CHEMICAL CHARACTERISATION, ENANTIOSELECTIVE PROFILING AND BIOACTIVITES OF MALAYSIAN *Citrus* SPP. LEAF EXTRACTS

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UNIVERSITI SAINS MALAYSIA

2025

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

August 2025

ACKNOWLEDGEMENT

All praise and thanks to Allah Subhanahu Wa Ta'ala for granting me the strength and ability to complete this thesis despite the many challenges. Alhamdulillah.

My deepest gratitude goes to my supervisor, Dr. Wong Yong Foo, for his continuous guidance, patience, and invaluable support throughout this research. Special thanks also to my co-supervisor, Dr. Siti Sarah Fazalul Rahiman, for her constant advice and encouragement.

I would like to sincerely thank my lab mates, Atiqah, Farizah, and Anas, as well as my PhD colleagues, Maram and Noor, for their kindness and support. My heartfelt appreciation goes to my beloved parents, Ibrahim and Muna, for their unconditional love, encouragement, and endless support in every step of my journey.

A special note of gratitude to my husband, Dr. Basel Bani Atta, for his unwavering belief in me, his encouragement, and support throughout my study. To my children, Wassan, Omar, Zain, and Celine—you are my inspiration and source of strength. Your love and patience motivated me to persevere.

I am also deeply thankful to my siblings, Maram, Areen, Salam, and Mohammad, for their love and support, and to my dear friends Besan, Tamara, and Lamees, for their encouragement and comfort during this journey.

Lastly, I extend my gratitude to the academic and administrative staff at the School of Chemical Sciences, and to everyone who contributed in one way or another to the completion of this work.

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celcius
go/mL	Microgram per milliliter
g	gram
egg	Microgram
IC_{50}	Half maximal inhibitory concentration
mg	Milligram
mg/mL	Milligram per milliliter
min	Minute
s	second
mL	Milliliter
µL	Microliter
m	Meter
cm	Centimeter
µm	Micrometer
mm	Millimeter
eV	Electronvolt

LIST OF ABBREVIATIONS

AE	Acyclic ester
AH	Acyclic hydrocarbon
AK	Acyclic ketone
CH	*C. hystrix*
CL	*C. limon*
CM	*C. microcarpa*
CP	*C. pyriformis*
^{1}D	First dimension column
^{2}D	Second dimension column
DH	Diterpene
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
*e*ggy	Enantioselective Gas chromatography
*e*tc.−FID	Enantioselective Gas chromatography with flame ionization detector
*e*GC×GC	Enantioselective comprehensive two-dimensional
EO	Essential oil
GC	Gas chromatography
GC×GC	Comprehensive two-dimensional gas chromatography
GC−MS	Gas chromatography-Mass spectrometry
HS-SPME	Headspace solid phase microextraction
IZD	Inhibition zone diameters
LMCS	Longitudinally modulated cryogenic system
MA	Monoterpene aldehyde

MAc	Monoterpenic acetate
MDGC	Multidimensional gas chromatography
MH	Monoterpenic hydrocarbon
MO	Monoterpenic oxide
M_R	Modulation ratio
m/z	Mass to charge ratio
NA	Not available
NIST	National Institute of Standards and Technology
OD	Oxygenated diterpenes
OM	Oxygenated monoterpene
OS	Oxygenated sesquiterpene
PC	Principal component
PCA	Principal component analysis
P_M	Modulation period
RI	Retention index
RI_{cal}	Calculated retention index
RI_{ref}	Reference retention index
RT	Retention time
SA	Sesquiterpenic aldehyde
SH	Sesquiterpenic hydrocarbon
TIC	Total ion chromatogram
^2t_R	Second dimension retention time
wb	Peak base width

PENCIRIAN KIMIA, PEMPROFILAN ENANTIOSELEKTIF DAN BIOAKTIVITI BAGI EKSTRAK DAUN *Citrus* SPP. DARI MALAYSIA

ABSTRAK

Pengautentikan dan kawalan kualiti minyak pati *Citrus* kekal mencabar disebabkan oleh komposisi kimia yang kompleks, kehadiran bahan pencemar jejak, serta ketiadaan metodologi analisis yang mantap. Walaupun minyak pati daripada kulit buah *Citrus* diiktiraf secara meluas kerana sifat terapeutik dan perubatan, pencirian secara sistematik minyak pati yang diekstrak daripada daun spesies *Citrus* serta aktiviti bio-loginya masih kurang diterokai. Dalam kajian ini, sebatian meruap dalam minyak pati yang disuling daripada daun *C. hystrix*, *C. limon*, *C. pyriformis*, dan *C. microcarpa* dari Malaysia dianalisis menggunakan kromatografi gas–spektrometri jisim kuadrupol (GC–qMS). Selain itu, teknik pengekstrakan mikro fasa pepejal ruang kepala (HS-SPME) digunakan untuk memprofilkan sebatian organik meruap biogenik (BVOC) yang dibebaskan daripada daun segar empat spesies *Citrus* tersebut, membolehkan pengukuran hampir masa nyata terhadap volatil daun tanpa menyebabkan tekanan fizikokimia.. Sebanyak 76 dan 80 sebatian sekunder telah dikenal pasti secara tentatif dalam *Citrus* spp. daun dan minyak suling wapnya, masing-masing mewakili 85.41–94.21% dan 84.88–97.99% daripada jumlah kiraan ion, meliputi kelas kimia hidrokarbon monoterpena, hidrokarbon seskuiterpena, monoterpena beroksigen dan seskuiterpena beroksigen. Persamaan dan perbezaan kimia antara profil metabolik minyak daun dan pelepasan meruap biogenik telah ditentukan. Analisis komponen utama bagi fitokonstituen yang dikenal pasti dan kuantiti relatifnya mendedahkan perbezaan metabolik spesifik spesies, dengan pembolehubah pemuatan menyumbang kepada pembezaan ini. *Citrus* spp. minyak daun dinilai untuk aktiviti antioksidan,

