

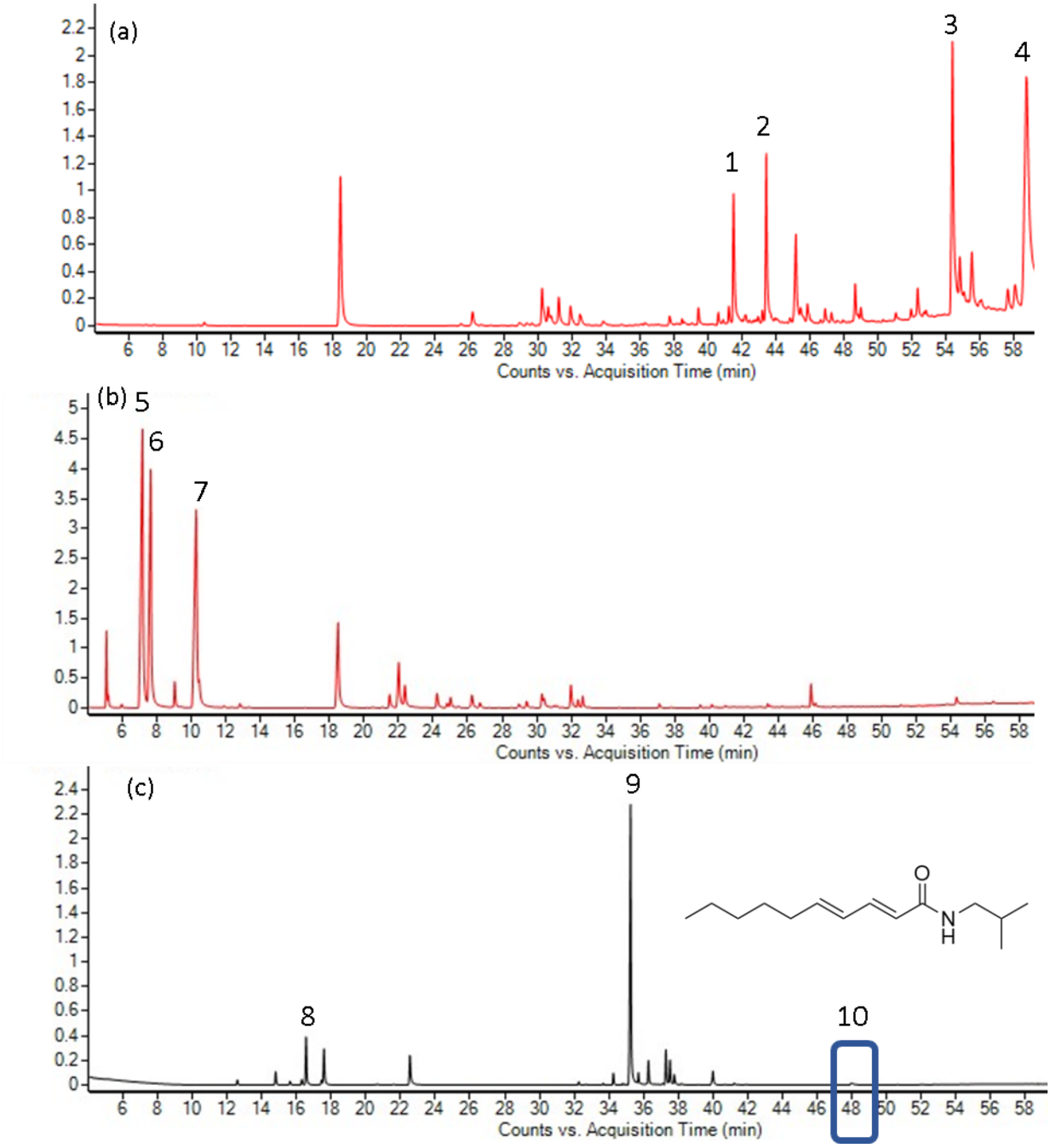


Figure 12: GC-MS chromatograms of CO_2 extracts of a) *A. kravanh*, b) *C. hystrix* (analysed on DB-HeavyWAX column) and c) *P. nigrum* 'Kampot' (analysed with HP-5 column). Peak number and compound names: **1**. eugenol acetate, **2**. tricosane, **3**. palmitic acid, **4**. oleic acid, **5**. β-pinene, **6**. sabinene, **7**. limonene, **8**. 3-carene, **9**. β-caryophyllene and **10**. pellitorine

