Bioactivity of Essential Oils of *Thymus serrulatus* and *Thymus schimperi* from Ethiopia: Hepatoprotective, Dental Caries Protective, Mosquitocidal and Acute Oral Toxicity

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GRADUATE PROGRAMMES

This is to certify that the Dissertation prepared by Destaw Damtie Yehualaw, entitled*:* Bioactivity of Essential Oils of *Thymus serrulatus* and *Thymus schimperi* from Ethiopia: Hepatoprotective, Dental Caries Protective, Mosquitocidal and Toxicity and submitted in fulfillment of the Requirements for the Degree of Doctor of Philosophy (Biology: Biomedical Sciences) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

This study was conducted to investigate; the ethnobotanical information, chemical composition, bioassays (antibacterial, mosquitocidal, larvicidal, and oviposition deterrent, hepatoprotective) and acute oral toxicity of the essential oils (EOs) of *Thymus* species collected from six localities in Ethiopia. Ethnobotanical information was collected using semistructured questionnaires, antibacterial test using disk diffusion technique, mosquitocidal activity using fumigation test, hepatoprotective activity in male Wistar rats and acute oral toxicity in female albino mice. *Thymus* species collected from Ofla (Ofl), Alamata (Ala), and Yilmana Densa (Yil) were identified as *Thymus serrulatus* and those collected from Tarmaber (Tar), Butajira (Buta), and Bale (Bal) as *Thymus schimperi*. Both species which are endemic to Ethiopia are traditionally used to treat different illnesses like blood pressure, general pain syndrome, liver diseases, influenza, abdominal pain, and against intestinal parasites. The major compounds in Ofl EO were thymol (49.55%), carvacrol (36.34%), and p-cymene (3.06%). In Ala EO, thymol was the dominant component (65.63%) followed by carvacrol (6.68%) and thymol methyl ether (6.55%). Yil EO on the other hand had carvacrol (80.84%), thymol (6.52%), and p-cymene (3.65) as its major components. Tar was the EO with thymol (48.84%), carvacrol (42.12%), and linalool (2.97%) as its major components. In the same way, the major components of Buta EO were (71.83%), thymol (15.77%), and p-cymene (3.75%). The predominant components of the last EO, Bal were thymol (53.57%), carvacrol (34.55%), and p-cymene (3.20%). Four of the EOs (Ofl, Ala, Tar, and Bal) were found to be thymol and the rest two (Yil and Buta) carvacrol chemotypes. All the essential oils inhibited cariogenic bacteria (*Streptococcus mutans* and *Lactobacillus*). The minimum inhibitory

concentration (MIC) and minimum bactericidal concentration (MBC) of the Bal EO against *S. mutans* was found to be 0.25 µL/mL and the MIC/MBC of all the rest EOs against this bacterium was 0.5 µL/mL. On the other hand, the MIC and MBC of all the EOs against *Lactobacillus* was at the dose of 0.5 µL/mL. Paracetamol-induced hepatotoxicity in Wistar rats was prevented by the application of EOs at 200 µL/Kg body weight. This was demonstrated by the reduced serum levels of marker enzymes; alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). In addition, it was seen from the histopathological tests that *Thymus* EOs prevented paracetamol-induced necrosis. *Thymus* EOs too showed larvicidal, mosquitocidal (fumigation test) and oviposition detterent activities against the mosquito *Anophelus arabiensis*. The essential oil concentrations that resulted in mortality of 50% of larvae (LD_{50}) were: (60.75 µL/L, Ala); (44 µL/L, Yil); and (41.75 µL/L, Tar). The LD_{50} values for the fumigation test, on the other hand, were: (17.19 µL/L, Ala); (14.92 µL/L, Yil); and (13.20 µL/L, Tar). The 200 µL/L, 100 µL/L, and 50 µL/L doses of Tar; the 200 µL/L and 100 µL/L doses of Ala; and the 200 µL/L dose of Yil resulted in complete oviposition detterent activity. Except some irritation responses, all the test EOs were found to be non-toxic to mice and had LD_{50} values in the range of 2000µL/kg body weight to 5000µL/kg body weight with corrected acute toxicity point (LD50) estimate of 2500µL/kg body weight. In conclusion, the different chemotypes of *T. serrulatus* and *T. shimperi* EOs resulted in antibacterial activities, hepatoprotective activities, mosquitocidal activities and were not toxic.

