CHEMICAL VARIABILITY OF CYPRESS (*Cupressus lusitanica*) ESSENTIAL OIL AND ITS INSECTICIDAL EFFICACY AGAINST *Musca domestica* L.

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Analytical Chemistry of Moi University

November 2022

DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced for other purpose than academic without the prior written permission of the author and/or Moi University.

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DEDICATION

This thesis is dedicated to the Almighty God for his daily guidance, and to my beloved family for their never-ending encouragement and moral support that have given me power to reach this far.

ABSTRACT

Housefly, *Musca domestica* Linnaeus (Muscidae) is a household pest that is primarily controlled by chemical insecticides that often affect the environment and promote insecticide resistance. As a safer alternative to the chemical insecticides, plants of Cupressaceae family are often used locally to repel houseflies (HFs) in different parts of Rwanda and Uganda. However, studies on their chemical characterization and bioactivity are limited. This study aimed at assessing the chemical variability and insecticidal efficacy of essential oils (EOs) from *C. lusitanica* Mill. (Cupressaceae) growing in three agro-ecological zones (AEZs) of Rwanda. The specific objectives were to: (i) Compare the yields of EOs from *C. lusitanica* leaves collected from three AEZs in Rwanda, (ii) analyze the major chemical components of obtained EOs, and (iii) evaluate their insecticidal activity against houseflies. Fresh leaves of *C. lusitanica* were judgmentally collected from Burera, Huye and Kayonza districts representing highlands (HLZ), midlands (MLZ) and lowlands (LLZ) zones, respectively. Steam distillation was used to extract the EOs and the average yields as per AEZ were calculated. The chemical components of EOs were analyzed using Fourier Transform Infrared (FT-IR) Spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS), while insecticidal activity was evaluated by exposing adult houseflies separately to test solutions (90, 70, 50 and 30% v/v) for 6, 12 and 24 h. Acetone and Dichlorvos (DDVP, 77%) insecticide served as test controls. The lethal doses (LD_{50} and LD_{90}) of tested solutions obtained via Probit analysis and Repellency percentages (PR) were recorded. Significantly higher (P= 0.02) EOs yield was from LLZ (Kayonza) (0.39 ± 0.01% v/w) when compared to HLZ (Burera) (0.27 ± 0.02% v/w) that was not significantly different (P= 0.13) from MLZ (Huye) EOs yield (0.34 ± 0.02% v/w). FT-IR results indicated the presence of C-H stretch for alkanes (2950-2850 cm^{-1}), C=O bend for aldehydes (1720-1740 cm^{-1}), Ketones (1720-1705 cm^{-1}), and Carboxylic acids (1725-1700 cm^{-1}), C=C for alkenes (~1640 cm^{-1}) and C-C stretch for Aromatics (900-800 cm^{-1}) in the EOs. GC-MS results revealed the dominance of Sabinene, Myrcene and α-Pinene for EOs from HLZ; Umbellulone, δ-3-Carene and Sabinene for EOs from MLZ; and γ-Terpinene, Umbellulone and Bornyl acetate for EOs from LLZ. DDVP insecticide showed significantly higher fumigant (LD_{50} of 0.015 to 0.002 ppm and LD_{90} of 0.52 to 0.20 ppm) and contact (LD_{50} of 0.01 to 0.001 ppm and LD_{90} of 1.03 to 0.19 ppm) toxicities against adult houseflies exposed for 6 to 24h. EOs from LLZ showed the highest contact toxicity on houseflies exposed for 24 h (LD_{50}=0.08 ppm; LD_{90}=16.26 ppm), while EOs from HLZ showed the lowest toxicity in 6 h of exposure (LD_{50} = 0.64 ppm; LD_{90} = 706.21ppm). EOs from MLZ showed toxicity with LD_{50} values of 0.41 to 0.15 ppm and LD_{90} of 453.24 to 22.01 ppm in 6 to 24 h of exposure. Fumigant toxicity followed a similar trend (LLZ: LD_{50}=0.15 ppm; LD_{90}=24.79 ppm for houseflies exposed for 24 h; HLZ: LD_{50}=1.90 ppm; LD_{90}=1250.21 ppm after 6 h of exposure; and MLZ EOs with LD_{50} of 0.66 to 0.28 ppm; and LD_{90} of 521.36 to 80.65 ppm in 6 to 24 h of houseflies' exposure. The repellent activity indicated that fewer adult houseflies were repelled at low dose (30% v/v) of EOs for 6 h, with MLZ EOs recording higher PR (42.77 ± 2.79) than LLZ (40.47 ± 0.62) or HLZ (36.81 ± 2.39). However, over 70% of houseflies were repelled at high dose (90% v/v) of EOs for 24 h (MLZ: 79.52 ± 3.30, LLZ: 75.62 ± 1.37, and HLZ: 70.38 ± 3.20%). At all tested doses, less than 14% and over 97% of houseflies were repelled by Acetone and DDVP, respectively. The variation of yields and chemical components as well as promising insecticidal activities were observed for EOs of *C. lusitanica* from the three study-based AEZs. The promising findings of this study call for further research into the biosafety of *C. lusitanica* EOs for use as safer insecticide against housefly.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE II-PTRE	Africa Center of Excellence II in Phytochemicals, Textiles and Renewable Energy
AEZs	Agro-Ecological Zones
DDVP	Dimethyl 2,2-Dichlorovinyl Phosphate insecticide
DMAPP	Dimethyl Allyl Pyrophosphate
EOs	Essential Oils
FAO	Food and Agriculture Organization
FT-IR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography- Mass spectroscopy
GPP	Geranyl Diphosphate
HLZ	Highlands zone
ICP	International Conference on Pesticides
IPP	Isopentenyl Pyrophosphate
LD_{50} /LD_{90}	Lethal dose/concentration that kills 50 or 90% of population in assay
LLZ	Lowlands zone
ANOVA	Analysis of Variance
MEP	2- Methyl Erythritol-4-Phosphate
MLZ	Midlands zone
REMA	Rwanda Environmental Management
SCFE	Supercritical fluid extraction
UNDP	United Nations Development Program
UR-CST	University of Rwanda – College of Science and Technology

ACKNOWLEDGEMENTS

First, I strongly thank the African Centre of Excellence in Phytochemistry, Textile and Renewable Energy (ACE II-PTRE) for granting me an opportunity to pursue Master of Science in Analytical Chemistry, and financed this research project.

My heartfelt thanks are particularly addressed to my supervisors; Dr. Jackson K. CHERUTOI, Dr. Jacqueline K. MAKATIANI and Prof. Théoneste MUHIZI for their constructive ideas and valuable advice during elaboration of this research project.

I would like to thank Laboratory Technicians, Mr. Frodouard HITIMANA, Mr. Védaste NYANDWI and Mr. Emmanuel NKUNDIMANA for their valuable assistance in essential oil extraction and FT-IR analysis. Special Thanks to Mr. Peter BIWOTT for his technical assistance during field collection and maintenance of houseflies.

I cannot forget to express my particular gratitude to my fellow colleagues, especially Mr. Timothy OMARA for daily encouragement and his scientific ideas during elaboration of this research project.

CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Pests are serious threats to the environment and public health due to their transmission of disease-causing pathogens (He *et al.*, 2021; Khamesipour *et al.*, 2018; Pierattini *et al.*, 2019). Among the pests, Insects are widespread in all types of environments, accounting for almost two-thirds of all known animal species, and can do harm in a variety of ways (Gizaw *et al.*, 2019). Many of insect pests feed on all kinds of plants including crop plants, and they also infest the food and other stored products and cause a huge food loss and deterioration of food quality, as well as spreading of vector-borne diseases (Gizaw *et al.*, 2019; Navarajan, 2007).

The housefly, *Musca domestica* Linnaeus (Diptera: Muscidae) is among the most successful pests on the planet due to its adaptation and arthropodal traits which enable it to compete successfully with the human population for scarce food and other natural resources (Ayo *et al.*, 2019). Moreover, houseflies are a threat to public health due to their vectorial capacity of pathogens from different sources including sewage, garbage and other sources of filth, where pathogens are picked up and transferred to foods and beverages, as well as household materials (Abbas *et al.*, 2013; Elbermawy *et al.*, 2011). Flies are also responsible for transmission of eye diseases like trachoma and skin diseases such as cutaneous diphtheria, mycoses, yaws and leprosy (Malik *et al.*, 2007).

The conventional method of pests management involves the use of chemical pesticides with the global annual usage reported by International Conference on Pesticides (ICP) of 2.5 million tons of pesticides in 2003, and probable increment of 1.5 million tons of pesticides by 2020 (Sharma *et al.*, 2019). Among chemical insecticides that are often used for control of the housefly population; organophosphates, pyrethroids, organochlorines and carbamates are well-known (Acevedo & Zapater, 2009). However, their environmental damage and promotion of insecticides resistance have necessitated the implementation of physical (like light-sticky and suction trap) and biological control techniques (Lushchak *et al.*, 2018; Ojianwuna *et al.*, 2011). In addition, the prior use of natural source pesticides has received a lot of attention due to their compatibility with environmental components, and they generally constitute the umbrella of green pesticides, which refer to all types of nature-oriented materials that contribute to the reduction of pest population (Chauhan *et al.*, 2017; Gaire *et al.*, 2017; Kamel *et al.*, 2019; Mossa, 2016; Sola *et al.*, 2014).

1.2. Statement of the Problem

Since the beginning of time, pests have been a source of enormous damage and transmission of some vector-borne diseases (Okwute, 2012). As pest, housefly (*Musca domestica*) is a public health threat due to its vectorial transmission of various disease-causing pathogens responsible for different vector-borne diseases including typhoid, dysentery and cholera (Geden, 2015; He *et al.*, 2021; Khamesipour *et al.*, 2018; Pierattini *et al.*, 2019). The pests control mostly involves the use of chemical pesticides that were reported to affect the environment and promote pesticide resistance (Kole *et al.*, 2019; Lushchak *et al.*, 2018). A large portion of used chemical pesticides is often reported to

reach destinations other than the targeted species and cause detrimental effects on environmental components as well as human health problems like disruption of reproductive and central nervous systems and increase the risk of developing different types of cancers include blood cancers; leukemia and lymphoma that significantly affect children (Lans-ceballos *et al.*, 2018; Mossa, 2016; Sarwar, 2016).

Different plant species (including Cupressaceae species in current study) have been documented to have great insecticidal activity against a variety of insects include houseflies, implying that they could be used as an alternative to chemical insecticides (Ebadollahi *et al.*, 2020; Giatropoulos *et al.*, 2013; Hasaballah *et al.*, 2018; Laxmishree & Nandita, 2017). Locally, Cupressaceae species (including *C. lusitanica* Mill.) are used to repel houseflies in Rwanda and Uganda (Baana *et al.*, 2018). As a result, there is a need to evaluate the effectiveness of *C. lusitanica* essential oils in controlling housefly and analyze the major chemical components that are likely linked to the essential oil's bioactivity.

1.3. Justification of the study

A survey conducted by Baana *et al.*(2018) revealed the local use of different plants of Cupressaceae family in different regions of Uganda for repelling houseflies in people's settlements. The modes of application include burning dry (dried in shade for a week) or fresh leaves and stem barks to generate smoke, place the fresh leaves and branchlets where flies are numerous or hang them on the roofs and walls of latrines and houses. In a similar manner, Cupressaceae species are locally used to fend off houseflies in different regions of Rwanda. In addition, the essential oils of different Cupressaceae species were reported in

different studies to exhibit the insecticidal activity against various insects due to their high terpene contents (Amria *et al.*, 2011; Aurora *et al.*, 2019; Tian, 2017). However, such terpene contents and other phytochemicals could vary from individual plant to another due to different factors linked to plant's ecological habitat and its connected environmental and climatic features that finally affect the bioactivity owned by plant species (Curado *et al.*, 2006; Karami *et al.*, 2020). Thus, *C. lusitanica* species in different ecological habitats could normally produce unequal amount of essential oils of different chemical profiles and bioactivities. Therefore, the determination of individual or population's chemical features and oil contents of *C. lusitanica* is very helpful to select the population or individual plant with higher oil content and distinct active components to fully utilize the bioactivity owned by this species. Furthermore, no similar scientific work was reported in open literature on essential oil of this species against houseflies in ecological zones of Rwanda.

1.4. Objectives

1.4.1. Main Objective

To characterize and assess insecticidal efficacy of essential oil from *C. lusitanica* leaves collected from Agro-ecological zones of Rwanda against adult housefly.

1.4.2. Specific objectives

i. To extract and compare the yields of essential oil from *C. lusitanica* leaves collected from three agro-ecological zones in Rwanda
ii. To analyze the major chemical components of essential oil of *C. lusitanica* leaves from three agro-ecological zones in Rwanda

iii. To evaluate the insecticidal potential of essential oil from *C. lusitanica* leaves against adult housefly.

1.5. Research Hypotheses

1. Fresh leaves of *C. lusitanica* from different ecological zones of Rwanda produce equal amount of essential oils.
2. Chemical components of essential oils from *C. lusitanica* do not vary as per agro-ecological zone.
3. Essential oils of *C. lusitanica* do not exhibit insecticidal activity against adult houseflies.

1.6. Significance of the study

The study provides data on the essential oil contents and chemical compositions, as well as on their efficiency as toxicant and repellent against adult houseflies; this has bridged the gap between the available knowledge on the local use of Cupressaceae species (especially *C. lusitanica*) as housefly repellents and insecticidal potential owned by EOs from these species against adult houseflies. Equally, the study provides data on insecticidal potential of EOs from *C. lusitanica* against adult housefly. Therefore, this EOs should be useful in development of new and safer plant-origin insecticide for housefly control in order to avoid the vector-borne diseases and other damages caused by this pest. This will also help to reduce the health risks associated to the use of chemical insecticides in controlling housefly, especially in people's settlements.

1.7. Limitation of the study

Despite the probable seasonal variation of phytochemicals among plant species, the fresh leaves of *C. lusitanica* were only sampled in a single season (rainy season) because of time limit. In addition, the method of extraction has an effect on the essential oil components and yield. Therefore, the use of different extraction methods is required for better comparisons, However, the financial and time limits have led to the use of only steam distillation, and the major components of essential oil were not individually isolated.

CHAPTER TWO

LITERATURE REVIEW

2.1. Plant ecology and phytochemicals variation

Global diversity of vegetation is noticeably influenced by different factors including climatic and ecological conditions of habitat through alteration of the life cycle, phytochemical composition and distribution of plant species, as well as development of new physical traits (Hufnagel & Garamvölgyi, 2014; Sampaio & Batista, 2018; Seth, 2004). Moreover, climatic and environmental changes are main sources of non-uniformity in production, accumulation and bioactivities of phytochemicals among same individual plant species from different ecological habitats. Therefore, environmental factors can be advantageous for the production of one chemotype but may exclude other chemotypes from plant population (Fongang & Bankeu, 2020; Hüsnü & Gerhard, 2010).

The abiotic factors such as light, altitude, temperature, soil properties, and precipitation (water availability) affect plant metabolites by altering the production pathways of a certain compound, leading to biosynthesis of different compound (Fernandes *et al.*, 2017; Kumar *et al.*, 2017). For example, previous studies have revealed that several plants of arid regions increase their phenolic contents, decrease proteins and carbon metabolites as an adaptive strategy to drought (Karami *et al.*, 2020; Mansour-Gueddes *et al.*, 2020; Mishra, 2016). It was reported that plants adapt and tolerate environmental stresses through variation in biosynthesis and regulation of their secondary metabolites, which is an adaptive complex mechanism of physiological and molecular programs that involves several genes and biosynthetic pathways (Barra, 2009; Kabtni *et al.*, 2020).

2.2. Biosynthesis and chemistry of plant essential oils

Essential oil is a complex mixture of more than five thousand chemical components with predominance of terpene hydrocarbons as well as their oxygenated derivatives like oxides, alcohols, aldehydes, ketones and acids (Butnariu & Sarac, 2018; Morsy, 2017; Sampaio & Batista, 2018; Yavari *et al.*, 2010). Chemical components of essential oil are classified into volatile fraction which constitutes 90-95% of total composition and consist of terpene hydrocarbons (Figure 2.1a) and their oxidative derivatives (Figure 2.1b), while non-volatile residues occupy 5-10% of constituents (Figure 2.1c), and they are made of fatty acids, waxes, sterols, flavonoids and carotenoids (Djilani & Dicko, 2014; Eslahi *et al.*, 2018; Shaaban *et al.*, 2012; Tongnuanchan & Benjakul, 2014).

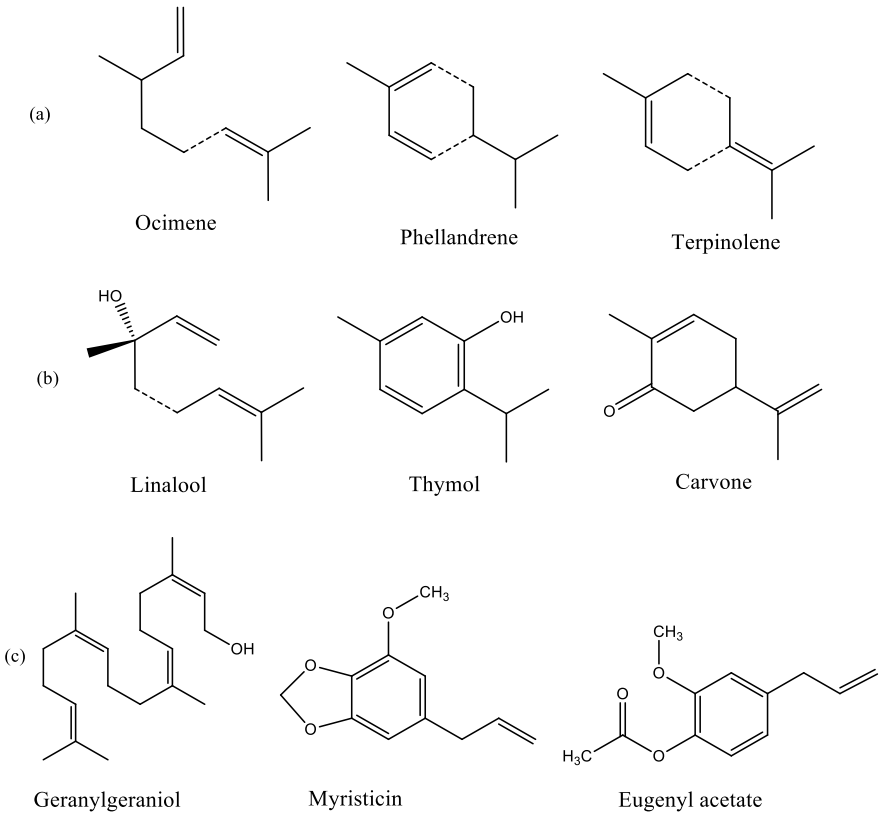


Figure 2.1. Chemical structures of: (a) terpenes, (b) their oxygenated derivatives, and (c) non-volatile chemical components of essential oils.

Essential oils (EOs) are widely distributed in the plant kingdom, but only aromatic plants contain extractable amounts, and they are accumulated in all parts of the plant with most amounts being less than 1% and rarely reach 15% of yield (Butnariu & Sarac, 2018). The term aromatic plant is attributed to plant species containing specialized secretory cells that store volatile oils and they are always emitting a blend of scent of characteristic odor (Dhifi *et al.*, 2016; Opender *et al.*, 2008; Sharifi-Rad *et al.*, 2017).

Qualitative and quantitative variations of essential oils among plant species occur due to the ecological habitat conditions (soil type, climate and altitude), maturity and genetic variety of plants (Djilani & Dicko, 2014; Lingan, 2018). Despite the climatic and environmental factors, the amount and quality of essential oil can also vary depending on the part of the plant used, harvesting time, and during sample processing and storage conditions (Butkienë *et al.*, 2015; Fongang & Bankeu, 2020; Juliani, 2017). The stated factors greatly influence the variation of phytochemicals and their bioactivities among the plant species growing in a certain ecological habitat (Curado *et al.*, 2006; Karami *et al.*, 2020).

It was reported that, aromatic plants synthetize their essential oil constituents through three biosynthetic pathways, which are the shikimic acid pathway leading to phenylpropenes and benzoic acid derivatives present in several essential oils; the methylerythritol pathway leading to mono- and di-terpenes, and the mevalonate pathway leading to sesquiterpenes (Moghaddam & Mehdizadeh, 2017). The oxidative derivatives of terpenes which are

highly odoriferous and responsible for the fragrance of certain plants species are derived through mevalonate and methylerythritol pathways (Morsy, 2017).

Normally, plants synthetize their secondary metabolites from primary metabolites through different pathways, including Glycolysis, Krebs cycle, Shikimate and Pentose phosphate pathways that result in intermediate precursors (Figure 2.2), however, the pathway and final compound may change in same or different parts of plant depending on different developmental stages (Aharoni & Galili, 2011; Barra, 2009).

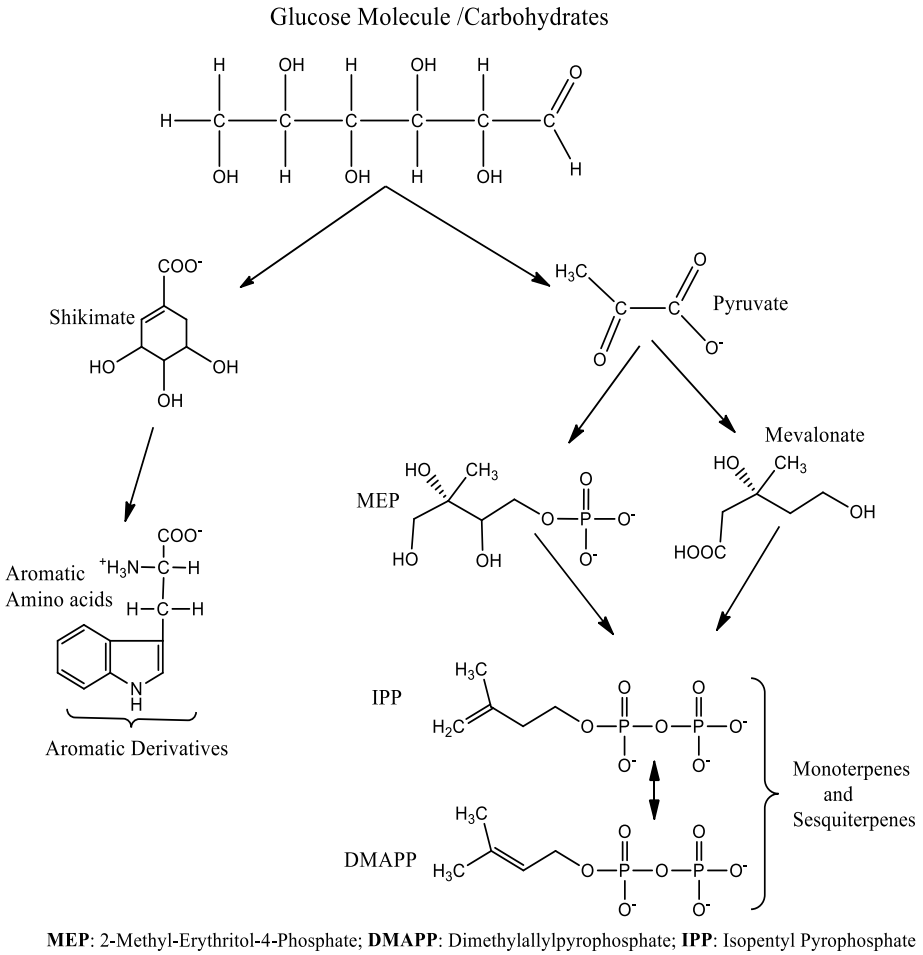


Figure 2.2. Summarized biosynthesis of precursors of essential oil constituents (adapted from Grausgruber-Gröger *et al.*, 2012).