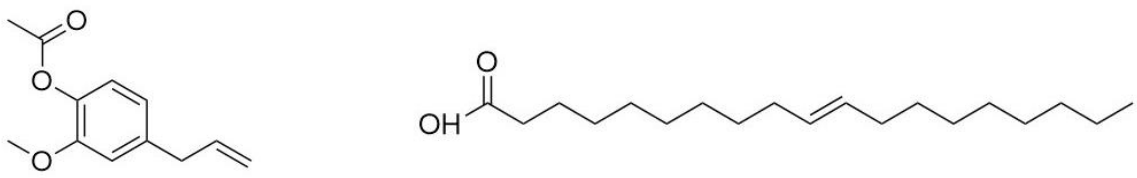


Figure 13: 1: Eugenol aceate and **4:** oleic acid

8. DISCUSSION

8.1. Physico-chemical characteristics of EOs and CO_2 extracts

In general, higher yields were obtained by SFE than hydrodistillation, with exception of *A. kravanh*, *C. cassia* fruits, *E. cardamomum* and *P. nigrum* 'Kampot'. From all samples, highest yield was acquired from *S. aromaticum* buds by SFE, which corresponds well with study conducted by Scopel et al. (2014). In their investigation, even higher yield was obtained by the SFE comprising to 19.6% v/w and containing higher content of eugenol than hydrodistilled EO. Among the EOs obtained by hydrodistillation, *A. kravanh* provided the highest yield followed by *E. cardamomum*. This finding agrees well with results obtained by Marongiu et al. (2004), where yields of hydrodistilled EOs and CO_2 extracts from *E. cardamomum* were compared. Their yields 5.0% of were only slightly higher for EOs than in our study. However, their CO_2 extract yield was higher, which can be attributed to different extraction parameters. Lowest yields from both extraction methods were obtained from *A. sativum* and *A. rusticana*, which were tested as positive spice controls. Our low yield values align with previously published studies regarding to EO yields of both species (Boukeria et al., 2016; Tomsone et al., 2013).

8.2. Screening of antimicrobial activity of EOs and CO_2 extracts

As far as the antimicrobial activity of spices tested is concerned, EOs and CO_2 extracts isolated from *C. cassia* and *C. verum* have exhibited the strongest effect against food pathogens. Generally, the *in vitro* growth inhibitory effect appeared slightly stronger in the liquid medium compared to the vapour phase or most EOs and CO_2 extracts tested. With sole exception of EO derived from *C. cassia* fruits, where the effect remained consistent across both phases. A similar pattern was observed in previous studies conducted by our team concerning certain plant volatile compounds (Houdkova et al., 2017), and EOs isolated from medicinal plants (Houdkova et al., 2018a; Houdkova et al., 2018b). The MIC values observed in our study for *C. cassia* bark and *C. verum* EOs in the liquid phase align closely with numerous previously published data. For instance, research by Ooi et al. (2006) determined the antibacterial effect of EO isolated from *C. cassia* bark against various pathogenic bacteria, including *E. coli* and *S. e.* Typhimurium, with MIC values of 300 µg/ml for both tested microorganisms. In another study by Unlu et al. (2010), *C. verum* EO inhibited the growth of *B. cereus*, *E. coli*, and *L. monocytogenes* with MIC values of 560, 1,120, and 560 µg/ml, respectively. Additionally, our results find support in the research conducted by Mith et al., (2014), where MICs for *C. verum* EO against most tested bacteria were lower than 1,000 µg/ml.

The disc volatilisation method is the most frequently employed method for evaluation of the *in vitro* growth inhibitory effect in the vapor phase. Such method was used during investigation carried out by Buckova et al. (2018), which assessed the antimicrobial potential of *C. cassia* EO vapours against several uropathogenic bacteria, including *E. coli*. A dose of 1 µl of EO/ml of airspace completely inhibited the growth of *E. coli* colonies on the Petri dish. Considering the density of *C. cassia* EO indicated in literature (1,030 µg/ml) (Haddi et al., 2017), this result is consistent with our findings. Conversely, study performed by Lopez et al. (2005) determined the growth inhibitory effects of *C. verum* vapours using a similar method and subsequently calculated MICs causing apparent inhibition (17.5 µl/l) of the atmosphere above microorganisms against *B. cereus* and *E. coli* and 34.9 µl/l against *L. monocytogenes.* Subsequent research conducted by Becerril et al. (2007) assessed the inhibitory concentration (IC) of *C. verum* against *E. coli* as 10 mg/l. In comparison to our results, their findings determined lower doses of EOs needed to inhibit the growth of tested bacteria. However, the findings of the disk volatilisation method providing qualitative results cannot be fully compared with quantitative data obtained using our technique. Moreover, variations in the age and segments of a tree stem, from which bark for the extraction of EO was obtained (Geng et al., 2011), diverse chemical composition of a headspace (Goni et al., 2009) and distinct bacterial strains used in the assay (Mith et al., 2014) could further contribute to the observed differences in values.

Only a few sporadic studies assessing the antimicrobial potential of CO_2 extracts are available. For example, research conducted by Kim et al. (2008) investigated the effect of *C. cassia* extract against food pathogens. MICs ranged from 250 to 500 µg/ml for *B. cereus*, *L. monocytogenes*, and *S. e*. Typhimurium, results similar to those presented in our study. Another examination evaluated the potential of CO_2 extracts from *C. cassia* fruits and bark against different bacterial pathogens. The MICs against *S. aureus* reached values of 300 and 500 µg/ml for fruits and bark, respectively (Yang et al., 2012). To the best of our knowledge, the antibacterial effect of CO_2 extracts from *C. cassia* and *C. verum* barks and fruit in the vapour phase was evaluated for the first time in our study. Moreover, this is the first report investigating the antibacterial properties of *C. cassia* fruit EO. Additionally, no quantitative literature data regarding CO_2 extract of *C. verum* and its growth inhibitory effect against foodborne pathogenic bacteria are currently available.

