CHEMICAL COMPOSITION, ANTIMICROBIAL AND LARVICIDAL PROPERTIES OF *Eucalyptus grandis* × *E. urophylla* LEAF EXTRACTS OBTAINED VIA HYDRODISTILLATION AND SUPERCRITICAL CARBON DIOXIDE FLUID EXTRACTION

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ABSTRACT

CHEMICAL COMPOSITION, ANTIMICROBIAL AND LARVICIDAL PROPERTIES OF *Eucalyptus grandis* × *E. urophylla* LEAF EXTRACTS OBTAINED VIA HYDRODISTILLATION AND SUPERCRITICAL CARBON DIOXIDE FLUID EXTRACTION

Yip See Cheng

The leaves of *Eucalyptus grandis* × *Eucalyptus urophylla*, a post-harvest residue from Malaysian timber plantations, are rich in bioactive compounds. This study evaluated the chemical composition, antimicrobial activity, mosquito larvicidal efficacy, and toxicity of leaf extracts obtained via hydrodistillation and supercritical carbon dioxide fluid extraction (SCE) from *Eucalyptus* hybrid trees of two age groups (17 to 31 months and 40 to 50 months). Optimal SCE conditions, identified using response surface methodology, were 79.8°C, 29.7 MPa, 116.2 min (younger trees) and 79.6°C, 30.0 MPa, 108.6 min (older trees). SCE (0.255% to 4.650% w/w) yielded significantly higher percentages than dried leaf hydrodistillation (0.191% w/w for younger trees; 0.046% w/w for older trees), with younger trees consistently producing higher yields across methods. Gas chromatography-mass spectrometry revealed that SCE extracts contained a broader range of chemical classes, including flavonoids, fatty alcohols, fatty aldehydes, and fatty acids. In contrast, hydrodistillation extracts were dominated by monoterpenoids, especially eucalyptol (10.10% to 26.67%) and α-terpinyl acetate (18.27% to 27.99%), with a

time-dependent shift towards sesquiterpenes and sesquiterpenoids. Bioassays demonstrated that SCE extracts exhibited stronger and broader-spectrum antimicrobial activity, inhibiting or killing most fungi and bacteria, including antibiotic-resistant clinical strains, at concentrations as low as 0.02 to 2.50 mg/mL. Conversely, hydrodistillation extracts displayed superior mosquito larvicidal activity against *Aedes aegypti* and *Aedes albopictus*, with median lethal concentration (LC_{50}) values ranging from 52.26 to 134.56 µg/mL after 24 h, which were lower than those observed in the brine shrimp lethality assay (LC_{50} = 209.24 to 222.24 µg/mL), indicating their potential for safe aquatic mosquito control. The findings of this study support the possible use of *Eucalyptus* SCE extracts as disinfectant and hydrodistillation extracts as natural mosquito larvicides, highlighting the valorisation potential of *Eucalyptus* hybrid leaf waste in agro-industrial applications. Future toxicological and mechanistic studies are required to develop safe formulations for practical applications.

Keywords: *Eucalyptus grandis* × *Eucalyptus urophylla*; hydrodistillation; supercritical fluid extraction; antibacterial; antifungal; *Aedes aegypti*; *Aedes albopictus*; *Artemia franciscana*

Subject Area: RS160-167 Pharmacognosy. Pharmaceutical substances (Plant, animal, and inorganic)

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
°C	Degree Celsius
df	Degree of freedom
DIZ	Diameter of inhibition zone
EC_{50}	Half-maximal effective concentration
ESBL	Extended-spectrum β-lactamase
EtOH	Ethanol
FDPM	Forestry Department of Peninsular Malaysia
FPPL	Fungal Priority Pathogens List
GC-MS	Gas chromatography-mass spectrometry
IC_{50}	Half-maximal inhibitory concentration
ISO	International Organization for Standardization
LC_{50}	Median lethal concentration
LC_{90}	90% lethal concentration
LC_{95}	95% lethal concentration
LPL	Lower prediction limit

MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MOPS	3-morpholinopropanesulfonic acid
MTIB	Malaysian Timber Industry Board
NIST	National Institute of Standards and Technology
PDA	Potato dextrose agar
pH	Potential of hydrogen
PPLH	Program Pembangunan Ladang Hutan
ppm	Parts per million
RI	Retention index
RPMI-1640	Roswell Park Memorial Institute-1640
RT	Retention time
SCE	Supercritical carbon dioxide fluid extraction
SFE	Supercritical fluid extraction
spp.	Species
VCRU	Vector Control Research Unit
UPL	Upper prediction limit
WHO	World Health Organization
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

1.1 Background and Problem Statement

Eucalyptus, a fast-growing tree in the *Myrtaceae* family, originates from Australia but has gained global attention due to its significant economic value (Gilles *et al*., 2010). Its remarkable attributes, such as a short growth cycle, high competitiveness, and high productivity, make it a prime species for timber plantations (Yahya, 2020a). Notably, advancements in hybridisation techniques have further led to the development of *Eucalyptus* clonal hybrids with enhanced growth and wood quality traits (Lu *et al*., 2014).

In Malaysia, government initiatives have promoted the cultivation of *Eucalyptus* to meet the increasing timber demands. Among the species cultivated, the hybrid *Eucalyptus grandis* × *Eucalyptus urophylla* has gained popularity due to its superior growth performance under local conditions (Yahya *et al*., 2020b). Consequently, large volumes of post-harvest *Eucalyptus* leaf are produced, often discarded as waste. Improper disposal may lead to environmental concerns (Phiri, Rangappa and Siengchin, 2024). However, these residual leaves of *Eucalyptus* are rich in volatile compounds and should not be undervalued. Extracts from *Eucalyptus* leaves are known for their diverse medicinal or therapeutic benefits,

including antiviral, antimicrobial, anticancer, anti-inflammatory, anti-spasmodic, antioxidant, antidiabetic, analgesic, mucolytic, and bronchodilatory effects. These effects stem from their complex chemical composition of monoterpenes, diterpenes, sesquiterpenes, and their oxygenated derivatives (Barbosa, Filomeno and Teixeira, 2016; Salehi *et al*., 2019; Chandorkar *et al*., 2021). These qualities support their use across aromatherapy, food and beverages, cosmetics, nutraceutical, and agriculture.

Furthermore, the chemical complexity of *Eucalyptus* leaf extracts may contribute to their potential translation into public health applications. The increasing resistance of human pathogenic bacteria and fungi to conventional therapeutic options highlights an urgent need for effective and safe alternative antimicrobial agents (Arip *et al*., 2022). Concurrently, mosquito-borne illnesses remain a significant health threat in tropical countries. In particular, dengue fever continues to pose a major public health challenge in Malaysia, as the increasing emergence of resistance to synthetic insecticides has been reported for the primary vectors, *Aedes aegypti* and *Aedes albopictus* (Gan *et al*., 2021). Therefore, *Eucalyptus* leaf extracts, which are rich in bioactive constituents, may exert multiple mechanisms against targeted organisms while reducing the likelihood of resistance development, making them promising candidates for natural antimicrobial and insecticidal agents.

Traditionally, extraction of plant material has been conducted through conventional methods such as hydrodistillation (Salehi *et al*., 2019). Nonetheless, supercritical carbon dioxide fluid extraction (SCE) has emerged as a green, sustainable, and efficient alternative. SCE offers key advantages, including high selectivity and the ability to preserve heat-sensitive compounds (Sodeifian and Sajadian, 2017). A comparative understanding of extraction methods is essential to optimise yield and bioactivity for industrial applications.

The yield and chemical composition of *Eucalyptus* leaf extracts can vary depending on several factors, including species, season, geographic location, climate, soil conditions, tree age, fertilisation, and extraction technique (de Oliveira *et al*., 2008; Zhang *et al*., 2010; Achmad *et al*., 2018). These variations can significantly influence the biological efficacy of the extracts, even within the same species.

Moreover, while *Eucalyptus* is predominantly cultivated in temperate regions such as Australia, its introduction to tropical countries, such as Malaysia, where conditions accelerated growth, due to the optimal growth temperature ranging between 18 to 22°C, raises questions regarding potential disparities in chemical composition between tropical and temperate-grown *Eucalyptus* trees (Queiroz *et al*., 2020). Therefore, a critical gap exists in exploring the *Eucalyptus* cultivated in tropical environments, such as the *E. grandis* × *E. urophylla* hybrid in

Malaysia, particularly in the chemical constituents and biological activities of their extracts.

1.2 Objectives

This study aimed to explore the potential applications of *E. grandis* × *E. urophylla* leaves, a post-harvest residue of timber production in Malaysia. The specific objectives were:

- To isolate leaf extracts from *Eucalyptus grandis* × *Eucalyptus urophylla* trees with different ages using hydrodistillation and supercritical carbon dioxide fluid extraction.
- To analyse and compare the yield and chemical composition of *Eucalyptus* hybrid extracts obtained through both extraction techniques.
- To evaluate the antimicrobial activity of the *Eucalyptus* hybrid extracts against selected human pathogenic bacteria and fungi.
- To determine the mosquito larvicidal efficacy of the *Eucalyptus* hybrid extracts against *Aedes aegypti* and *Aedes albopictus*.
- To evaluate the toxicity of the *Eucalyptus* hybrid extracts using the brine shrimp lethality assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial Threats to Human Health

A comprehensive understanding of medically relevant microorganisms forms the foundation for effective diagnosis, treatment, and prevention strategies. Bacteria and fungi are two major classes of microorganisms that play critical roles in human health and are implicated in diverse clinical contexts. Ranging from normal flora to harmful pathogens, their capacity for rapid adaptation and evolution presents substantial challenges to healthcare systems worldwide and underscores the importance of continued research to combat their infectious threats (Bliven and Maurelli, 2016).

2.1.1 Bacteria

Bacteria represent a diverse group of single-celled, prokaryotic microorganisms. They are broadly classified into two major categories, Gram-positive and Gram-negative, based on the variations in their cell wall structures, as revealed by the Gram staining technique. Gram-positive bacteria appear purple or blue under microscopic examination, attributable to the ability of their thick peptidoglycan layer to retain the crystal violet dye. Conversely, Gram-negative bacteria display a red or pink colouration, as their thin peptidoglycan layer fails to

retain the violet stain and instead takes up the counterstain, safranin (Murray, Rosenthal and Pfaller, 2021).

The Gram-positive bacteria tested in this study are *Bacillus cereus*, *Enterococcus hirae* and *Staphylococcus aureus*. *Bacillus cereus* is a rod-shaped bacterium that exhibits aerobic metabolism. Commonly found in soil, food, and on human skin, it is a well-known causative agent of foodborne illness, producing two distinct forms of poisoning, which are diarrhoeal and emetic (Schoeni and Wong, 2005). Beyond foodborne illness, *B. cereus* also acts as an opportunistic pathogen responsible for a wide range of local and systemic infections, including bacteraemia, cutaneous infections, endocarditis, endophthalmitis, pneumonia, septicaemia, salpingitis, and meningitis, particularly in immunocompromised individuals (Glasset *et al*., 2018). Although this species has shown susceptibility to chloramphenicol, aminoglycosides, imipenem, vancomycin, and fluoroquinolones, the production of β-lactamase contributes to its resistance to ampicillin via the degradation of β-lactam ring structure (Woh and Ng, 2024).

Enterococcus hirae, a spherical Gram-positive facultative anaerobe, is typically a commensal inhabitant of the gastrointestinal tracts of both humans and animals. While it is more commonly associated with zoonotic infections, it has gained attention as an emerging nosocomial pathogen capable of causing severe human diseases, including peritonitis, bacteraemia, endocarditis, acute pancreatitis,

pyelonephritis, and septic shock (Sim *et al*., 2012; Di Lodovico *et al*., 2017). The scarcity of documented human infection is due to the difficulty in accurately identifying *E. hirae* and the limited availability of comprehensive clinical reports on its characteristics and treatment (Pãosinho *et al*., 2016). Moreover, the resistance of this species to streptomycin and tetracycline has been reported (Chajęcka-Wierzchowska, Zadernowska and Łaniewska-Trokenheim, 2016).

Staphylococcus aureus is a facultative anaerobic bacterium that exists as part of the normal skin microbiota, but it is also the principal causative agent of numerous infections, including staphylococcal abscesses, bacteraemia, endocarditis, pneumonia, and osteomyelitis. Its clinical significance has increased with the emergence of methicillin-resistant *S. aureus* (MRSA) strains, which pose a major burden in healthcare due to their resistance to multiple antibiotics (Ahmad-Mansour *et al*., 2021).

Besides, Gram-negative *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* were also evaluated in this study. *Klebsiella pneumoniae*, a rod-shaped, lactose-fermenting member of the *Enterobacteriaceae* family, exhibits facultative anaerobic growth and is commonly found in the environment and in human intestines. Additionally, it poses a significant threat as nosocomial pathogen due to its ability in causing pneumonia, urinary tract infections, and intra-abdominal infections (Madigan and Martinko, 2006). Meanwhile, the increasing prevalence of

K. pneumoniae resistant strains that produce *β*-lactamase and carbapenemase is particularly concerning healthcare challenge worldwide (Asokan *et al*., 2025).

Pseudomonas aeruginosa, a rod-shaped bacterium belonging to the family *Pseudomonadaceae*, grows under aerobic conditions and can be isolated from diverse environments, including soil, plants, fruits, and water. This Gram-negative opportunistic pathogen is a major cause of severe infections, particularly in the respiratory tract, urinary tract, outer ear, burns, wounds, and bloodstream (Mielko *et al*., 2019). The clinical management of *P. aeruginosa* infections is challenging due to its proficient adaptive resistance mechanisms, which reduce susceptibility to most antibiotics, including *β*-lactams, aminoglycosides, and fluoroquinolones (Breidenstein, de la Fuente-Núñez and Hancock, 2011).

Escherichia coli, another rod-shaped bacterium belonging to *Enterobacteriaceae* family, exhibits facultatively anaerobic and is the most predominant commensal of the human gastrointestinal tract as well as one of the leading causes of intestinal and extra-intestinal infections, including traveler's diarrhoea, urinary tract infections, bacteraemia, and meningitis. Despite the availability of antibiotics for treatment, *E. coli* infections are often complicated by its resistance mechanisms. For instant, resistance to *β*-lactams in *E. coli* is mediated by the secretion of *β*-lactamase enzymes, which inactivate penicillins and cephalosporins (Allocati *et al*., 2013). Notably, extended-spectrum *β*-lactamase

(ESBL)-producing *E. coli* strains have been increasingly reported worldwide in both healthy individuals and clinical settings, with frequently observed co-resistance to other antibiotic classes such as fluoroquinolones (Kawamura *et al*., 2017). Moreover, infections caused by ESBL-producing *E. coli* are often associated with poorer clinical outcomes than *β*-lactam-susceptible strains, as intestinal carriage of these strains is usually persistent and asymptomatic (Bezabih *et al*., 2021).

2.1.2 Fungi

Fungi are eukaryotes characterised by the presence of a membrane-bound nucleus. They are generally larger than bacteria. One distinguishing feature of fungi is the presence of a rigid cell wall, primarily composed of glucan and chitin, which distinguishes them from cellulose-based plant cell wall (Bowman and Free, 2006). Fungi, comprising more than one form, are identified based on their morphology and growth behaviour. Yeasts exist as independent single cells, while filamentous fungi grow as long hyphal threads. Both groups play crucial ecological and industrial roles (Walker and White, 2017). Yeasts are renowned for their significance in fermentation and food production. Conversely, filamentous fungi contribute to organic matter decomposition and food spoilage.

Despite most fungi are harmless to healthy individuals due to limited virulence (de Pauw, 2011), certain species pose global health concerns, particularly among immunocompromised populations. Recognising this, in 2022, the World

Health Organization (WHO) introduced the Fungal Priority Pathogens List (FPPL) to highlight invasive fungal pathogens of major clinical importance, particularly those associated with antifungal resistance. The aim is to recognise the unmet research needs and encourage further research efforts, combating the significant public health impact caused by these pathogens (WHO, 2022).

Candida species are commensal organisms inhabiting the human gastrointestinal and genitourinary tracts, but some are opportunistic pathogens capable of causing various infections, ranging from mild skin conditions to invasive candidiasis, infiltrating deep tissues and bloodstream (Pfaller *et al*., 2019). Among them, *Candida albicans* and *Candida auris* are categorised in the critical priority group of the FPPL. *Candida albicans* is the leading cause of candidiasis worldwide, attributable to its multiple virulence factors such as the ability to transit between yeast, pseudohyphal, and hyphal forms in response to environment, immune evasion, and pervasive presence in healthcare settings (Pristov and Ghannoum, 2019). Moreover, despite the availability of diagnostic methods, treatment options remain limited to echinocandins and azoles, and infection preventability is still a challenge. The antifungal resistance in this species is relatively uncommon, but rising resistance rates have been reported (WHO, 2022). On the other hand, *Candida auris* is an emerging nosocomial pathogen, spreading rapidly in healthcare settings. This yeast is particularly concerning because it can persistently colonise the skin and cause difficult-to-treat invasive infections with high mortality due to

its intrinsic resistance to multiple antifungal drugs (Chowdhary, Sharma and Meis, 2017; WHO, 2022).

Besides, *Candida tropicalis, Candida parapsilosis,* and *Nakaseomyces glabratus* (previously named as *Candida glabrata*) are ranked as the high priority group in the FPPL (WHO, 2022). *Nakaseomyces glabratus* is the second most common cause of candidiasis, after *C. albicans*. Its pathogenicity is attributed to the secretion of hydrolytic and proteolytic enzymes to adhere to human cells and medical devices. Furthermore, its aggressiveness as a human pathogenic fungus is strengthened by its ability to form biofilm, which contributes to its high resistance rates against current antifungal therapies (Frías-De-León *et al*., 2021). Invasive candidiasis caused by *N. glabratus* carries 30-day mortality rates of up to 50%, with rising resistance to echinocandins and azoles further complicating treatment (WHO, 2022). Similarly, *C. parapsilosis*, a member of the psilosis complex, has the ability to proliferate in hyperalimentation solutions and form biofilms for adhesion, so it frequently causes nosocomial infections in patients who have undergone invasive medical procedures (Tóth *et al*., 2019; Branco, Miranda and Rodrigues, 2023). Although generally considerate moderately resistant, there have been recorded cases of biofilm-mediated resistance to several drugs such as amphotericin B, fluconazole, ravuconazole, and voriconazole (Kuhn *et al*., 2002; Katragkou *et al*., 2008; Cavalheiro and Teixeira, 2018). *Candida tropicalis* is a potent biofilm-producing species, which is also regarded as one of the highly virulent *Candida* species due to its virulence factors, such as lytic enzyme secretion, strong adherence

to epithelial and endothelial cells, bud-to-hyphae transition, along with phenotypic switching, which enable it to adapt and survive under varying environmental conditions (Zuza-Alves, Silva-Rocha and Chaves, 2017). Invasive infections caused by this species are associated with alarmingly high mortality rates, ranging from 55% to 60%. Moreover, antifungal resistance, particularly to azoles, has been reported in *C. tropicalis*, making the empirical use of echinocandins as a preferred treatment option (WHO, 2022).

In addition to the *Candida* species, the critical priority group of FPPL also includes *Cryptococcus neoformans* and *Aspergillus fumigatus* (WHO, 2022). *Cryptococcus neoformans*, predominantly encountered as a haploid yeast, is commonly found in soil and plant. It is encapsulated by polysaccharides that contribute to virulence. Furthermore, *C. neoformans* stands out as a prominent causative agent of systemic fungal infections, Cryptococcosis. Infection typically occurs via inhalation, leading to pulmonary infection in immunocompromised individuals, which can progress to pneumonia and subsequently disseminate via the bloodstream. *Cryptococcus neoformans* possesses the capability to breach the blood-brain barrier, causing fatal cryptococcal meningitis (Chun and Madhani, 2010). Currently, the antifungal resistance of this species is still poorly understood, but the diminished susceptibility to fluconazole has been reported (WHO, 2022).

Filamentous fungus *Aspergillus fumigatus* is commonly found in soil and compost. As the most ubiquitous airborne saprophytic fungus, it poses significant health risks as the causative agent behind various pulmonary diseases in humans, birds, and other mammals (Morelli, Kerkaert and Cramer, 2021). This fungus sporulates frequently and disperses the conidia into the air. These tiny conidia are readily inhaled, reaching alveoli, where they are usually cleared by healthy immune systems, resulting in only occasional infections like aspergilloma and allergic bronchopulmonary aspergillosis. However, in immunocompromised patients, invasive aspergillosis can occur with mortality rates of 47% to 88% (Sales-Campos *et al*., 2013; WHO, 2022). The effective agents against *A. fumigatus* are typically azoles and liposomal amphotericin B but resistance is rising due to the widespread agricultural use of azole fungicides.

Dermatophyte *Trichophyton rubrum* belongs to a class of filamentous fungi reliant on keratin for growth. They can infect keratinised tissues, leading to superficial infection of the skin, hair, and nails. However, due to their dependence for keratin, these fungi do not invade mucosal surfaces (Hainer, 2003). *Trichophyton rubrum*, an anthropophilic fungus, is accountable for over 60% of dermatophytosis cases such as tinea corporis, tinea inguinalis, tinea pedis, tinea unguium, and deep dermal infections (Zheng *et al*., 2020). While not life-threatening, they can still significantly impact the quality of life due to their chronic, recurrent natures, and are easily transmissible. Present therapeutic methods primarily rely on oral administration and topical application of antifungal agents.

However, systemic antifungals remain burdened by drawbacks, including prolonged treatment duration, risk of hepatotoxicity, and potential drug interactions (Ma *et al*., 2022). Furthermore, the emergence of resistant strains to terbinafine, the gold-standard treatment for dermatophytosis, has recently been reported in human patients worldwide (Kano *et al*., 2022).

2.1.3 Antimicrobial Agents

The widespread of multidrug-resistant microorganisms has become a major global health threat. The rapid dissemination of resistance genes worsens the severity of this issue and underscores the need for international cooperation to protect public health. To fight against the rise of multidrug-resistant strains, legislative actions have been implemented to restrict the use of antibiotics in both prophylaxis and treatment. In addition, expanding research efforts in searching for new antimicrobial agents is essential. The currently implemented strategies to address antimicrobial resistance include the use of bacteriophages and the development of next-generation vaccines (Urban-Chmiel *et al*., 2022). There is also increasing attention directed toward plant-derived substances as alternative antimicrobial agents.

Plants synthesise a wide range of secondary metabolites to protect themselves from infections. Among these, phenolics, which are broadly categorised into flavonoids and non-flavonoids based on their chemical structures, represent a major class. Terpenes are another large family that contains over 40,000

compounds with various biological activities. They are classified according to their number of isoprene units. Alkaloids are nitrogen-containing compounds derived from amino acids and common secondary metabolites in plants (Arip *et al.*, 2022). Many of these secondary metabolites have been reported for their antimicrobial activity. For instance, flavonoids isolated from *Eucalyptus maculata* exhibited noticeable inhibitory effects against both bacterial and fungal pathogens (Takahashi, Kokubo and Sakaino, 2004). Therefore, these plant metabolites pose potential to be isolated as natural antimicrobials.

Extraction is a moderately selective process. As a result, plant extracts, without involving any extensive purification, often contain complex mixtures of secondary metabolites. Therefore, they differ significantly from synthetic drugs by possessing greater molecular complexity, ring system diversity, scaffold variety, and stereochemical variation. This chemical complexity may help to reduce the resistance development and enable the plant extracts to exhibit their antimicrobial effect through multiple mechanisms (Arip *et al.*, 2022). One prominent example is the promising antimicrobial properties demonstrated by essential oils extracted from various botanical sources, such as *Eucalyptus* spp., *Artemisia* spp., *Citrus* spp., and *Cymbopogon* spp., against both antibiotic-sensitive and antibiotic-resistant strains (Thielmann, Muranyi and Kazman, 2019; Mangalagiri, Panditi and Jeevigunta, 2021).

2.1.4 Antimicrobial Assays

In vitro antimicrobial susceptibility testing of a substance can be performed using numerous methods, such as diffusion method, dilution method, and time-kill assay. Organisations like the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have contributed to the refinement and standardisation of these testing initiatives (Balouiri, Sadiki and Ibnsouda, 2016). Among them, liquid dilution assays are particularly suitable for screening non-polar antimicrobial agents.

Broth microdilution method is widely used to determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of antimicrobial substances. This standardised and precise method provides essential information on the lowest concentrations of antimicrobial agents required to inhibit the growth and to kill 99.9% of test microorganisms, thereby determining microbial susceptibility or resistance (Golus *et al*., 2016). The broth microdilution method usually utilises 96-well microtiter plates to serially dilute the antimicrobial substances in a suitable growth medium, enabling the simultaneous testing of multiple agents (Gajic *et al*., 2022). In recent years, this method has been further refined. Instead of relying solely on turbidity measurement after incubation, growth indicators such as iodonitrotetrazolium chloride (INT) and resazurin dye have been introduced to facilitate visual detection of microbial growth. These indicators act as electron acceptors, and are reduced to coloured products in the presence of metabolically

active microbial cells, thereby enhancing both sensitivity and ease of interpretation (Braissant *et al*., 2020).

2.2 Mosquitoes

Mosquitoes are insects of the *Culicidae* family and have existed on Earth for over 200 million years. There are approximately 3,500 recognised species, thriving in diverse ecological settings such as deserts, marshes, jungles, and urban centres (Baik and Carlson, 2020). Despite their small size, mosquitoes are among the most important disease vectors, transmitting various pathogens, including arboviruses, protozoans, filariae, and, to a lesser extent, bacteria (Tandina *et al*., 2018).

2.2.1 Mosquito Biology

The mosquito life cycle comprises four stages, which are egg, larva, pupa, and adult. Both larval and pupal stages are aquatic, while the adult stage is aerial (Bar and Andrew, 2013). Development from larva to adult is accelerated in warmer climates, which increases the frequency of blood feeding by female mosquitoes and subsequently enhances pathogen transmission (Githeko *et al*., 2000). Elevated temperatures also facilitate arbovirus replication and dissemination, while increased outdoor human activity in warm weather further intensifies human-mosquito contact (Bi, Zhang and Parton, 2007; Kilpatrick *et al*., 2008). Therefore, Malaysia, a tropical country with high average temperatures, provides an ideal breeding environment for mosquitoes, making the local residents particularly

susceptible to mosquito-borne diseases. The most common mosquito genera in Malaysia are *Anopheles, Culex*, and *Aedes* (Saleeza, Norma-Rashid and Azirun, 2013).

The yellow fever mosquito, *Aedes aegypti*, is native to Africa but is now widespread in tropical and subtropical regions (Nava and Debboun, 2020). In contrast, the Asian tiger mosquito, *Aedes albopictus*, originated from East Asia and several islands in the western Pacific and Indian Oceans but has since expanded globally, except Antarctica (Caminade *et al*., 2012).

The larvae of *Ae. aegypti* and *Ae. albopictus* share certain morphological similarities. They possess a pair of antennae and a pair of mouth brushes at the head, which connects to the rounded thorax by a narrow neck. Lateral hairs arise from the thorax and the abdomen. Their abdomen consists of eight cylindrical segments, with a row of comb spines and a siphon attached to the terminal segment. The abdomen terminates in an anus, while the respiratory openings and spiracles are at the end of the siphon (Supriyono *et al*., 2023). At this stage, the two species can be distinguished by features of the head, lateral hairs, comb spine, and siphon. Specifically, *Ae. aegypti* larvae have a rounder head and longer lateral hairs on the thorax and abdomen, while *Ae. albopictus* exhibit a more elongated head and shorter, less branched lateral hairs (Martins *et al.*, 2023). In addition, subapical denticles exist only at the end of *Ae. aegypti* comb spine, whereas three sharp

denticles are present only on the pecten teeth of *Ae. albopictus* siphon (Sivanathan, 2006; Supriyono *et al*., 2023).

At the adult stage, the body of both *Aedes* species is dark brown in colour and characterised by the white stripes on their legs and body. Their antennae consist of 13 segments, each separated by a pigmented ring. Their thorax is covered with bristles, with denser hairs on the dorsal than on the lateral part (Supriyono *et al.*, 2023). Despite these similarities, *Ae. albopictus* is usually smaller and has a single longitudinal stripe on its scutum (Lwande *et al*., 2020), while *Ae. aegypti* exhibits a lyre-shaped pattern and a pair of median longitudinal white lines of scales on its scutum (Bucton *et al*., 2019).

2.2.2 Mosquito-borne Diseases

Mosquito-borne diseases such as dengue, malaria, yellow fever, chikungunya, and Zika fever, pose major global health challenges, with malaria alone responsible for more than 500,000 deaths annually (Jones *et al*., 2021; WHO, 2023). In Malaysia, the commonly reported mosquito-borne diseases include dengue fever, malaria, and chikungunya. Among these, dengue fever emerges as a primary concern, being endemic in the country (Muhammad Ghazali *et al*., 2025). This arboviral infection manifests in various clinical forms, ranging from asymptomatic infection to fatal dengue haemorrhagic fever and shock. Dengue haemorrhagic fever is characterised by intensified vascular permeability, resulting in plasma leakage and bleeding tendencies due to cytokine storm, an abnormal

immune response with excessive cytokine production (Kularatne and Dalugama, 2022). Dengue fever, caused by four serotypes of RNA virus (DENV-1, DENV-2, DENV-3, and DENV-4), is mainly transmitted by *Aedes* mosquitoes, particularly *Ae. aegypti* and *Ae. albopictus* (Pongsiri, Themboonlers and Poovorawan, 2012; Shafie *et al*., 2016).

Aedes aegypti is the primary urban vector for dengue due to its anthropophilic nature, females preferentially feed on humans to obtain nutrients for egg development. This increases the risk of arbovirus transmission as infected mosquitoes can readily pass the virus to human hosts (Matthews, 2019). Conversely, *Ae. albopictus* ranks as the secondary dengue vector, due to differences in feeding behaviour (Gratz, 2004). Unlike *Ae. aegypti*, which feeds on multiple hosts within a reproductive cycle, *Ae. albopictus* tends to feed repeatedly on the same host, thereby reducing opportunities for dengue virus transmitting (Chow-Shaffer *et al*., 2000; Scott *et al*., 2000; Richards *et al*., 2006).

Nonetheless, *Ae. albopictus* has a wider host range, feeding on humans, other mammals, such as cats and dogs, and avian species such as chickens and northern cardinals (Richards *et al*., 2006). Additionally, it inhabits both urban and rural areas, unlike *Ae. aegypti* which is largely confined to domestic environments with high population density (Valerio *et al*., 2010; Nava *et al*., 2020). Importantly, *Ae. albopictus* is a highly competent vector for chikungunya virus, due to a single

amino acid mutation in the envelope 1 gene, which enhances its viral adaptability compared with *Ae. aegypti* (Tsetsarkin *et al*., 2007). This species played a primary role in chikungunya epidemics in the Indian Ocean Islands from 2004 to 2007 and in Italy (2007 and 2017) (Gloria-Soria *et al*., 2020).

2.2.3 Mosquito Control Strategy

Effective mosquito control is imperative in reducing disease transmission and public health concerns. The primary focus of mosquito control is vector-based interventions. Traditionally, this has relied on the use of chemical or biological agents targeting immature and adult mosquito stages, as well as environmental management to eliminate mosquito breeding sites. For controlling immature stages, the organophosphate larvicide temephos is commonly applied in household water. Temephos exerts neurotoxic effects through the cholinergic pathway (George *et al*., 2015; Satriawan, Sindjaja and Richardo, 2019). In contrast, adult mosquito control frequently relies on pyrethroid insecticides, which act on voltage-gated sodium ion channels and reduce acetylcholinesterase activity (Hołyńska-Iwan and Szewczyk-Golec, 2020). Additionally, synthetic repellents like N, N-diethyl-meta-toluamide (DEET) are commonly used, functioning as both odourant and tastant agents that interfere with host-sensing behaviour (Legeay *et al*., 2018).

Nevertheless, the uncontrolled use of synthetic chemicals has led to growing environmental concerns. Their effectiveness is also increasingly undermined by the emergence of resistance in mosquito populations via

mechanisms such as enhanced metabolic detoxification and target-site insensitivity (Liu, 2015). Ishak *et al*. (2015) evaluated the insecticide resistance profiles of *Aedes* species in Malaysia, reporting moderate resistance to temephos in F_2 generation larvae of *Ae. aegypti* and *Ae. albopictus* collected from Penang and Kuala Lumpur. The same study also observed high pyrethroid resistance to permethrin in *Ae. aegypti* populations across Malaysia, whereas only moderate resistance was detected in *Ae. albopictus* populations from Kuala Lumpur. Similarly, Rosilawati *et al.* (2017) found that 70% of the progeny from Malaysian *Ae. aegypti* was highly resistant to permethrin, further confirming the widespread pyrethroid resistance within Malaysian *Aedes* populations. Furthermore, these chemicals are associated with adverse health effects in humans, such as allergies and asthma. For instance, temephos has been reported to induce permanent DNA damage in human HepG2 cells and apoptotic effect in human lymphocytes at a concentration of 10 µM (Benitez-Trinidad *et al*., 2015). Similarly, Skolarczyk, Pekar and Nieradko-Iwanicka (2017) demonstrated that exposure to pyrethroids reduced plasma levels of anti-inflammatory IL-10 in children, highlighting their potential immunosuppressive effect and underscoring the urgent need for safer alternatives.

To address these challenges, researchers have increasingly explored plant-based insecticides as sustainable alternatives. Plant-derived insecticides, with complex chemical compositions, exert their toxic effect towards mosquitoes through multiple mechanisms while minimising the likelihood of resistance development (Isman, 2000; Gnankiné and Bassolé, 2017; Pawar *et al*., 2025).

Numerous studies have reported the efficacy of plant extracts against mosquito genera such as *Aedes, Anopheles,* and *Culex*. Examples include extracts from mint (Govindarajan *et al*., 2012), eucalyptus (Vivekanandhan *et al*., 2020), ylang ylang (Soonwera, 2015), and ginger (Huong *et al*., 2020). Essential oils, in particular, form a monomolecular film on the water surface, lowering surface tension and blocking spiracular openings, thereby suffocating larvae by inhibiting tracheal respiration (Kamaraj *et al*., 2023).

Beyond botanicals, innovative molecular interventions are emerging as next-generation tools for mosquito-borne disease prevention. One approach involves releasing mosquitoes infected with *Wolbachia*, a maternally inherited endosymbiotic bacterium. *Wolbachia* reduces viral transmission potential and induces cytoplasmic incompatibility, which causes embryonic death when uninfected females mate with *Wolbachia*-infected males, leading to population suppression (Hu *et al*., 2021). Additionally, another promising strategy is gene editing using CRISPR-Cas9 to target survival-related genes in mosquitoes. For instance, loss-of-function mutations in the flight-regulating genes *AeAct-4* (actin) and *myo-fem* (myosin) in female *Ae. aegypti* have produced genetically modified "flightless females", which are unable to mate or blood-feed (O'Leary and Adelman, 2020).

2.2.4 Larvicidal Assays

The assessment of the effectiveness of new mosquito control agents depends heavily on reliable insecticidal assays, which provide essential data on their efficacy and potential for integration into mosquito management programmes. A range of methodologies are employed to assess the efficacy of insecticides against different life stages of mosquitoes. Among these, bioassays targeting immature stages are particularly important, as they evaluate the ability of test substances to disrupt the mosquito life cycle at its early phase. Larvicidal bioassays are more commonly used than pupicidal bioassays, since mosquitoes pass through four larval instars, providing a longer testing period compared to the relatively short pupal stage. The World Health Organization (2005) larval bioassay is one of the most widely applied methods for assessing mosquito larvicidal activity. In this assay, mosquito larvae of known instars are exposed to various concentrations of a test agent, and larval mortality is observed over a specified period. Typically, four or five concentrations are prepared to generate mortality outcomes ranging from 0% to 100%, allowing researchers to estimate suitable lethal concentrations for potential field application (Wang, Li and Liu, 2023). Due to its simplicity, efficiency, and minimal sample requirement, the WHO larval bioassay has been extensively employed to assess the efficacy of novel larvicides against different mosquito species (Uragayala *et al*., 2015; Kaushik *et al*., 2024).

2.3 *Eucalyptus*

The name of *Eucalyptus* is derived from the Ancient Greek words "*eu*," which means "true," and "*calyptus*," which means "cover", referring to its cap-like operculum that seals the flower buds before blooming (Chandorkar *et al*., 2021). *Eucalyptus* spp. are evergreen trees belonging to the family *Myrtaceae*. The *Eucalyptus* genus comprises approximately 730 species and has been introduced into more than 120 countries for forest plantation (Nicolle and Jones, 2018; Zaiton *et al*., 2020).

Globally, over 90% of cultivated *Eucalyptus* plantations are dominated by the “big nine” species, namely *E. grandis*, *E. camaldulensis*, *E. urophylla, E. pellita*, *E. globulus*, *E. saligna*, *E. tereticornis*, *E. dunnii*, and *E. nitens* (Stanturf *et al*., 2013). In addition to pure species, hybridisation techniques have led to the development of clonal hybrids with enhanced timber quality and disease resistance, while retaining beneficial attributes such as rapid growth and high adaptability to diverse ecological conditions (Lu *et al*., 2014; Prasetyo *et al*., 2019). Clonal hybrids derived from *E. tereticornis*, *E. urophylla*, *E. deglupta*, *E. grandis*, *E. globulus*, *E. citriodora*, *E. alba,* and *E. camaldulensis* have been cultivated extensively in numerous countries, including Argentina, Brazil, China, Indonesia, Malaysia, and South Africa (Madhibha *et al*., 2013; Sseremba, Mugabi and Banana, 2016; Chahomchuen, Insuan and Insuan, 2020; Tine *et al*., 2020; Diloksumpun *et al*., 2022).

2.3.1 *Eucalyptus* Cultivation in Malaysia

Historically, the Malaysian wood-processing industry has depended heavily on timber from natural forests. However, ongoing depletion of forest resources poses a significant challenge to meet the growing demand. According to the Forestry Department of Peninsular Malaysia (FDPM), the forested land in Peninsular Malaysia decreased from 5.83 million hectares in 2013 to 5.74 million hectares in 2023, leading to a drop in log production from 4.08 million cubic meters to 2.82 million cubic meters (FDPM, 2025).

To address this shortage, the expansion of forest plantations through afforestation or reforestation has been recognised by the Malaysian government as an important strategy to overcome the scarcity of timber supply. The Forest Plantation Development Programme (Program Pembangunan Ladang Hutan, PPLH) implemented via the Malaysian Timber Industry Board (MTIB), provides financial assistance to eligible private companies, government agencies, cooperatives, and organisations to establish commercial plantations of four hectares or more (MTIB, 2021). Among the species listed under PPLH, *Eucalyptus* has emerged as a leading choice for large-scale cultivation due to its fast growth rate, limited space occupancy, high phenotypic plasticity, and adaptability to diverse climates and soils (Yahya, 2020a). Its wood is widely used in the production of paper pulps, plywood, furniture, poles, and sawn timber (Lu *et al*., 2014).

Malaysia's tropical climate makes it particularly suitable for the cultivation of *Eucalyptus* due to its hot and humid weather throughout the year. *Eucalyptus* has low cold tolerance and requires high nutrient and water supply during its growth and maintenance, conditions that are readily met in Malaysia (Zhang and Wang, 2021). Additionally, the spread of *Ceratocystis* disease that led to significant mortality among *Acacias* plantations (10% to 20%) has prompted many forestry companies in Malaysia to transition towards *Eucalyptus* (Yahya, 2020a).

Currently, the most widely planted *Eucalyptus* species in Malaysia are *Eucalyptus pellita* and the hybrid *Eucalyptus grandis* × *Eucalyptus urophylla* because of their high survival rate and favourable stem morphology (Yahya, 2020a). Notably, *E. grandis* × *E. urophylla* has been reported to outperform *E. pellita* in terms of tree diameter and height after 18 months of planting in Peninsular Malaysia (Yahya *et al*., 2020b). *Eucalyptus grandis* is valued for its rapid growth and long, straight trunk, making it ideal for the timber and paper pulp industries, especially in subtropical regions (Ogunwande *et al*., 2003). However, it is susceptible to the fungal pathogens such as *Crysoporthe austroafricana* and *Coniothyrium* spp., which cause canker diseases in tropical areas. In contrast, *Eucalyptus urophylla* boasts superior disease tolerance (Van den Berg *et al*., 2015). The hybrid *E. grandis* × *E. urophylla* (Figure 2.1) successfully combines the desirable traits from both parental species, which are the rapid growth and ease of propagation of *E. grandis*, with the enhanced disease resistance, improved environmental adaptability, and higher wood density of *E. urophylla* (Wingfield, 2003; Kullan *et al*., 2012). This

hybrid was first introduced into Malaysia in 2008 using clones selected from Southern China and is now cultivated in Sabah and parts of Peninsular Malaysia (Yahya, 2020a).

Figure 2.1: *Eucalyptus grandis* × *Eucalyptus urophylla* trees.

2.4 *Eucalyptus* Leaf Extracts

Plant extracts are natural products obtained from plant materials through various extraction methods involving the use of solvents. They are recognised as a valuable source of diverse bioactive compounds (Abdullahi *et al*., 2022). Among these, essential oils represent a prominent class, aromatic, hydrophobic liquids that are only slightly soluble in water but readily soluble in organic solvents (da Silva *et al*., 2020). *Eucalyptus* is a rich source of phytochemicals present in multiple parts of the plant, including leaves, fruits, flowers, and bark (Surbhi *et al*., 2021). Traditionally, extracts obtained from different parts of *Eucalyptus* tree, including

leaf, have been employed by indigenous communities and traditional healers to manage respiratory ailments such as nasal congestion, bronchitis, asthma, common colds, and pulmonary tuberculosis (Mieres-Castro *et al*., 2021).

2.4.1 Yield

Studies on *Eucalyptus* have shown that extraction yield is significantly influenced by various factors, including the extraction method, plant age, and environmental factors. The leaves of *E. grandis* × *E. urophylla* grown in Sichuan Province, China produced a yield of 7.86% via supercritical carbon dioxide fluid extraction (SCE), compared to only 0.70% via steam distillation (Zhou *et al*., 2016; Zhou *et al*., 2021). Similarly, a study by Herzi *et al*. (2013) reported that SCE significantly outperformed hydrodistillation in terms of efficiency and extraction yield. Specifically, under conditions of 90 bar and 40°C for 30 min, SCE increased the extraction yield of *E. cinerea* and *E. camaldulensis* by 20% and 40%, respectively, compared to a 240 min hydrodistillation process. Other than extraction method, variations in the yield of *Eucalyptus* leaf extracts could be affected by factors such as geographical origin and tree age. For instance, hydrodistillation of *E. urophylla* leaves from Brazil yielded 0.20%, while leaves from the same *Eucalyptus* species grown in Thailand yielded 0.60% (Alves *et al*., 2021; Insuan *et al*., 2021). In Ethiopia, 4-year-old *E. globulus* trees from Entoto showed higher extraction yield (2.36%) than 2-year-old trees (1.69%) using hydrodistillation (Fikremariam *et al*., 2019). However, a contrasting trend was observed in Ankober, Ethiopia, where the highest yield (1.32%) was obtained from

the youngest (3-year-old) trees, while the oldest (100-year-old) trees showed the lowest yield (0.95%) (Shiferaw *et al*., 2019). These discrepancies underscore the influence of genetic and environmental factors on extraction outcomes.

2.4.2 Chemical Composition

Eucalyptus leaf extracts are typically rich in monoterpenes such as eucalyptol (1,8-cineole), *α*-pinene, phellandrene, limonene, citronellal, citronellol, and *p*-cymene, as well as sesquiterpenes such as globulol, spathulenol, virdiflorol, and *β*-caryophyllene (Zhou *et al*., 2021; Yip *et al*., 2024). Among these, eucalyptol is the most frequently reported major component, making up a substantial portion of the chemical profile in many species (Elaissi *et al*., 2012; Goldbeck *et al*., 2014; Reyes *et al*., 2019; Chahomchuen, Insuan and Insuan, 2020; da Silva *et al*., 2020; Li *et al*., 2020; Alves *et al*., 2021; Abbas *et al*., 2022; Diloksumpun *et al*., 2022; Farag *et al*., 2024).

The chemical composition of *Eucalyptus* leaf extracts is highly variable and affected by several factors including extraction techniques, tree age, species, and geographical location (de Oliveira *et al*., 2008; Zhang *et al*., 2010; Achmad *et al*., 2018, Siramon, Ohtani and Ichiura, 2013; Sebei *et al*., 2015; Barbosa, Filomeno and Teixeira, 2016; Almas *et al*., 2019). For example, eucalyptol (36.2%) was reported as the major component for steam-distilled extract of *E. grandis* × *E. urophylla* from Herval City, Brazil (Goldbeck *et al*., 2014). However, leaf extracts

of the same species from Sichuan Province and Hainan Province, China were rich in *α*-pinene (17.0%) and allo-ocimene (43.2%), respectively, despite employing the same extraction method (Liu *et al*., 2008; Zhou *et al*., 2021). The extraction technique also plays a crucial role in shaping the chemical profile. Hydrodistilled extracts of *E. camaldulensis* were dominated by eucalyptol (45.7%) and *p*-cymene (17.1%), whereas SCE extracts were richer in compounds such as 8,14-cedranoxide (43.8%) and elemol (6.3%) (Herzi *et al*., 2013). Furthermore, Shiferaw *et al*. (2019) has proven the influence of age on the content of *Eucalyptus* leaf extracts, as they observed that as *E. globulus* tree aged from 3 to 100 years, the abundance of eucalyptol (61.0% to 73.9%) increased, while that of *α*-pinene (19.4% to 13.7%) declined.

