CHEMICAL COMPOSITION AND NANOENCAPSULATION OF *CYNOMETRA CAULIFLORA* ESSENTIAL OILS AND THEIR BIOLOGICAL ACTIVITY STUDIES

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UNIVERSITI SAINS MALAYSIA

2023

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by

BENEDICT ANAK SAMLING

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

February 2023

ACKNOWLEDGEMENT

Firstly, I am very thankful to my supervisor, Dr. Tan Wen Nee for her great assistance, advice, support, and encouragement throughout the research study. Without her guidance, this research would not have been successful. Next, I would like to express my sincere gratitude to my co-supervisor, Prof. Dr. Zaini Assim for his valuable guidance and advice.

I am grateful to the Dean of the School of Distance Education, USM for equipping me with the research facilities throughout the research study. Also, I would like to thank the Dean of the Institute of Postgraduate Studies, USM for giving me a chance to pursue my postgraduate study. I would also like to acknowledge the late Mr. Hidir Marzuki and Mr. Sekudan Tedong from Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS) for plant identification. I would also like to express my gratitude to Dr. Syarifah Ab Rashid and Ms. Musthahimah Muhamad for their assistance in biological studies. The USM Short Term Grant (304.PJJAUH.6315245) and the Sarawak Biodiversity Centre (SBC-2021-RDP-30-TWN) are also acknowledged.

I am indebted to the Dean of the Faculty of Resource Science and Technology and Human Resource Department, UNIMAS for supporting and approving my unrecorded leave. I would like to thank Assoc. Prof. Dr. Sim Siong Fong, Assoc. Prof. Dr. Tay Meng Guan and Dr. Teng Sing Tung for their assistance. Thanks also to the laboratory staff of UNIMAS, in particular, Mr. Wahap Marni, Mr. Shafri Semawi, Ms. Limjatai, Madam Sheela, and Ms. Syida for their assistance during the course of this research study. I would like to take this opportunity to thank my colleagues, Madam

Ting Woei, Ms. Zeti Akhtar, Mr. Leo Bulin, Mr. Tomy Bakeh and Mr. Amin Mangi for their support and cooperation throughout the research study.

Lastly, I would like to express my deepest gratitude to my lovely family members. Thank you for giving me support and endless love throughout this study.

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LIST OF SYMBOLS

g	gram
h	hour
mg	milligram
mg/mL	milligram per milliliter
Min	minute
α	alpha
β	beta
γ	gamma
%	per cent
m	meter
mm	millimetre

LIST OF ABBREVIATIONS

CCEO	Cynometra cauliflora essential oil
CCEO-CSNP	*Cynometra cauliflora* essential oil - loaded chitosan nanoparticles
CS	Chitosan
EE	Encapsulation efficiency
EO	Essential oil
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
LC	Loading capacity
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NP	Nanoparticles
pH	Potential of hydrogen
Rpm	Rotation per minutes
RI	Retention index

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KOMPOSISI KIMIA DAN NANOENKAPSULASI MINYAK PATI *CYNOMETRA CAULIFLORA* DAN KAJIAN AKTIVITI BIOLOGI

ABSTRAK

Cynometra cauliflora L., dikenali sebagai "nam-nam" atau "katak puru" di Malaysia adalah tergolong dalam keluarga Fabaceae. Pokok ini ditanam di kawasan kampung dan telah digunakan secara tradisional untuk merawat penyakit seperti kehilangan selera makan, masalah kulit, kencing manis, dan masalah berkaitan kolesterol. Setakat kini, kajian terhadap *C. cauliflora* adalah terhad. Objektif kajian ini adalah untuk mengenalpasti juzuk kimia minyak pati dari daun, ranting dan buah *C. cauliflora* dan mengenkapsulasikan minyak pati dalam nanopartikel kitosan (CSNPs). Aktiviti antioksidan, antimikrob dan ketoksikan minyak pati dan minyak pati terenkapsulasi telah dinilai. Juzuk minyak telah dikenalpasti dengan menggunakan GC dan GC-MS kapilari. Aktiviti pengautan radikal 1,1-difenil-2-pikrilhidrazil (DPPH) telah dilakukan untuk menilai aktiviti antioksidan. Aktiviti antimikrob telah dinilai dengan menggunakan cerakin Kirby Bauer dan mikropencairan kaldu ke atas *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Candida utilis*, and *Candida albicans*. Mengenai sitotoksisiti, cerakin proliferasi sel telah dijalankan dengan menggunakan 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida (MTT) ke atas sel kanser payudara manusia (MCF-7 dan MDA-MB-231) dan sel normal payudara manusia (MCF-10A). Keputusan menunjukkan minyak pati daun, ranting dan buah *C. cauliflora* terdiri daripada dua puluh enam, tujuh belas dan lima puluh juzuk kimia masing-masing. Minyak pati daun, ranting dan buah telah didominasi oleh hidrokarbon monoterpena (54.15%), monoterpena beroksigen

(69.53%) dan seskuiterpena beroksigen (65.48%) masing-masing. Minyak pati ranting memberikan kuasa pengautan radikal bebas tertinggi (37.12 ± 2.84 µg/mL) berbanding dengan minyak pati daun (207.17 ± 2.95 µg/mL) dan minyak pati buah (461.88 ± 12.61 µg/mL). Dalam cerakin antimikrob, minyak pati ranting merencat semua mikroorganisma dengan zon perencatan berjulat dari 10.3 hingga 29.7 mm. Minyak pati ranting menunjukkan kepekatan perencatan minimum (MIC) dan kepekatan keracunan bakteria minimum (MBC) yang paling rendah terhadap *Staphylococcus aureus* dan MRSA (MIC 125.0 µg/mL dan MBC 250 µg/mL). Mengenai sitotoksisiti, minyak pati ranting menunjukkan kesan anti-proliferasi terhadap sel MCF-7. Namun, tiada kesan yang ketara terhadap sel MDA-MB-231. Minyak pati *C. cauliflora* telah dienkapsulasikan dalam CSNPs dengan menggunakan teknik pengegelan emulsi-ionik. Mikroskopi elektron penghantaran (TEM) mendedahkan nanopartikel minyak pati *C. cauliflora* (CCEOs-CSNPs) adalah berbentuk sfera. Saiz purata CCEOs-CSNPs adalah kurang daripada 100 nm. Kejayaan nanoenkapsulasi telah disahkan oleh analisis spektroskopi inframerah transformasi Fourier (FTIR), penyerakan cahaya dinamik (DLS) dan pembelauan sinar-X (XRD). Kecekapan enkapsulasi (EE) dan kapasiti pembebanan (LC) masing-masing berjulat dari 38.83% hingga 44.16% dan dari 32.55% hingga 33.73%. Aktiviti antioksidan CCEOs-CSNPs telah dipertingkat berbanding dengan minyak pati yang tidak terenkapsulasi. CCEOs-CSNPs memberikan IC_{50} berjulat dari 21.65 hingga 259.13 µg/mL dalam aktiviti pengautan radikal DPPH. Tambahan pula, CCEOs-CSNPs menunjukkan kesan aktiviti antimikrob yang memuaskan ke atas mikroorganisma. CCEOs-CSNPs menunjukkan kesan sitotoksisiti terhadap sel MCF-7 dan MDA-MB-231 dengan nilai IC_{50} berjulat dari 3.72 hingga 17.81 µg/mL dan dari 16.24 hingga 17.65 µg/mL masing-masing.