Apart from the *Cinnamomum* samples, weak antimicrobial effect was observed for CO_2 extract derived from *A. officinarum*, *A. rusticana,* and *C. zedoaria* EOs and both EO and extract of *S.*

aromaticum. In case of *A. officinarum* CO_2 extract, no report assessing its antibacterial effects is currently available. However, in research conducted by Srividya et al. (2010), such potential has been determined for hydroalcoholic extracts. Their MICs were 250 μg/ml against *B. cereus* and *E. coli*, which is much lower values than in our study. Such disparity between the MIC values can be explained by the different extraction method. Our results for EO derived from *A. rusticana* are in contrast with previously published study conducted by Kloucek et al. (2012), where antimicrobial potential of several spices in both liquid and vapour phase was assessed. In their results, *A. rusticana* EO exhibited the strongest antimicrobial effects from 69 EOs tested with MIC value 31.25 μl/l against all tested microorganisms. Such discrepancy can be attributed to different bacterial strains and method used in both studies. EO obtained from *C. zedoaria* rhizome exhibited weak antimicrobial potential against *B. cereus* (in both phases) and *L. monocytogenes* (in broth) with MICs 1024 μg/ml. Compared to other studies evaluating the antibacterial potential in liquid phase (Lai et al., 2004; Thin et al., 2022), our MIC values are higher. In these investigations, *C. zedoaria* EO inhibited growth of *B. cereus* at concentrations 500 and 200 μg/ml, respectively. Such discrepancies can be attributed to different extraction method and antimicrobial assay. Last sample exhibiting weak antimicrobial effect was EO isolated from *S. aromaticum*. Our MIC value is however higher than in some previously published studies (Radunz et al., 2019). In their study, EO isolated from *S. aromaticum* has exhibited inhibitory effect at a concentration of 304 μg/ml against *E. coli*, *L. monocytogenes* and *S. e.* Typhimurium. Our higher MIC concentration can again be attributed to different extraction method for obtaining the EO (steam distillation) and distinctive antibacterial assessment. For the assessment of *S. aromaticum* EO antimicrobial potential in vapour phase, study performed by Lopez et al. (2005) reported much lower MICs, 17.5 μg/ml against both *B. cereus* and *L. monocytogenes* and 26.2 μg/ml against *E. coli*. Similarly to our results for *Cinnamomum* samples, results obtained from disc volatilisation assay are more qualitative results with indicative value that cannot be fully compared with quantitative data obtained using our method. For CO_2 extracts, a few studies are available. Research conducted by Scopel et al. (2014) determined the effect of *S. aromaticum* CO_2 extract with respect to *E. coli* with the lowest MIC value 1000 μg/ml and such result is in consonance to our study. Study conducted by Das et al. (2021) evaluated the antibacterial potential of CO_2 extract against *S. aureus* and *S. dysenteriae* and the MIC values were 2500 and 3000 μ/ml. Although different bacterial strains were tested, our MIC values were lower.

Although no other EOs or CO_2 extracts have demonstrated antimicrobial effects in our study, some of them have previously been reported to produce high antibacterial effect. For example, *A. sativum* EO has been extensively researched for its growth inhibitory effects against bacteria in both liquid and vapour phases (Benkeblia, 2004; Kloucek et al., 2012; Nedorostova et al., 2009) and this spice is generally acknowledged as natural antibiotic for centuries (Harris et al., 2001). Furthermore, many Zingiberaceae species have previously been appraised for antimicrobial potential but did not inhibit any bacterial strains in our study. For instance, *B. rotunda* EO has previously shown promising antimicrobial effect against pneumonia causing *H. influenzae* (Houdkova et al., 2018b) or with respect to *B. cereus* exhibiting very promising MIC 62.5 μg/ml in liquid phase (Tasfiyati et al., 2023). Such disparity can be caused by different extraction method used in our study, since only CO_2 extract has been obtained in our study. Moreover, different geographical origin and antimicrobial assay could also contribute to such different results. *A. kravanh* EO has previously been reported to exhibit growth inhibitory effect on *E. coli* and *S. enterica* Typhimurium by Diao et al. (2014) with MIC values 2500 μg/ml. Since such MIC value is high, it was not even tested in our study and therefore can be in accordance with our results. EO from *A. subulatum*, another representative from Amomum genus, has also previously demonstrated antimicrobial effect against *B. cereus* and *E. coli* in investigation carried out by Satyal et al. (2012). In their research, seed and rind EOs inhibited growth of *B. cereus* at concentrations of 625 and 313 μg/ml, respectively and showed MIC values against *E. coli* of 625 and 1215 μg/ml, respectively. In our study, the EO was obtained from fruits including seeds simultaneously, which could account for the different findings along with different hydrodistillation type (Likens-Nickerson apparatus) performed during their study. *E. cardamomum* EO has also previously been reported to exhibit growth-inhibitory effect against food pathogenic bacteria. In study conducted by Singh et al. (2008), *E. cardamomum* EO showed apparent inhibition zone against the growth of *B. cereus*, *E. coli* and *S. typhi* at a concentration of 3 μg/ml per agar well during agar well diffusion method assessment. While these results differ from our study, a direct comparison cannot be made due to differences in methodology. Furthermore, study performed by Al-Zereini et al. (2022) showed much higher MIC (3750 μg/ml) against *B. subtilis*. Since such high concentration was not tested during our investigation such result supports our study. In case of *C. hystrix*, neither EO nor CO_2 extract provided antimicrobial activity, which agrees with study conducted by Waikedre et al. (2010). In their study, no antibacterial effect was observed against *B. subtilis* and *E. coli*, although EO inhibited growth of certain fungi. Such finding can be further supported by investigation conducted by Sreepian et al. (2023), who tested antimicrobial activity of *C. hystrix* EO by agar