antiproliferatif, dan antimikrob. Keputusan menunjukkan bahawa *C. limon* menunjukkan keupayaan penghapusan radikal DPPH tertinggi (IC_{50}: 29.14 ± 1.97 mg/mL), manakala *C. hystrix* menunjukkan aktiviti paling rendah (IC_{50}: 279.03 ± 10.37 mg/mL). Semua minyak daun mempamerkan kesan antiproliferatif yang kuat terhadap garisan sel kanser serviks HeLa (IC_{50}: 11.66 − 87.17 μg /mL) dan aktiviti antimikrob yang ketara terhadap *Staphylococcus hominis*, dengan *C. hystrix* mencapai perencatan lengkap. Seterusnya, taburan enansiomer bagi sebatian terpenik kiral terpilih (α-pinene, limonene, sitronelal, linalool, dan terpinen-4-ol) dalam *Citrus* spp. minyak daun disiasat menggunakan kromatografi gas enantioselektif (*e*GC) dan kromatografi gas dua dimensi komprehensif enantioselektif (*e*GC×GC) ditambah dengan pengesan pengionan nyalaan (FID). Lajur enantioselektif dimensi pertama (^{1}D) telah dipilih dengan menilai prestasi empat fasa pegun kiral pada resolusi enansiomer monoterpena kiral yang disasarkan. Gabungan lajur enantioselektif ^{1}D (MEGA-DEX DMP-Beta) dan lajur dimensi kedua kutub (SUPELCOWAX®10) memberikan pengasingan bebas gangguan bagi bahan larut kiral yang disasarkan. Kesan nisbah modulasi pada pecahan enansiomerik telah dinilai dengan mengubah tetapan tempoh modulasi. Pemisahan dua dimensi yang cepat telah dicapai menggunakan keadaan kromatografi yang dioptimumkan, memastikan pemisahan enansiomerik bebas gangguan yang mencukupi bagi bahan larut yang disasarkan. Keputusan menunjukkan variasi yang besar untuk pecahan enansiomer terpenes kiral. (+)-limonene dikenal pasti sebagai enansiomer utama (60.3−98.9%) dalam semua *Citrus* spp. minyak daun, (+)-linalool ialah enantiomer utama dalam *C. microcarpa* (95.9%), (–)-terpenin-4-ol ialah isomer utama dalam *C. microcarpa (66.4%)* dan *C. pyriformis* (61.1%), (–)-α-pinene ialah antipoda yang dominan dalam *C. limon* (55.5%) dan *C. microcarpa* (92.1%). *C. hystrix* mengandungi (–)-sitronelal (100%)

sebagai enansiomer tulen, manakala *C. limon* dan *C. pyriformis* mempunyai perkadaran yang lebih rendah (9.0–34.6%), dan sitronelal tiada dalam *C. microcarpa*. Pendekatan kromatografi gas×gas enantioseparatif (eGC×GC) menunjukkan kelebihan ketara berbanding enantio-GC (eGC) konvensional dari segi masa analisis dan kuasa pemisahan, dengan mencapai pemisahan enantiomerik sepenuhnya dalam masa 47 minit. Kelima-lima pasangan enantiomer berjaya dipisahkan pada tahap asas ($Rs \geqslant 1.5$) tanpa gangguan matriks, manakala eGC 1D hanya berjaya memisahkan α-pinena sepenuhnya dan menunjukkan ko-elusi kritikal bagi limonena dan terpinen-4-ol.

CHEMICAL CHARACTERISATION, ENANTIOSELECTIVE PROFILING AND BIOACTIVITES OF MALAYSIAN *Citrus* SPP. LEAF EXTRACTS

ABSTRACT

Authentication and quality control of *Citrus* essential oils remain challenging due to their complex chemical composition, the presence of trace adulterants, and the lack of robust analytical methodologies. While *Citrus*-derived peel essential oils are widely recognized for their therapeutic and medicinal applications, systematic characterization of leaf-derived *Citrus* spp. essential oils rand their bioactivity remains underexplored. This study examined the volatile constituents of essential oil distilled from the leaves of Malaysian *C. hystrix*, *C. limon*, *C. pyriformis*, and *C. microcarpa*, using gas chromatography–quadrupole mass spectrometry. Additionally, headspace solid-phase microextraction (HS-SPME) was used to profile the biogenic volatile organic compounds emitted from the freshly collected leaves of these four *Citrus* spp. to allow near-real-time measurement of leaf volatiles without physicochemical stress. A total of 76 and 80 secondary compounds were tentatively identified in *Citrus* spp. leaf and their steam-distilled oils, representing 85.41–94.21% and 84.88–97.99% of the total ion count, respectively, covering the chemical classes of monoterpene hydrocarbons, sesquiterpenes hydrocarbons, oxygenated monoterpenes, and oxygenated sesquiterpenes. The chemical similarities and differences between the metabolic profiles of leaf oils and biogenic volatile emissions were determined. Principal component analysis of the identified phytoconstituents and their relative quantities revealed species-specific metabolic differences, with loading variables contributing to this differentiation. The *Citrus* spp. leaf oils were evaluated for their antioxidant, antiproliferative, and antimicrobial activities. Results indicated that *C.*

limon showed the highest DPPH radical scavenging ability (IC_{50}: 29.14 ± 1.97 mg/mL), while *C. hystrix* exhibited the lowest activity (IC_{50}: 279.03 ± 10.37 mg/mL). All leaf oils exhibited significant antiproliferative effects against the HeLa cervical cancer cell line (IC_{50}: 11.66 − 87.17 µg /mL), and significant antimicrobial activity against *Staphylococcus hominis*, with *C. hystrix* achieving complete inhibition. Next, the enantiomeric distributions of selected chiral terpenic compounds (α-pinene, limonene, citronellal, linalool, and terpinen-4-ol) in *Citrus* spp. leaf oils were investigated using enantioselective gas chromatography (*e*GC) and enantioselective comprehensive two-dimensional gas chromatography (*e*GC×GC) coupled with flame ionisation detector (FID). The first dimension (1D) enantioselective column was selected by evaluating the performance of four chiral stationary phases on the enantiomeric resolution of the targeted chiral monoterpenes. A combination of 1D enantioselective column (MEGA-DEX DMP-Beta) and polar second dimension column (SUPELCOWAX®10) provided interference-free separation of the targeted chiral solutes. The effect of modulation ratio on the enantiomeric fractions was evaluated by altering the modulation period setting. A rapid two-dimensional separation was achieved using the optimised chromatographic conditions, ensuring adequate interference-free enantiomeric separation of the targeted solutes. Results indicated considerable variations for the enantiomeric fractions of the chiral terpenes. (+)-limonene was identified as the predominant enantiomer (60.3–98.9%) in all *Citrus* spp. leaf oils, (+)-linalool was the major enantiomer in *C. microcarpa* (95.9%), (–)-terpenin-4-ol was the major isomer in *C. microcarpa* (66.4%) and *C. pyriformis* (61.1%), (–)-α-pinene was the dominant antipode in *C. limon* (55.5%) and *C. microcarpa* (92.1%). *C. hystrix* contained (–)-citronellal (100%) as the pure enantiomer, while *C. limon* and *C. pyriformis* have lower proportions (9.0–34.6%),