Sebaliknya, tiada kesan sitotoksik diperhatikan terhadap sel MCF-10A. Secara keseluruhan, kajian ini menunjukkan potensi CCEOs-CSNPs sebagai agen terapeutik.

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ABSTRACT

Cynometra cauliflora L., locally known as "*nam-nam*" or "*katak puru*" in Malaysia belongs to the Fabaceae family. The tree is cultivated in villages and has been used traditionally to treat appetite loss, skin disorders, diabetic, and cholesterol related problems. Thus far, limited studies have been conducted on *C. cauliflora*. The present study aimed to identify the essential oils (EOs) composition of the leaf, twig and fruit of *C. cauliflora* and to encapsulate the EOs in chitosan nanoparticles (CSNPs). Antioxidant, antimicrobial and cytotoxic activities were performed to assess the free and encapsulated EOs. The identification of EOs constituents was made using capillary GC and GC-MS. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was employed to assess the antioxidant activity. Antimicrobial activity was assessed using Kirby Bauer assay and broth microdilution assay against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Candida utilis*, and *Candida albicans*. Regarding the cytotoxicity, a cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed on human breast cancer (MCF-7 and MDA-MB-231) and human breast normal (MCF-10A) cells. Results showed that the leaf, twig and fruit EOs of *C. cauliflora* consisted of twenty-six, seventeen and fifty constituents, respectively. The leaf, twig and fruit EOs were dominated by monoterpenes hydrocarbon (54.15%), oxygenated monoterpenes (69.53%) and oxygenated sesquiterpenes (65.48%),

respectively. The twig EO gave the highest free radical scavenging power (37.12 ± 2.84 µg/mL) as compared to the leaf EO (207.17 ± 2.95 µg/mL) and fruit EO (461.88 ± 12.61 µg/mL). In the antimicrobial assay, the twig EO inhibited all microorganisms with inhibition zones ranging from 10.3 to 29.7 mm. The twig EO displayed the lowest minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Staphylococcus aureus* and MRSA (MIC: 125.0 µg/mL; MBC: 250 µg/mL). Regarding cytotoxicity, the twig EO showed an anti-proliferative effect against MCF-7 cells. However, no remarkable cytotoxic activity was noticed against MDA-MB-231 cells. *C. cauliflora* EOs were encapsulated in CSNPs using the emulsion-ionic gelation technique. Transmission electron microscopy (TEM) revealed the spherical shape of *C. cauliflora* EOs-loaded CSNPs (CCEOs-CSNPs). The average size of CCEOs-CSNPs was less than 100 nm. The success of nanoencapsulation was validated by Fourier transform infrared (FTIR), dynamic light scattering (DLS) and X-ray diffraction (XRD) analyses. The encapsulation efficiency (EE) and loading capacity (LC) of CCEOs-CSNPs ranged from 38.83% to 44.16%, and from 32.55% to 33.73%, respectively. The antioxidant activity of CCEOs-CSNPs was enhanced as compared to the free EOs. CCEOs-CSNPs gave IC_{50} ranging from 21.65 to 259.13 µg/mL in DPPH radical scavenging activity. Additionally, CCEOs-CSNPs displayed an appreciable antimicrobial activity on the test microorganisms. The CCEOs-CSNPs showed cytotoxicity against MCF-7 and MDA-MB-231 cells with IC_{50} values ranging from 3.72 to 17.81 µg/mL, and from 16.24 to 17.65 µg/mL, respectively. On the contrary, no cytotoxic effect was observed against MCF-10A cells. Overall, the present study highlights the potential of CCEOs-CSNPs as therapeutic agents.

CHAPTER 1

INTRODUCTION

1.1 Background study

Natural products refer to organic substances that exist in nature. They are derived from living organisms such as plants, animals, microorganisms (Orhan, 2014), insects (Ratcliffe et al., 2011; Seabrooks & Hu, 2017) and marine organisms (Estrella Parra et al., 2022). Natural products are gaining much interest due to their beneficial attributes. Generally, natural products are divided into primary and secondary metabolites. Primary metabolites include carbohydrates, lipids, nucleic acids, amino acids and sugars. These metabolites occur in cells and play an essential role in metabolism and cell regeneration. Meanwhile, polyketides, fatty acids-derived compounds, terpenoids, phenylpropanoids, and alkaloids are among the key secondary metabolites (Li et al., 2016; Wu et al., 2021). Natural products have wide applications in medicine, food, cosmetic, pharmaceutical, and textile industries (Calixto, 2019). According to Wangchuk (2018), approximately 75% of modern drugs are derived from natural products. For instance, morphine which is used as a painkiller was first isolated from *Papaver somniferum* (opium poppy). Quinine, an anti-malarial drug, was isolated from the bark of *Cinchona succirubra*. In addition to that, quinine is used in the treatment of fever, stomach and throat discomforts (Talapko et al., 2019). On the contrary, penicillin is a popularly known antibiotic derived from a microbial natural product (Abdel-Razek et al., 2020). Thus, natural products are an important source of drug leads (Calixto, 2019).

Among the natural products, plants are recognised as the primary source of new drugs. Plants produce various natural products with diverse chemical entities and

biological activities (Zhu et al., 2021). The interest in plant-based remedies and botanical products is currently seeing a global increase. The utilisation of plants for health-promoting effects is prevalent in the population. Approximately 25% of drugs are originated from plants (Garcia, 2020). Phytochemicals are chemical substances obtained from plants that exert beneficial health effects. Studies have demonstrated that phytochemicals may be effective in treating various diseases owing to their potent biological activities (Ahmad Khan & Ahmad, 2019). *Curcuma longa*, popularly known as turmeric, is widely used in the treatment of various health complications. The phytochemical responsible for the yellow colour of turmeric is known as curcumin. Curcumin possesses various biological properties, including anti-inflammatory, antioxidant, anticancer, antifungal, antiviral, and antisteatotic properties (Lin & Lee, 2006; Ramos-Tovar & Muriel, 2019). Thus, plant-based natural products are promising candidates with a wide range of therapeutic properties (Thomas et al., 2021).