disk diffusion method. Concentration needed to inhibit the growth of *E. coli* was 13 300 µg/ml and such high concentration was not even tested in our study. Similarly to previous samples, also *P. nigrum* 'Kampot' EO and CO_2 extract did not show any growth-inhibitory effects against tested bacteria. Such findings agree with research conducted by Seo et al. (2015), who assessed antibacterial effect of certain EOs by airtight disc apparatus. In their study, *P. nigrum* EO vapours inhibited the growth of *E. coli* at a 2.5 µl/ml, which is higher concentration (2500 µg/ml) then tested in our experiments. Contrastingly, study conducted by Morsy & Abd El-Salam (2017) reported MIC value of *P. nigrum* EO against *E. coli* as 1.95 µg/ml. However, since different cultivar of *P. nigrum* in different antimicrobial assay were investigated in our study, our results cannot be directly compared. For CO_2 extracts, number of studies is limited.

Gram-positive bacteria (*B. cereus* and *L. monocytogenes*) were slightly more susceptible to EOs and CO_2 extracts than Gram-negative (*E. coli* and *S.e.* Typhimurium) ones in our study, which is attributed to the nature of their cell walls and additional outer membrane. Presence of such membrane prevents the diffusion of hydrophobic compounds through the lipopolysaccharide layer (Burt, 2004; Smith-Palmer et al., 1998).

8.3. Chemical composition of EOs and supercritical CO_2 extracts from *Cinnamomum* spp. barks and fruits in liquid and vapour phase

The results of analyses of chemical composition of EOs and supercritical CO_2 extracts obtained from *Cinnamomum* spp. showed that (*E*)-cinnamaldehyde was the predominant constituent in all examined samples. With exception of report of Chang et al. (2013), who described *cis*-methoxycinnamic acid (43.06%) as a principal component present in *C. cassia* bark EO, such finding is in accordance with numerous previously published studies (Hameed et al., 2016; Sandner et al., 2018). Moreover, this constituent has been proposed to exhibit antimicrobial activity in both direct contact (Firmino et al., 2018; Unlu et al., 2010) and in vapour phase (Lopez et al., 2007). However, subsequent research indicates that vapours from cinnamon EO exert similar inhibitory effects as pure cinnamaldehyde vapours, suggesting the involvement of other minor compounds (Becerril et al., 2007). Slight variations in the content of other major compounds have also been noted in the literature. For instance, Ooi et al. (2006) reported 2-methoxycinnamaldehyde (8%) as the second most abundant compound, while cinnamyl acetate ranked second in our sample. Chemical composition of *C. cassia* supercritical extract varied substantially from the EO. Although (*E*)-cinnamaldehyde and 2-methoxycinnamaldehyde,

which has also been suggested to possess antimicrobial effect, were present in highest levels, coumarin has been detected by HP-5 column as the second most abundant compound after (*E*)-cinnamaldehyde. Such finding is consistent with finding of Yang et al. (2012), who reported its high content (20.69%) after 10 min of extraction by supercritical CO_2. Moreover, we reported for first time chemical composition of EO isolated from fruits of *C. cassia*, which contained highest level of (*E*)- cinnamaldehyde in comparison with all samples. Chemical profile of EO from *C. verum* has been a subject of multiple studies and mostly, higher amount of the main compound was detected than in our sample. For example, research performed by Unlu et al. (2010) determined 68.95% of (*E*)-cinnamaldehyde, followed by 9.94% of benzaldehyde and 7.44% of cinnamyl acetate. Supercritical extract of *C. verum* was richer in (*E*)-cinnamaldehyde than EO which is agreement with available literature, however, β-caryophyllene was detected as second main compound (Marongiu et al., 2007; Tateo & Chizzini, 1989). Factors such as extraction conditions, geographical origin, and plant material age significantly influence the chemical composition of EOs and CO_2 extracts (Chang et al., 2013; Ooi et al., 2006), elucidating discrepancies between reported literature and our findings. The potential toxicity of cinnamaldehyde has been a subject of multiple investigations and the compound has been concluded as safe when consumed within the acceptable daily intake (ADI = 0–1.25 mg/kg). However higher than nutritional levels of cinnamaldehyde may induce genotoxic and hepatotoxic effects. Therefore, caution is advised when cinnamaldehyde is taken as a preventive measure (WHO, 1984; Zhu et al., 2017).

In pursuit of identifying the primary constituents responsible for the antimicrobial properties of the most potent vapours, we investigated the chromatographic profile of the headspace above *C. cassia* fruits' EO at the MIC concentration. Sesquiterpenoids emerged as the predominant chemical group, followed by aldehydes, with a higher concentration of recovered monoterpenoids compared to EO diluted in hexane. While no prior studies have examined the atmosphere generated by *C. cassia* fruit EO, previous research has explored the composition of the headspace above *C. verum* or *C. cassia* barks. For instance, Miller et al. (1996) reported the difference between chemical composition of supercritical CO_2 extracts and headspace above ground fresh samples of *C. verum* and *C. cassia* barks. Difference between chromatographic profiles was attributed to higher presence of minor compounds detected in EO than in CO_2 extracts using SPME analysis. The difference is likely to be caused by lower recovery of (*E*)-cinnamaldehyde and coumarin and such finding is in accordance with our results. Additionally, Goni et al. (2009) investigated the composition of an atmosphere enhanced with *C. verum* EO