During biosynthesis of essential oil constituents, the active isoprene units; isopentenyl pyrophosphate (IPP) repeatedly joins with its isomer, dimethyl allyl pyrophosphate (DMAPP) in a head-to-tail manner to form a C_{10} compound, Geranyl diphosphate (GPP) also known as Geranyl pyrophosphate (Figure 2.3), that is the precursor of all terpenes and terpenoids (Prins *et al.*, 2010; Rehman *et al.*, 2016).

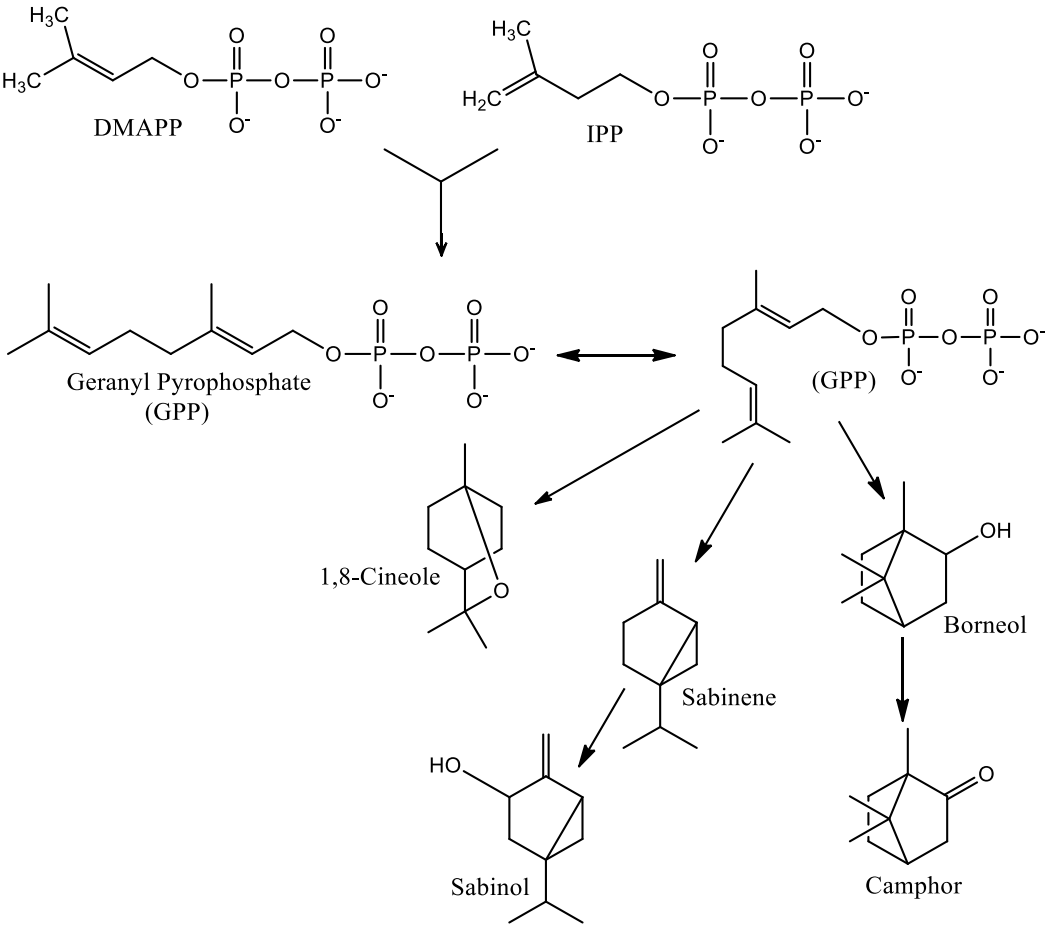


Figure 2.3. Summary of biosynthetic mechanisms of essential oil constituents. (Adapted from Grausgruber-Gröger *et al.*, 2012).

2.3. Ecological adaptations and importance of *Cupressus lusitanica* Mill.

The genus *Cupressus* is part of Cupressaceae family, which comprises about 30 genera and 130 species of evergreen coniferous trees (Farjon, 1993). *C. lusitanica* Mill. also known as Mexican white cedar, is a coniferous tree belongs to Pinopsida Class and Spermatophya Phylum native to Mexico and Central America (Kimutai & Mainya, 2016; Kuiate *et al.*, 2006; Tesfaye *et al.*, 2020). It grows fast and reach up to 35 m in favorable sites and tends to be invasive at high altitude regions (Kuiate *et al.*, 2006). The morphological characteristics of *C. lusitanica* is more distinguishable; it has scale-like leaves, rough sub-cylindrical branchlets aligned along a single plane, and sub-ovulate cones with six to ten scales (Brink *et al.*, 2007; Mamo & Adilo, 2004) (Figure 2.4).

Figure 2.4. *Cupressus lusitanica* Mill. plant in its habitat.
(***Source***: Photo taken from the field during sampling)

The ecological features favorable for *C. lusitanica* include moist climates and altitude of 1,000 to 4,000 m, with average annual rainfall ranges from 800 to 4,000 mm and mean annual temperature of 12 to 30 ^{o}C (Mamo & Adilo, 2004). *C. lusitanica* can also tolerate short dry season and drought, but it cannot withstand waterlogging, and it is very adaptive to deep, fertile, drained and moist soil with neutral to little acidic property (Orwa *et al.*, 2009).

Cupressus lusitanica was firstly introduced in Rwanda around 20th Century and it is very adaptive to the hilly soil of Rwanda, which is characterized by volcanic mountains of more than 4,000 m above the sea level at the North-West, wavy hills in most central regions, whereas the East region is relatively flat with less than 1,500 m above the sea level (Kamatenesi-Mugisha *et al.*, 2013; Nduwamungu, 2011; Nsabimana *et al.*, 2008). Thus, the above topographic pattern is very responsible to the moderate and cool climate of Rwanda characterized by average annual temperature and precipitation of 20 ^{o}C and 1,200 mm, respectively (Nduwamungu, 2011; Rwanda Environment Management Authority [REMA], 2011).

Cupressus lusitanica has become the major plantation in Eastern Africa and it has crucial economic and medicinal uses, like timber production (furniture, construction), fire wood production, wind breaking, and it is used as degraded-land reclamation plants (Kimutai & Mainya, 2016). It has been traditionally used to fend off insect pests from stored grains, and as flies repellent in rural areas (Almasa *et al.*, 2019; Baana *et al.*, 2018; Hassanzadeh *et al.*, 2010). *C. lusitanica* leaves have medical importance in curing headache, flu, catarrh and some skin diseases caused by fungi, while its essential oil is very useful in treatment

of cough and rheumatism. Moreover, *C. lusitanica* is mostly used for ornamental purpose as Christmas tree, and it is also useful in making various cosmetics like deodorants, perfumes and soaps (Kamatenesi-Mugisha *et al.*, 2013; Kimutai & Mainya, 2016).

2.4. Phytochemistry of Cupressaceae family

Studies have revealed a variety of phytochemicals isolated from different species of Cupressaceae family include flavonoids, tannins, saponins, phenolic compounds and terpenoid that makes a larger portion of essential oil components (Al-Snafi, 2016). The chemical features of essential oils from cypress species have been studied and reported to be largely dominated by monoterpene hydrocarbons and their oxidative compounds (Langsi *et al.*, 2018; Nouri *et al.*, 2015; Teke *et al.*, 2013) (Figure 2.5). Of all the monoterpene hydrocarbons, α-pinene, limonene, sabinene and γ-3-carene were frequently reported as dominants, while terpinen-4-ol, linalool, α-terpineol and umbellulone are the most reported oxygenated monoterpenes (Hassanzadeh *et al.*, 2010; Langsi *et al.*, 2018; Nouri *et al.*, 2015; Teke *et al.*, 2013).

Pierre-leandri *et al.*(2003) have investigated seven Cupressus species and revealed myrcene, sabinene and cymene as major monoterpene components in their essential oils, whereas the oxidative monoterpenes were terpinolene, terpinen-4-ol and bornyl acetate. Bett *et al.*(2017) and Kuiate *et al.*(2006) reported the dominant of α-pinene, sabinene and limonene in essential oil extracted from the leaves of *C. lusitanica*, however umbellulone, terpinen-4-ol and linalool were dominant oxygenated monoterpenes. The sesquiterpenes (cadrene, α-amorphene, germacrene, calamenene and cadinene) and their oxidative

derivatives (Cadrol, α-Cadinol, α-Acorenol and Geranyl acetone) were regularly reported as minor constituents in essential oil of Cupressaceae family (Filho *et al.*, 2011; Langsi *et al.*, 2018; Nouri *et al.*, 2015; Teke *et al.*, 2013).

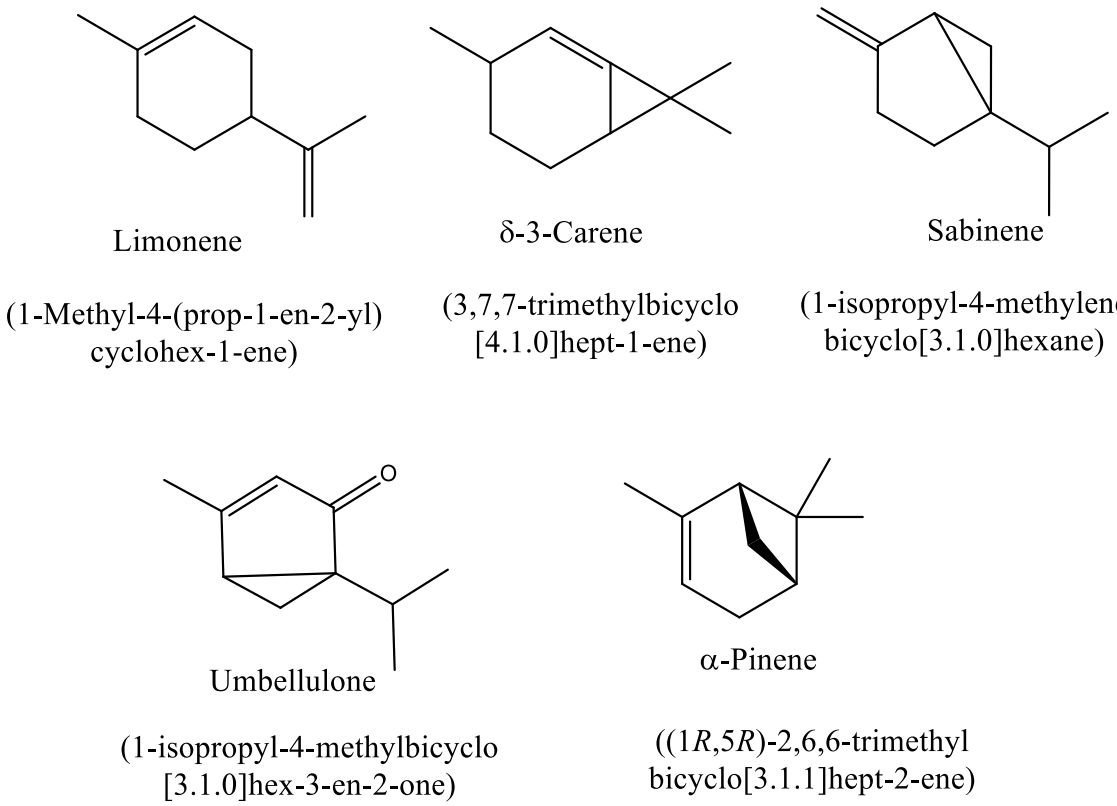


Figure 2.5. Structures of some compounds reported in essential oil of different Cupressaceae species (Bett *et al.*, 2017; Kuiate *et al.*, 2006; Teke *et al.*, 2013)

2.5. Essential oils extraction methods

Essential oils are extracted from different parts of aromatic plants including the leaves, petals, stems, seeds, and even the roots via various techniques including conventional techniques like hydro-distillation (water distillation), direct steam distillation, water-steam distillation, soxhlet extraction, maceration and effleurage (Danlami *et al.*, 2014; Doughari,

2014). Although they are widely used, those techniques are classified as traditional techniques and some are time and energy-consuming with lower yields of production (Ayvaz *et al.*, 2008; Aziz *et al.*, 2018; Butnariu & Sarac, 2018; Naeem *et al.*, 2018).

Modern techniques like solid phase micro-extraction (SPME), Supercritical fluid extraction (SFE), and Microwave-energy based methods have been developed for effective extraction of phytochemicals including essential oils (Rassem *et al.*, 2016). However, they are valuable in certain situation like production of overpriced essential oils due to their costly equipment (Handa *et al.*, 2008; Rassem *et al.*, 2016).

Steam distillation method is worth an estimated 93% of the efficiency production in essential oils extraction (Aziz *et al.*, 2018; Dixit, 2007), and its system consists of a packed bed of the plant materials that is connected to the steam source (Figure 2.6) and allows only steam to pass through it while boiling water is not mixed with plant materials (Tongnuanchan & Benjakul, 2014).

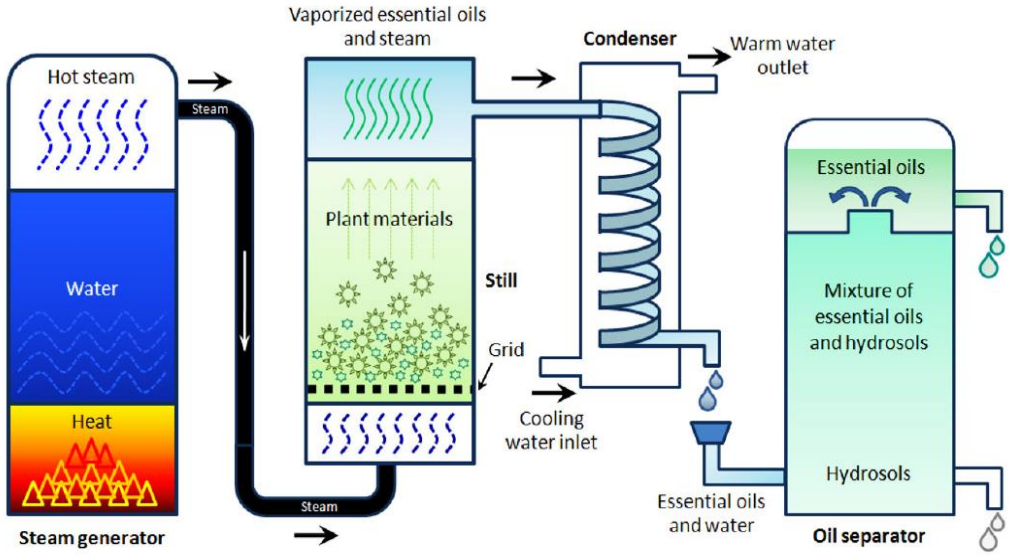


Figure 2.6. Diagram illustrating industrial steam distillation for essential oil extraction. (*Source:* Tongnuanchan & Benjakul, 2014).

Steam distillation is mainly used for extraction of essential oils at temperatures below 100 °C and it is the most widely accepted process for the production of essential oils on large scale due to its relative advantage of preventing thermal degradation of chemical components (Nasardin *et al.*, 2018; Tongnuanchan & Benjakul, 2014).

In contrast, water distillation (commonly called hydro distillation) consists of packing plant materials in still with addition of sufficient water. By boiling, the steam and hot water influence the freed of essential oil from glands in plant tissues and form vapor mixture with water which is automatically distilled-off via separator after condensation (Dilworth *et al.*, 2017; Zhang-Wen *et al.*, 2018). Regardless its slowness and degradation of required compounds due to prolonged heat, water distillation is facile and less expensive (Attokaran, 2017; Butnariu & Sarac, 2018; Kimutai & Mainya, 2016).

Supercritical fluid extraction (SCFE) is a modern extraction technique involves the use of supercritical fluids as solvent due to their interesting properties, such as low viscosity, high diffusivity and density closer to liquids at their critical temperature and pressure (Doughari, 2014). Carbon dioxide (CO_2) is the most useful supercritical solvent in extraction processes because of its suitable properties like inflammability and inertness, relatively low critical temperature and pressure, high purity and easy recovery (Doughari, 2014; Rassem *et al.*, 2016). Supercritical fluid extraction has been slow to find commercial applications because it requires sophisticated and expensive high-pressure equipment and technology (Parhi & Suresh, 2013; Zhang-Wen *et al.*, 2018).

On the other hand, the microwave energy based techniques are the most developed methods of essential oils and natural products extraction with much attention that has been devoted to their applications with some advantages over other methods like hydro-distillation, including high yield and purity of extracted oil, rapidity and the lower energy consumption (Akhtar *et al.*, 2019).

Some of microwave-energy based techniques that are mostly used for essential oils extraction are microwave dielectric heating extraction, vacuum microwave hydro-distillation, microwave steam distillation, solvent-free microwave extraction, microwave-accelerated steam distillation and microwave-assisted extraction (Desai *et al.*, 2010). Microwave-energy based techniques use the non-ionizing electromagnetic waves of frequency between 0.3 to 300 GHz, that reach the inner glandular, trichomes and vascular systems of the plant materials to vaporize volatile materials which increases the internal

pressure of the cell and finally causes the cell rupture (Doughari, 2014; Rassem *et al.*, 2016). The use of microwave energy in essential oil extraction provides more valuable essential oil with higher amounts of oxygenated components, but it causes fewer chemical changes of original plant components such as rearrangement, dehydration and isomerization (Desai *et al.*, 2010).

2.6. Storage of essential oils

After extraction, the essential oil should be stored in appropriate container, at lower temperature (4-8 °C) to retain their quality and quantity before analysis (Rowshan *et al.*, 2016). The lower temperature prevents evaporation of compounds of lower boiling temperature. They should also be kept away from sunlight to prevent deterioration due to photo-degradation (Filho *et al.*, 2011; Rao *et al.*, 2011). The plastic containers are not suitable for essential oil storage due to the absorption that may take place and alter the properties, as well as the compositions of essential oil (Edris, 2016). Since some essential oils are more reactive and prone to oxidation, the small amount of essential oil should not be kept in large bottles with large air space above the oil (Edris, 2016; Filho *et al.*, 2011; Umereweneza *et al.*, 2019). Normally, the, the dark, brown or blue-colored glass bottles of fitting size are good for storage of any type of essential oil (Dixit, 2007).

2.7. Housefly

2.7.1. Housefly biology and adaptation

The housefly, *Musca domestica* L. (Diptera: Muscidae), also known as common fly, is the most widespread fly species on the planet. It is believed to have probably originated from

the steppes of central Asia in the Middle East and is now inhabited all over the world where it adapts in different regions and climatic conditions (Khamesipour *et al.*, 2018). The warm climatic conditions are usually very favorable for reproduction and faster development of the housefly. Thus, more than twenty generations can be produced per year in tropical and subtropical regions, whereas only about ten to twelve generations of houseflies may occur in temperate regions (Sanchez-arroyo & Capinera, 2020).

Housefly preferably feeds on all kinds of human food found in human settlements (milk, water, sugar, blood and meat) and garbage, but the mouthparts structure can only allow housefly to feed on liquid foodstuffs and food that are readily soluble in the salivary gland secretions. Hence, the dry substances are firstly dissolved in housefly saliva before ingestion (Keiding, 2011; Sarwar, 2020). In unconfined areas, the female flies frequently fly long distances following the source of odors like cattle manure, poultry dung, rotting foodstuff, and decomposing organic materials to locate the isolated and secure breeding media (Tian, 2017).

It takes about 16 days to complete the housefly life cycle under optimal conditions, which undergoes four distinct stages from egg, larva (maggot), pupa and finally to adult fly (Figure 2.7). However, under less favorable conditions, the lifecycle may reach to 42 days due to the lower rate of development, and flies of subnormal size can emerge (Kelling, 2001). A female housefly can lay approximately 450 eggs in masses on organic matter such as manure and garbage in various batches. Then, the hatching process begins to give out larvae within 8 to 24 h that grow faster passing through three larval stages.

The first and second larval instars last about one day each, while the third, in three days, develops into a creamy white maggot that forms a capsule-like case, the puparium, within that the transformation from larva to adult takes place (Keiding, 2011).