and citronellal is absent in *C. microcarpa*. Evidently, *e*GC×GC approach was found to give significant advantages over *e*GC with regards to analysis times and resolving power, completing a full enantiomeric separation in 47 min. All five enantiomeric pairs were baseline-resolved ($Rs \geq 1.5$) without matrix interference, whereas 1D *e*GC fully resolved only α-pinene and showed critical co-elutions for limonene and terpinen-4-ol.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The genus *Citrus* belongs to the *Rutaceae* family, which comprises of 33 genera and approximately 162 species (according to the Tanaka system) that are distributed in tropical and subtropical regions throughout the world (Saunt, 2000; Tanaka, 1977). Apart from culinary uses, *Citrus* spp. plants have been cultivated and exploited for their nutritional and therapeutic values. To date, *Citrus* has been highly regarded as one of the most vital fruits for commercial cultivation. The worldwide output of lemons and limes was over 9 million tons around 2021–2022 (United States Department of Agriculture. *Citrus*: World Markets and Trade, 2022). Furthermore, the essential oil obtained from these plants has gained great importance in medicine and the economy, as it can be used in the food, perfume, cosmetic, and drug sectors (Palazzolo, Laudicina, & Germanà, 2013; Reddy, 2019).

Citrus essential oils, derived from various fruits under the *Citrus* genus, are widely recognized as the most prevalent natural essential oils. These oils hold a significant share in the market for commercial natural tastes and perfumes (Sawamura, 2011). Essential oils (EO) from *Citrus* plants have been found to demonstrate diverse medicinal qualities and favorable pharmacological effects, including antibacterial (Klimek-szczykutowicz, Szopa, & Ekiert, 2020; Z. H. Li, Cai, Liu, Sun, & Luo, 2019; Lin et al., 2021), anticancer (Raut & Karuppayil, 2014; Spadaro et al., 2012), anti-inflammatory (Alinejhad, Asayesh, & Asayesh, 2016; Dalia Hamdan et al., 2011), and antioxidant (Bertuzzi, 2013; Frassinetti, Caltavuturo, Cini, Croce Della, & Maserti, 2011; Nguyen et al., 2018), anti-fungal, anti-microbial, anti-spasmodic, anti-diabetic,

anti-dermatophyte activities (Bora, Kamle, Mahato, Tiwari, & Kumar, 2020; Dosoky & Setzer, 2018; Z. H. Li et al., 2019; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008). These effects have been attributed to their ability to induce oxidative stress, leading to the disruption of cell membranes and cytoplasmic structures. Additionally, certain monoterpenes and sesquiterpenes exhibit radical scavenging properties, thereby functioning as antioxidant molecules (Agus, 2021; Sharmeen, Mahomoodally, Zengin, & Maggi, 2021).

The complex array of phytoconstituents in *Citrus* EO is responsible for the ascribed bioactivities and organoleptic attributes. These phytoconstituents vary according to parameters such as genetic variability or variety, climate, geographical origin, seasonal changes, and other environmental influences (Hosni et al., 2010). The phytochemistry of *Citrus* spp. EOs are comprised of monoterpenic and sesquiterpenic hydrocarbons, and their oxygenated derivatives, which include alcohols, aldehydes, ketones, esters, and others (Butnariu, 2021). Characteristic essential oil profiles of *Citrus* spp. have been reported to identify species/cultivars, determine genetic diversity, and interpret sensory attributes (Ferrer et al., 2021). Given the availability of *Citrus* plants around the world and their associated pharmacological potentials, numerous studies have been targeted to investigate the chemical composition of EOs derived from different *Citrus* species and/or varieties that confer distinct pharmacological properties (Koziol et al., 2014).

Volatile organic compounds (VOCs) exist in diverse forms, encompassing a wide range of sources, such as freshly harvested or dried plants, extracts, and herbal preparations that may include EOs produced by various fragrant plants. Essential oils derived from *Citrus* spp. mostly consist of volatile molecules responsible for their characteristic fragrance. The aromatic compounds present in the freshly harvested

leaves of *Citrus* spp. contribute significantly to the overall sensory characteristics and flavour profile of the culinary preparations. These VOCs find application in various domains of daily existence, such as medicine, food, and cosmetics. The distinct olfactory characteristics exhibited by various *citrus* fruits and leaves are attributed to the intricate amalgamation. Plant VOCs consist primarily of terpenes, including monoterpenes and sesquiterpenes, as well as phenylpropane derivatives (A. Mahdavi, Moradi, & Mastinu, 2020; Moghaddam & Mehdizadeh, 2017). The essential oils owe their scent, flavor, and biological qualities to these compounds, which have a notable impact on the sensory perception of consumers. These volatile substances offer therapeutic benefits, such as expectorant, antibacterial, diuretic, and digestive stimulant effects (Zárybnický, Boušová, Ambrož, & Skálová, 2018). However, it is important to note that some of these substances have harmful impacts (Gershenzon & Dudareva, 2007).

The olfactory perception of a substance is influenced by the specific chemicals that are emitted into the surrounding atmosphere as a result of their vapor pressure. Consequently, these volatile entities can be determined through the utilization of headspace gas chromatography. Nevertheless, it is crucial to acknowledge that the molecules responsible for generating the fragrance may exist in concentrations that are either close to or significantly lower than the limit of detection in gas chromatography (Kolb & Ettre, 2006). Since the constituent compounds are volatile, the essential oils can be analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC−MS). However, since there is very little structural difference between the compounds, the mass spectra obtained from the GC−MS are similar, making identifying the compounds difficult (Oprean, Oprean, Tamas, Sandulescu, & Roman, 2001).

Notably, the demand for genuine *Citrus* spp. EOs are continuously increasing worldwide, with selected *Citrus* spp. oils fetch higher market values (e.g., *Citrus* hystrix EO (>150 USD per kg) compared to *Citrus* sinensis EO (3.5−5.0 USD per kg) (da Camara, Akhtar, Isman, Seffrin, & Born, 2015; Efendi, Budiarto, Poerwanto, Santosa, & Agusta, 2021). Consequently, many reports have been made on the occurrences of mislabeling or adulteration incidents, such as blending with other inexpensive EOs or the addition of lower-cost synthetic components (Cuchet et al., 2019; Juliani et al., 2006; Ojha et al., 2022; Wang, Zhao, Ali, Avonto, & Khan, 2021). In light of these phenomena, many analytical approaches (e.g., gas chromatography, isotope ratio mass spectrometry, and others) have been developed to authenticate and safeguard the quality of *Citrus* EOs (Bounaas et al., 2018; Dosoky, Satyal, & Setzer, 2022; Masson et al., 2016; Schipilliti, Dugo, Santi, Dugo, & Mondello, 2011).