Cynometra cauliflora is from the family of Fabaceae. It is an underutilised fruit tree native to Malaysia and widely cultivated in villages (Lim, 2012). The tree can grow up to 15 m tall with flowers and fruits on its trunk. The fruit is locally known as "nam-nam" or "katak puru" with a greenish-yellow to brown colour. The fruit resembles kidney-shaped and has rough and wrinkled skin (Tajudin et al., 2012). The mature fruit can be consumed as fruit salad, made into a juice or pickled, cooked with sugar, or fried in batter. In traditional medicine, fruits are used to treat appetite loss while seed oil is used in the treatment of skin diseases (Lim, 2012; Ado et al., 2015).

EOs are volatile aromatic liquids obtained from various plant parts, such as leaf, fruit, seed, bark, root, and flower. They are natural oils with many applications due to their remarkable biological activities. EOs are known to possess anti-

inflammatory, antioxidant, antibacterial, antiviral, anticancer, and tissue regenerative activities. These extracted aromatic oils are widely used in cosmetics, flavours and fragrances, household products, food, agriculture, and medicinal purposes (Falleh et al., 2020; Chen et al., 2021).

Nanoencapsulation is a technique used to encapsulate active constituents with coating materials to form nano-sized particles (Chaudhari et al., 2021). The active constituents are known as core materials whereas the coating materials are referred to as wall materials. Nanoparticles can be synthesised to have different properties depending on the preparation techniques (Murthy et al., 2018). Nanoencapsulation protects EOs from external environmental factors and improves their stability and efficacy (Chaudhari et al., 2021).

1.2 Problem statement

EOs are known to possess a wide range of biological activities, such as antioxidant, antimicrobial, and anticancer owing to the presence of bioactive constituents. However, the volatility and stability of EOs have limited their applications. Encapsulation of EOs could be employed to protect the bioactive constituents from oxidation and degradation (Chaudhari et al., 2021). Previous studies have shown that extracts from *C. cauliflora* were rich in carotenoids, tannic acids, terpenes, and flavonoids (Khoo et al., 2016). The leaf extract of *C. cauliflora* was reported to possess antioxidant and anti-diabetic activities (Aziz & Iqbal, 2013; Abd Aziz et al., 2017). Additionally, the methanolic fruit extract of *C. cauliflora* exhibited cytotoxic activity toward human promyelocytic leukaemia HL-60 cells, inducing the cells into apoptotic cell death (Tajudin et al., 2012). To date, there is no literature regarding the chemical composition and nanoencapsulation of the EOs of *C.*

cauliflora, and their biological activities. Thus, this study aims to address the gap in the literature regarding the chemical composition of the EOs of *C. cauliflora* and their biological activities. Further, nanoencapsulation is employed to overcome the limitations of EOs for better stability, efficacy and controlled release.

1.3 Objectives

The objectives of this study are;

- To extract and characterise the EOs from the leaves, twigs, and fruits of *C. cauliflora*.
- To synthesise and characterise the *C. cauliflora* essential oils loaded-chitosan nanoparticles (CCEOs-CSNPs).
- To evaluate the biological activities of the free and nanoencapsulated CCEOs.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural products

Since ancient times, natural products have been used traditionally in the treatment of diseases. These bioactive natural products possess therapeutic potential for the treatment of health disorders and complications. At present, natural products have wide applications in flavouring, colouring, cosmetic, pharmaceutical, drug discovery and development (Chopra & Dhingra, 2021). In this aspect, natural products derived from plants are an important source of novel bioactive constituents (Mushtaq et al., 2018). *Psoralea corylifolia, Curcuma longa* and *Solanum lycopersicum* are among the popular plants known for their bioactive constituents. Bakuchiol obtained from *P. corylifolia* was reported to exhibit anticancer properties while curcumin isolated from *C. longa* possesses anti-inflammatory activity. On the contrary, *S. lycopersicum* yielded antioxidant lycopene (Chopra & Dhingra, 2021).

Natural products are classified into two major categories, namely, primary and secondary metabolites (Ramakrishna et al., 2021). Primary metabolites are important in plant growth, development and cell reproduction. Besides, they play an essential role in energy production for plant primary metabolism and maintaining regular physiological processes. Proteins, carbohydrates, lipids and nucleic acids are examples of primary metabolites (Zaynab et al., 2019). These groups of primary metabolites are resulted from catabolic and anabolic pathways and assembled into macromolecules (Buenz et al., 2018). Meanwhile, secondary metabolites are not directly involved in the plant's primary metabolism. They are organic molecules with low molecular weights (Gantait et al., 2021). Secondary metabolites are vital in biochemical functions

to provide plant fitness and survival. They are produced when a plant is exposed to environmental stresses and extrinsic forces. They serve as defence metabolites to protect plants from insect herbivorous and phytopathogens. It was reported that the yield and content of secondary metabolites are influenced by the plants' physiological and plant developmental stages (Thakur et al., 2019). Thus far, approximately 200,000 secondary metabolites have been discovered in the plant kingdom (Gorlenko et al., 2020). Secondary metabolites are divided into three major groups, namely, alkaloids, terpenes and phenolics. Alkaloids are naturally occurring organic constituents containing nitrogen atom(s). On the contrary, terpenes comprise isoprene units. Each isoprene unit is made up of five carbon atoms with double bonds. Phenolics are plant metabolites consisting of an aromatic ring having one or more hydroxyl groups. Phenolic constituents play a key role in plant propagation and germination (Panchawat & Ameta, 2021). Plant secondary metabolites have been shown to possess various biological effects with wide applications. They have been employed in pharmaceutical, agrochemical, flavour and fragrance, colouring, additive and pesticides (Ivănescu et al., 2021; Mele et al., 2021; Yeshi et al., 2022).

2.2 The Fabaceae family

There are approximately 400,000 vascular plants on Earth (Kersey, 2019). Among the higher plants, the Fabaceae family comprises 730 genera and 19,400 species. Plants from Fabaceae are distributed worldwide, especially in tropical rainforests (Centeno-González et al., 2021). The Fabaceae family is a plant family under the kingdom of Plantae, the division of Magnoliophyta, the class of Magnoliopsida, and the order of Fabales. The Fabaceae (Leguminosae) is also known as legume, pea or bean family. It is the third largest family of vascular plants. The

Fabaceae is typically found in terrestrial tropical ecosystems with diverse habitats and morphology, ranging from woody rainforest trees to shrubs and herbaceous plants. Most of the Fabaceae species possess symbiotic root nodules correlated with bacteria fixing nitrogen. The symbiosis capability has improved the nitrogen cycle and the production of agricultural products (Zhao et al., 2021).