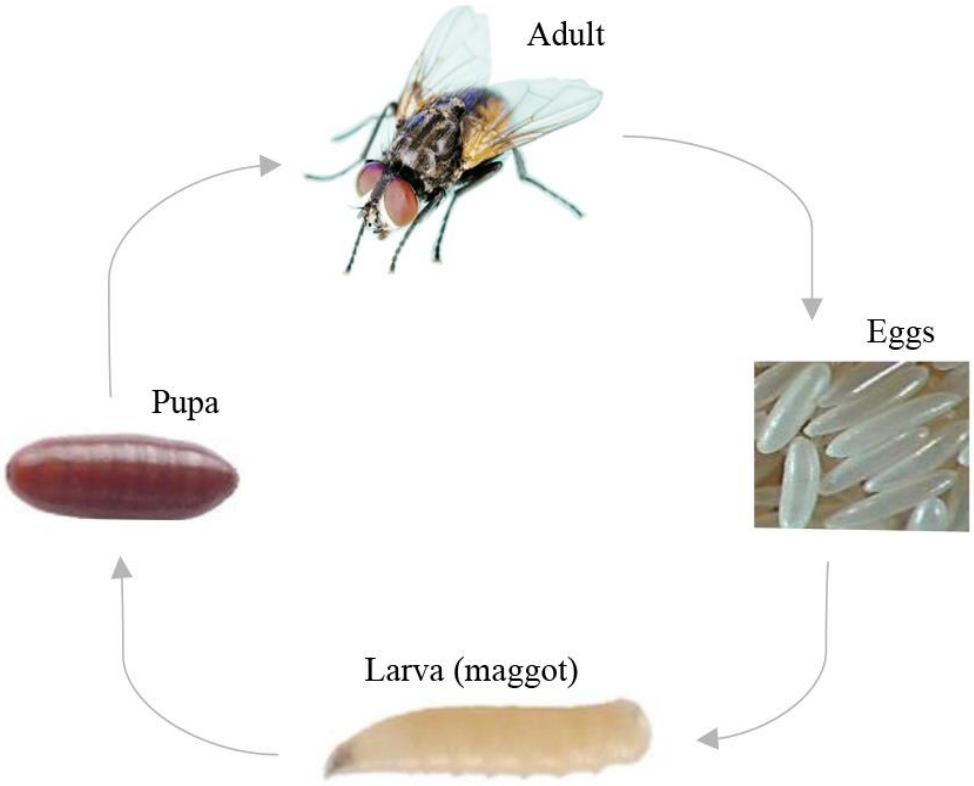


Figure 2.7. Life cycle of housefly (*Musca domestica* Linnaeus)
(Adapted from Keiding, 2011).

A fly emerges in about five days after pupation, then spreads its wings shortly after emergence and the body dries and hardens. Flies become sexually mature after 2-3 days and females deposit their first batch of eggs 4 days after copulation (Keiding, 2011; Kelling, 2001). The lifespan of adult housefly varies from 14 to 21 days, and it may extend to 60 days in cooler conditions (Keiding, 2011).

2.7.2. Housefly as a pest and its management strategies

Housefly is among the most successful pests on earth due to their adaptation and arthropodal characteristics that favor their successful competitions with human population for scarce food and other ecological resources (Ayo *et al.*, 2019). Housefly is considered as successful insect due to its fecundity and ability to multiply rapidly (Baana *et al.*, 2018). Housefly has a worldwide distribution, and it is a potential vector of many pathogens like *Escherichia coli*, *Shigella* spp. and *Salmonella* spp., accountable for protozoan (amoebic dysentery), bacterial (shigellosis, salmonellosis, cholera) and helminthic (round worms, hookworms, pinworms and tapeworms) infectious diseases (Abbas *et al.*, 2013; Malik *et al.*, 2007; Soonwera & Sinthusiri, 2014; Wang *et al.*, 2019).

The housefly control strategies include physical methods that consist of improving environmental sanitation and hygiene, like removal of fly breeding sites and sources that attract flies, and proper disposal of biodegradable wastes (Abbas *et al.*, 2013). The physical methods of housefly control are usually easy and safer, however they are not very effective at combating a high density of houseflies (Malik *et al.*, 2007; Wang *et al.*, 2019).

On the other hand, the chemical insecticides are effective and work quickly for housefly control (Acevedo & Zapater, 2009). Chemical insecticides work by disrupting insect body's functions of endocrine, reproductive and nervous systems, whereas some affect energy production and water balance systems of insect pest (Asid *et al.*, 2015; Betancur, 2018). When insecticide enters an insect body, it may involve in metabolic processes, and produce physiological and biochemical changes (Sparks *et al.*, 2020).

The well-known synthetic insecticides like organophosphates, organochlorines, pyrethroids and carbamates, generally act on insect nervous system, which leads to an abnormal function of neurotransmitters, due to the bind of insecticide molecules on neurotransmitter sites, and directly deregulates the function of specific cellular channels (Lushchak *et al.*, 2018; Robea *et al.*, 2018).

Although chemical pesticides are more effective, their long term use have been reported to cause many environmental problems, including toxicity on non-target organisms, bioaccumulation and higher persistence in environment (Aktar *et al.*, 2009; Gangemi *et al.*, 2016; Gill & Garg, 2014; Jairoce *et al.*, 2016).

The FAO (2021) reported the increment of pesticide uses between 1990 and 2019 with global estimate of 4.2 million tonnes by 2019. This huge usage of pesticides results in a worldwide annual unintentional and acute pesticide poisoning of about 385 million cases with around 220,000 deaths (Boedeker *et al.*, 2020; Sharma *et al.*, 2019). It has been reported that only about 0.1% of the pesticides is estimated to reach the target pests while the remaining bulk contaminates the surrounding environmental components, including water, air as well as soil, where they could persist and bio-accumulate in higher tropic level of food chain (Sharma *et al.*, 2019). Moreover, the public health sectors frequently report the interconnection between pesticides exposure and many acute health effects include stinging eyes, rashes, blindness, nausea, dizziness, diarrhea and death, as well as chronic health effects like cancers, birth defects and disruption of the endocrine system (Mossa, 2016; Sarwar, 2016).

Some pests, including housefly have been reported to develop resistance which leads to the failure of chemical pesticides (Lushchak *et al.*, 2018). In general, the pesticides resistance can be developed through metabolism, alteration of target-site, penetration resistance or behavioral resistance mechanisms of pest (Kole *et al.*, 2019). In this context, much emphasis has been placed on the use of plant-origin products, among them, essential oils from various plant species that have shown pesticidal activity against a variety of pests including houseflies (Hikal *et al.*, 2017; Mohan *et al.*, 2011; Showler, 2018; Soonwera & Sinthusiri, 2014). Furthermore, the control of houseflies can also involve the use of biological agents, like fungal and bacterial pathogens, parasitoids among others, which are components of environment and are of less impacts (Elkattan *et al.*, 2011; Laxmishree & Nandita, 2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Description of study areas

Rwanda is a hilly and mountainous country, geographically located in central Africa (Figure 3.1) between 1° 04’ and 2°51’ latitude South, 28° 45’ and 31° 15’ longitude East with 26,338 km^2 of surface area and altitude variation of 900 to 4,507 m above sea level (Mupenzi *et al.*, 2011; Nteziyaremye & Omara, 2020; Twagiramungu, 2006; United Nations Development Program [UNDP], 2007).

The six major agro-ecological zones of Rwanda (Figure 3.1) are grouped into three altitudinal regions (Iiyama *et al.*, 2018a, 2018b; Mupenzi *et al.*, 2011); The North-West of Rwanda is a part of highlands region occupied by Congo-Nile crest, Buberuka highland and volcanic highland zones, with more than 2,000 m above sea level, while the East is part of lowlands region, which is relatively flat with altitudes below 1,500 m and it consists of Eastern savanna and Eastern plateau zones. The central plateau is part of midlands that consist of wavy hills with altitude of 1,500 to 1,900 m above sea level (Rwanda Environment Management Authority [REMA], 2011). Therefore, such topographic pattern is responsible for the moderate and cool climate of the country, with the annual average temperature and precipitation of 20 ^{o}C and 1,250 mm, respectively (Mukuralinda *et al.*, 2016; Ocimati *et al.*, 2014; REMA, 2011; Verdoodt & Ranst, 2003).

The current study was based on three sampling sites within each of three agro-ecological zones (Figure 3.1).

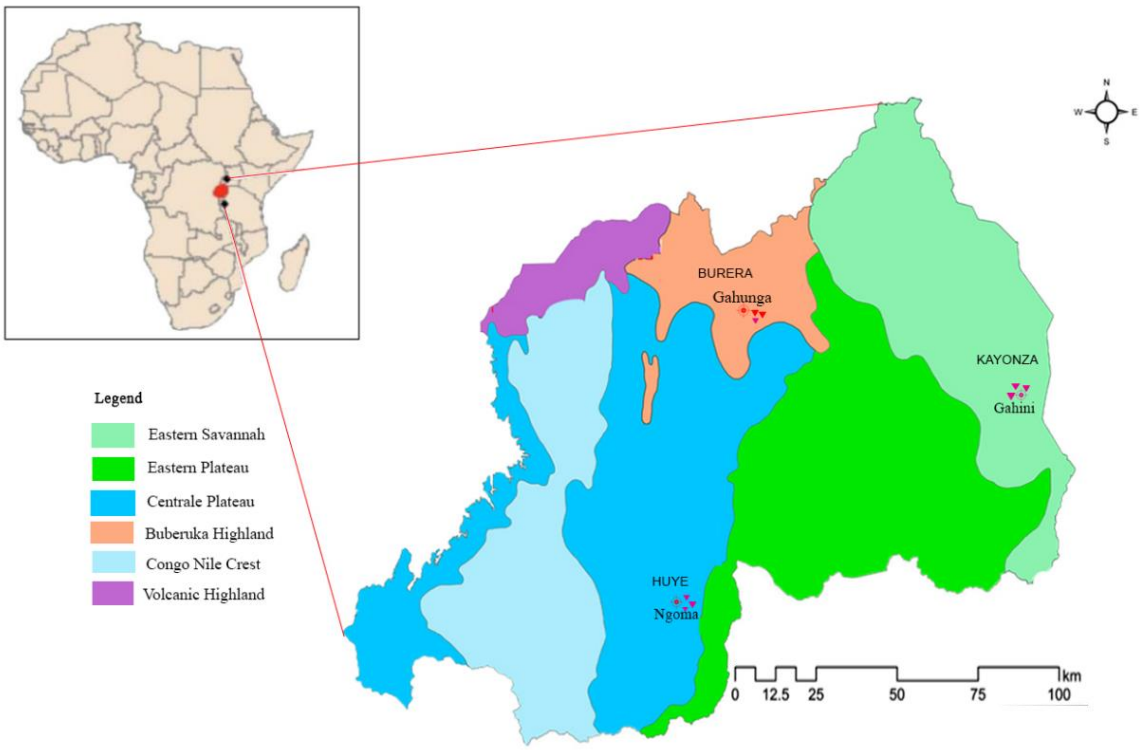


Figure 3.1. Map showing Agro-Ecological Zones of Rwanda with areas of the study (Adapted from Mukuralinda *et al.*, 2016)

3.2. Materials, Apparatus, Chemicals and Reagents

3.2.1. Chemicals and Reagents

Chemicals of Analytical grade, including Acetone and Anhydrous sodium sulphate (Na_2SO_4) were sourced from Millipore Sigma (St. Louis, USA), while Dichlorvos (DDVP: Dimethyl 2, 2-dichlorovinyl phosphate, 77%) insecticide was sourced from LOBA CHEMIE PVT Ltd (Mumbai, India). The Milk Powder and sugar were produced by Highland Creamers & Foods Ltd and Mumias Sugar Company Ltd, Kenya, respectively.

3.2.2. Materials and Apparatus

The materials used in this study were: Aluminium foil, Petri dishes with lid (90mm Diameter x 15mm Deep), Filter papers (Whatman-110 mm No.1) and Cotton pads, Disposal Rubber gloves and Conical flasks (purchased from Kindo Lab Enterprises), Digital Hygrometer (HTC-2 Model, 10%~ 99% RH, accuracy: ± 10% RH, ± 1°C, manufactured by Narayann Scientific Instrument Co. Ltd, New Delhi, India), Cooler box and Plastic jars (manufactured by Tokyo Plast. International Ltd., Japan), Amber glass vials (4.0 mL, purchased from ISOLAB Laborgeräte GmbH, Wertheim, Germany), Boiling flask and Biomass flask (sourced from LOBA CHEMIE PVT Ltd, Mumbai, India) Micropipette (0-20 µL) (sourced from MICROLIT, India), Insect rearing cages (50×34×37cm) and bioassay cages (22×15×17cm).

3.2.3. Equipment

Gas Chromatography (Hewett-Packard GC, Agilent 8890A with Agilent 5977 mass selective Detector) hyphenated to a Mass spectroscopy (HP-5 MS with ultra-inert column of 30 m length × 0.25 mm internal diameter × 0.25 µm film thickness) and FT-IR spectrometer (Bruker Alpha II, 111311, Germany) equipped with a Diamond Crystal ATR (Attenuated Total Internal Reflectance) accessory were used for analysis of essential oil.

3.3. Methods and Research Design

The study was designed to start with the collection of leaves of *C. lusitanica* and extraction of the essential oil using steam distillation method. Characterization of major chemical components of the essential oil with Gas Chromatography-Mass spectroscopy (GC-MS)

and Fourier Transform Infrared spectroscopy (FT-IR) was followed by evaluation of insecticidal potential of essential oil against housefly (*M. domestica*) as it is summarized in Figure 3.2.

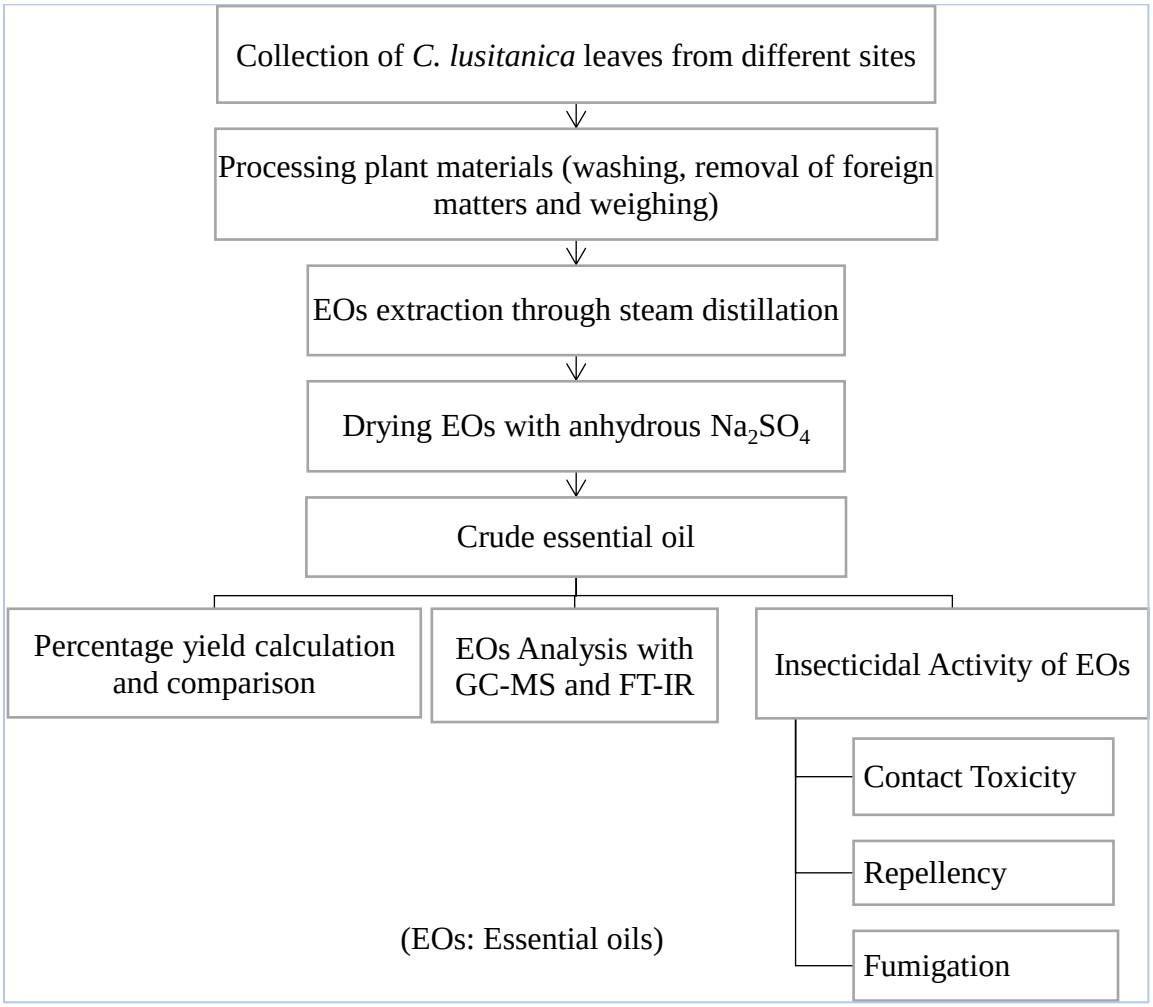


Figure 3.2. A summarized outline of research activities and experimental methods.

3.3.1. Plant material collection and plant species identification

Fresh leaves of wild mature *C. lusitanica* were collected from three selected Agro-ecological zones of Rwanda by judgemental sampling. Three locations from each

ecological zones including Eastern savannah (Kayonza District in lowlands zone, LLZ), Central plateau (Huye District in Midlands zone, MLZ) and Buberuka Highland (Burera District in Highlands zone, HLZ) were selected to sample fresh leaves of mature *C. lusitanica*, then the samples were mixed together to form a composite sample from each AEZ.

The sampling locations were characterized by their geographic coordinates taken using google map, which are 1°51'26.2"S 30°29'22.8"E in Gahini sector (Kayonza, LLZ); 2°35'30.3"S 29°43'59.3"E in Ngoma sector (Huye, MLZ) and 1°27'10.2"S 29°41'53.7"E in Gahunga sector (Burera, HLZ). The samples were then taken to University of Rwanda, College of Science and Technology Chemistry Laboratory and refrigerated at 4 ^{o}C until the extraction process on next day.

The plant species were identified by a botanist in the Department of Biology, University of Rwanda – College of Science and Technology and the voucher specimens (14427/001, 14427/002, 14427/003) were compared to those deposited under the name "Colete Nuyt 141", and deposited in the National herbarium of Rwanda at the University of Rwanda, Centre of Excellence in Biodiversity and Natural Resource Management (CoEB), Rwanda.

3.3.2. Houseflies' collection and maintenance

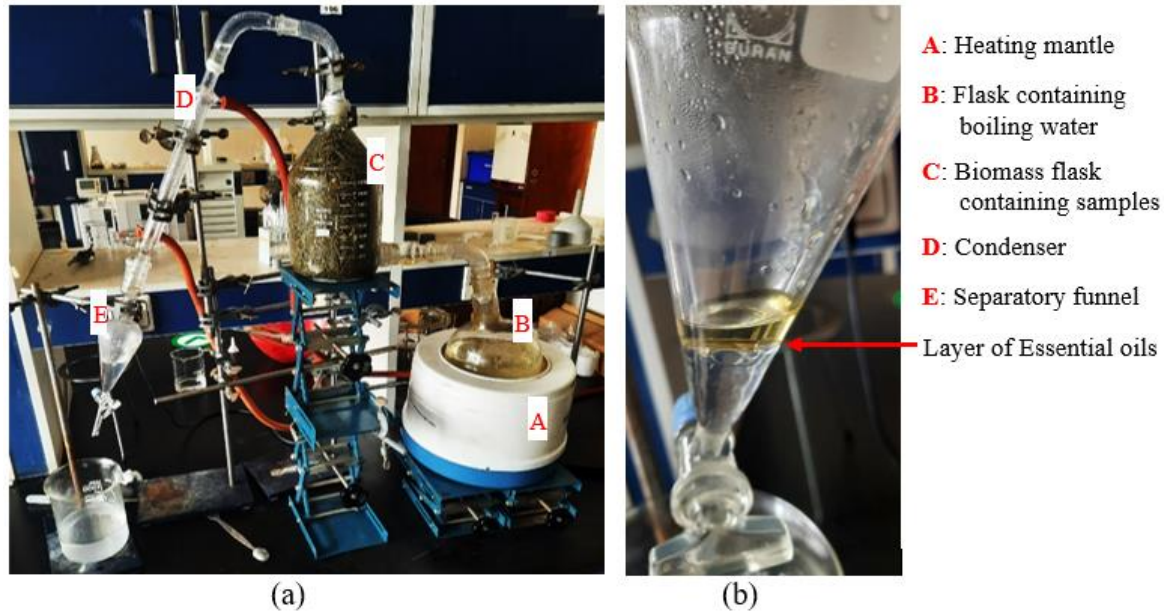
The starter colony of adult houseflies (*M. domestica)* were collected from a slaughterhouse at the market of Moi university, Kesses, using sweep net and plastic jars (3.5 L), then taken to Biological Sciences Laboratory of Moi University for identification and breeding. The collected adults houseflies were reared in cages (50×34×37 cm) and provided with different foodstuffs according to the method described by Khater and Geden (2019) and

Chintalchere *et al.*(2013). The mixture of milk powder and granulated sugar at 1:1 ratio, bread soaked in the fresh milk, wheat flour and boiled eggs were provided for adults and replaced every two days, while tap water was provided daily. Both, foodstuffs and water were provided to houseflies using plastic petri dishes (90mm Diameter x 15mm Deep). Cow dung placed in the transparent plastic box (20 × 15 cm) were served as breeding media and larval development substrate, while the pupae were kept in separate jars for adult emergence. The rearing and experimental conditions were maintained at 65±5% relative humidity (RH) and temperature of 20 ± 2 °C, and adult houseflies were continuously available for the experiments.

3.3.3. Extraction of *Cupressus lusitanica* essential oils

From each ecological zone, a total mass of 2.40 Kg of fresh leaves of *C. lusitanica* was separately subjected to steam distillation in four replicates for 3 h.

Following the procedural steps described by Campolo *et al.*(2018) and Ahmet and Ergin **(**2019): The weighed 600 g (composite sample made of leaves from sampled locations in each zone) of fresh leaves of *C. lusitanica* were packed into biomass flask (2,000 mL), then connected to boiling flask (2,000 mL) that contained 1,750 mL of water. The water in boiling flask were boiling and allowed the steam to pass through the plant samples for 3 h (Figure 3.3a). The mixture of water and essential oils vapors passing through the condensation flask cooled down and collected in a separatory funnel where the essential oil floated on top of water (Figure 3.3b), then collected in amber vials (Figure 3.3c).



(c)

Figure 3.3. (a) Steam distillation set up, (b) and (c) essential oils collected in separatory funnel and amber vials, respectively.

3.4. Yield evaluation of essential oils from *Cupressus lusitanica* leaves

The distilled essential oils from each agro-ecological zone were dried over anhydrous sodium sulfate and stored in amber glass vials (4.0 mL) in fridge at 4 °C.

The yields of essential oils were then calculated using Equation 1.

$$\text{Percentage Yield} = \frac{m_1}{m_0} \times 100 \quad \text{(v/w)} \qquad \text{(Equation.1)}$$

with m_0 and m_1, the mass of fresh leaves packed in biomass flask (g) and volume of extracted essential oil (mL), respectively.