It is known that plant terpenes are biosynthesized via a series of biogenetic pathways, and many of these compounds are present as different stereoisomers (Jahangeer et al., 2021; Ludwiczuk, Skalicka-Woźniak, & Georgiev, 2017; Ninkuu et al., 2021). The assessment of the enantiomeric compositions of chiral terpene mixtures in plant-derived extracts is important in understanding the physiological and ecological roles of the biologically active enantiomers (Kamaitytė-Bukelskienė, Ložienė, & Labokas, 2021; Luxová, Urbanová, Valterová, Terzo, & Borg-Karlson, 2004). The chiral ratios of specific terpenic molecules may provide the possibility to detect adulteration of EOs that are typically performed by admixture of synthetic aromatic compounds. In this respect, enantioselective gas chromatography (*e*GC) has been used as one of the powerful tools for the determination of enantiomeric ratio (ER) of chiral terpenes in plant EOs (Chanotiya, Pragadheesh, Yadav, Gupta, & Lal, 2021; Chanotiya & Yadav, 2009; Krupčík, Gorovenko, Špánik, Armstrong, & Sandra, 2016;

Wong, Davies, Chin, Larkman, & Marriott, 2015). In particular, the development of various versatile chiral stationary phases in *e*GC has greatly facilitated the stereo-differentiation of chiral terpenes from a variety of EOs (Bicchi et al., 2010; Huang, Zhang, & Armstrong, 2010; König et al., 1988; Schurig, 2010). Albeit *e*GC regularly provides sufficient resolution for chiral discrimination of chiral volatile organic compounds in EOs, a few studies have highlighted the applicability of enantioselective comprehensive two-dimensional gas chromatography (*e*GC×GC) for the interference-free ascertain of ER or enantiomeric excess (EE) of volatile racemates (Krupčík et al., 2016; Shellie & Marriott, 2002). The second dimension (2D) consists of achiral column phases that allow additional separation to eliminate non-specific co-elution concerning the enantiomer of interest. A commonly utilized *e*GC×GC setup is the first-dimension (1D) chiral stationary phase column and the second-dimension (2D) columns packed with achiral columns separated by the inlet liner.

1.2 Problem Statement

Citrus spp. plant are highly valued and has been in increasing demand due to their wide medical and therapeutic applications (Alsarhan et al., 2014). However, there is a growing concern regarding the lack of standardized protocols for the quality control and authentication of *Citrus* spp. leaf essential oils, particularly those derived from Malaysian cultivars. In Malaysia, there are approximately 60 species across 19 genera (Chooi, 1994), many of which exhibits similar morphological characteristics that are not sufficiently distinctive to discriminate among different varieties. This ambiguity enables unethical traders to take advantages by selling lower-quality *Citrus* spp. essential oils as premium products, leading to economic deception and potential safety risks (Schmidt, 2016). Adulterants are usually added at a trace levels to avoid

detection by conventional analytical methods. Besides, the complex chemical profiles of *Citrus* spp. essential oils limit the selection of reliable chemical markers for evaluating authenticity. In order to protect customer safety and ensure product integrity, it is imperative to establish a robust strategy to assess the quality and authenticity of *Citrus* spp. essential oils. Despite interest in the pharmacological properties of essential oils (EOs) has grown, research specifically targeting the chemical composition and bioactivities of leaf-derived EOs from Malaysian *Citrus* species remains scarce (González-Mas, Rambla, López-Gresa, Amparo Blázquez, et al., 2019). Although *Citrus* essential oils are broadly recognized for their biological activities, significant knowledge gaps persist regarding leaf oils, particularly from species such as *Citrus hystrix* (kaffir lime), *Citrus limon* (lemon), *Citrus pyriformis* (ponderosa lemon), and *Citrus microcarpa* (calamansi lime). Comparative studies on their phytochemical constituents and associated biological activities, such as antioxidant (Gursoy et al., 2010; Lu et al., 2019), antiproliferative (Saengha et al., 2022), and antimicrobial (Frassinetti et al., 2011) effects are limited. These gaps hinder a comprehensive understanding of how variations in secondary metabolites contribute to differences in biological activity and therapeutic potential, thereby limiting broader pharmacological and industrial applications (Alsarhan et al., 2014). Therefore, this study aims to systematically characterize the phytochemical profiles and associated biological activities of *Citrus* spp. leaf essential oils. A specific research problem lies in the extraction of volatile compounds from *Citrus* leaves. Although traditional steam distillation is a widely recognized solvent-free extraction method, it is associated with drawbacks, such as lengthy extraction times and the potential degradation of heat-sensitive compounds (Mahato et al., 2019). These challenges highlight the necessity for evaluating alternative solvent-free extraction methods such as headspace solid-

phase microextraction (HS-SPME). Despite its potential advantages in rapid extraction, high sensitivity, and minimal thermal degradation of volatiles, HS-SPME has not been comprehensively applied for profiling volatile secondary metabolites in Malaysian *Citrus* leaves (Cordero et al., 2012; Tinjan & Jirapakkul, 2007). Thus, addressing this methodological gap could lead to improved, efficient, and sustainable protocols suitable for routine phytochemical analysis of *Citrus* leaf volatiles.

Furthermore, while general chiral analyses of *Citrus* peel essential oils have been reported (Mondello et al., 2002), detailed investigations into the enantiomeric composition of key bioactive terpenes (e.g., α-pinene, limonene, citronellal, linalool, and terpinen-4-ol) in the leaf oils of Malaysian *Citrus* species are lacking (Cuchet et al., 2021). Current analytical techniques for enantiomeric analysis, including conventional comprehensive two-dimensional gas chromatography (GC×GC), typically rely on cryogenic modulators that use liquid nitrogen. These techniques are associated with high costs, operational complexity, and intensive maintenance requirements. To address these limitations, the present study evaluates a novel, cryogen-free, thermal modulation-based enantioselective comprehensive two-dimensional gas chromatography-flame ionization detection (*e*GC×GC–FID) method for high-resolution enantiomeric analysis. This approach eliminates the dependency on cryogenic fluids, thereby reducing operational complexity and costs while maintaining high chromatographic resolution and sensitivity. The application of this technique is expected to improve the accuracy and efficiency of chiral terpene analysis in *Citrus* leaf essential oils, addressing a critical technical limitation in existing analytical protocols.

In summary, this research seeks to comprehensively address existing gaps in the phytochemical characterization, biological activity assessment, and

enantioselective analysis of Malaysian *Citrus* leaf essential oils. By systematically evaluating their chemical profiles, bioactivity potential, and stereochemical compositions, this study will substantially contribute to scientific understanding of the pharmacological potential and industrial applicability of *Citrus* spp. leaf-derived essential oils.