by SPME, identifying eugenol as the most abundant constituent, followed by β-caryophyllene, accompanied by terpenes in trace levels, with negligible amounts of (*E*)-cinnamaldehyde in the headspace. Although our study focuses on different botanical species, the low recovery of (*E*)-cinnamaldehyde from the headspace corroborates our findings. Moreover, the percentage of this aldehyde has gradually been decreasing over time during our experiments and the most abundant peak has been replaced by peaks of δ-cadinene and γ-muurolene. Even though we did not perform headspace analysis of *C. cassia* fruits EO at various concentrations, its quantitative composition will probably be concentration dependent as it has previously been reported for cinnamaldehyde (Wang et al., 2008). The increasing presence of δ-cadinene and γ-muurolene suggests their potential contribution to the high antimicrobial efficacy of our samples, though no reports investigating such effects of individual compounds are currently available. Nevertheless, these sesquiterpenes have been identified in plant EOs or extracts with proven antimicrobial or antifungal activity, such as *Z. officinale* (Sasidharan & Menon, 2010), *Hypericum linaroides* (Cakir et al., 2005), and *Ocimum gratissimum* (Silva et al., 2010). Although the exact cause of gradual decreasing of (*E*)-cinnamaldehyde content in the headspace remains unclear, it could partially be explained by its potential ability to pass back to the liquid growth medium. Research conducted by Novy et al. (2014) determined such an ability with thymoquinone, another constituent with proven antibacterial effect against foodborne pathogens. To the best of our knowledge, this is the first report investigating the chemical composition of *C. cassia* fruit EO both diluted in hexane and the vapours in the headspace above it.

Traditionally, immature fruits of *C. cassia* have found use as flavouring agents in various Asian cuisines, including curries, pickles, chutneys, poached fruit, apple desserts, and local pastries. From a sensory perspective, this commodity bears resemblance to *C. cassia* bark, yet minor compounds contribute floral, woody, nut-like, cool, and sweet impressions, primarily due to the presence of γ-muurolene and δ-cadinene (Guoruoluo et al., 2017; Santos et al., 2017). Moreover, chemical analysis revealed significantly lower content of coumarin compared to *C. cassia* bark, thus presenting fewer concerns regarding potential toxicity associated with its culinary use. In several previous studies, the EO from *C. cassia* bark has been well-received or neutrally accepted by sensory panellists when used as a preservative in various animal-based foods, such as Cheddar cheese, ham, salmon, and lean pork. With exception of lean salmon, where addition of cinnamon EO into MAP or vacuum packaging slightly increased microbial growth (Van Haute et al., 2016), the inclusion of cinnamon EOs or extracts was generally met

with neutral or positive reactions among sensory panellists (Tayel et al., 2015) and improving microbial shelf life (Dussault et al., 2014). Other studies have focused on plant food products, such as the research led by (Sandner et al., 2018), which investigated the effects of edible coatings enriched with *C. cassia* EO on minimally processed apple slices. While significant improvements were observed in shelf life and browning index, sensory descriptors received slightly lower evaluations from panellists. Similar effects have been noted in other fruit species, such as kiwis and melons (Muche & Rupasinghe, 2011; Dos Santos et al., 2018). These findings suggest that despite the distinctive flavour and aroma of cinnamon, *C. cassia* EOs and CO_2 extracts can be effectively utilized for food preservation, especially when combined with suitable products such as cheese and meat. Additionally, there is minimal direct contact with the food matrix when used in combination with MAP, thereby reducing the risk of negative effects on sensory properties and safety profiles. However, further experiments focused on diverse food models are necessary, and special attention should be paid to fatty fish products.

8.4. Chemical composition of EOs and supercritical CO_2 extracts from *Amomum kravanh*, *Citrus hystrix,* and *Piper nigrum* 'Kampot'

GC-MS analysis of chemical composition of EOs and supercritical CO_2 extracts isolated from phytochemically less explored Cambodian spices showed that eucalyptol was the dominant constituent of *A. kravanh* EO. This finding is in accordance with previously published studies investigating the chemical composition of EO from this plant (Diao et al., 2014; Feng et al., 2011), as well as from other species from *Amomum* genus (Sabulal & Baby, 2021; Yu et al., 2018). Consistently with Zhang et al. (2020), β-pinene and α-terpineol were abundant compounds in the analysed *A. kravanh* EO sample. Contrastingly, Diao et al. (2014) reported relatively lower amounts of α-pinene (5.71%) and β-pinene (2.41%), with terpinyl acetate (11.2%) and dipentene (6.1%) as abundant EO components, likely due to differences in geographical origins of the samples. Corresponding with Zhang et al. (2020), limonene was identified as the third-most prevalent compound of the EO from *A. kravanh* when utilising the DB-HeavyWAX column. The current literature only reports the use of the DB-HeavyWAX column, the results of which differ from those reported in the present study. According to Yothipitak et al. (2009), eucalyptol (71.45%), β-pinene (8.64%), and limonene (4.77%) were the three dominant constituents of the *A. kravanh* extract obtained by SFE. These discrepancies can be caused by the different extraction parameters (33 °C and 175 bars) used during the SFE process and by the distinct geographical origin (Thailand) of the plant sample. Different main

constituents identified during the HP-5 column and DB-HeavyWAX column analyses could be the result of the stronger detection sensitivity and ability of the polar DB-HeavyWAX column to separate and quantify fatty acids and their methylesters from the rest of the sample compared to the non-polar HP-5 column. Furthermore, polar columns based on polyethylene glycol have more accurate results regarding the identification of fatty acid saturation and therefore are commonly employed in the analyses of complex fats and oils (David et al., 2005; Woo et al., 2012). To confirm and further analyse the fatty acid profile of *A. kravanh* CO_2 extract, derivatization prior to analysis is highly encouraged to be conducted. Suggested methods for derivatization include methylation, silylation and alkylation (Topolewska et al., 2015).