Generally, the leaves of Fabaceae are pinnate, bipinnately, or trifoliolate compounds. Occasionally, the leaves are unifoliolate and spiral. The inflorescence varies with bracteate flowers. The flowers are bisexual, though, occasionally they are unisexual. The perianth of Fabaceae is biseriate, dichlamydeous, and sometimes contains a hypanthium. Aposepalous or synsepalous of calyx and corolla are noted with five sepals and petals, respectively. At the base of the ovary, nectaries are present as a ring. Generally, the fruits of Fabaceae are legumes. Occasionally, the fruits are indehiscent, winged, drupe-like, or diverged into transverse partitions (Wanda et al., 2015).

Among the Fabaceae family, *Astragalus* is the largest plant genus with 2,500 to 3,000 species (Su et al., 2021), followed by *Acacia* with 1,000 to 1,350 species (Zheleva-Dimitrova et al., 2021; Atta et al., 2022), *Crotalaria* and *Indigofera* with 700 species, and *Mimosa* with approximately 500 species (Atta et al., 2022). The Fabaceae plant species had been extensively explored due to their significant economic and ecological impacts. Plants from Fabaceae possess potent pharmacological properties such as antibacterial, antioxidant, anticancer, antifungal (Ferraz et al., 2021; Zaak et al., 2022), anti-inflammatory, antidiabetic, anti-malarial, antipyretic, antinociceptive, anti-depressant (Oladeji et al., 2021) and antiviral (Ahovègbé et al., 2021; Bisht et al., 2022).

2.2.1 The genus *Cynometra*

The genus *Cynometra* is widely distributed in the tropics region. Approximately 85 plant species of *Cynometra* are distributed in the regions of Mexico, the Caribbean, Argentina, Africa, Madagascar, Comoros Island, and Fiji (Radosavljevic, 2019). They can be found in a forest from sea level to 1,300 m above sea level. On the African continent, *Cynometra* has the most diverse species. Meanwhile, it is present in small numbers in Australia and the western Pacific (Cooper, 2015). It was reported that 26, 22, and 12 species of *Cynometra* were found in Africa, Asia, Madagascar and the Comoros Island, respectively (Radosavljevic et al., 2017).

2.2.2 *Cynometra cauliflora*

Cynometra cauliflora is from the family of Fabaceae. It is an underutilised fruit tree native to Malaysia. The tree is widely cultivated in villages (Abd Aziz & Iqbal, 2013; Ado et al., 2019). Table 2.1 shows the taxonomy classification of *C. cauliflora*. According to the literature, *C. cauliflora* is also cultivated in India, Sri Lanka, and Indonesia (Lim, 2012; Abd Aziz & Iqbal, 2013; Ado et al., 2019). Figure 2.1 shows the illustration of *C. cauliflora.* The tree of *C. cauliflora* is small with flowers and fruits on its trunk (Lim, 2012). The leaf has an oblique base and emarginate apex (Ding et al., 1996). The flowers are small with white or pink calyx grow on the hard trunk (Ding et al., 1996). *C. cauliflora* is a non-seasonal fruit tree, nonetheless, it gives better fruit yield during the dry season (Lim, 2012). The fruit is locally known as "nam-nam" or "katak puru". It resembles a kidney shape and has rough and wrinkled skin (Ding et al., 1996). The fruits will turn greenish-yellow during ripening. The unripe fruit has a sour taste, whilst the mature fruit is a sourish-sweet taste. In the local community, mature fruits can be eaten raw or consumed as fruit salad, made into juice or pickled,

cooked with sugar or fried in batter (Tajudin et al., 2012). Plant parts of *C. cauliflora* have been used traditionally to treat diseases. For instance, fruits have been used to increase appetite while seed oil is used to treat skin disorders. Leaves are used in the treatment of diabetic and reduce the cholesterol level (Seyedan et al., 2019).

Table 2.1 Taxonomy classification of *C. cauliflora* (Orrell, 2022)

Kingdom	**Plantae**
Phylum	Tracheophytes
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	*Cynometra*
Specific epithet	*cauliflora*
Binomial name	*Cynometra cauliflora* L.

Figure 2.1 Illustration of *C. cauliflora* (Ding et al., 1996)

2.2.3 Previous studies on *C. cauliflora*

The fruits of *C. cauliflora* have been previously studied by Rabeta and Faraniza (2013). Extraction of fruits was carried out using methanol and water. The proximate composition, mineral content, total phenolic content and antioxidant activity of the fruit extracts were assessed. The fruits of *C. cauliflora* contained 87.27% moisture, 1.41% total ash content, 0.66% protein, 0.18% fat, 1.72% crude fibre, and 8.77% carbohydrate content. It was found that calcium (6.14 mg), zinc (0.46 mg), iron (1.01 mg) and sodium (0.55 mg) were detected in 100 g of fruits. The total phenolic content was 847.21 mg and 98.79 mg for methanolic and water extracts, respectively. A ferric

reducing antioxidant power (FRAP) assay was performed to assess the antioxidant activity. It was found that the methanolic extract (19397.22 µM/g) possessed higher antioxidant activity than the water extract (7197.22 µM/g). Therefore, *C.cauliflora* could be employed as a natural antioxidant (Rabeta & Faraniza, 2013).

In another study, the leaf of *C. cauliflora* was investigated for its antiacetylcholinesterase, antityrosinase, antioxidant and α-glucosidase activities (Ado et al., 2015). Different solvents were used in the extraction, namely, *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol, methanol, and water. Based on the findings, the total phenolic contents recorded 214.37, 185.18, 154.68, 122.15, 71.85, and 73.57 mg GAE/g extract for methanolic, *n*-butanol, water, ethyl acetate, *n*-hexane and dichloromethane extracts, respectively. Solvent polarity plays a key role in the different total phenolic contents observed in the study. High polarity solvents are capable of extracting phenolics as compared to low polarity solvents. Antioxidant activity was carried out using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and FRAP assays. In the DPPH assay, the methanolic, ethyl acetate, *n*-butanol and water extracts showed better free radical scavenging activity than the positive control, butylated hydroxyanisole (BHA). Meanwhile, dichloromethane and *n*-hexane extracts exhibited weaker antioxidant activity than the positive control. In FRAP assay, leaf methanolic and ethyl acetate extracts displayed significant ferric ion-reducing activity at a concentration of 0.5 mg/mL. It was reported that the reducing ability of the leaf extract was decreased when the sample concentration decreases. In antiacetylcholinesterase activity, IC_{50} values ranging from 0.011 to 0.186 mg/mL were recorded for the leaf extracts as compared to the positive control, tacrine (IC_{50}: 0.006 mg/mL). A highly polar extract was found to exhibit better antiacetylcholinesterase activity than the less polar extract. In tyrosinase inhibitory activity, none of the leaf