1.3 Objectives of Study

Citrus spp. is a highly valued plant that has been in high demand. However, there is a growing concern regarding the lack of an approbated standard for the quality control and authentication of Malaysian *Citrus* spp. leaf essential oils. Several morphologies can be observed, but they are not sufficiently distinctive to discriminate among different varieties, which allows traders to take unfair advantages by selling low-quality lookalikes of *Citrus* spp. essential oil at a high price. Moreover, some types of *Citrus* spp. oil adulteration can cause safety issues. Adulterants are usually added at a low level to avoid detection by conventional analytical methods. Besides, the high complexity of the *Citrus* oil chemical profile impedes the number of markers employed in evaluating quality and authenticity. In order to protect customer rights and safety, it is imperative to set a strategy to assess *Citrus* spp. oil quality and its authenticity effectively.

Therefore, the major objectives of this project are as follows:

i. To characterize the chromatographic fingerprints and differentiate secondary metabolites in the leaf essential oils of *C. hystrix*, *C. limon*, *C. pyriformis*, and *C. microcarpa* using GC-MS combined with chemometric analysis.

ii. To compare the efficiency and effectiveness of headspace solid-phase microextraction (HS-SPME) with conventional steam distillation for solvent-free extraction and characterization of volatile compounds from leaves of selected Malaysian *Citrus* species.

iii. To develop enantioselective comprehensive two-dimensional gas chromatography (*e*GC×GC) approaches for the stereoisomeric analysis of α-pinene, limonene, citronellal, linalool, and terpinen-4-ol in *Citrus* spp. leaf essential oils.

iv. To evaluate and compare the antioxidant, antiproliferative, and antimicrobial activities of *C. hystrix*, *C. limon*, *C. pyriformis*, and *C. microcarpa* leaf essential oils.

1.4 Scope of Work

This work focuses specifically on the chemical characterization, bioactivity assessment, and stereochemical profiling of leaf essential oils obtained from four selected Malaysian *Citrus* species: *Citrus hystrix* (kaffir lime), *Citrus limon* (lemon), *Citrus pyriformis* (ponderosa lemon), and *Citrus microcarpa* (calamansi lime). Initially, gas chromatography-mass spectrometry (GC–MS) was utilized for the separation, detection, and identification of secondary volatile organic compounds (VOCs) present in these leaf essential oils. Identification of VOCs was conducted by calculating retention indices and comparing them with literature values. Key chemical markers unique to each *Citrus* species were identified, which allows discrimination according to their species. Subsequently, multivariate statistical analyses, specifically principal component analysis (PCA), were performed to classify and differentiate the *Citrus* leaf oils based on qualitative variations in their chromatographic fingerprints.

The biological activities of these leaf essential oils were systematically evaluated through DPPH radical scavenging (for antioxidant activity), MTT assays on HeLa cells (antiproliferative effects), and antimicrobial assays against selected bacterial strains. The volatile profiles were also examined using headspace solid-phase microextraction (HS-SPME), a solvent-free extraction technique, and compared with conventional steam distillation to evaluate extraction efficiency, sensitivity, and chemical profiles of recovered compounds. Furthermore, the stereochemical compositions of selected chiral monoterpenes (α-pinene, limonene, citronellal, linalool, and terpinen-4-ol) in the leaf oils were investigated using enantioselective gas chromatography (*e*GC) and enantioselective comprehensive two-dimensional gas chromatography (*e*GC×GC) coupled with flame ionization detection (FID). This high-resolution approach incorporates an enantioselective first-dimension column and a polar second-dimension column, enhanced chromatographic resolution and enabled accurate determination of enantiomeric ratios by effectively resolving co-eluting peaks from the targeted chiral terpenic compounds.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant Metabolomics

Plants comprise a wide range of structurally diverse metabolites, which have a major role in plant growth, development, and environmental responses. These plant metabolites are generally categorized as primary and secondary compounds (Zandalinas et al., 2017). Primary metabolites, including carbohydrates, proteins, lipids, and vitamins, are important to plant growth, development, and reproduction. Secondary metabolites aren't necessary for plant growth but are vital to the survival of a plant in stressful conditions, such as terpenes, phenolics, nitrogen-containing compounds and sulfur-containing compounds (Zandalinas et al., 2017). These low molecular weight metabolites (< 1500 Da) are also important as a nutrition, energy source, and untapped source for drug discovery for human beings. Therefore, the comprehensive analysis of plant metabolites has received huge interest, and it is currently termed plant metabolomics. The analysis of plant metabolites using different analytical methods falls under the umbrella of metabolomics studies. Likewise, the term genome describes the entire gene set in the biological sample. A new concept of metabolome refers to all the metabolites found in the studied plant extract (Kueger et al., 2012). Hence, when considering plant metabolomics, it's also referred to as metabolome characterization (Kueger et al., 2012).

It is approximated that the plant kingdom constitutes for about 200,000 different metabolites, which differ markedly in their chemical structure and physical properties (Payum, 2016). Therefore, many analytical techniques for high-throughput analysis of plant metabolites have emerged over the past decade. Plant metabolic

consists of three different approaches: metabolic profiling, metabolic fingerprinting, or targeted metabolic analysis (Wolfender et al., 2015). Targeted metabolic analysis results provide a qualitative and quantitative analysis of known metabolites. In addition to the quantification advantage, this method offers a low detection limit and a high throughput. However, this technique requires prior knowledge of the selected compound with authentic reference standards, which limits the applicability of this approach to the determination of metabolic changes in plants and unknown metabolites (Ghosson et al., 2018). On the other hand, metabolite profiling or untargeted metabolic analysis allows the evaluation of a wide range of samples of known and unknown metabolites. This technique offers medium throughput and semi-quantitative observations but does not identify certain major peaks. Metabolic profiling is the most abundant method of metabolomics analysis for even unknown metabolites (Ghosson et al., 2018; Oikawa et al., 2015). Torras-Claveria et al. used the gas chromatography−mass spectrometry (GC−MS) approach to apply a metabolic profiling approach for the bioactive alkaloid fraction of *Pancratium canariense* extract for the investigation of known and unknown bioactive compounds as anti-acetylcholinesterase inhibitors (Torras-Claveria et al., 2010).

Deferentially, metabolic fingerprints are the most high-performance tool that recognizes a single known or unknown metabolite. It also provides a complete overview of the unique metabolism status and all detectable metabolites in the examined sample (Silveira-Sotelo et al., 2015). Thus, a good knowledge of the metabolic composition of the selected plant and the research goal, therefore, governs the best method to use for every single research.