Key words: Bioactivity, Toxicity, *Thymus serrulatus*, *Thymus schimperi*, Hepatoprotective, Caries causing (cariogenic) bacteria, Mosquitocidal.

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List of abbreviations and acronyms

%ER – Percent Effective Repellency

AChE - Acetylcholinesterase

AFRO -World Health Organization Regional Office for Africa

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

APAP - Paracetamol (Paracetamol)

ASA - Acetyl Salicylic Acid

ASE - Accelerated Solvent Extraction

AST – Aspartate aminotransferase

CAM -Complementary and Alternative Medicine

CAT - Catalase

CCE - Counter-Current Extraction

CCl_4 - Carbon Tetrachloride

$CDCl_3$ - deuterated chloroform

CHD - Comparison between classical hydrodistillation

CID -Controlled instantaneous decomposition

COX-2 - Cyclooxygenase-2

CYP450 - Cytochrome P450

DAP - Diastolic Atrial Pressure

DAs - Development Agents

DMFT - Decayed, Missing and Filled Teeth score

DMSO - dimethylsulfoxide

DT - Distillation time

EO –Essential Oil

EPA - United States Environmental Protection Agency

EPHI - Ethiopian Public Health Institute

ESCMID - European Society of Clinical Microbiology and Infectious Diseases

Eto - evapotranspiration

EUCAST -European Committee for Antimicrobial Susceptibility Testing

FAO -Food and Agriculture Organization of the United Nations

GC-MS – Gas chromatography Mass Spectrophotometry

GHS - Globally Harmonized System of Classification and Labeling of Chemicals

GRAS - Generally Recognised As Safe

GSH – Glutathione

KI - Kovat's Index

LD – Lethal Dose

LMW - Low-Molecular-Weight

MAE - Microwave Assisted Extraction

MAPs - Medicinal and Aromatic Plants

MBC – Minimum Bactericidal Concentration

MDGs – Millenium Development Goals

MHD - Microwave hydrodistillation

MIC - Minimum Inhibitory Concentration

MRS agar - de Man, Rogosa and Sharpe agar

MS agar - Mitis Salivarius Agar

NAPQI - N-acetyl-p-benzoquinoimine

NMR – Nuclear Magnetic Resonance

OAI - Oviposition Activity Index

OM – Outer Membrane

PCV- Packed Cell Volume

PLE - Pressurized Liquid Extraction

RAPD - Randomized Amplified Polymorphic DNA

RCTs - Randomized Controlled Clinical Trials

SAP - Systolic Atrial Pressure

SDE -Simultaneous distillation extraction

SFAs - Saturated Fatty Acids

SFE - Supercritical Fluid Extraction

SNNPR -Southern Nations, Nationalities, and Peoples' Regional state

SOD - Superoxide Dismutase

SPME -Solid phase micro-extraction

T-80 - tween 80

TEO – *Thymus* Essential Oil

TM - Traditional Medicine

UFAs - Unsaturated Fatty Acids

WHO- World Health Organization

Chapter 1- Introduction

Chapter 1: Introduction

1.1. Background

1.1.1. Traditional medicine

Traditional and/or complementary and alternative medicine (majorly of plant sources) are in use by developing and developed nations respectively to fulfill parts of their primary healthcare systems. The majority of healthcare systems in Ethiopia too are dependent on the use of herbal medicines (Endashaw Bekele, 2007; Kalayu Mesfin *et al.,* 2013).

The term "Traditional medicine" (TM) is a broad term including knowledge, skills, and practices based on the theories, beliefs, and experiences native to various cultures. They are used for maintenance of health and for prevention, diagnosis, improvement or treatment of physical and mental illnesses (WHO, 2013). TMs include both medication and non-medication therapies. The former involves the use of herbal medicines, animal parts, and/or minerals and the later do not use medication and includes practices like traditional Chinese Acupuncture, Indian Ayurveda, Arabic unani medicine, manual therapies and spiritual therapies (WHO, 2002). TMs are also called homeopathic or conventional medicines if they are parts of a country's own traditional medicine and are completely incorporated into the dominant health care system.