In *C. hystrix* EO, β-pinene emerged as the most abundant constituent, consistent with previously published analyses where the percentages of β-pinene ranged from 25.93 to 47.93% (Jantan et al., 1996; G. Li et al., 2021; Sato et al., 1990; Tran et al., 2021). Jantan et al. (1996) and Tran et al. (2021) also reported also reported limonene as the second-most dominant compound, comprising almost 15 and 20% of the sample, respectively. Sabinene was the third-most abundant constituent in the present study, which agrees with the above-mentioned study of Tran et al. (2021). However, a slight discrepancy arises when comparing to the study conducted by Sato et al. (1990), where sabinene accounted for more than 20% of the extract and was the second-most dominant compound. Moreover, the *C. hystrix* EO analysed in the present study was lower in citronellal in comparison to previously published data. Since the samples from previously published studies were collected in Malaysia (Jantan et al., 1996), Vietnam (Le et al., 2020) and Thailand (Sato et al., 1990), differences in chemical composition can be attributed their different geographical origins. Furthermore, in the case of the study conducted by Sato et al. (1990), steam distillation was used as the extraction method. In addition, maturity of the fruit and processing of the sample before extraction are factors which can affect the chemical composition of the EO (Suresh et al., 2021). While CO_2 extraction was previously conducted from the leaves of this species by Norkaew et al. (2013), to the best of our knowledge, this is the first report investigating the chemical composition of CO_2 extract isolated from the peel of *C. hystrix*. Due to the presence of large secretory cavities in *Citrus* spp. fruit rinds, their EOs are traditionally obtained by cold pressing, comprising a volatile fraction with mono and sesquiterpenes and their oxygenated derivatives. However, a non-volatile fraction represented by coumarins, psoralens, and other oxygen heterocyclic compounds is also present in cold-pressed oils (Lu et al., 2019; Russo et al., 2021). Although investigation of *C. hystrix* cold-pressed EO is currently not available in the literature, several studies compared cold-pressed

and hydrodistilled EOs from more common *Citrus* species. The most common conclusion was that there is a higher recovery of terpene hydrocarbons in cold-pressed oils, which are compounds responsible for the typical aroma of *Citrus* oils (Ferhat et al., 2007; Le et al., 2020). Therefore, the comparison of cold-pressed *C. hystrix* EO with other extraction methods is highly encouraged for future research related to chemical composition or bioactivity assessment.

The primary distinction between the *P. nigrum* 'Kampot' EO and CO_2 extract lies in the presence of pellitorine, belonging to the piperamides, which constituted more than 1% of the total CO_2 extract. This nitrogen-containing compound has also been detected by Luca et al. (2023) in much lower amounts (0.18%); however, other piperamides like piperine, piperettine, and guineesine were also present in their extracts. This slight dissimilarity can be attributed to the different *P. nigrum* cultivar assessed in in the present study and the different extraction conditions of the SFE process, where higher pressure (up to 300 bars) was used for the selective recovery of piperazines. The primary compound in the EO was sesquiterpene β-caryophyllene, consistent with numerous previously published studies assessing the composition of *P. nigrum* EO. This sesquiterpene ranged from 29.9 to 62.3% of the volatile oil (Andriana et al., 2019; Kapoor et al., 2009; Y. Li et al., 2020). Other major constituents in the *P. nigrum* EO within the present study were 3-carene and limonene. This corresponds well with Li et al. (2020), where 3-carene and limonene were present in maximal respective amounts of 26.84 and 25.83% in the various EOs obtained from black and white peppercorns of Chinese origin. However, slightly different components were identified by Andriana et al. (2019), where β-thujene and β-selinene accounted for 20.58 and 5.59% of the sample, respectively. Furthermore, Kapoor et al. (2009) and Bagheri et al. (2014) reported limonene (13.2%), β-pinene (7.9%), and sabinene (5.9%) as the predominant compounds of the EO. According to the ISO guideline, EO from black peppercorns should contain 25–26% of β-caryophyllene, followed by limonene (11.5–13.4%), sabinene (9.1–9.6%) and α-pinene (8.4–9.7%) (Andrade et al., 2017). These slight disparities in the main components can be attributed to a different cultivar of *P. nigrum* assessed in the present study, along with varying harvest and post-harvest handling of the fruits used to produce dried red Kampot peppercorns (Morm et al., 2020). In the CO_2 extract, the amount of β-caryophyllene was even higher than in the EO, which is in accordance with Bagheri et al. (2014), where the recovery of this sesquiterpene was also higher than in the hydrodistilled EO. Moreover, a higher recovery of sesquiterpenes and their oxygenated derivatives for CO_2 extracts opposed to EOs has also been previously reported (Andrade et al., 2017; Luca et al.,

2023). The main components in the *P. nigrum* 'Kampot' CO_2 extract were monoterpenes 3-carene and limonene, and such findings correspond well with Topal et al., (2008). These same compounds amounted to 10.32 and 5.4% in the *P. nigrum* CO_2 extract, respectively. A slight discrepancy can be observed compared to the results of Luca et al. (2023), where sabinene was present in 8.61% and limonene comprised 8.21% of the total *P. nigrum* extract, which can again be attributed to the different cultivar researched in the present study. Additionally, to the best of our knowledge, the chemical composition of *P. nigrum* 'Kampot' EO and CO_2 extract has been assessed for the first time in the current report.