It is also noteworthy that *Eucalyptus* hybrids tend to display a composite chemical profile reflecting both parental species. In a study by Lu *et al*. (2014), the hydrodistilled extract of *E. grandis* leaves was characterised by *α*-pinene (21.8%) and eucalyptol (23.6%), whereas the hydrodistilled extract of *E. urophylla* leaves contained mainly eucalyptol (57.1%) and *α*-cubebene (9.7%). Accordingly, the hybrid *E. grandis* × *E. urophylla* extract obtained in the same study exhibited a combined profile with eucalyptol (48.2%), *α*-pinene (15.6%), and *α*-cubebene (9.9%).

2.4.3 Antibacterial Effects

Numerous studies have demonstrated the antibacterial activity of *Eucalyptus* extracts against various human pathogens. Leaf extracts obtained from *Eucalyptus globulus, E. grandis, E. citriodora*, *E. tereticornis*, and *E. camaldulensis* showed diameters of inhibition zones (DIZs) ranging from 7.7 to 34.7 mm against Gram-positive bacteria, such as *Streptococcus mutans, Staphylococcus epidermidis, S. aureus, Bacillus subtilis, B. cereus,* and *Listeria monocytogenes.* These extracts also inhibited Gram-negative bacteria, including *Acinetobacter calcoaceticus, Escherichia coli*, *Serratia marcescens, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa,* and *Citrobacter freundii* with DIZs ranging from 7.0 to 29.0 mm (Elaissi *et al*., 2011; Damjanović *et al*., 2011; Elaissi *et al*., 2012; Soyingbe *et al*., 2013; Goldbeck *et al*., 2014; Dogan *et al*., 2017; Diloksumpun *et al*., 2022).

Eucalyptus hybrids have also yielded leaf extracts with potential in inhibiting bacterial growth. For instance, Ambrosio *et al*. (2017) reported a DIZ of 13.1 mm for *E. grandis* × *E. urophylla* extract against *Salmonella enterica* serotype Enteritidis, while Goldbeck *et al*. (2014) found that the same hybrid extract inhibited *Streptococcus mutans* with an MIC of 0.025 mg/mL and a DIZ of 23.0 mm, outperforming 0.1% sodium fluoride solution, with a DIZ of 11 mm. Furthermore, *E. urophylla* × *E. camaldulensis* extracts were effective against eight bacterial strains, with DIZs between 11.8 to 24.2 mm, MICs ranged from 0.3 to 8.0 mg/mL, and MBCs ranged from 0.5 to 8.0 mg/mL (Diloksumpun *et al*., 2022).

Among the tested bacteria, Gram-positive *Streptococcus pyogenes* was the most sensitive, while Gram-negative *E. coli* was the most resistant. Despite the growing number of research on the antibacterial properties of *Eucalyptus* leaf extracts, most studies have focused on single extraction techniques, while comparative evaluations between extraction methods remain limited.

The antibacterial activity of *Eucalyptus* leaf extracts is attributed to their bioactive constituents. Moreira *et al*. (2005) suggested that phenolics disrupt bacterial cell membranes by increasing permeability and promoting intracellular leakage. Moreover, molecular docking studies of Sharma *et al*. (2024) showed that eucalyptol and *α*-pinene bind to bacterial DNA gyrase B, potentially inhibiting DNA replication and transcription.

2.4.4 Antifungal Effects

Research on the antifungal activity of *Eucalyptus* extracts against human pathogenic fungi remains limited, but available literature suggests their promising antifungal potential. *Candida* species, which cause candidiasis, are susceptible to extracts from *E. globulus*, *E. citriodora,* and *E. camaldulensis.* For example, Musyimi and Ogur (2008) reported the complete suppression of *C. albicans* growth by undiluted *E. citriodora* hydrodistilled extracts, while *E. camaldulensis* leaf and fruit extracts at 30 µg/mL produced DIZs of 23 mm and 22 mm, respectively, against *C. tropicalis* (Dogan *et al*., 2017). The MICs for *E. globulus* extracts against various *Candida* species, such as *C. parapsilosis, C. albicans, C. utilis*, *C.*

dubliniensis, and *C. tropicalis,* ranged from 0.01 to 4.00 mg/mL (Lim and Shin, 2008; Damjanović *et al*., 2011; Noumi *et al*., 2011; El-Ahmady, El-Shazly and Milad, 2013). Notably, Hasibuan, Agusnar and Taufik (2022) demonstrated that a mouthwash formulation containing *E. grandis* leaf extract showed a dose-dependent effect on *C. albicans* growth. Specifically, the formulation containing a higher extract volume (0.3 mL) yielded a larger DIZ (12.2 mm) compared to the formulation incorporating only 0.1 mL extract, which produced a DIZ of 11.6 mm.

Moreover, the *Eucalyptus* extracts have also shown efficacy against ringworm-causing dermatophytes, such as *Trichophyton* and *Microsporum* species. Lim and Shin (2008) assessed the susceptibility of six *Trichophyton* strains to *E. globulus* extracts. The lowest MIC (31.3 µg/mL) was observed for *T. mentagrophytes*, followed by 62.5 µg/mL for *T. rubrum*, 125.0 µg/mL for *T. tonsurans, T. schoenleinii*, and *T. erinacei*, and 250.0 µg/mL for *T. soudanense*. Musyimi and Ogur (2008) reported that *E. citriodora* hydrodistilled extract achieved complete inhibition of *T. mentagrophytes* at 100% concentration, whereas *E. globulus* extract only inhibited 23% of fungal growth at the same concentration. Similarly, against *Microsporum gypseum*, 100% fungal inhibition was achieved using *E. citriodora* extract, compared to 46% by *E. globulus* (Musyimi and Ogur, 2008). Likewise, Tolba *et al*. (2015) confirmed the fungicidal activity of *E. citriodora* leaf extract, with MFCs of 5.00 µL/mL against *M. gypseum* and 1.25 µL/mL against *M. canis*. Despite these promising findings, only the antifungal

properties of non-hybrid species have been documented, highlighting the need of extending research efforts to *Eucalyptus* hybrids.

The antifungal effects of *Eucalyptus* leaf extracts are mainly due to terpenoids such as limonene, citronellol, and citronellal, which disrupt fungal proteins, inhibit protein synthesis, and impair ergosterol biosynthesis (Pereira *et al*., 2015; Cai *et al*., 2019; OuYang *et al*., 2021). Additionally, the hydrophobic nature of hydrodistilled extracts promotes their penetration into fungal membranes, resulting in membrane leakage and electrolyte efflux, thereby disturbing fungal cell homeostasis (Salvatori *et al*., 2023).

2.4.5 Insecticidal Effects

The potential of *Eucalyptus* leaf extracts as botanical insecticides has attracted growing interest due to their eco-friendly profile. Mosquitoes of the genera *Culex*, *Anopheles*, and *Aedes,* are vectors of several diseases, such as malaria, dengue, chikungunya, and Zika. The larvicidal effectiveness of leaf extracts obtained from *E. nitens* was evaluated against *Aedes* mosquitoes in the study of Alvarez Costa *et al*. (2017). A higher median lethal concentration (LC_{50}) of 52.83 ppm was reported for *Ae. aegypti*, in comparison to 28.19 ppm for *Ae. albopictus,* suggesting *Ae. albopictus* showed a higher sensitivity to the larvicidal effects of *E. nitens* extracts. In a follow-up study, they examined the extracts from the same *Eucalyptus* species for its effect on the survival and swimming ability of the larvae of *An. pseudopunctipennis* and *Ae. aegypti* (Alvarez Costa *et al*., 2018). It was

found that *An. pseudopunctipennis* had a higher tolerance to the *E. niten* extracts than *Ae. aegypti* as only *Ae. aegypti* larvae showed a concentration-dependent decrease in their swimming ability. This finding was further evidenced by the higher LC_{50} of 87.881 ppm observed for *An. pseudopunctipennis,* compared to 43.846 ppm for *Ae. aegypti*.

Eucalyptus globulus leaf extracts also demonstrated larvicidal activity against *Ae. aegypti, An. stephensi,* and *Cx. quinquefasciatus,* with LC_{50} and 90% lethal concentration (LC_{90}) values of 13.578 ppm and 106.755 ppm, 30.198 ppm and 103.389 ppm, and 7.469 ppm and 32.454 ppm, respectively, after 24 h post-treatment (Vivekanandhan *et al*., 2020). These values were comparable to those of the organophosphate larvicide, temephos, which yielded LC_{50} and LC_{90} values ranging from 6.736 to 29.790 ppm and 13.338 to 75.864 ppm, respectively, after 24 h post-treatment. Farag *et al*. (2024) further reported the adulticidal and oviposition deterrent effects of *E. globulus* extract (6%) against *Cx. pipiens* mosquitoes. Under both laboratory and field settings, the corresponding oviposition deterrent indices were -0.82 and -0.80, respectively, indicating a strong deterrent effect. The extract showed noteworthy adulticidal activity (LC_{50} = 0.33%) after 24 h of exposure. Geographical origin also influences insecticidal potency of *Eucalyptus* leaf extracts. *E. citriodora* extracts from Colombia and Vietnam showed larvicidal effects against *Ae. aegypti*, with LC_{50} values of 70 µg/mL (Vera *et al*., 2014) and 104.4 µg/mL (Manh *et al*., 2020), respectively. However, while the impact of geographical origin has been shown in literature, the effect of extraction techniques

on the insecticidal efficacy of *Eucalyptus* leaf extracts remains insufficiently explored.

Beyond mosquitoes, *Eucalyptus* leaf extracts were also effective against the sandfly *Lutzomyia longipalpis* which can transmit the parasite *Leishmania infantum* and cause visceral leishmaniasis. Maciel *et al*. (2010) reported that *E. staigeriana*, *E. globulus,* and *E. citriodora* extracts inhibited egg hatching and killed sandfly larvae and adults, with half-maximal effective concentration (EC_{50}) values of 3.60, 2.63, and 0.59 mg/mL for *E. staigeriana*; 9.23, 25.29, and 7.78 mg/mL for *E. globulus* and 9.44, 1.78, and 5.04 mg/mL for *E. citriodora*, correspondingly. These effects are attributed to both direct and indirect mechanisms exerted by bioactive constituents, including eucalyptol, limonene, and pinene. These compounds inhibit insect acetylcholinesterase, γ-aminobutyric acid, and octopamine receptors, while also inducing oxidative stress that leads to cellular damage and eventual cell death (Chaudhari *et al*., 2021).

2.5 Extraction Methods

Extraction is the process of separating soluble plant metabolites from insoluble plant matter (marc) using selective solvents under standardised procedures (Azwanida, 2015). Various plant parts such as barks, peels, leaves, buds, seeds, and flowers may be used as raw materials. The choice of extraction method depends largely on the nature and form of the botanical material. Importantly, the selected technique significantly affects the extract quality, as inappropriate method

may degrade or alter the phytochemical profile, resulting in reduced bioactivity and compromised properties. In extreme cases, changes in colour, odour, and viscosity of plant extract may occur (Tongnuanchan and Benjakul, 2014). Generally, extraction methods are categorised into conventional and advanced techniques, each with distinct principles, advantages, and limitations.

2.5.1 Distillation

Distillation is a process that separates components based on differences in vapour pressure (Dekebo, 2019). According to the International Organization for Standardization (2021), essential oils are natural products obtained from botanical sources through hydrodistillation, steam distillation, dry distillation, or, in the case of citrus fruits, a mechanical pressing, followed by physical separation of the aqueous phase.

Hydrodistillation uses boiling water to release volatile compounds from plant materials. The vapour, rich in essential oils, is condensed, and the essential oil is physically separated from the water. This method is cost-effective and easy to operate, but has drawbacks such as potential loss of volatile components due to prolonged heating and longer extraction times (El Asbahani *et al*., 2015; Aziz *et al*., 2018).

Steam distillation, another conventional extraction technique, involves exposing plant material to steam, inducing the vaporisation, condensation, and collection of volatile oils. However, thermolabile constituents may degrade under high temperatures (Fornari *et al*., 2012). Moreover, this method requires large quantities of raw material, but yield is relatively lower compared to hydrodistillation, and its ability to fully capture the full aromatic and medicinal profile of the plant is often questioned (Khandge *et al*., 2018; Pheko-Ofitlhile and Makhzoum, 2024).

2.5.2 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) has emerged as a sustainable and eco-friendly alternative to conventional distillation, despite its higher equipment costs. Carbon dioxide (CO_2) is the preferred solvent due to its low critical pressure (7.38 MPa), low critical temperature (31.10°C), non-toxicity, non-flammability, and cost-effectiveness. The non-polar nature of CO_2 makes it suitable for extracting lipophilic compounds, while its poor solubility for polar compounds can be enhanced with co-solvents such as ethanol and methanol (Fornari *et al*., 2012; Azwanida, 2015).

In supercritical carbon dioxide fluid extraction (SCE), CO_2 is utilised beyond its critical temperature and pressure, where it exhibits both gas-like and liquid-like properties. This unique state enables efficient penetration and

solubilisation of plant constituents. Additionally, CO_2 can be recycled through repeated compression and decompression cycles, reducing environmental waste generation. When reverted to its gaseous state, CO_2 leaves behind little-to-no residues in the extract. Moreover, the accelerated extraction and enhanced solute-solvent mass transfer rates also result in good yields and high extract quality (Chai *et al*., 2021). Another significant advantage of SCE over conventional extraction methods is its adjustable selectivity, which enables targeted isolation of desired compounds through optimisation of extraction parameters using mathematical modelling (Pheko-Ofitlhile and Makhzoum, 2024).

Optimisation of SCE conditions is essential to balance yield and cost-efficiency. The key parameters include operating pressure, temperature, extraction duration, and CO_2 flow rate (Ara and Raofie, 2016; Chai *et al*., 2020; Gawron *et al*., 2021). Due to the complexity of these variables and their interactions, statistical tools such as response surface methodology (RSM) are widely used. RSM employs a polynomial model to predict optimal operating conditions. Basically, it estimates the response of interest based on the behaviour of acquired data set and thereby determines the levels of those variables to reach the finest system performance (Bezerra *et al*., 2008; Akhbari *et al*., 2018; Djimtoingar *et al*., 2022).

2.6 Toxicity

A common misconception is that natural products are always safer and cause lesser side effects than synthetic drugs due to their natural origin (Fennell *et*

al., 2004; Street, Stirk and Van Staden, 2008). However, unlike synthetic medications, which typically consist of a single or a few active ingredients, plant extracts often comprise hundreds of chemical compounds (George, 2011). While this chemical complexity may result in synergistic effects that enhance bioactivity, it may also increase the risk of adverse reactions. Therefore, the assumption that natural products are inherently safe is unfounded.

Despite the widespread use of *Eucalyptus* leaf extracts in daily life, their toxicity remains unexplored. In the brine shrimp lethality assay, *E. globulus* leaf extracts exhibit toxic potential, with LC_{50} of 67.6 µg/mL (Atmani-Merabet *et al*., 2018) and 9.59 µL/mL (Akolade *et al*., 2012) against *Artemia salina.* In contrast, a skin sensitisation assessment conducted by Moreira *et al*. (2022) revealed that the hydrodistilled *E. globulus* leaf extracts did not cause significant effect on the expression of co-stimulatory molecules or Nrf2-dependent Hmox-1 gene, while maintaining tissue viability above 50%. These findings indicate that *Eucalyptus* leaf extracts may be safe for topical use, as they did not cause notable skin irritation or sensitisation. Compared to *E. globulus*, the toxicological profiles of other *Eucalyptus* species are less well-characterised. Alves *et al*. (2021) assessed the genotoxicity and mutagenicity of leaf extracts from *E. urophylla* and *E. urophylla* × *E. camaldulensis.* Their study revealed that *E. urophylla* exhibited higher mutagenic potential than the hybrid, although both extracts induced nuclear and chromosomal abnormalities in the lettuce root meristematic cells. Notably, no

studies have investigated the toxicity profile of *E. grandis* × *E. urophylla*, despite the increasing economic value of this hybrid.

Nonetheless, poisoning from *Eucalyptus* extract exposure has been reported, with symptoms including seizures, decreased consciousness, ataxia, and vomiting (Flaman *et al*., 2001; Karunakara and Jyotirmanju, 2012; Ittyachen *et al*., 2019). Therefore, toxicity studies are essential in any research involving the therapeutic or commercial use of *Eucalyptus* leaf extracts in humans.

2.6.1 Toxicity Screening Assays

Many aromatic plants have not been scientifically tested for their safety profile. Plant-derived compounds may exhibit toxicological properties that could restrict their safe application (Roy *et al*., 2026). Therefore, research on toxicity of plant extracts is crucial for the development of plant-based antimicrobial or insecticidal agents. Several approaches can be used to evaluate the potential toxicity of plant extracts.

In vitro studies are widely adopted for the toxicity screening of plant extracts. These laboratory investigations typically test on cell lines of interest to predict potential adverse effects *in vivo*. Understanding the effect of plant extracts on cell viability, proliferation, and membrane integrity can provide valuable information for further formulation (Ghica *et al*., 2024). Common cell-based assays

include colourimetric assays, luminometric assays, dye exclusion test, and fluorometric assays. The selection of assay depends on the suspected mechanism of action, extract concentration, and exposure duration of the plant extract, as each assay measures different parameters to assess the cellular response (Gavanji *et al*., 2023).

In addition, screening for the safety of plant extracts can be performed via *in vivo* experiments using laboratory animals. Acute toxicity tests, which measure the mortality of tested organism after short-term exposure (Roy *et al*., 2026), are frequently applied for rapid toxicity assessment. These tests determine dose-response relationships to calculate the median lethal dose, which estimates the amount needed to kill 50% of the test population (Chan *et al*., 2021).

The brine shrimp (*Artemia* spp.) is one of the commonly used model organisms for *in vivo* toxicity screening instead of more ecologically relevant species because its lethality assay is simple and free from ethical concerns (Chan *et al*., 2021). In the brine shrimp lethality assay, nauplii are preferred instead of adult organisms due to their higher sensitivity to toxic substances (Banti and Hadjikakou, 2021). Previous studies have demonstrated a positive correlation between brine shrimp lethality and toxicity in rodent models (Parra *et al*., 2001; Naidu, Ismail and Sasidharan, 2014), supporting its suitability as a toxicity screening model. However, this assay remains a simplified biological model and

doubts have been raised regarding its reliability in fully predicting the toxic effects of physiologically differed vertebrates (Chan *et al*., 2021). As a result, vertebrate models such as zebrafish have gained increasing attention due to their histological, morphological, and physiological similarities to mammals (Roy *et al*., 2026).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Equipment

The chemicals (Table A1) and equipment (Table A2) used in this study are listed in Appendix A. The preparation of reagents in this research is indicated in Appendix B.

3.2 Overview of Experimental Design

The overview of the experimental design for this study is shown in Figure 3.1. The research started with the collection of plant leaf samples, which were subsequently subjected to extraction using two methods, hydrodistillation and supercritical carbon dioxide fluid extraction (SCE), to enable comparison of conventional technique with green and sustainable alternative. Next, the chemical composition of the obtained extracts was then analysed using gas chromatography-mass spectrometry (GC-MS). After that, the antimicrobial potential of the extracts was assessed using the colourimetric broth microdilution method, while their insecticidal activity was tested through mosquito larvicidal assays. The toxicity of the extracts was further evaluated using the brine shrimp lethality assay. Finally, the experimental data were analysed with R programming and SPSS Statistics 27, with statistical significance threshold set at $p < 0.05$.

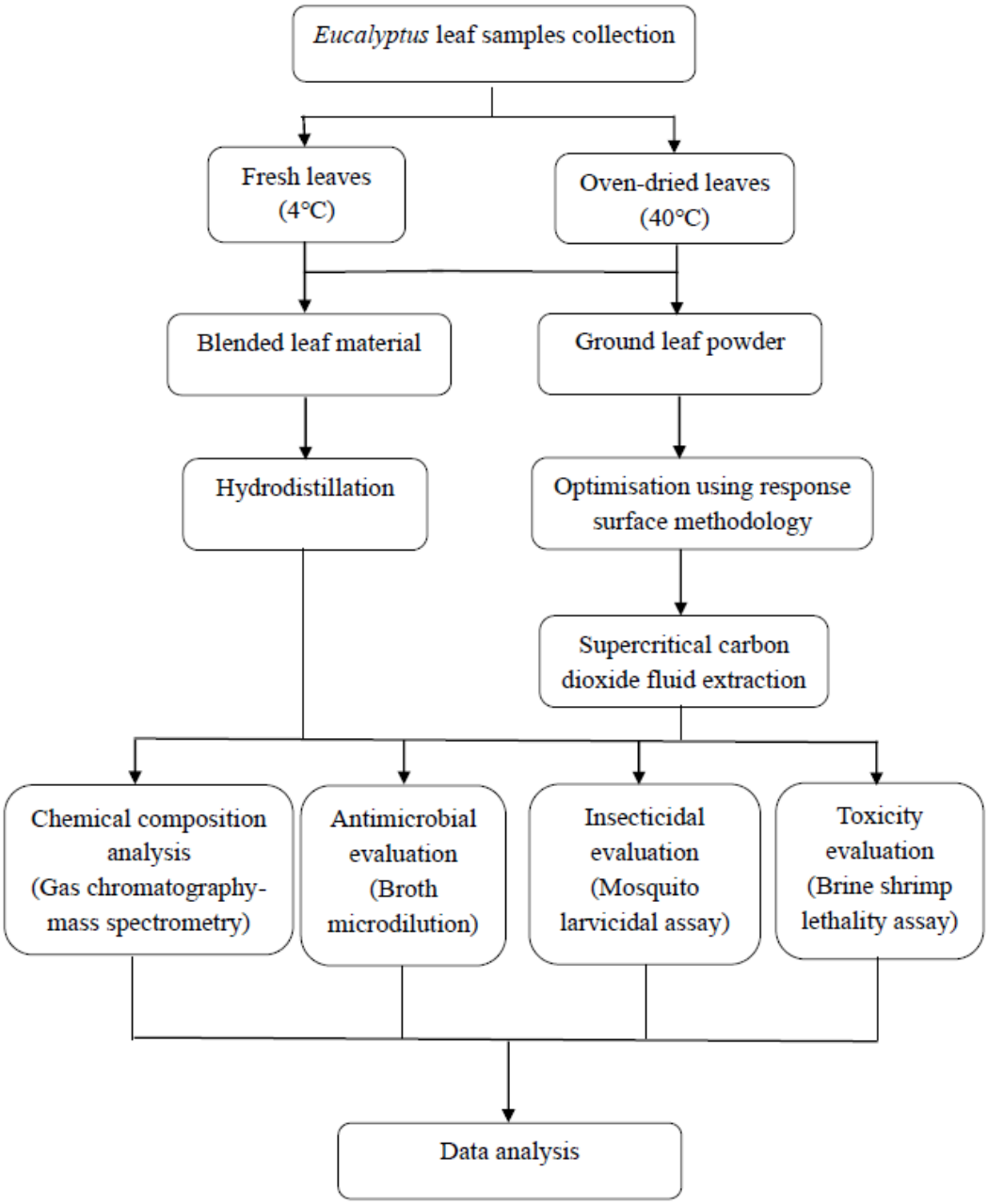


Figure 3.1: The overview of experimental design.

3.3 Plant Sample Collection

The plant samples used in this study were supplied by Edubest Plantation Holdings Sdn. Bhd. From February 2023 to April 2024 (Table A3), fresh leaves were collected from *Eucalyptus* trees of two age groups in Gua Musang, Kelantan, Malaysia (Figure 3.2). Trees cultivated in the Blau plantation (4° 39' 48" N, 101° 36' 58" E) were aged 17 to 31 months, while those from the Pulai plantation (4° 48' 192" N, 101° 55' 15" E) were aged 40 to 50 months at the time of harvesting. The *Eucalyptus grandis* × *Eucalyptus urophylla* hybrid Clone 3229 analysed in this project originated from tissue culture materials provided by the Chinese Academy of Forestry, Guangzhou, China. The prepared voucher specimens were kept at the Faculty of Science, Universiti Tunku Abdul Rahman, Malaysia with voucher code: UTAR/FSC/23/001.

Upon arrival at the laboratory, the fresh leaves were rinsed with water and separated into two batches. One batch was stored at 4°C to preserve freshness, while the other batch was oven-dried at 40°C for 5 to 7 days until they became brittle and could be easily crushed to ensure complete dehydration (Figure 3.3). The fresh leaves were used exclusively for hydrodistillation, while the dried leaves were subjected to both hydrodistillation and SCE.

(a)

(b)

Figure 3.2: *Eucalyptus* plantations in Blau (a) and Pulai (b), Gua Musang, Kelantan.

(a)

(b)

Figure 3.3: Fresh (a) and dried (b) *Eucalyptus* leaves.

3.4 Supercritical Carbon Dioxide Fluid Extraction (SCE)

Supercritical carbon dioxide fluid extraction (SCE) was carried out at Universiti Teknologi PETRONAS, Seri Iskandar, Perak (Figure 3.4). Prior to extraction, the dried leaves were ground into powder and sieved through a 250 µm mesh (Figure 3.5). For each run, 5.0 g of leaf powder was carefully placed into a 50 mL extraction vessel, which was then heated to the selected extraction temperature using an oven. The extractive solvent, CO_2 gas, cooled to 0°C, was pumped into the extraction vessel at a flow rate of 8 mL/min using a preparative pump. The extraction pressure was regulated automatically by a back-pressure regulator. After leaving the extraction vessel, the CO_2 carrying the dissolved compounds underwent depressurisation and vaporisation, leaving the extracts deposited in the collection vials. The extracts were stored at 4°C. The extraction yield was calculated using the following formula:

$$\text{Extraction yield (\% w/w)} = \frac{Mass\ of\ extract}{Mass\ of\ leaf\ powder} \times 100$$

To evaluate the effect of a modifier, the SCE under optimum conditions, identified by response surface methodology (RSM), was repeated with 10% ethanol as a co-solvent. Each ethanol-assisted extraction was performed in triplicate. For clarity, the extracts from trees aged 17 to 31 months using pure CO_2 and ethanol-assisted SCE were labelled as A and A10, respectively, while the extracts from trees aged 40 to 50 months using pure CO_2 and ethanol-assisted SCE were labelled as B and B10.

Figure 3.4: Setup for SCE.

Figure 3.5: Sieved *Eucalyptus* leaf powders.

3.4.1 SCE Optimisation

The maximum SCE yield was evaluated by optimising three factors, which were extraction pressure (10 to 30 MPa), temperature (40 to 80°C) and duration (30 to 150 min). Optimisation was performed using response surface methodology (RSM) with central composite design (CCD) in Design Expert version 13. A total of 20 randomised experimental runs consisting of 6 axial points, 6 centre points, and 8 factorial points was conducted to investigate the significance of extraction temperature, pressure, and duration on the yield of *Eucalyptus* leaf extract. The acquired data were fitted to a second-order polynomial model derived from the quadratic design (Zhao and Zhang, 2014):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} {X_i}^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j$$

Where Y is the predicted response (extraction yield), β_0 is the constant intercept, X_i and X_j are the independent variables, and β_i, β_{ii}, and β_{ij} are the linear coefficients, quadratic coefficients, and interaction coefficients, respectively.

The three-dimensional response surface plots were generated to illustrate the response patterns. The adequacy of the prediction model was then evaluated by Analysis of Variance (ANOVA) at $\alpha = 0.05$. Finally, three confirmation tests were run under the optimised conditions to validate the model prediction and assess repeatability.

3.5 Hydrodistillation

Fresh and dried *Eucalyptus* leaves were subjected to hydrodistillation using a 10 L stainless-steel distiller (Figure 3.6). The ratio of leaf sample to distilled water was maintained at 1:4. Specifically, 1 kg of blended leaf material was immersed in 4 L of distilled water and boiled on an induction cooker at 800 W. The vapour was condensed using a distillation chiller, and the extract was collected in a separating funnel. The extract was then dried using anhydrous sodium sulphate, transferred into glass vials protected from light, and stored at 4°C. The hydrodistillation process lasted 8 h, with extracts collected at three time points (4, 6, and 8 h). The extraction yield was calculated based on the following equation:

$$\text{Extraction yield (\% w/w)} = \frac{\text{Weight of extract}}{\text{Weight of leaf material}} \times 100$$

For dried leaves, the yield was calculated based on dry weight of plant material, while for fresh leaves, it was calculated based on fresh weight. Each extraction was performed in triplicate, and extracts collected at three time points were pooled prior to bioassays. While hydrodistillation products are typically classified as essential oils according to the International Organization for Standardization (2021), the products of SCE do not meet this definition. To maintain clarity and consistency, the term “extract” is used throughout this study to refer to products from both extraction techniques. For sample designation, fresh leaf hydrodistillation extract from *Eucalyptus* trees aged 17 to 31 months and 40 to

50 months were labelled as HfA and HfB, respectively, while the corresponding dried leaf extracts were labelled as HdA and HdB, respectively.

Figure 3.6: Setup for hydrodistillation.

3.6 Chemical Composition Analysis

The chemical composition of the extracts was analysed using gas chromatography–mass spectrometry (GC-MS) due to its high sensitivity in separating and identifying volatile compounds. Given the complexity of *Eucalyptus* leaf extracts, two types of capillary columns were employed for component separation: a non-polar column (5% diphenyl-95% dimethyl polysiloxane, 30.0 m × 0.25 mm × 0.25 µm) and a polar column (crossbond carbowax polyethylene glycol, 60.0 m × 0.25 mm × 0.25 µm). Helium gas served as the mobile phase.

Prior to injection, all extracts were prepared at 1 mg/mL in appropriate solvents and filtered through 0.45 μm nylon syringe filters. For analyses with the non-polar column, acetone was used for hydrodistillation extracts and ethanol-assisted SCE extracts, while hexane was used for pure CO_2 SCE extracts. For analyses with the polar column, both hydrodistillation and SCE extracts were dissolved in acetone. The injection volume was set at 1 μL, and the electron impact ionisation (EI) of the mass spectrometer was applied at 70 eV. The component identification was performed by comparison with the National Institute of Standards and Technology 23 Mass Spectral Library, Smart Aroma Database, and co-injection of pure standards. Only compounds with a similarity index ≥ 86% were reported. Detailed instrumental settings and component identification procedures for both columns are summarised in Table 3.1. The relative abundance of each identified compound was calculated using a normalisation method, based on peak areas in the total ion chromatogram (TIC). The retention index of components analysed using non-polar column was determined with respect to C7 to C40 alkanes and compared with those in National Institute of Standards and Technology 23 Mass Spectral Library to further confirm the identification.

Table 3.1: GC-MS settings and component identification method applied for chemical composition analysis using non-polar and polar columns.

Settings	Non-polar column		Polar column
	SCE	Hydrodistillation	
Oven temperature	60°C for 5 min, increased at 5°C/min to 280°C, held for 5 min	50°C for 5 min, increased at 5°C/min to 200°C, held for 5 min	40°C for 5 min, increased at 3°C/min to 250°C, held for 15 min
Analysis time	54 min	40 min	90 min
Injector port temperature	280°C	200°C	250°C
Ion source temperature	200°C	200°C	200°C
Interface temperature	280°C	200°C	250°C
Injection mode	Split	Split	Split
Split ratio	20	20	5
Helium gas linear velocity	36.5 cm/sec	36.3 cm/sec	25.5 cm/sec
Column flow	1.00 mL/min	1.00 mL/min	1.00 mL/min
Mass-to-charge range for mass spectrum	35–600	35–600	40–500
Component identification	NIST, STD	NIST, STD	NIST, Smart Aroma Database

NIST: National Institute of Standards and Technology 23 Mass Spectral Library

STD: Standards of α-pinene, limonene, β-caryophyllene, α-terpineol, borneol, eucalyptol, and aromadendrene injected under identical conditions to verify.

3.7 Antimicrobial Testing

In this study, the antimicrobial activity of *Eucalyptus* leaf extracts was investigated against a diverse range of microorganisms using the colourimetric broth microdilution method. This method is widely accepted for quantitatively evaluating the inhibitory and killing effects of non-polar plant extracts, while allowing direct comparisons between the tested extracts. For the antibacterial assays, ten bacterial strains were tested, comprising five Gram-positive strains: *Bacillus cereus* ATCC® 1457™, *Enterococcus hirae* ATCC® 10541™, and *Staphylococcus aureus* ATCC® 6538™, together with two methicillin-resistant clinical isolates SA-LWE23#1 and SA-LWE23#2. Two MRSA strains were included as they were originated from different patients, thereby representing distinct clinical sources. Additionally, five Gram-negative strains were examined: *Escherichia coli* ATCC® 35218™ and ESBL-producing clinical isolate EC-LWE23#1, *Klebsiella pneumoniae* ATCC® 13883™ and carbapenem-resistant clinical isolate KP-LWE23#1, as well as *Pseudomonas aeruginosa* ATCC® 15442™.

For the antifungal assays, eight fungal species were included, consisting of six yeasts (*Candida albicans* ATCC® 90028™, *Candida auris* CDC B11903, *Nakaseomyces glabratus* ATCC® MYA-2950™, *Candida parapsilosis* ATCC® 22019™, *Candida tropicalis* ATCC® 750™, and *Cryptococcus neoformans* ATCC® 13690™), along with two filamentous fungi: *Aspergillus fumigatus*

ATCC® 204305™ (non-dermatophyte) and *Trichophyton rubrum* ATCC® 28188™ (dermatophyte).

3.7.1 Antibacterial Assays

Bacterial inocula were prepared according to the guidelines of Clinical Laboratory Standards Institute (CLSI, 2012). Bacteria were cultured on Mueller Hinton agar (MHA) at 37ºC for 18 to 24 h. Several colonies were then transferred into a tube containing 3 mL of Mueller Hinton broth (MHB) to prepare a bacterial suspension, which turbidity adjusted to 0.5 McFarland standard using a densitometer, corresponding to an absorbance of 0.08 to 0.13 at 625 nm, equivalent to approximately 1-2×10^8 CFU/mL. The suspension was further diluted with MHB to reach a working concentration of 1×10^6 CFU/mL.

Based on the procedure modified from Sit *et al*. (2017), a 10 mg/mL stock solution of the extract was prepared by dissolving 10 mg of extract in 1 mL of ethanol-water mixture (2:1, v/v), followed by sterilisation through a 0.45 µm nylon syringe filter. The resultant stock solution was serially two-fold diluted with MHB in 96-well microplates to yield eight concentrations (0.02, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, and 2.50 mg/mL). Chloramphenicol, with concentrations ranging from 1 to 128 µg/mL, served as the positive control. For each well, 50 µL of bacterial inoculum was added to 50 µL of the test or control solution, resulting in a final volume of 100 µL. Sterility controls with MHB only, negative controls with extract

only, and growth controls with bacterial inoculum only were included to confirm sterility of reagents and viability of the bacteria. The microplates were incubated at 37°C for 24 h. After incubation, 20 µL of 0.4 mg/mL INT was added to each well to determine the MIC. Bacterial growth was assessed by the appearance of a pink formazan precipitate. To determine the MBC, 20 µL from wells with no visible bacterial growth was inoculated onto MHA plates and incubated for 24 h. Colony formation was recorded, and MBC values were determined accordingly. All antibacterial assays were performed in triplicate.

3.7.2 Antifungal Assays

Fungal inocula were prepared following the guidelines of the CLSI (CLSI, 2008a). Yeasts were cultured on potato dextrose agar (PDA) at 35°C. *Candida* species and *N. glabratus* were incubated for 48 h, while *C. neoformans* was incubated for 72 h. Colonies were suspended in sterile RPMI-1640 medium, and the turbidity of the suspension was adjusted to an absorbance of 0.45 to 0.55 at 530 nm using a spectrophotometer, corresponding to $1\text{-}5\times10^{6}$ CFU/mL. The suspension was further diluted in RPMI-1640 medium to reach a working concentration of $0.5\text{-}2.5\times10^{3}$ CFU/mL.

According to CLSI (2008b), the non-dermatophyte *A. fumigatus* was cultured on PDA and incubated at 35°C for 72 h. One millilitre of sterile saline containing a drop of Tween 80 was added to the fresh mould culture, and conidia

were harvested with a sterile swab. The resulting suspension was transferred to a centrifuge tube containing 4 mL of saline, vortexed, and allowed to settle for 10 min. One millilitre of the supernatant was then transferred into a cuvette for absorbance measurement, with saline serving as the blank. The absorbance was adjusted to 0.09 to 0.13 at 530 nm, corresponding to a concentration of $1\text{-}5\times10^6$ cells/mL. The suspension was further diluted with RPMI-1640 medium to obtain a working concentration of $0.4\text{-}5\times10^4$ cells/mL.

For the dermatophyte *T. rubrum,* cultures were grown on oatmeal agar at 30°C for 5 days. A suspension was prepared by adding 5 mL of sterile saline to the culture and harvesting conidia with a cotton swab. The mixture was transferred to a centrifuge tube containing 2 mL of saline, vortexed, and allowed to settle for 10 min. Ten microlitre of the supernatant was mixed with 10 µL of 0.4% trypan blue and loaded into a haemocytometer. Cells were observed under a light microscope at 400× magnification. Viable cells, which were unstained, were counted, while dead cells stained blue were excluded. Cells on the left and top borderlines were included, whereas those touching the right and bottom borderlines were excluded. The cell concentration was calculated using the following formula:

$$\text{Cell count} = \text{Average viable cells per square} \times \text{Dilution factor} \times 1000$$

The suspension was diluted to achieve a final working concentration of $1\text{-}3\times10^3$ cells/mL.

Antifungal activity of the extracts was evaluated using the modified colourimetric broth microdilution method of Sit *et al*. (2017). A 10 mg/mL stock solution was prepared by dissolving 10 mg of extract in 1 mL of ethanol-water mixture (2:1, v/v), followed by sterilisation through a 0.45 µm nylon syringe filter. In a 96-well microplate, 50 µL of RPMI-1640 medium was dispensed into each well. Then, 50 µL of extract was added to the first well of each column. Two-fold serial dilution was performed by transferring 50 µL sequentially down the column (row A-G), producing eight concentrations ranging from 0.02 to 2.50 mg/mL. For positive controls, 5-fluorocytosine with concentrations ranging from 0.06 to 8 µg/mL was used for yeast, whereas itraconazole with concentrations ranging from 0.25 to 32 µg/mL was used for *A. fumigatus* and *T. rubrum*. Fifty microlitre of fungal inoculum was added to each well, making the final volume of 100 µL per well. Sterility controls with medium only, negative controls with extract only, and growth controls with fungal inoculum only were included in each plate. The microplates were incubated under conditions recommended by CLSI (CLSI, 2008a; 2008b). After incubation, 20 µL of 0.4 mg/mL INT was pipetted to each well to determine the MIC. To determine the MFC, 20 µL from wells without visible pink precipitate were spread on PDA plates using the spread plate technique and incubated. The lowest concentration of extract that completely inhibited fungal growth on agar was recorded as the MFC. All antifungal assays were conducted in triplicate.

3.8 Mosquito Larvicidal Assays

The mosquito larvicidal activity of the extracts was evaluated following the World Health Organization guidelines (WHO, 2005) with minor modifications to ensure methodological standardisation and result comparability. Eggs of laboratory-reared *Aedes aegypti* and *Aedes albopictus* mosquitoes were purchased from the Vector Control Research Unit (VCRU) of Universiti Sains Malaysia (USM), Malaysia. Eggs were immersed in dechlorinated tap water supplemented with ground pet food pellets and maintained at room temperature to encourage hatching and development into third instar larvae.

A 40 mg/mL stock solution of each extract was prepared in methanol and diluted with distilled water to produce five concentration levels (25, 50, 100, 200, and 400 µg/mL). Each concentration was prepared in round plastic containers with a final volume of 100 mL. Twenty third instar larvae were transferred into individual containers and acclimatised for 1 h holding period prior to treatment. Larval mortality was recorded at 24 and 48 h post-treatment. Larvae were considered dead if no movement was observed when prodded with a pipette tip in the cervical region. The negative control consisted of 1% (v/v) methanol, whereas the positive control was 100 µg/mL temephos, a synthetic larvicide. The percentage mortality was calculated using the following formula:

$$\text{Mortality (\%)} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100$$

If mortality in the negative control ranged from 5% to 20%, the corrected mortality rate was calculated using Abbott's formula (Abbott, 1987):

$$\text{Corrected mortality (\%)} = \frac{Lc - Lt}{Lc} \times 100$$

where L_c is the number of survivors in the negative control and L_t is the number of survivors in the treated group.

Mortality data was analysed using Probit analysis to obtain the median lethal concentration (LC_{50}) and 95% lethal concentration (LC_{95}). All treatments were performed in quadruplicate, and morphological changes in dead larvae were examined under a stereo microscope at 30× magnification.

3.9 Brine Shrimp Lethality Assay

The toxicity of extracts was assessed via brine shrimp lethality assay, modified from McLaughlin, Rogers and Anderson (1998). This bioassay was employed due to its cost-effectiveness, simplicity, and the short incubation period required for brine shrimp. *Artemia franciscana* cysts (2 g), supplied by the Institute of Climate Adaptation and Marine Biotechnology (ICAMB) of Universiti Malaysia Terengganu (UMT), were added to an aquarium tank containing 2 L of artificial seawater and incubated at room temperature for up to 48 h to allow hatching and development into second instar nauplii (Figure 3.7). Once the nauplii began to emerge after 12 h, 1 mL of *Artemia* PKC-Nutri+® (Tiong *et al*., 2024) was added

to prevent mortality due to starvation. Prior the testing, nauplii were collected with a glass pipette, transferred to a beaker and held for 1 h holding period, during which they were fed again.

Figure 3.7: Second instar *Artemia franciscana* nauplius (magnification: 400×).

For SCE samples, a 10,000 µg/mL stock solution was produced by dissolving 0.05 g of extract in 5 mL of absolute ethanol. For hydrodistilled samples, the same concentration was achieved by dissolving 0.05 g of extract in 5 mL of 10% ethanol-artificial seawater mixture. Stock solutions were serially diluted with artificial seawater to prepare test concentrations of 1, 10, 100, 500, and 1000 µg/mL. SCE samples were sonicated for 30 min in an ultrasonic water bath to improve

solubility. After the 1 h holding period, 10 nauplii were transferred into each plastic container containing 5 mL of the test solution.

Artificial seawater containing ethanol (1% (v/v) for hydrodistilled extracts; 1%, 5%, and 10% (v/v) for SCE extracts) served as the negative control, while potassium dichromate (1, 10, 100, 500, and 1000 µg/mL) was used as the positive control. After 24 h of incubation at room temperature, live nauplii were counted, and mortality was calculated. If mortality was observed in the negative control, Abbott's correction was applied (Banti and Hadjikakou, 2021). Each treatment was performed in triplicate. Median lethal concentration (LC_{50}) and 95% lethal concentration values (LC_{95}) were determined using Probit analysis.

3.10 Statistical Analysis

All experiments were conducted in replicates. Extraction yields (n = 3), mosquito larvicidal assays (n = 4), and brine shrimp lethality assays (n = 3) were expressed as means ± standard deviations, while antimicrobial data were presented as means or ranges. Microsoft Excel 2019 was used to calculate means and standard deviations. Probit analysis was performed to estimate the lethal concentrations (LC_{50} and LC_{95}) of extracts against mosquito larvae and brine shrimp nauplii.

Statistical analyses were conducted using R version 4.5.1 and IBM Statistical Package for the Social Sciences version 27. Results with $p < 0.05$ were

considered statistically significant. Student *t*-test was performed to compare the extraction yield between pure CO_2 SCE and ethanol-assisted SCE. Different types of analysis of variance (ANOVA) were applied depending on the dataset. Specifically, the non-parametric Aligned Rank Transform (ART) ANOVA followed by Tukey's post-hoc test was employed to analyse non-normally distributed hydrodistillation yield data (Mangiafico, 2016; Kay *et al*., 2025). Three-way ANOVA was performed to evaluate the effects of extraction pressure, temperature, and time on SCE yield, while two-way and four-way ANOVA followed by Tukey's post-hoc test were applied to brine shrimp mortality and mosquito larval mortality, respectively. Non-normal distributions of mosquito larval and brine shrimp mortality data were anticipated in dose-response bioassays, as values were bounded between 0% to 100% to determine effective concentration ranges and contained a significant proportion (> 50%) of extreme values (0% or 100% mortality). However, the relatively large sample sizes used in this study (n = 640 for mosquito larvicidal assays; n = 120 for brine shrimp lethality assays) enhanced the robustness of parametric ANOVA to moderate deviations from normality, thereby supporting the validity of the statistical outcomes.

CHAPTER 4

RESULTS

4.1 *Eucalyptus* Leaves Collection and Preparation

Fresh leaves of *Eucalyptus* trees were collected from two plantations in Gua Musang, Malaysia. A temperature of 40°C was chosen for sample drying to avoid the loss or alteration of potential thermolabile active constituents while eliminating trapped water molecules. Drying and hardening of the leaf samples eased the subsequent crushing and grinding process, allowing easier breakage of the leaves. The resultant leaf powders were also sieved. This pretreatment is essential for SCE to decrease the particle size of the leaf sample so that a larger surface area is available for sufficient contact with the solvent, resulting in lower mass transfer resistance and thereby enhancing the efficiency of extraction (Yousefi *et al*., 2019).