Plant metabolomics studies provide a comprehensive overview of the chemical composition of plants that are affected by processing time, environmental conditions,

sample preparation, and sample extraction processes. Moreover, metabolomics provides a good understanding of the complex biological metabolic pathway affected by external factors by characterizing the entire metabolome changes (Piasecka et al., 2019). Consequently, the detection of metabolic changes at the different stages of development helps to recognize a specific metabolite at a specific stage of development. In the same way, plant metabolism may help to recognize a particular biomarker that combines genetic background and environmental effects. The identified biomarker can be applied as a diagnostic metabolite for a particular sample (Hong et al., 2016). A recent study demonstrated the role of metabolomics in explaining the biological pathway alteration in tobacco cell culture model after exposure to phytohormones of auxins and cytokinins with light (Vasilev et al., 2016). As a result, metabolomics is being considered a valuable method for the extensive characterization of the metabolome of herbal samples and food preparation and to ensure their quality and safety.

Several analytical methods and instrument technologies have been developed for the study of highly complex plant metabolome, each of which must be selected according to particular interests and the type of sample used. These usually involve nuclear magnetic resonance spectroscopy (NMR), liquid chromatography–mass spectrometry (LC–MS), and GC–MS (Hakeem et al., 2016). Additionally, recent higher-order separation technologies, namely, multidimensional gas chromatography (MDGC), have shown notable advantages for metabolomics data exploration by offering higher resolution and higher peak capacity to resolve many more compounds (Dymerski, 2018).

2.2 Essential Oils

Plant essential oil constitutes a complex combination of secondary compounds with low molecular weights (usually less than 300 Daltons) that are extracted from diverse plant materials (Dhifi et al., 2016). These plant-derived oils are blends of lipophilic aromatic oily substances made up of natural, organic compounds that are volatile and semi-volatile. The expression "essential oil" is derived from "quinta essential," which was the name given by Paracelsus von Hohenheim, a Swiss medical reformer in the 16th century, to the active component of a drug, because it was originally believed that each oil represented the plant essence (HAAGEN-SMIT & NIMMO, 1963). They are called essential because it was once thought that each oil represented the essence (Fonseca et al., 2021; Guenther, 1950). According to the International Organization for Standardization (ISO), the phrase “essential oil” is reserved for a “product obtained from vegetable raw material, either by distillation with water or steam, or from the shuck of *Citrus* fruits by a mechanical process, or by dry distillation” (ISO 9235, 1997) that is, by physical means only. Plants produce these compounds as secondary metabolites, which serve a specific purpose in mediating ecological roles that are essential for the growth or survival of plants (Turek & Stintzing, 2013).

In early times, specifically in ancient Eastern countries, the analysis of essential oils began, but the information remained insufficient and ambiguous. This remained the case until the Catalan physician Arnaldo Da Villanova made a quantum leap in the thirteenth century through the practice of distillation in European therapy. Then, between the nineteenth and twentieth centuries, scientists investigated the research, isolation, and identification of essential oils' separate components (Nakatsu et al., 2000). As a result of the growing global demand for natural products, the

application of essential oils expanded beyond aromatherapy to encompass various industrial sectors, including cosmetics, perfumery, beverages, and food production. In recent times, the analysis of essential oils has evolved to incorporate various instrumental approaches, ranging from conventional to advanced chromatographic and spectroscopic techniques.

2.3 Composition and Bioactivity of Essential Oil

Essential oils consist of intricate combinations of organic molecules that contribute to the distinctive aroma and flavor of the plant. They are naturally occurring compounds biosynthesized by plants for purposes other than nutrition, such as for defense or attractiveness (Nieto, 2017). Essential oils can be extracted from various plant parts, including bark, wood, roots, buds, flowers, leaves, stems, seeds, or fruits. These compounds are typically stored in specialized cells such as secretory cells, cavities, epidermal cells, canals, or glandular trichomes (Bozin et al., 2006). According to Haagen-Smit (1948), essential oils can be categorized into four three distinct structural families based on their hydrocarbon skeleton: i) non-terpenoid hydrocarbons, ii) terpenoids (which include monoterpene and sesquiterpene hydrocarbons, as well as their oxygenated derivatives such as lactones, esters, alcohols, aldehydes, ketones, and ethers), iii) phenylpropanoids (such as phenols and phenol ethers), and iv) miscellaneous (Haagen-Smit, 1948; Kubeczka, 1979; Mollaei et al., 2023). Sesquiterpenes are biosynthesized via the mevalonate pathway, monoterpenes via the methyl-erythritol pathway and phenylpropanoids via the Shikimic acid pathway (Rehman et al., 2016; Vogt, 2010).

2.3.1 Non Terpenoid (Hydrocarbons)

These compounds consist solely of carbon and hydrogen atoms exhibiting significant variations in both complexity and size. These chemicals have excellent solubility in lipids while demonstrating insolubility in water (Carson & Hammer, 2011). Hydrocarbons can be classified as either saturated or unsaturated, with the latter containing numerous bonds; each double bond leads to a reduction of two hydrogen atoms compared to the saturated equivalent (hence, four fewer hydrogen atoms for triple bonds), and as a result, is in a higher oxidation state (Brielmann et al., 2006). The hydrocarbons known as benzenoids, alkenes, and alkanes are classified as non-terpenoid compounds (Zuzarte & Salgueiro, 2015). Compounds that include aromatic rings typically exhibit enhanced stability. Highly fragrant chemicals may exhibit decreased solubility in typical organic solvents due to more powerful intermolecular interactions.

2.3.2 Terpenes

Terpenes are the most extensive category of secondary metabolites found in plants. Terpenoids, a class of naturalist chemicals, are derived from C5 (isoprene) units. Figure 2.1 shows the structure of the isoprene unit.

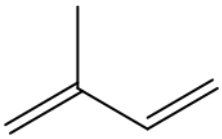

Figure 2.1 Chemical structure of isoprene unit (C_5H_8).

The isoprene rule, as proposed by Leopold Ruzicka, involves the concatenation of isoprene units that are connected in a head-to-tail fashion (Ruzicka, 1953). Terpenes are composed of connected isoprene units (C5); when one unit is present, it is referred

to as hemiterpenes (C_5H_8). Monoterpenes ($C_{10}H_{16}$), which are generated through the conjugation of two isoprene units, each containing ten carbon atoms (C10), account for approximately 90% of essential oils. Many of these molecules differ in structure, number, nature, and their pure variety. Plant essential oils also contain sesquiterpenes ($C_{15}H_{24}$), which result from three isoprene units, and diterpenes ($C_{20}H_{32}$), a conjunction of four units of isoprene (C20). The molecules sesquiterpenes and diterpenes, triterpenes (C30), and tetraterpenes (C40) are also situated in distinct but variable quantities (Bakkali et al., 2008).