9. CONCLUSIONS

In summary, this study reports antimicrobial activity of 17 CO_2 extracts and 14 EOs isolated from spices originating from various parts of Asia, with special focus on species traditional used in Cambodia as spices and food condiments. Their antimicrobial efficacy was assessed using broth microdilution volatilisation method against four foodborne pathogens, namely *B. cereus*, *E. coli*, *L. monocytogenes* and *S. e*. Typhimurium. Results indicate that EOs and CO_2 extracts isolated from *Cinnamomum* spp. exerted certain degree of antimicrobial potential in both liquid and vapour phase. Particularly, EO isolated from immature fruits of *C. cassia* demonstrated the strongest potential against food pathogens. The most susceptible bacterial strain to our samples was Gram-positive *B. cereus*. These findings suggest the potential application of EO and CO_2 extract from *C. cassia* fruits as volatile antimicrobial agents in food industry, particularly as a part of MAP enhancement. The most promising approach in food preservation would be incorporation of EOs and CO_2 extract into the active packaging with over-time release of active constituents. Furthermore, incorporation into nanoemulsions also presents an interesting research area (Ribeiro-Santos et al., 2017; Sanchez-Gonzalez et al., 2011; Singh et al., 2022). However, further experiments addressing organoleptic acceptance and effectiveness in appropriate food models are necessary to verify their potential commercial use in food preservation. Furthermore, the broth microdilution volatilisation method has been validated in use to determine susceptibility of food pathogenic bacteria in vapour phase.

With aim to characterize chemistry of spices producing antimicrobial effects, chromatographic profiles of EOs and CO_2 extracts have been analysed using two columns with different polarity. Across all *Cinnamomum* samples, (*E*)-cinnamaldehyde has been detected as the most abundant constituent accompanied with sesquiterpenoids, esters and monoterpenoids. Subsequent time

series headspace sampling and analysis of vapours above the mixture of growth medium and *C. cassia* fruits EO revealed a notable decrease in content of (*E*)-cinnamaldehyde in the headspace that has gradually been replaced by peaks of δ-cadinene and γ-muurolene. Proposed mechanism behind this phenomenon is ability of (*E*)-cinnamaldehyde to pass back into the liquid medium from the headspace, however, further investigation would be necessary to confirm or disprove this hypothesis. Moreover, antimicrobial potential of δ-cadinene and γ-muurolene merits additional investigations.

Although none of the EOs or CO_2 extracts obtained from Cambodian samples displayed comparable antimicrobial effect to *Cinnamomum* samples, their chromatographic profiles were examined to compare both extraction methods and efficacy of both columns of different polarity. EOs and CO_2 extracts from fruits of *A. kravanh*, *C. hystrix* and red peppercorns of *P. nigrum* 'Kampot' have been subjected to GC-MS chemical analysis. The chromatograms of EOs and CO_2 extracts from all spice species differed and the most significant difference was seen for the EO and CO_2 extracts from *A. kravanh* fruits. EO was rich in eucalyptol while CO_2 extract comprised of oleic and palmitic acids and tricosane. *C. hystrix* and *P. nigrum* 'Kampot' CO_2 extracts were generally richer in sesquiterpenes and their oxygenated derivatives in comparison to their EOs, where monoterpenes were more abundant. Furthermore, fatty acid derivatives, nitrogen-containing compounds, and other non-volatile constituents were also more prevalent in the CO_2 extracts. To the best of our knowledge, this study describes the first isolation and chemical analysis of EO and CO_2 extract from *P. nigrum* 'Kampot' fruits, and CO_2 extract from the peel of *C. hystrix*. These findings suggest that supercritical CO_2 can potentially be used for the extraction of Asian spices like *A. kravanh, C. cassia* and *P. nigrum* 'Kampot' with aim to extract different constituents than present in hydrodistilled EOs. Nevertheless, further research determining the most efficient extraction parameters tailored to desired constituents is needed before its commercial application in spice-processing practices.

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Yu, G.-W., Cheng, Q., Nie, J., Wang, X.-J., Wang, P., Li, Z.-G., & Lee, M.-R. (2018). Microwave hydrodistillation based on deep eutectic solvent for extraction and analysis of

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11. APPENDICES

List of the appendices

Appendix 1: Curriculum vittae

Appendix 2: List of authors publications

Appendix 3: Purchasing and collecting plant material

Appendix 4: Drying plant material, EOs distillation and CO_2 extraction

Appendix 1:

Curriculum Vitae

Name: Kateřina Vihanová
Telephone: (+ 420) 775 123 505
E-mail: vihanova@ftz.czu.cz

Education:

10/2017-05/2024 Doctoral
Czech University of Life Sciences Prague
Faculty of Tropical AgriSciences
Doctoral study program Tropical Agrobiology and Bioresource Management
Dissertation thesis title: Chemical composition and antimicrobial activity of essential oils and supercritical carbon dioxide extracts of Asian spices against food pathogens in liquid and vapour phase