We call traditional medicines "complementary" or "alternative" or "nonconventional" medicines if; (1) they are not part of a country's own traditional medicine and are not completely incorporated into the dominant health-care system, (2) the dominant health care system of a country is based on allopathic (modern) medicine and traditional

medicine is not incorporated into the national health care system (WHO, 2002; WHO, 2013).

Generally, “traditional medicine” is used when referring to Africa, Latin America, South-East Asia, and/or the Western Pacific, whereas “complementary and alternative medicine (CAM)” is used when referring to Europe, North America, and Australia. When referring in a general sense to all of these regions, the comprehensive TM/CAM is used (WHO, 2002).

Of the traditional medicines, herbal medicines (medicinal plants) have been utilized all through mankind's history starting from the beginning of life on earth. Usually, there is no sharp dividing line separating plant uses as food or drug. Thus, the edible plants have been used as sources of both food and medicine in different cultures (Halberstein, 2005).

Through his long history of evolution, man has identified and classified plants suitable for use in fulfilling life requirements like disease prevention and treatment. The evolution of plant-based medicine resulted in the development of the presently well-known conventional medicines native to India, China, Tibet, North America, Amazonia, and local systems within Africa (Mamedov, 2012). Irrespecive of their ancient nature, medicinal plants are reported by the World Health Organization (WHO) as they are still in use by a huge number of the population of most developing countries (ftp://ftp.fao.org/docrep/fao/008/af285e/af285e00.pdf).

The term “herbal medicine” incorporates herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients (WHO, 2002). Irrespective to the

rise and advancement of synthetic chemistry and biotechnology, the therapeutic and preventive role of plants is still fundamental. A lot of species are perceived as having medicinal value, and many of them are usually used to treat and prevent particular illnesses and diseases (Srivastava *et al.,* 1996) especially in developing countries (Indrakumar *et al.,* 2012).

Surprizingly, 62 – 80% of the world's population is still dependent on traditional medicines for the treatment of common illness and more than 50% of all modern clinical drugs are of natural origin (Indrakumar *et al.,* 2012). Around 25% of the active compounds in currently manufactured medications were initially from plant sources (Halberstein, 2005).

By the year 2002, up to 80% of the African populations were using TMs to meet their primary healthcare needs and 90% of the Ethiopian population was dependent on traditional medicine followed by the population of Benin (80%), Rwanda (70%), Tanzania (60%), and Uganda (60%) (WHO, 2002). According to recent reports, about 80% of Ethiopian population is still depending on traditional medicine to treat different types of human ailments (Kalayu Mesfin *et al.,* 2013). The health of around 90% of livestock population in Ethiopia is also dependent on medicinal plants (Endashaw Bekele, 2007).

1.1.2. History of medicinal plant use

Plants were considered as the main sources of natural products to sustain human health till the nineteenth century when the German chemist Friedrich Wöhler accidentally synthesized urea in 1828. This situation began the time of synthetic compounds and drugs (Mendonça-Filho, 2006).

Man may have discovered the curative properties of certain plants; through instinct (Mendonça-Filho, 2006), trial and error (Banso and Adeyemo, 2006), spiritual learning, and watching how primates and different creatures use those plants (Mamedov, 2012).

Once the first traditional healers recognized plants for therapeutic purposes, they protected and passed their understandings about medicinal plants from generation to generation through oral conventions and learnings (Mamedov, 2012). Many of the Ethiopian people believe that the skill of traditional health practitioners is to be 'given by God' and this knowledge passes orally from father to a favorite child, usually a son or is acquired by some spiritual procedures (Kebede Deribe *et al.*, 2006).

As mentioned above, man had interest on medicinal plants starting from the time of immemorial and every civilization has a history of medicinal plant use (Banso and Adeyemo, 2006; Sundaram *et al.*, 2011; Lakshmi and Reddy, 2012). Archeological findings reveal that the history of the use of medicinal plants dates back thousands of years ago. The ancient Egyptians were using medicinal plants through the years 3000 to 6000 back. The ancient Greece through 1500 to 3000 years back; China, India, and Tibet

through 1000 to 2000 years back; and healers in the Aztec and Maya Indian cultures of Mexico and Central America around 1000 years ago (Halberstein, 2005).