4.2 Optimisation of Supercritical Carbon Dioxide Fluid Extraction

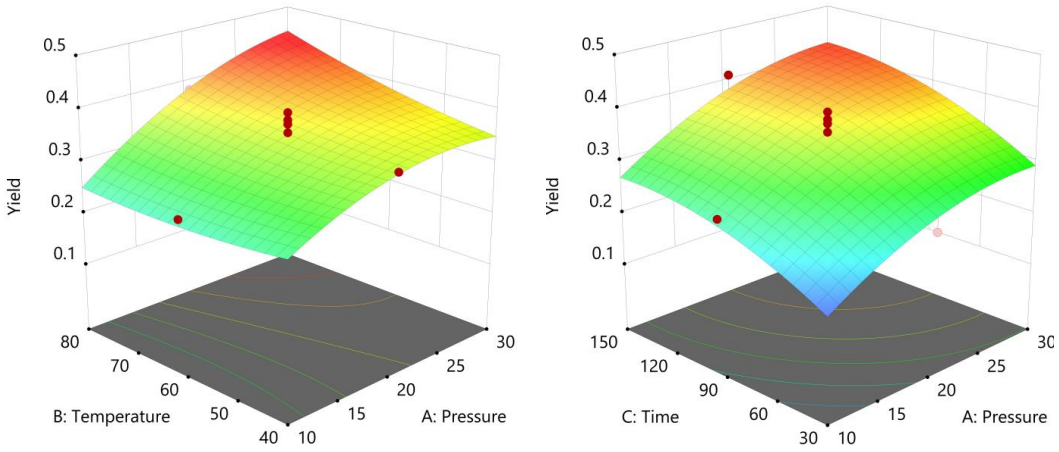
The optimisation of the SCE method was accomplished by employing response surface methodology (RSM) using Design Expert software. Table 4.1 displays the different experimental conditions used in SCE following the central composite design and their corresponding yields. The SCE parameters investigated included pressure (10 to 30 MPa), temperature (40 to 80°C), and extraction time (30 to 150 min), with extraction yield (percentage) as the response variable. The

extraction yields for younger *Eucalyptus* (A) from Run 1 to Run 20 ranged from 0.150% to 0.436% (w/w), whereas the older *Eucalyptus* (B) yielded from 0.034% to 0.250% (w/w).

Table 4.1: Experimental design and response surface methodology for SCE of *Eucalyptus* from two age groups.

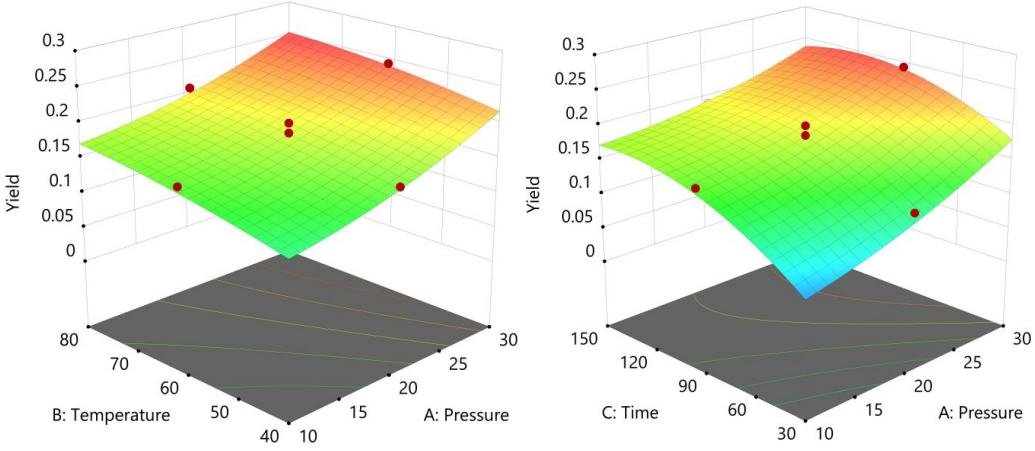
Runs	Factors			Extraction yield, % (w/w)	
	Pressure (MPa)	Temperature (°C)	Time (min)	Younger *Eucalyptus* (A)	Older *Eucalyptus* (B)
1	10	40	30	0.150	0.034
2	10	40	150	0.308	0.138
3	10	60	90	0.260	0.160
4	10	80	30	0.204	0.078
5	10	80	150	0.232	0.194
6	20	40	90	0.346	0.160
7	20	60	30	0.236	0.126
8	20	60	90	0.380	0.170
9	20	60	90	0.394	0.186
10	20	60	90	0.356	0.200
11	20	60	90	0.318	0.168
12	20	60	90	0.302	0.176
13	20	60	90	0.372	0.182
14	20	60	150	0.410	0.190
15	20	80	90	0.382	0.208
16	30	40	30	0.212	0.150
17	30	40	150	0.430	0.218
18	30	60	90	0.376	0.244
19	30	80	30	0.414	0.192
20	30	80	150	0.436	0.250

The impact of SCE parameters on extraction yield is illustrated through three-dimensional response surface plots (Figures 4.1 and 4.2), where two parameters are varied while the third is fixed at its central value. Figures 4.1(a) and 4.2(a) show the combined influence of pressure and temperature on the yield at 90 min. An increase in both parameters led to higher SCE yields, with the maximum yield observed at the upper ends of the pressure and temperature ranges. Notably, the positive impact of increasing pressure on the yield of A was more pronounced at elevated temperatures, as evidenced by the steeper yield increment at 80°C compared to 40°C when pressure rose from 10 to 30 MPa. Figures 4.1(b) and 4.2(b) illustrate the combined effect of pressure and time on the extraction yield. While the positive influence of pressure remained evident over time, SCE yield increased steadily as the extraction time extended from 30 to 90 min. However, the yield plateaued beyond 90 min and even slightly declined after 120 min for B.



(a) (b)

Figure 4.1: Response surface plots for SCE yield of younger *Eucalyptus* (A) in relation to (a) temperature and pressure at 90 min extraction (b) pressure and time at 60°C.



(a) (b)

Figure 4.2: Response surface plots for SCE yield of older *Eucalyptus* (B) in relation to (a) temperature and pressure at 90 min extraction (b) pressure and time at 60°C.

The optimised SCE parameters (temperature, pressure, extraction time) determined by the software for maximum extraction yield of A were 79.8°C, 29.7 MPa, and 116.2 min, while 79.6°C, 30.0 MPa, and 108.6 min were identified for B. These values were rounded during practical application due to instrument limitations. The extraction yield (Yield) can also be predicted using second-order polynomial equations derived from the quadratic optimisation model in terms of coded variables, where X = pressure, Y = temperature, and Z = extraction time:

For A:

$$Yield = 0.3536 + 0.0714X + 0.0222Y + 0.0600Z + 0.0287XY + 0.0067XZ - 0.0408YZ - 0.0355X^2 + 0.0105Y^2 - 0.0305Z^2$$

For B:

$$Yield = 0.1842 + 0.0450X + 0.0222Y + 0.0410Z - 0.0033XY - 0.0118XZ + 0.0002YZ + 0.0120X^2 - 0.0060Y^2 - 0.0320Z^2$$

The optimisation model generated using the software was further analysed using three-way analysis of variance (ANOVA), and the results are displayed in Table C1 for A and Table C2 for B (Appendix C). The models developed were statistically significant ($p \leq 0.0001$). Coefficients of determination close to 1 (R^2 = 0.9301 for A and 0.9734 for B) indicate a strong correlation between the model predictions and experimental data. In addition, ANOVA revealed that all three parameters (pressure, temperature, and extraction time) exerted significant independent effects on extraction yield ($p < 0.05$).

4.3 Supercritical Carbon Dioxide Fluid Extraction (SCE)

To verify the model predictions, three repetitive tests were conducted under the acquired optimal process conditions, yielding an average extract of 0.475% ± 0.029% (w/w) for A and 0.255% ± 0.024% (w/w) for B (Table 4.2). The actual yields fell within their respective predicted ranges (A: 0.388% to 0.522%; B: 0.234% to 0.285%) and were generally consistent with the predicted yields, confirming the good fit of the generated equations under actual experimental conditions and the suitability of this model for optimising the SCE process. As shown in Figure 4.3, SCE of *E. grandis* × *E. urophylla* leaves generated viscous, opaque green extracts that adhered to the wall of the vials.

The optimised SCE conditions were also tested in triplicate with the addition of 10% ethanol as a co-solvent to evaluate its influence on extraction efficiency. The results clearly demonstrated the positive effect of co-solvent addition on SCE performance. Specifically, the average extract yield improved approximately ten-fold to 4.650% ± 0.327% (w/w) for A (A10) and 2.640% ± 0.139% (w/w) for B (B10), after ethanol additions, which was significantly ($p < 0.001$) higher than that of pure CO_2 extraction. Interestingly, higher extraction yields were consistently obtained from younger *Eucalyptus* (A and A10) compared to older *Eucalyptus* (B and B10).

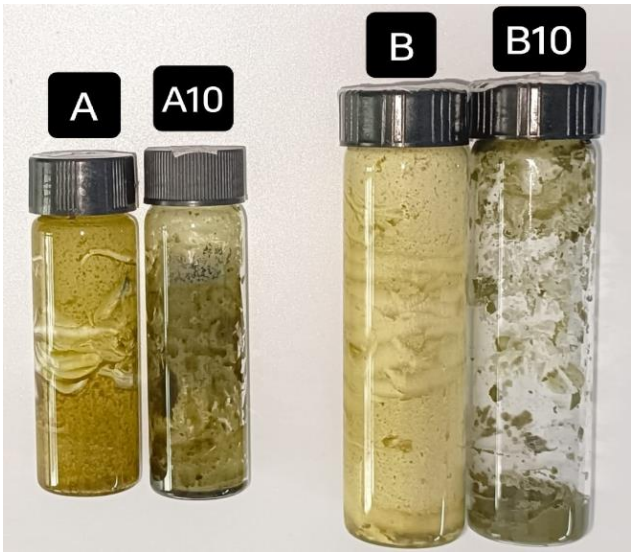


Figure 4.3: Extracts obtained using pure CO_2 SCE and 10%-ethanol-assisted extraction. A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus.*

Table 4.2: Extraction yields of pure CO_2 SCE and 10% ethanol-assisted extraction.

Extracts	Yield, % (w/w) (n = 3)
A	0.475 ± 0.029^{a}
A10	4.650 ± 0.327^{b}
B	0.255 ± 0.024^{a}
B10	2.640 ± 0.139^{b}

Different superscript represents significant ($p < 0.001$) difference of extraction yield within each age group.

A: SCE of younger *Eucalyptus*
A10: Ethanol-assisted SCE of younger *Eucalyptus*
B: SCE of older *Eucalyptus*
B10: Ethanol-assisted SCE of older *Eucalyptus*

4.4 Hydrodistillation

The majority of the extract was obtained during the first 4 h of distillation, yielding 0.104% ± 0.011%, 0.173% ± 0.026%, 0.015% ± 0.000%, and 0.038% ± 0.003% (w/w) for HfA, HdA, HfB, and HdB, respectively (Table 4.3). These values corresponded to 60.00% to 90.58% of the total content. Only slight increase in yield, ranging from 0.002% to 0.010% (w/w), were detected with each additional 2-h extension of distillation time, corresponding to 4.19% to 32.00% of the total yield. Notably, similar to the SCE results, younger *Eucalyptus* (HfA and HdA) consistently produced higher yields than older *Eucalyptus* (HfB and HdB) under all leaf conditions and time points. The highest total yield, 0.191% (w/w), was observed in the hydrodistillation of dried leaves from younger *Eucalyptus*.

Hydrodistillation of the leaves of *E. grandis* × *E. urophylla* produced clear yellow extracts (Figure 4.4). The densities were relatively similar across all samples, calculated as 0.963 ± 0.001, 0.966 ± 0.004, 0.951 ± 0.009, and 0.965 ± 0.005 g/mL for HfA, HdA, HfB, and HdB, respectively. The total extraction yields from fresh *Eucalyptus* leaves were 0.118% (w/w) for HfA and 0.025% (w/w) for HfB (fresh weight basis). In contrast, dried leaves yielded 0.191% (w/w) for HdA and 0.046% (w/w) for HdB (dry weight basis). Correspondingly, for 1 kg of leaf samples, the extraction volume obtained were 1.225, 1.977, 0.263, and 0.477 mL for HfA, HdA, HfB, and HdB, respectively.

Aligned Rank Transform (ART) ANOVA (Tables C3 and C4) (Appendix C) was conducted to analyse the effects of hydrodistillation duration and tree age on the extraction yields of dried and fresh leaf samples, respectively. The result showed significant ($p < 0.01$) main effects of both tree age and duration, as well as a significant interaction between them, on the extraction yields, regardless of whether calculated on a fresh weight or dried weight basis.

Table 4.3: Extraction yields of hydrodistillation.

***Eucalyptus* age (month)**	**Time (h)**	**Fresh leaf**		**Dried leaf**	
		Yield, % (w/w)	**Overall (%)**	**Yield, % (w/w)**	**Overall (%)**
17 to 31	4th	0.104 ± 0.011[a]	88.14	0.173 ± 0.026[a]	90.58
	6th	0.009 ± 0.003[bc]	7.63	0.008 ± 0.003[ce]	4.19
	8th	0.005 ± 0.002[cd]	4.24	0.010 ± 0.001[be]	5.24
	Total	0.118	100	0.191	100
40 to 50	4th	0.015 ± 0.000[ab]	60.00	0.038 ± 0.003[ab]	82.61
	6th	0.008 ± 0.001[bc]	32.00	0.005 ± 0.001[cd]	10.87
	8th	0.002 ± 0.001[d]	8.00	0.003 ± 0.001[d]	6.52
	Total	0.025	100	0.046	100

Superscripts (a to e) represent significant ($p < 0.05$) differences of extraction yield across times among age groups within each leaf condition.

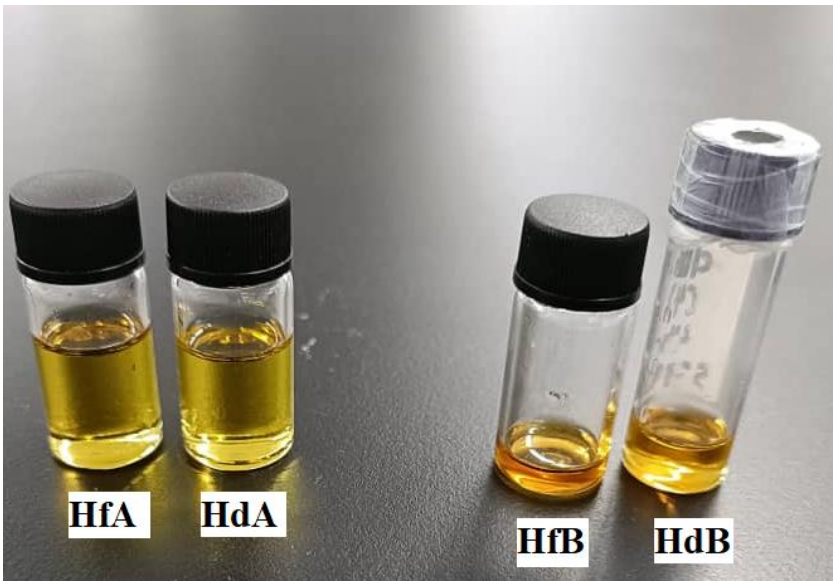


Figure 4.4: Extracts obtained using hydrodistillation. HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

4.5 Chemical Composition Analysis

The chemical compositions of the *Eucalyptus* hybrid extracts were analysed using gas chromatography–mass spectrometry (GC-MS). The total ion chromatograms of the extracts are shown in Appendix D. The SCE extracts contained only 8 to 22 identified components when analysed via GC-MS equipped with a non-polar column, covering 72.26% to 93.73% of the total peak areas (Table 4.4), but revealed 4 to 34 components with the polar column, accounting for 83.07% to 100.00% of the total peak areas (Table 4.5). Monoterpenoids remained the predominant class in A (36.80%; 29.19%) across both the non-polar and polar columns. Specifically, A consistently showed high abundances of eucalyptol (18.82%; 13.56%) and globulol (12.69%; 11.64%). α-Terpinyl acetate also

constituted 10.86% of A in the non-polar column analysis. However, α-terpinyl acetate and α-terpineol co-eluted at the same retention time and exhibited similar similarity indices when analysed using the polar column. Therefore, they were reported together as α-terpinyl acetate/α-terpineol, accounting for 11.64% of A. Notably, based on the non-polar column results where both compounds were well resolved, α-terpinyl acetate was consistently identified as the more abundant compound, suggesting it was the predominant contributor in the co-eluted peak. Although a considerable presence of monoterpenoids is also detected in B regardless of column, it was dominated by eucalyptin (16.46%), 1-hexacosanol (12.24%), and octacosanal (11.51%) in non-polar column analysis, but octadecanoic acid (22.61%), α-terpinyl acetate/α-terpineol (22.17%), and elaidic acid (17.90%) when the polar column is used. In contrast, the ethanol-assisted SCE extracts (A10 and B10) displayed different chemical profiles, demonstrating an increase in more polar and higher molecular weight sesquiterpenoids, along a decrease in monoterpenes, sesquiterpenes, and monoterpenoids. Particularly, eucalyptol and octacosanal disappeared when comparing the pure CO_2 extracts with ethanol-assisted extracts. A10 showed a substantially high percentage of globulol (non-polar column: 34.05%; polar column: 20.99%) and introduced more polar compounds such as 1-heptacosanol (5.96%), 1,2,4-nonadecanetriol, 3TFA derivative (1.16%), 5,5-dimethyl-4-(3-oxobutyl)dihydro-2(3H)-furanone (1.31%), and (1R,2R,4R)-4-(2-hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol (2.79%). Compared to B, B10 revealed a relatively higher abundance of eucalyptin (32.71%), 1-hexacosanol (12.48%), and α-terpinyl acetate (7.31%) when analysed with the

non-polar column. The high percentage of α-terpinyl acetate/α-terpineol (22.81%) in B10 was also observed in the polar column analysis, along with abundant phytol (45.57%), spathulenol (18.65%), and ethyl palmitate (12.97%).

Table 4.4: Chemical composition analysis of extracts obtained from SCE using GC-MS with a non-polar column.

No	Components	Peak area (%)			
		A	A10	B	B10
1.	1,2,3-Butanetriol	-	-	-	2.66
2.	Limonene*	1.33	-	-	-
3.	Eucalyptol*	**18.82**	-	2.96	-
4.	Fenchol	-	-	1.07	-
5.	Sabinol	0.89	-	-	-
6.	Borneol*	0.86	-	4.91	2.81
7.	*trans-p*-Mentha-1(7),8-dien-2-ol	0.86	-	-	-
8.	α-Terpineol*	3.40	-	6.81	4.10
9.	2-Hydroxycineole	-	-	1.05	-
10.	Isocarveol	1.11	-	-	-
11.	α-Terpinyl acetate	**10.86**	4.25	7.09	**7.31**
12.	α-Cubebene	1.26	-	-	-
13.	β-Gurjunene	1.30	-	-	-
14.	Aromadendrene*	10.83	5.84	-	-
15.	Humulen-(v1)	2.55	-	-	-
16.	1-Dodecanol	-	-	2.97	-
17.	(1R,2R,4R)-4-(2-Hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol	-	-	-	4.54
18.	2,4-Di-tert-butylphenol	-	-	1.92	-
19.	*trans*-Calamenene	1.15	-	-	-
20.	Epiglobulol	2.08	5.25	-	-
21.	Ledol	1.24	-	-	-
22.	Spathulenol	1.87	3.38	2.34	4.28
23.	Globulol	**12.69**	**34.05**	1.34	-
24.	Viridiflorol	2.97	**7.70**	-	-
25.	Rosifoliol	2.21	-	-	-
26.	1,10-Di-epi-cubenol	2.40	5.83	-	-
27.	Neophytadiene	-	-	-	2.67
28.	Hexahydrofarnesyl acetone	-	-	-	2.54
29.	*n*-Hexadecanoic acid	-	-	1.62	-
30.	Phytol	1.57	-	1.64	2.71
31.	1-iodo-Docosane	-	-	2.08	-

Table 4.4 (cont.): Chemical composition analysis of extracts obtained from SCE using GC-MS with a non-polar column.

No	Components	Peak area (%)			
		A	**A10**	**B**	**B10**
32.	Squalene	-	-	3.75	-
33.	1-Hexacosanal	-	-	4.78	-
34.	1-Heptacosanol	-	**5.96**	-	-
35.	1-Hexacosanol	-	-	**12.24**	**12.48**
36.	Octacosanal	2.35	-	**11.51**	-
37.	Eucalyptin	-	-	**16.46**	**32.71**
38.	Octacosanol	-	-	7.19	-
Identified components (%)		84.60	72.26	93.73	78.81
Monoterpenes (%)		1.33	-	-	-
Sesquiterpenes (%)		15.94	5.84	-	-
Monoterpenoids (%)		36.80	4.25	23.89	18.76
Sesquiterpenoids (%)		26.61	56.21	3.68	4.28
Flavonoids (%)		-	-	16.46	32.71
Fatty alcohol (%)		-	5.96	22.40	12.48
Fatty aldehyde (%)		2.35	-	16.29	-
Others (%)		1.57	-	11.01	10.58

'*': components that are confirmed using pure standard. Major components are indicated in bold.

A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus.*

Table 4.5: Chemical composition analysis of extracts obtained from SCE using GC-MS with a polar column.

No.	Components	Peak area (%)			
		A	A10	B	B10
1.	α-Pinene	0.29	-	-	-
2.	Limonene	1.12	-	-	-
3.	Eucalyptol	**13.56**	-	2.97	-
4.	Copaene	0.70	-	-	-
5.	Fenchol	0.60	-	-	-
6.	β-Gurjunene	1.01	-	-	-
7.	β-Humulene	0.77	-	-	-
8.	Aromadendrene	8.84	6.53	-	-
9.	Alloaromadendrene	2.32	1.61	-	-
10.	Pinocarveol	0.73	-	-	-
11.	α-Terpinyl acetate / α-Terpineol	**11.64**	-	**22.17**	**22.81**
12.	Borneol	0.81	-	7.46	-
13.	epi-Bicyclosesquiphellandrene	0.92	1.50	-	-
14.	β-Selinene	0.35	-	-	-
15.	Bicyclogermacren	0.38	-	-	-
16.	*trans-p*-Mentha-1(7),8-dien-2-ol	0.69	-	-	-
17.	*trans*-Calamenene	1.31	1.15	-	-
18.	2-Hydroxycineole	-	-	2.26	-
19.	Isocarveol	0.73	-	-	-
20.	Palustrol	0.37	-	-	-
21.	(1aR,3aS,7S,7aS,7bR)-1,1,3a,7-Tetramethyldecahydro-1H-cyclopropa[a]naphthalen-7-ol	0.51	-	-	-
22.	Epiglobulol	2.11	3.55	-	-
23.	Gleenol	1.46	-	-	-
24.	Cubeban-11-ol	1.11	1.85	-	-
25.	1,10-Di-epi-cubenol	2.39	-	-	-
26.	Globulol	**11.64**	**20.99**	-	-
27.	Viridiflorol	2.47	3.72	-	-
28.	Rosifoliol	1.11	-	-	-
29.	Hexahydrofarnesyl acetone	0.84	**6.89**	1.62	-
30.	Spathulenol	1.99	2.41	4.46	**18.65**
31.	Muurola-4,10(14)-dien-1β-ol	1.00	-	-	-
32.	1,2,4-Nonadecanetriol, 3TFA derivative	-	1.16	-	-
33.	Ethyl palmitate	-	2.34	-	12.97
34.	Sobrerol 8-acetate	0.43	2.03	-	-
35.	3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate	1.00	-	-	-
36.	Phytol	5.86	-	11.64	**45.57**
37.	5,5-Dimethyl-4-(3-oxobutyl)dihydro-2(3H)-furanone	-	1.31	-	-
38.	Tetrapentacontane	-	-	1.86	-

Table 4.5 (cont.): Chemical composition analysis of extracts obtained from SCE using GC-MS with a polar column.

No.	Components	Peak area (%)			
		A	**A10**	**B**	**B10**
39.	(1R,2R,4R)-4-(2-Hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol	-	2.79	-	-
40.	Squalene	-	-	5.05	-
41.	Octadecanoic acid	9.92	**23.24**	**22.61**	-
42.	Elaidic acid	-	-	**17.90**	-
Identified components (%)		90.98	83.07	100.00	100.00
Monoterpenes (%)		1.41	-	-	-
Sesquiterpenes (%)		15.29	9.64	-	-
Monoterpenoids (%)		29.19	4.82	34.86	22.81
Sesquiterpenoids (%)		27.47	33.67	4.46	18.65
Diterpenoids (%)		6.86	-	11.64	45.57
Fatty acids (%)		9.92	23.24	40.51	-
Others (%)		0.84	11.70	8.53	12.97

Major components are indicated in bold.
A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*.

For the 8-h hydrodistillation extracts, 26 to 28 peaks separated using the non-polar column were successfully identified, accounting for 95.63% to 99.67% of the total peak areas (Table 4.6). When analysed with the polar column, 38 to 42 components were identified, representing 95.69% to 98.52% of the total peak areas (Table 4.7). All hydrodistillation extracts (HfA, HdA, HfB, and HdB) were characterised by high monoterpenoid content (47.97% to 65.37%). Eucalyptol and α-terpinyl acetate were consistently the major constituents in all four extracts. Using the non-polar column, eucalyptol ranged from 13.05% to 26.67%, while α-terpinyl acetate ranged from 18.27% to 26.13%. Similarly, polar column analysis showed high abundances of 10.10% to 26.14% for eucalyptol and 24.41% to 27.99% for α-terpinyl acetate. In addition to these major components, α-pinene also appeared as a main component in HfA (non-polar: 13.92%; polar: 9.63%) and HdA (non-polar: 15.68%; polar: 7.68%). However, this trend was not observed in HfB and HdB. These two extracts were made up of high percentages of α-terpineol (9.22%; 9.34%) in non-polar column analysis and borneol (6.67%; 5.61%) in polar column analysis.

Table 4.6: Chemical composition analysis of extracts obtained from 8-h hydrodistillation using GC-MS with a non-polar column.

No	Components	Peak area (%)			
		HfA	**HdA**	**HfB**	**HdB**
1.	*α*-Pinene*	**13.92**	**15.68**	1.36	7.84
2.	Fenchene	-	0.51	-	0.48
3.	Isoamyl isobutyrate	0.35	-	-	-
4.	*o*-Cymene	0.75	0.79	0.74	1.40
5.	Limonene*	5.92	8.24	3.06	5.53
6.	Eucalyptol*	**22.87**	**26.67**	**13.05**	**21.88**
7.	Isoterpinolene	-	0.79	-	0.48
8.	Fenchol	1.57	1.56	2.54	3.03
9.	*α*-Campholenal	0.64	0.60	0.63	0.71
10.	Sabinol	0.97	0.80	1.43	1.59
11.	Pinocarvone	0.42	0.59	-	0.57
12.	Borneol*	3.58	3.07	6.62	7.46
13.	Terpinen-4-ol	0.63	-	-	-
14.	*α*-Terpineol*	7.67	6.31	**9.22**	**9.34**
15.	Isocarveol	0.39	-	-	-
16.	Bornyl acetate	0.50	-	-	-
17.	*α*-Terpinyl acetate	**26.13**	**19.73**	**24.63**	**18.27**
18.	*α*-Cubebene	0.36	-	-	-
19.	*β*-Caryophyllene*	1.32	1.34	4.98	3.00
20.	Aromadendrene*	1.95	1.79	-	-
21.	Humulene	-	-	0.81	-
22.	Humulen-(v1)	0.74	0.72	1.23	0.89
23.	Viridiflorene	-	0.38	1.28	0.88
24.	*δ*-Cadinene	-	-	1.12	0.68
25.	*trans*-Calamenene	0.53	0.55	1.52	1.13
26.	Epiglobulol	0.41	0.53	-	-
27.	Ledol	-	0.37	0.83	-
28.	Spathulenol	0.78	0.88	4.02	2.56
29.	Caryophyllene oxide	-	-	1.64	0.93
30.	Globulol	3.50	3.69	5.07	2.62
31.	Viridiflorol	0.79	0.77	2.49	1.22
32.	Cubeban-11-ol	-	0.47	-	0.71
33.	Rosifoliol	0.58	0.48	1.49	-
34.	*β*-Eudesmol	-	0.82	1.61	0.74
35.	1,10-Di-epi-cubenol	0.82	0.87	1.32	0.67
36.	*τ*-Muurolol	-	-	0.72	-
37.	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl) propan-2-ol	0.74	0.67	1.98	1.02
38.	Butyl isobutyl phthalate	-	-	1.02	-
Identified components (%)		98.83	99.67	96.41	95.63

Table 4.6 (cont.): Chemical composition analysis of extracts obtained from 8-h hydrodistillation using GC-MS with a non-polar column.

Components	Peak area (%)			
	HfA	**HdA**	**HfB**	**HdB**
Monoterpenes (%)	19.84	25.22	4.42	14.33
Sesquiterpenes (%)	4.37	4.23	9.42	5.45
Monoterpenoids (%)	65.37	59.33	58.12	62.85
Sesquiterpenoids (%)	8.15	10.10	22.69	11.60
Others (%)	1.10	0.79	1.76	1.40

'*': components that are confirmed using pure standard. Major components are indicated in bold.
HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

Table 4.7: Chemical composition analysis of extracts obtained from 8-h hydrodistillation using GC-MS with a polar column.

No	Components	Peak area (%)			
		HfA	**HdA**	**HfB**	**HdB**
1.	*α*-Pinene	**9.63**	**7.68**	1.04	4.48
2.	Camphene	0.35	0.40	-	0.38
3.	3-Methylbutyl ester, butanoic acid	0.30	0.27	-	-
4.	Limonene	5.23	6.31	2.62	2.63
5.	Eucalyptol	**16.85**	**26.14**	**10.79**	**10.10**
6.	*p*-Cymene	0.74	0.84	0.74	0.73
7.	*δ*-Terpinene	-	0.53	-	-
8.	Isopentyl isovalerate	-	0.29	-	-
9.	*α*-Campholenal	0.34	0.36	-	-
10.	*α*-Gurjunene	-	-	-	0.42
11.	Pinocarvone	0.47	0.71	0.64	0.58
12.	Bornyl acetate	0.41	0.32	0.40	0.45
13.	Fenchol	1.58	2.28	2.67	2.18
14.	*β*-Caryophyllene	1.81	1.41	3.80	4.45
15.	Terpinen-4-ol	0.96	0.86	0.72	0.62
16.	Aromadendrene	2.23	1.47	0.48	0.54
17.	Alloaromadendrene	0.98	0.69	1.05	1.32
18.	Pinocarveol	1.02	1.65	1.60	1.32
19.	*α*-Humulene	-	-	0.62	0.62
20.	*α*-Terpinyl acetate	**27.99**	**24.41**	**26.79**	**26.75**
21.	Borneol	3.54	5.15	**6.67**	**5.61**
22.	*α*-Muurolene	-	-	0.40	-
23.	*β*-Cadinene	0.60	0.42	1.25	1.19
24.	Citronellol	0.35	-	-	-
25.	6,6-Dimethylbicyclo[3.1.1]hept-2-ene-2-methanol	-	0.25	-	-
26.	*trans*-*p*-Mentha-1(7),8-dien-2-ol	0.39	0.56	0.50	0.36
27.	Geranyl isobutyrate	0.36	-	0.38	-
28.	*trans*-Calamenene	1.32	-	1.81	1.86
29.	Isocarveol	0.33	0.47	0.42	-
30.	Palustrol	-	0.23	0.48	0.45
31.	Caryophyllene oxide	0.65	-	1.16	1.37
32.	(1aR,3aS,7S,7aS,7bR)-1,1,3a,7-Tetramethyldecahydro-1H-cyclopropa[a]naphthalen-7-ol	-	-	0.41	-
33.	Epiglobulol	0.79	0.70	0.41	-
34.	Gleenol	1.03	0.69	1.15	0.99
35.	Cubeban-11-ol	0.91	0.73	1.87	1.55
36.	epi-Cubenol	-	-	0.50	0.48
37.	1,10-Di-epi-cubenol	1.54	1.15	1.41	1.30
38.	Globulol	5.01	3.95	4.96	3.97

Table 4.7 (cont.): Chemical composition analysis of extracts obtained from 8-h hydrodistillation using GC-MS with a polar column.

No	Components	Peak area (%)			
		HfA	**HdA**	**HfB**	**HdB**
39.	Viridiflorol	1.36	0.94	2.94	2.58
40.	Rosifoliol	0.69	0.56	1.15	0.94
41.	Hexahydrofarnesyl acetone	-	-	-	0.77
42.	(+)-Spathulenol	1.66	1.34	4.35	4.74
43.	Muurola-4,10(14)-dien-1*β*-ol	0.49	0.36	-	-
44.	γ-Eudesmol	0.50	0.30	-	0.67
45.	Valerianol	0.34	-	-	0.44
46.	*τ*-Muurolol	0.43	0.29	0.65	0.64
47.	*δ*-Cadinol	0.36	0.23	0.56	0.48
48.	m-Cymen-5-ol	-	-	0.37	-
49.	*α*-Eudesmol	0.36	-	-	-
50.	(-)-Spathulenol	-	-	0.87	0.81
51.	Cadalene	-	-	0.42	0.44
52.	*β*-Eudesmol	1.17	0.60	1.56	1.37
53.	3,7-Dimethyl-6-octenoic acid	0.50	-	-	-
54.	Isophytol	-	-	0.38	-
55.	*trans*-Farnesol	0.73	-	-	-
56.	Phytol	2.22	0.98	3.94	3.81
57.	Dibutyl phthalate	-	-	1.98	1.30
Identified components (%)		98.52	96.52	96.91	95.69
Monoterpenes (%)		15.95	15.76	4.40	8.22
Sesquiterpenes (%)		5.62	3.99	8.02	8.98
Monoterpenoids (%)		54.59	63.16	51.95	47.97
Sesquiterpenoids (%)		19.34	12.07	26.24	24.64
Others (%)		3.02	1.54	6.30	5.88

Major components are indicated in bold.
HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

Some components were unique to a specific tree age group, extraction method, or leaf condition, although most of them were present in low abundance. For GC-MS analysis using the non-polar column (Tables 4.4 and 4.6), leaf extracts obtained from younger *Eucalyptus* uniquely contained aromadendrene (A: 10.83%; A10: 5.84%), epiglobulol (A: 2.08%; A10: 5.25%), viridiflorol (A: 2.97%; A10: 7.70%), and 1,10-di-epi-cubenol (A: 2.40%; A10: 5.83%) when extracted using pure CO_2 and ethanol-assisted SCE, but only aromadendrene (HfA: 1.95%; HdA: 1.79%) and epiglobulol (HfA: 0.41%; HdA: 0.53%) remained unique when extracted using hydrodistillation. Conversely, leaf extracts obtained from older *Eucalyptus* exclusively contained 1-hexacosanol (B: 12.24%; B10: 12.48%) and eucalyptin (B: 16.46%; B10: 32.71%) when extracted using SCE; as well as δ-cadinene (HfB: 1.12%; HdB: 0.68%) and caryophyllene oxide (HfB: 1.64%; HdB: 0.93%) using hydrodistillation. For GC-MS analysis using the polar column (Tables 4.5 and 4.7), 12 components were found exclusively in younger tree extracts regardless of extraction method. They were aromadendrene (A: 8.84%; A10: 6.53%), alloaromadendrene (A: 2.32%; A10: 1.61%), epi-bicyclosesquiphellandrene (A: 0.92%; A10: 1.50%), *trans*-calamenene (A: 1.31%; A10: 1.15%), epiglobulol (A: 2.11%; A10: 3.55%), cubeban-11-ol (A: 1.11%; A10: 1.85%), globulol (A: 11.64%; A10: 20.99%), viridiflorol (A: 2.47%; A10: 3.72%), sobrerol 8-acetate (A: 0.43%; A10: 2.03%), 3-methylbutyl ester, butanoic acid, (HfA: 0.30%; HdA: 0.27%), α-campholenal (HfA: 0.34%; HdA: 0.36%), and muurola-4,10(14)-dien-1β-ol (HfA: 0.49%; HdA: 0.36%). Older tree extracts also uniquely contained five compounds when hydrodistillation was used: α-humulene

(HfB: 0.62%; HdB: 0.62%), epi-cubenol (HfB: 0.50%; HdB: 0.48%), (-)-spathulenol (HfB: 0.87%; HdB: 0.81%), cadalene (HfB: 0.42%; HdB: 0.44%), and dibutyl phthalate (HfB: 1.98%; HdB: 1.30%).

Furthermore, hydrodistillation extracts (HfA, HdA, HfB, HdB) contained five constituents that absent in SCE extracts when analysed using the non-polar column (Table 4.6): *α*-pinene (1.36% to 15.68%), *o*-cymene (0.74% to 1.40%), *α*-campholenal (0.60% to 0.71%), *β*-caryophyllene (1.32% to 4.98%), and 2-(4a,8-dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol (0.67% to 1.98%), and nine unique constituents when analysed using the polar column (Table 4.7): *p*-cymene (0.73% to 0.84%), pinocarvone (0.47% to 0.71%), bornyl acetate (0.32% to 0.45%), *β*-caryophellene (1.41% to 4.45%), terpinen-4-ol (0.62% to 0.96%), *β*-cadinene (0.42% to 1.25%), *τ*-muurolol (0.29% to 0.65%), *δ*-cadinol (0.23% to 0.56%), and *β*-eudesmol (0.60% to 1.56%). Similarly, disparities were also apparent when comparing the hydrodistilled extracts obtained from leaves of different conditions. For example, fenchene (HdA: 0.51%; HdB: 0.48%), isoterpinolene (HdA: 0.79%; HdB: 0.48%), and cubeban-11-ol (HdA: 0.47%; HdB: 0.71%) were unique in hydrodistilled extracts obtained from dried leaves when analysed using the non-polar column (Table 4.6), whereas fresh leaf samples analysed using the polar column (Table 4.7) uniquely included geranyl isobutyrate (HfA: 0.36%; HfB: 0.38%).

GC-MS analysis employing a non-polar column also identified 24 to 29 peaks in the hydrodistilled samples collected at three different time points, accounting for 88.85% to 100.00% of the total peak areas (Table 4.8). Certain components demonstrated time-specific patterns. For the fresh and dried leaf extracts derived from both age groups, monoterpenes and monoterpenoids such as *α*-pinene, *o*-cymene, limonene, *α*-campholenal, and pinocarvone were collected only in the early stages of hydrodistillation (4 h), while sesquiterpenes and sesquiterpenoids, such as humulene and *τ*-muurolol, emerged only at later stages (6 to 8 h). This trend aligned with the shift in component dominance in the extracts. At 4 h, monoterpenoids such as eucalyptol (28.79%; 29.70%) dominated HfA and HdA, followed by *α*-terpinyl acetate (14.13%; 17.80%). However, *α*-terpinyl acetate (28.88%; 28.80%) became predominant by 6 h, along with increased abundances of sesquiterpenes and sesquiterpenoids like globulol (13.44%; 13.18%) and aromadendrene (6.19%; 5.32%). At 8 h, *α*-terpinyl acetate (13.19%; 23.36%) and globulol (16.42%; 13.79%) continued to appear prominently in the extracts. Similarly, eucalyptol (25.43%) was the most abundant constituent in the first 4 h of distillation for HdB, shifting to *α*-terpinyl acetate at 6 h (26.43%) and 8 h (23.59%). For HfB, *α*-terpinyl acetate consistently remained the prominent constituent throughout all durations, with 24.75%, 23.88%, and 23.45% recorded at the 4^{th}, 6^{th}, and 8^{th} hours, respectively. Notably, the prominent emergence of sesquiterpenes and their oxygenated derivatives after 4 h was also observed in samples from older *Eucalyptus*, particularly globulol and *β*-caryophyllene appeared as 8.91%, 5.69%

and 8.54%, 6.25% at 6 h, as well as 11.01%, 7.62% and 4.57%, 4.90% at 8 h for HfB and HdB, respectively.

Table 4.8: Chemical composition analysis of hydrodistillation extracts collected at three time points using GC-MS with a non-polar column.

No	Components	Peak area (%)											
		HfA (h)			HdA (h)			HfB (h)			HdB (h)		
		4	6	8	4	6	8	4	6	8	4	6	8
1.	*α*-Pinene*	7.56	-	-	**18.70**	-	-	1.57	-	-	**11.37**	-	-
2.	Fenchene	-	-	-	0.56	-	-	-	-	-	0.72	-	-
3.	Isoamyl isobutyrate	-	-	-		-	-	-	-	-	0.49	-	-
4.	*o*-Cymene	1.09	-	-	0.89	-	-	1.13	-	-	1.63	-	-
5.	Limonene*	7.23	-	-	8.70	-	-	4.26	-	-	6.87	-	-
6.	Eucalyptol*	**28.79**	-	-	**29.70**	-	-	**19.27**	-	-	**25.43**	0.68	-
7.	Isoterpinolene		-	-	0.86	-	-		-	-	0.62	-	-
8.	Isopentyl isovalerate	-	-	-	0.34	-	-	0.54	-	-	-	-	-
9.	Fenchol	2.06	0.82	-	1.65	-	0.80	3.89	-	0.56	3.06	2.44	1.64
10.	*α*-Campholenal	0.49	-	-	0.62	-	-	1.05	-	-	0.73	-	-
11.	Sabinol	1.67	0.61	-	0.75	-	-	2.13	-	-	1.64	1.28	0.91
12.	Pinocarvone	1.07	-	-	0.59	-	-	1.08	-	-	0.96	-	-
13.	Borneol*	4.23	3.22	1.32	2.92	2.30	3.25	9.28	2.07	2.26	7.33	**7.26**	6.05
14.	Terpinen-4-ol	0.56	-	-	0.31	-	-	0.56	-	-	-	-	-
15.	*trans-p*-Mentha-1(7),8-dien-2-ol	0.71	-	-	-	-	-	0.48	-	-	-	-	-
16.	*α*-Terpineol*	**8.33**	**8.79**	3.23	5.76	**6.86**	**9.27**	**12.50**	3.66	3.67	8.82	**10.80**	**9.06**
17.	*cis*-Carveol	0.42	-	-	-	-	-	0.52	-	-	-	-	-
18.	Isocarveol	0.93	0.61	-	-	-	-	0.70	-	-	-	0.60	-
19.	Bornyl acetate	-	-	-	0.33	-	-	-	-	-	-	-	-
20.	*α*-Terpinyl acetate	**14.13**	**28.88**	**13.19**	**17.80**	**28.80**	**23.36**	**24.75**	**23.88**	**23.45**	**16.04**	**26.43**	**23.59**
21.	*α*-Cubebene	-	0.74	1.35	-	-	0.70	-	-	-	-	-	-
22.	*α*-Gurjunene	-	-	0.70	-	-	-	-	0.58	-	-	0.63	-

Table 4.8 (cont.): Chemical composition analysis of hydrodistillation extracts collected at three time points using GC-MS with a non-polar column.

No	Components	Peak area (%)											
		HfA (h)			HdA (h)			HfB (h)			HdB (h)		
		4	6	8	4	6	8	4	6	8	4	6	8
23.	*β*-Caryophyllene*	1.70	2.91	4.49	1.15	3.32	3.16	3.64	**8.54**	4.57	2.06	6.25	4.90
24.	*β*-Gurjunene	0.43	0.67	1.42	-	0.49	0.62	-	-	-	-	-	-
25.	Aromadendrene*	3.39	6.19	**13.55**	1.38	5.32	6.10	-	1.29	0.57	-	0.99	0.83
26.	Humulene	-	-	0.62	-	0.52	-	-	1.55	1.00	-	0.67	0.62
27.	Humulen-(v1)	1.15	2.40	4.49	0.53	2.28	2.48	0.78	2.19	1.44	0.62	2.02	1.74
28.	*β*-Selinene	-	0.51	1.10	-	0.59	0.78	-	-	-	-	-	-
29.	Viridiflorene	0.56	0.98	2.24	0.32	1.72	1.56	0.87	2.64	1.61	0.65	1.99	2.11
30.	*α*-Muurolene	-	-	-	-	0.44	-	-	0.94	-	-	-	-
31.	*γ*-Cadinene	-	-	0.57	-	-	-	-	-	-	-	-	-
32.	*δ*-Cadinene	-	0.86	1.71	-	1.23	1.58	0.63	2.00	1.45	0.40	1.45	1.41
33.	*trans*-Calamenene	-	2.00	3.09	0.30	2.77	2.97	0.67	2.73	1.88	0.64	2.23	2.38
34.	Epiglobulol	0.79	1.96	2.64	0.35	2.02	2.14	-	-	0.39	-	-	-
35.	Ledol	0.58	1.26	1.43	-	1.33	1.33	-	1.59	1.74	-	-	1.29
36.	Spathulenol	1.29	3.11	3.40	0.64	3.09	2.85	2.50	6.66	**7.29**	1.98	5.17	6.38
37.	Caryophyllene oxide	-	1.38	1.72	-	2.09	2.11	-	3.13	2.76	0.45	2.03	2.33
38.	Globulol	5.79	**13.44**	**16.42**	2.58	**13.18**	**13.79**	2.39	**8.91**	**11.01**	1.70	5.69	**7.62**
39.	Viridiflorol	1.35	2.93	3.94	0.54	2.69	2.48	1.32	5.09	5.95	0.82	2.25	3.55
40.	Cubeban-11-ol	0.57	1.59	2.07	0.31	2.32	2.52	0.76	2.82	3.49	0.48	2.04	2.53
41.	2-Methyl-,3,7-dimethyl-2,6-octadienyl ester, (E)-butanoic acid	-	-	-	-	0.54	-	-	-	-	-	-	-
42.	Rosifoliol	0.81	2.28	2.40	-	1.78	1.76	-	2.49	3.24	-	1.32	2.10
43.	*β*-Eudesmol	-	-	-	-	3.07	3.06	-	2.66	3.23	-	-	2.34

Table 4.8 (cont.): Chemical composition analysis of hydrodistillation extracts collected at three time points using GC-MS with a non-polar column.