3.5. Analyses of major chemical components of *Cupressus lusitanica* essential oils

3.5.1. Fourier Transform Infrared spectroscopy analysis

The FT-IR spectra of essential oils were recorded in the spectral range of 4000 to 400 cm^{-1} with the scanning resolution set to 2.0 cm^{-1} for 24 scans on each essential oil sample. The analysis was repeated twice for confirmation of spectra. The liquid sample (2 drops ~ 0.1 mL) of essential oil was put on diamond crystal plate and allowed the infrared beams to pass through the essential oil sample. The functional groups and chemical bonding corresponding to significant peaks on FT-IR spectra of essential oils were determined by comparing their wavenumbers with data on FT-IR correlation chart (Zhang-Da *et al.*, 2016), IR guide of Bruker Optics (Germany) and the data from previous studies (Adinew, 2014; Li *et al.*, 2013; Michelina *et al.*, 2019).

3.5.2. Identification of EOs chemical components by Gas Chromatography-Mass spectroscopy

Gas chromatography-mass spectroscopy (GC-MS) analysis of *C. lusitanica* EOs was performed using a Hewett-Packard GC with mass selective detector equipped with a MS ultra-inert column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness) and a mass system with ionization energy of 70 ev. Helium was the carrier gas at a flow

rate of 1 ml/min. Injector and MS transfer line temperatures were set at 250 ºC and 280 ºC, respectively. The oven temperature was programmed from 110 ºC with an increase of 10 ºC/min to 200 ºC, and finally to 280 °C at 5 °C/min. Diluted samples (1:100 v/v in hexane) of 1.0 µl were injected manually in the split-less mode. The components were identified by comparing their relative retention times and mass spectra with those of standards library (NIST 11) and installed Mass Hunter Software, as well as the data reported in literature. Results were further confirmed by comparing the elution order of the compounds with their relative retention indices on non-polar phases.

3.6. Evaluation of Insecticidal potential of essential oil against housefly

The insecticidal potential of *C. lusitanica* essential oil against houseflies was evaluated in three sections of assay that are repellency, fumigant and contact toxicity. All bioassays were performed by exposing thirty (30) adult houseflies of mixed sex separately to the test solutions of 90, 70, 50 and 30% v/v concentrations of essential oils from each AEZ and Dichlorvos (DDVP, 77%) insecticide for 6, 12 and 24 h. The concentrations were prepared by dilution with Acetone solvent. Dichlorvos (DDVP, 77%) insecticide and Acetone solvent served as positive and negative test controls, respectively.

3.6.1. Contact toxicity bioassay

The contact toxicity was evaluated by following the method described by Suwannayod *et al.*(2019) and Tian (2017) with slight modifications on experimental conditions, exposure time and method of anaesthetizing houseflies.

A batch of 30 male and female adult houseflies (3–5 days old) were put in plastic jars (3.5 L) covered with a mesh and then anaesthetized by placing the jar in the fridge at 7-8 °C for 3 to 5 minutes. The anaesthetized houseflies were removed from fridge and put on white paper. One microliter (1.0 µL) from each test solution and controls was applied to the pronotum of each anesthetized housefly using micro-pipette (0-20 µL). The treated houseflies were transferred to bioassay cages (22×15×17 cm) and provided with sugar-milk solution (10%). Adult houseflies' mortality was recorded after 6, 12 and 24 h of exposure to the test solutions. The housefly was defined as dead when it did not exhibit any movement after being prodded with a small brush (Paramasivam & Selvi 2017). Mortality between 5% and 20% in the negative control assay was corrected using Abbott's formula (Abbott 1925) (Equation 2), while above 20% of mortality was rejected and the bioassay repeated. Three replicates per test solution were done.

$$\text{Corrected mortality}(\%) = \frac{\text{X}-\text{Y}}{100-\text{Y}} \times 100 \qquad \text{(Equation 2)}$$

Where:

Y: Mortality (%) in negative control;

X: Observed mortality (%).

The lethal doses, LD_{50} and LD_{90} of each test solution against houseflies were obtained via Probit analysis of dose-mortality relationship.

3.6.2. Fumigant toxicity bioassay

According to the method described by El-Sherbini & Osman (2014) and Bande-borujeni *et al.*(2018), fumigant toxicity was evaluated by placing thirty male and female adult houseflies in a 5 L plastic jar covered with mesh to facilitate ventilation. The filter papers

cut in the same diameter with jar caps were separately impregnated with 100.0 µL of EOs and Dichlorvos insecticide test solution (90, 70, 50 and 30% v/v) and Acetone (Negative control). Each treated filter paper was attached to the inner surface of jar cap and protected with a piece of mesh in the way that prevented its direct contact with houseflies. The mortality of housefly was recorded in 6, 12 and 24 h, where the housefly considered dead when it did not exhibit any movement. Three replications per test solution were done.

The mortality percentages were corrected using Abbott's formula in equation 2 (Abbott 1925), then converted into Probit values for calculation of lethal doses required to kill 50% (LD_{50}) and 90% (LD_{90}) of housefly population (Lopes *et al.*, 2019).

3.6.3. Repellency bioassay

The repellency potential of *C. lusitanica* essential oil against houseflies was evaluated using the modified method described by Chauhan *et al.*(2017). The experimental setup consisted of two same size chambers made of transparent jars of 3.5L with interconnecting passage where the houseflies were introduced (Figure 3.4).

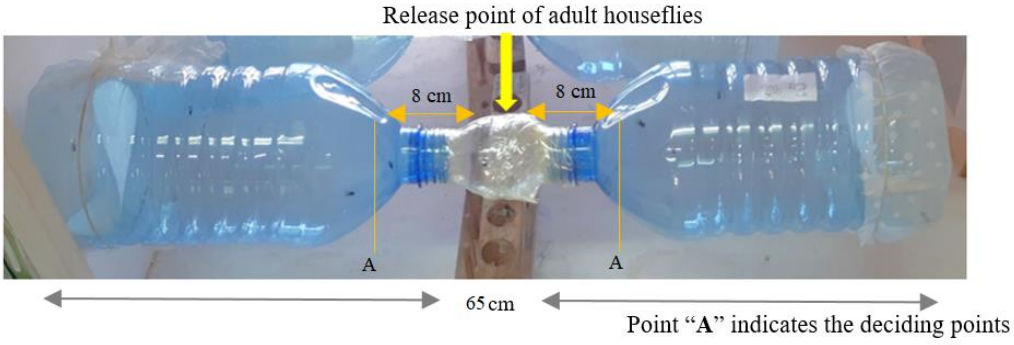


Figure 3.4. Bioassay set up for repellency of *Cupressus lusitanica* essential oil against houseflies.

The filter papers (Whatman-110 mm thickness) impregnated with 50 µL of each EOs concentrations (90, 70, 50 and 30% v/v) were separately placed inside the end of one chamber, while the filter paper impregnated with 50 µL of acetone (Negative control) was placed inside the end of opposite chamber. Similar procedure as EOs treatment assays was set for Dichlorvos insecticide which served as positive control.

Thirty (30) adult houseflies (3-5 days old) of mixed sex were knocked down (by placing the jar in fridge at 7-8 °C) and then introduced at release point (in the half-way of the two chambers) (Figure 3.4) to allow the movement of their choice between two chambers. The housefly's movement was monitored and recorded in 6, 12 and 24 h of exposure by counting the number of houseflies reached the deciding points (Point A) (Figure 3.4) from the release point toward either the chamber with test solution treated filter paper or the one with Acetone (negative control) treated filter paper. The number of houseflies moved toward the chamber treated with test solution (N_t) were considered as repelled by negative control, while the houseflies in opposite direction (N_c) were considered as they were repelled by test solution, and both were expressed in percentages (Equation 3 and 4). All the experiments were conducted in triplicates, and the repellency percentages (PR) were calculated using Equation 5.

$$N_t = \frac{\text{No. of HFs moved toward EOs treated chamber}}{\text{No. of total HFs in assay}} \times 100 \qquad \text{(Equation 3)}$$

$$N_c = \frac{\text{No. of HFs moved toward Acetone treated chamber}}{\text{No. of total HFs in assay}} \times 100 \qquad \text{(Equation 4)}$$

$$\%\ PR = \frac{N_c - N_t}{N_c + N_t} \times 100 \qquad \text{(Equation 5)}$$

With N_t and N_c, the percent of houseflies moved toward the test solution and Acetone treated chambers, respectively.

Repellency data were corrected using Abbott's formula (Abbott, 1925) (Equation 6) when the flies attracted to negative control treated chamber were between 5 and 20%, while for more than 20% were rejected and the experiment repeated (Paramasivam & Selvi, 2017).

$$\text{Corrected repellancy percentage} = \frac{N_t - N_c}{100 - N_c} \times 100 \qquad \text{(Equation 6)}$$

3.7. Statistical analysis

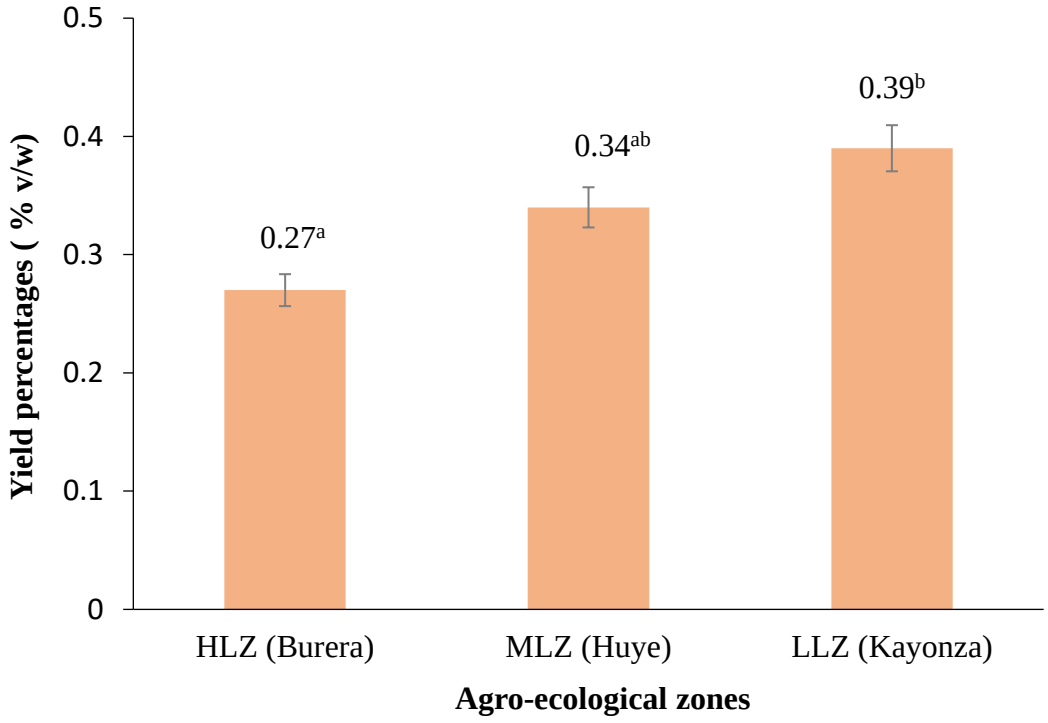
The results were expressed as means ± standard error of four replicates for yields data and three replicates for repellency and mortality (for fumigant and contact toxicity) data. Significant difference between mean values were established through Tukey's honest significant difference (HSD) test (one-way ANOVA). The mortality and repellency data between 5% and 20% were relatively corrected using Abbott's formula (Abbott, 1925), and the relationship between EOs concentrations and housefly mortality was determined using Probit regression to estimate lethal doses (LD_{50} and LD_{90}) in 6, 12, and 24 h post-treatment. All analyses were performed at 95% confidence interval using Minitab statistical software (Release 17, Minitab Inc., USA).

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1. Percentage yield of essential oil from *C. lusitanica* leaves

The average of 2.4 kilograms of *C. lusitanica* fresh leaves from each Agro-ecological zone were subjected to steam distillation in four replicates for 3 h, and the total amount of 9.30, 8.10 and 6.50 mL of essential oils (corresponded to the calculated yields in Figure 4.1) were obtained as per samples from lowland zone (Kayonza), midland zone (Huye) and highland zone (Burera), respectively.



HLZ: Highlands, MLZ: Midlands, LLZ: Lowlands zones. Mean values of four replicates; the values followed by same letter are not significantly different at *P=0.05*

Figure 4.1. Comparison of average yield in percentages of *Cupressus lusitanica* essential oil from the three agro-ecological zones of Rwanda.

The average yield of essential oil of *C. lusitanica* leaves from highland zone (0.27 ± 0.02%, v/w) was significantly lower (P = 0.02) than that from lowland zone (0.39 ± 0.01%, v/w)), however it showed no statistical difference (P = 0.13) to the yield of EO from midland zone (0.34 ± 0.02) (Figure 4.1). Similarly, the average yield of EOs from midland zone was also lower than that from lowland zone, but the two were not significantly different (P = 0.27). The yield percentages of *C. lusitanica* EOs in the current study were a little higher than the yields of 0.35 and 0.125% obtained for hydro-distilled essential oils from fresh leaves of *C. lusitanica* in Kenya (Bett, 2015; Kimutai & Mainya, 2016). Hassanzadeh *et al.*(2010) also reported a yield ranging from 0.28 to 0.58% for hydro-distilled oil in Costa Rica, while by hydro-distillation, Kuiate *et al.*(2006) reported a yield of 0.33% for *C. lusitanica* fresh leaves from Cameroon.

The higher yields of EOs obtained in the current study could be attributed to the extraction method used (no data were previously reported on use of steam distillation method for EOs extraction from *C. lusitanica*) or the use of fresh leaves rather than dried ones (Almasa *et al.*, 2019; Bett, 2015). In addition, the synthesis and accumulation of EOs are also affected by various factors, that are linked to the physiology of the plant species itself or variability in characteristics and components of plant habitat (Moghaddam & Mehdizadeh, 2017). The production of essential oils in numerous aromatic plants was demonstrated to increase under water-stress conditions (Isah, 2019). Different authors reported the increase of monoterpene amounts as a response to drought, water stress and hot climate, that enable many aromatic plants under stressed conditions to lower their photosynthetic activities and significantly reduce the emission of their terpene compounds, which results in their

accumulation within secretory glands (El-Zaeddi *et al.*, 2016; Nowak *et al.*, 2010). The statement was also supported by findings of El Hamrouni-Aschi *et al.*(2013) who reported the higher yields of EOs from *Cupressus simpervirens* in semi-arid regions of Tunisia.

Consistent with the previous findings of El-Zaeddi *et al.* (2016), Isah (2019) and Nowak *et al.* (2010), results of the present study showed a correlation of essential oil yields with geo-climatic conditions where higher yield were obtained at lower altitude region (Kayonza). This confirms the influence of high temperature and insufficient precipitation on the aromatic plants in this region. On the other hand, the lower yield of essential oil was obtained at higher altitude region (Burera) which is characterized by the cooler climatic conditions with lower annual temperature and abundant rainfall. Furthermore, the observed variations in essential oil yields could also be the result of interactions between *C. lusitanica* species with various biotic factors including plant genetics, maturity and stage of growth, and abiotic factors like soil properties and chemical elements within habitats and then, such interactions may influence the production of plant essential oil (Jeshni *et al.*, 2017; Kumar *et al.*, 2017).

4.2. FT-IR analysis results of *C. lusitanica* essential oils

The results of FT-IR analysis of essential oils indicated the presence of different functional groups and chemical bonding correspond to different groups of compounds. All essential oil samples showed almost similar FT-IR spectra (appendix 3a, 3b and 3c) and their comparison are shown in Figure 4.2. The significant peaks appeared at around 2923-2933 cm^{-1}, 1710-1723 cm^{-1}, around 1450 cm^{-1}, 1370-1373 cm^{-1} and 875-878 cm^{-1}.

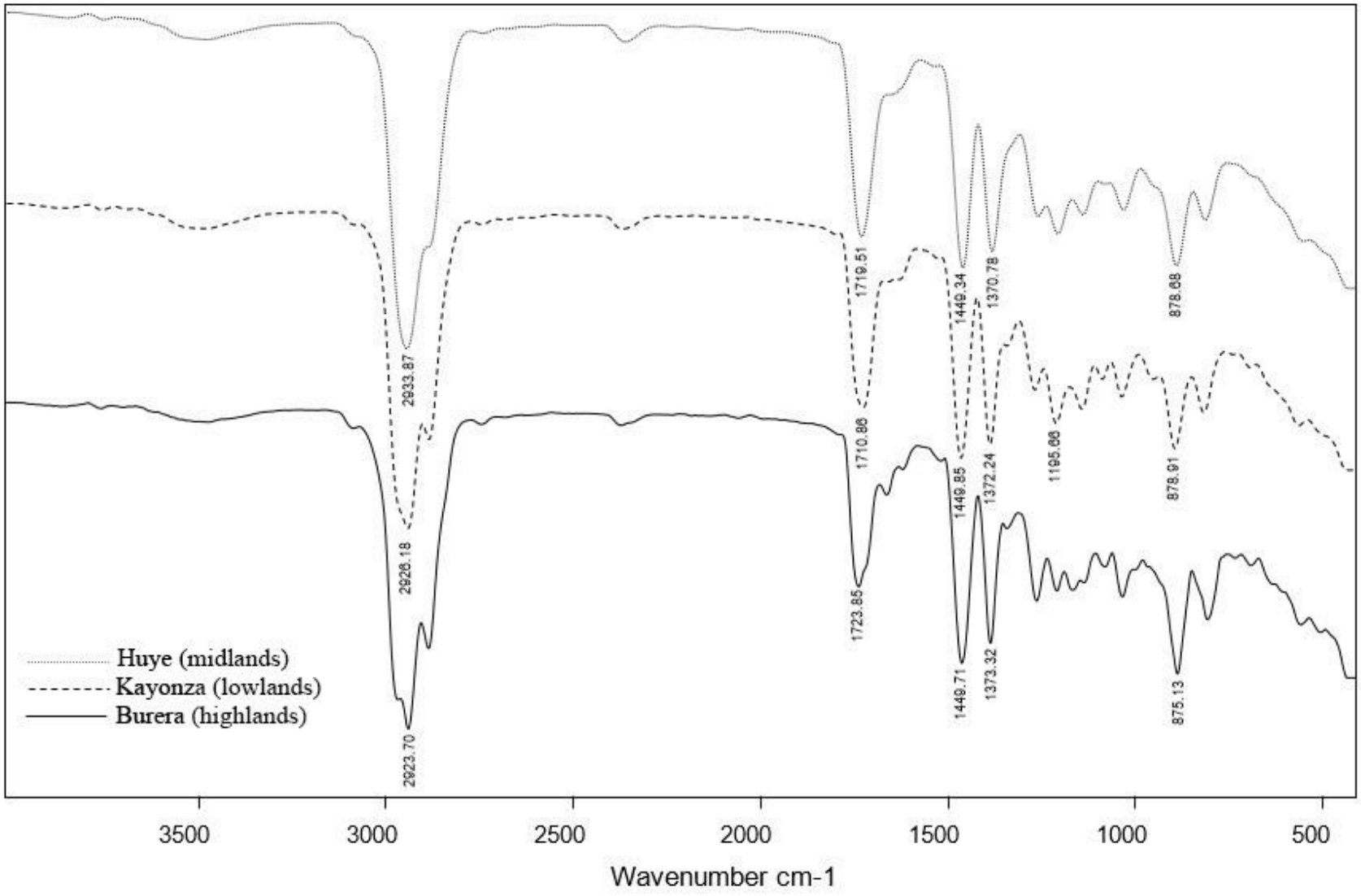


Figure 4.2. Comparative FT-IR spectra of *Cupressus lusitanica* essential oils from three ecological zones of Rwanda.

According to the IR guide of Bruker optics (Germany) and FT-IR correlation chart (Zhang-Da *et al.*, 2016), the significant peak on FT-IR spectra of *C. lusitanica* essential oil around absorption band of 2940-2920 cm^{-1}, is indicating the presence of asymmetrical and symmetrical C-H stretches in CH_3, and CH_2 for alkanes, like aliphatic group of terpenes. Peaks located around 1725-1700 cm^{-1} and around 1720-1705 cm^{-1} corroborate with carbonyl group (C=O) of the carboxylic acids and saturated ketones, respectively. The presence of saturated aldehydes carbonyls is indicated by picks at 1720-1740 cm^{-1}. Other significant peaks were observed at 1450 cm^{-1}, 1375-1370 cm^{-1} and are indicating the C-OH stretch for tertiary alcohols and –C-O-CH_3 for ethers (alkyl substituted ethers), respectively. The vibrational frequency at ~1190 cm^{-1} confirmed the presence of -CH_2- stretch (methylene-cyclohexane ring) (Morar *et al*., 2017). The peaks around 900-800 cm^{-1} are attributed to the vibrations of out-of-plane bending patterns of aromatic rings and alkenes such as monocyclic and bicyclic terpenes, whereas the absorption bands at 1166 and 1111 cm^{-1} suggested the presence of terpenes with tertiary and secondary alcoholic functions (Berechet *et al*., 2015; Michelina *et al*., 2019).

4.3. GC-MS analysis results of *C. lusitanica* essential oils

The GC-MS analysis led to the identification and quantification of 37, 36 and 30 compounds corresponding to 97.47%, 96.65% and 97.44% of the total components of essential oils from *C. lusitanica* leaves collected from highland zone (Burera), midland zone (Huye) and lowland zone (Kayonza), respectively. The identified compounds and their relative abundances (Appendix 4a, 4b, 4c) are listed in Table 4.1.

Table 4.1. Major chemical components in essential oils from the leaves of *C. lusitanica* from three ecological zones of Rwanda.