It undergoes extra amendments through cyclization responses and specific rearrangements via oxidation, reduction, and hydroxylation. It is, therefore, the addition of monoterpenes, sesquiterpenes, and their mono- and sesquiterpenoids account for 85% to 90% of the oil (Moghaddam & Mehdizadeh, 2017). Terpenes, on the other hand, can be cyclic or acyclic, and their chemical structures become more varied as the length of the isoprene chain increases. Terpenoids, which are built from the shared precursor isopentenyl diphosphate (IPP), are the most prevalent volatile element of essential oil. Terpenes, a subclass of terpenoids derived from vinyl pyrophosphate isoprene units produced through the acetate mevalonate pathway, are further diversified through a variety of enzymatic procedures, including isomerization and oxidation, after formation (Degenhardt et al., 2009; Q. Zhang et al., 2019).

2.3.3 Phenylpropanoids

Phenylpropanoids are common aromatic molecules in essential oils. Their chemical structure is a phenyl ring linked to a three-carbon propene side chain. Figure 2.2 shows the chemical structure of phenylpropanoids. These chemicals add flavour and fragrance to essential oils due to their pleasing smell. Phenylpropanoids are found

in many plant species and are extracted by steam distillation or solvent extraction. This group of organic compounds includes many of those found in the greatest number of plant species. It is produced in response to biotic or abiotic stresses to protect plants from exposure to ozone and ultraviolet irradiation, wounds, and herbivores as well as infection (Korkina, 2007). They are synthesized from the amino acid phenylalanine, which is converted into cinnamic acid. Further, the reduction of a carboxylic acid group present in the cinnamic acid will produce an aldehyde (cinnamaldehyde), and with further reduction, a monolignol forms, which is phenylpropene (e.g., eugenol and safrole) (Başer & Demirci, 2007; Sá et al., 2014).

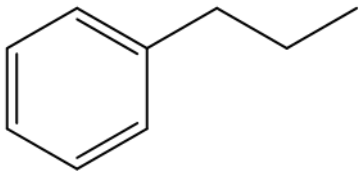

Figure 2.2 Phenylpropane skeleton

Sometimes, the hydroxyl (OH) group is attached to a benzene ring, typically accompanied by an isopropyl tail like thymol. These phenolic compounds are believed to be the primary contributors to the antioxidant properties of essential oils (Tisserand & Young, 2013). The relative composition of essential oils is influenced by various factors, including climate conditions, geographic location, and other environmental factors.

2.3.4 Miscellaneous

These clusters of compounds are different from the previously listed groupings and are regarded as reactive molecules found in only a few essential oils. These plant metabolites are incidental and limitedly distributed to particular plants (Tisserand & Young, 2013). These highly reactive and strongly scented compounds are present in

only a limited number of essential oils. They possess relatively uncomplicated compositions and lack terpene or phenylpropanoid hydrocarbon components. with the possibility of nitrogen or sulfur inclusion (Hüsnü et al., 2007). Furthermore, hydrocyanic acid is an inorganic acid that is present in bitter almond oil (Mollaei et al., 2023).

2.4 Biological Activities of Essential Oils

Essential oils typically consist of 2-3 predominant components that are present in relatively high amounts, ranging from 20% to 70%, in comparison to other components that are present in trace amounts. For instance, the main constituents of the essential oil derived from *Thymus vulgaris L.* are thymol (48.1%) and p-cymene (11.7%), which exhibit antimicrobial, antifungal, antioxidant, analgesic, and anti-inflammatory activities (Galovičová et al., 2021). Moreover, carvacrol (50,0%) in Savory oil exhibited strong inhibitory activities against fungi (Stević et al., 2014). In addition, Chamomile essential oil (Matricaria) contains the sesquiterpene alcohol α-bisabolol, which has the ability to inhibit glioma cells (Uedo et al., 1999). Aromatherapists use the essential oil from the bergamot fruit (*Citrus bergamia, Risso*) containing linalool as an important constituent to alleviate cancer pain, moderate mood disorders, anxiety caused by stress, and other similar conditions (Bagetta et al., 2010).

Numerous studies have investigated the fact that if other metabolites are combined at the same time, they will have a greater effect on the magnitudes of biological activities (Fürstenberg-Hägg et al., 2013). For example, the essential oil derived from *Salvia hispanica L.* and α-thujone exhibited insecticidal properties against third-instar larvae of the beet armyworm (Spodoptera exigua Hübner). Furthermore, various combinations of the four major constituents (α-thujone, (+)-

camphor, α-caryophyllene, and 1,8-cineole) extracted from *S. hispanica* demonstrated synergistic effects in terms of their insecticidal activity (Y. Chen et al., 2021). The effect has also been described of the *Foeniculum vulgare* essential oil on the growth rate and survival of *S. dysenteriae* bacteria (Diao et al., 2014). Moreover, there is a growing trend in utilizing essential oils as a means to extend the longevity of food items, as customers are increasingly mindful of the potential health issues associated with certain synthetic preservatives (Teixeira et al., 2013). For instance, the utilization of quinoa as a carrier polymer in the production of edible films, together with the incorporation of essential oils such as lemon and sage oil, which possess significant biological activity. The application of lemon oil and quinoa demonstrated the highest efficacy in inhibiting lipid oxidation in rainbow trout fillets. Sage oil and quinoa exhibit enhanced antimicrobial capabilities. The study revealed that the incorporation of lemon and sage essential oil into quinoa-based edible film resulted in improved quality and extended shelf life of rainbow trout fillets (Alak et al., 2019). In addition, literature provides a wide range of extracted essential oils of medicinal and herbal plants that have the potential to treat diseases (Kokoska et al., 2019; Mittal et al., 2018; Sharifi-Rad et al., 2017). For many centuries, essential oils have been utilized primarily in the aromatherapy and industry sectors, so deep insight into the chemical composition of essential oils and their biological response is of paramount significance.

Although *Citrus* peel EOs have been extensively studied for their health benefits, comparatively, limited research has been conducted on *Citrus* leaf-derived essential oils, representing a clear knowledge gap addressed in this work. Therefore, the focus on antioxidant, antiproliferative, and antimicrobial bioactivities is justified

by their alignment with traditional uses, potential commercial applications, and mechanistic relevance to the major terpene constituents in *Citrus* leaves.