2013-2015 master's degree
Czech University of Life Sciences Prague
Faculty of Agrobiology, Food and Natural Resources
MSc study program Food and Nutrition,

2008 – 2012 bachelor's degree
Czech University of Life Sciences Prague
Faculty of Agrobiology, Food and Natural Resources
BSc studies,

Abroad placements:

07/2023-08/2023
PhD students Summer Science Camp in Indonesia
Kristen University Satya Wacana, Salatiga
Role: Research presentation, teaching and agenda organization in cooperation with UKSW Salatiga

03-04/2019
Student mobility in Cambodia
Royal University of Agricultural, Phnom Penh
Role: Plant sample purchasing and collection

04-06/2018
Student mobility in the Philippines
Visayas State University, Baybay
Role: Plant sample purchasing and collection, essential oils distillation

08/2012-07/2013
Erasmus study exchange program
Wageningen University, the Netherlands
Nutrition and Health field of study, winter and summer semester

Project participation:

- 2018 Chemical composition and biological activity of medicinal and edible tropical plants (IGA 20185019)
- 2020 Evaluation of biological effects and chemical analysis of compounds from tropical plants (IGA 20205001)
- 2021 Evaluation of biological effect and chemical analysis of compounds from tropical plants (IGA 20213109)
- 2021 Proof-of-concept activities 2 at CULS Prague, Technology Grant Agency of the Czech Republic (TP01010050)

Other education:
07/2018, 08/2019 and 08/2022 – Spanish courses in Barcelona, Valencia, and San Sebastian

09/2012 -04/2013 – Social Dutch language courses (Wageningen University)

07- 08/2010 and 07/2011
Language courses in Malta – General English Courses

Work experiences:

07/2022-08/2023
Czech University of Life Sciences Prague
Faculty of Forestry and Wood Sciences
Role: International Relations Office
Responsibilities: Screening for potential international partnership universities, agenda with incoming post-graduate students and employees, organization of international events (summer schools, workshops)
Full-time job

Language skills:

English – fluent knowledge
Spanish – intermediate knowledge
Dutch-pre-intermediate knowledge
German – passive knowledge

Laboratory work skills: Extraction of natural compounds, antimicrobial activity testing, chemical analysis (GC-MS)

Interests: Beach volleyball, fitness, traveling, animals, learning foreign languages, drawing

Appendix 2:

List of authors publications:

PUBLICATIONS IN SCIENTIFIC JOURNALS

Vihanova, K., Urbanova, K., Nguon, S., Kokoska, L., (2023), Chemical composition of essential oils and supercritical carbon dioxide extracts from *Amomum kravanh*, *Citrus hystrix* and *Piper nigrum* 'Kampot', *Molecules* **2023**, *28* (23), 7748;(IF= 4.6)

Vihanova, K., Houdkova, M., Promgool, T., Urbanova, K., Kanokmedhakul, S., & Kokoska, L. (2021). In vitro growth inhibitory effect of essential oils and supercritical carbon dioxide extracts from Cinnamomum spp. barks and fruits against food bacterial pathogens in liquid and vapor phase. *Journal of Food Safety* **2021**, *41*(4), (IF = 2.449)

CONFERENCE CONTRIBUTIONS

- **Oral presentation**
- **Vihanova, K.,** Houdkova, M., Promgool, T., Horak, O., Kanokmedhakul, S., Kokoska L. Evaluation of in vitro growth-inhibitory effect of spice essential oils and supercritical carbon dioxide extracts on food pathogenic bacteria in liquid and vapor phase using broth microdilution volatilization method, ICAAP conference, 10.-11.10. 2019, Novi Sad, Serbia.

- **Poster communications**

- **Vihanova, K**., Houdkova, M., Promgool, T., Urbanova, K., Kanokmedhakul S., Kokoska L. Evaluation of in vitro growth inhibitory effect of cinnamon essential oils and supercritical carbon dioxide extracts against food pathogenic bacteria in liquid and vapor phase, 35th EFoST International Conference, 1.-4.11. 2021, Lausanne, Switzerland.
- **Vihanova K.**, Houdkova M., Horak O., Kokoska L. Evaluation of in vitro growth-inhibitory effect of spice essential oils on food pathogenic bacteria in liquid and vapor phase using broth microdilution volatilization method.,32nd EFFoST International Conference, 6. 11 - 8. 11. 2018, Nantes, France.

Appendix 3:

Purchasing and collecting plant material and Cambodian species

Local spice store, Orussey Market, Phnom Penh, Cambodia (Vihanova, 2019)

La Plantation pepper farm, Kampot, Cambodia (Vihanova, 2019)

Local herbal market, Phnom Penh, Cambodia (Vihanova, 2019)

P. nigrum 'Kampot' (Vihanova, 2019)

Mondulkiri, Cambodia (Korytakova, 2019)

A. Kravanh fruits (Traditional Chinese Medicine, 2016)

E. littoralis (Korytakova, 2019)

C. hystrix fruits (Vihanova, 2019)

Appendix 4:

Drying plant material, EOs distillation and supercritical CO_2 extraction

Baybay, Philippines (Netopilova, 2018) from @lee_lab_prague

Hydrodistillation of *C. microcarpa*, Baybay, Philippines (Fiserova, 2018) from @lee_lab_prague

Supercritical fluid extraction of *K. galanga* (Vihanova, 2021) (Chaure,2021)

Amomum kravanh EO and CO_2 extract (Vihanova, 2021)