extracts showed comparable activity to the positive control, kojic acid (IC_{50}: 0.0034 mg/mL). It was observed that methanolic, ethyl acetate and *n*-butanol extracts exhibited higher inhibition activity than *n*-hexane, dichloromethane and water extracts. In the anti-*α*-glucosidase assay, ethyl acetate, methanolic, and *n*-butanol extracts showed significant inhibition with IC_{50} values of 0.030, 0.042, and 0.044 mg/mL, respectively. Owing to the significant bioactivity displayed by the ethyl acetate and *n*-butanol extracts, LC-MS/MS analysis was carried out to characterise their chemical constituents. In total, 14 and 13 peaks were detected in ethyl acetate and *n*-butanol extracts, respectively. Procyanidin, catechin, taxifolin, vitexin, isovitexin, kaempferol, and quercetin derivatives were tentatively identified in the ethyl acetate extract of *C. cauliflora*. Meanwhile, Procyanidin, apigenin, taxifolin, vitexin, isovitexin, kaempferol, quercetin, and isorhamnetin derivatives were tentatively assigned in the *n*-butanol extract of *C. cauliflora*. These constituents were believed to play a key role in the observed antiacetylcholinesterase, antityrosinase, antioxidant and anti-α-glucosidase activities (Ado et al., 2015). In a study conducted by Choudhary and Kumar (2015), the methanolic, *n*-butanolic and aqueous extracts of *C. cauliflora* exhibited antiacetylcholinesterase activity with IC_{50} values of 0.065 ± 0.0008, 0.031 ± 0.021 and 0.011 ± 0.005 mg/mL, respectively. Thus, *C. cauliflora* could be potentially developed into nutraceutical and herbal products.

In 2016, Perera and co-workers performed *in vitro* anti-inflammatory and antioxidant activities on the leaves of *C. cauliflora* collected in Sri Lankan to justify its medicinal uses on inflammation. Ethanol was used as the extraction solvent in the study. The anti-inflammatory activity was carried out using anti-arachidonate 5-lipoxygenase (A5-LOX), anti-hyaluronidase, xanthine oxidase inhibitory, and nitric oxide (NO) production inhibitory assays. The ethanol leaf extract of *C. cauliflora*

showed an IC_{50} value of 77.21 μg/mL as compared to the standard, baicalein (IC_{50}: 1.76 μg/mL) in the anti-A5-LOX assay. Regarding the anti-hyaluronidase activity, *C. cauliflora* exhibited inhibition of 49.33% when compared with the standard, tannic acid (90.28% inhibition). In the xanthine oxidase inhibitory assay, the leaf extract of *C. cauliflora* displayed an enzyme inhibition of 48.86%. The NO production inhibitory activity gave an inhibition of 14.65% with no observed cytotoxicity at a concentration of 500 μg/mL. In assessing the antioxidant activity, various assays were used. The leaf extract of *C. cauliflora* gave 12.46 μg/mL, 15.67 μg/mL, 2760 mg TE/g, and 1317 mg TE/g in the DPPH free radical scavenging activity, ferrous ion chelating (FIC) activity, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC), respectively. In addition, the total polyphenolic contents and total flavonoid contents gave 173.0 mg GAE/g extract and 33.51 mg QE/g extract, respectively, for *C. cauliflora* leaf extract. Hence, *C. cauliflora* is worth for further study to assess its potential in the pharmaceutical industry (Perera et al., 2016).

The leaf ethanol extract of *C. cauliflora* and its major compound, vitexin have been investigated for their anti-obesity and lipid-lowering effects by Seyedan et al. (2019). High-performance liquid chromatography (HPLC) was employed to quantify vitexin. Based on the results, vitexin was eluted at a retention time of 14.7 min with a concentration of 23.53 ± 1.38 mg/g extract. The total saponin test gave 629.1 ± 2.6 mg diosgenin equivalents/g extract of *C. cauliflora.* The total flavonoids and phenolic contents were found at 55.0 mg rutin equivalents/g and 113.2 mg gallic acid equivalents/g of *C. cauliflora* extracts. In GC-MS analysis, phytol, vitamin E and *β*-sitosterol were detected in the ethanol extract of *C. cauliflora*. The ethanol leaf extract of *C. cauliflora* (400 and 200 mg/kg) and vitexin were found to significantly decreased the body and liver weights, adipose tissue and lipid accumulation in the liver as

compared to the control group in the *in vivo* study. Furthermore, *C. cauliflora* extract also reduced the levels of serum triglyceride, low-density lipoprotein, lipase, interleukin 6, peptide YY, and resistin. Thus, the study suggested the potential of *C. cauliflora* for obesity management (Seyedan et al., 2019).

In a recent study, fruits of *C. cauliflor*a from Sri Lanka were investigated for their contents of ascorbic acid, total vitamin C, total phenolic and flavonoid, total iron, and antioxidant capacities. The fruit gave total vitamin C content of 37.9 mg/100 g extract. According to the classification, the total Vitamin C content was divided into high (>100 mg/100 g extract), medium (50 - 100 mg/100 g extract), and low (<50 mg/100 g extract). Thus, the reported total vitamin C content value was considered as low based on the classification. Meanwhile, ascorbic acid and dehydroascorbic acid contents were 31.3 and 7.7 mg/100 g extract, respectively. The total iron recorded content of 0.4 mg/100 g extract. Regarding the total phenolic and total flavonoid contents, the *C. cauliflora* fruit extract showed 428.5 mg gallic acid equivalent/100 g extract and 26.1 mg quercetin equivalent/100 g extract, respectively. In antioxidant activity, *C. cauliflora* fruit extract exhibited an IC_{50} of 8.7 mg/mL in the DPPH assay. Meanwhile, the FRAP assay gave 63.2 μmol $FeSO_4$/g extract for *C. cauliflora*. Based on the findings, the nutritional values possessed by *C. cauliflora* suggested its potential as a valuable fruit in promoting human health (Abeysuriya et al., 2020).