No	Components	Peak area (%)											
		HfA (h)			HdA (h)			HfB (h)			HdB (h)		
		4	6	8	4	6	8	4	6	8	4	6	8
44.	1,10-Di-epi-cubenol	1.09	3.25	3.90	0.61	3.75	3.56	0.63	2.22	2.46	-	1.57	1.89
45.	γ-Eudesmol	-	-	-	-	1.92	1.91	-	-	-	-	-	-
46.	*τ*-Muurolol	-	1.24	0.72	-	0.79	0.99	-	1.10	1.34	-	0.81	1.08
47.	*δ*-Cadinol	-	0.53	-	-	-	0.53	-	-	0.68	-	-	-
48.	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl) propan-2-ol	-	2.42	-	-	3.60	3.17	0.79	3.60	4.73	0.69	2.25	3.11
49.	Cadalene	-	-	0.83	-	0.63	0.66	-	-	-	-	-	-
50.	Hexahydrofarnesyl acetone	-	-	-	-	0.56	0.51	-	-	-	-	-	0.73
51.	Butyl isobutyl phthalate	-	-	-	-	-	-	-	2.35	4.21	-	-	0.71
Identified components (%)		98.77	95.58	92.54	99.19	100.00	100.00	98.69	94.69	94.98	96.20	88.85	90.90
Monoterpenes (%)		14.79	-	-	28.82	-	-	5.83	-	-	19.58	-	-
Sesquiterpenes (%)		7.23	15.26	33.07	3.38	16.54	17.64	5.92	19.73	10.64	3.73	14.00	11.61
Monoterpenoids (%)		63.39	42.93	17.74	60.43	37.96	36.68	76.21	29.61	29.94	64.01	49.49	41.25
Sesquiterpenoids (%)		12.27	37.39	41.73	5.33	44.40	45.17	9.06	43.00	50.19	6.76	25.36	36.60
Others (%)		1.09	-	-	1.23	1.10	0.51	1.67	2.35	4.21	2.12	-	1.44

'*': components that are confirmed using pure standard. Major components are indicated in bold.

HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

4.6 Antibacterial Testing

The antibacterial assays evaluated the efficacy of eight *Eucalyptus* leaf extracts against selected pathogenic bacteria, including Gram-positive bacteria (three ATCC strains and two clinical isolates) and Gram-negative bacteria (three ATCC strains and two clinical isolates). Tables 4.9 and 4.10 summarise the MICs and MBCs of the extracts against these bacteria. Chloramphenicol was used as the positive control, and its activity against the 10 tested bacteria, with MIC values ranging from 1.00 to 128.00 μg/mL, confirmed the validity of assay.

SCE extracts demonstrated MIC values ranging from 0.02 to 1.25 mg/mL and MBC values between 0.02 to 2.50 mg/mL for Gram-positive bacteria, whereas their MIC and MBC values for Gram-negative bacteria varied from 0.31 to 2.50 mg/mL and 0.63 to 2.50 mg/mL, respectively. Among the four tested clinical isolates, two isolates of methicillin-resistant *S. aureus* were inhibited by all four SCE extracts, particularly, MICs of 0.04 mg/mL were observed for the SA-LWE23#2 strain when treated with B and B10, showing that SCE extracts worked well against drug-resistant bacteria. Notably, B10 exhibited the broadest antibacterial spectrum among the extracts, inhibiting all tested strains with MIC values ranging from 0.04 to 2.50 mg/mL. It also exhibited bactericidal activity against all strains except *Escherichia coli* ATCC® 35218™, with MBC values ranging from 0.08 to 2.50 mg/mL.

Hydrodistillation extracts, on the other hand, displayed relatively higher MIC and MBC values. They required concentrations ranging between 0.16 to 2.50 mg/mL to inhibit the growth of the tested Gram-positive bacteria and concentrations between 0.31 to 2.50 mg/mL to achieve the bactericidal effects. Particularly, HdB displayed slightly stronger antibacterial activity, inhibiting the growth of Gram-positive ATCC strains at lower concentrations compared to the other hydrodistillation extracts. Of the five Gram-negative bacterial strains tested, only *Klebsiella pneumoniae* ATCC® 13883™ showed susceptibility to all four hydrodistillation extracts, with MIC values ranging from 1.25 to 2.50 mg/mL, and with bactericidal effects observed only for HfB at 2.50 mg/mL.

Table 4.9: Minimum inhibitory concentrations and minimum bactericidal concentrations of the eight *Eucalyptus* leaf extracts towards Gram-positive bacteria.

Bacterial species	MIC (mg/mL)									MBC (mg/mL)							
	A	**A10**	**B**	**B10**	**HfA**	**HdA**	**HfB**	**HdB**	**CMP (µg/mL)**	**A**	**A10**	**B**	**B10**	**HfA**	**HdA**	**HfB**	**HdB**
Bacillus cereus ATCC® 14579™	0.08 – 0.16	0.02	0.04	0.04 – 0.08	0.63	0.31	0.31	0.16 – 0.31	2.00–4.00	0.08	0.02	0.04	0.08	0.63	0.31	0.31	0.31
Enterococcus hirae ATCC® 10541™	0.16	1.25	0.31	0.16	1.25 – 2.50	1.25	1.25 – 2.50	0.63	32.00–64.00	0.16	1.25	1.25	0.31	NA	NA	2.50	0.63
Staphylococcus aureus ATCC® 6538™	0.08	0.31 – 0.63	0.02	0.63	0.31 – 0.63	0.31 – 0.63	0.31 – 0.63	0.31	1.00–2.00	0.31	0.63	0.31	1.25	1.25	1.25	1.25	0.63
Staphylococcus aureus SA-LWE23#1	0.08 – 0.16	1.25	0.04 – 0.08	0.08	NA	2.50	2.50	2.50	16.00	0.16	2.50	0.16	0.08	-	NA	NA	2.50
Staphylococcus aureus SA-LWE23#2	0.08 – 0.16	1.25	0.04	0.04	NA	2.50	2.50	2.50	32.00–64.00	1.25	NA	0.63	0.16	-	NA	NA	NA

MIC and MBC values are expressed as mean or range of three consistent replicates. NA: no activity; "-": MBC testing is not conducted due to no inhibition is observed for MIC; CMP: Chloramphenicol

A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

Table 4.10: Minimum inhibitory concentrations and minimum bactericidal concentrations of the eight *Eucalyptus* leaf extracts towards Gram-negative bacteria.

Bacterial species	MIC (mg/mL)									MBC (mg/mL)							
	A	**A10**	**B**	**B10**	**HfA**	**HdA**	**HfB**	**HdB**	**CMP (µg/mL)**	**A**	**A10**	**B**	**B10**	**HfA**	**HdA**	**HfB**	**HdB**
Escherichia coli ATCC® 35218™	NA	NA	2.50	1.25	NA	NA	NA	NA	64.00–128.00	-	-	NA	NA	-	-	-	-
Escherichia coli EC-LWE23#1	NA	NA	NA	1.25 – 2.50	NA	NA	NA	NA	64.00–128.00	-	-	-	2.50	-	-	-	-
Klebsiella pneumoniae ATCC® 13883™	1.25 – 2.50	1.25	0.63	0.31 – 0.63	2.50	2.50	1.25 – 2.50	1.25 – 2.50	1.00–2.00	2.50	2.50	1.25	0.63	NA	NA	2.50	NA
Klebsiella pneumoniae KP-LWE23#1	NA	2.50	1.25 – 2.50	1.25	NA	NA	NA	NA	8.00	-	NA	2.50	1.25	-	-	-	-
Pseudomonas aeruginosa ATCC® 15442™	1.25	1.25	1.25 – 2.50	0.63	NA	NA	NA	NA	16.00–32.00	2.50	1.25	2.50	1.25	-	-	-	-

MIC and MBC values are expressed as mean or range of three consistent replicates. NA: no activity; "-": MBC testing is not conducted due to no inhibition is observed for MIC; CMP: Chloramphenicol

A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

4.7 Antifungal Testing

The antifungal activity of the eight *Eucalyptus* leaf extracts obtained from two age groups using two methods, was evaluated against eight fungal species. The lowest concentrations of the samples required to inhibit fungal growth (MICs) and kill the fungi (MFCs), determined using a broth microdilution method, are listed in Table 4.11. Although all tested extracts showed a certain degree of antifungal activity, their efficacy varied depending on the fungal species and extraction method. To check the assay validity and consistency, positive controls with 5-fluorocytosine for yeasts and itraconazole for filamentous fungi were included. They produced MIC values ranging from 0.06 to 8.00 µg/mL and from 8.00 to 32.00 µg/mL, respectively, which were stronger than the inhibitory effect observed for the tested extracts.

The assessments against yeasts demonstrated that extracts obtained via SCE (A, A10, B, B10) generally exhibited lower MICs and MFCs compared to hydrodistillation extracts (HfA, HdA, HfB, HdB). Specifically, SCE extracts exhibited MIC values ranging from 0.02 to 1.25 mg/mL and MFC values ranging from 0.04 to 2.50 mg/mL against *Candida* spp., *Nakaseomyces glabratus*, and *Cryptococcus neoformans*, lower than the MIC and MFC values of hydrodistillation extracts, which varied from 0.16 to 1.25 mg/mL and 0.31 to 2.50 mg/mL, respectively. Remarkably, although B10 was the only SCE extract active against *Nakaseomyces glabratus*, it demonstrated a fungicidal effect at 0.63 mg/mL, lower than that of the hydrodistillation extracts. Interestingly, the ethanol-assisted

extracts (A10 and B10) exhibited MIC values that were comparable or lower than those of their corresponding pure CO_2 extracts (A and B), suggesting that ethanol-assisted SCE enhanced the antifungal activity of the resulting extracts. For example, B10 showed the lowest MIC (0.02 mg/mL) and MFC (0.04 mg/mL) across all treatments in the bioassay against *Candida tropicalis*. Another strong performance was observed with A10, which exerted similarly low MIC and MFC values of 0.08 mg/mL against *Cryptococcus neoformans*.

In contrast, filamentous fungi displayed different trends in their susceptibility tests. *Aspergillus fumigatus* exhibited higher resistance, with no inhibitory effects observed for any of the *Eucalyptus* leaf extracts. On the other hand, stronger antifungal activity was observed against *Trichophyton rubrum,* where hydrodistillation extracts exhibited MIC and MFC values ranging from 0.04 to 0.08 mg/mL, lower than the range of 0.16 to 0.31 mg/mL obtained for SCE extracts.

Table 4.11: Minimum inhibitory concentrations and minimum fungicidal concentrations of the eight *Eucalyptus* leaf extracts against fungal species.

Fungal species	MIC (mg/mL)									MFC (mg/mL)							
	A	A10	B	B10	HfA	HdA	HfB	HdB	PC (µg/mL)	A	A10	B	B10	HfA	HdA	HfB	HdB
Yeasts																	
Candida albicans	0.63 – 1.25	0.63	1.25	0.31– 0.63	0.63 – 1.25	0.63	0.63	0.31 – 0.63	4.00– 8.00	1.25	1.25	1.25	0.63	1.25	0.63	0.63	0.63
Candida auris	0.31	0.08	0.31	0.31	1.25	1.25	1.25	1.25	0.25– 0.50	0.31	0.31	0.63	0.31	2.50	2.50	1.25	1.25
Candida parapsilosis	0.63	0.31	0.63	0.31	1.25	1.25	1.25	1.25	2.00– 4.00	2.50	NA	NA	0.63	2.50	1.25	2.50	2.50
Candida tropicalis	1.25	0.31	0.31	0.02– 0.04	0.31	0.16– 0.31	0.16– 0.31	0.63	2.00– 4.00	1.25	0.63	0.31	0.04	0.31	0.31	0.31	0.63
Nakaseomyces glabratus	NA	NA	NA	0.31– 0.63	1.25	0.63	1.25	1.25	0.06– 0.13	-	-	-	0.63	1.25	2.50	2.50	NA
Cryptococcus neoformans	0.16	0.08	0.16 – 0.31	0.16– 0.31	0.31	0.31	0.31	0.31	2.00– 4.00	0.16	0.08	0.31	0.31	0.31	0.31	0.31	0.31
Filamentous fungi																	
Aspergillus fumigatus	NA	NA	NA	NA	NA	NA	NA	NA	8.00– 16.00	-	-	-	-	-	-	-	-
Trichophyton rubrum	0.16	0.16	0.31	0.16	0.08	0.08	0.04	0.04	16.00– 32.00	0.16	0.16	0.31	0.16	0.08	0.08	0.04	0.04

MIC and MFC values are expressed as mean or range of three consistent replicates. NA: no activity; "-": MFC testing is not conducted due to no inhibition is observed for MIC; PC: positive control with 5-fluorocytosine for yeasts and itraconazole for filamentous fungi.
A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

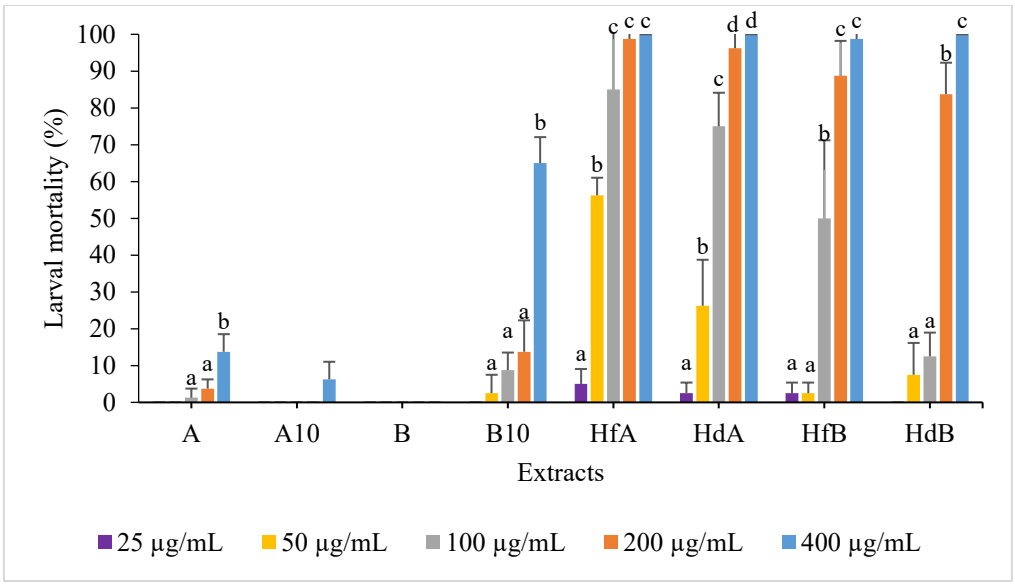
4.8 Mosquito Larvicidal Testing

In this study, *Eucalyptus* leaf extracts obtained via two methods were evaluated for their larvicidal activity against third instar larvae of *Aedes aegypti* and *Aedes albopictus* using five concentrations (25 to 400 µg/mL) for 24 and 48 h. The resultant larval mortality for each treatment with 1% (v/v) methanol as negative control is included in Tables E1 and E2 (Appendix E). The mortality trends shown in Figures 4.5 and 4.6 revealed that the larvicidal activity of the samples depended on both concentration and exposure period. Hydrodistillation extracts (HfA, HdA, HfB, and HdB) outperformed SCE extracts (A, B, A10, and B10) in killing *Ae. aegypti* and *Ae. albopictus* larvae. They achieved 100% larval mortality for both mosquito species within 24 h at 400 µg/mL, with the only exception observed for the HfB treatment against *Ae. aegypti* larvae, which reached 98.75% ± 2.50% mortality. Extending the treatment for another 24 h further increased mortality, with more than 50% of larvae of both species killed by hydrodistillation extracts at 100 µg/mL or above. The positive control, temephos resulted in 100% mortality for both species at 24 h post-treatment and is excluded from the graphs for visual clarity.

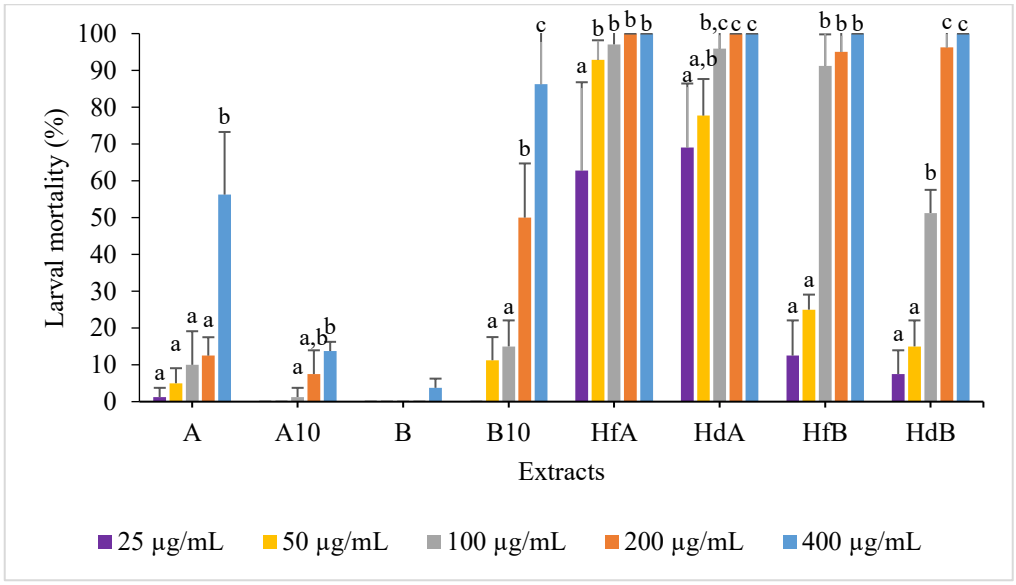
In contrast, SCE extracts demonstrated weaker larvicidal activity, with no complete larval mortality observed. Even at the highest concentration (400 µg/mL) for 48 h, the highest mortality recorded was only 86.25% ± 15.48% for B10 against *Ae. aegypti*. For most of the SCE extracts (A, B, and A10), the *Ae. aegypti* and *Ae. albopictus* larval mortality remained low (< 15%) after an additional 24 h of treatment at concentrations of 200 µg/mL or below. Interestingly, although the

larvicidal activity of B10 was weaker than that of the hydrodistillation extracts, its mortality rates were higher than those of the other SCE extracts. This result suggests that B10 may uniquely contain certain active larvicidal compounds that were not present in the other SCE extracts.

Analysis of mosquito larval mortality using a four-way ANOVA (Table C5) (Appendix C) demonstrated that mortality rates were significantly ($p < 0.05$) affected by extract type, exposure time, mosquito species, and concentration. Moreover, interactions among these variables were also statistically significant ($p < 0.001$).

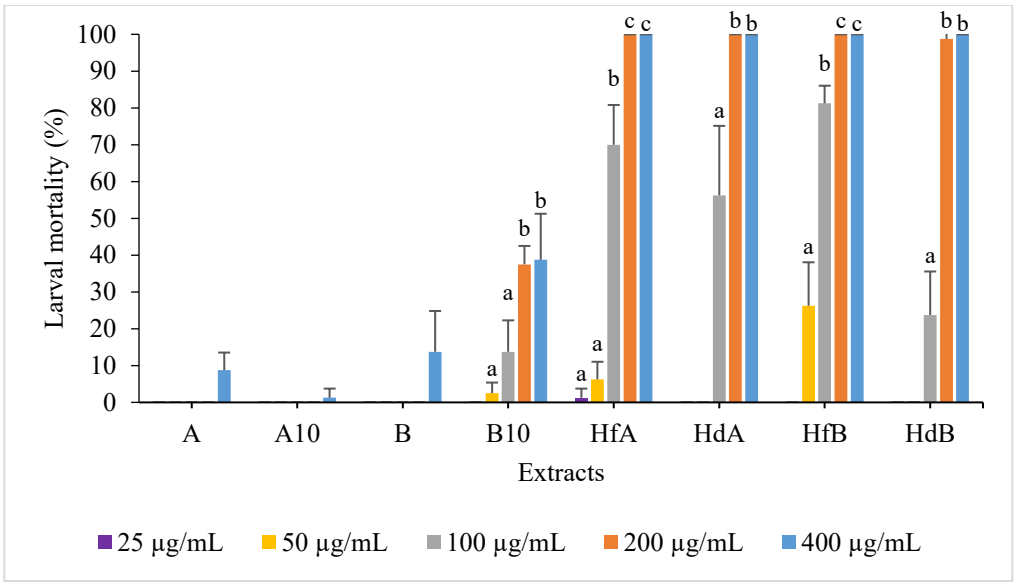


(a)

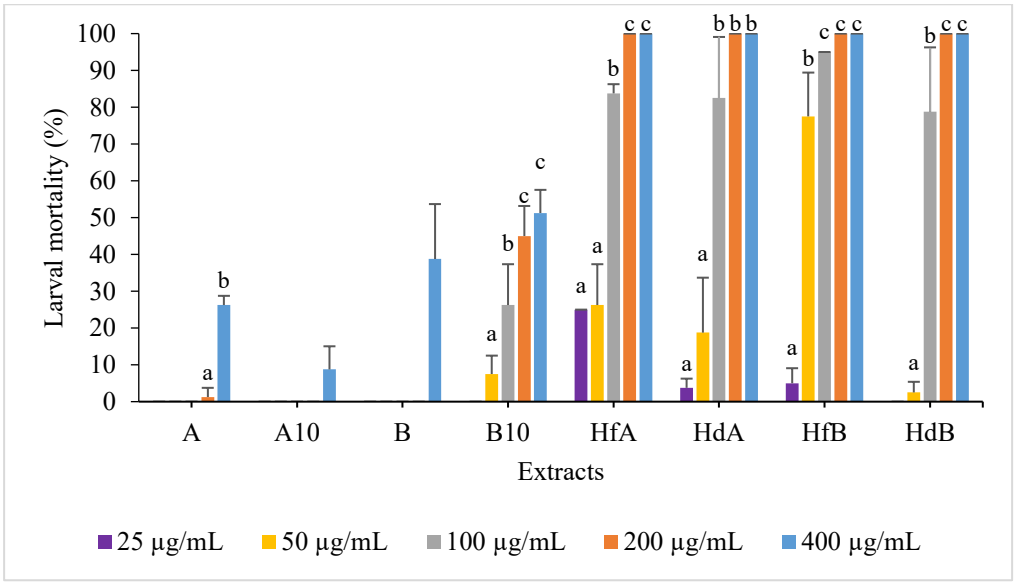


(b)

Figure 4.5: Larval mortality (*n* = 4) of *Aedes aegypti* treated with *Eucalyptus* leaf extracts for 24 h (a) and 48 h (b). Bars with different letters (a to d) represent significant differences ($p < 0.05$) between concentrations for each sample. A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*



(a)



(b)

Figure 4.6: Larval mortality (*n* = 4) of *Aedes albopictus* treated with *Eucalyptus* leaf extracts for 24 h (a) and 48 h (b). Bars with different letters (a to c) indicate significant differences ($p < 0.05$) between concentrations for each sample. A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

Extracts that produced over 50% mortality against both tested mosquito species were selected for Probit analysis (Tables C7 and C8) (Appendix C) to determine the median lethal concentrations (LC_{50}) and 95% lethal concentrations (LC_{95}). These included the four hydrodistillation extracts and one SCE extract. In the tests against *Ae. aegypti* (Table 4.12), the hydrodistillation extracts showed low LC_{50} (52.26 to 134.56 µg/mL) and LC_{95} (129.83 to 296.55 µg/mL) values after 24 h. These values further decreased after 48 h (LC_{50}: 14.27 to 84.87 µg/mL and LC_{95}: 71.92 to 240.21 µg/mL). However, the SCE extract B10 resulted in much higher LC_{50} and LC_{95} values against *Ae. aegypti*, which were 341.96 µg/mL and 1317.96 µg/mL, respectively, after 24 h exposure, and 187.02 µg/mL and 719.24 µg/mL, respectively, after 48 h exposure.

Similarly, the hydrodistillation extracts demonstrated superior activity against *Ae. albopictus*, with LC_{50} and LC_{95} values ranging from 67.27 to 119.79 µg/mL and 131.07 to 187.11 µg/mL, respectively, at 24 h post-exposure (Table 4.13). For 48 h post-exposure, the LC_{50} values of the hydrodistillation extracts ranged from 42.13 to 81.35 µg/mL and the corresponding LC_{95} values ranged from 81.19 to 169.69 µg/mL. In contrast, much higher concentrations (≥ 294.48 µg/mL) were needed to kill more than 50% of *Aedes albopictus* larvae when the larvicidal tests were conducted using B10. The lower LC_{50} and LC_{95} values, combined with the higher mortality rates recorded for the hydrodistillation extracts against both *Aedes* spp., suggest that hydrodistillation extracted more potent larvicidal compounds compared to SCE.

Table 4.12: Lethal concentrations of five *Eucalyptus* leaf extracts against *Aedes aegypti* larvae after 24 h and 48 h post-treatments.

Extracts	Time (h)	LC_{50}, µg/mL (LCL-UCL)	LC_{95}, µg/mL (LCL-UCL)	Regression coefficient ± Standard error
B10	24	341.96 (219.63 – 1241.01)	1317.96 (581.63 – 80460.10)	2.807 ± 0.327
	48	187.02 (126.41 – 318.92)	719.24 (390.49 – 3941.01)	2.812 ± 0.252
HfA	24	52.26 (40.37 – 66.01)	129.83 (95.36 – 242.33)	4.162 ± 0.386
	48	14.27 (7.60 – 19.76)	71.92 (56.29 – 110.02)	2.342 ± 0.432
HdA	24	70.87 (63.95 – 78.46)	174.24 (148.56 – 215.47)	4.210 ± 0.366
	48	14.99 (8.25 – 20.82)	91.76 (70.60 – 142.57)	2.090 ± 0.353
HfB	24	105.34 (71.23 – 155.67)	261.18 (171.55 – 817.15)	4.171 ± 0.348
	48	59.34 (32.95 – 100.91)	164.51 (97.88 – 1165.21)	3.714 ± 0.317
HdB	24	134.56 (72.48 – 283.08)	296.55 (180.17 – 6130.61)	4.793 ± 0.423
	48	84.87 (53.85 – 137.44)	240.21 (145.41 – 1091.70)	3.640 ± 0.297

LC_{50}: Median lethal concentration; LC_{95}: 95% lethal concentration; LCL: Lower confidence limit; UCL: Upper confidence limit

B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus*.

Table 4.13: Lethal concentrations of five *Eucalyptus* leaf extracts against *Aedes albopictus* larvae after 24 h and 48 h post-treatments.

Extracts	Time (h)	LC_{50}, µg/mL (LCL-UCL)	LC_{95}, µg/mL (LCL-UCL)	Regression coefficient ± Standard error
B10	24	-	-	-
	48	294.48 (176.22 – 1065.91)	2461.18 (797.60 – 194283.33)	1.784 ± 0.208
HfA	24	82.00 (50.34 – 138.76)	151.27 (103.83 – 1345.08)	6.185 ± 0.624
	48	53.36 (18.39 – 118.67)	169.69 (88.90 – 19866.57)	3.274 ± 0.295
HdA	24	96.32 (89.34 – 103.52)	149.92 (133.26 – 184.27)	8.559 ± 1.336
	48	67.42 (53.55 – 85.56)	140.24 (105.06 – 260.63)	5.171 ± 0.481
HfB	24	67.27 (61.61 – 73.42)	131.07 (114.52 – 158.28)	5.678 ± 0.572
	48	42.13 (27.43 – 60.12)	81.19 (57.75 – 296.38)	5.774 ± 0.584
HdB	24	119.79 (111.47 – 129.42)	187.11 (166.69 – 223.31)	8.493 ± 1.053
	48	81.35 (75.43 – 87.30)	125.41 (113.75 – 144.51)	8.750 ± 1.035

"-": Probit analysis is not conducted as mortality in this treatment was below 50%; LC_{50}: Median lethal concentration; LC_{95}: 95% lethal concentration; LCL: Lower confidence limit; UCL: Upper confidence limit

B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

Morphological deformities were observed in the treated larvae under a stereo microscope at 30× magnification, in contrast to the untreated control group. Larvae of both *Ae. aegypti* (Figure 4.7) and *Ae. albopictus* (Figure 4.8) exposed to *Eucalyptus* leaf extracts at the highest concentration (400 µg/mL) showed notable elongation of the neck region and blackening of the midgut.

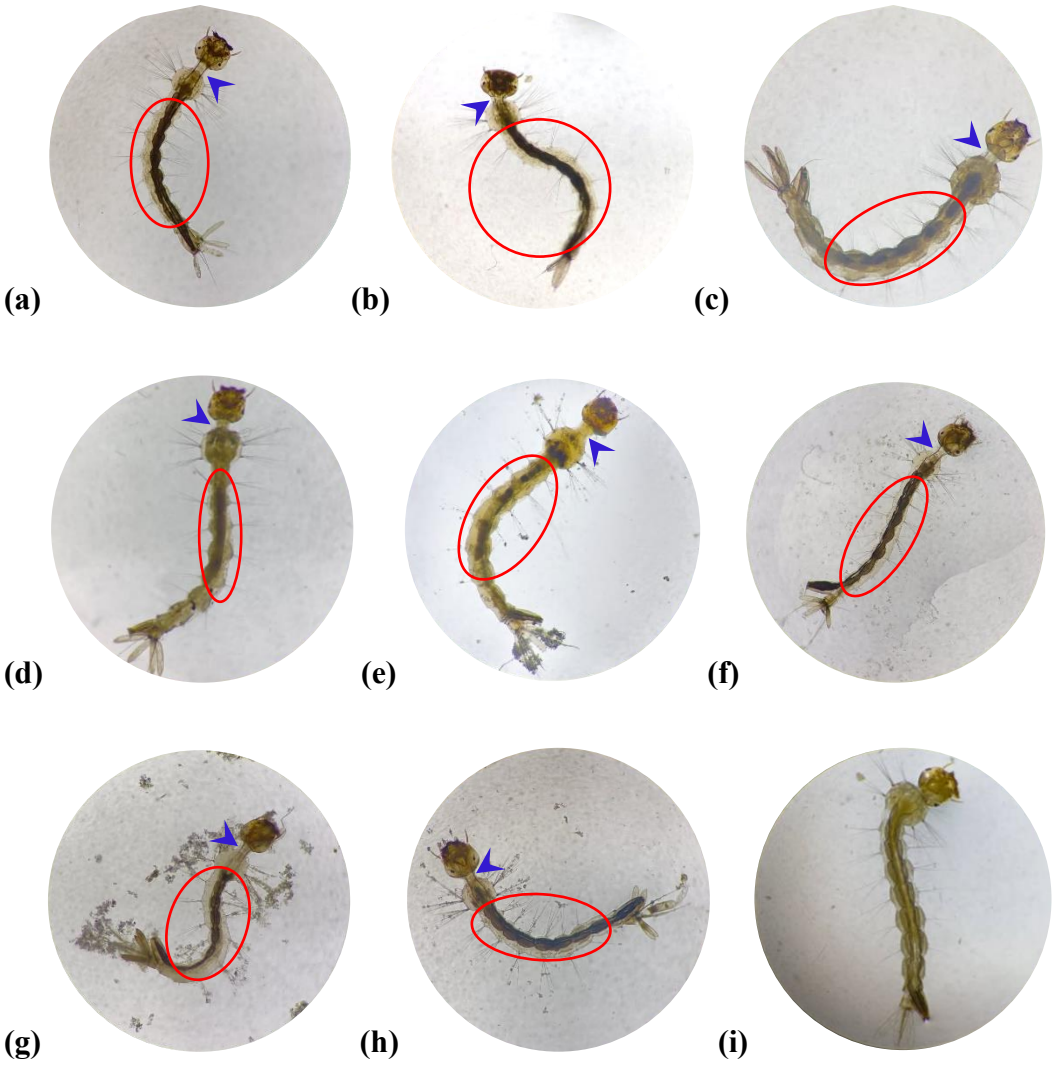


Figure 4.7: Elongated necks (blue arrow) and blackened midguts (red circle) observed in *Aedes aegypti* larvae treated with eight *Eucalyptus* leaf extracts at 400 µg/mL (magnification: 30×). (a) Fresh leaf from younger *Eucalyptus*; (b) Dried leaf from younger *Eucalyptus*; (c) Fresh leaf from older *Eucalyptus*; (d) Dried leaf from older *Eucalyptus*; (e) SCE of younger *Eucalyptus*; (f) Ethanol-assisted SCE of younger *Eucalyptus*; (g) SCE of older *Eucalyptus*; (h) Ethanol-assisted SCE of older *Eucalyptus*; (i) Normal, untreated *Ae. aegypti* larva.

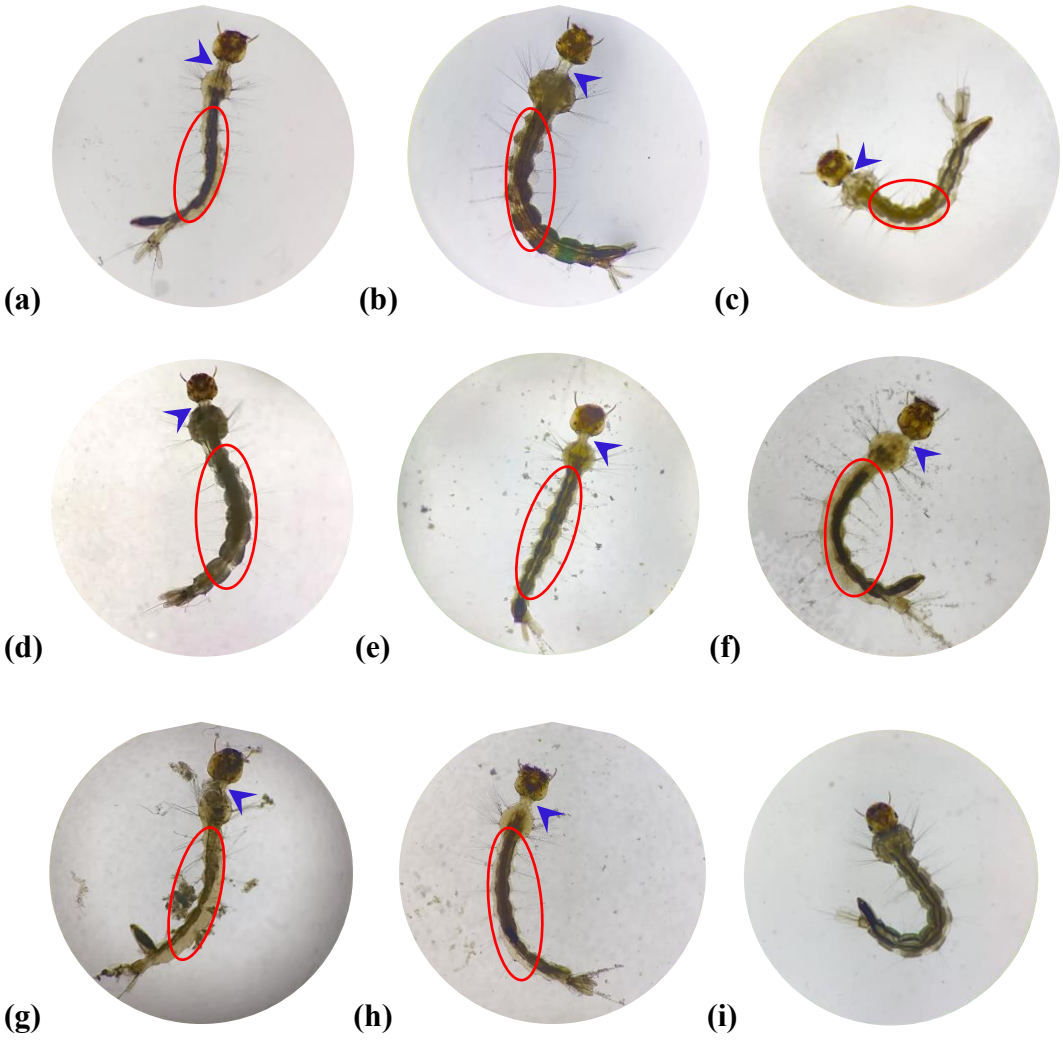


Figure 4.8: Elongated necks (blue arrow) and blackened midguts (red circle) observed in *Aedes albopictus* larvae treated with eight *Eucalyptus* leaf extracts at 400 µg/mL (magnification: 30×). (a) Fresh leaf from younger *Eucalyptus*; (b) Dried leaf from younger *Eucalyptus*; (c) Fresh leaf from older *Eucalyptus*; (d) Dried leaf from older *Eucalyptus*; (e) SCE of younger *Eucalyptus*; (f) Ethanol-assisted SCE of younger *Eucalyptus*; (g) SCE of older *Eucalyptus*; (h) Ethanol-assisted SCE of older *Eucalyptus*; (i) Normal, untreated *Ae. albopictus* larva.

4.9 Brine Shrimp Lethality Assay

Brine shrimp lethality assay was used to test the toxicity of *Eucalyptus* leaf extracts based on their ability to kill laboratory-cultured *Artemia franciscana* nauplii. Figure 4.9 illustrates the mortality rate of brine shrimp nauplii following 24 h exposure to various concentrations of eight *Eucalyptus* leaf extracts. The respective nauplii mortality for each treatment, positive control (potassium dichromate) and negative controls is shown in Table E3 (Appendix E). Because mortality was observed in the 5% and 10% (v/v) ethanol, the Abbott formula was applied to correct the nauplii mortality at 500 µg/mL and 1000 µg/mL.

A similar mortality trend was observed across all extracts, with 100% survival of exposed nauplii at the lower concentrations of 1 and 10 µg/mL. Nauplii mortality began to emerge for some extracts as the concentration increased to 100 µg/mL. Specifically, HfA, HdA, and HfB caused no mortality, whereas A and HdB resulted in slight mortality of 3.33% ± 5.77%. In contrast, B, B10, and A10 showed mortalities of 13.33% ± 5.77%, 23.33% ± 5.77%, and 26.67% ± 11.55%, respectively. At 500 µg/mL, B, B10, and A10 each caused around 80% mortality, whereas the remaining extracts resulted in complete mortality. Subsequently, complete nauplii mortality was observed for all extracts at the highest concentration of 1000 µg/mL. These results demonstrate a clear concentration-dependent toxicity profile.

The results of the two-way ANOVA (Table C6) (Appendix C) indicated that there was no significant difference in mortality rates among the eight *Eucalyptus* leaf extracts (p = 0.971). However, significant differences were observed for concentration levels, as well as the interaction between extract type and concentration ($p < 0.001$).

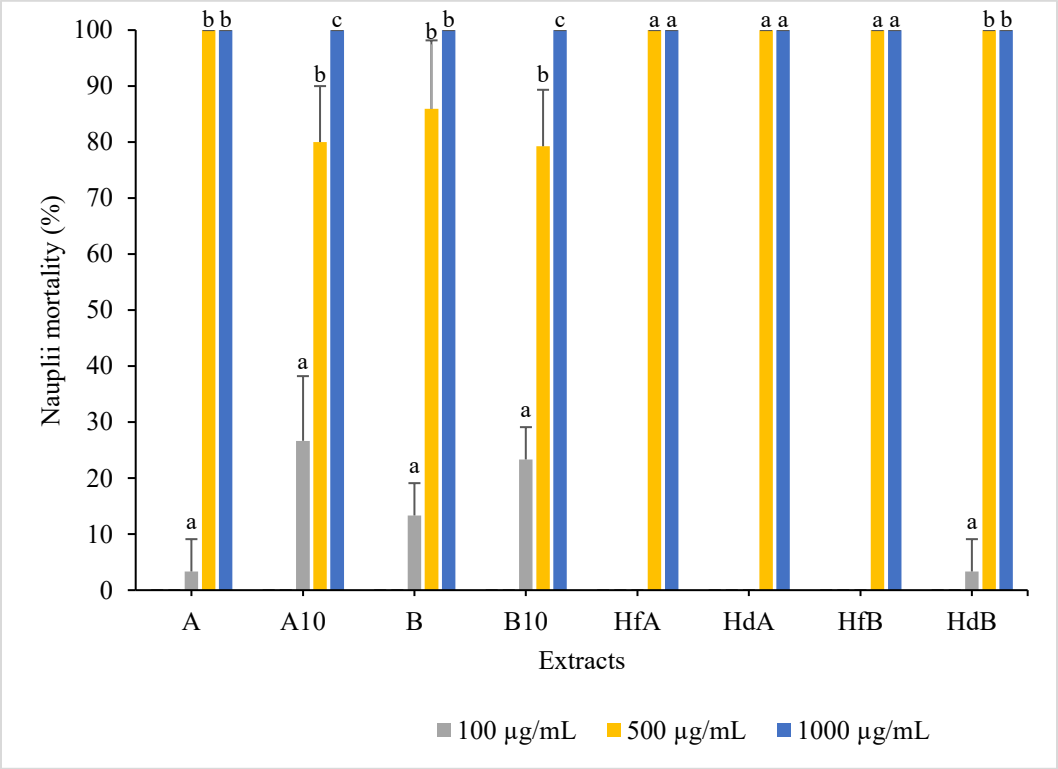


Figure 4.9: Mortality of *Artemia franciscana* nauplii exposed to *Eucalyptus* leaf extracts for 24 h. No nauplii mortality was observed at the two lowest concentration levels so bars for 1 and 10 µg/mL are not shown. Bars with different letters (a to c) represent significant differences ($p < 0.05$) across concentrations for each extract. A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus*.

Probit analysis was conducted for the eight *Eucalyptus* leaf extracts (Table C9) (Appendix C) and Table 4.14 provides the LC_{50} and LC_{95} of the *Eucalyptus* leaf extracts and potassium dichromate (positive control) against brine shrimp nauplii. Among the eight *Eucalyptus* leaf extracts, the LC_{50} values were relatively similar, ranging from 187.43 to 222.24 µg/mL. In particular, the ethanol-assisted SCE extracts, A10 (187.43 µg/mL) and B10 (198.61 µg/mL), exhibited slightly lower LC_{50} values compared with both hydrodistillation and pure CO_2 SCE extracts, which ranged from 209.24 to 222.24 µg/mL. However, differences were observed in the LC_{95} values. The ethanol-assisted SCE extracts, A10 (834.45 µg/mL) and B10 (825.06 µg/mL), reported higher LC_{95} values than those of the hydrodistillation extracts and their pure CO_2 counterparts. These results suggest that ethanol-assisted extracts exhibited higher acute toxicity at low concentrations but lower toxicity at higher concentrations. Notably, the positive control, potassium dichromate, showed a much lower LC_{50} of 20.15 µg/mL and LC_{95} of 172.20 µg/mL, highlighting the relatively low acute toxicity of the *Eucalyptus* leaf extracts.

Table 4.14: Lethal concentrations of eight *Eucalyptus* leaf extracts against *Artemia franciscana* nauplii after 24 h post-treatment.

Extracts	LC_{50}, µg/mL (LCL-UCL)	LC_{95}, µg/mL (LCL-UCL)	Regression coefficient ± Standard error
A	209.24 (158.10-286.80)	437.84 (314.16-774.59)	5.129 ± 0.911
A10	187.43 (129.49-254.42)	834.45 (557.81-1655.60)	2.536 ± 0.417
B	217.29 (161.26-284.38)	671.97 (482.44-1140.03)	3.355 ± 0.517
B10	198.61 (139.65-267.08)	825.06 (559.82-1585.24)	2.660 ± 0.432
HfA	222.24 (163.67-300.49)	467.82 (340.24-785.32)	5.088 ± 0.901
HdA	222.24 (163.67-300.49)	467.82 (340.24-785.32)	5.088 ± 0.901
HfB	222.24 (163.67-300.49)	467.82 (340.24-785.32)	5.088 ± 0.901
HdB	209.24 (158.10-286.80)	437.84 (314.16-774.59)	5.129 ± 0.911
Potassium dichromate (Positive control)	20.15 (12.47-32.02)	172.20 (93.77-451.25)	1.765 ± 0.254

LC_{50}: Median lethal concentration; LC_{95}: 95% lethal concentration; LCL: Lower confidence limit; UCL: Upper confidence limit.

A: SCE of younger *Eucalyptus*; A10: Ethanol-assisted SCE of younger *Eucalyptus*; B: SCE of older *Eucalyptus*; B10: Ethanol-assisted SCE of older *Eucalyptus*; HfA: Fresh leaf from younger *Eucalyptus*; HdA: Dried leaf from younger *Eucalyptus*; HfB: Fresh leaf from older *Eucalyptus;* HdB: Dried leaf from older *Eucalyptus.*

CHAPTER 5

DISCUSSION

5.1 Optimisation of Supercritical Carbon Dioxide Fluid Extraction

Knowing the optimum extraction parameters is crucial to minimise sample loss, reduce operating cost, save time, and increase extraction efficiency. In this study, RSM was used to construct a mathematical model linking the independent variables, extraction temperature, pressure, and time, to the response variable, extraction yield, based on data obtained from experimental runs conducted according to the central composite design. As detailed in Section 4.2, all three independent variables had a significant impact on the SCE yield, and the experimental dataset presented curvature, indicating that the change in response variable associated with a one-unit shift in the independent variable was not constant. Therefore, quadratic response surfaces were recommended by the software for analysis (Djimtoingar *et al*., 2022). Furthermore, the three-dimensional surface plots indicated that A (Figure 4.1) and B (Figure 4.2) showed similar overall trends, where higher temperatures, pressures, and longer extraction times generally improved extraction yields.