Writen manuscripts were other ways of transferring knowledge of medicinal plants to the coming generations.The ancient Egyptians, Greeks, Indians, Chinese, Arabs, the Jewish and explorers have passed knowledge about medicinal plants to generations through their manuscripts. Some of these manuscripts are; the 5000 years old manuscript found on a Sumerian clay slab from Nagpur (India), the Chinese book "Pen T'Sao," written around 2500 BC,the Indian holy books Vedas, the Ebers Papyrus dating to 1550 BC, the Bible and the sacred Jewish book the Talmud (Petrovska, 2012).

Furthermore, different writers have mentioned about and listed medicinal plants in their manuscripts. Examples are: Homer in his stories "The Iliad and The Odysseys" (800 BC); Herodotus (500 BC); Hippocrates (459–370 BC); Theophrastus (371-287 BC); Celsus (25 BC–50 AD); Dioscorides (40 – 90 AD); and the Roman physician and pharmacist, Galen (131–200 AD) (Petrovska, 2012).

In the seventh century (AD) the Slavic people used plants as medications, insecticides, and poisons in hunting (e.g. *Aconitum napellus*). In the Middle Ages (5^{th} to the 15^{th} century) (http://en.wikipedia.org/wiki/Middle_Ages), people in monasteries took the responsibility to cultivate medicinal plants, prepare drugs, and heal patients. In the same era, the Arabs imported many medicinal plants from India and developed knowledge about the use of these medicinal plants. This knowledge from the Arabs was consulted by

European physicians; John Mesue (850 AD), Avicenna (980-1037), and Ibn Baitar (1197-1248).

Furthermore, expeditions had their own contributions on the transfer of knowledge and medicinal plants from one part of the world to the other. For example, Marco Polo's expeditions (1254-1324) in tropical Asia, China, and Persia; the discovery of America (1492); and Vasco De Gama's trips to India (1498) took medicinal plants to Europe. Consequently, development of botanical gardens and cultivation of medicinal plants originated either from domestic or taken from the new and old worlds expanded in Europe (Petrovska, 2012).

Between 16^{th} and 18^{th} centuries, medicinal plants were used as simple pharmaceutical forms. After this time, the need of compound drugs including animal and plant origin increased. Early nineteenth century acted as a turning point in medicinal plant use history due to the beginning of scientific pharmacy following the detection, verification, and isolation of alkaloids and glycosides from various plants. This time was also known for the development of chemical methods which assisted the discovery of other active substances such as tannins, saponosides, etheric oils, vitamins, hormones, etc. Further in the nineteenth century, use of plant pharmaceuticals was progressively replaced by their pure isolates like therapeutics, alkaloids and glycosides. In the early twentieth century, researchers developed techniques to maintain fresh and labile medicinal plants and their components. Research works were also done on the techniques of manufacturing of herbal medicinal products and cultivation of medicinal plants (Petrovska, 2012).

Presently, people use informal drugs through self-medication or with the prescription of a physician or pharmacist. The application of these drugs is based on knowledge from popular medicine (traditional or folk medicine) or on scientific or experimental results. They are used either independently or in combination with synthetic drugs (complementary medicine). Knowledge about the precise diagnosis of the illness and the pharmacological effect of medicinal plant components is essential for adequate and successfull therapy (Petrovska, 2012).

The use of medicinal plants has persisted as ‘‘treatment of choice’’ irrespective to the increasing use of factory-made synthetic drugs for numerous health problems in populations throughout the world (Halberstein, 2005). Currently, around 7 billion people and about 250,000 plants co-exist on our planet (Mamedov, 2012). Many of these plants have healing properties in addition to their nutritional values (Bhandari, 2009). Around 35,000 to 70,000 species of these plants have been used as remedies and only 17% of them have been scientifically examined for their medicinal potentials. Their chemical and biological diversity makes plants limitless renewable sources for the development of new pharmaceuticals. In today’s global market, tropical plants serve as sources of more than 50% of major drugs (Mamedov, 2012)

The bulk of medicinal plants traded are still wild and only very small number of species are cultivated (Kuipers, 1997). At least four out of every five of those plants are collected from the wild and most of them are from floras of developing countries (Srivastava *et al.*, 1996). Of all the plants on our globe, medicinal and aromatic plants (MAPs) are those

which play significant roles in meeting the demands for traditional medicines (ftp://ftp.fao.org/docrep/fao/008/af285e/af285e00.pdf).