2.3 Essential oils (EOs)

EOs are secondary metabolites stored in plant secretory cells (D'Addabbo & Avato, 2021). Flowers, buds, leaves, seeds, stems, fruits (Falleh et al., 2020), roots, rhizomes (Lunz & Stappen, 2021), barks (Lee et al., 2022), and tubers (Ouakouak et al., 2021) are commonly extracted for their EOs. EOs are odoriferous organic

constituents with low molecular weights. Generally, EOs are soluble in organic solvents but insoluble in water (Vianna et al., 2021; Ezeorba et al., 2022).

EOs are known to possess a wide range of biological activities, notably, antibacterial, antiviral, antifungal, anticancer, antimutagenic, anti-inflammatory, antioxidant (Lammari et al., 2020), antimycotic, antiparasitic, (Falleh et al., 2020) and antidiabetic (Stevens & Allred, 2022). EOs are used as flavouring agents in foods, beverages, perfumes, fragrances, cosmetics, disinfectants, insecticides, and oral products (Fuentes et al., 2021). For instance, EOs obtained from *Citrus*, *Lavender*, *Eucalyptus* and tee trees are commonly used in fragrances (Sharmeen et al., 2021). Besides, EO from *Cola*, *Cinnamon*, *Myristica* and *Vanilla* is used in soft drinks manufacturing (Ameh & Obodozie-Ofoegbu, 2016). EOs from *Mentha piperita* are widely employed as the primary ingredient in the manufacturing of toothpaste, confectionary, analgesic balms, mouth wash, chewing gums and tobacco (Gholamipourfard et al., 2021).

2.4 Extraction of EOs

Various techniques are available to extract EOs, namely, hydrodistillation, steam distillation, cold expression, organic solvent extraction, supercritical carbon dioxide extraction, enfleurage, microwave-assisted extraction, microwave hydrodiffusion and gravity extraction, high-pressure solvent extraction, ultrasonic extraction, solvent-free microwave extraction and phytonic extraction (Tongnuanchan & Benjakul, 2014; Stratakos & Koidis, 2016; Moghaddam & Mehdizadeh, 2017). Hydrodistillation is a conventional method used to extract EOs as described by the European Pharmacopoeia (Chen et al., 2021). It is widely used owing to its simplicity and not involving organic solvents (Rezaei et al., 2021). Figure 2.2 shows the general

set-up of the hydrodistillation using the Clevenger-type apparatus (Samadi et al., 2017; Mahanta et al., 2021). Throughout the distillation process, the plants' materials interact with the boiling water and release the EOs through evaporation (Stratakos & Koidis, 2016). The EOs was collected using distilled *n*-pentane while nitrogen gas was used to concentrate the EOs at room temperature (Tan et al., 2020).

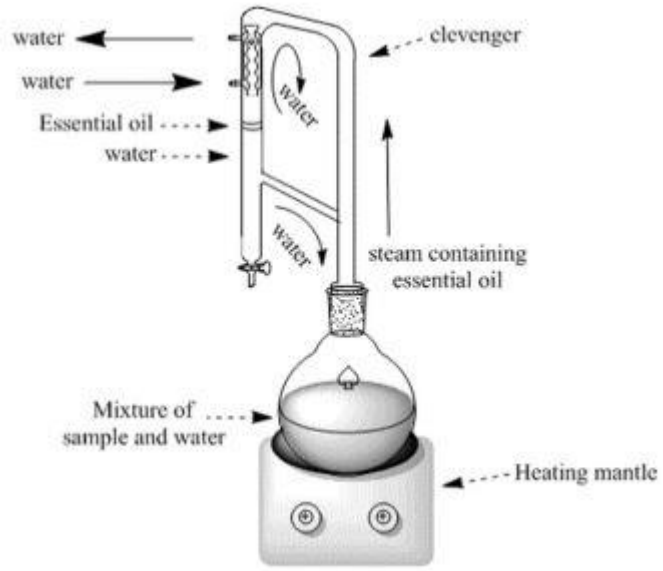

Figure 2.2 Hydrodistillation using Clevenger-type Apparatus (Samadi et al., 2017)

2.4.1 Chemical composition of EOs

EOs consists of more than 300 volatile constituents with molecular weights less than *m/z* 300 (El-Tarabily et al., 2021). Typically, EOs mainly comprised of terpenes and phenylpropanoids (Ni et al., 2021). Owing to the beneficial effects of terpenes and terpenoids, they have been extensively studied for their vital roles in human health and other applications (Masyita et al., 2022).

2.4.1(a) Terpenes and terpenoids

Terpenes are one of the largest groups of plant secondary metabolites. They are categorised based on the number of isoprene units in their structure (Figure 2.3). The C5 isoprene units will undergo rearrangement or head-to-tail condensation to produce a variety of terpenes. The resulting terpenes are categorised into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, sesquarterpenes, and tetraterpenes which corresponded to C5, C10, C15, C20, C25, C30, C35, and C40, respectively. Meanwhile, terpenoids are oxygen-containing terpenes which formed via biochemical modifications (Wani et al., 2021). They are grouped into alcohols, aldehydes, esters, ether, epoxides, ketones and phenols (Masyita et al., 2022).

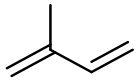

Figure 2.3 Chemical structure of isoprene unit

Hemiterpenes are the simplest among the terpenes. They are considered a minor group in EOs with the molecular formula C_5H_{10}. They are commonly emitted from plants of conifers, oaks, and poplars. Tiglic acid, angelic acid, isovaleric acid, senecioic acid, and isoamyl alcohol are among the common hemiterpenes found in EOs (Figure 2.4) (Masyita et al., 2022).

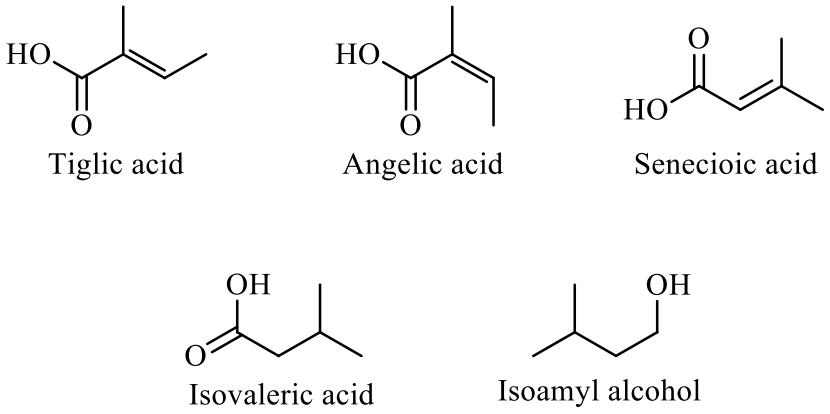

Figure 2.4 Chemical structures of hemiterpenoids

Monoterpenes constitute two units of isoprene with the molecular formula $C_{10}H_{16}$. They made up almost 90% of total EOs constituents (Kashyap et al., 2022). Myrcene and ocimene are examples of acyclic monoterpenes while limonene and pinenes are cyclic monoterpenes (Figure 2.5) (Kang & Lee, 2016). Most of the monoterpenes are responsible for the emission of specific scents from plants. On the contrary, citral, geraniol, and linalool are examples of acyclic monoterpenoids. Cyclic monoterpenoids are commonly represented by α-terpineol, thymol, menthol, and eucalyptol (Figure 2.6) (Wani et al., 2021).