Pressure plays a major role in the SCE mechanism. Maximum yields were obtained at higher pressures because elevated pressure resulted in high CO_2 fluid

density, allowing more effective mass transfer and solute release by exerting stronger compression forces on the plant material (Zhao and Zhang, 2014; Yousefi *et al*., 2019). Thus, raising the pressure improved both the solvent power of supercritical CO_2 and the solubility of solutes. The enhancement effect of increasing pressure on the SCE yield of vegetal samples has also been reported in the literature for *Pinus gerardiana* (chilghoza pine) and *Eucalyptus loxophleba* (Zhao and Zhang, 2014; Amani, Ardestani and Honarvar, 2021).

Extraction time also significantly impacted the yield. Extended durations provided more time for supercritical CO_2 to penetrate the plant material, dissolve the solute, and subsequently diffuse it out of the matrix (Zhao and Zhang, 2014). However, when the extraction time was excessively prolonged, the yield reached a plateau and even showed negative quadratic effects, leading to unnecessarily high costs and time wastage. Therefore, a duration ranging from 90 to 120 min is preferable as a desirable extraction time. Similar observations were made by Zhao and Zhang (2014) in their SCE of *Eucalyptus loxophleba* leaves, where extraction beyond 120 min had minimal impact on yield.

Elevated temperature can induce contradictory effects on extraction yield. Higher temperature decreases the density of supercritical CO_2 and reduces its ability to dissolve solutes. Conversely, the vapour pressure of solutes in the plant material increases with temperature, enhancing their solubility in the solvent

(Yousefi *et al*., 2019; de Melo *et al*., 2020). Thus, the overall influence of temperature on extraction yield relies on a complex balance between solvent density and solute volatility. The current study observed that increasing temperature consistently improved the SCE yield within the tested range. This suggests that the elevated solute vapour pressure at higher temperatures effectively compensated for the concurrent reduction in CO_2 density. Comparable findings were reported in a study using SCE on *E. loxophleba*, where the yield increased from 3.3% to 4.4% when the temperature was elevated from 40 to 80°C at a pressure of 30 MPa (Zhao and Zhang, 2014).

5.2 Extraction Yield

A co-solvent, 10% ethanol was employed for SCE in this study because extraction yield was shown to plateau at ethanol concentrations above 10% (Zhao and Zhang, 2014). The enhancing effect of 10% ethanol on the SCE yield aligns with the well-known ability of this polar modifier to form hydrogen bonds with solutes, thereby improving extraction efficiency (Chai *et al*., 2020). At the same time, polar modifiers can bind to active sites of the plant matrix, disrupting solute-matrix interactions and accelerating solute desorption (Khajeh, 2011).

However, the SCE yields achieved for *E. grandis* × *E. urophylla* in the present investigation (0.255% to 4.650% w/w), with and without co-solvent addition, were markedly lower than the 7.86% reported by Zhou *et al*. (2014) for

8-h SCE conducted at 80°C and 40 MPa using only pure CO_2. Similarly, when comparing the hydrodistillation yields in this study with the 1.03% (w/w) obtained by da Silva *et al*. (2020), who hydrodistilled fresh leaves of same *Eucalyptus* hybrid species cultivated in Brazil for 4 h, the current yields (0.025% to 0.191% w/w) were much lower, despite using a longer hydrodistillation period of 8 h. Extraction yield in plants has been known to be influenced by a complex group of factors, the observed variations are likely due to differences in extraction conditions, equipment settings, and geographical origin (Malaka *et al.,* 2022; Yip *et al.,* 2024). Conversely, the densities of the hydrodistillation extracts from *E. grandis* × *E. urophylla* Clone 3229 in the present investigation (0.951 to 0.966 g/mL) were comparatively higher than those reported by da Silveira *et al*. (2021) for hydrodistilled extracts isolated from two distinct clones of *E. grandis* × *E. urophylla* (Clone GG100: 0.8909 g/mL; Clone 1144: 0.8603 g/mL).

Since the hydrodistillation yields for fresh and dried leaves in this study were calculated using different weight bases (fresh weight and dry weight, respectively), direct comparison between their yields is not applicable. However, the overall percentages calculated for the yield based on the time-dependent collection revealed that dried leaf extracts (HdA: 90.58%; HdB: 82.61%) had higher initial percentages at 4 h than fresh leaf extracts (HfA: 88.14%; HfB: 60.00%). This may suggest that drying could potentially enhance and accelerate extraction by improving heat transfer (Sewanu *et al*., 2015).

This study also highlighted the influence of tree age on extraction yield, as the leaves harvested from older *Eucalyptus* trees yielded significantly less extract. Essential oils accumulate in specialised glands during leaf development but may diminish after full leaf expansion via evaporation and leakage (Fikremariam *et al*., 2019). In addition, branches become wider and denser as trees grow, which may reduce sunlight exposure to the leaves, impairing photosynthesis and reducing carbon flux for secondary metabolite production (Fajar *et al.*, 2019).

The low yield increment after 4 h and the overall percentages for hydrodistillation yield shown in Table 4.3 indicate that over 50% of the extract in *Eucalyptus* hybrid leaves was released in the first 4 h of extraction, showing that prolonged extraction may be inefficient. These outcomes agreed with the findings of Anh *et al*. (2019), who reported that *Ocimum gratissimum* (clove basil) leaf extract production increased from the first to third hour of hydrodistillation but did not further increase even extending the time to the fifth hour. This demonstrated that a short distillation time may not be enough for solute diffusion out of the plant material, whereas prolonged exposure to heat may not improve yield and may even modify the quality of the products.

5.3 Chemical Composition of *Eucalyptus* Leaf Extracts

The GC-MS analysis using both non-polar and polar columns revealed notable differences in extract composition, influenced by tree age, distillation

duration, and leaf condition. The impact of tree age on chemical composition was indicated by the presence of unique compounds detected in extracts from trees of either age group (Tables 4.4, 4.5, 4.6, and 4.7). A similar compositional pattern was reported for *Cryptomeria japonica* (Japanese cedar) leaf extracts. While 16-kaurene and elemol remained dominant across three age groups, Cheng *et al*. (2009a) noted variations in minor constituents, where *cis*-piperitol was only detected in plants aged 26 and 42 years, whereas cedrol was only present in plants aged 58 years. Moreover, aromadendrene was consistently unique in SCE extracts obtained from trees aged 17 to 31 months at an abundance > 5% (Tables 4.4 and 4.5), suggesting it may serve as chemical marker to distinguish between the two tree age groups in this study.

Differences between fresh and dried leaf samples indicated that drying altered the chemical composition of *Eucalyptus* leaf extracts. Similar observations were also reported by other researchers comparing chemical profiles of extracts derived from fresh and dried plant samples, where extracts of dried basil and thyme contained compounds absent in the fresh samples (Ghasemi Pirbalouti, Mahdad and Craker, 2013; Rahimmalek and Goli, 2013). Variations between fresh and dried leaf samples may arise from degradation or transformation of compounds via oxidation, glycoside hydrolysis, dehydration, and esterification during the drying process, as well as rupture of plant cells storing volatile compounds (Díaz-Maroto *et al*., 2004; Sewanu *et al*., 2015). However, the low abundance (< 1%) of unique

components reported for either leaf condition in this study reflects their inconsistent detectability, limiting their applicability as markers.

As shown in Table 4.8, the appearance of time-specific components aligned with the shift from monoterpenes and monoterpenoids to sesquiterpenes and sesquiterpenoids in hydrodistillation extracts over time, reflecting the volatility of compounds. Monoterpenes and their oxygenated derivatives, composed of two isoprene units, have shorter carbon chains and lower molecular weights than sesquiterpenes and sesquiterpenoids, which are composed of three isoprene units. Consequently, monoterpenes and monoterpenoids have lower boiling points, are more volatile, and are rapidly released during hydrodistillation. These findings were further corroborated by similar trends observed for extracts of lemon thyme (Jurevičiūtė *et al*., 2019) and coriander (Zheljazkov, Astatkie and Schlegel, 2014), where extending hydrodistillation time reduced the extraction of low-boiling-point constituents but favoured the extraction of high-boiling-point compounds.

The addition of 10% ethanol as a co-solvent for SCE also altered the chemical profiles of SCE extracts by enhancing the extraction of compounds with higher polarity (Tables 4.4 and 4.5). Ethanol's polar nature promotes dissolvability of compounds with higher polarity and overcomes the limited selectivity of non-polar CO_2 (Uwineza and Waśkiewicz, 2020). Concurrently, the introduction of 10% ethanol as a co-solvent reduced the content of monoterpenes and their oxygenated

derivatives in the extracts, including the complete loss of eucalyptol. A similar trend was noticed in the study of Zhao and Zhang (2014), which found that the eucalyptol content in *Eucalyptus loxophleba* leaf extracts decreased from 77.0% to 46.1% when ethanol concentration increased from 5% to 10%.

The *α*-terpinyl acetate, a monoterpenoid, was consistently present in considerable amounts in all hydrodistillation extracts across all time points (Tables 4.6, 4.7, and 4.8) and in all SCE extracts (Tables 4.4), indicating its relatively high stability and dominance in *Eucalyptus* leaf extracts across different extraction methods and plant ages. However, notable discrepancies were found between our SCE results and those observed by Zhou *et al*. (2014). Major compounds such as *α*-pinene (24.8%) and 1,1-diethoxy-3-methyl-butane (8.6%) identified in their study were absent in this study, while new dominant constituents like eucalyptin and globulol emerged. This highlights how chemical profiles can vary even within the same hybrids due to methodological factors, cultivation practices, and environmental variations, as plant density, soil type, and air temperature have also been reported to affect secondary metabolite production (Tuttolomondo *et al.*, 2016; Malaka *et al.*, 2022). Conversely, the predominant constituents reported by da Silva *et al.* (2020) for their 4-h hydrodistilled *E. grandis* × *E. urophylla* leaf extract in Brazil, which were eucalyptol (41.34%), *α*-pinene (27.66%), *α*-terpinyl acetate (7.95%), and *α*-terpineol (6.90%), were also found in considerable abundance in the current 4-h extracts, except for HfB, which exhibited a lower α-pinene content.

5.4 Comparison of Yield and Chemical Composition between Dried Leaf Hydrodistillation and SCE

When comparing extraction yields from dried leaves using hydrodistillation and SCE, SCE demonstrated superior efficiency, producing higher yields (Table 4.2) for both samples in less than 2 h, compared with hydrodistillation, which continued for 8 h (Table 4.3). Notably, the variation in sample preparation in this study, at which ground leaf powder for SCE, versus blended samples for hydrodistillation, may have partially contributed to the enhanced SCE performance. Nevertheless, previous studies employing ground dried leaf powder for both techniques have also reported the enhanced extraction efficiency of SCE over hydrodistillation in a shorter duration when applied to *Eucalyptus cinerea* (Herzi *et al*., 2013), *E. camaldulensis* (Herzi *et al*., 2013), and *E. loxophleba* (Zhao and Zhang, 2014), suggesting that the superior performance of SCE is not solely due to sample preparation.

In terms of chemical composition, hydrodistillation predominantly produced extracts rich in terpenes and terpenoids, although low abundances of non-terpenoid acids and esters were also detected. Conversely, SCE, especially with 10% ethanol as a co-solvent, captured a broader range of chemical classes, extracting not only terpenes and terpenoids but also non-terpenoid acids, alcohols, alkanes, esters, and other high-molecular-weight constituents. A similar trend has been reported in the literature. Specifically, da Cruz Francisco *et al*. (2001) and Zhao and Zhang (2014) observed that SCE produced extracts with lower eucalyptol

content (32% to 39% and 46%, respectively) but higher amounts of high-molecular-weight fatty acids, esters, and waxy-like compounds for dried leaves of *Eucalyptus camaldulensis* and *E. loxophleba*, respectively, compared with hydrodistillation, which yielded extracts with 43% and 70% eucalyptol, respectively. Although the differences in eucalyptol content in the current study were less pronounced, a consistent pattern was observed, with eucalyptol levels ranging from 2.96% to 18.82% in SCE extracts and from 10.10% to 26.67% in hydrodistillation extracts.

Differences in extraction yield and chemical composition between SCE and hydrodistillation can be explained by their isolation mechanisms. Hydrodistillation relies on heating leaf samples in water at 100°C. Volatile compounds readily evaporate with water at this temperature, but the evaporation of heavier compounds is limited by their higher boiling points (Zhao and Zhang, 2014). In contrast, SCE employs pressurised supercritical CO_2 to flow through the reactor filled with leaf powders and dissolve extractable components from the plant matrix (Uwineza and Waśkiewicz, 2020). The continuous diffusion of solvent into the plant matrix enables both volatile and non-volatile compounds to solubilise (Zhao and Zhang, 2014). Additionally, when polar ethanol is added as a co-solvent, it overcomes the limitation of CO_2, which preferentially extracts non-polar components.

5.5 Antibacterial Activity of *Eucalyptus* Leaf Extracts

In terms of bacterial susceptibility, Gram-positive bacteria were more susceptible to both SCE and hydrodistillation extracts than Gram-negative bacteria. This difference can be explained by the presence of a lipopolysaccharide outer membrane in Gram-negative bacteria, which acts as a hydrophilic barrier against the penetration of hydrophobic compounds (Nazzaro *et al*., 2013). In contrast, the cell wall of Gram-positive bacteria, which consists of a thick peptidoglycan layer but lacks lipopolysaccharides, is more permeable and allows the hydrophobic compounds in the *Eucalyptus* leaf extracts to penetrate more easily and interact with the phospholipid bilayer. This interaction increases ion permeability, causes leakage of intracellular components, and disrupts bacterial enzymes (Silva *et al*., 2011; Barbosa, Filomeno and Teixeira, 2016). The greater sensitivity of Gram-positive bacteria to *Eucalyptus* extracts has been commonly reported in the literature (Gilles *et al*., 2010; Ben Marzoug *et al*., 2011; Silva *et al*., 2011; Tyagi and Malik, 2011; Diloksumpun *et al*., 2022), further supporting the notion that the active components of *Eucalyptus* leaf extracts, especially the SCE extracts with higher antibacterial efficacy, may target the bacterial cell membrane.

While the fundamental mechanism of membrane disruption typically affects both antibiotic-sensitive and antibiotic-resistant strains, higher concentrations of *Eucalyptus* leaf extracts were generally required to inhibit the growth of resistant strains in this study. This reduced bacterial susceptibility is likely attributed to their adaptive defence mechanisms. Antibiotic-resistant bacteria usually employ

strategies such as enzymatic modification of antimicrobial agents, alteration of target sites, active drug efflux, and reduced drug uptake to enhance their survival (Munita and Arias, 2016). These resistance mechanisms may also facilitate the inactivation or elimination of bioactive constituents present in the *Eucalyptus* leaf extracts, thereby weaken their antibacterial effectiveness (Abers *et al*., 2021).

Little studies have explored the antibacterial activity of *E. grandis* × *E. urophylla* extracts. In a related study using steam-distilled extracts from the same hybrid, Zhou *et al*. (2021) reported an MIC of 0.045 mg/mL against *B. cereus*, which is comparable to those obtained for SCE extracts (0.02 to 0.08 mg/mL) in the present study but lower than those observed for hydrodistilled extracts (0.16 to 0.63 mg/mL). In addition, the steam-distilled extracts exhibited stronger inhibitory action towards *E. coli* and *P. aeruginosa* at 0.091 mg/mL and 0.023 mg/mL, respectively. In contrast, in the current study, growth inhibition against both bacterial species was only observed for SCE extracts, with MIC values ranging from 1.25 to 2.50 mg/mL and 0.63 to 2.50 mg/mL, respectively. These discrepancies could be attributed to variations in bacterial strains or differences in chemical composition due to extraction conditions. When comparing with the parental *Eucalyptus* species, the antimicrobial potency of *E. grandis* × *E. urophylla* in this study stands out notably. Their MIC and MBC values were predominantly lower than those reported for *E. grandis* (Soyingbe *et al*., 2013) and *E. urophylla* (Diloksumpun *et al*., 2022), particularly against Gram-positive strains. This suggests that hybridization may improve the production of antimicrobial

constituents. However, the well-studied species, *E. globulus*, exhibited stronger antibacterial activity than our *Eucalyptus* hybrid, with lower MICs of 2.07 µg/mL and 2.23 µg/mL as well as MBCs of 4.26 µg/mL and 4.21 µg/mL against *S. aureus* and *P. aeruginosa*, respectively (Nasir Shah *et al*., 2023).

It has been demonstrated that compounds found in *Eucalyptus* leaf extracts, including eucalyptol, *α*-terpinyl acetate, and *α*-pinene, exhibit inhibitory activity against bacteria (Marei, Rabea and Badawy, 2019; Ivanov *et al*., 2021; Melkina *et al*., 2021). In the literature, compounds such as *α*-terpinyl acetate (0.125 mg/mL), *α*-pinene (0.686 mg/mL), and eucalyptol (0.600 mg/mL) exhibited stronger antibacterial activity against *E. coli* when tested individually, with MICs lower than the 1.25 to 2.50 mg/mL reported for *Eucalyptus* leaf extracts in this study (Wang, Chen and Hou, 2019; Andrade-Ochoa *et al.*, 2021; Vaičiulytė *et al*., 2021). Similarly, Badr, Badawy and Taktak (2021) revealed that *α*-terpinyl acetate inhibited *S. aureus* at an MIC of 0.80 mg/mL, lower than that of *α*-terpinyl acetate-rich lavender extract (3.00 mg/mL). In contrast, Marei, Rabea and Badawy (2019) observed an MIC of 0.79 mg/mL for *α*-terpinyl acetate against *S. aureus* ATCC® 6538™, which is higher than those obtained in the present study. In the same study, *α*-pinene exhibited an MIC of 0.60 mg/mL against *S. aureus*, comparable with current MIC values (0.31 to 0.63 mg/mL) reported for hydrodistillation and ethanol-assisted SCE extracts, but much higher than the MIC values of pure CO_2 SCE extracts (A: 0.08 mg/mL; B: 0.02 mg/mL). Additionally, eucalyptol in the study of Hendry *et al*. (2009) demonstrated weaker antibacterial activity, with MIC values exceeding

4 mg/mL against *S. aureus* and *P. aeruginosa,* compared to *E. grandis* × *E. urophylla* SCE extracts used in the current work, which exhibited MIC values below 2.50 mg/mL. Moreover, Mulyaningsih *et al*. (2010) reported synergistic antibacterial effects between aromadendrene and eucalyptol in inhibiting methicillin-resistant *S. aureus*. These findings indicate that the antibacterial efficacy of *Eucalyptus* leaf extracts likely arises from interactions among multiple constituents rather than solely from the presence of individual compounds.

The crucial role of interactions between multiple compounds in determining the overall antibacterial effectiveness of *Eucalyptus* leaf extracts was also reflected in the current study, where the terpene-rich hydrodistilled *E. grandis* × *E. urophylla* extracts did not correlate with superior antibacterial activity. They generally required higher concentrations to inhibit the tested bacteria and were active against only a narrow range of bacteria compared to SCE extracts, despite SCE extracting terpenes in relatively lower abundance. Consistent with the current findings, Abbas *et al*. (2022) investigated the antibacterial activity of *E. camaldulensis* leaf extracts obtained using SCE and hydrodistillation. They found that the SCE extract had better antibacterial activity against *E. coli* (MIC: 0.083 mg/mL) and *S. aureus* (MIC: 0.129 mg/mL) compared to hydrodistillation extracts (MIC: 0.121 mg/mL for *E. coli* and 0.134 mg/mL for *S. aureus*).

Among the four SCE extracts, B10 showed the broadest antibacterial spectrum. This is likely explained by its chemical composition, which contains several major constituents previously reported to possess strong antibacterial activity. Eucalyptin exhibited inhibitory activity against methicillin-resistant *S. aureus, B. cereus, E. faecalis*, and *P. acnes*, with MIC values ranging from 0.001 to 0.004 mg/mL in the study of Takahashi, Kokubo and Sakaino (2004). 1-Hexacosanol has been reported to show inhibitory effects against *S. aureus, P. aeruginosa*, and *E. coli* (Oscar *et al*., 2018). Spathulenol in the research of Bougatsos *et al.* (2004) also showed inhibitory activity against *S. aureus*, *S. epidermidis*, and *E. coli*, with MIC values ranging from 1.35 to 8.50 mg/mL. Moreover, Inoue *et al.* (2005) revealed that phytol induced potassium cation leakage in *S. aureus* cells by disrupting their cell membranes, thereby resulting in a clear bactericidal effect. On the other hand, contrary to previous research by Sewanu *et al*. (2015), which reported greater antibacterial effectiveness for fresh *Eucalyptus grandis* leaf hydrodistilled extract compared to dried leaf extract, our results showed comparable antibacterial efficacy between fresh and dried leaf hydrodistilled extracts. This implies that the unique minor components present at low abundances (< 1%) in dried leaf extracts may not play a key role in antibacterial activity. Similarly, no pronounced difference was observed between extracts obtained from the leaves of younger and older trees, indicating that tree age had minimal influence on antibacterial activity.

5.6 Antifungal Activity of *Eucalyptus* Leaf Extracts

Limited studies have examined the antifungal activity of *E. grandis* × *E. urophylla* extracts against common human pathogenic fungal strains. In the agar dilution assay conducted by Salvatori *et al.* (2023), steam-distilled *E. grandis* × *E. urophylla* extracts showed inhibitory activity against *C. albicans*, with an MIC of 0.312% and an MBC of 0.625%. Comparisons were also made with the parental species, *E. grandis*. Notably, Chikowe, Mpala and Cock (2020) reported that water extract of *E. grandis* leaves had no activity against *C. albicans*, while in this study, the extracts exhibited MIC values ranging from 0.31 to 1.25 mg/mL, suggesting enhanced antifungal properties in the *Eucalyptus* hybrid. When compared to the broader literature, the present extracts exhibited more potent antifungal activity than hydrodistilled *E. globulus* leaf extract. Specifically, *E. globulus* leaf extract inhibited *C. albicans*, *C. parapsilosis*, *C. neoformans*, and *T. rubrum* at 2.50 to 5.00 mg/mL (Moreira *et al.*, 2022), whereas our extracts achieved the same effect at 0.04 to 1.25 mg/mL.

The current findings suggest that SCE, especially with ethanol as a co-solvent, is a superior method for producing *Eucalyptus* leaf extracts that are active against yeasts. Other studies have also highlighted differences in antifungal efficacy of plant extracts based on extraction methods. For instance, *Cymbopogon flexuosus* (lemongrass) extracts isolated using hydrodistillation and SCE showed potential antifungal effects against nine fungal strains, including *C. parapsilosis*, *C. auris, C. albicans*, *C. tropicalis*, *N. glabratus*, and *T. rubrum,* with the

hydrodistilled extract showing comparable or slightly lower MIC values than SCE-based extracts (Jaradat, 2023).

Furthermore, the antifungal activity of *Eucalyptus* leaf extracts is attributed to their bioactive compounds, which have been documented to disrupt fungal cell membranes and interfere with metabolic processes. For instance, Singh *et al.* (2024) revealed that eucalyptol induced the production of reactive oxygen species, which triggered oxidative damage to cellular components, including lipids, RNA, DNA, and proteins, ultimately leading to fungal cell death. In addition, eucalyptol has been shown to destabilise monolayers formed by phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and ergosterol which are lipids and sterols commonly found in fungal membranes (Hąc-Wydro *et al*., 2017; Połeć *et al*., 2019). These findings highlight that eucalyptol may incorporate into the fungal membrane, thereby altering its organisation. Moreover, Konuk and Ergüden (2022) demonstrated the ability of α-pinene to disrupt yeast cell membrane integrity, leading to ion leakage. The lipophilic properties of aromadendrene allow it to disrupt biomembrane permeability, while its exocyclic methylene group and cyclopropane ring enable it to alkylate proteins, leading to conformational disturbances (Mulyaningsih *et al*., 2011). Globulol has also been reported to inhibit fungal N-myristoyl transferase, an essential enzyme for fatty acid translocation and membrane structure modification (Khuntia *et al*., 2023). Notably, *E. grandis* × *E. urophylla* SCE extract was found to inhibit the expression of enzymes involved in fungal metabolism and cell wall biosynthesis, indicating that components of

Eucalyptus leaf extracts may compromise both fungal energy production and cell wall integrity (Zhou *et al*., 2016).

While *Eucalyptus* leaf extracts do contain antifungal constituents, compound interactions also play an important role in determining the overall antifungal activity. In this study, the *Eucalyptus* leaf extracts did not exhibit inhibitory activity against *A. fumigatus*. However, Vaičiulytė *et al*. (2021) reported a low MIC value of 0.002 mg/mL for *α*-terpinyl acetate, while Alves *et al*. (2019) documented MICs of 1.100 mg/mL and 8.900 mg/mL for *α*-pinene and eucalyptol, respectively, against *A. fumigatus*. For *C. albicans*, the MICs of the current *Eucalyptus* leaf extracts ranged from 0.31 to 1.25 mg/mL, which is lower than the 8.90 mg/mL reported for eucalyptol (Alves *et al*., 2019), comparable to the 0.570 to 1.110 mg/mL reported for *α*-pinene (Alves *et al*., 2019), but much higher than the 0.008 mg/mL reported for *α*-terpinyl acetate (Vaičiulytė *et al*., 2021). The differences in the antifungal efficacy between pure components with the extracts strongly suggest the possibility of both synergistic and antagonistic interactions among the various compounds present in the complex mixture of *Eucalyptus* leaf extracts.

Consistent with the antibacterial results, superior anti-yeast activity was observed for SCE extracts compared to hydrodistillation extracts. This superior antifungal activity is likely due to the diverse range of constituents extracted by

SCE. Long-chain fatty alcohols under the class of polycosanols, including octacosanol in B and 1-hexacosanol in both B and B10, have been documented for their antifungal activity. Specifically, octacosanol was effective against *C. neoformans*, *T. ajelloi*, and *T. rubrum* with MFCs ranging from 4.0 to 125.0 µg/mL (Tchakam *et al*., 2012), while 1-hexacosanol killed *C. krusei* at a concentration of 250.0 µg/mL (Tcheuko *et al*., 2021). Furthermore, eucalyptin, the dominant flavonoid identified in B and B10, was reported by Takahashi, Kokubo and Sakaino (2004) to inhibit *Trichophyton mentagrophytes* with an MIC of 0.031 mg/mL. Notably, the monoterpenoids borneol and *α*-terpinyl acetate, present in all SCE extracts, demonstrated antifungal effects against *C. albicans*, with MICs ranging from 0.008 to 2.000 mg/mL (Liu *et al*., 2017; Vaičiulytė *et al*., 2021; Wang, Liu and Zhan, 2022). Moreover, Fernandes *et al.* (2023) reported the antifungal efficacy of spathulenol against *C. neoformans*, with MIC of 0.200 mg/mL. Likewise, phytol, the major diterpenoid in B and B10, inhibited the growth of *C. albicans* at an MIC of 0.063 mg/mL and showed binding affinity to *C. albicans* lanosterol synthase, suggesting its potential to inhibit ergosterol synthesis (Ghaneian *et al*., 2015; de Souza *et al*., 2022). The saturated fatty acid, octadecanoic acid, which predominated in A, A10, and B, has been found to inhibit *C. albicans* at a concentration of 312.50 µg/ml (Choi *et al*., 2013). Neophytadiene, another unique diterpene detected in B10, was also predominant in *Apium graveolens* (wild celery) aerial extracts (34.60%), which demonstrated antifungal activity against *C. neoformans, Candida*, *Aspergillus,* and *Trichophyton* strains, with MIC values ranging from 0.04 to 0.64 µg/mL (Marongiu *et al*., 2012).

In contrast, the tested filamentous fungi in this study displayed different susceptibility patterns. *Trichophyton rubrum* was more susceptible to hydrodistillation extracts than SCE extracts. The variation in susceptibility between yeasts and filamentous fungi to *Eucalyptus* leaf extracts may be attributed to their cell wall compositional differences. Chitin accounts for only 1% to 2% of the yeast cell wall, which is lower than 10% to 20% in filamentous fungi (Bowman and Free, 2006). Therefore, the susceptibility of *T. rubrum* suggests that hydrodistillation extracts may contain antifungal constituents that target chitin. Monoterpenes such as limonene and α-pinene, together with the monoterpenoids α-terpinyl acetate, borneol, and eucalyptol, which are abundant in hydrodistillation extracts, have been reported to exhibit binding affinities ranging from -5.569 to -6.352 kcal/mol toward VdCHS1, a chitin synthase enzyme (Azenzem, Koussa and Alfeddy, 2025). Besides, *A. fumigatus* was resistant to both SCE and hydrodistillation extracts due to its rigid and complex cell wall, which contains not only chitin but also β-1,3-glucan cross-linked to α-1,3-glucan, β-1,3-1,4-glucans, galactosaminogalactan, and galactomannan. Additionally, the rodlet layer of its conidial cell wall consists of hydrophobins and dihydroxynaphthalene-melanin to form a hydrophobic protective barrier against bioactive molecules (Garcia-Rubio *et al*., 2020).

Interestingly, a study conducted by Vaičiulytė *et al*. (2021) reported a remarkably low MFC of 0.0016 mg/mL for *Thymus pulegioides* (lemon thyme)

hydrodistillation extract against *T. rubrum*, demonstrating an even stronger antifungal effect than the standard drug, itraconazole. This remarkable activity was attributed to its high *α*-terpinyl acetate content (64%), evidenced by the ability of this component to kill *T. rubrum* at a concentration as low as 0.0004 mg/mL in the microdilution assay. In present study, the same strain of *T. rubrum* (ATCC® 28188™) was sensitive to all eight *Eucalyptus* leaf extracts tested, with the lowest MIC being recorded at 0.04 mg/mL (Table 4.11). Remarkably, *α*-terpinyl acetate was the only component consistently present in noticeable amounts across all the extracts, further supporting that the observed sensitivity of *T. rubrum* could also be linked to the presence of *α*-terpinyl acetate.

5.7 Mosquito Larvicidal Activity of *Eucalyptus* Leaf Extracts

Aedes aegypti larvicidal activity of leaf extracts from *E. grandis* × *E. urophylla* was previously reported by Gallon *et al.* (2020). At 100 µg/mL, their hydrodistillation extracts caused 100% mortality in third- to fourth-instar larvae after 24 h post-treatment, indicating stronger activity than the hydrodistillation extracts obtained in the present study. Comparisons with hydrodistilled extracts from parental *Eucalyptus* species further showed that the 24-h LC_{50} values against *Ae. aegypti* in this study (Table 4.12) were slightly higher than the 32.4 µg/mL reported for *E. grandis* (Lucia *et al.*, 2007), but were comparable to *E. urophylla*, which demonstrated an LC_{50} of 95.5 µg/mL (Cheng *et al.*, 2009b). In the larvicidal tests against *Ae. albopictus*, however, the LC_{50} values in the current study (Table

4.13) showed stronger larvicidal efficacy than the 285.8 µg/mL reported for *E. urophylla* from Cheng *et al.* (2009b).

Moreover, morphological deformities observed in the treated *Aedes* larvae, such as blackened midguts and elongated necks, are consistent with previous studies and may reflect underlying physiological damage. For instance, Seye *et al.* (2021) reported intestinal tissue degradation, muscular disruption, and damaged microvilli in *Ae. aegypti* larvae treated with *Cymbopogon citratus* (lemongrass) extracts. Similarly, Soonwera and Phasomkusolsil (2016) also reported multiple deformities in *Ae. aegypti* larvae treated with extracts of *C. citratus* and *Syzygium aromaticum* (clove), including elongated necks, enlarged thorax, degraded respiratory tracheae and digestive tract, which suggested possible disruption of hormonal regulation and chitin synthesis during molting process.

Based on larval mortality rates as well as LC_{50} and LC_{95} values (Section 4.8), the hydrodistilled *Eucalyptus* leaf extracts outperformed the SCE extracts in larvicidal activity against both *Ae. aegypti* and *Ae. albopictus*. Their superior effectiveness is likely related to their phytochemical composition. Hydrodistilled extracts contained a relatively higher proportion of terpenes and terpenoids previously evaluated for mosquito larvicidal activity. Specifically, Araujo *et al*. (2003) demonstrated that eucalyptol induced 100% larval mortality in *Ae. aegypti* at 100 µg/mL after 24 h, while Perumalsamy, Kim and Ahn (2009) reported

larvicidal activities of eucalyptol, limonene, α-pinene, and α-terpineol against third instar *Ae. aegypti* larvae, with LC_{50} values ranging from 24.5 to 111.8 µg/mL. Cheng *et al*. (2009a) also documented LC_{50} of 79.1 µg/mL and 74.0 µg/mL for α-pinene against *Ae. aegypti* and *Ae. albopictus* larvae, respectively. Nonetheless, the potential interaction among constituents in the extracts should not be overlooked, as variations in the mechanism of action among components may influence the overall toxicity of a combination against mosquito larvae. Sarma *et al*. (2019) found that binary combinations of eucalyptol and α-pinene at a 1:1 ratio demonstrated synergistic larvicidal activity against *Ae*. *aegypti* by achieving > 90% larval mortality after 24 h, whereas the combination of limonene with eucalyptol or α-pinene showed antagonistic effects.

In addition, B10 exhibited lower LC_{50} values against larvae of both mosquito species compared to the other SCE extracts. B10 showed relatively higher abundances of eucalyptin and 1-hexacosanol, along with the unique presence of 1,2,3-butanetriol, (1R,2R,4R)-4-(2-hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol, and neophytadiene in the non-polar column analysis. However, most of these compounds, particularly the major component eucalyptin, have not been previously investigated for mosquito larvicidal activity and thus represent a potential area to be explored. Among them, 1-hexacosanol has demonstrated *ex vivo* acetylcholinesterase inhibitory activity on larval homogenates of *Ae. aegypti* in the study of Gade *et al*. (2017). However, B, which displayed lower larvicidal activity in this study, also contained comparable proportions of this compound,

suggesting that 1-hexacosanol alone may not fully account for the larvicidal efficacy of B10. Furthermore, B10 contained a relatively high abundance of spathulenol (18.65%) in the polar column analysis. This sesquiterpenoid has been reported to kill the first instar *Ae. aegypti* larvae with an LC_{50} of 48.70 µg/mL (Ali *et al*., 2023). Similarly, ethyl palmitate, detected at a high percentage (12.97%) in B10, was previously screened for its mosquito larvicidal efficacy (Wang *et al*., 2016). It was active against third instar larvae of wild *Ae. albopictus* (LC_{50}: 52.40 µg/mL) and insecticide-susceptible *Ae. aegypti* (LC_{50}: 53.43 µg/mL). Although no studies have investigated the larvicidal activity of phytol against *Aedes* larvae, Luo *et al*. (2022) demonstrated its toxicity against *An. sinensis* larvae, with an LC_{50} of 16.03 µg/mL. Given that this diterpenoid alcohol was one of the major constituents (45.57%) of B10 in the present study and considering its prevalence in plant extracts with established mosquito larvicidal properties, such as *Ageratum houstonianum* (19.39% to 52.10%; Hadidy *et al*., 2022) and *Salvia splendens* (41.46%; Mathew and Thoppil, 2011), phytol may contribute to the larval toxicity.

5.8 Toxicity of *Eucalyptus* Leaf Extracts

The mortality trend of *A. franciscana* nauplii (Figure 4.9) showed a concentration-dependent pattern, indicating that concentration is a critical factor in determining toxicity. Tables 4.12 and 4.13 indicate that the LC_{50} values for the hydrodistillation extracts against *Aedes* mosquito larvae were lower than those for *A. franciscana* nauplii (Table 4.14). Given that the brine shrimp lethality assay is widely used to assess the toxicity of substances towards aquatic organisms, the

current findings imply that *Eucalyptus* hydrodistillation extracts demonstrate greater selectivity and toxicity towards *Ae. aegypti* and *Ae. albopictus* larvae but not towards *A. franciscana* nauplii. This highlights their potential as natural mosquito larvicides that are relatively safe for non-target aquatic ecosystems.

The *Eucalyptus* hybrid used in this study has not been previously investigated for brine shrimp toxicity. Therefore, comparisons were made with one of its parental species, *E. grandis*. All hybrid extracts were more toxic (LC_{50} between 187.43 to 222.24 µg/mL) than the methanolic and water extracts from *E. grandis* (LC_{50} > 1000 µg/mL), which were classified as non-toxic against *A. franciscana* (Chikowe, Mpala and Cock, 2020).

Furthermore, the LC_{50} value of potassium dichromate obtained in this study (20.15 µg/mL) was consistent with prior studies for *A. franciscana* nauplii, which reported values ranging from 9.12 to 37.00 µg/mL (Ocaranza-Joya *et al*., 2019; Ruiz-González *et al*., 2023; Abdolhay, Mehrdadi and Pardakhti, 2024). This finding supports the reliability and reproducibility of the brine shrimp lethality assay employed in the current study, further strengthening the accuracy of the findings and reinforcing that potassium dichromate remains a trustworthy reference point for assessing the relative safety of extracts.

CHAPTER 6

CONCLUSION

In summary, this study successfully obtained leaf extracts from *Eucalyptus grandis* × *Eucalyptus urophylla* trees of two age groups using hydrodistillation and supercritical carbon dioxide fluid extraction (SCE). SCE optimisation via response surface methodology confirmed that extraction pressure, temperature, and time significantly influenced extraction yield. The optimal conditions were identified as 29.7 MPa, 79.8°C, and 116.2 min for trees aged 17 to 31 months, while trees aged 40 to 50 months reached peak yield at 30.0 MPa, 79.6°C, and 108.6 min. In contrast, a distillation time of 6 h was sufficient for hydrodistillation to produce 92.00% to 95.77% of extracts from the leaves. Younger trees consistently produced higher yields than older trees, regardless of the extraction method or leaf condition.

Tree age influenced extraction yield but had minimal impact on the chemical composition and bioactivity compared with the extraction technique. SCE yielded leaf extracts with greater chemical variability, comprising terpenes, terpenoids, alkanes, aldehydes, alcohols, and other high-molecular-weight compounds. Conversely, 8-h hydrodistillation primarily extracted volatile terpenes and terpenoids, such as eucalyptol and α-terpinyl acetate. A notable shift from monoterpenes and monoterpenoids to sesquiterpenes and sesquiterpenoids was

observed in the chemical profiles of hydrodistillation extracts as distillation time increased from 4 to 8 h, due to the variations in boiling points and volatility of components.

The bioactivities and toxicity of the *Eucalyptus* leaf extracts were concentration-dependent. SCE extracts demonstrated superior antimicrobial efficacy against human pathogenic bacteria and fungi, whereas hydrodistillation extracts exhibited greater larvicidal toxicity against *Ae. aegypti* and *Ae. albopictus* larvae, with LC_{50} values ranging from 52.26 to 134.56 µg/mL and 67.27 to 119.79 µg/mL, respectively, after 24 h. Importantly, hydrodistillation extracts achieved effective larval mortality at concentrations lower than those required to produce toxicity in the brine shrimp toxicity assessments (LC_{50}: 209.24 to 222.24 µg/mL), suggesting a favourable safety margin for non-target aquatic organisms. The observed bioactivities are likely due to the known antimicrobial and larvicidal properties of major components, as well as the possible synergistic interactions with minor components.

As a result, it can be concluded that *Eucalyptus* leaf extracts obtained via SCE show potential as natural disinfectants, while *Eucalyptus* leaf extracts obtained via hydrodistillation have possible application as natural insecticides. These findings align with the Sustainable Development Goal (SDG) 3: Good Health and Well-Being as well as SDG 12: Responsible Consumption and Production. The

investigation into the antimicrobial and larvicidal potential of *E. grandis* × *E. urophylla* leaf extracts offers a low-risk alternative to synthetic chemicals for reducing the spread of infectious and vector-borne diseases. Furthermore, the valorisation of agro-industrial wastes such as *Eucalyptus* leaves into natural disinfectants and insecticides encourages the sustainable application of natural resources in Malaysia.

6.1 Limitations of Study

This study has several limitations. Firstly, it examined only two age groups of *Eucalyptus* trees, with a relatively narrow age gap between them (17 to 31 months versus 40 to 50 months). This limited age range may have constrained the detection of more pronounced age-related variations in extraction yield, chemical composition, and bioactivity. Secondly, the toxicity screening employed *Artemia franciscana* (brine shrimp) as a non-human model. Although the brine shrimp lethality assay is rapid and cost-effective, its results may not fully represent complex human toxicological responses due to inherent biological differences between invertebrate and mammalian systems. Additionally, given the chemical complexity of *Eucalyptus* leaf extracts, the individual constituents in the extracts were not extensively evaluated, leaving uncertainties regarding their safety profile. Another key limitation of this study is the absence of molecular analysis to elucidate the mechanisms underlying the observed antimicrobial and larvicidal effects. While the extracts demonstrated microbicidal effects based on MBC and MFC determinations, additional confirmation using imaging techniques is essential.

Consequently, the translational potential of the current findings is confined to *in vitro* observations and requires further validation to outline the possible application of the *Eucalyptus* leaf extracts.

6.2 Further Works

Future research should consider expanding the age range of *Eucalyptus* trees for leaf collection to provide a more comprehensive understanding of how tree age influences plant metabolites. In addition, incorporating human cell lines, such as skin or lung cells, in subsequent toxicity assessments would yield more accurate insights into potential effects on human health and assist in defining appropriate application pathways. Furthermore, isolation techniques are recommended to identify potential toxic compounds, thereby clarifying whether the removal of specific constituents is necessary prior to application. To translate the current findings toward practical application, advanced imaging techniques, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM), should be employed to visualise the morphological and structural deformities in treated microbes and mosquito larvae, providing evidence of the extent of damage. Collectively, these approaches could enhance the reliability of safety evaluations, deepen the understanding of underlying mechanisms, and confirm the intended use of *Eucalyptus* leaf extracts as natural insecticides and disinfectants, ultimately supporting the development of effective formulations.

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APPENDIX A

Table A1: List of chemicals and media with their respective manufacturers.

Chemicals/ Media	Manufacturers
Acetone, spectroscopy grade	Merck, Germany
Alkanes (C7 – C40)	Sigma-Aldrich, USA
α-Pinene	Sigma Aldrich, USA
α-Terpineol	Sigma Aldrich, USA
Aromadendrene	ChemFaces, China
Borneol	Sigma Aldrich, USA
Artemia franciscana cyst	Great Salt Lake Inc., USA
Artemia PKC-Nutri+® food	Universiti Malaysia Terengganu, Malaysia
Carbon dioxide gas	Linde, Malaysia
Chloramphenicol	Duchefa Biochemie, Netherlands
Dimethyl sulfoxide, AR grade	Fisher Scientific, UK
Ethanol, AR grade	Rank Synergy, Malaysia
Eucalyptol	Sigma Aldrich, USA
5-Fluorocytosine	Acros Organics, China
Helium gas	Linde, Malaysia
Hexane, HPLC grade	Fisher Scientific, Korea
Itraconazole	Sigma Aldrich, USA
Methanol, AR grade	Rank Synergy, Malaysia
3-Morpholinopropanesulfonic acid	Bio Basic, Canada
Mueller-Hinton agar	HiMedia, India
Mueller-Hinton broth	HiMedia, India
Iodonitrotetrazolium chloride	Sigma Aldrich, USA
Oatmeal agar	Laboratorios Conda S.A., Spain
Potassium dichromate	Systerm Chemical, Malaysia
Potato dextrose agar	Liofilchem, Italy
Roswell Park Memorial Institute-1640 medium with L-glutamine, without bicarbonate	Biowest, France
R-(+)-Limonene	Sigma Aldrich, USA
Instant Ocean® sea salt	Spectrum Brands, USA
Sodium chloride	John Kollin Corporation, USA
Sodium sulphate	Merck, Germany
Temephos	Dr. Ehrenstorfer GmbH, Germany
(-)-*trans*-Caryophyllene	Sigma Aldrich, USA
Trypan blue, 0.4%	Invitrogen, New Zealand
Tween 80	Systerm Chemical, Malaysia

Table A2: Equipment used with their respective manufacturers.