Peak No.	Retention Time (min)	Retention Index	Compounds	Composition (%)		
				Burera (highlands)	**Huye (midlands)**	**Kayonza (lowlands)**
1	5.66	938	Thujene	0.12	tr	0.10
2	5.78	943	α-Pinene	**10.23**	**8.21**	**5.24**
3	5.93	949	Tricyclene	0.11	0.11	tr
4	6.46	969	Sabinene	**20.84**	**10.54**	**4.05**
5	6.71	978	β-Pinene	2.58	2.26	1.52
6	7.07	992	Myrcene	**19.63**	1.31	**7.20**
7	7.21	997	α-Phellandrene	1.06	0.73	0.66
8	7.33	1001	β-Phellandrene	1.04	0.39	0.41
9	7.42	1004	δ-3-Carene	**10.13**	**16.76**	3.13
10	7.45	1005	α-Terpinene	**6.72**	**5.84**	2.88
11	7.89	1017	*p*-Cymene	2.11	1.73	1.32
13	8.55	1036	(Z-), β-Ocimene	2.08	0.65	0.42
14	8.92	1047	Limonene	1.27	2.08	**5.53**
16	9.69	1069	γ-Terpinene	*3.14*	0.65	**18.77**
18	10.08	1080	Terpinolene	tr	-	tr
32	14.76	1198	δ-2-Carene	tr	-	tr
Monoterpene hydrocarbons				*81.06*	*51.26*	*51.23*
12	8.38	1031	1,8 -Cineole	1.22	2.36	0.71
15	9.23	1056	Sabinene hydrate	0.14	0.26	-
17	9.87	1074	Linalool	**6.83**	2.10	**8.71**
19	10.23	1084	Linalool oxide	-	0.27	-
20	10.63	1090	2-Nonanone	0.11	-	-
21	11.38	1115	Camphor	tr	1.21	-
22	11.53	1118	α-Thujone	tr	0.39	-
23	11.83	1121	Borneol	tr	tr	tr
24	12.23	1135	Camphene hydrate	1.38	**3.47**	2.33
25	13.23	1160	*p*-Cymen-8-ol	tr	0.15	-

26	13.61	1169	Benzyl alcohol	1.07	2.33	2.13
27	13.84	1175	Umbellulone	3.23	**24.21**	**18.16**
28	14.24	1185	Terpinen-4-ol	0.53	2.08	1.50
29	14.35	1188	menth-2-en-1-ol	0.47	tr	-
30	14.50	1191	α-Terpineol	0.32	3.18	0.38
31	14.55	1192	*cis*-Carveol	tr	tr	-
33	14.93	1202	γ-Terpinen-7-al	0.43	0.52	0.50
34	15.02	1204	Verbenone	0.43	1.71	0.54
35	15.24	1209	Peperitol	-	0.12	-
36	15.69	1220	Eucarvone	-	-	0.81
37	15.97	1226	bornyl acetate	tr	-	**9.72**
38	16.16	1231	Peperitone	tr	0.92	0.57
41	21.02	1344	α-Terpinyl acetate	tr	-	-
	Oxygenated Monoterpenes			*16.16*	*45.28*	*46.06*
39	16.83	1247	α-Cubebene	0.12	-	-
40	20.80	1341	β-Cedrene	0.13	tr	0.15
42	21.89	1367	β- Elemene	-	0.11	-
	Sesquiterpene hydrocarbons			*0.25*	*0.11*	*0.15*
	Total identified compounds			***97.47%***	***96.65%***	***97.44%***

tr, trace < 0.10%; (-) not detected; retention index calculated from retention times in relation to the series n- alkanes on a HP-5 MSUI Capillary column. Compounds are listed in elution order, and the % composition in **bold** represents the major compounds.

The chemical components of *C. lusitanica* essential oils was generally dominated by monoterpene hydrocarbons and oxygenated monoterpenes (Table 4.1), and some of major compounds identified are presented in Figure 4.3. The essential oils of *C. lusitanica* leaves from highland zone (Burera) was dominated by hydrocarbons and oxygenated monoterpenes corresponding to 80.06% and 16.16%, respectively, with Sabinene (20.84%), Myrcene (19.63%), α-Pinene (10.23%) and δ-3-Carene (10.13%) as major monoterpene hydrocarbons found, while the oxygenated monoterpenes were mainly

Linalool (6.83%), Umbellulone (3.23%), and Camphene hydrate (1.38%). On the other hand, the chemical components of essential oils of *C. lusitanica* leaves from midland zone (Huye) was dominated by monoterpene hydrocarbons (51.26%), followed by oxygenated monoterpenes (45.28%). Major monoterpene hydrocarbons were δ-3-Carene (16.76%), Sabinene (10.54%), α-Pinene (8.21%), and α-Terpinene (5.84%), whereas Umbellulone (24.21%), Camphene hydrate (3.47%), α-Terpineol (3.18%), 1,8-Cineole (2.36%) and Linalool (2.16%) were dominant oxygenated monoterpenes. Interestingly, γ-Terpinene (18.77%), Myrcene (7.20%), Limonene (5.53%), α-Pinene (5.24%) and Sabinene (4.05%) were dominant monoterpene hydrocarbons that constituted about 51.00% of chemical components of essential oils from *C. lusitanica* growing in lowland zone (Kayonza), while about 46.00% portion was occupied by oxygenated monoterpenes, with major compounds; Umbellulone (18.16%), Bornyl acetate (9.72%), Linalool (8.71%) and Camphene hydrate (2.30%).

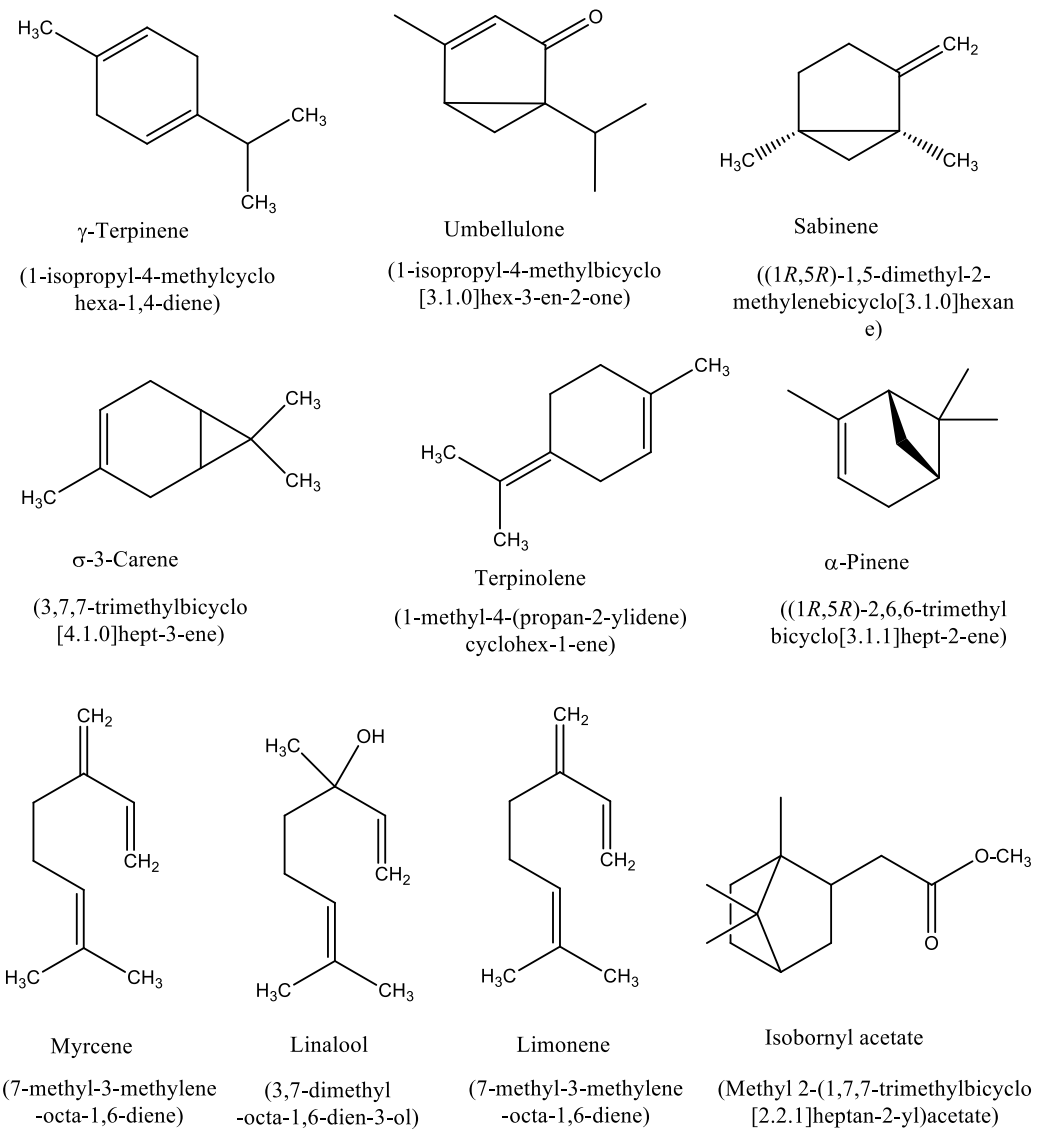


Figure 4.3. Some of the major compounds identified in essential oils of *C. lusitanica* growing in Rwanda.

Different reports have often pointed out umbellulone, α-pinene, germacrene-D, limonene and terpinen-4-ol as the major compounds in the essential oils of *C. lusitanica* growing in different regions of the world (Bett *et al.*, 2017; Filho *et al.*, 2011; Hassanzadeh *et al.*, 2010; Kuiate *et al.*, 2006; Teke *et al.*, 2013). However, according to different authors (Aziz *et al.*, 2018; 2020; Tongnuanchan & Benjakul, 2014), the concentrations of components

vary from region to another due to the influence of many factors, including harvest season, climate, soil type, age of the plants and the extraction method. For example, Bett *et al.*(2017) reported the dominance of oxygenated monoterpenes in the leaf EOs of *C. lusitanica* growing in Kenya with umbellulone (18.38%), α-pinene (9.97%), Sabinene (8.16%) and Limonene (7.91%) as major compounds. Almost similar results were reported by Kuiate *et al.*(2006) for EOs from *C. lusitanica* leaves in Cameroon with dominance of umbellulone (18.30%), germacrene-D (8.20%), α-pinene (7.40%), *epi*-zonarene (5.0%), limonene (3.5%) and terpinen-4-ol (2.6%). However, the oil was dominated by sesquiterpenes (34.70%) followed by oxygenated monoterpenes (28.0%). Different findings were however reported in Cameroon with the dominance of sesquiterpenes like germacrene-D (18.5%), *epi*-zonarene (8.2%), *cis*-calamenene (8.2%), and oxygenated monoterpenes like terpinen-4-ol (6.30%), linalool (6.0%) and umbellulone with 6.0% (Teke *et al.*, 2013). In contrast to the foregoing findings from Cameroon (Kuiate *et al.*, 2006; Teke *et al.*, 2013), Kenya (Bett *et al.*, 2017) and Costa Rica (Hassanzadeh *et al.*, 2010), the EOs of *C. lusitanica* growing in Brazil was reported to contain β-pinene, and β-(Z)-ocimene as major monoterpenes and oxygenated monoterpenes like *endo*-fenchol, whereas the main sesquiterpenes were α-acoradiene, α-amorphene, thujopsan-2α-ol and 7α-*epi*-selinene (Filho *et al.*, 2011).

A strong justification for this variation could not be only related to different climatic and edaphic conditions across different regions, which directly influence the metabolism of the plants, but also due to exposure to different biotic components and age of plants (Filho *et al.*, 2011).

The current results of chemical components of EOs of *C. lusitanica* leaves from Rwanda are comparable to the findings in previous reports in different countries. However, compounds like Germacrene-D and some sesquiterpenes and their oxidative compounds (*epi*-zonarene, *cis*-calamenene, amorphene, *endo*-fenchol and thujopsan-2α-ol) reported by different authors (Bett, 2015; Hassanzadeh *et al.*, 2010; Kuiate *et al.*, 2006; Teke *et al.*, 2013) were not detected in all essential oils of *C. lusitanica* from three studied agro-ecological zones.

The GC- MS results showed that there is an intraspecific variation in the chemical profile of leaf EOs of *C. lusitanica* growing in different geographical regions of Rwanda, which is obviously contradictive to the stated hypothesis. The observed variations could be the result of interactions between *C. lusitanica* with various biotic and abiotic factors as well as climate and environmental conditions that influence the production or alter the chemical profile of plant essential oils.

4.4. Insecticidal potential of *C. lusitanica* essential oils

C. lusitanica essential oils were found to have potent insecticidal activity against adult housefly, *M. domestica* with activity varied from one agro-ecological zone to another.

4.4.1. Contact toxicity of *C. lusitanica* essential oils against houseflies

The contact toxicity results of *C. lusitanica* essential oil against houseflies showed that mortality of houseflies increased with the concentrations of essential oils and the time that exposed houseflies to these chemicals.

The mortality results are presented in Table 4.2, Table 4.3 and Table 4.4. Among all tested essential oil concentrations, the essential oil of *C. lusitanica* from lowland zone (Kayonza) showed the most potent contact toxicity than others with considerably low LD_{50} and LD_{90} values of 0.28 and 191.33 ppm, respectively in 6 h post treatment (Table 4.2). The toxicity of Dichlorvos insecticide was much greater compared to most potent essential oil (Kayonza).

On the other hand, the least lethal effect was observed for the essential oil of *C. lusitanica* from highland zone (Burera) that resulted in lethal dose values (LD_{50} and LD_{90}) of 0.64 and 706.21 ppm, respectively which were much higher than lethal doses of Dichlorvos insecticide (0.008 and 1.03 ppm) within 6 h. The essential oil of *C. lusitanica* from midland zone (Huye) exhibited moderate contact toxicity against houseflies with lethal dose values, LD_{50} of 0.41 ppm and LD_{90} of 453.24 ppm in 6 h of post treatment (Table 4.2).

Table 4.2. Contact toxicity of *C. lusitanica* essential oils from three ecological zones and controls (DDVP and Acetone) against houseflies within 6 h post treatment.

Treatments (EOs and controls)	**Mortality percentages (mean ±SE) per treatment concentrations**				**LD_{50} (ppm)**	**LD_{90} (ppm)**
	30%	**50%**	**70%**	**90%**		
Burera (HLZ)	44.22 ± 3.81	53.01 ± 5.59	59.31 ± 1.83	65.71 ± 2.19	0.64[a]	706.21[a]
Huye (MLZ)	42.89 ± 1.44	60.61 ± 1.55	62.04 ± 5.24	69.46 ± 2.52	0.41[a]	453.24[b]
Kayonza (LLZ)	51.63 ± 4.02	66.84 ± 5.87	69.58 ± 3.58	73.26 ± 2.56	0.28[a]	191.33[c]
DDVP	81.68 ± 2.11	91.17 ± 2.50	93.78 ± 2.45	96.20 ± 2.14	0.01[a]	1.03[c]
Acetone	11.11 ± 2.94	14.44 ±2.94	11.11±1.12	13.33±1.92	-	-

HLZ: Highlands, MLZ: Midlands and LLZ: Lowlands zones, Positive control: Dichlorvos (Dimethyl 2,2-dichlorovinyl phosphate, DDVP 77%) insecticide; Negative control: Acetone. Means ± SE of three replicates. Lethal dose values followed by the same letter in column are not significantly different at $P = 0.05$ (Tukey's HSD test).

The statistical comparison of lethal concentrations (LD_{50}) showed that the essential oil of *C. lusitanica* from Kayonza (lowland) exhibited higher toxicity to houseflies than the essential oil of *C. lusitanica* from other regions in 6, post treatment. However, the difference in toxicity against houseflies were statistically not significant ($P > 0.05$) among all tested essential oils concentrations and also in comparison to Dichlorvos (DDVP 77%) insecticide, taken as positive control. The LD_{90} values showed the significant differences in toxicity against houseflies among of all tested essential oils, and when compared to Dichlorvos insecticide at all concentrations, except the toxicity showed by essential oil from lowland (Kayonza) that was lower and not significant compared to that of Dichlorvos insecticide ($P = 0.09$).

After 12 h of exposure, the higher toxicity was also observed for essential oil of *C. lusitanica* from lowland zone (Kayonza) followed by essential oils from midland zone (Huye) and highland zone (Burera) with LD_{50} and LD_{90} values of 0.12 and 37.02 ppm for Kayonza, 0.18 and 127.90 ppm for Huye and 0.34 and 176.37 ppm for Burera, respectively (Table 4.3).

Table 4.3. Contact toxicity of *C. lusitanica* essential oils from three ecological zones and controls (DDVP and Acetone) against houseflies within 12 h, post treatment.

Treatment (EOs and controls)	Mortality percentages (mean ±SE) per treatment concentrations				LD_{50} (ppm)	LD_{90} (ppm)
	30%	50%	70%	90%		
Burera (HLZ)	49.70 ± 2.30	57.59 ± 2.81	64.26 ± 2.09	70.90 ±3.31	0.34^{a}	176.37^{a}
Huye (MLZ)	50.98 ± 1.52	68.23 ± 3.89	70.90 ± 3.31	76.16 ± 2.31	0.18^{b}	127.90^{ab}
Kayonza (LLZ)	57.59 ± 4.89	68.21 ± 0.21	77.49 ± 5.80	81.44 ± 2.72	0.12^{b}	37.02^{ab}
DDVP	86.54 ± 1.41	92.45 ± 2.10	96.25 ± 2.14	97.43 ± 1.29	0.01^{c}	0.54^{b}
Acetone	16.67±1.92	17.78 ± 1.14	14.44 ± 1.10	15.56 ± 1.13	-	-

HLZ: Highlands, MLZ: Midlands and LLZ: Lowlands zones, Positive control: Dichlorvos (Dimethyl 2,2-dichlorovinyl phosphate, DDVP 77%) insecticide; Negative control: Acetone. Mean ± SE of three replicates. Lethal dose values followed by the same letter in column are not significantly different at $P = 0.05$ (Tukey's HSD test).

In 12 h of post-treatment, the toxicity (LD_{50}) of *C. lusitanica* essential oil from highland zone (Burera) against houseflies was significantly lower than that produced by essential oils from lowland zone (Kayonza) ($P = 0.00$), midland zone (Huye) ($P = 0.002$) and Dichlorvos insecticide ($P = 0.00$). The toxicity of essential oil of *C. lusitanica* from Huye against houseflies was also lower compared to the toxicity of essential oil from Kayonza, though they showed no statistical difference ($P = 0.18$). There was no statistical difference observed in toxicity (with respect to LD_{90}) among all tested essential oils and Dichlorvos insecticide concentrations against adult houseflies, except for the essential oil of *C. lusitanica* from Burera that showed a significantly lower toxic effect against houseflies compared to the Dichlorvos insecticide ($P = 0.00$).

The highest toxicity of *C. lusitanica* essential oil were observed for all essential oil after 24 h of exposure time (Table 4.4), with toxicity of essential oil from Kayonza (LD_{50} = 0.08 ppm, LD_{90} = 16.26 ppm) being higher than essential oil from Burera (LD_{50} = 0.24 ppm, LD_{90} = 73.49 ppm) and Huye (LD_{50} = 0.15 ppm, LD_{90} = 22.01 ppm).

Table 4.4. Contact toxicity of *C. lusitanica* essential oils from three ecological zones and controls (DDVP and Acetone) against houseflies within 24 h, post treatment.

Treatment (EOs and controls)	Mortality percentages (mean ± SE) per treatment concentrations				LD_{50} (ppm)	LD_{90} (ppm)
	30%	50%	70%	90%		
Burera (HLZ)	53.77 ± 3.41	66.03 ± 4.79	70.09 ± 3.47	80.99 ± 3.50	0.24^{a}	73.49^{a}
Huye (MLZ)	57.82 ± 1.38	70.09 ± 2.58	78.26 ± 3.50	85.05 ± 1.29	0.15^{ab}	22.01^{ab}
Kayonza (LLZ)	61.87 ± 3.77	72.79 ± 1.37	82.35 ± 4.86	85.00 ± 2.81	0.08^{bc}	16.26^{ab}
DDVP	91.39 ± 1.46	96.15 ± 2.22	97.48 ± 1.26	98.67 ± 1.33	0.00^{c}	0.19^{b}
Acetone	18.89 ± 1.12	17.78 ± 1.11	18.89 ± 1.14	17.78 ± 1.12	-	-

HLZ: Highlands, MLZ: Midlands and LLZ: Lowlands zones, Positive control: Dichlorvos (Dimethyl 2,2-dichlorovinyl phosphate, DDVP 77%) insecticide; Negative control: Acetone. Mean ± SE of three replicates. Lethal dose values followed by the same letter in column are not significantly different at $P = 0.05$ (Tukey's HSD test).

The comparison of LD_{50} values obtained in 24 h showed that the lethal effect of essential oil against houseflies was significantly higher for *C. lusitanica* from lowland zone (Kayonza) compared to *C. lusitanica* from highland zone (Burera) ($P = 0.03$). The essential oil of *C. lusitanica* from midland zone (Huye) exhibited lower lethal effect than essential oil from lowland zone (Kayonza), but still was higher than toxicity of essential oil from highland zone (Burera) against houseflies, though all showed no statistical difference ($P > 0.05$) in their toxicity against houseflies. The Dichlorvos insecticide showed a significantly

higher toxicity against houseflies compared to all tested essential oils, contrarily to the essential oil from lowland zone (Kayonza) that showed no statistical difference in toxicity against houseflies, but still low compared to the toxicity produced by Dichlorvos insecticide ($P = 0.35$). In consideration of lethal concentrations (LD_{90}), the difference in lethal effects was not significant among all tested essential oils and when compared to Dichlorvos insecticide at all concentrations against houseflies, with exception to the essential oil of *C. lusitanica* from highland zone (Burera) that produced significantly lower contact toxicity against houseflies compared to Dichlorvos insecticide ($P = 0.04$).

4.4.2. Repellency capacity of *C. lusitanica* essential oils against houseflies

The adult houseflies were more tolerant at low concentrations of essential oils (30% v/v) and post-treatment time (6 h), but became more susceptible at higher concentration (90% v/v) and 24h post treatment time. The result of repellency of *C. lusitanica* essential oils against houseflies within 6 to 24 h post treatment is presented in the Table 4.5.

Table 4.5. Repellent activity of *C. lusitanica* essential oils from three ecological zones and controls (DDVP and Acetone) against housefly.