2.4.1 Antioxidant Activity

Reactive oxygen species (ROS) contribute to cellular ageing, inflammation, and various chronic diseases. Natural antioxidants mitigate oxidative stress by neutralising ROS, and protecting biomolecules from oxidative damage (Halliwell & Gutteridge, 2015). Monoterpenes such as limonene and terpinene, which are abundant in *Citrus* leaves, have demonstrated significant radical-scavenging abilities (Cerrón-Mercado et al., 2022). In-vitro antioxidant capacity is commonly assessed using assays such as DPPH, $ABTS^{+}$, ORAC, and FRAP, which measure electron- or hydrogen-donating ability. The DPPH assay was chosen for this study due to its speed, simplicity, and reproducibility (Brand-Williams et al., 1995). Previous studies reported that *C. hystrix* leaf EO exhibited an IC_{50} >250 µg mL^{-1} in DPPH assays, outperforming its peel oil (Wungsintaweekul et al., 2010). Similarly, *C. microcarpa* EO demonstrated a potent IC_{50} value of ~ 0.05 mg/ml (Nguyen et al., 2018). Loizzo et al. reported an IC_{50} value of 6.47 ± 1.0 mg/ml for *C. limon* leaf EO that was extracted by hydro-distillation (Loizzo et al., 2016).

2.4.2 Antiproliferative Activity

Cancer remains a major global health burden, and natural products are a vital source of anticancer agents (Newman & Cragg, 2020). Certain *Citrus*-derived terpenes (e.g., limonene, citronellal) have been reported to induce apoptosis or cell-cycle arrest in tumour cells. Several scientific investigations have revealed that EOs have the ability to cause cell death through both intrinsic (depending on mitochondria) and

extrinsic (dependent on death receptors) mechanisms (Mohamed Abdoul-Latif et al., 2023). The MTT assay, a standard colorimetric method, quantifies mitochondrial dehydrogenase activity as a proxy for cell viability using the tetrazolium salt MTT (Mosmann, 1983). The HeLa cervical cancer line was selected for its reproducibility, well-characterised biology, and sensitivity to monoterpene-induced cytotoxicity (Peng et al., 2020). Previous studies reported that Italian *C. limon* leaf EO, extracted by hydrodistillation, exhibited an IC_{50} of 56 μg mL^{-1} against HeLa cells (Petretto et al., 2023), yet, comparable data for Malaysian cultivars are lacking.

2.4.3 Antimicrobial Activity

The rise of antibiotic resistance necessitates the need for alternative novel antimicrobial agents. Essential oils are known to disrupt microbial cell membranes and interfere with cellular metabolism (Burt, 2004). *Citrus* leaf EOs, especially those rich in citral, citronellal, or linalool, have demonstrated significant antibacterial activity (Fisher & Phillips, 2008). Previous studies have shown that *C. limon* EO showed antibacterial activity against *S. carnosus* and *E. gergoviae*, with inhibition zone diameter 15.65 mm, and 15.93 mm, respectively (Viuda-Martos et al., 2007).

2.5 Authenticity of Essential Oil

The utilization of natural extracts is widely regarded as a significant marketing benefit in many domains, such as tastes, fragrances, cosmetics, and the healthcare sector. However, the cost of natural extracts frequently surpasses that of synthetic components, resulting in numerous instances of adulteration (Balekundri & Mannur, 2020). The subject of adulteration, specifically in the context of essential oils, has garnered increasing attention. Adulteration of essential oils can occur as a result of

various factors, including the consideration of cost, the growing prevalence of their consumption, and, in certain cases, their limited availability, which stands in contrast to the continuously rising demand (Salgueiro et al., 2010). The chemical composition of essential oils can exhibit variations due to a range of circumstances, including the season of harvest, the habitat in which the plants are grown, methods of drying, procedures employed for extraction and isolation, as well as other relevant variables (Kumari et al., 2014).

Authenticity is crucial for consumers, as it ensures the absence of foreign bodies or impurities in the raw material. Regulatory standards have established quality standards through quality labels specifying the chemical composition of each essential oil. Authenticity is of critical importance to customers, regulatory frameworks, and economic considerations. avoid unfair competition and disrupt the local and national economies of producing countries. Analytical techniques encompassing physical, chemical, chromatographic, spectroscopic, and thermal procedures are employed to identify and detect established adulterants inside essential oils. The control of authenticity in monographs is achieved by the utilization of quantitative values (KE & DG, 2015). Several instances of adulteration have been documented, such as the inclusion of non-volatile substances, the addition of synthetic or natural compounds, and the substitution or addition of more affordable and less expensive essential oils. To avoid being detected by currently used analytical methods, adulterants are frequently added in a low concentration range of 5−8% (Pellati et al., 2013).

Additionally, essential oils may undergo dilution by incorporating a non-volatile component in order to mitigate costs. For instance, vegetable or mineral oils are commonly added due to their affordability, accessibility, similar density to essential oils, and comparable greasy texture (Baser & Buchbauer, 2009; McHale,

2002). The prevalent methods of adulteration involve the use of inexpensive oils derived from either mineral or vegetable sources, along with the utilization of synthetic solvents, with the primary objective of maximizing financial gain. Diverse studies reported evidence of adulteration of the essential oil derived from *Rosa Damascena Mill.*, which is renowned for its exquisite fragrance. This rose oil is susceptible to adulteration with other oils, such as *C. martini,* an oil that is less expensive (Pellati et al., 2013). In the same context, adulteration of sandalwood oil derived from the *Santalum ssp.* plant by adding poly (ethylene glycol) has also been reported (John et al., 1991). A further instance of this type of deception involves the dilution of *Citronella* oil from *C. winterianus* with dipropylene glycol (Cerceau et al., 2020). The act of adulteration can give rise to adverse consequences and unfavorable characteristics, such as the potential for allergic reactions or toxic effects, hence raising concerns related to safety and security. Consequently, various international organizations, such as the ISO, the European Pharmacopoeia (Ph. Eur.), the Codex Alimentarius Commission, the Food Chemical Codex, the Flavor and Extract Manufacturers Association (FEMA), and the Research Institute for Fragrance Material (RIFM), have established standards and specifications for many plant essential oils (Bouin & Wierer, 2014; Experts & Committee, 2010; Heires, 2008).

2.6 Citrus spp.

The genus *Citrus* belongs to the *Rutaceae* family, which consists of over 160 genera and around 162 species (according to the Tanaka classification system). These species are distributed throughout tropical and subtropical regions worldwide (Talon et al., 2020; Tanaka, 1977). According to the Food and Agriculture Organisation, *Citrus* farming is presently practiced in over 80 countries. *Citrus* fruits have been