Equipment	Manufacturers
Aluminium foil	Diamond, Malaysia
Autoclave machine	Hirayama, Japan
Analytical balance	OHAUS Corporation, USA
Bacterial incubator	Ecocell, Germany
Biosafety cabinet	Telstar, Spain
Bunsen burner	Portagaz, China
Compound microscope	Leica DM500, Germany
5% diphenyl-95% dimethyl polysiloxane capillary column	Shimadzu, SH-I-5Sil MS, Japan
Densitometer	Grant Instruments, England
Distillation chiller	BÜCHI, Switzerland
Filter top, 500 mL (0.22 µm, polyethersulfone membrane)	Techno Plastic Products AG, Switzerland
Fungal incubator	Memmert, Germany
Gas chromatography–mass spectrometer	Shimadzu, QP2020 NX, Japan
Haemocytometer	Hecht Glaswarenfabrik GmbH & Co KG, Germany
Hot plate	LMS, Japan
Induction cooker	Philips, China
Inverted microscope	Nikon, Eclipse Ts2, Japan
Laminar flow cabinet	Esco Lifesciences, Singapore
Microplate Reader	Thermo Fisher Scientific, USA
Oven	Memmert, Germany
pH meter	Eutech Instruments, Singapore
UV/Vis Spectrophotometer	Thermo Fisher Scientific, USA
Test sieve, 250 µm	Retsch Gmbh, Germany
Stereo microscope	Motic, SMZ-16, China
Supercritical fluid extraction set	Jasco, Japan
Stainless-steel distiller, 10 L	Laboratory & Scientific Enterprise, Malaysia
Syringe filter (0.45 µm, nylon)	Bioflow Lifescience, Malaysia
Ultrasonic water bath	Crest Group, USA
Vortex mixer	VELP Scientifica, Italy

Table A3: Fresh weights of *Eucalyptus grandis* × *Eucalyptus urophylla* leaves collected on seven different days.

Location	Collection date	Leaf sample weight (kg)
Blau	16th February 2023	0.8 kg
	12th July 2023	1.2 kg
	20th September 2023	1.0 kg
	4th January 2024	3.5 kg
	25th April 2024	6.0 kg
Pulai	9th March 2023	1.5 kg
	17th October 2023	3.0 kg
	4th January 2024	5.0 kg

APPENDIX B

Reagent and media preparations

Agar Preparation

Mueller-Hinton agar (MHA) was prepared for the antibacterial assays. A total of 38 g of MHA powder was dissolved in 1 L of distilled water. For the antifungal assays, potato dextrose agar (PDA) was used for yeast and *A. fumigatus* whereas oatmeal agar was used for *T. rubrum*. PDA and oatmeal agar were prepared by dissolving 42 g and 72.5 g of powder, respectively, in 1 L of distilled water. Heating with frequent agitation, followed by boiling for 1 min were required to completely dissolve the oatmeal agar powder. Once the agar powders were mixed thoroughly in the distilled water, they were autoclaved at 121℃ for 15 min to sterilise them before pouring into petri dishes.

Broth Medium Preparation

The broth medium for antibacterial assay was Mueller-Hinton broth (MHB) while RPMI-1640 was used as broth medium in antifungal assay. For 1 L of MHB, 21 g of MHB powder was dissolved in 1 L of distilled water and autoclaved at 121 ºC for 15 min. For 1 L of RPMI-1640, 20.8 g of RPMI-1640 powder supplemented with L-glutamine was added to 69.08 g of MOPS. The mixture was dissolved in 0.80 L of distilled water, adjusted to pH 7.0 using 0.1 M sodium hydroxide solution,

and sterilised by membrane filtration using 0.22 µm polyethersulfone membrane filter top.

Growth Indicator Preparation

Four mg of iodonitrotetrazolium chloride was dissolved in 10 mL of distilled water and filter-sterilised using a 0.45 µm nylon syringe filter. The prepared solution was stored at 4°C until use.

Chloramphenicol Preparation

Chloramphenicol of 512 mg was dissolved in 10 mL of 95% ethanol to generate a 51200 µg/mL drug solution and sterilised using a 0.45 µm syringe filter. The drug solution was then diluted with MHB to generate a 512 µg/mL stock.

Antifungal Drugs Preparation

5-Fluorocytosine (32 mg) was dissolved in distilled water (10 mL), while itraconazole (32 mg) was dissolved in dimethyl sulfoxide (10 mL) to reach a concentration of 3200 µg/mL. The solutions were sterilised using a 0.45 µm syringe filter and diluted with RPMI-1640 to produce a 32 µg/mL stock for yeast and a 128 µg/mL stock for filamentous fungi, respectively.

Saline Preparation

Sodium chloride of 0.85 g was weighed and added into 100 mL distilled water. The mixture was autoclaved to generate a sterile saline.

Temephos Preparation

Temephos (60 mg) was dissolved in 6 mL of distilled water to produce a 10,000 µg/mL stock solution. To obtain the working concentration of 100 µg/mL, 1 mL of the stock solution was diluted with 99 mL of distilled water.

Artificial Seawater Preparation

Artificial seawater was prepared by mixing 35 g Instant Ocean® sea salt in 1 L of distilled water.

Potassium Dichromate Preparation

Potassium dichromate of 60 mg was weighed and dissolved in 6 mL of 10% ethanol to form a 10,000 µg/mL stock solution, which then diluted with distilled water to to produce 1, 10, 100, 500, and 1000 µg/mL solution.

APPENDIX C

Statistical analysis employed in this study.

Table C1: Three-way ANOVA for SCE of *Eucalyptus* leaves obtained from trees aged 17 to 31 months.

Source	Sum of squares	df	Mean square	F-value	*p*-value
Model	0.1260	9	0.0140	14.79	0.0001
X-Pressure	0.0510	1	0.0510	53.87	< 0.0001
Y-Temperature	0.0049	1	0.0049	5.21	0.0456
Z-Time	0.0360	1	0.0360	38.04	0.0001
XY	0.0066	1	0.0066	6.99	0.0246
XZ	0.0004	1	0.0004	0.3852	0.5487
YZ	0.0133	1	0.0133	14.04	0.0038
X^2	0.0035	1	0.0035	3.65	0.0850
Y^2	0.0003	1	0.0003	0.3232	0.5823
Z^2	0.0026	1	0.0026	2.70	0.1317
Residual	0.0095	10	0.0009		
Lack of Fit	0.0029	5	0.0006	0.4331	0.8101
Pure Error	0.0066	5	0.0013		
Corrected Total	0.1354	19			
R^2	0.9301				

Table C2: Three-way ANOVA for SCE of *Eucalyptus* leaves obtained from trees aged 40 to 50 months.

Source	Sum of squares	df	Mean square	F-value	*p*-value
Model	0.0476	9	0.0053	40.65	< 0.0001
X-Pressure	0.0203	1	0.0203	155.61	< 0.0001
Y-Temperature	0.0049	1	0.0049	37.87	0.0001
Z-Time	0.0168	1	0.0168	129.18	< 0.0001
XY	0.0001	1	0.0001	0.6494	0.4391
XZ	0.0011	1	0.0011	8.49	0.0155
YZ	0.0000	1	0.0000	0.0038	0.9518
X^2	0.0004	1	0.0004	3.04	0.1117
Y^2	0.0001	1	0.0001	0.7608	0.4035
Z^2	0.0028	1	0.0028	21.64	0.0009
Residual	0.0013	10	0.0001		
Lack of Fit	0.0006	5	0.0001	0.8608	0.5633
Pure Error	0.0007	5	0.0001		
Corrected Total	0.0489	19			
R^2	0.9734				

Table C3: Aligned Rank Transform ANOVA for the yields of fresh leaf hydrodistillation.

Source	Degree of freedom	F-value	Pr(>F)	Sig.
Time	2	33.491	< 0.001	< 0.001
Age	1	37.278	< 0.001	< 0.001
Time * Age	2	74.000	< 0.001	< 0.001

Table C4: Aligned Rank Transform ANOVA for the yields of dried leaf hydrodistillation.

Source	Degree of freedom	F-value	Pr(>F)	Sig.
Time	2	12.474	0.001	0.001
Age	1	38.145	< 0.001	< 0.001
Time * Age	2	43.570	< 0.001	< 0.001

Table C5: Four-Way ANOVA for mosquito larval mortality.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1071768.580[a]	159	6740.683	153.253	0.000
Intercept	798868.167	1	798868.167	18162.734	0.000
Extract	493101.172	7	70443.025	1601.563	0.000
Concentration	306925.412	4	76731.353	1744.532	< 0.001
Time	19375.456	1	19375.456	440.512	< 0.001
Species	2035.633	1	2035.633	46.281	< 0.001
Extract * Concentration	164617.312	28	5879.190	133.667	< 0.001
Extract * Time	5253.030	7	750.433	17.062	< 0.001
Extract * Species	16042.648	7	2291.807	52.106	< 0.001
Concentration * Time	2228.750	4	557.187	12.668	< 0.001
Concentration * Species	3587.686	4	896.921	20.392	< 0.001
Time * Species	1355.492	1	1355.492	30.818	< 0.001
Extract * Concentration * Time	22140.199	28	790.721	17.978	< 0.001
Extract * Concentration * Species	25597.218	28	914.186	20.785	< 0.001
Extract * Time * Species	1750.368	7	250.053	5.685	< 0.001
Concentration * Time * Species	900.190	4	225.048	5.117	< 0.001
Extract * Concentration * Time * Species	6858.015	28	244.929	5.569	< 0.001
Error	21112.279	480	43.984		
Total	1891749.026	640			
Corrected Total	1092880.859	639			

a. R Squared = 0.981 (Adjusted R Squared = 0.974)

Table C6: Two-Way ANOVA for the mortality of brine shrimp nauplii.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	258900.813[a]	39	6638.482	429.601	< 0.001
Intercept	195661.739	1	195661.739	12661.998	< 0.001
Concentration	254413.621	4	63603.405	4116.013	< 0.001
Extract	27.068	7	3.867	0.250	0.971
Concentration * Extract	4460.123	28	159.290	10.308	< 0.001
Error	1236.214	80	15.453		
Total	455798.765	120			
Corrected Total	260137.027	119			

a. R Squared = 0.995 (Adjusted R Squared = 0.993)

Table C7: Probit analysis of five *Eucalyptus* leaf extracts against *Aedes aegypti* larvae after 24 h and 48 h post-treatments.

Extracts	Time (h)	Chi Square, χ^2	Degree of freedom	*p*-value
B10	24	10.374	3	0.016
	48	9.638	3	0.022
HfA	24	5.426	3	0.143
	48	1.950	3	0.583
HdA	24	0.392	3	0.942
	48	4.380	3	0.223
HfB	24	12.062	3	0.007
	48	17.162	3	< 0.001
HdB	24	22.919	3	< 0.001
	48	14.304	3	0.003

Heterogeneity factor is used in the calculation of confidence limits when *p*-value < 0.150.

B10: Ethanol-assisted SCE of older *Eucalyptus*
HfA: Fresh leaf from younger *Eucalyptus*
HdA: Dried leaf from younger *Eucalyptus*
HfB: Fresh leaf from older *Eucalyptus*
HdB: Dried leaf from older *Eucalyptus*

Table C8: Probit analysis of five *Eucalyptus* leaf extracts against *Aedes albopictus* larvae after 24 h and 48 h post-treatments.

Extracts	Time (h)	Chi Square, χ^2	Degree of freedom	*p*-value
B10	48	8.012	3	0.046
HfA	24	17.189	3	< 0.001
	48	23.837	3	< 0.001
HdA	24	0.878	3	0.831
	48	6.173	3	0.103
HfB	24	1.611	3	0.657
	48	12.719	3	0.005
HdB	24	0.946	3	0.814
	48	0.166	3	0.983

Heterogeneity factor is used in the calculation of confidence limits when *p*-value < 0.150.

B10: Ethanol-assisted SCE of older *Eucalyptus*
HfA: Fresh leaf from younger *Eucalyptus*
HdA: Dried leaf from younger *Eucalyptus*
HfB: Fresh leaf from older *Eucalyptus*
HdB: Dried leaf from older *Eucalyptus*

Table C9: Probit analysis of eight *Eucalyptus* leaf extracts against *Artemia franciscana* nauplii after 24 h post-treatment.

Extracts	Chi Square, χ^2	Degree of freedom	*p*-value
A	0.989	3	0.804
A10	2.009	3	0.571
B	0.535	3	0.911
B10	1.824	3	0.610
HfA	2.363	3	0.500
HdA	2.363	3	0.500
HfB	2.363	3	0.500
HdB	0.989	3	0.804
Potassium dichromate	2.302	3	0.512

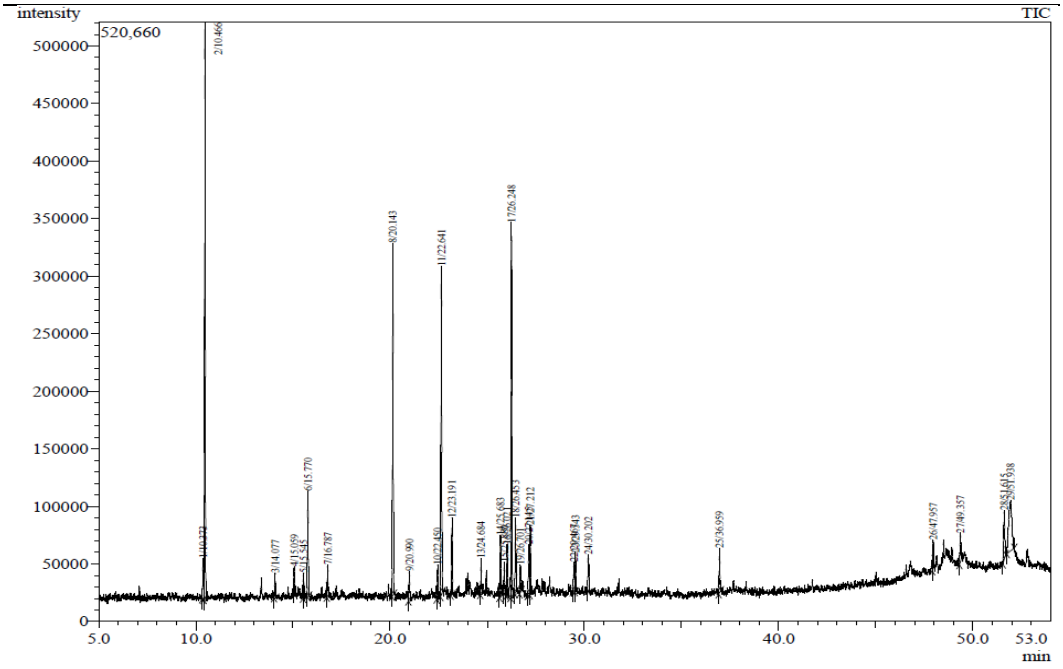
No heterogeneity factor is used in the calculation of confidence limits as *p*-values > 0.150

A: SCE of younger *Eucalyptus*
A10: Ethanol-assisted SCE of younger *Eucalyptus*
B: SCE of older *Eucalyptus*
B10: Ethanol-assisted SCE of older *Eucalyptus*
HfA: Fresh leaf from younger *Eucalyptus*
HdA: Dried leaf from younger *Eucalyptus*
HfB: Fresh leaf from older *Eucalyptus*
HdB: Dried leaf from older *Eucalyptus*

APPENDIX D

Chemical composition and total ion chromatogram of all extracted samples.

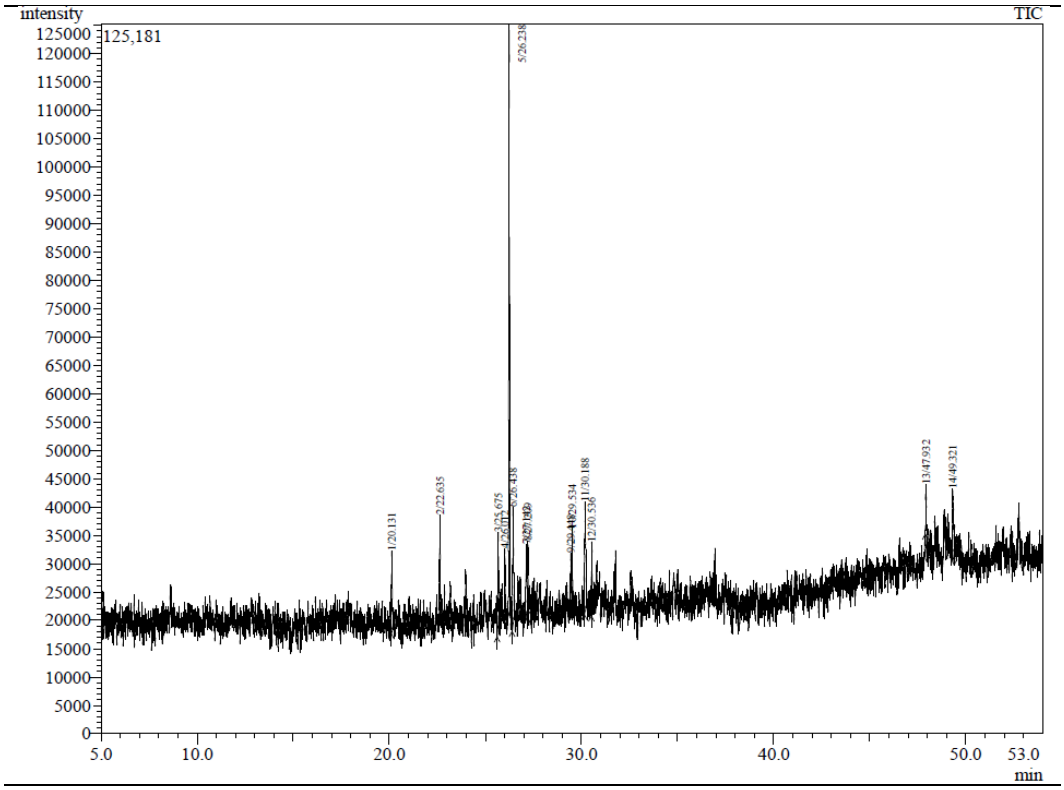
Table D1: Chemical composition and total ion chromatogram of A analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	10.373	1029	90756	1.33	Limonene	94
2	10.466	1032	1282262	18.82	Eucalyptol	96
3	14.077	1140	60866	0.89	Sabinol	89
4	15.059	1171	58722	0.86	Borneol	94
5	15.545	1187	58907	0.86	*trans-p*-Mentha-1(7),8-dien-2-ol	90
6	15.770	1194	231600	3.40	α-Terpineol	96
7	16.787	1228	75606	1.11	Isocarveol	90
8	20.143	1346	739989	10.86	α-Terpinyl acetate	95
9	20.990	1377	85599	1.26	α-Cubebene	92
10	22.450	1433	88452	1.30	β-Gurjunene	96
11	22.641	1441	738132	10.83	Aromadendrene	96
12	23.191	1462	173715	2.55	Humulen-(v1)	92
13	24.684	1522	78042	1.15	*trans*-Calamenene	91
14	25.683	1564	141714	2.08	Epiglobulol	95

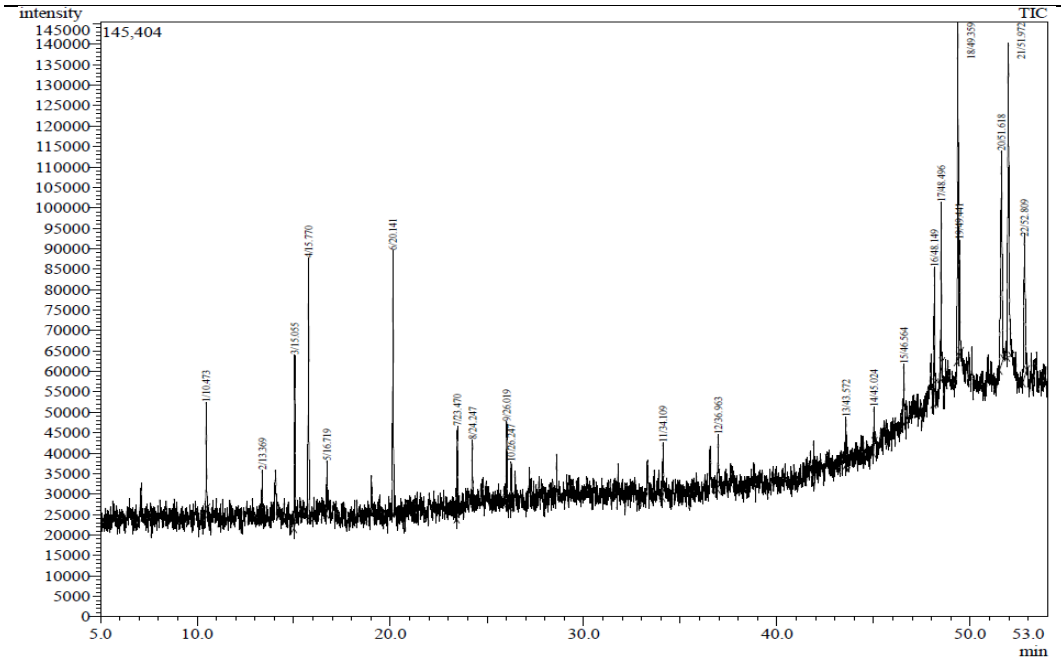
15	25.880	1572	84437	1.24	Ledol	87
16	26.021	1578	127207	1.87	Spathulenol	94
17	26.248	1588	864391	12.69	Globulol	95
18	26.453	1596	202170	2.97	Viridiflorol	92
19	26.701	1607	88332	1.30	Unknown	-
20	27.145	1627	150858	2.21	Rosifoliol	87
21	27.212	1630	163344	2.40	1,10-Di-epi-cubenol	94
22	29.467	1731	73460	1.08	Unknown	-
23	29.543	1734	85534	1.26	Unknown	-
24	30.202	1765	126571	1.86	Unknown	-
25	36.959	2107	107237	1.57	Phytol	95
26	47.957	2799	89899	1.32	Unknown	-
27	49.357	2900	87783	1.29	Unknown	-
28	51.615	3039	160453	2.35	Octacosanal	91
29	51.938	3057	497490	7.30	Unknown	-

Table D2: Chemical composition and total ion chromatogram of A10 analysed using non-polar column.



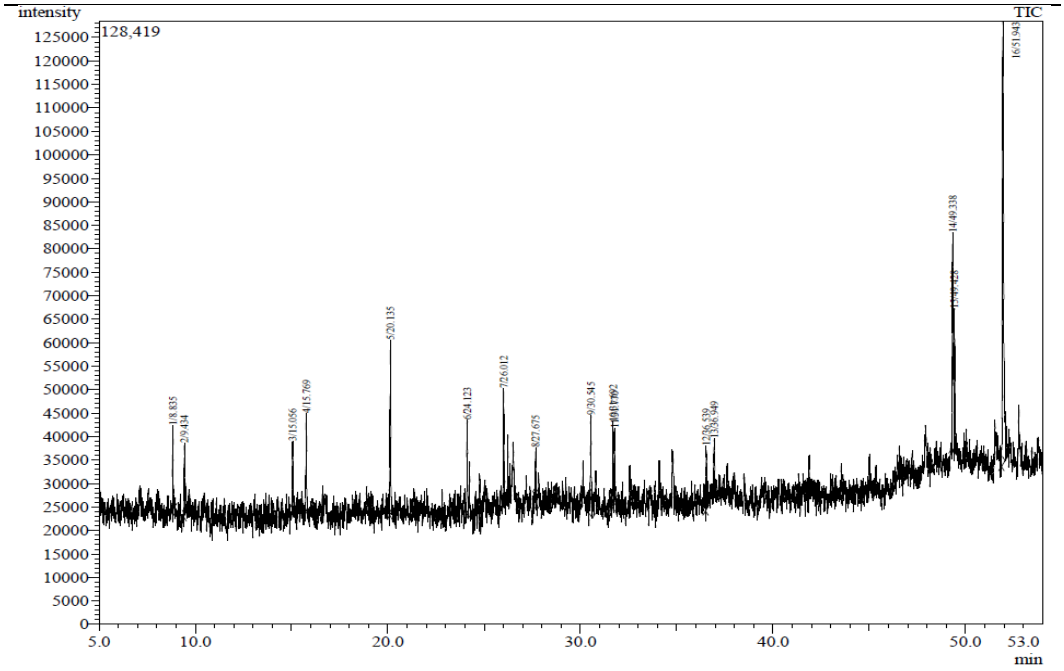
Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	20.131	1346	33067	4.25	α-Terpinyl acetate	88
2	22.635	1440	45433	5.84	Aromadendrene	94
3	25.675	1564	40845	5.25	Epiglobulol	93
4	26.012	1578	26327	3.38	Spathulenol	86
5	26.238	1587	264940	34.05	Globulol	95
6	26.438	1596	59956	7.70	Viridiflorol	90
7	27.142	1627	31853	4.09	Unknown	-
8	27.209	1629	45378	5.83	1,10-Di-epi-cubenol	87
9	29.448	1730	27413	3.52	Unknown	-
10	29.534	1734	30883	3.97	Unknown	-
11	30.188	1764	61260	7.87	Unknown	-
12	30.536	1780	32122	4.13	Unknown	-
13	47.932	2797	32307	4.15	Unknown	-
14	49.321	2897	46390	5.96	1-Heptacosanol	86

Table D3: Chemical composition and total ion chromatogram of B analysed using non-polar column.



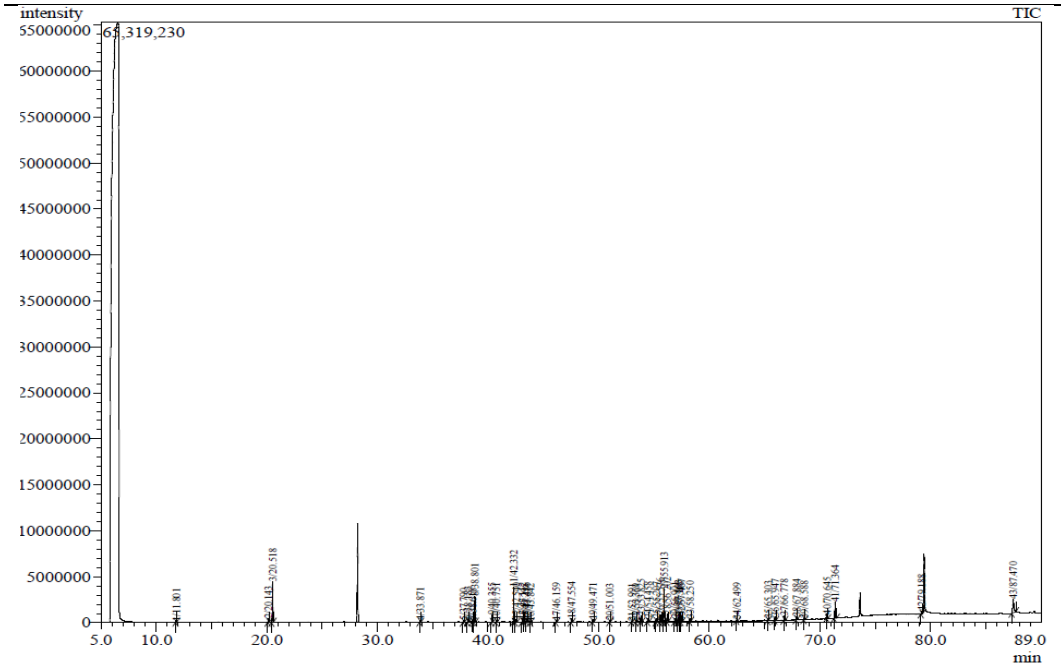
Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	10.473	1032	65278	2.96	Eucalyptol	96
2	13.369	1118	23510	1.07	Fenchol	88
3	15.055	1171	108423	4.91	Borneol	96
4	15.770	1194	150346	6.81	α-Terpineol	96
5	16.719	1226	23120	1.05	2-Hydroxycineole	89
6	20.141	1346	156419	7.09	α-Terpinyl acetate	95
7	23.470	1473	65592	2.97	1-Dodecanol	90
8	24.247	1504	42363	1.92	2,4-Di-tert-butylphenol	93
9	26.019	1578	51722	2.34	Spathulenol	94
10	26.247	1588	29475	1.34	Globulol	87
11	34.109	1956	35743	1.62	n-Hexadecanoic acid	86
12	36.963	2108	36238	1.64	Phytol	92
13	43.572	2500	35697	1.62	Unknown	-
14	45.024	2596	20162	0.91	Unknown	-
15	46.564	2700	45817	2.08	1-iodo-Docosane	86
16	48.149	2812	82669	3.75	Squalene	92
17	48.496	2837	105554	4.78	1-Hexacosanal	92
18	49.359	2900	269980	12.24	1-Hexacosanol	94
19	49.441	2905	82754	3.75	Unknown	-
20	51.618	3039	253898	11.51	Octacosanal	90
21	51.972	3058	363086	16.46	Eucalyptin	87
22	52.809	3104	158553	7.19	Octacosanol	92

Table D4: Chemical composition and total ion chromatogram of B10 analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.835	984	48898	3.93	Unknown	-
2	9.434	1001	33155	2.66	1,2,3-Butanetriol	90
3	15.056	1171	35043	2.81	Borneol	93
4	15.769	1194	51058	4.10	α-Terpineol	94
5	20.135	1346	90992	7.31	α-Terpinyl acetate	95
6	24.123	1499	56511	4.54	(1R,2R,4R)-4-(2-Hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol	92
7	26.012	1578	53299	4.28	Spathulenol	93
8	27.675	1650	18644	1.50	Unknown	-
9	30.545	1781	56219	4.52	Unknown	-
10	31.692	1836	33260	2.67	Neophytadiene	90
11	31.770	1840	31612	2.54	Hexahydrofarnesyl acetone	90
12	36.539	2085	39296	3.16	Unknown	-
13	36.949	2107	33736	2.71	Phytol	90
14	49.338	2898	155442	12.48	1-Hexacosanol	94
15	49.428	2904	100665	8.09	Unknown	-
16	51.943	3057	407233	32.71	Eucalyptin	86

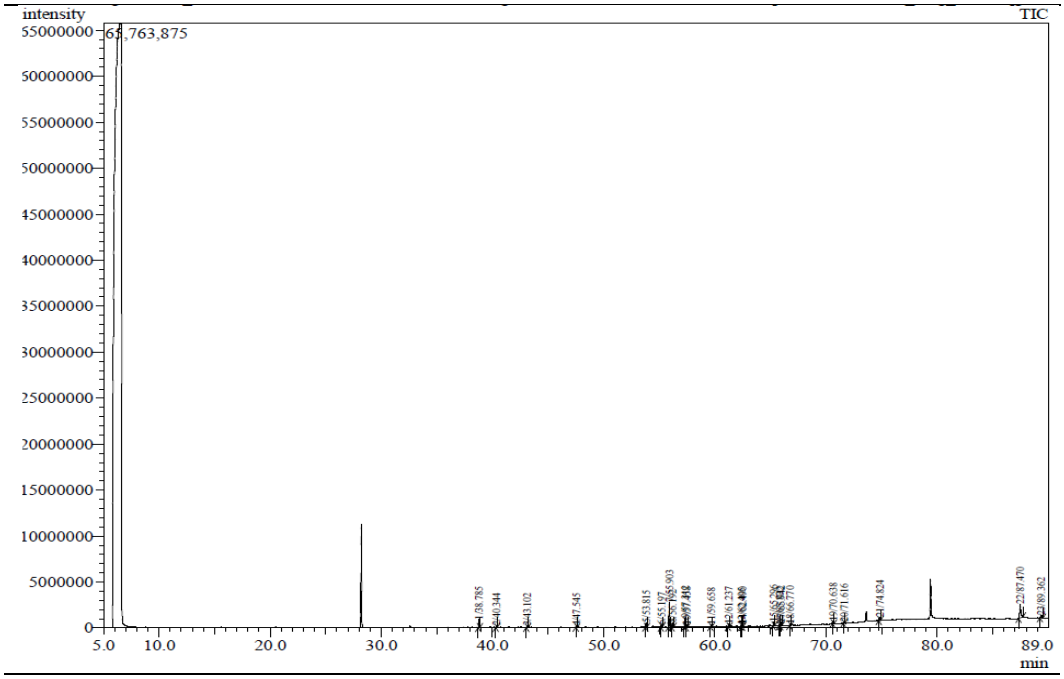
Table D5: Chemical composition and total ion chromatogram of A analysed using polar column.



Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	11.801	337519	0.29	α-Pinene	97
2	20.143	1316870	1.12	Limonene	94
3	20.518	15939037	13.56	Eucalyptol	97
4	33.871	820537	0.70	Copaene	93
5	37.790	707936	0.60	Fenchol	96
6	38.183	1185670	1.01	β-Gurjunene	94
7	38.618	902091	0.77	β-Humulene	88
8	38.801	10394233	8.84	Aromadendrene	94
9	40.355	2729067	2.32	Alloaromadendrene	95
10	40.751	856764	0.73	Pinocarveol	94
11	42.332	13686165	11.64	α-Terpinyl acetate / α-Terpineol	98 / 88
12	42.540	946307	0.81	Borneol	98
13	43.112	1085002	0.92	epi-Bicyclosesquiphellandrene	94
14	43.295	415126	0.35	β-Selinene	95
15	43.486	670859	0.57	Unknown	-
16	43.842	444319	0.38	Bicyclogermacren	93
17	46.159	816196	0.69	*trans*-*p*-mentha-1(7),8-dien-2-ol	90
18	47.554	1543046	1.31	*trans*-Calamenene	91
19	49.471	852285	0.73	Isocarveol	91
20	51.003	439183	0.37	Palustrol	91
21	52.991	423176	0.36	Unknown	-

22	53.369	605173	0.51	(1aR,3aS,7S,7aS,7bR)-1,1,3a,7-Tetramethyldecahydro-1H-cyclopropa[a]naphthalen-7-ol	91
23	53.825	2483163	2.11	Epiglobulol	95
24	54.458	1715027	1.46	Gleenol	87
25	55.207	1307878	1.11	Cubeban-11-ol	93
26	55.576	2815125	2.39	1,10-Di-epi-cubenol	96
27	55.913	13687594	11.64	Globulol	91
28	56.202	2908861	2.47	Viridiflorol	95
29	56.901	1308627	1.11	Rosifoliol	91
30	57.183	1658261	1.41	Unknown	-
31	57.318	985796	0.84	Hexahydrofarnesyl acetone	92
32	57.467	2344766	1.99	Spathulenol	94
33	58.250	1175219	1.00	Muurola-4,10(14)-dien-1β-ol	88
34	62.499	507252	0.43	Sobrerol 8-acetate	86
35	65.303	824189	0.70	Unknown	-
36	65.947	1645350	1.40	Unknown	-
37	66.778	1687937	1.44	Unknown	-
38	67.884	1173948	1.00	3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate	93
39	68.588	695117	0.59	Unknown	-
40	70.645	1703791	1.45	Unknown	-
41	71.364	6883974	5.86	Phytol	97
42	79.188	1259309	1.07	Unknown	-
43	87.470	11663297	9.92	Octadecanoic acid	94

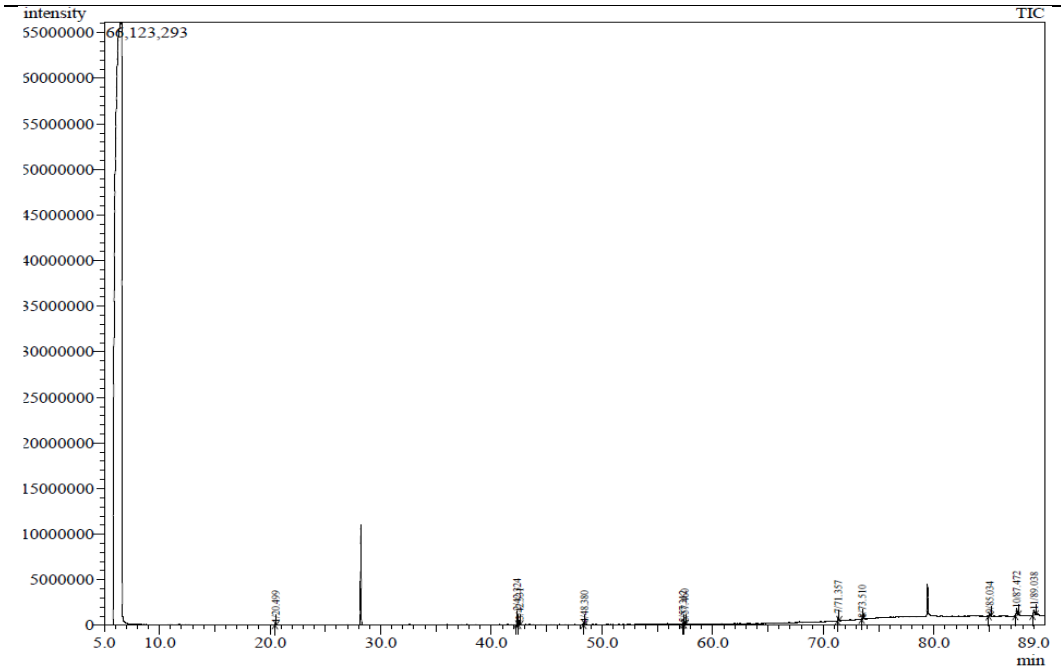
Table D6: Chemical composition and total ion chromatogram of A10 analysed using polar column.



Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	38.785	3119299	6.53	Aromadendrene	95
2	40.344	766764	1.61	Alloaromadendrene	95
3	43.102	718752	1.50	epi-Bicyclosesquiphellandrene	94
4	47.545	547919	1.15	*trans*-Calamenene	95
5	53.815	1693730	3.55	Epiglobulol	95
6	55.197	882173	1.85	Cubeban-11-ol	93
7	55.903	10028488	20.99	Globulol	91
8	56.192	1775975	3.72	Viridiflorol	95
9	57.312	3289625	6.89	Hexahydrofarnesyl acetone	93
10	57.458	1153228	2.41	Spathulenol	94
11	59.658	553359	1.16	1,2,4-Nonadecanetriol, 3TFA derivative	87
12	61.237	1116918	2.34	Ethyl palmitate	95
13	62.405	709028	1.48	Unknown	-
14	62.490	971893	2.03	Sobrerol 8-acetate	86
15	65.296	1367889	2.86	Unknown	-
16	65.832	608838	1.27	Unknown	-
17	65.942	1157249	2.42	Unknown	-
18	66.770	715782	1.50	Unknown	-
19	70.638	1238155	2.59	Unknown	-

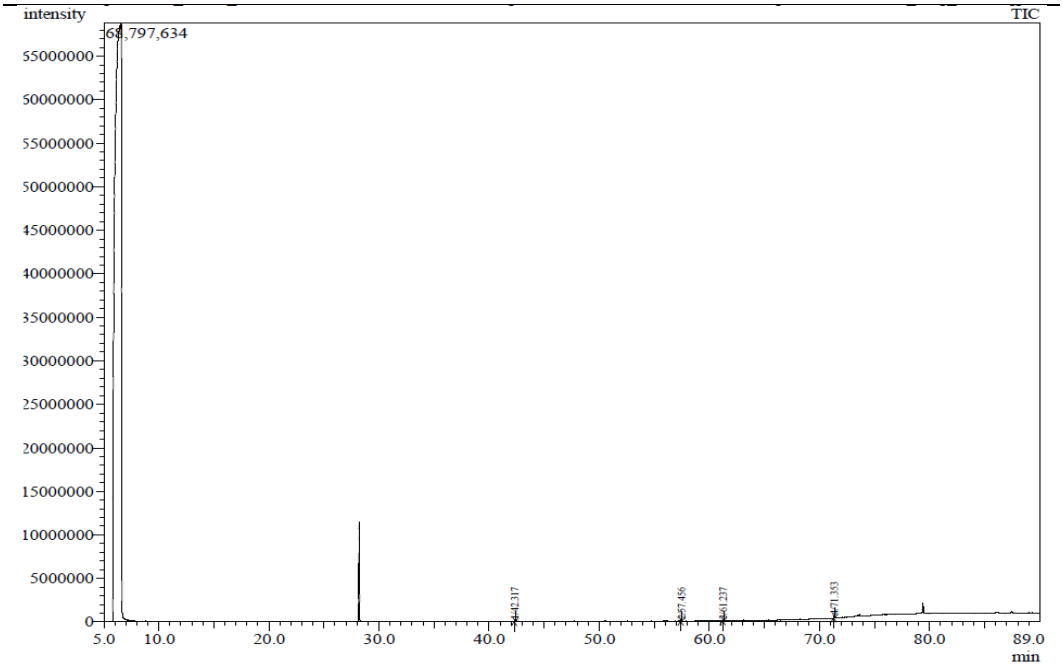
20	71.616	624869	1.31	5,5-Dimethyl-4-(3-oxobutyl)dihydro-2(3H)-furanone	93
21	74.824	1332655	2.79	(1R,2R,4R)-4-(2-Hydroxypropan-2-yl)-1-methylcyclohexane-1,2-diol	92
22	87.470	11102799	23.24	Octadecanoic acid	95
23	89.362	2293518	4.80	Unknown	-

Table D7: Chemical composition and total ion chromatogram of B analysed using polar column.



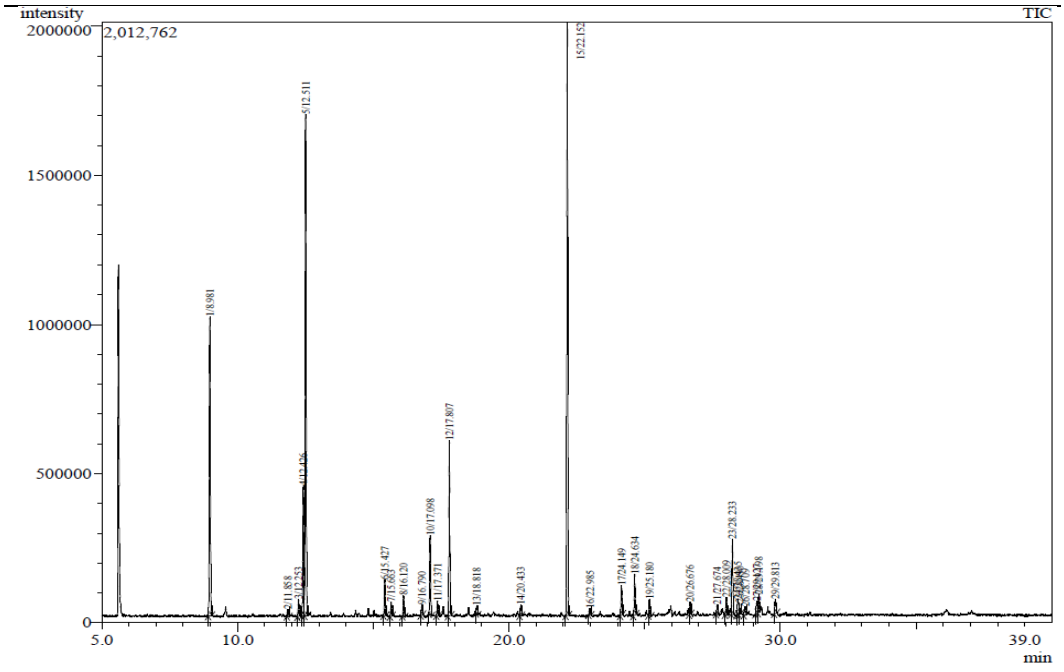
Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	20.499	714314	2.97	Eucalyptol	97
2	42.324	5361338	22.17	α-Terpinyl acetate / α-Terpineol	95 / 92
3	42.531	1803371	7.46	Borneol	98
4	48.380	547081	2.26	2-Hydroxycineole	94
5	57.312	390536	1.62	Hexahydrofarnesyl acetone	92
6	57.460	1078639	4.46	Spathulenol	94
7	71.357	2815065	11.64	Phytol	97
8	73.510	449270	1.86	Tetrapentacontane	86
9	85.034	1222081	5.05	Squalene	94
10	87.472	5467634	22.61	Octadecanoic acid	94
11	89.038	4328732	17.90	Elaidic acid	91

Table D8: Chemical composition and total ion chromatogram of B10 analysed using polar column.



Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	42.317	683935	22.81	α-Terpinyl acetate / α-Terpineol	97 / 90
2	57.456	559419	18.65	Spathulenol	92
3	61.237	389149	12.97	Ethyl palmitate	97
4	71.353	1366855	45.57	Phytol	97

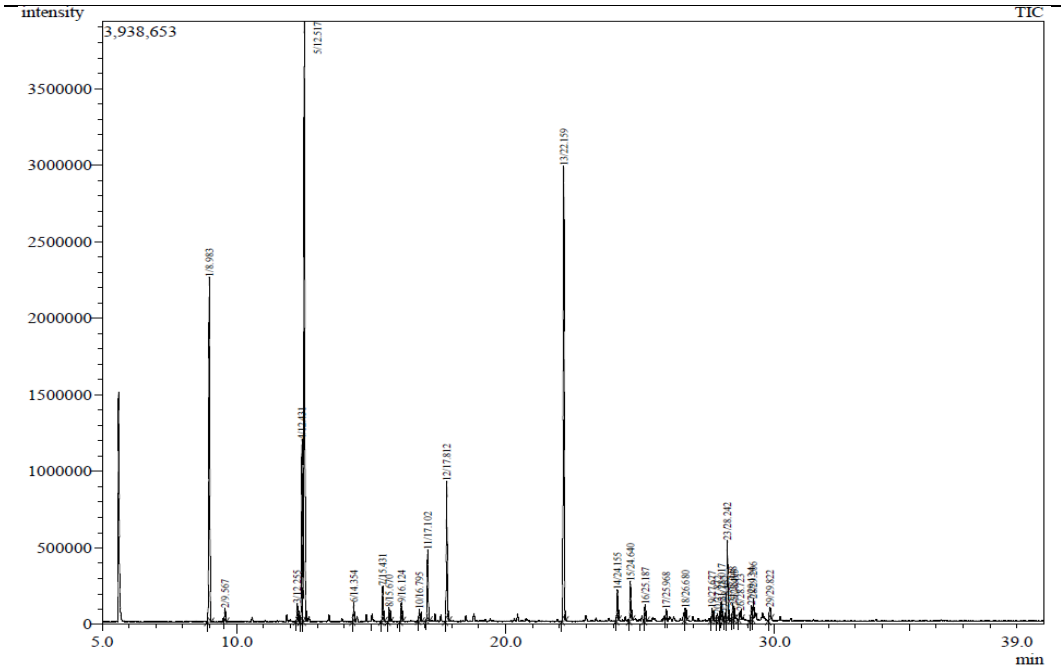
Table D9: Chemical composition and total ion chromatogram of 8-h hydrodistilled HfA analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.981	932	2586272	13.92	α-Pinene	96
2	11.858	1011	64327	0.35	Isoamyl isobutyrate	94
3	12.253	1023	139608	0.75	*o*-Cymene	96
4	12.426	1028	1098910	5.92	Limonene	95
5	12.511	1030	4247391	22.87	Eucalyptol	95
6	15.427	1117	292380	1.57	Fenchol	97
7	15.663	1125	119070	0.64	α-Campholenal	94
8	16.120	1139	179643	0.97	Sabinol	91
9	16.790	1161	77691	0.42	Pinocarvone	93
10	17.098	1170	665338	3.58	Borneol	96
11	17.371	1179	117186	0.63	Terpinen-4-ol	94
12	17.807	1193	1425590	7.67	α-Terpineol	95
13	18.818	1227	71635	0.39	Isocarveol	86
14	20.433	1283	92861	0.50	Bornyl acetate	94
15	22.152	1345	4852792	26.13	α-Terpinyl acetate	95
16	22.985	1376	66917	0.36	α-Cubebene	90
17	24.149	1421	244888	1.32	β-Caryophyllene	96
18	24.634	1440	361502	1.95	Aromadendrene	96
19	25.180	1461	136754	0.74	Humulen-(v1)	92
20	26.676	1522	98795	0.53	*trans*-Calamenene	94
21	27.674	1563	76195	0.41	Epiglobulol	95
22	28.009	1577	145730	0.78	Spathulenol	95

23	28.233	1587	649767	3.50	Globulol	95
24	28.435	1595	147148	0.79	Viridiflorol	90
25	28.490	1598	86925	0.47	Unknown	-
26	28.709	1607	107945	0.58	Rosifoliol	94
27	29.127	1625	132203	0.71	Unknown	-
28	29.198	1629	151409	0.82	1,10-Di-epi-cubenol	95
29	29.813	1656	137717	0.74	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90

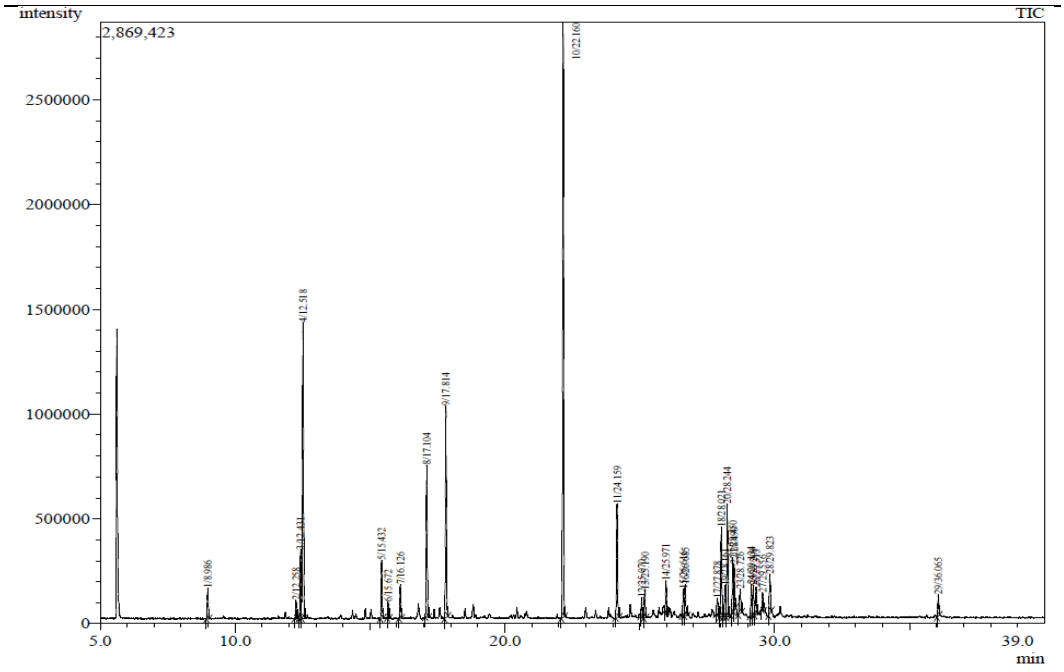
Table D10: Chemical composition and total ion chromatogram of 8-h hydrodistilled HdA analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.983	932	5771020	15.68	*α*-Pinene	96
2	9.567	948	187421	0.51	Fenchene	88
3	12.255	1023	289337	0.79	*o*-Cymene	96
4	12.431	1028	3031437	8.24	Limonene	96
5	12.517	1031	9816256	26.67	Eucalyptol	95
6	14.354	1084	291471	0.79	Isoterpinolene	95
7	15.431	1117	574048	1.56	Fenchol	96
8	15.670	1125	220759	0.60	*α*-Campholenal	94
9	16.124	1139	293532	0.80	Sabinol	91
10	16.795	1161	218193	0.59	Pinocarvone	94
11	17.102	1170	1130747	3.07	Borneol	96
12	17.812	1193	2320906	6.31	*α*-Terpineol	95
13	22.159	1346	7259896	19.73	*α*-Terpinyl acetate	95
14	24.155	1421	492172	1.34	*β*-Caryophyllene	96
15	24.640	1440	657092	1.79	Aromadendrene	95
16	25.187	1462	264836	0.72	Humulen-(v1)	92
17	25.968	1493	140363	0.38	Viridiflorene	94
18	26.680	1522	203486	0.55	*trans*-Calamenene	95
19	27.677	1564	196125	0.53	Epiglobulol	95
20	27.872	1572	136090	0.37	Ledol	86
21	28.017	1578	322436	0.88	Spathulenol	95
22	28.162	1584	126094	0.34	Unknown	-

23	28.242	1587	1357506	3.69	Globulol	94
24	28.448	1596	281978	0.77	Viridiflorol	90
25	28.493	1598	172051	0.47	Cubeban-11-ol	90
26	28.723	1608	176877	0.48	Rosifoliol	94
27	29.134	1626	302414	0.82	*β*-Eudesmol	87
28	29.206	1629	318649	0.87	1,10-Di-epi-cubenol	95
29	29.822	1656	248211	0.67	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90

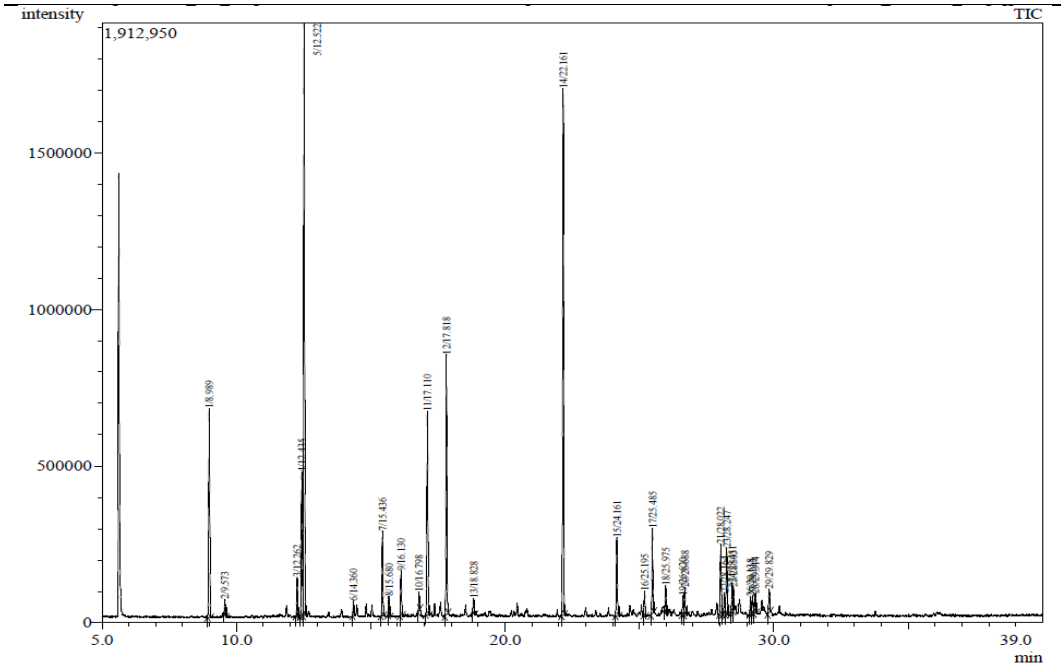
Table D11: Chemical composition and total ion chromatogram of 8-h hydrodistilled HfB analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.986	932	378096	1.36	*α*-Pinene	96
2	12.258	1023	205597	0.74	*o*-Cymene	96
3	12.431	1028	853782	3.06	Limonene	95
4	12.518	1031	3635007	13.05	Eucalyptol	95
5	15.432	1117	707843	2.54	Fenchol	97
6	15.672	1125	175797	0.63	*α*-Campholenal	94
7	16.126	1139	399727	1.43	Sabinol	91
8	17.104	1171	1845950	6.62	Borneol	96
9	17.814	1193	2570425	9.22	*α*-Terpineol	95
10	22.160	1346	6863245	24.63	*α*-Terpinyl acetate	95
11	24.159	1421	1388972	4.98	*β*-Caryophyllene	96
12	25.070	1457	225504	0.81	Humulene	95
13	25.190	1462	343082	1.23	Humulen-(v1)	92
14	25.971	1493	356803	1.28	Viridiflorene	93
15	26.616	1519	312672	1.12	*δ*-Cadinene	91
16	26.685	1522	423319	1.52	*trans*-Calamenene	95
17	27.878	1572	232415	0.83	Ledol	88
18	28.021	1578	1118746	4.02	Spathulenol	96
19	28.161	1584	455682	1.64	Caryophyllene oxide	89
20	28.244	1587	1412611	5.07	Globulol	94
21	28.450	1596	693380	2.49	Viridiflorol	92

22	28.499	1598	558933	2.01	Unknown	-
23	28.726	1608	414128	1.49	Rosifoliol	95
24	29.134	1626	448438	1.61	*β*-Eudesmol	86
25	29.209	1629	368323	1.32	1,10-Di-epi-cubenol	95
26	29.313	1634	439230	1.58	Unknown	-
27	29.556	1644	201341	0.72	*τ*-Muurolol	91
28	29.823	1656	551946	1.98	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	88
29	36.065	1946	282977	1.02	Butyl isobutyl phthalate	96

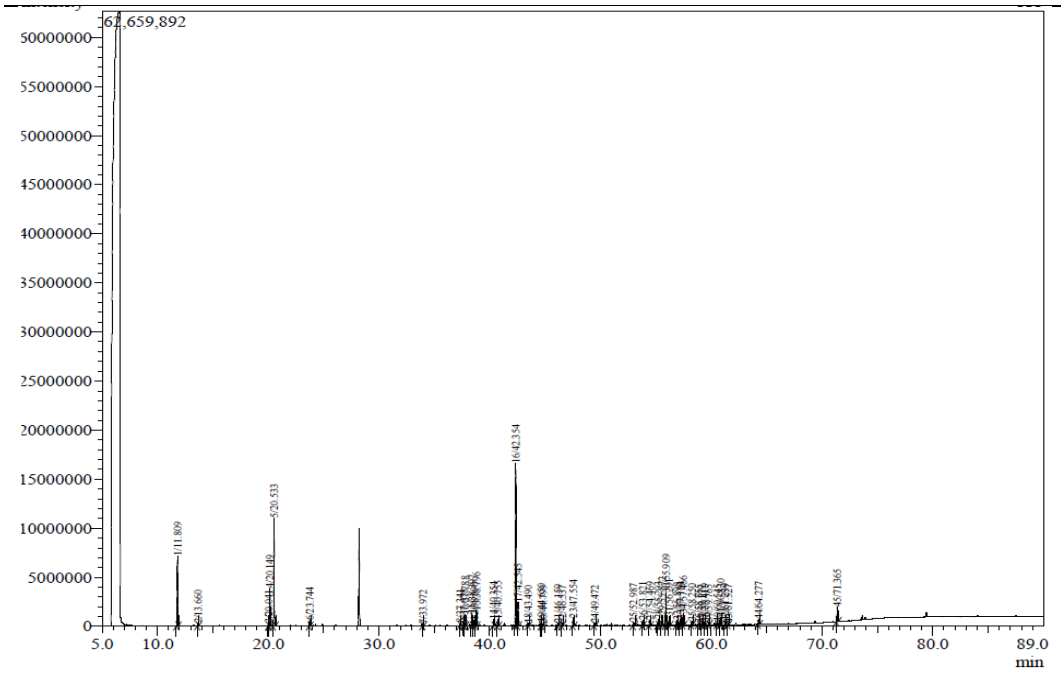
Table D12: Chemical composition and total ion chromatogram of 8-h hydrodistilled HdB analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.989	932	1725627	7.84	*α*-pinene	96
2	9.573	948	106137	0.48	Fenchene	88
3	12.262	1023	307888	1.40	*o*-Cymene	96
4	12.435	1028	1216249	5.53	Limonene	95
5	12.522	1031	4813150	21.88	Eucalyptol	95
6	14.360	1084	105888	0.48	Isoterpinolene	94
7	15.436	1117	666965	3.03	Fenchol	97
8	15.680	1125	155613	0.71	*α*-Campholenal	94
9	16.130	1139	350374	1.59	Sabinol	91
10	16.798	1161	125954	0.57	Pinocarvone	93
11	17.110	1171	1640863	7.46	Borneol	96
12	17.818	1193	2054584	9.34	*α*-Terpineol	95
13	18.828	1228	120316	0.55	Unknown	-
14	22.161	1346	4018717	18.27	*α*-Terpinyl acetate	95
15	24.161	1421	660669	3.00	*β*-Caryophyllene	96
16	25.195	1462	196541	0.89	Humulen-(v1)	92
17	25.485	1473	643835	2.93	Unknown	-
18	25.975	1493	193968	0.88	Viridiflorene	94
19	26.620	1519	149462	0.68	*δ*-Cadinene	91
20	26.688	1522	247827	1.13	*trans*-Calamenene	96
21	28.022	1578	564086	2.56	Spathulenol	96

22	28.164	1584	204986	0.93	Caryophyllene oxide	87
23	28.247	1587	576850	2.62	Globulol	94
24	28.451	1596	267775	1.22	Viridiflorol	89
25	28.503	1598	155260	0.71	Cubeban-11-ol	88
26	29.138	1626	161774	0.74	*β*-Eudesmol	86
27	29.213	1629	148211	0.67	1,10-Di-epi-cubenol	93
28	29.314	1634	196810	0.89	Unknown	-
29	29.829	1656	224285	1.02	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	89

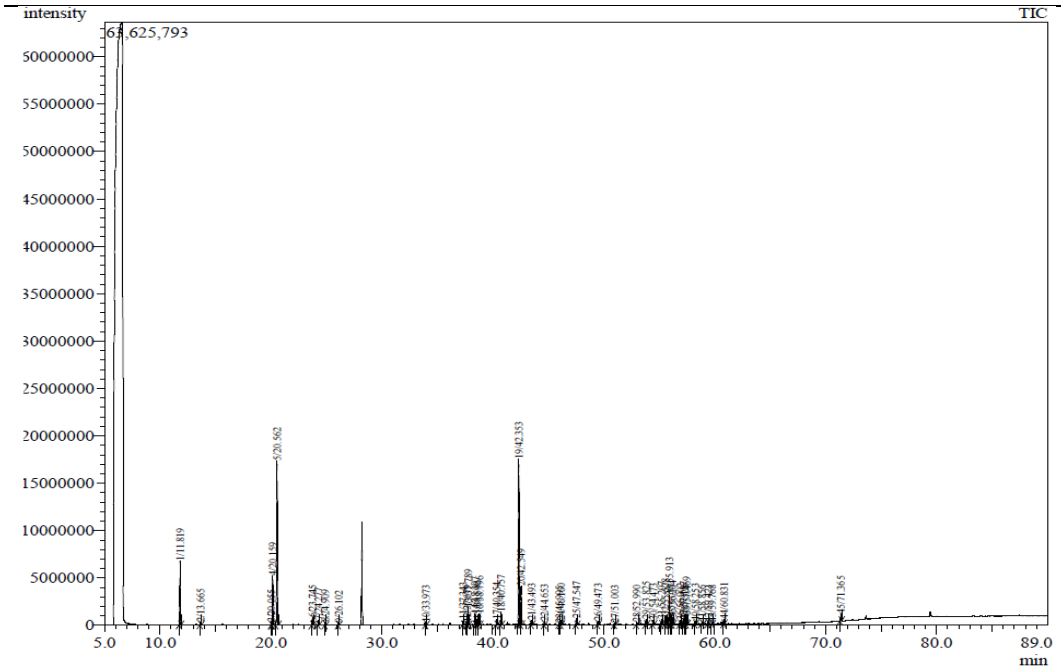
Table D13: Chemical composition and total ion chromatogram of 8-h hydrodistilled HfA analysed using polar column.



Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	11.809	24676923	9.63	*α*-Pinene	97
2	13.660	885824	0.35	Camphene	94
3	20.041	758250	0.30	3-Methylbutyl ester, butanoic acid	95
4	20.149	13395816	5.23	Limonene	93
5	20.533	43176689	16.85	Eucalyptol	97
6	23.744	1902760	0.74	*p*-Cymene	96
7	33.972	871171	0.34	*α*-Campholenal	96
8	37.341	1211636	0.47	Pinocarvone	92
9	37.678	1055673	0.41	Bornyl acetate	95
10	37.788	4051845	1.58	Fenchol	96
11	38.397	4641356	1.81	*β*-Caryophyllene	95
12	38.590	2464653	0.96	Terpinen-4-ol	93
13	38.796	5720356	2.23	Aromadendrene	94
14	40.354	2508185	0.98	Alloaromadendrene	95
15	40.755	2616791	1.02	Pinocarveol	94
16	42.354	71726724	27.99	*α*-Terpinyl acetate	97
17	42.545	9081770	3.54	Borneol	98
18	43.490	971411	0.38	Unknown	-
19	44.650	1532711	0.60	*β*-Cadinene	93
20	44.799	901024	0.35	Citronellol	86
21	46.159	994731	0.39	*trans-p*-Mentha-1(7),8-dien-2-ol	90

22	46.537	913078	0.36	Geranyl isobutyrate	88
23	47.554	3385592	1.32	*trans*-Calamenene	91
24	49.472	840882	0.33	Isocarveol	90
25	52.987	1672902	0.65	Caryophyllene oxide	96
26	53.821	2016098	0.79	Epiglobulol	94
27	54.469	2647858	1.03	Gleenol	89
28	55.203	2340263	0.91	Cubeban-11-ol	93
29	55.572	3951404	1.54	1,10-Di-epi-Cubenol	96
30	55.909	12839909	5.01	Globulol	92
31	56.201	3491962	1.36	Viridiflorol	95
32	56.898	1776664	0.69	Rosifoliol	93
33	57.184	2089437	0.82	Unknown	-
34	57.466	4245411	1.66	(+)-Spathulenol	94
35	58.250	1262227	0.49	Muurola-4,10(14)-dien-1β-ol	88
36	58.855	1284282	0.50	γ-Eudesmol	93
37	59.179	859617	0.34	Valerianol	92
38	59.419	1094224	0.43	τ-Muurolol	91
39	59.765	911091	0.36	δ-Cadinol	93
40	60.545	916938	0.36	α-Eudesmol	92
41	60.830	3003081	1.17	β-Eudesmol	88
42	61.239	1276190	0.50	3,7-Dimethyl-6-octenoic acid	88
43	61.527	751400	0.29	Unknown	-
44	64.277	1883489	0.73	*trans*-Farnesol	95
45	71.365	5700019	2.22	Phytol	97

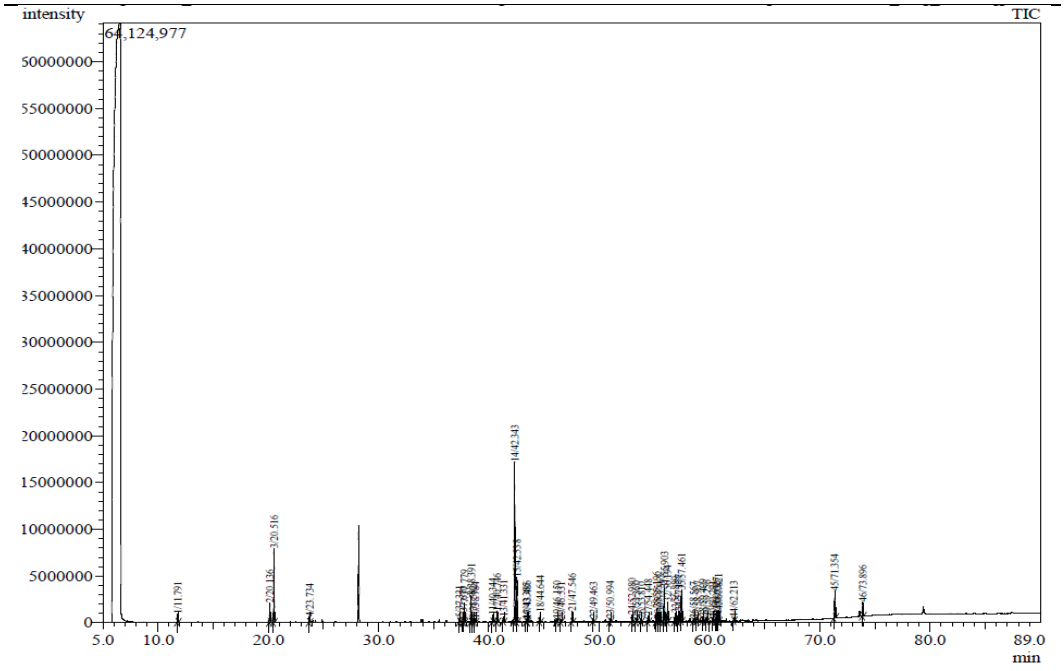
Table D14: Chemical composition and total ion chromatogram of 8-h hydrodistilled HdA analysed using polar column.



Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	11.819	22823426	7.68	α-Pinene	97
2	13.665	1175912	0.40	Camphene	95
3	20.055	801316	0.27	3-Methylbutyl ester, butanoic acid	95
4	20.159	18740293	6.31	Limonene	93
5	20.562	77691342	26.14	Eucalyptol	97
6	23.745	2496404	0.84	*p*-Cymene	96
7	24.277	1566563	0.53	δ-Terpinene	96
8	24.909	873485	0.29	Isopentyl isovalerate	95
9	26.102	687720	0.23	Unknown	-
10	33.973	1057810	0.36	α-Campholenal	96
11	37.343	2124308	0.71	Pinocarvone	92
12	37.679	936398	0.32	Bornyl acetate	94
13	37.789	6767187	2.28	Fenchol	97
14	38.397	4180983	1.41	β-Caryophyllene	95
15	38.590	2570092	0.86	Terpinen-4-ol	94
16	38.796	4373050	1.47	Aromadendrene	94
17	40.354	2045320	0.69	Alloaromadendrene	95
18	40.757	4901218	1.65	Pinocarveol	95
19	42.353	72544474	24.41	α-Terpinyl acetate	97
20	42.549	15299637	5.15	Borneol	98
21	43.493	1724061	0.58	Unknown	-
22	44.653	1246819	0.42	β-Cadinene	92

23	45.995	728270	0.25	6,6-Dimethyl-bicyclo[3.1.1]hept-2-ene-2-methanol	94
24	46.160	1675819	0.56	*trans-p*-Mentha-1(7),8-dien-2-ol	89
25	47.547	2986336	1.00	Unknown	-
26	49.473	1386153	0.47	Isocarveol	91
27	51.003	689332	0.23	Palustrol	91
28	52.990	1029462	0.35	Unknown	-
29	53.825	2091830	0.70	Epiglobulol	93
30	54.473	2063828	0.69	Gleenol	90
31	55.207	2159464	0.73	Cubeban-11-ol	93
32	55.578	3410219	1.15	1,10-Di-epi-cubenol	95
33	55.913	11725716	3.95	Globulol	91
34	56.077	1130639	0.38	Unknown	-
35	56.204	2803471	0.94	Viridiflorol	95
36	56.902	1663810	0.56	Rosifoliol	93
37	57.187	2090150	0.70	Unknown	-
38	57.328	769442	0.26	Unknown	-
39	57.469	3995968	1.34	(+)-Spathulenol	94
40	58.253	1068827	0.36	Muurola-4,10(14)-dien-1β-ol	89
41	58.856	892558	0.30	γ-Eudesmol	91
42	59.421	868656	0.29	τ-Muurolol	90
43	59.768	685516	0.23	δ-Cadinol	93
44	60.831	1780270	0.60	β-Eudesmol	89
45	71.365	2904100	0.98	Phytol	97

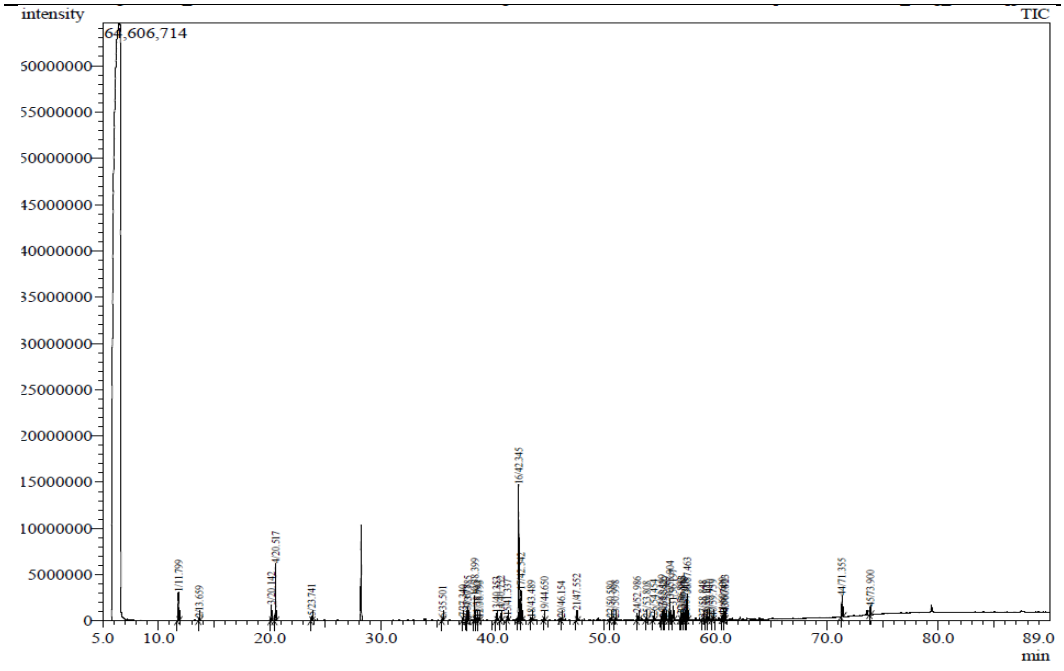
Table D15: Chemical composition and total ion chromatogram of 8-h hydrodistilled HfB analysed using polar column.



Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	11.791	2845508	1.04	*α*-Pinene	97
2	20.136	7201171	2.62	Limonene	93
3	20.516	29635751	10.79	Eucalyptol	97
4	23.734	2030067	0.74	*p*-Cymene	96
5	37.331	1763557	0.64	Pinocarvone	92
6	37.670	1093521	0.40	Bornyl acetate	94
7	37.779	7321198	2.67	Fenchol	96
8	38.391	10440704	3.80	*β*-Caryophyllene	96
9	38.582	1980464	0.72	Terpinen-4-ol	92
10	38.784	1312511	0.48	Aromadendrene	94
11	40.344	2883034	1.05	Alloaromadendrene	95
12	40.746	4388036	1.60	Pinocarveol	95
13	41.331	1715568	0.62	*α*-Humulene	95
14	42.343	73562372	26.79	*α*-Terpinyl acetate	97
15	42.538	18309599	6.67	Borneol	98
16	43.385	1094824	0.40	*α*-Muurolene	89
17	43.486	1580962	0.58	Unknown	-
18	44.644	3424763	1.25	*β*-Cadinene	93
19	46.150	1366873	0.50	*trans*-*p*-mentha-1(7),8-dien-2-ol	89
20	46.531	1046591	0.38	Geranyl isobutyrate	89
21	47.546	4969947	1.81	*trans*-Calamenene	91

22	49.463	1140725	0.42	Isocarveol	90
23	50.994	1331222	0.48	Palustrol	92
24	52.980	3186212	1.16	Caryophyllene oxide	96
25	53.360	1115701	0.41	(1aR,3aS,7S,7aS,7bR)-1,1,3a,7-Tetramethyldecahydro-1H-cyclopropa[a]naphthalen-7-ol	88
26	53.810	1124271	0.41	Epiglobulol	91
27	54.448	3158274	1.15	Gleenol	86
28	55.196	5148674	1.87	Cubeban-11-ol	93
29	55.351	1365667	0.50	epi-Cubenol	89
30	55.565	3867143	1.41	1,10-Di-epi-cubenol	95
31	55.903	13612359	4.96	Globulol	91
32	56.194	8080492	2.94	Viridiflorol	96
33	56.889	3148174	1.15	Rosifoliol	93
34	57.177	3814783	1.39	Unknown	-
35	57.461	11944894	4.35	(+)-Spathulenol	94
36	58.567	1026328	0.37	Unknown	-
37	58.907	2099136	0.76	Unknown	-
38	59.409	1793520	0.65	τ-Muurolol	91
39	59.756	1539349	0.56	δ-Cadinol	93
40	60.291	1026001	0.37	m-Cymen-5-ol	90
41	60.627	2390113	0.87	(-)-Spathulenol	88
42	60.730	1151227	0.42	Cadalene	96
43	60.821	4271924	1.56	β-Eudesmol	89
44	62.213	1054904	0.38	Isophytol	95
45	71.354	10817242	3.94	Phytol	97
46	73.896	5431064	1.98	Dibutyl phthalate	96

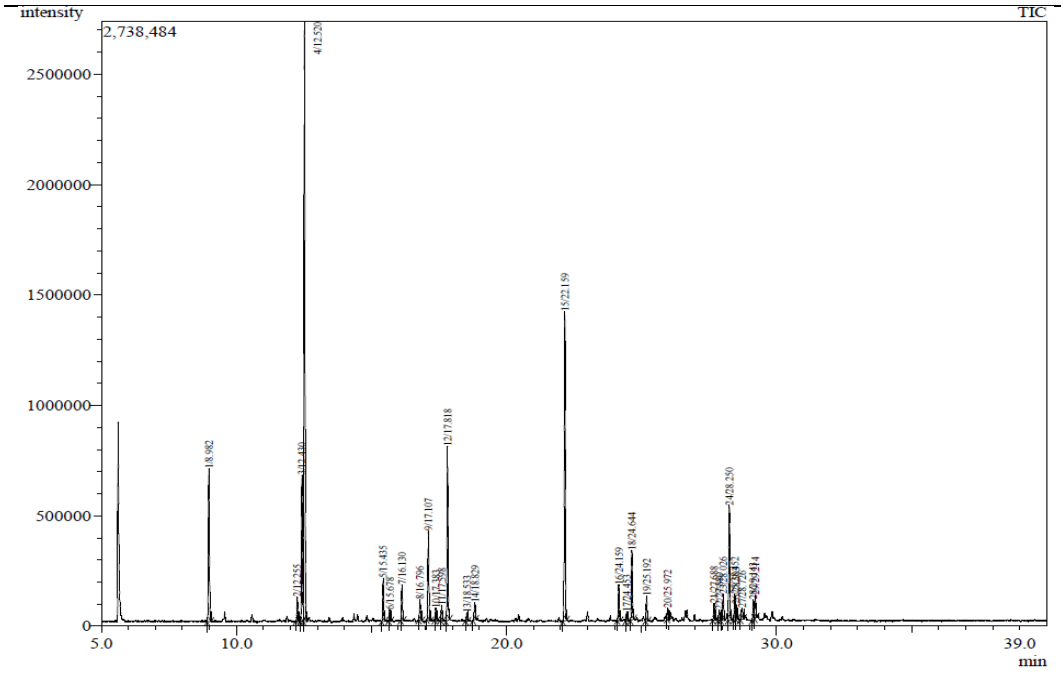
Table D16: Chemical composition and total ion chromatogram of 8-h hydrodistilled HdB analysed using polar column.



Peak no.	RT (min)	Peak area	Peak area %	Component name	% similarity
1	11.799	10064962	4.48	*α*-Pinene	97
2	13.659	853609	0.38	Camphene	94
3	20.142	5917342	2.63	Limonene	93
4	20.517	22695326	10.10	Eucalyptol	97
5	23.741	1629694	0.73	*p*-Cymene	96
6	35.501	940284	0.42	*α*-Gurjunene	94
7	37.340	1313591	0.58	Pinocarvone	92
8	37.678	1009551	0.45	Bornyl acetate	95
9	37.785	4895187	2.18	Fenchol	96
10	38.399	9991727	4.45	*β*-Caryophyllene	96
11	38.592	1401636	0.62	Terpinen-4-ol	89
12	38.793	1208966	0.54	Aromadendrene	94
13	40.353	2968094	1.32	Alloaromadendrene	95
14	40.752	2966113	1.32	Pinocarveol	95
15	41.337	1401319	0.62	*α*-Humulene	95
16	42.345	60095679	26.75	*α*-Terpinyl acetate	97
17	42.542	12607509	5.61	Borneol	98
18	43.489	1338623	0.60	Unknown	-
19	44.650	2667417	1.19	*β*-Cadinene	93
20	46.154	810924	0.36	*trans*-*p*-Mentha-1(7),8-dien-2-ol	89
21	47.552	4168407	1.86	*trans*-Calamenene	92
22	50.580	1210334	0.54	Unknown	-

23	50.998	1013075	0.45	Palustrol	92
24	52.986	3082951	1.37	Caryophyllene oxide	97
25	53.808	975858	0.43	Unknown	-
26	54.454	2219549	0.99	Gleenol	87
27	55.199	3478486	1.55	Cubeban-11-ol	93
28	55.352	1084718	0.48	epi-Cubenol	90
29	55.569	2919006	1.30	1,10-Di-epi-cubenol	95
30	55.904	8926665	3.97	Globulol	92
31	56.197	5795623	2.58	Viridiflorol	95
32	56.893	2104145	0.94	Rosifoliol	93
33	56.983	3574783	1.59	Unknown	-
34	57.179	2558867	1.14	Unknown	-
35	57.318	1727842	0.77	Hexahydrofarnesyl acetone	89
36	57.463	10647353	4.74	(+)-Spathulenol	94
37	58.848	1515873	0.67	γ-Eudesmole	93
38	59.175	992889	0.44	Valerianol	91
39	59.410	1436125	0.64	τ-Muurolol	90
40	59.759	1080677	0.48	δ-Cadinol	93
41	60.630	1810147	0.81	(-)-Spathulenol	88
42	60.740	981867	0.44	Cadalene	95
43	60.823	3073466	1.37	β-Eudesmol	90
44	71.355	8564209	3.81	Phytol	97
45	73.900	2923857	1.30	Dibutyl phthalate	96

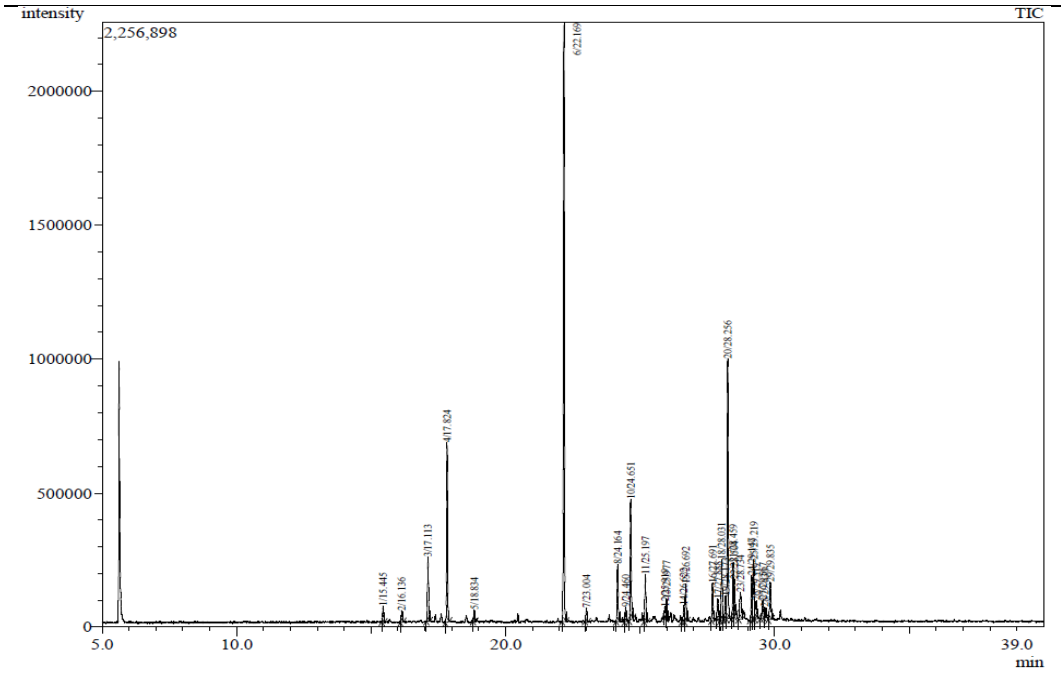
Table D17: Chemical composition and total ion chromatogram of HfA collected at 4th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.982	932	1807572	7.56	*α*-Pinene	96
2	12.255	1023	261577	1.09	*o*-Cymene	97
3	12.430	1028	1728476	7.23	Limonene	96
4	12.520	1031	6881468	28.79	Eucalyptol	96
5	15.435	1117	492962	2.06	Fenchol	96
6	15.678	1125	118126	0.49	*α*-Campholenal	95
7	16.130	1139	399894	1.67	Sabinol	91
8	16.796	1161	255414	1.07	Pinocarvone	94
9	17.107	1171	1009750	4.23	Borneol	97
10	17.383	1179	133211	0.56	Terpinen-4-ol	94
11	17.598	1186	170744	0.71	*trans-p*-Mentha-1(7),8-dien-2-ol	93
12	17.818	1193	1991495	8.33	*α*-Terpineol	97
13	18.533	1217	99814	0.42	*Cis*-carveol	91
14	18.829	1228	222891	0.93	Isocarveol	89
15	22.159	1346	3377300	14.13	*α*-Terpinyl acetate	91
16	24.159	1421	405831	1.70	*β*-Caryophyllene	97
17	24.453	1433	102364	0.43	*β*-Gurjunene	94
18	24.644	1440	810705	3.39	Aromadendrene	96
19	25.192	1462	275035	1.15	Humulen-(v1)	92
20	25.972	1493	133110	0.56	Viridiflorene	94
21	27.688	1564	188476	0.79	Epiglobulol	96

22	27.880	1572	138713	0.58	Ledol	89
23	28.026	1578	308910	1.29	Spathulenol	96
24	28.250	1587	1383935	5.79	Globulol	96
25	28.452	1596	321454	1.35	Viridiflorol	91
26	28.503	1598	135818	0.57	Cubeban-11-ol	87
27	28.726	1608	194167	0.81	Rosifoliol	91
28	29.143	1626	287937	1.20	Unknown	-
29	29.214	1629	261201	1.09	1,10-Di-epi-cubenol	93

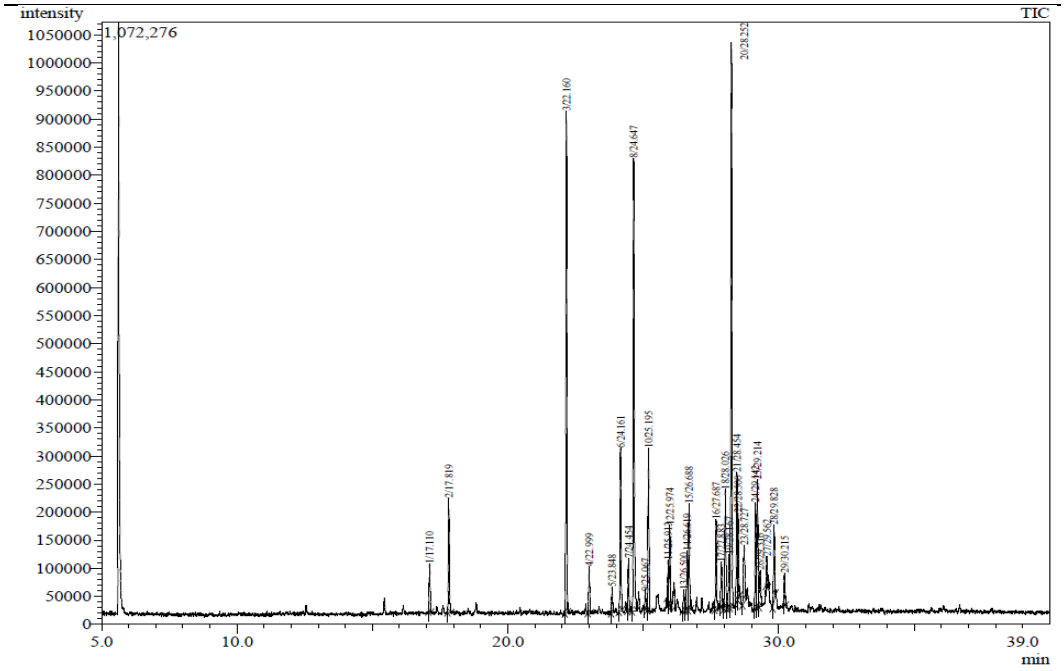
Table D18: Chemical composition and total ion chromatogram of HfA collected at 6th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	15.445	1118	153567	0.82	Fenchol	97
2	16.136	1140	114050	0.61	Sabinol	90
3	17.113	1171	606404	3.22	Borneol	97
4	17.824	1193	1655557	8.79	α-Terpineol	97
5	18.834	1228	115184	0.61	Isocarveol	90
6	22.169	1346	5439803	28.88	α-Terpinyl acetate	92
7	23.004	1377	140214	0.74	α-Cubebene	93
8	24.164	1421	548515	2.91	β-Caryophyllene	96
9	24.460	1433	126935	0.67	β-Gurjunene	95
10	24.651	1441	1166439	6.19	Aromadendrene	96
11	25.197	1462	452710	2.40	Humulen-(v1)	91
12	25.919	1491	96819	0.51	β-Selinene	91
13	25.977	1493	184920	0.98	Viridiflorene	91
14	26.622	1519	162287	0.86	δ-Cadinene	91
15	26.692	1522	376130	2.00	*trans*-Calamenene	96
16	27.691	1564	368546	1.96	Epiglobulol	95
17	27.888	1572	236594	1.26	Ledol	89
18	28.031	1578	586120	3.11	Spathulenol	97
19	28.173	1584	259549	1.38	Caryophyllene oxide	87
20	28.256	1588	2530697	13.44	Globulol	96
21	28.459	1596	551715	2.93	Viridiflorol	93
22	28.504	1598	298729	1.59	Cubeban-11-ol	89

23	28.734	1608	429596	2.28	Rosifoliol	92
24	29.147	1626	549971	2.92	Unknown	-
25	29.219	1630	612799	3.25	1,10-Di-epi-Cubenol	94
26	29.319	1634	282890	1.50	Unknown	-
27	29.567	1645	233886	1.24	*τ*-Muurolol	92
28	29.630	1648	99825	0.53	*δ*-Cadinol	93
29	29.835	1657	454957	2.42	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	88