Treatments (EOs and Controls)	Exposure time	Repellency percentages (PR) per treatment concentrations			
		30%	50%	70%	90%
Burera (HLZ)	6h	36.81 ± 2.39^{a}	42.10 ± 3.99^{a}	47.62 ± 0.60^{ab}	54.14 ± 1.60^{b}
	12h	42.93 ± 4.46^{a}	46.00 ± 3.42^{ab}	52.95 ± 2.62^{ab}	58.98 ± 3.21^{b}
	24h	46.77 ± 2.77^{a}	53.70 ± 3.25^{ab}	60.18 ± 2.53^{bc}	70.38 ± 3.20^{c}
Huye (MLZ)	6h	42.77 ± 2.79^{a}	48.24 ± 4.50^{ab}	54.73 ± 2.52^{b}	62.32 ± 1.60^{b}
	12h	46.87 ± 1.64^{a}	52.71 ± 4.16^{ab}	61.51 ± 2.78^{b}	69.20 ± 3.89^{b}
	24h	50.57 ± 2.73^{a}	62.83 ± 3.21^{ab}	67.55 ± 3.86^{bc}	79.52 ± 3.30^{c}
Kayonza (LLZ)	6h	40.47 ± 0.62^{a}	45.77 ± 4.18^{ab}	51.15 ± 1.59^{b}	58.83 ± 3.09^{b}
	12h	44.35 ± 2.86^{a}	48.47 ± 5.68^{ab}	57.80 ± 1.53^{b}	64.02 ± 2.04^{b}
	24h	48.10 ± 1.11^{a}	58.84 ± 3.96^{b}	63.88 ± 1.73^{b}	75.62 ± 1.37^{c}
DDVP	6h	97.78 ± 2.23^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	12h	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	24h	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Acetone	6h	6.67 ± 1.92^{a}	7.78 ± 1.10^{a}	6.67 ± 1.92^{a}	5.56 ± 1.11^{a}
	12h	12.24 ± 1.12^{a}	12.22 ± 1.92^{a}	7.78 ± 1.14^{b}	13.33 ± 1.92^{a}
	24h	12.20 ± 1.40^{a}	13.30 ±1.60^{a}	7.78 ± 1.40^{b}	13.33 ± 1.92^{a}

HLZ: Highlands, MLZ: Midlands and LLZ: Lowlands zones, Positive control: Dichlorvos (Dimethyl 2,2-dichlorovinyl phosphate, DDVP 77%) insecticide; Negative control: Acetone. Mean (PR) ± SE of three replicates. PR values followed by the same letter in row are not significantly different at $P = 0.05$ (Tukey's HSD test).

The repellency rates of essential oil against houseflies were dose-exposure time-dependent with an exception to Dichlorvos (DDVP 77%) insecticide, a positive control that showed 97.78% of repellency at concentration of 30% v/v in 6 h of exposure time and 100% of repellency at all tested concentrations within 24 h post treatment (Table 4.5).

The higher repellency potentials were observed for the essential oil of *C. lusitanica* from midland (Huye) at all tested concentrations with 42.77 ± 2.79, 48.24 ± 4.50, 54.73 ± 2.52 and 62.32 ± 1.60% at concentrations of 30%, 50%, 70% and 90% v/v, respectively in 6 h post treatment (Table 4.5). At the same concentrations and exposure time, the essential oil of *C. lusitanica* from lowland (Kayonza) exhibited the moderate repellency capacity with 40.47 ± 0.62, 45.77 ± 4.18, 51.15 ± 1.59 and 58.83 ± 3.09%, while the essential oil from highland (Burera) showed lower repellency capacity with 36.81 ± 2.39, 42.10 ± 3.99, 47.62 ± 0.60 and 54.14 ± 1.60 repellency percentages at the increasing order of EOs concentrations, respectively.

In 6 h of exposure time, all tested essential oils of *C. lusitanica* showed the significantly higher repellent activity against houseflies at concentration of 90% v/v, compared to the repellencies produced at 30% (P= 0.008, 0.006, and 0.005 for Huye, Kayonza and Burera, respectively) and 50% v/v (P= 0.04, 0.039 and 0.036 for Huye, Kayonza and Burera, respectively). However, there was no significance difference between repellencies produced at concentration of 90% and 70% v/v (P= 0.35, 0.27 and 0.31 for Huye, Kayonza and Burera, respectively). The repellencies of houseflies observed at essential oil concentration of 50% v/v were higher than that produced at 30% v/v for all tested essential oils, but still lower than the repellency observed at 70% v/v, however, all were not significantly different.

In 12 h post treatment, 46.87 ± 1.64, 52.71 ± 4.16, 61.51 ± 2.78, and 69.20 ± 3.89% of repellence were respectively recorded at 30, 50, 70 and 90% v/v of essential oil from Huye,

whereas the same concentrations of essential oil from Burera exhibited the lower repellency potential with 42.93 ± 4.46, 46.00 ± 3.42, 52.95 ± 2.62 and 58.98 ± 3.21 percent, respectively (Table 4.5). The moderate repellency potential against houseflies was observed for *C. lusitanica* essential oil from Kayonza with repellence percentages of 44.35 ± 2.86, 48.47 ± 5.68, 57.80 ± 1.53 and 64.02 ± 2.04 at 30, 50, 70 and 90% v/v, respectively.

The statistical analysis proved that, the repellency capacity of *C. lusitanica* essential oil against houseflies was significantly higher at 90% v/v than the repellencies observed at 30% (P= 0.006, 0.05 and 0.02 for Huye, Kayonza and Burera, respectively) and at 50% v/v (P= 0.03 and 0.05 for Huye and Kayonza, respectively) after 12 h of post treatment, with the exception of essential oil of *C. lusitanica* from Burera that showed no statistical difference between repellency capacities observed at concentrations of 90% and 50% v/v (P= 0.11). Although, they were higher than the repellencies observed at 30 and 50% v/v, the repellency produced at 70% showed no statistical difference for all tested EOs.

Similarly, the higher repellency potential was observed for essential oil from Huye (midland) in 24 h, with the recorded repellency of 50.57 ± 2.73, 62.83 ± 3.21, 67.55 ± 3.86 and 79.52 ± 3.30 percent, respectively at 30, 50, 70 and 90% v/v of EOs concentrations. The moderate repellency capacity of essential oil from Kayonza (lowland) were presented by 48.10 ± 1.11, 58.84 ± 3.96, 63.88 ± 1.73 and 75.62 ± 1.37% of repellency, while the lower repellency potential was observed for essential oil from Burera (highland) with repellency percentages of 46.77 ± 2.77, 53.70 ± 3.25, 60.18 ± 2.53 and 70.38 ± 3.20 at 30, 50, 70 and 90% v/v, respectively.

Generally, after 24 h of exposure time, the repellency potentials of *C. lusitanica* essential oil observed at concentration of 30% v/v against houseflies were significantly lower than the repellencies observed at 90% v/v (P = 0.001, 0.00 and 0.002 for Huye, Kayonza and Burera, respectively) and at 70% (P = 0.03, 0.006 and 0.05 for Huye, Kayonza and Burera, respectively) for all tested essential oils. The repellency capacities observed at essential oil concentration of 50% v/v were also higher than the repellencies produced at 30% v/v with significance difference for essential oil from Kayonza (P= 0.047), but still lower than the repellency potentials observed at 70% v/v. Compare to the repellencies produced at concentration of 90% v/v, the observed repellency at 50% v/v were significantly lower (P= 0.03, 0.004, and 0.017 for Huye, Kayonza and Burera, respectively).

4.4.3. Fumigant toxicity of essential oils against houseflies

The fumigant effects of *C. lusitanica* essential oils against housefly in 6, 12 and 24 h of exposure time are presented in Table 4.6, Table 4.7 and Table 4.8, respectively. The mortality results produced in 6 h of exposure time (Table 4.6) showed that the essential oil of *C. lusitanica* from lowland (Kayonza) was the most potent with lower LD_{50} and LD_{90} values of 0.51 and 111.43 ppm, respectively. On the other hand, the lower fumigant toxicity against houseflies was exhibited by essential oil of *C. lusitanica* from highland (Burera) with higher LD_{50} and LD_{90} values of 1.90 and 1250.21 ppm whereas the oil of *C. lusitanica* collected from midland (Huye) showed considerably moderate fumigant toxicity against adult housefly with LD_{50} and LD_{90} values of 0.66 and 521.36 ppm, respectively in 6 h of exposure time.

Table 4.6. Fumigant toxicity of *C. lusitanica* essential oils from three ecological zones and controls (DDVP and Acetone) against houseflies in 6 h post treatment.

Treatments (EOs and controls)	Mortality percentages (mean ± SE) per treatment concentration				LD_{50} (ppm)	LD_{90} (ppm)
	30%	50%	70%	90%		
Burera (HLZ)	38.30 ± 3.09	44.62 ± 1.79	54.71 ± 0.49	62.18 ± 4.10	1.90[a]	1250.21[a]
Huye (MLZ)	48.39 ± 1.75	52.19 ± 2.56	60.93 ± 4.80	71.04 ± 1.53	0.66[b]	521.36[b]
Kayonza (LLZ)	49.63 ± 2.98	53.45 ± 1.35	68.48 ± 4.74	76.05 ± 2.72	0.51[b]	111.43[c]
DDVP	86.63 ± 2.26	92.40 ± 2.22	93.83 ± 3.27	94.96 ± 3.25	0.01[c]	0.52[c]
Acetone	8.89 ± 2.23	12.22 ± 1.11	11.11 ± 1.13	14.44 ± 2.22	-	-

HLZ: Highlands, MLZ: Midlands and LLZ: Lowlands zones, Positive control: Dichlorvos (Dimethyl 2,2-dichlorovinyl phosphate, DDVP 77%) insecticide; Negative control: Acetone. Mean ± SE of three replicates. Lethal dose values followed by the same letter in column are not significantly different at $P = 0.05$ (Tukey's HSD test).

The statistical analysis of lethal concentrations (LD_{50}) proven that the toxic effects of essential oils against houseflies were statistically different among all tested oils concentrations and also compared to the Dichlorvos insecticide with the exception for the essential oil of *C. lusitanica* from midland (Huye) that produced lower fumigant toxicity against houseflies compared to that produced by essential oil of *C. lusitanica* from lowland (Kayonza) ($P = 0.63$) within 6 h of exposure time. The LD_{90} values proved a significant difference in toxicity among all tested essential oil concentrations and compared to Dichlorvos insecticide against houseflies except for the essential oils from lowland (Kayonza) that showed no statistical difference in fumigant toxicity compared to Dichlorvos insecticide ($P = 0.61$).

In 12 h post treatment (Table 4.7), the higher fumigant toxicity of *C. lusitanica* essential oil against houseflies was recorded for lowland (Kayonza) with 0.29 ppm and 81.47 ppm of LD_{50} and LD_{90}, respectively. The poorer lethal effect was observed with the *C. lusitanica* oil from highland (Burera), which resulted in LD_{50} of 0.81 ppm and LD_{90} of 766.80 ppm, while the *C. lusitanica* essential oil from midland (Huye) exhibited the moderate fumigant toxicity against houseflies with LD_{50} and LD_{90} values of 0.39 ppm and 159.35 ppm, respectively.

Table 4.7. Fumigant toxicity of *C. lusitanica* essential oil from three ecological zones and controls (DDVP and Acetone) against houseflies in 12 h post treatment.

Treatment (EOs and controls)	**Mortality percentages (mean ± SE) per treatment concentrations**				**LD_{50} (ppm)**	**LD_{90} (ppm)**
	30%	**50%**	**70%**	**90%**		
Burera (HLZ)	44.68 ± 2.79	48.64 ± 2.70	59.23 ± 0.80	65.74 ± 1.76	0.81^{a}	766.80^{a}
Huye (MLZ)	48.69 ± 2.11	57.95 ± 2.06	63.11 ± 1.78	73.70 ± 1.05	0.39^{b}	159.35^{b}
Kayonza (LLZ)	52.61 ± 0.68	56.60 ± 1.96	70.97 ± 2.98	76.31 ± 0.34	0.29^{bc}	81.47^{b}
DDVP	90.29 ± 2.30	94.97 ± 1.19	95.06 ± 2.47	98.77 ± 1.23	0.00^{c}	0.36^{b}
Acetone	13.33 ± 1.92	16.67 ± 1.92	14.44 ± 1.11	17.78 ± 2.22	-	-

HLZ: Highlands, MLZ: Midlands and LLZ: Lowlands zones, Positive control: Dichlorvos (Dimethyl 2,2-dichlorovinyl phosphate, DDVP 77%) insecticide; Negative control: Acetone. Mean ± SE of three replicates. Lethal dose values followed by the same letter in column are not significantly different at $P = 0.05$ (Tukey's HSD test).

The LD_{50} values obtained in 12 h revealed that the essential oil of *C. lusitanica* from Kayonza (lowland) exhibited a significantly higher fumigant toxicity against houseflies compared to the toxicity produced by that from Huye (midland) ($P = 0.006$). Although its fumigant toxicity to houseflies was lower and not significant compared to the fumigant

toxicity exhibited by *C. lusitanica* oil from lowland (Kayonza) ($P = 0.79$), the essential oil of *C. lusitanica* from Huye (midland) exhibited a significantly higher fumigant toxicity against adult houseflies than the essential oil of *C. lusitanica* from highland (Burera) ($P = 0.02$). Considering lethal concentrations (LD_{90}), the essential oil of *C. lusitanica* from highlands (Burera) produced a significantly lower fumigant toxicity against houseflies than the toxicity produced by other tested essential oils and Dichlorvos insecticide ($P < 0.05$) at all concentrations. Moreover, the fumigant toxicity produced by essential oil of *C. lusitanica* from Kayonza (lowland) against adult houseflies was not significantly different from the fumigant toxicity exhibited by essential oil from Huye (midland) ($P = 0.55$) and Dichlorvos insecticide ($P = 0.51$).

The fumigant toxicity observed in 24 h of exposure time for *C. lusitanica* essential oil against houseflies were higher compared to the toxicity recorded in 6 and 12 h of exposure time (Table 4.8). The results indicated that the lethal concentrations (LD_{50} = 0.15 ppm and LD_{90} = 24.79 ppm) for *C. lusitanica* essential oil from Kayonza (lowland) were much lower than that recorded for other regions (LD_{50} = 0.28 ppm and LD_{90} = 80.65 ppm for Huye and LD_{50} = 0.38 ppm and LD_{90} = 220.32 ppm for Burera), which implies the higher fumigant toxicity against houseflies.

Table 4.8. Fumigant toxicity of *C. lusitanica* essential oil from three ecological zones and controls (DDVP and Acetone) against houseflies in 24 h post treatment.

Treatments (EOs and Controls)	Mortality percentages (mean ± SE) per treatment concentrations				LD_{50} (ppm)	LD_{90} (ppm)
	30%	50%	70%	90%		
Burera (HLZ)	52.39 ± 1.13	55.00 ± 2.53	65.97 ± 1.55	72.80 ± 1.23	0.38^{a}	220.32^{a}
Huye (MLZ)	52.39 ± 1.13	59.21 ± 2.12	68.68 ± 2.90	76.88 ± 1.25	0.28^{a}	80.65^{b}
Kayonza (LLZ)	59.21 ± 2.12	65.94 ± 3.81	75.51 ± 0.14	83.67 ± 0.10	0.15^{b}	24.79^{bc}
DDVP	93.96 ± 1.10	94.97 ± 1.19	97.53 ± 2.47	98.77 ± 1.23	0.00^{c}	0.20^{c}
Acetone	17.78 ± 2.22	20.00 ± 0.00	16.67 ± 1.92	18.89 ± 1.11	-	-

HLZ: Highlands, MLZ: Midlands and LLZ: Lowlands zones, Positive control: Dichlorvos (Dimethyl 2,2-dichlorovinyl phosphate, DDVP 77%) insecticide; Negative control: Acetone. Mean ± SE of three replicates. Lethal dose values followed by the same letter in column are not significantly different at $P = 0.05$ (Tukey's HSD test).

The significant difference in fumigant toxicities were observed among all tested essential oils concentrations against adult houseflies in 24 hours of exposure time ($P < 0.05$), with the highest toxic effect being observed for the essential oil from Kayonza, followed by the essential oil from midland (Huye) that exhibited higher toxic effect, but not significant compared to that produced by essential oil from highland ($P = 0.06$). The fumigant toxicities produced by all tested essential oils of *C. lusitanica* against houseflies were significantly lower than the fumigant toxicities caused by Dichlorvos insecticide ($P < 0.05$). The comparison of the lethal concentrations (LD_{90}) values proved a statistical difference between fumigant toxicity against houseflies among all tested essential oil from *C. lusitanica* and Dichlorvos insecticide ($P < 0.05$) with the exception of the essential oil from Kayonza that showed no statistical difference in toxic effect against houseflies compared to the essential oil of *C. lusitanica* from Huye ($P = 0.18$) and Dichlorvos ($P = 0.75$).

The current study showed variable promising results as per agro-ecological zone and they are in agreement with a number of studies that have reported the insecticidal activity of essential oils from species of Cupressaceae family via topical application, fumigation and repellency against various pests, including houseflies (Baana *et al.*, 2018; Bett, 2015; Elbermawy *et al.*, 2011; Giatropoulos *et al.*, 2013; Langsi *et al.*, 2018; Pavela, 2008; Teke *et al.*, 2013). However, the data on the bioactivity of *C. lusitanica* essential oil against houseflies are scarce, and it is difficult to make exact comparisons with other studies due to the difference in chemical components of *C. lusitanica* essential oil, target insect, mode of application, different concentrations used and length of exposure time considered. The ethnobotanical survey conducted by Baana *et al.*(2018) revealed the use of *Cupressus simpervirens* (very similar to *C. lusitanica*) for repelling houseflies by placing the fresh leaves in areas where flies are numerous or hanged in the roof and walls of latrines and house. In similar study, the essential oil of *C. sempervirens* showed repellent potential and larvicidal activity towards larvae of houseflies (Elbermawy *et al*., 2011), while Yang *et al.* (2020) observed the higher contact toxicity (LD_{50}=1.23 g/cm^2) and lower fumigant toxicity (LD_{50} =556.80 g/cm^2) for essential oil of *Cupressus sempervirens* against stored product pest (*Sitophilus zeamais Motschulsky*). Among the eight Cupressus species investigated by Giatropoulos *et al.* (2013), the essential oil of *Cupressus benthamii* showed potent toxicity (LC_{50} =37.5 mg/L) with the major composition, umbellulone, limonene, δ-3-carene and α-pinene while the others species provided rather moderate toxicity against larvae and adult mosquitoes *(Aedes albopictus)* (LC_{50} = 47.90 to 70.60 mg/L). The results reported by Lee *et al.*(2015) confirmed the strong repellent behavioral responses of fruit flies and houseflies to the essential oil from Hinoki cypress (*Chamaecyparis obtusa*) after 5 h of exposure

duration, while the oil of *Juniperus communis*, *Juniperus virginiana* and *Thuja occidentalis* of Cupressaceae family were also reported to exhibit lethal effects on houseflies in 24-h with LD_{50} values of 86.0, 24.0 and 42.0 µg/fly after topical application and 10.80, 80.0 and 6.30 µg/cm^3 after fumigant test, respectively (Pavela, 2008).

In most cases, the insecticidal constituents of many plant extracts and essential oils are monoterpenoids due to their anti-cholinesterasic properties which cause high levels of mortality of insects at higher concentrations, however, such monoterpenoids can be lost after long post-treatment time due to their high volatility (Chen *et al.*, 2018). The toxic and repellent effects of the terpenes such as α- pinene, β-pinene, limonene, linalool, myrcene, α-terpineol, terpinen-4-ol, α-,γ-terpinene, 1,8-cineole, Bornyl acetate and terpinolene against houseflies have been previously demonstrated (Haselton *et al.*, 2015; Palacios *et al.*, 2009; Tian, 2017; Urzúa *et al.*, 2010; Zhang *et al.*, 2017). Palacios *et al.*(2009) reported the low and moderate fumigant toxicity of α-pinene and β-pinene against adult *M. domestica*, whereas the study conducted by Urzúa *et al.* (2010) demonstrated the moderate toxicity of α-pinene and limonene against houseflies (*M. domestica*) with lethal concentrations (LC_{50}) of 12.10 and 5.0 mg/dm^3 in 30 minutes, respectively. Terpinolene, ρ-cymene and other 11 monoterpenes exhibited strong fumigant activity against *M. domestica* in the study conducted by Zhang *et al.*(2017), while El-sherbini & Osman (2014) reported the variable mortality of houseflies from 65 to 100%, 55 to 100% and 75 to 100% in 6 to 24 h of exposure to monoterpenes; α-pinene, myrcene and limonene, respectively. In previous study by Haselton *et al.* (2015), the α-pinene was proven to be the antenna-stimulatory and it exhibited the baseline repellent properties against houseflies under

laboratory conditions. Moreover, the study conducted on the insecticidal potential of essential oil components including limonene, γ-terpinene, linalool, verbenone and camphor demonstrated the moderate toxic effect against adult female houseflies via topical application with lethal doses of 226.63, 236.47, 238.05, 426.67 and 512.12 µg/fly, respectively in 24 h, and 213.36, 221.55, 209.73, 409.93 and 477.91 µg/fly, respectively in 48 h (Tian, 2017). In the same study, p-cymene showed a significant higher repellency at low and high concentrations (0.1µg/µL and 100 µg/µL), while limonene, γ-terpinene and linalool exhibited significant higher repellency against houseflies (*M. domestica*) only at high concentrations (10 and 100 µg/µL).

Despite not being tested directly, the insecticidal effects of *C. lusitanica* essential oil could be linked to the presence of individual major chemical components of essential oil such as γ-terpinene (18.77%), umbellulone (18.16%), Bornyl acetate (9.72%), linalool (8.71%), myrcene (7.20%), limonene (5.53%), α-pinene (5.24%) and sabinene (4.05%) for *C. lusitanica* from lowland (Kayonza); umbellulone (24.21%), δ-3-carene (16.76%), sabinene (10.54%), α-pinene (8.21%), α-terpinene (5.84%), camphene hydrate (3.47%), and α-terpineol (3.18%) for *C. lusitanica* from midland (Huye) and sabinene (20.84%), myrcene (19.63%), α-pinene (10.23%), δ-3-carene (10.13%), linalool (6.83%), umbellulone (3.23%) and γ-terpinene (3.14%) for *C. lusitanica* from highland (Burera). In addition, the insecticidal activity of *C. lusitanica* essential oil can also be explained by synergistic action of essential oils' components (Bett *et al.*, 2017)**.**

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The results of present study showed variation in yield and chemical components of essential oils from fresh leaves of *C. lusitanica* growing in three agro-ecological zones of Rwanda, and the observed variations are attributed to the influence of climatic and environmental conditions, or plant genetics, maturity and stage of growth. However, it could also be attributed to the result of interactions between *C. lusitanica* plant with various biotic and abiotic factors within ecological habitats that influence the production or alter the chemical components of plant essential oils.

The essential oils of *C. lusitanica* showed a promising insecticidal potential that varied from one region to another against adult houseflies with higher fumigant and contact toxicity being exhibited by the essential oil of *C. lusitanica* from lowlands (Kayonza), followed by that from midlands (Huye), while essential oil from highlands (Burera) showed lower toxicity against housefly. Essential oils of *C. lusitanica* from midland zone (Huye) showed the highest repellency activity against housefly, while the lowest repellency activity was exhibited by essential oil from highland zone (Burera). The observed variation in insecticidal activities of *C. lusitanica* essential oils against housefly among investigated agro-ecological zones is due to unequal distribution of compounds like limonene, γ-terpinene, α-pinene, δ-3-carene, α-terpineol, γ-terpinene, linalool, Sabinene and Bornyl acetate in essential oils that were previously reported in different studies to exhibited insecticidal activity against houseflies.