1.1.3. Medicinal value of aromatic plants

Aromatic plants enhance the desirable flavor and aroma of foods and drinks so that humans enjoy eating and drinking. Thanks to the use of herbs in folk medicine, common diseases such as cold and diarrhea couldn't make life miserable. These uses and activities of aromatic plants are owing to their complex mixtures of compounds known as essential oils (EOs) (Sebsebe Demissew and Nigist Asfaw, 1994).

The *Lamiaceae* is one of these aromatic plant families consisting of plants that provide some of the essential ingredients of life mentioned above. In Ethiopia, the family is represented by over 20 genera, *Thymus* and *Ocimum* being the two most known (Sebsebe Demissew and Nigist Asfaw, 1994).

Ethiopia has two representative species of the genus *Thymus*, *T. schimperi* and *T. serrulatus* both of which are endemic to the country (Fichtl and Adi, 1994, Woldemedhin Zebene, 2011). Both species are perennial herbs, woody at the base and 5-40 cm high. The inflorescence is commonly crowded into globose and oblong heads with pink corollas. Their flowers are purplish-pink in terminal heads and the whole plant gives off the characteristic smell of thyme (Fichtl and Adi, 1994).

T. schimperi has ovate to elliptic leaves with entire margins. It is comparatively widespread in central, eastern and northern Ethiopia. Perhaps, it might have been collected by a German traveler, W.G. Schimperi (1837-1878) who collected a lot of plant

specimens from northern Ethiopia (Fekadu Fullas, 2009). It is locally known as *Tosign (Amh) (*Sebsebe Demissew and Nigist Asfaw, 1994).

T. serrulatus has obovate to oblanceolate leaf-laminas with weakly crenate margins and is restricted to northern Ethiopia. It is locally known as *Tesni* or *Thasne (Tig)* and *Tosign (Amh)* (Sebsebe Demissew and Nigist Asfaw, 1994).

1.1.4. Medicinal value of *Thymus* species in Ethiopia

In traditional medicine, *Thymus* species in Ethiopia are used to treat different illnesses like gonorrhea, cough, liver disease (Kunert, 2000); Gara Bokoyso (Oromipha) (stomach pain) (Haile Yineger *et al.*, 2008); hypertension (Nurya Abdurhman, 2010; Parvez and Yadav, 2010); Kidney problem (Behailu Etana, 2010; Parvez and Yadav, 2010); dermal fungi (Nigussie Doni *et al.*, 2012); *Tinea capitis* (Parvez and Yadav, 2010); and abdominal cramps (Andemariam, 2010). Research works also revealed the antihelimenthic (Jemal Hussien *et al.*, 2011); antibacterial and fungicidal activities of *T. schimperi* (Chewaka Tura, 2009; Mohammed Nasir, 2010; Pagiotti *et al.*, 2010; Shewaye Lakew, 2011). The boiled leaves are drunk to treat the illnesses mentioned (Sebsebe Demissew and Nigist Asfaw, 1994).

1.1.5. Distribution of *Thymus* species in Ethiopia

The two endemic *Thymus* species in Ethiopia are distributed in Ethiopian highlands growing on edges of roads, in open grassland, on bare rocks and on slopes, between 2200-4000 m above sea level (Sebsebe Demissew and Nigist Asfaw, 1994).

T. serrulatus species are found in Northern Ethiopia especially Tigray [Degua Temben (Mahbere Silassie), Alamata, and Ofla districts (weredas)] (Atey G/Medhin, 2008) and Yilmana Densa Wereda of the Amhara Region (Collected and identified for the sake of this research). On the other hand, *T. schimperi* is widely distributed in Amhara, Oromia, and Southern Nations Nationalities and Peoples Region. It is found in Denkoro Forest (Debresina Wereda of South Wello) (Abate Ayalew, 2003), Chancho (Chewaka Tura, 2009), Ankober (Nigussie Doni *et al.,* 2012), Menz Gera Midir (Guassa) (GAGMP, 2007), and Tarmaber weredas of North Shewa as well as Bure Wereda of West Gojjam (Tessega Belie, 2009) and Gondar areas (Nigist Asfaw *et al.,* 2000).