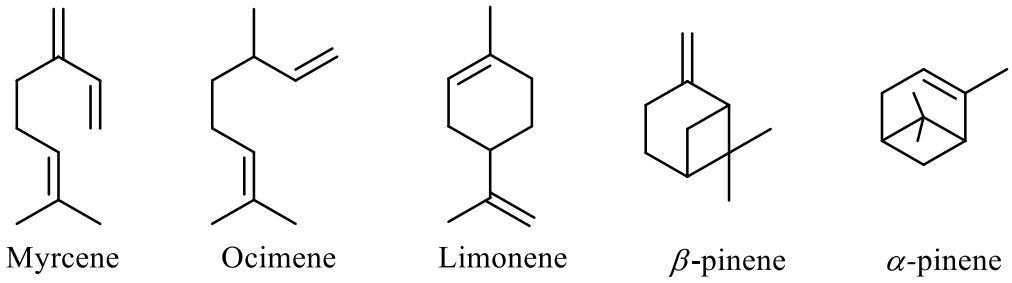

Figure 2.5 Chemical structures of acyclic and cyclic monoterpenes

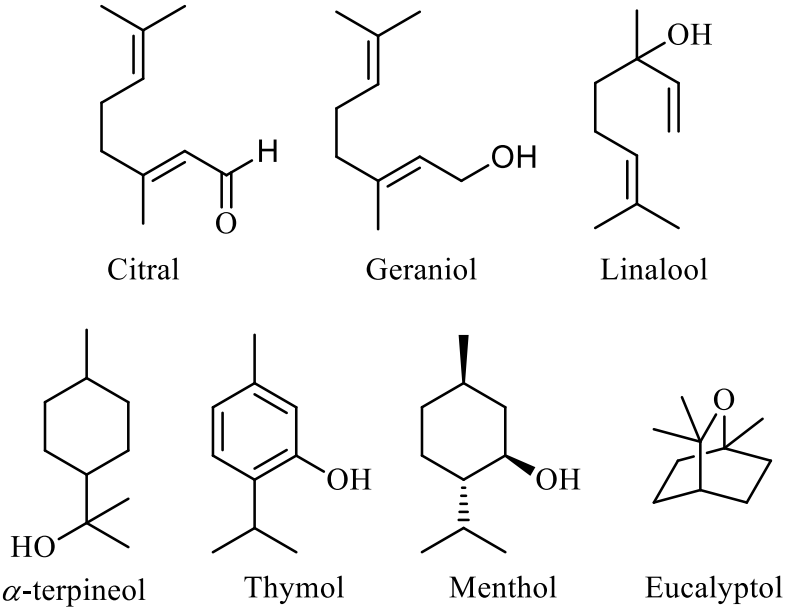

Figure 2.6 Chemical structures of acyclic and cyclic monoterpenoids

Sesquiterpenes are among the most widely occurring terpenoids. They are represented by three units of isoprene with molecular formula $C_{15}H_{24}$. Similar to other terpenes, sesquiterpenes can be found in acyclic and cyclic forms. Many sesquiterpenes and their derivatives are detected in plant EOs. They are gaining increasing interest due to their attractive odour and flavour characteristics. Examples of popularly known sesquiterpenes and sesquiterpenoids are β-caryophyllene, α-humulene, farnesol, and patchoulol (Figure 2.7) (Pragadheesh et al., 2020).

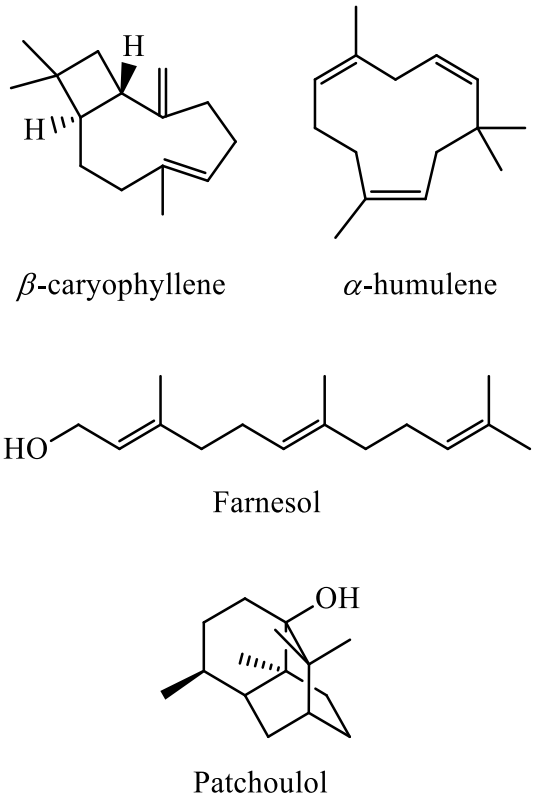

Figure 2.7 Chemical structures of common sesquiterpenes and sesquiterpenoids.

2.4.2 Bioactivity of EOs

2.4.2(a) Antioxidant activity

Over the past few decades, there has been a growing interest in the use of EOs as natural antioxidants. By definition, antioxidants are compounds which are able to slow down or inhibit the oxidation process. Studies have reported that naturally occurring antioxidants are able to prevent the damage caused by free radicals (Dziąbowska-Grabias et al., 2021). Free radicals such as hydroxyl and superoxide are reactive oxygen species (ROS) (Demirci-Çekiç et al., 2022). ROS are reactive molecules resulting from the unpaired electron in oxygen molecules ROS are oxidising agents responsible for oxidative damage on biomolecules such as lipids, DNA and proteins (de Fatima Alves Nonato et al., 2022). The development of various degenerative diseases may occur if ROS is produced overwhelmingly. Thus, a balance

between ROS and antioxidants is needed for normal physiological function. Owing to the potentially harmful effects caused by synthetic antioxidants, the search for antioxidants from natural sources is urgently needed. In this aspect, plant-based natural products, particularly EOs, are being extensively studied for antioxidant activity. Terpenoids, the principal components in plant EOs, are comprised of phenolics. They can react with ROS by scavenging and neutralising them. They reduce or inhibit cellular damage through their free radical scavenging property (Bhavaniramya et al., 2019; Demirci-Çekiç et al., 2022). EOs obtained from cinnamon, clove and thyme are widely known for their antioxidant properties (Bhavaniramya et al., 2019). Antioxidant activity can be assessed using electron transfer or hydrogen atom assays. In this aspect, DPPH and FRAP are examples of electron transfer assays while oxygen radical antioxidant capacity (ORAC) is hydrogen atom assay (Xiao et al., 2020).