In general, the promising insecticidal activities observed for all investigated essential oils of *C. lusitanica* against houseflies provide a safer prophylactic measure for control of adult housefly population.

5.2. Recommendations

Apart from the steam distillation method used in current study, the study recommends the use and comparison of different essential oil extraction methods, especially modern techniques like solid phase micro-extraction (SPME), Supercritical fluid extraction (SFE), and Microwave-energy based methods for optimization of extraction yield and it also would provide insight to the qualitative and quantitative variations linked to the essential oil extraction methods. Isolation of pure compounds for major composition of C. *lusitanica* essential oils, and evaluation of insecticidal activity against adult houseflies for individual pure compounds isolated from EOs could be the future perspective in academic research.

In addition to the current study on adult houseflies, the future study could look into toxicity and the effectiveness of *C. lusitanica* essential oils at each stage of housefly development (egg, larvae and pupae). Moreover, the promising findings of this study call for further research into the biosafety of *C. lusitanica* EOs for use as insecticide against housefly.

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APPENDICES

Appendix 1: (a) collection, (b) preparation and (c) packaging of fresh leaves of *C. lusitanica*

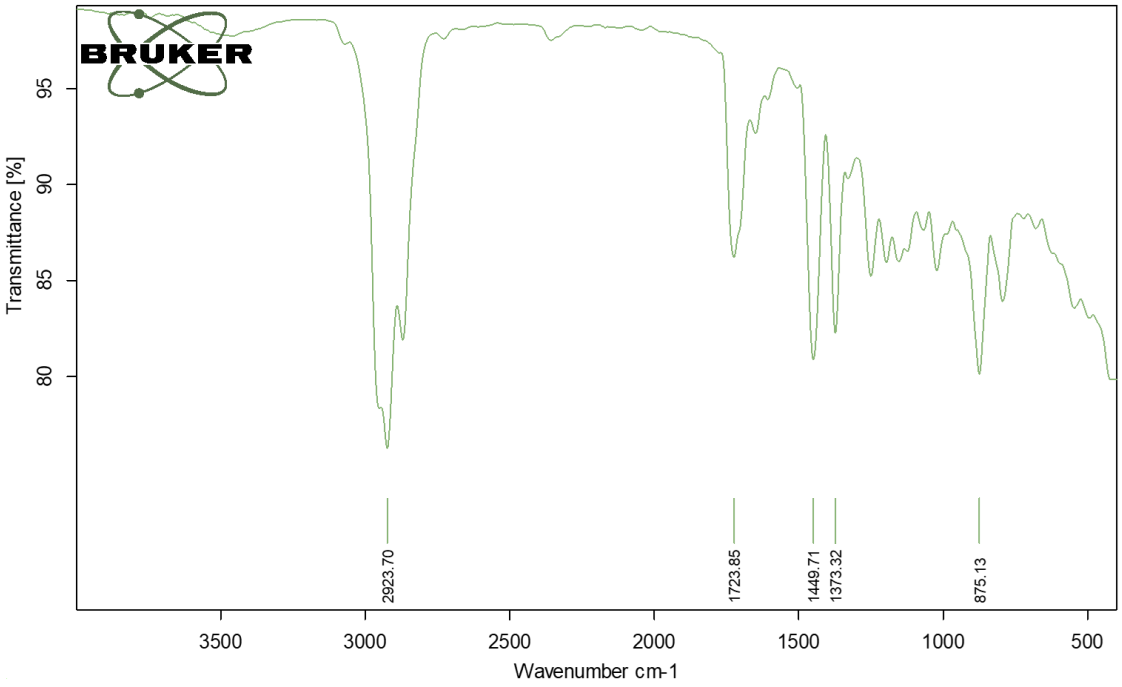
(a) (b) (c)

Appendix 2: Table. Tests for differences of means of EOs yields as per ecological zone

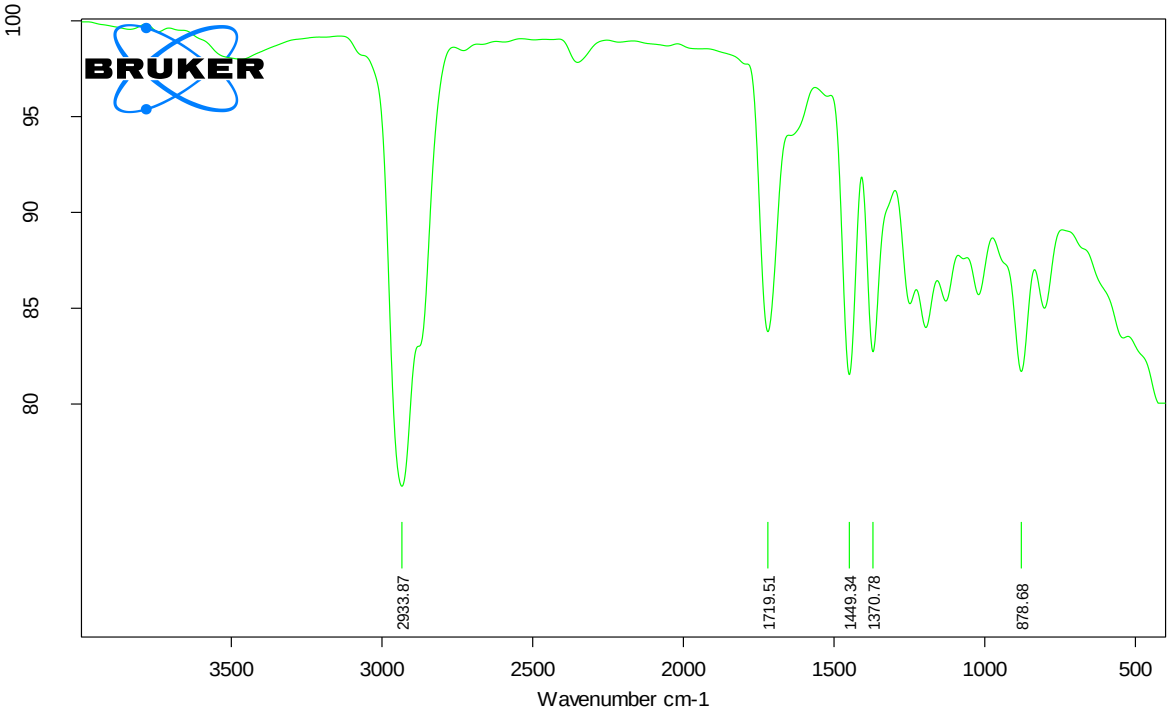
Factor	*Mean*	*St.Dev*	*95% CI*	*Difference of levels*	*Difference of Means*	*T-Value*	*Adjusted P-Value*
Burera	0.27	0.04	(0.22, 0.33)	Huye - Burera	0.07	2.30	0.13
Huye	0.34	0.04	(0.29, 0.39)	Kayonza - Burera	0.12	4.05	0.02*
Kayonza	0.39	0.03	(0.34, 0.45)	Kayonza - Huye	0.05	1.75	0.27

* *Significant difference of means by Tukey's HSD test at 95% CI (one way- ANOVA, Minitab 17)*

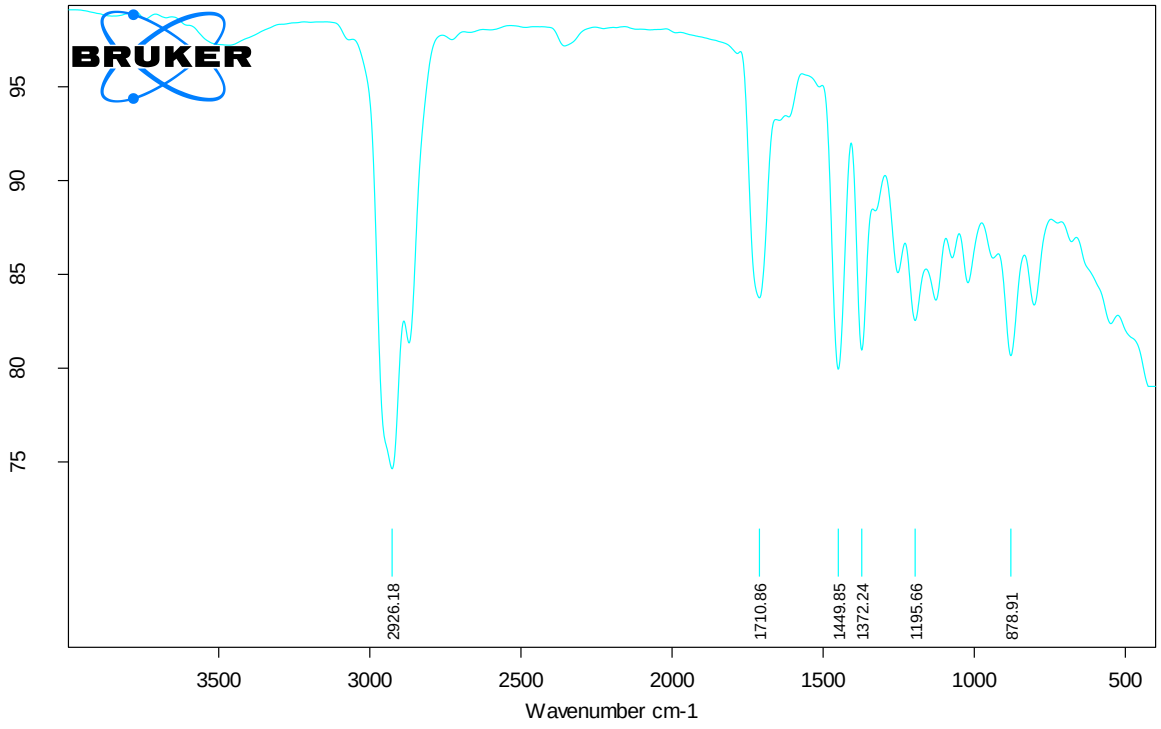
Appendix 3a: FT-IR spectrum of essential oils of *C. lusitanica* leaves from Burera (highlands)



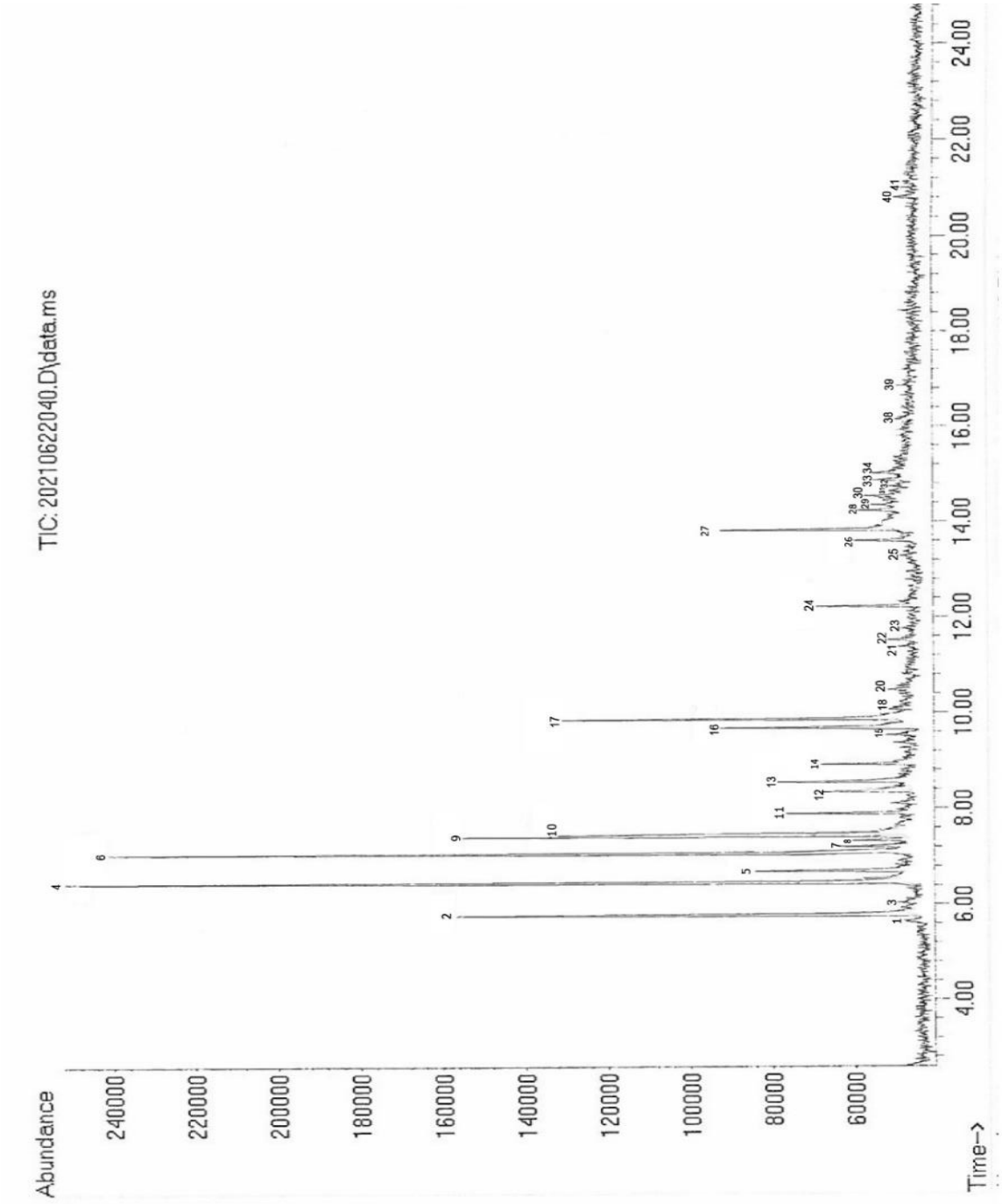
Appendix 3b. FT-IR spectrum of essential oil of *C. lusitanica* leaves from Huye (midlands)



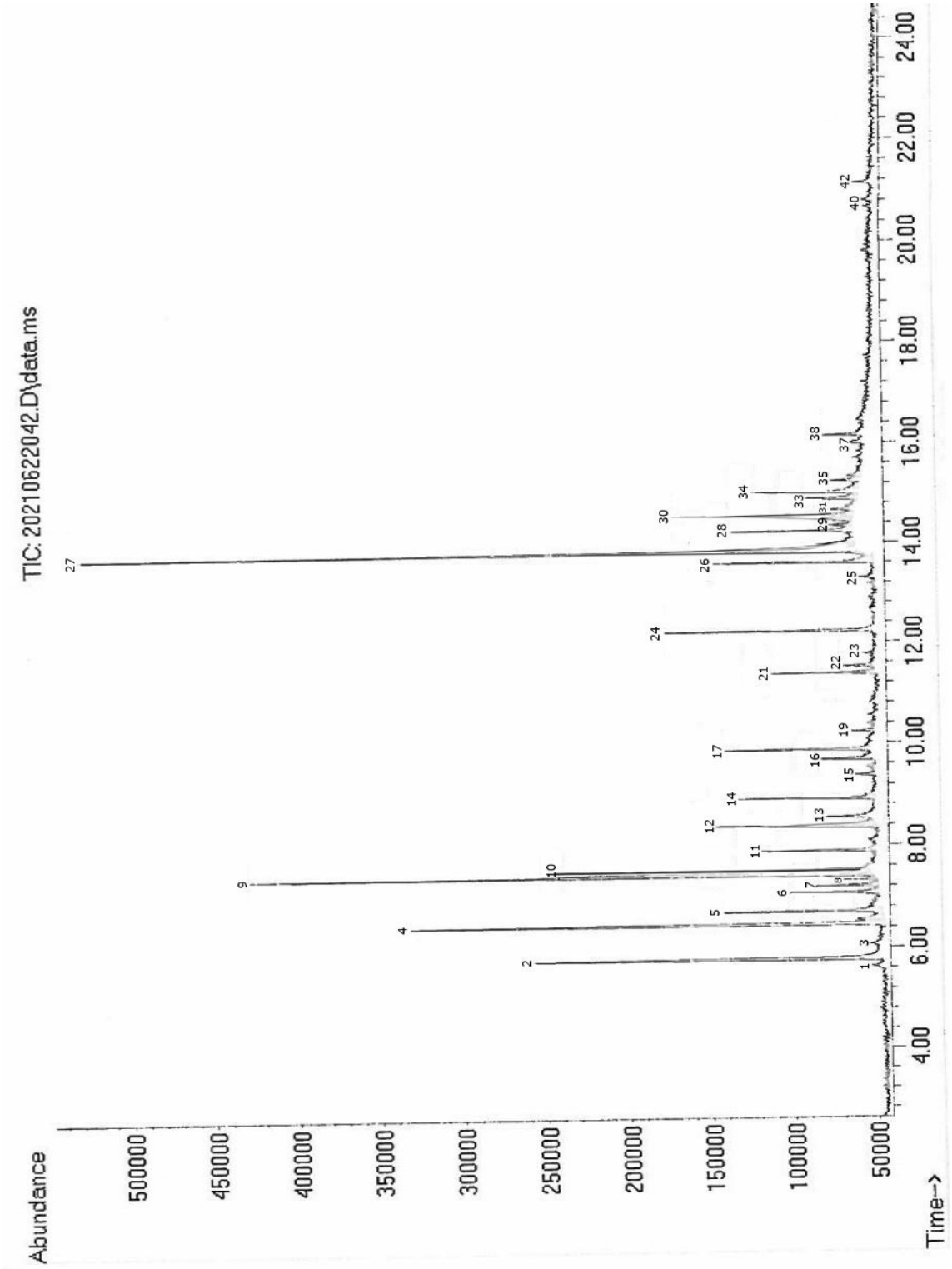
Appendix 3c. FT-IR spectrum of essential oil of *C. lusitanica* leaves from Kayonza (lowlands)



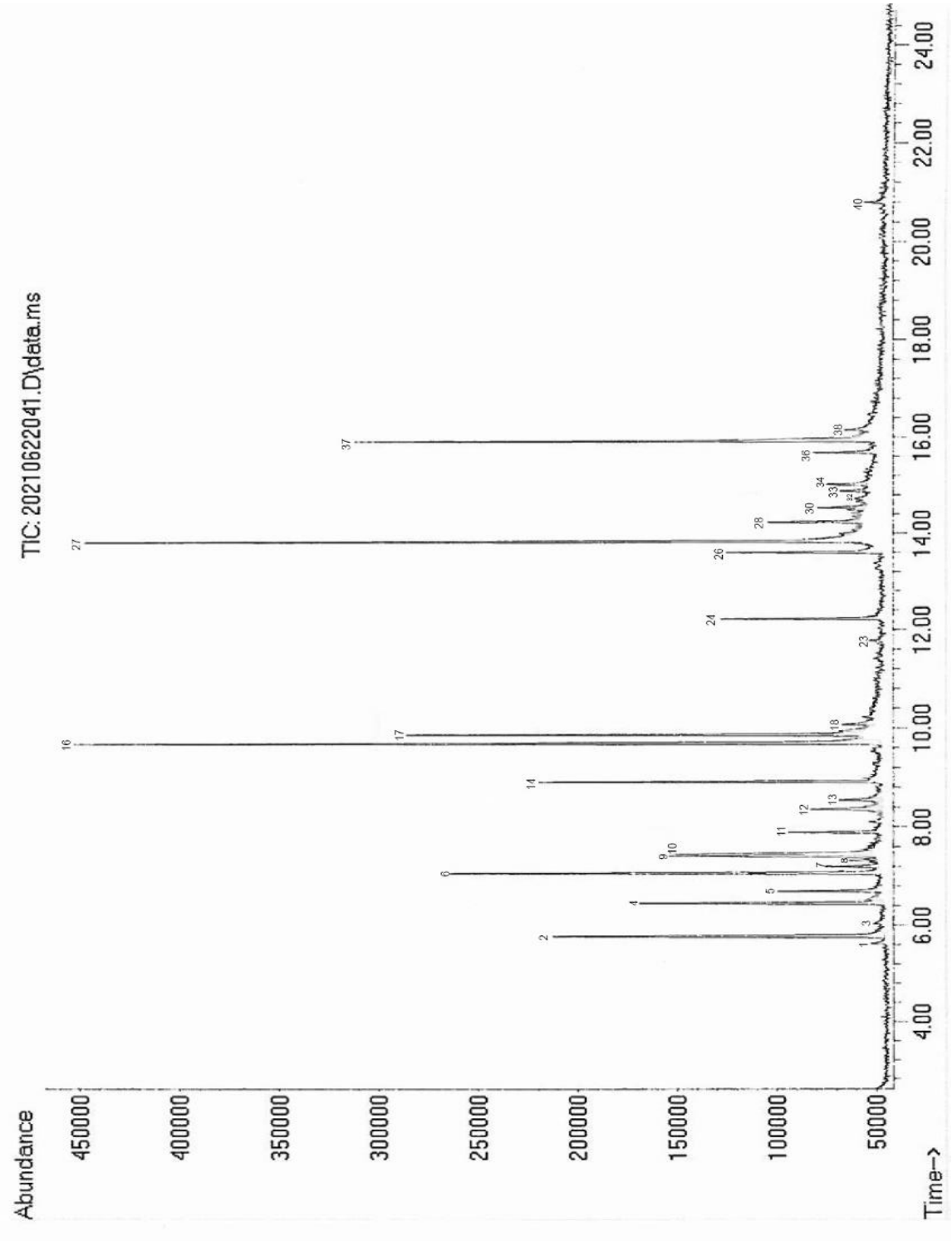
Appendix 4a: GC-MS chromatogram of essential oil from *C. lusitanica* leaves collected from Burera (highlands).



Appendix 4b: GC MS chromatogram of essential oil from *C. lusitanica* leaves collected from Huye (midlands).