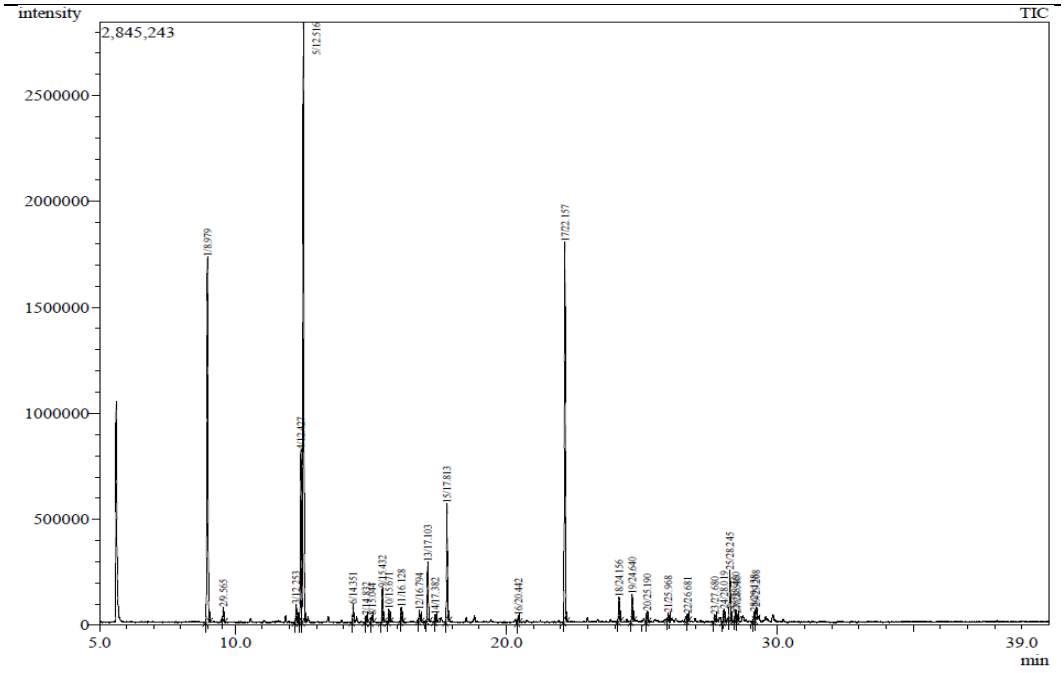
Table D19: Chemical composition and total ion chromatogram of HfA collected at 8th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	17.110	1171	213804	1.32	Borneol	96
2	17.819	1193	521940	3.23	*α*-Terpineol	97
3	22.160	1346	2129894	13.19	*α*-Terpinyl acetate	92
4	22.999	1377	217587	1.35	*α*- Cubebene	92
5	23.848	1409	112776	0.70	*α*-Gurjunene	94
6	24.161	1421	724716	4.49	*β*-Caryophyllene	97
7	24.454	1433	230072	1.42	*β*-Gurjunene	96
8	24.647	1440	2188273	13.55	Aromadendrene	96
9	25.067	1457	100440	0.62	Humulene	95
10	25.195	1462	725712	4.49	Humulen-(v1)	92
11	25.913	1490	178387	1.10	*β*-Selinene	93
12	25.974	1493	361123	2.24	Viridiflorene	93
13	26.500	1514	92656	0.57	*γ*-Cadinene	95
14	26.619	1519	275573	1.71	*δ*-Cadinene	92
15	26.688	1522	499543	3.09	*trans*-Calamenene	96
16	27.687	1564	426417	2.64	Epiglobulol	97
17	27.883	1572	231458	1.43	Ledol	88
18	28.026	1578	549403	3.40	Spathulenol	96
19	28.167	1584	276955	1.72	Caryophyllene oxide	86
20	28.252	1588	2651046	16.42	Globulol	96
21	28.454	1596	636053	3.94	Viridiflorol	93
22	28.500	1598	333583	2.07	Cubeban-11-ol	89

23	28.727	1608	386863	2.40	Rosifoliol	94
24	29.142	1626	583846	3.62	Unknown	-
25	29.214	1629	629244	3.90	1,10-Di-epi-cubenol	94
26	29.316	1634	239364	1.48	Unknown	-
27	29.562	1645	115917	0.72	τ-Muurolol	92
28	29.828	1656	379623	2.35	Unknown	-
29	30.215	1673	134304	0.83	Cadalene	96

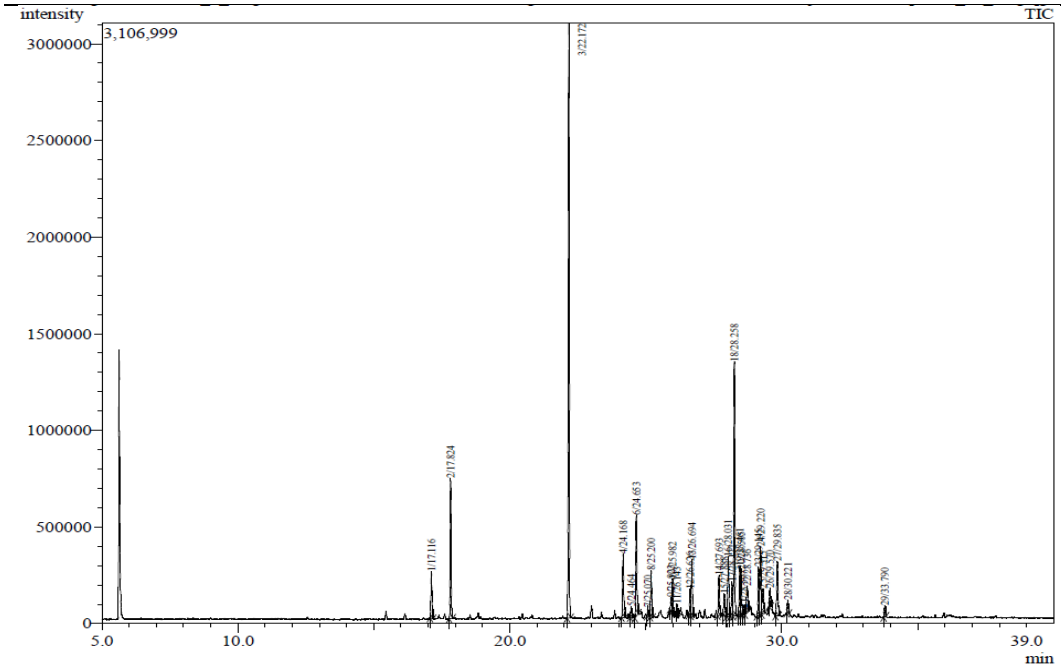
Table D20: Chemical composition and total ion chromatogram of HdA collected at 4th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.979	932	4495230	18.70	*α*-Pinene	97
2	9.565	948	133819	0.56	Fenchene	89
3	12.253	1023	214098	0.89	*o*-Cymene	97
4	12.427	1028	2091445	8.70	Limonene	96
5	12.516	1031	7140468	29.70	Eucalyptol	96
6	14.351	1084	206936	0.86	Isoterpinolene	95
7	14.832	1098	67623	0.28	Unknown	-
8	15.044	1105	82372	0.34	Isopentyl isovalerate	96
9	15.432	1117	397622	1.65	Fenchol	96
10	15.671	1125	148961	0.62	*α*-Campholenal	95
11	16.128	1139	179376	0.75	Sabinol	91
12	16.794	1161	141909	0.59	Pinocarvone	94
13	17.103	1171	702931	2.92	Borneol	97
14	17.382	1179	74764	0.31	Terpinen-4-ol	94
15	17.813	1193	1383897	5.76	*α*-Terpineol	97
16	20.442	1283	79395	0.33	Bornyl acetate	95
17	22.157	1346	4279070	17.80	*α*-Terpinyl acetate	93
18	24.156	1421	276099	1.15	*β*-Caryophyllene	96
19	24.640	1440	330973	1.38	Aromadendrene	95
20	25.190	1462	127011	0.53	Humulen-(v1)	91
21	25.968	1492	78048	0.32	Viridiflorene	92
22	26.681	1522	72644	0.30	*trans*-Calamenene	94

23	27.680	1564	83460	0.35	Epiglobulol	94
24	28.019	1578	154680	0.64	Spathulenol	96
25	28.245	1587	621205	2.58	Globulol	96
26	28.450	1596	130765	0.54	Viridiflorol	91
27	28.500	1598	73761	0.31	Cubeban-11-ol	89
28	29.138	1626	126039	0.52	Unknown	-
29	29.208	1629	146257	0.61	1,10-Di-epi-cubenol	94

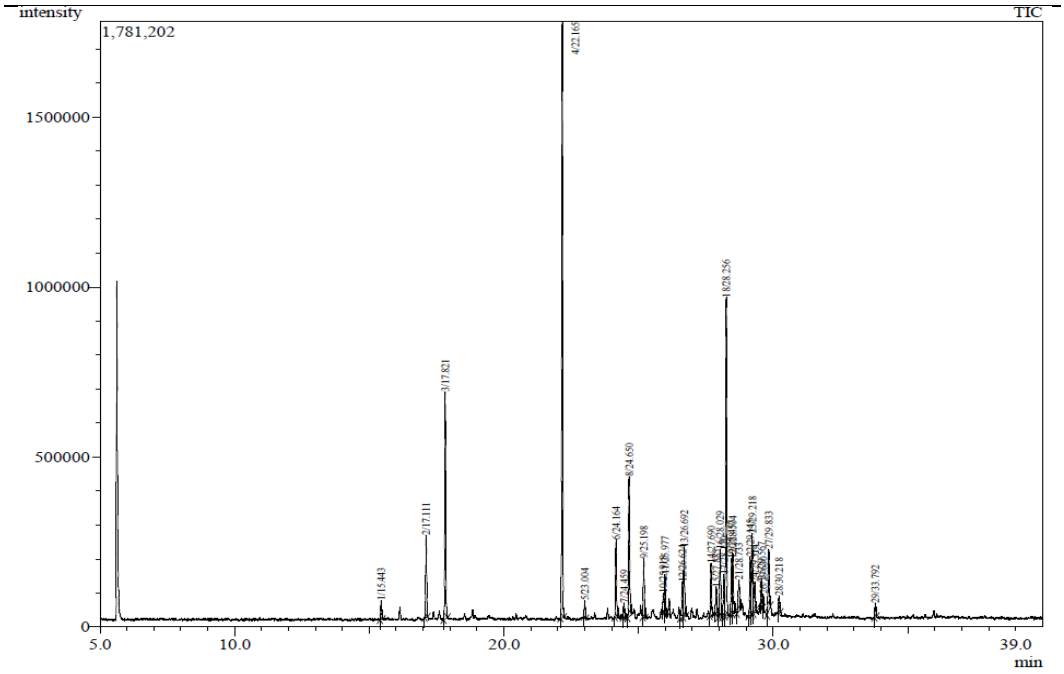
Table D21: Chemical composition and total ion chromatogram of HdA collected at 6th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	17.116	1171	601977	2.30	Borneol	96
2	17.824	1193	1797864	6.86	*α*-Terpineol	95
3	22.172	1346	7551332	28.80	*α*-Terpinyl acetate	95
4	24.168	1422	871383	3.32	*β*-Caryophyllene	97
5	24.464	1433	128377	0.49	*β*-Gurjunene	95
6	24.653	1441	1394269	5.32	Aromadendrene	96
7	25.070	1457	135307	0.52	Humulene	94
8	25.200	1462	596766	2.28	Humulen-(v1)	92
9	25.923	1491	154410	0.59	*β*-Selinene	92
10	25.982	1493	450608	1.72	Viridiflorene	93
11	26.143	1499	115626	0.44	*α*-Muurolene	94
12	26.626	1520	321920	1.23	*δ*-Cadinene	92
13	26.694	1522	732948	2.77	*trans*-Calamenene	96
14	27.693	1564	529915	2.02	Epiglobulol	95
15	27.888	1572	348231	1.33	Ledol	86
16	28.031	1578	809046	3.09	Spathulenol	96
17	28.172	1584	548834	2.09	Caryophyllene oxide	89
18	28.258	1588	3456563	13.18	Globulol	94
19	28.461	1596	706396	2.69	Viridiflorol	90
20	28.505	1598	608689	2.32	Cubeban-11-ol	89
21	28.597	1602	142516	0.54	2-Methyl-, 3,7-dimethyl-2,6-octadienyl ester, (E)-butanoic acid	86

22	28.736	1608	466611	1.78	Rosifoliol	95
23	29.145	1626	804394	3.07	*β*-Eudesmol	86
24	29.220	1630	982848	3.75	1,10-Di-epi-cubenol	95
25	29.315	1634	504463	1.92	*γ*-Eudesmol	87
26	29.570	1645	207622	0.79	*τ*-Muurolol	89
27	29.835	1657	943043	3.60	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	89
28	30.221	1674	164556	0.63	Cadalene	96
29	33.790	1840	146688	0.56	Hexahydrofarnesyl acetone	94

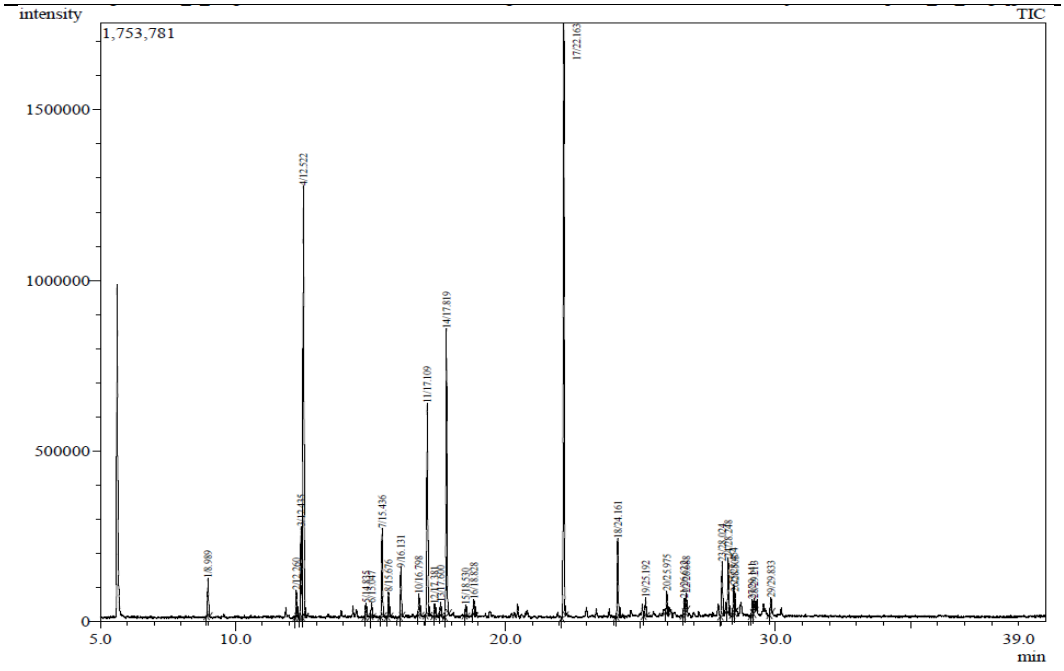
Table D22: Chemical composition and total ion chromatogram of HdA collected at 8th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	15.443	1118	143779	0.80	Fenchol	96
2	17.111	1171	586018	3.25	Borneol	97
3	17.821	1193	1673396	9.27	α-Terpineol	96
4	22.165	1346	4216887	23.36	α-Terpinyl acetate	95
5	23.004	1377	127062	0.70	α-Cubebene	91
6	24.164	1421	571069	3.16	β-Caryophyllene	96
7	24.459	1433	112488	0.62	β-Gurjunene	95
8	24.650	1441	1100461	6.10	Aromadendrene	96
9	25.198	1462	446801	2.48	Humulen-(v1)	92
10	25.918	1491	140315	0.78	β-Selinene	93
11	25.977	1493	282453	1.56	Viridiflorene	93
12	26.624	1519	284898	1.58	δ-Cadinene	91
13	26.692	1522	536954	2.97	*trans*-Calamenene	96
14	27.690	1564	387088	2.14	Epiglobulol	96
15	27.885	1572	240261	1.33	Ledol	86
16	28.029	1578	514965	2.85	Spathulenol	96
17	28.170	1584	380364	2.11	Caryophyllene oxide	89
18	28.256	1588	2488552	13.79	Globulol	94
19	28.457	1596	447320	2.48	Viridiflorol	90
20	28.504	1598	454478	2.52	Cubeban-11-ol	90
21	28.733	1608	317435	1.76	Rosifoliol	95
22	29.145	1626	551743	3.06	β-Eudesmol	86

23	29.218	1630	643417	3.56	1,10-Di-epi-cubenol	95
24	29.314	1634	344296	1.91	*γ*-Eudesmol	91
25	29.567	1645	178105	0.99	*τ*-Muurolol	91
26	29.630	1648	96328	0.53	*δ*-Cadinol	94
27	29.833	1657	572817	3.17	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	89
28	30.218	1674	118214	0.66	Cadalene	95
29	33.792	1840	92542	0.51	Hexahydrofarnesyl acetone	94

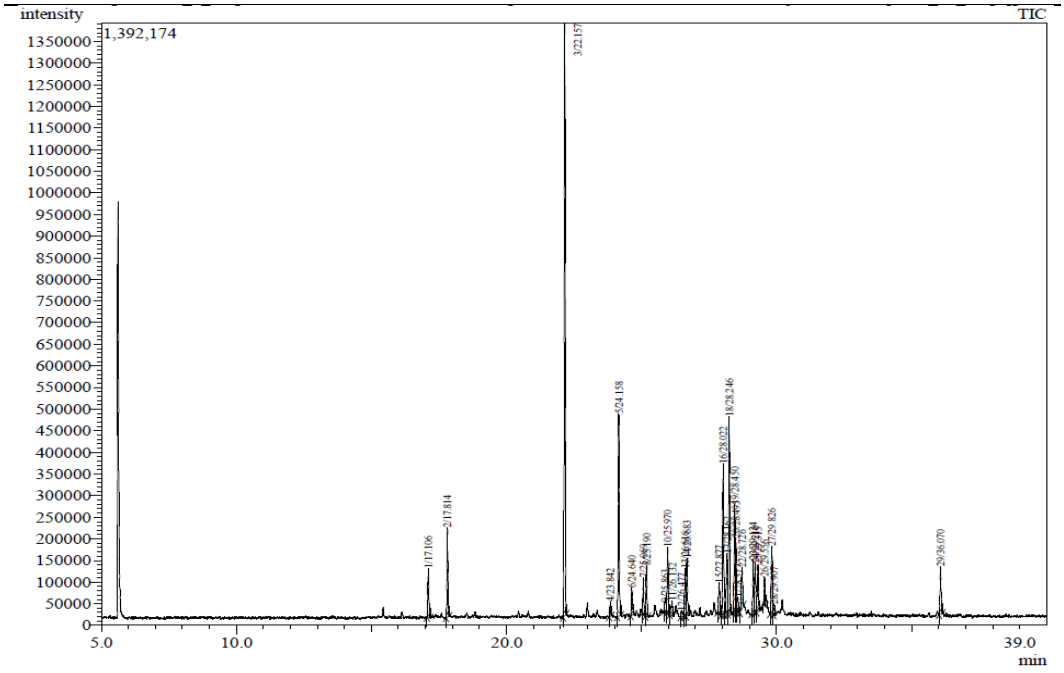
Table D23: Chemical composition and total ion chromatogram of HfB collected at 4th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	8.989	932	265895	1.57	*α*-Pinene	96
2	12.260	1023	190721	1.13	*o*-Cymene	97
3	12.435	1028	720346	4.26	Limonene	95
4	12.522	1031	3260216	19.27	Eucalyptol	95
5	14.835	1098	97289	0.58	Unknown	-
6	15.047	1105	91668	0.54	Isopentyl isovalerate	96
7	15.436	1117	657461	3.89	Fenchol	97
8	15.676	1125	177706	1.05	*α*-Campholenal	94
9	16.131	1140	360124	2.13	Sabinol	91
10	16.798	1161	182561	1.08	Pinocarvone	94
11	17.109	1171	1569579	9.28	Borneol	97
12	17.381	1179	95410	0.56	Terpinen-4-ol	94
13	17.600	1186	80686	0.48	*trans-p*-Mentha-1(7),8-dien-2-ol	91
14	17.819	1193	2113658	12.50	*α*-Terpineol	96
15	18.530	1217	87175	0.52	*cis*-Carveol	91
16	18.828	1228	118983	0.70	Isocarveol	88
17	22.163	1346	4185892	24.75	*α*-Terpinyl acetate	95
18	24.161	1421	615051	3.64	*β*-Caryophyllene	96
19	25.192	1462	131106	0.78	Humulen-(v1)	91
20	25.975	1493	146724	0.87	Viridiflorene	94

21	26.622	1519	105816	0.63	*δ*-Cadinene	92
22	26.688	1522	112885	0.67	*trans*-Calamenene	95
23	28.024	1578	423117	2.50	Spathulenol	97
24	28.248	1587	404592	2.39	Globulol	95
25	28.454	1596	222970	1.32	Viridiflorol	92
26	28.503	1598	128781	0.76	Cubeban-11-ol	88
27	29.141	1626	129006	0.76	Unknown	-
28	29.213	1629	105874	0.63	1,10-Di-epi-cubenol	94
29	29.833	1657	133363	0.79	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90

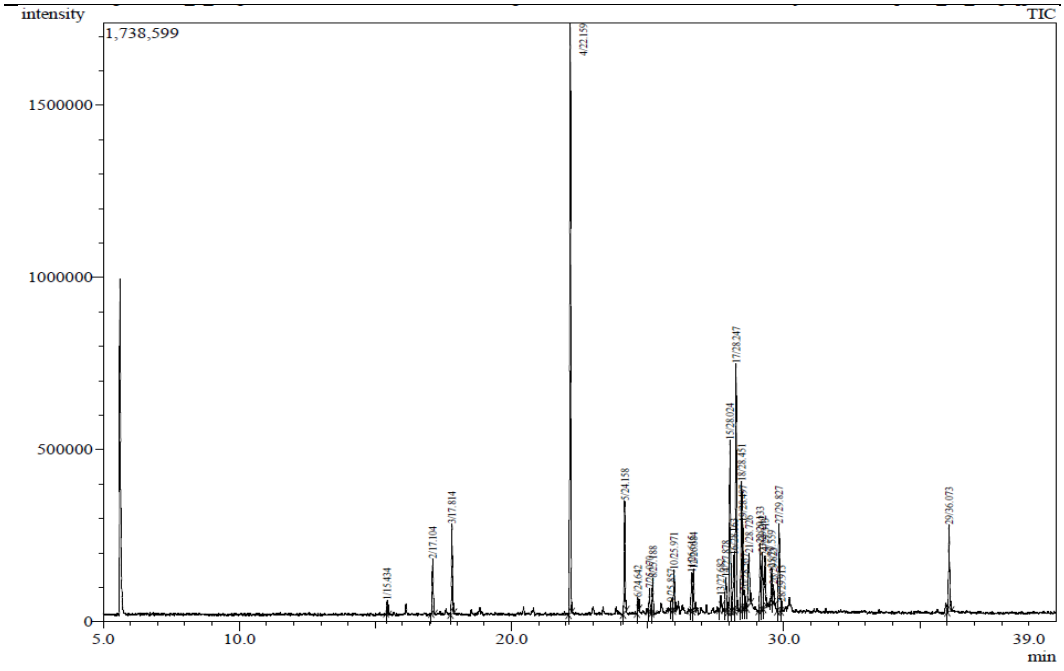
Table D24: Chemical composition and total ion chromatogram of HfB collected at 6th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	17.106	1171	287409	2.07	Borneol	96
2	17.814	1193	509030	3.66	*α*-Terpineol	96
3	22.157	1346	3317362	23.88	*α*-Terpinyl acetate	94
4	23.842	1409	80931	0.58	*α*-Gurjunene	93
5	24.158	1421	1186003	8.54	*β*-Caryophyllene	96
6	24.640	1440	179138	1.29	Aromadendrene	96
7	25.069	1457	215190	1.55	Humulene	96
8	25.190	1462	304342	2.19	Humulen-(v1)	92
9	25.863	1488	148321	1.07	Unknown	-
10	25.970	1493	366644	2.64	Viridiflorene	93
11	26.132	1499	130529	0.94	*α*-Muurolene	96
12	26.477	1513	64823	0.47	Unknown	-
13	26.616	1519	277449	2.00	*δ*-Cadinene	92
14	26.683	1522	379176	2.73	*trans*-Calamenene	96
15	27.877	1572	221454	1.59	Ledol	88
16	28.022	1578	925425	6.66	Spathulenol	96
17	28.162	1584	435211	3.13	Caryophyllene oxide	90
18	28.246	1587	1237289	8.91	Globulol	95
19	28.450	1596	706766	5.09	Viridiflorol	91
20	28.495	1598	391053	2.82	Cubeban-11-ol	87
21	28.577	1601	86360	0.62	Unknown	-
22	28.726	1608	345686	2.49	Rosifoliol	95

23	29.134	1626	369815	2.66	*β*-Eudesmol	86
24	29.210	1629	308715	2.22	1,10-Di-epi-cubenol	94
25	29.315	1634	372508	2.68	Unknown	-
26	29.556	1644	153000	1.10	*τ*-Muurolol	91
27	29.826	1656	500202	3.60	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90
28	29.907	1660	65478	0.47	Unknown	-
29	36.070	1946	326094	2.35	Butyl isobutyl phthalate	96

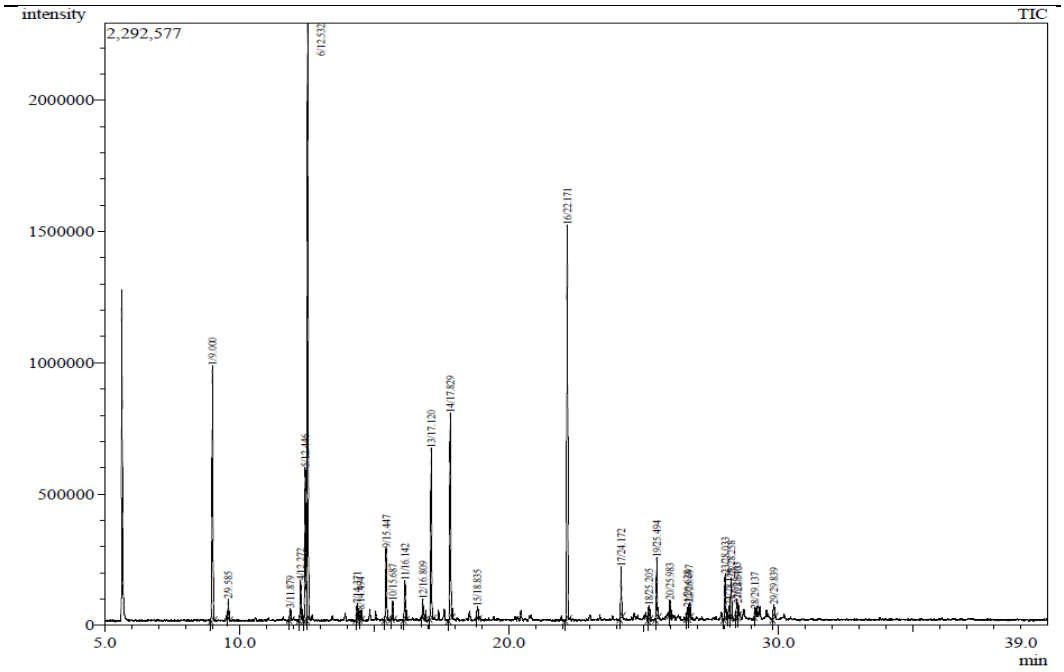
Table D25: Chemical composition and total ion chromatogram of HfB collected at 8th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	15.434	1117	98885	0.56	Fenchol	96
2	17.104	1171	397930	2.26	Borneol	97
3	17.814	1193	647197	3.67	*α*-Terpineol	96
4	22.159	1346	4132607	23.45	*α*-Terpinyl acetate	95
5	24.158	1421	806207	4.57	*β*-Caryophyllene	97
6	24.642	1440	100305	0.57	Aromadendrene	95
7	25.070	1457	176046	1.00	Humulene	96
8	25.188	1462	254492	1.44	Humulen-(v1)	92
9	25.857	1488	149361	0.85	Unknown	-
10	25.971	1493	283511	1.61	Viridiflorene	91
11	26.615	1519	255996	1.45	*δ*-Cadinene	91
12	26.684	1522	330990	1.88	*trans*-Calamenene	96
13	27.682	1564	68529	0.39	Epiglobulol	91
14	27.878	1572	306932	1.74	Ledol	89
15	28.024	1578	1284340	7.29	Spathulenol	97
16	28.163	1584	485577	2.76	Caryophyllene oxide	89
17	28.247	1587	1940262	11.01	Globulol	95
18	28.451	1596	1049162	5.95	Viridiflorol	91
19	28.497	1598	615569	3.49	Cubeban-11-ol	87
20	28.567	1601	121661	0.69	Unknown	-
21	28.726	1608	570429	3.24	Rosifoliol	95
22	29.133	1626	569519	3.23	*β*-Eudesmol	87

23	29.212	1629	433424	2.46	1,10-Di-epi-cubenol	95
24	29.313	1634	536129	3.04	Unknown	-
25	29.559	1645	236415	1.34	*τ*-Muurolol	92
26	29.623	1647	120350	0.68	*δ*-Cadinol	95
27	29.827	1656	834446	4.73	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	89
28	29.913	1660	76136	0.43	Unknown	-
29	36.073	1946	741211	4.21	Butyl isobutyl phthalate	96

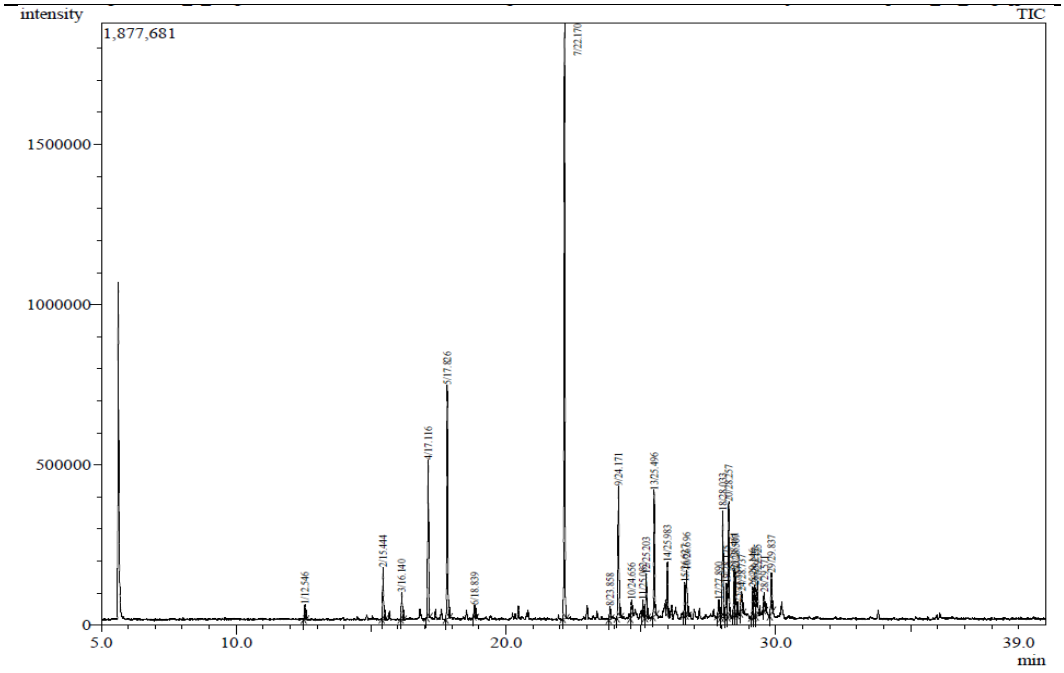
Table D26: Chemical composition and total ion chromatogram of HdB collected at 4th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	9.000	933	2564433	11.37	*α*-Pinene	96
2	9.585	949	162459	0.72	Fenchene	88
3	11.879	1012	110221	0.49	Isoamyl isobutyrate	95
4	12.272	1024	367918	1.63	*o*-Cymene	96
5	12.446	1029	1549523	6.87	Limonene	95
6	12.532	1031	5736660	25.43	Eucalyptol	95
7	14.371	1085	139321	0.62	Isoterpinolene	94
8	14.494	1088	92132	0.41	Unknown	-
9	15.447	1118	691462	3.06	Fenchol	97
10	15.687	1125	164902	0.73	*α*-Campholenal	94
11	16.142	1140	369715	1.64	Sabinol	91
12	16.809	1161	216244	0.96	Pinocarvone	94
13	17.120	1171	1653695	7.33	Borneol	96
14	17.829	1194	1989318	8.82	*α*-Terpineol	95
15	18.835	1228	116900	0.52	Unknown	-
16	22.171	1346	3618332	16.04	*α*-Terpinyl acetate	94
17	24.172	1422	464647	2.06	*β*-Caryophyllene	95
18	25.205	1462	140836	0.62	Humulen-(v1)	92
19	25.494	1474	553742	2.45	Unknown	-
20	25.983	1493	147099	0.65	Viridiflorene	94
21	26.628	1520	90449	0.40	*δ*-Cadinene	91
22	26.697	1523	143886	0.64	*trans*-Calamenene	94

23	28.033	1578	447507	1.98	Spathulenol	95
24	28.176	1584	101355	0.45	Caryophyllene oxide	86
25	28.258	1588	382893	1.70	Globulol	94
26	28.463	1596	184875	0.82	Viridiflorol	90
27	28.513	1598	107398	0.48	Cubeban-11-ol	87
28	29.137	1626	97688	0.43	Unknown	-
29	29.839	1657	155094	0.69	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90

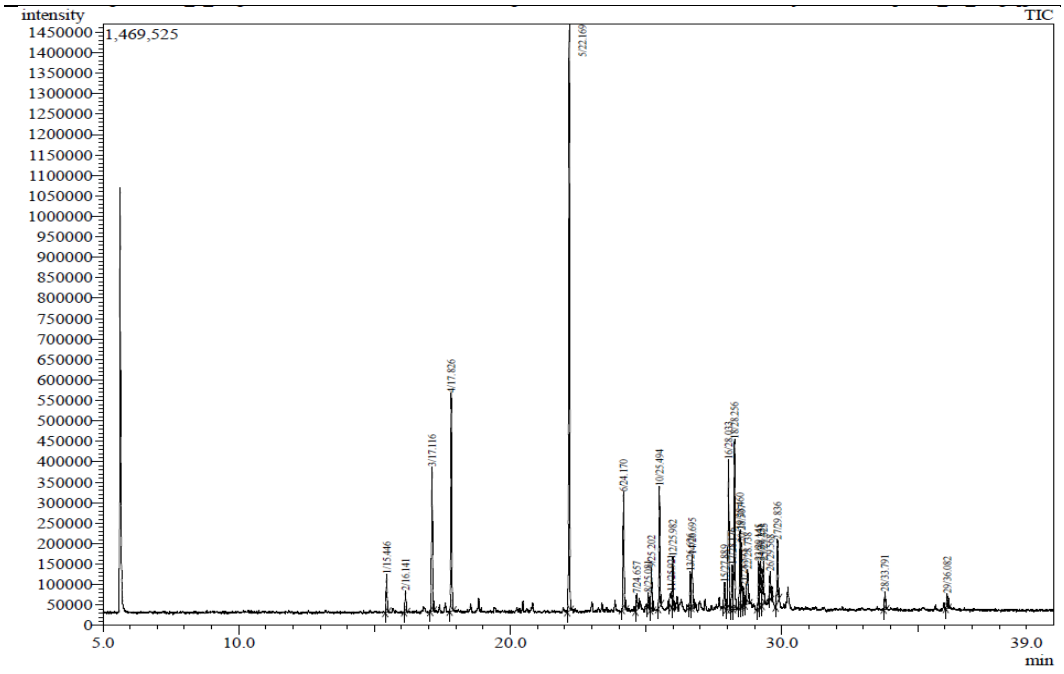
Table D27: Chemical composition and total ion chromatogram of HdB collected at 6th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	12.546	1032	114393	0.68	Eucalyptol	96
2	15.444	1118	412876	2.44	Fenchol	97
3	16.140	1140	216636	1.28	Sabinol	91
4	17.116	1171	1227394	7.26	Borneol	97
5	17.826	1194	1826514	10.80	*α*-Terpineol	96
6	18.839	1228	101535	0.60	Isocarveol	86
7	22.170	1346	4471162	26.43	*α*-Terpinyl acetate	95
8	23.858	1409	105948	0.63	*α*-Gurjunene	94
9	24.171	1422	1057325	6.25	*β*-Caryophyllene	96
10	24.656	1441	167145	0.99	Aromadendrene	95
11	25.082	1458	112991	0.67	Humulene	95
12	25.203	1462	342132	2.02	Humulen-(v1)	92
13	25.496	1474	959747	5.67	Unknown	-
14	25.983	1493	336011	1.99	Viridiflorene	93
15	26.627	1520	245909	1.45	*δ*-Cadinene	91
16	26.696	1523	377698	2.23	*trans*-Calamenene	96
17	27.890	1572	163534	0.97	Unknown	-
18	28.033	1578	874847	5.17	Spathulenol	96
19	28.175	1584	344093	2.03	Caryophyllene oxide	86
20	28.257	1588	963307	5.69	Globulol	93
21	28.461	1596	380737	2.25	Viridiflorol	91

22	28.507	1598	345003	2.04	Cubeban-11-ol	89
23	28.587	1602	110186	0.65	Unknown	-
24	28.737	1608	223044	1.32	Rosifoliol	94
25	29.146	1626	285237	1.69	Unknown	-
26	29.222	1630	266201	1.57	1,10-Di-epi-cubenol	94
27	29.325	1634	367051	2.17	Unknown	-
28	29.571	1645	136423	0.81	*τ*-Muurolol	92
29	29.837	1657	380423	2.25	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90

Table D28: Chemical composition and total ion chromatogram of HdB collected at 8th hour analysed using non-polar column.



Peak no.	RT (min)	RI	Peak area	Peak area %	Component name	% similarity
1	15.446	1118	243741	1.64	Fenchol	96
2	16.141	1140	134285	0.91	Sabinol	90
3	17.116	1171	896589	6.05	Borneol	97
4	17.826	1194	1342138	9.06	*α*-Terpineol	96
5	22.169	1346	3495886	23.59	*α*-Terpinyl acetate	95
6	24.170	1422	726858	4.90	*β*-Caryophyllene	96
7	24.657	1441	123488	0.83	Aromadendrene	95
8	25.081	1458	91958	0.62	Humulene	95
9	25.202	1462	257499	1.74	Humulen-(v1)	92
10	25.494	1474	706720	4.77	Unknown	-
11	25.921	1491	154833	1.04	Unknown	-
12	25.982	1493	313056	2.11	Viridiflorene	93
13	26.626	1520	209421	1.41	*δ*-Cadinene	91
14	26.695	1522	352739	2.38	*trans*-Calamenene	96
15	27.889	1572	191864	1.29	Ledol	88
16	28.033	1578	945804	6.38	Spathulenol	96
17	28.176	1584	345056	2.33	Caryophyllene oxide	86
18	28.256	1588	1129619	7.62	Globulol	94
19	28.460	1596	525597	3.55	Viridiflorol	89
20	28.507	1598	375630	2.53	Cubeban-11-ol	88
21	28.573	1601	125826	0.85	Unknown	-
22	28.738	1608	311123	2.10	Rosifoliol	95

23	29.145	1626	346412	2.34	β-Eudesmol	86
24	29.221	1630	280476	1.89	1,10-Di-epi-cubenol	95
25	29.323	1634	360043	2.43	Unknown	-
26	29.568	1645	160584	1.08	τ-Muurolol	90
27	29.836	1657	460313	3.11	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90
28	33.791	1840	108820	0.73	Hexahydrofarnesyl acetone	93
29	36.082	1946	104548	0.71	Butyl isobutyl phthalate	94

APPENDIX E

Observed mortality of mosquito larvae and brine shrimp nauplii.

Table E1: *Aedes aegypti* larval mortality after exposed to *Eucalyptus* leaf extracts for 24 h and 48 h.

Extracts	Concentration (µg/mL)	Observed Mortality							
		24 h				48 h			
		1	2	3	4	1	2	3	4
A	25	0	0	0	0	0	1	0	0
	50	0	0	0	0	2	1	0	1
	100	0	0	1	0	1	0	3	4
	200	1	1	0	1	2	2	2	4
	400	2	4	3	2	8	16	10	11
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
A10	25	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	1	0
	200	0	0	0	0	0	3	2	1
	400	0	2	1	2	2	3	3	3
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
B	25	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0
	400	0	0	0	0	1	1	0	1
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
B10	25	0	0	0	0	0	0	0	0
	50	0	0	0	2	4	2	1	2
	100	2	3	1	1	3	5	2	2
	200	2	1	5	3	10	9	14	7
	400	14	14	13	11	17	20	19	13
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
HfA	25	2	1	1	0	17	11	10	17
	50	12	11	12	10	18	18	19	20
	100	20	13	15	20	20	18	20	20
	200	19	20	20	20	20	20	20	20
	400	20	20	20	20	20	20	20	20
	1% (v/v) Methanol (negative control)	0	0	0	0	1	3	4	3
HdA	25	0	1	0	1	19	12	13	14
	50	6	8	2	5	18	14	17	15
	100	13	17	14	16	20	18	20	19

	200	20	20	17	20	20	20	20	20
	400	20	20	20	20	20	20	20	20
	1% (v/v) Methanol (negative control)	0	0	0	0	1	1	3	3
HfB	25	0	0	1	1	1	3	1	5
	50	1	0	1	0	5	4	5	6
	100	15	7	6	12	20	19	18	16
	200	15	19	19	18	16	20	20	20
	400	19	20	20	20	20	20	20	20
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
HdB	25	0	0	0	0	2	1	0	3
	50	4	0	1	1	5	2	3	2
	100	4	2	1	3	10	9	10	12
	200	16	17	19	15	18	20	20	19
	400	20	20	20	20	20	20	20	20
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
Temephos (positive control)	100	20	20	20	20	20	20	20	20

Total number of larvae per replicate is 20.
A: SCE of younger *Eucalyptus*
A10: Ethanol-assisted SCE of younger *Eucalyptus*
B: SCE of older *Eucalyptus*
B10: Ethanol-assisted SCE of older *Eucalyptus*
HfA: Fresh leaf from younger *Eucalyptus*
HdA: Dried leaf from younger *Eucalyptus*
HfB: Fresh leaf from older *Eucalyptus*
HdB: Dried leaf from older *Eucalyptus*

Table E2: *Aedes albopictus* larval mortality after exposed to *Eucalyptus* leaf extracts for 24 h and 48 h.

Extracts	Concentration (µg/mL)	Observed Mortality							
		24 h				48 h			
		1	2	3	4	1	2	3	4
A	25	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	1	0
	400	1	1	3	2	6	5	5	5
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
A10	25	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0
	400	1	0	0	0	2	0	3	2
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
B	25	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0
	400	2	1	2	6	7	4	9	11
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
B10	25	0	0	0	0	0	0	0	0
	50	0	0	1	1	1	1	3	1
	100	2	5	3	1	6	8	3	4
	200	8	8	8	6	9	11	9	7
	400	8	5	11	7	9	10	12	10
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
HfA	25	0	0	1	0	5	5	5	5
	50	1	0	2	2	4	6	3	8
	100	15	11	16	14	17	17	17	16
	200	20	20	20	20	20	20	20	20
	400	20	20	20	20	20	20	20	20
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
HdA	25	0	0	0	0	1	1	0	1
	50	0	0	0	0	3	1	3	8
	100	12	15	12	6	19	19	16	12
	200	20	20	20	20	20	20	20	20
	400	20	20	20	20	20	20	20	20
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
HfB	25	0	0	0	0	1	1	0	2
	50	7	5	2	7	17	17	12	16
	100	16	15	17	17	19	19	19	19
	200	20	20	20	20	20	20	20	20
	400	20	20	20	20	20	20	20	20

	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
HdB	25	0	0	0	0	0	0	0	0
	50	0	0	0	0	1	0	1	0
	100	3	8	3	5	14	17	12	20
	200	19	20	20	20	20	20	20	20
	400	20	20	20	20	20	20	20	20
	1% (v/v) Methanol (negative control)	0	0	0	0	0	0	0	0
Temephos (positive control)	100	20	20	20	20	20	20	20	20

Total number of larvae per replicate is 20.
A: SCE of younger *Eucalyptus*
A10: Ethanol-assisted SCE of younger *Eucalyptus*
B: SCE of older *Eucalyptus*
B10: Ethanol-assisted SCE of older *Eucalyptus*
HfA: Fresh leaf from younger *Eucalyptus*
HdA: Dried leaf from younger *Eucalyptus*
HfB: Fresh leaf from older *Eucalyptus*
HdB: Dried leaf from older *Eucalyptus*

Table E3: *Artemia franciscana* mortality after exposed to *Eucalyptus* leaf extracts for 24 h.

Extracts	Concentration (µg/mL)	Observed Mortality		
		1	2	3
A	1	0	0	0
	10	0	0	0
	100	1	0	0
	500	10	10	10
	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
	5% (v/v) Ethanol (negative control)	0	0	0
	10% (v/v) Ethanol (negative control)	0	1	1
A10	1	0	0	0
	10	0	0	0
	100	4	2	2
	500	9	8	7
	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
	5% (v/v) Ethanol (negative control)	0	0	0
	10% (v/v) Ethanol (negative control)	0	1	1
B	1	0	0	0
	10	0	0	0
	100	2	1	1
	500	10	8	8
	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
	5% (v/v) Ethanol (negative control)	0	1	0
	10% (v/v) Ethanol (negative control)	0	0	2
B10	1	0	0	0
	10	0	0	0
	100	3	2	2
	500	9	8	7
	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
	5% (v/v) Ethanol (negative control)	0	1	0
	10% (v/v) Ethanol (negative control)	0	2	0
HfA	1	0	0	0
	10	0	0	0
	100	0	0	0
	500	10	10	10
	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
HdA	1	0	0	0
	10	0	0	0
	100	0	0	0
	500	10	10	10
	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
HfB	1	0	0	0
	10	0	0	0
	100	0	0	0
	500	10	10	10

	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
HdB	1	0	0	0
	10	0	0	0
	100	1	0	0
	500	10	10	10
	1000	10	10	10
	1% (v/v) Ethanol (negative control)	0	0	0
Potassium dichromate (positive control)	1	1	0	0
	10	3	3	1
	100	9	9	9
	500	10	10	10
	1000	10	10	10

Total number of brine shrimp nauplii per replicate is 10.
A: SCE of younger *Eucalyptus*
A10: Ethanol-assisted SCE of younger *Eucalyptus*
B: SCE of older *Eucalyptus*
B10: Ethanol-assisted SCE of older *Eucalyptus*
HfA: Fresh leaf from younger *Eucalyptus*
HdA: Dried leaf from younger *Eucalyptus*
HfB: Fresh leaf from older *Eucalyptus*
HdB: Dried leaf from older *Eucalyptus*