2.4.2(b) Antimicrobial activity

In general, an antimicrobial is defined as a substance which is able to inhibit or kill the growth of microorganisms (Burnett-Boothroyd & McCarthy, 2011). The antimicrobial action of EOs has been studied extensively. EOs exert antimicrobial activity through various mechanisms, such as cell wall destruction, membrane protein damage, cytoplasm coagulation, hydrolysis of adenosine triphosphate (ATP), proton motive force reduction, cytoplasmic membrane damage, and cell permeability. For instance, EOs can travel through the lipids of bacterial cell membranes and disturb the cell wall structure owing to their hydrophobicity. This has increased the cell membrane permeability, leading to the ion leakage and other cellular substances (Bhavaniramya et al., 2019). The bioactive constituents present in the EOs may adhere on the cell surface and enter the phospholipid bilayer of the cell membrane. The accumulation of

the bioactive constituents disturbs the structural integrity of the cell membrane. Subsequently, it negatively affects the cell metabolism and resulted in bacterial cell death (Chouhan et al., 2017). For instance, *trans*-cinnamaldehyde-rich EOs inhibit the growth of *Escherichia coli* and *Salmonella typhimurium* by lowering the ATP level. Subsequently, the bioactive constituents penetrate to the cell periplasm and interrupt the cell matrix (Bhavaniramya et al., 2019).

Among the bioassays, disc diffusion and broth/agar dilution are widely used to assess antimicrobial activity. In disc diffusion method, agar plates are inoculated with an inoculum of the test microorganism. The test substance is then deposited on a filter paper disc placed on the agar surface. The agar plates are incubated and the diameter of the inhibition zones are then determined. On the contrary, broth dilution method is the most common assay used for the determination of minimum inhibitory concentration (MIC). MIC is defined as the lowest concentration of the test substance that inhibits the microorganism growth. The dilution method involves the preparation of two-fold dilutions of the test substance in a liquid growth medium. Then, each well is inoculated with a microbial inoculum and subjected to incubation. In determining the minimum bactericidal concentration (MBC), a sample from the well is sub-cultured to determine the number of surviving cells after 24 h of incubation. MBC is defined as the lowest concentration of the test substance needed to kill 99.9% of the final microbial inoculum (Hudzicki, 2009; Balouiri et al., 2016).

2.4.2(c) Cytotoxicity

Globally, cancer is one of the leading causes of death with approximately ten million deaths reported in year 2020 (WHO, 2022). The most commonly diagnosed cancer is breast cancer with high morbidity and mortality. It is a heterogeneous disease

and associated with hormone estrogens. Besides, it was reported that about 5-10% of breast cancers are related to gene mutations. A high incidence of breast cancer occurs in the age ranging from 65 to 80 years. Nonetheless, invasive breast cancer incidence is noticed in women with ages less than 50 years. Studies have reported that advanced breast cancer is generally incurable while an early-stage breast cancer is curable in almost 70% of patients (Coughlin, 2019). Chemotherapy, radiation therapy, and surgery are currently the main treatments for breast cancer. Nevertheless, the adverse side effects of the standard treatments have urged researchers to look for alternate approaches (Sambi et al., 2019). In this regard, plant EOs are considered a promising therapeutic agent for anticancer owing to the presence of various bioactive constituents. Plant EOs is postulated to reduce the size of cancer cell. In addition, plant EOs have been reported to trigger the death of cancer cells through apoptosis, necrosis, cell cycle arrest, and cell organelles dysfunction (Sharma et al., 2022). Clark et al. (2021) revealed that the tea tree oil showed significant cytotoxicity against human breast MCF-7 cancer cells in dose-dependent manner. This could possibly due to the presence of high amount of bioactive oxygenated monoterpenes. According to Silva et al. (2021), oxygenated monoterpenes act as cell cycle blockers and apoptosis inducers in MCF-7 cells. At high concentration, they could induce cancer cell death through necrosis.

2.5 Applications of EOs

The use of EOs in industrial and pharmaceutical applications has gained much interest recently owing to their potent pharmacological activity. In food industry, EOs are mainly employed for food preservation (Bhavaniramya et al., 2019). *Artemisia fragrans* EOs has been incorporated into the coating material of meat products in food

industry. The counts of microorganisms were significantly lower in *A. fragrans* EOs-coated meat products compared to the uncoated meat products. The coated meat products exhibited inhibition on coliforms, molds, and yeasts. This beneficial attribute has increased the shelf life of meat products during storage (Yaghoubi et al., 2021). For centuries, EOs has gained attention in aromatherapy. Aromatherapy uses EOs via inhalation and external application to provide mental and physical balance. Numerous studies have shown that aromatherapy could alleviate stress and revitalise the body. Lemon oil is used to strengthen the immune system. On the contrary, cineole-rich eucalyptus EOs is employed to regulate the nervous system to treat debility, headache, and neuralgia (Ali et al., 2015). In cosmetic industry, the use of EOs as ingredients has improved the properties and the commercial image of the cosmetic product due to its natural origin. EOs of *Rosmarinus officinalis* has been incorporated in the preparation of terpolymeric capsules for antifungal attribute (González-Minero et al., 2020). In pharmaceutical setting, the use of EOs as therapeutic agents is greatly studied. Semi-solid formulations, such as creams and ointments have been prepared using EOs. For instance, EOs of *Hypericum perforatum* was incorporated in an ointment formulation for wound healing purpose. The ointment showed better bactericidal and candidal effects than the standard ointment (Baptista-Silva et al., 2020).

2.6 Limitations of EOs

Despite a wide application of EOs possessed, their utilisation is limited by volatility, hydrophobicity, and instability. EOs constituted approximately 95% of volatile constituents and about 5% of non-volatile constituents. The volatile constituents are easily influenced by external factors. Air, heat, and irradiation are among the external factors which may affect the quality of EOs. Meanwhile, the