Appendix 4c: GC MS chromatogram of essential oil from *C. lusitanica* leaves collected from Kayonza (lowlands)

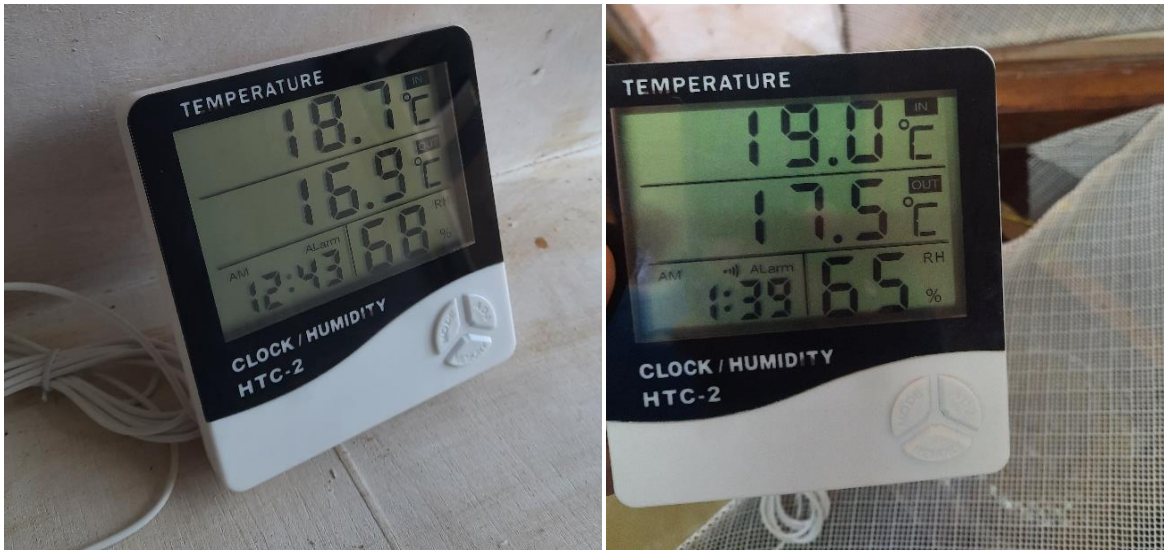


Appendix 5. Collection and Maintenance of houseflies.

(a) Field collection of the starter houseflies (*Musca domestica)* using a sweep net and plastic jars covered with mesh.

(b) Laboratory rearing of the collected houseflies for breeding

(c) Monitoring of houseflies rearing conditions (temperature and relative humidity) using Digital hygrometer.



(d) The cow dung used as breeding media of houseflies

(e) The appearance of the white maggots of houseflies in breeding media

(f) (1) Pupa in dry breeding media (cow dung) and (2) the pupa separated from breeding media and placed in the cage until the emergence of adult houseflies.

(1)

(2)

Appendix 6: Experimental set ups of bioassays.

(a) Experimental bioassay for Fumigant toxicity testing for EOs against Houseflies

(b) Experimental bioassay for Contact toxicity testing for EOs against Houseflies

(c) Experimental bioassay for Repellency capacity testing for EOs against Houseflies

Appendix 7: Output tables of statistical analysis for repellency potential of EOs against houseflies

Table A7.1: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Burera at different concentrations against houseflies after 6 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	5.30	3.51	(-5.93, 16.53)	1.51	0.474
at 70% - at 30%	10.81	3.51	(-0.42, 22.04)	3.08	0.059
at 90% - at 30%	17.34	3.51	(6.11, 28.56)	4.95	0.005
at 70% - at 50%	5.51	3.51	(-5.72, 16.74)	1.57	0.444
at 90% - at 50%	12.04	3.51	(0.81, 23.27)	3.43	0.036
at 90% - at 70%	6.53	3.51	(-4.70, 17.76)	1.86	0.314

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.2: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Burera at different concentrations against houseflies after 12 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	3.08	4.94	(-12.74, 18.89)	0.62	0.922
at 70% - at 30%	10.03	4.94	(-5.78, 25.84)	2.03	0.253
at 90% - at 30%	16.06	4.94	(0.24, 31.87)	3.25	0.047
at 70% - at 50%	6.95	4.94	(-8.86, 22.77)	1.41	0.528
at 90% - at 50%	12.98	4.94	(-2.84, 28.79)	2.63	0.112
at 90% - at 70%	6.03	4.94	(-9.79, 21.84)	1.22	0.632

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.3: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Burera at different concentrations against houseflies after 24 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	6.93	4.18	(-6.46, 20.32)	1.66	0.402
at 70% - at 30%	13.41	4.18	(0.02, 26.80)	3.21	0.050
at 90% - at 30%	23.61	4.18	(10.22, 37.00)	5.65	0.002
at 70% - at 50%	6.48	4.18	(-6.91, 19.87)	1.55	0.454
at 90% - at 50%	16.68	4.18	(3.29, 30.07)	3.99	0.017
at 90% - at 70%	10.20	4.18	(-3.19, 23.59)	2.44	0.146

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.4: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Huye at different concentrations against houseflies after 6 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	5.47	4.30	(-8.30, 19.24)	1.27	0.603
at 70% - at 30%	11.96	4.30	(-1.81, 25.73)	2.78	0.091
at 90% - at 30%	19.55	4.30	(5.78, 33.32)	4.55	0.008
at 70% - at 50%	6.49	4.30	(-7.28, 20.26)	1.51	0.475
at 90% - at 50%	14.08	4.30	(0.31, 27.85)	3.27	0.045
at 90% - at 70%	7.59	4.30	(-6.18, 21.36)	1.77	0.354

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.5: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Huye at different concentrations against houseflies after 12 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	5.85	4.63	(-8.97, 20.66)	1.26	0.608
at 70% - at 30%	14.64	4.63	(-0.17, 29.46)	3.17	0.053
at 90% - at 30%	22.34	4.63	(7.52, 37.15)	4.83	0.006
at 70% - at 50%	8.80	4.63	(-6.02, 23.61)	1.90	0.299
at 90% - at 50%	16.49	4.63	(1.68, 31.31)	3.57	0.030
at 90% - at 70%	7.70	4.63	(-7.12, 22.51)	1.66	0.400

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.6: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Huye at different concentrations against houseflies after 24 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	12.26	4.67	(-2.69, 27.21)	2.63	0.112
at 70% - at 30%	16.98	4.67	(2.03, 31.92)	3.64	0.027
at 90% - at 30%	28.95	4.67	(14.00, 43.89)	6.20	0.001
at 70% - at 50%	4.72	4.67	(-10.23, 19.66)	1.01	0.748
at 90% - at 50%	16.69	4.67	(1.74, 31.63)	3.58	0.030
at 90% - at 70%	11.97	4.67	(-2.97, 26.92)	2.57	0.123

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.7: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Kayonza at different concentrations against houseflies after 6 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	5.30	3.87	(-7.09, 17.69)	1.37	0.549
at 70% - at 30%	10.68	3.87	(-1.71, 23.07)	2.76	0.093
at 90% - at 30%	18.36	3.87	(5.96, 30.75)	4.74	0.006
at 70% - at 50%	5.38	3.87	(-7.01, 17.77)	1.39	0.538
at 90% - at 50%	13.06	3.87	(0.67, 25.45)	3.38	0.039
at 90% - at 70%	7.68	3.87	(-4.72, 20.07)	1.98	0.269

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.8: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Kayonza at different concentrations against houseflies after 12 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	4.12	4.84	(-11.40, 19.64)	0.85	0.829
at 70% - at 30%	13.45	4.84	(-2.06, 28.97)	2.78	0.091
at 90% - at 30%	19.67	4.84	(4.15, 35.19)	4.06	0.015
at 70% - at 50%	9.33	4.84	(-6.18, 24.85)	1.93	0.290
at 90% - at 50%	15.55	4.84	(0.03, 31.06)	3.21	0.050
at 90% - at 70%	6.21	4.84	(-9.30, 21.73)	1.28	0.597

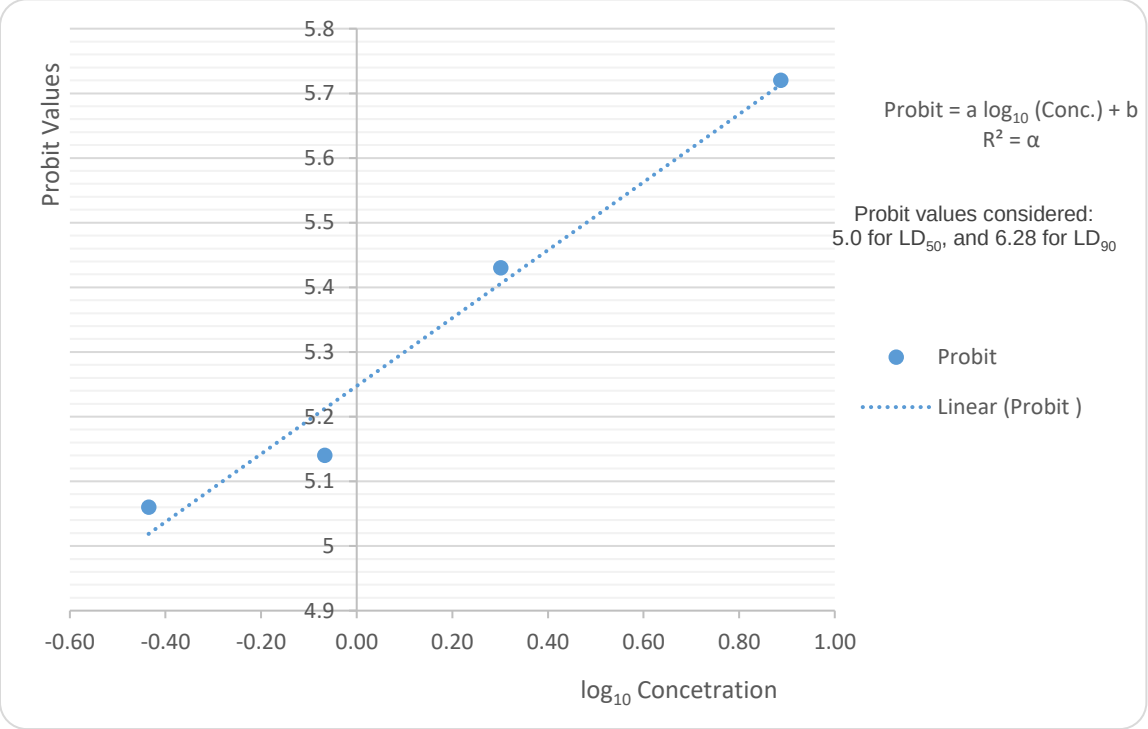
SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A7.9: Tests for Differences of Means of repellency capacity of *C. lusitanica* EO from Kayonza at different concentrations against houseflies after 24 h of exposure time

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
at 50% - at 30%	10.74	3.30	(0.16, 21.32)	3.25	0.047
at 70% - at 30%	15.79	3.30	(5.21, 26.37)	4.78	0.006
at 90% - at 30%	27.52	3.30	(16.94, 38.10)	8.33	0.000
at 70% - at 50%	5.05	3.30	(-5.53, 15.63)	1.53	0.465
at 90% - at 50%	16.78	3.30	(6.20, 27.36)	5.08	0.004
at 90% - at 70%	11.73	3.30	(1.15, 22.31)	3.55	0.031

SE: standard error of three replicates, N=30, P< 0.05 meant significant difference of values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Appendix 8: General regression equation for calculation of lethal dose values (LD_{50} and LD_{90}) for both contact and fumigant toxicities testing.



Appendix 9: Output tables of statistical comparisons of lethal concentrations (LD_{50} and LD_{90}) of *C. lusitanica* essential oil for Fumigant toxicity against houseflies.

Table A9.1: Tests for Differences of Means of LD_{50} values from Fumigant toxicity testing of *C. lusitanica* EO against houseflies after 6-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD50 - B-LD50	-1.242	0.122	(-1.631, -0.852)	-10.21	0.000
K-LD50 - B-LD50	-1.390	0.122	(-1.780, -1.000)	-11.43	0.000
DDVP-6 - B-LD50	-1.889	0.122	(-2.278, -1.499)	-15.53	0.000
K-LD50 - H-LD50	-0.148	0.122	(-0.538, 0.241)	-1.22	0.633
DDVP-6 - H-LD50	-0.647	0.122	(-1.037, -0.257)	-5.32	0.003
DDVP-6 - K-LD50	-0.499	0.122	(-0.888, -0.109)	-4.10	0.015

B-LD_{50}, H-LD_{50}, K-LD_{50} and DDVP represent the lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30, P<0.05 meant significant difference of LD_{50} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A9.2: Tests for Differences of Means of LD_{90} values from Fumigant toxicity testing of *C. lusitanica* EO against houseflies after 6-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD90 - B-LD90	-728.9	89.1	(-1014.2, -443.5)	-8.18	0.000
K-LD90 - B-LD90	-1138.8	89.1	(-1424.1, -853.5)	-12.79	0.000
DDVP-6 - B-LD90	-1249.7	89.1	(-1535.0, -964.4)	-14.03	0.000
K-LD90 - H-LD90	-409.9	89.1	(-695.2, -124.6)	-4.60	0.008
DDVP-6 - H-LD90	-520.8	89.1	(-806.1, -235.5)	-5.85	0.002
DDVP-6 - K-LD90	-110.9	89.1	(-396.2, 174.4)	-1.25	0.618

B-LD_{90}, H-LD_{90}, K-LD_{90} and DDVP represent the lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30. P<0.05 meant significant difference of LD_{90} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A9.3: Tests for Differences of Means of LD_{50} values from Fumigant toxicity testing of *C. lusitanica* EO against houseflies after 12-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD50 - B-LD50	-0.417	0.108	(-0.762, -0.072)	-3.87	0.020
K-LD50 - B-LD50	-0.518	0.108	(-0.863, -0.173)	-4.81	0.006
DDVP-12 - B-LD50	-0.806	0.108	(-1.151, -0.461)	-7.48	0.000
K-LD50 - H-LD50	-0.101	0.108	(-0.446, 0.244)	-0.94	0.785
DDVP-12 - H-LD50	-0.390	0.108	(-0.735, -0.045)	-3.62	0.028
DDVP-12 - K-LD50	-0.288	0.108	(-0.633, 0.057)	-2.68	0.105

B-LD_{50}, H-LD_{50}, K-LD_{50} and DDVP represent the lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30, P<0.05 meant significant difference of LD_{50} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A9.4: Tests for Differences of Means of LD_{90} values from Fumigant toxicity testing of *C. lusitanica* EO against houseflies after 12-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD90 - B-LD90	-607.4	56.5	(-788.5, -426.4)	-10.74	0.000
K-LD90 - B-LD90	-685.3	56.5	(-866.4, -504.2)	-12.12	0.000
DDVP-12 - B-LD90	-766.4	56.5	(-947.5, -585.4)	-13.56	0.000
K-LD90 - H-LD90	-77.9	56.5	(-259.0, 103.2)	-1.38	0.545
DDVP-12 - H-LD90	-159.0	56.5	(-340.1, 22.1)	-2.81	0.087
DDVP-12 - K-LD90	-81.1	56.5	(-262.2, 100.0)	-1.43	0.514

B-LD_{90}, H-LD_{90}, K-LD_{90} and DDVP represent the lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30. P<0.05 meant significant difference of LD_{90} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A9.5: Tests for Differences of Means of LD_{50} values from Fumigant toxicity testing of *C. lusitanica* EO against houseflies after 24-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD50 - B-LD50	-0.1028	0.0329	(-0.2081, 0.0026)	-3.13	0.056
K-LD50 - B-LD50	-0.2338	0.0329	(-0.3392, -0.1285)	-7.11	0.000
DDVP-24 - B-LD50	-0.3807	0.0329	(-0.4860, -0.2754)	-11.58	0.000
K-LD50 - H-LD50	-0.1310	0.0329	(-0.2364, -0.0257)	-3.98	0.017
DDVP-24 - H-LD50	-0.2779	0.0329	(-0.3833, -0.1726)	-8.45	0.000
DDVP-24 - K-LD50	-0.1469	0.0329	(-0.2522, -0.0415)	-4.47	0.009

B-LD_{50}, H-LD_{50}, K-LD_{50} and DDVP represent the lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30, P<0.05 meant significant difference of LD_{50} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A9.6: Tests for Differences of Means of LD_{90} values from Fumigant toxicity testing of *C. lusitanica* EO against houseflies after 24-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD90 - B-LD90	-139.7	24.2	(-217.1, -62.2)	-5.78	0.002
K-LD90 - B-LD90	-195.5	24.2	(-273.0, -118.1)	-8.09	0.000
DDVP-24 - B-LD90	-220.1	24.2	(-297.6, -142.7)	-9.10	0.000
K-LD90 - H-LD90	-55.9	24.2	(-133.3, 21.6)	-2.31	0.175
DDVP-24 - H-LD90	-80.5	24.2	(-157.9, -3.0)	-3.33	0.042
DDVP-24 - K-LD90	-24.6	24.2	(-102.0, 52.9)	-1.02	0.745

B-LD_{90}, H-LD_{90}, K-LD_{90} and DDVP represent the lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30. P<0.05 meant significant difference of LD_{90} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Appendix 10: Output tables of statistical comparisons of lethal concentrations (LD_{50} and LD_{90}) of *C. lusitanica* essential oil for Contact toxicity against houseflies.

Table A10.1: Tests for Differences of Means of LD_{50} values from Contact toxicity testing of *C. lusitanica* EO against houseflies after 6-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD50 - B-LD50	-0.228	0.155	(-0.857, 0.402)	-1.47	0.526
K-LD50 - B-LD50	-0.361	0.155	(-0.990, 0.269)	-2.33	0.233
DDVP-6 - B-LD50	-0.627	0.155	(-1.257, 0.002)	-4.06	0.051
K-LD50 - H-LD50	-0.133	0.155	(-0.763, 0.497)	-0.86	0.825
DDVP-6 - H-LD50	-0.400	0.155	(-1.029, 0.230)	-2.59	0.183
DDVP-6 - K-LD50	-0.267	0.155	(-0.896, 0.363)	-1.72	0.418

B-LD_{50}, H-LD_{50}, K-LD_{50} and DDVP represent the topical lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30, P<0.05 meant significant difference of LD_{50} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A10.2: Tests for Differences of Means of LD_{90} values from Contact toxicity testing of *C. lusitanica* EO against houseflies after 6-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD90 - B-LD90	-253.0	56.4	(-482.9, -23.1)	-4.48	0.037
K-LD90 - B-LD90	-514.9	56.4	(-744.8, -285.0)	-9.12	0.003
DDVP-6 - B-LD90	-705.2	56.4	(-935.1, -475.3)	-12.49	0.001
K-LD90 - H-LD90	-261.9	56.4	(-491.8, -32.0)	-4.64	0.033
DDVP-6 - H-LD90	-452.2	56.4	(-682.1, -222.3)	-8.01	0.005
DDVP-6 - K-LD90	-190.3	56.4	(-420.2, 39.6)	-3.37	0.089

B-LD_{90}, H-LD_{90}, K-LD_{90} and DDVP represent the topical lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30. P<0.05 meant significant difference of LD_{90} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A10.3: Tests for Differences of Means of LD_{50} values from Contact toxicity testing of *C. lusitanica* EO against houseflies after 12-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD50 - B-LD50	-0.1578	0.0275	(-0.2459, -0.0696)	-5.73	0.002
K-LD50 - B-LD50	-0.2213	0.0275	(-0.3094, -0.1331)	-8.04	0.000
DDVP-12 - B-LD50	-0.3319	0.0275	(-0.4200, -0.2437)	-12.06	0.000
K-LD50 - H-LD50	-0.0635	0.0275	(-0.1516, 0.0246)	-2.31	0.175
DDVP-12 - H-LD50	-0.1741	0.0275	(-0.2622, -0.0860)	-6.33	0.001
DDVP-12 - K-LD50	-0.1106	0.0275	(-0.1987, -0.0225)	-4.02	0.016

B-LD_{50}, H-LD_{50}, K-LD_{50} and DDVP represent the topical lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30, P<0.05 meant significant difference of LD_{50} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A10.4: Tests for Differences of Means of LD_{90} values from Contact toxicity testing of *C. lusitanica* EO against houseflies after 12-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD90 - B-LD90	-48.5	35.5	(-193.1, 96.1)	-1.37	0.576
K-LD90 - B-LD90	-139.4	35.5	(-284.0, 5.3)	-3.92	0.056
DDVP-12 - B-LD90	-175.9	35.5	(-320.5, -31.2)	-4.95	0.026
K-LD90 - H-LD90	-90.9	35.5	(-235.5, 53.7)	-2.56	0.188
DDVP-12 - H-LD90	-127.4	35.5	(-272.0, 17.2)	-3.59	0.074
DDVP-12 - K-LD90	-36.5	35.5	(-181.1, 108.1)	-1.03	0.745

B-LD_{90}, H-LD_{90}, K-LD_{90} and DDVP represent the topical lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30. P<0.05 meant significant difference of LD_{90} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A10.5: Tests for Differences of Means of LD_{50} values from Contact toxicity testing of *C. lusitanica* EO against houseflies after 24-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD50 - B-LD50	-0.0880	0.0432	(-0.2263, 0.0503)	-2.04	0.251
K-LD50 - B-LD50	-0.1557	0.0432	(-0.2940, -0.0174)	-3.61	0.028
DDVP-24 - B-LD50	-0.2324	0.0432	(-0.3707, -0.0941)	-5.38	0.003
K-LD50 - H-LD50	-0.0677	0.0432	(-0.2060, 0.0706)	-1.57	0.446
DDVP-24 - H-LD50	-0.1444	0.0432	(-0.2827, -0.0061)	-3.35	0.041
DDVP-24 - K-LD50	-0.0767	0.0432	(-0.2150, 0.0616)	-1.78	0.349

B-LD_{50}, H-LD_{50}, K-LD_{50} and DDVP represent the topical lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30, P<0.05 meant significant difference of LD_{50} values (Tukey's HSD test, One-way ANOVA, Minitab 17).

Table A10.6: Tests for Differences of Means of LD_{90} values from Contact toxicity testing of *C. lusitanica* EO against houseflies after 24-h post treatment

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
H-LD90 - B-LD90	-51.5	16.3	(-117.8, 14.8)	-3.16	0.108
K-LD90 - B-LD90	-57.2	16.3	(-123.5, 9.1)	-3.51	0.079
DDVP-24 - B-LD90	-73.3	16.3	(-139.6, -7.0)	-4.50	0.036
K-LD90 - H-LD90	-5.8	16.3	(-72.1, 60.6)	-0.35	0.983
DDVP-24 - H-LD90	-21.8	16.3	(-88.1, 44.5)	-1.34	0.588
DDVP-24 - K-LD90	-16.1	16.3	(-82.4, 50.2)	-0.99	0.765

B-LD_{90}, H-LD_{90}, K-LD_{90} and DDVP represent the topical lethal doses of essential oil from Burera, Huye and Kayonza, and Dichlorvos insecticide (Dimethyl 2, 2-dichlorovinyl phosphate, DDVP) against houseflies; respectively. SE: standard error of means of three replicates, N=30. P<0.05 meant significant difference of LD_{90} values (Tukey's HSD test, One-way ANOVA, Minitab 17).