In Oromia Region *T. schimperi* is found in Adaba Dodola area (Kunert, 2000), Dinsho (Ermias Dagne *et al.,* 1998; Ermias Lulekal *et al.,* 2011), and Sanetti Mountains (Bussmann *et al.,* 2011; Tariku Mekonnen *et al.,* 2011) (Bale Zone). *T. schimperi* in Oromia Region is also found in Goma (Behailu Etana, 2010), Asendabo (Parvez and Yadav, 2010), and areas around Jimma (Jemal Hussien *et al.,* 2011) (Jimma Zone) as well as Debre Zeyit (Mohammed Nasir, 2010), Awash National Park (Tinsae Bahru *et al.,* 2010), and Menagesha Suba State Forest (Abate Zewdie, 2007).

1.2. Hypothesis and objectives

1.2.1. Hypothesis

EOs of *T. serrulatus* and *T. shimperi* have bioactivity and therapeutic potentials in that they demonstrate antibacterial property, hepatoprotective activity, mosquitocidal activity, and are not toxic.

1.2.2. Objectives

General objective

To investigate the dental caries protective, hepatoprotective, and mosquitocidal activities as well as toxicity of the different chemotypes of *Thymus serrulatus* and *Thymus schimperi* EOs.

Specific objectives

- To document the ethno-botanical information of *T. serrulatus* and *T. shimperi* from the dominantly growing areas in Ethiopia.
- To determine the chemotypes of *T. serrulatus* and *T. schimperi* essential oils.
- To test the *in vitro* antibacterial activities of *T. serrulatus* and *T. shimperi* essential oils against dental caries causing bacteria (oral *Streptococci* and *Lactobacilli*).
- To evaluate hepatoprotective effect of *T. serrulatus* and *T. shimperi* essential oils in laboratory-bred Wistar rats.
- To test the mosquitocidal activities of *T. serrulatus* and *T. shimperi* essential oils against *An. arabiensis*.

- To evaluate the acute oral toxicity of *T. serrulatus* and *T. shimperi* essential oils in laboratory-bred mice.

General guide to the dissertation content

- The citations and references of Ethiopian Names are written without initials (e.g. Nigist Asfaw *et al.,* 2000, not Asfaw *et al.,* 2000) due to the department's rule and lack of family names with the Ethiopian naming system.
- The chemical composition, antibacterial and acute oral toxicity tests were done for the six EOs (Ofl, Ala, Yil, Tar, Buta, and Bal)
- The hepatoprotective and mosquitocidal activities were done only for three of the EOs (Ala, Yil, and Tar). These EOs were selected because they were significantly different in their chemotypes. Ala was found to be strongly thymol chemotype (thymol = 65.63% and carvacrol =6.68%), Yil was strongly carvacrol chemotype (thymol = 6.52% and carvacrol =80.84%), and Tar was thymol chemotype but with close proportions of thymol and carvacrol (thymol = 48.84% and carvacrol =42.12%).
- **Chapter 2** deals with literature review, **Chapter 3** deals with the materials and methods section, **Chapter 4** with the results section, and **Chaptor 5** with the discussion, conclusion, and recommendation sections.

Chapter 2 - Literature Review

Chapter 2: Literature review

A large proportion of the higher plants are able to produce EOs, which are parts of SMs. Around 17,500 aromatic plant species of higher plants are known to produce EOs. Of the 3,000 EOs known, about 300 are commercially important for pharmaceuticals, cosmetics and perfume industries and have pesticidal potentials (Tripathi *et al.,* 2009). Plant families with capability of producing EOs include *Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae* and *Piperaceae* (Tripathi *et al.,* 2009). These families may possess EOs in one or more of their different parts such as flowers, leaves, bark, wood, roots, seeds, fruits, rhizomes and gums or oleoresin exudations (Handa, 2008).

2.1. Lamiaceae

2.1.1. Overview

Lamiaceae also called the mint family are among the most diverse plant families with cosmopolitan distribution (Pistelli, 2006; Said-Al Ahl and Abdou, 2009; Derakhshani *et al.*, 2012; Çali, 2014). This family is particularly well represented in the temperate regions of the world, such as the Mediterranean region and in tropical upland savannas (Pistelli, 2006). The *Lamiaceae* is a large plant family represented by about 236 genera and above 7200 species (Hedberg *et al.,* 2006).

Many members of the *Lamiaceae* family are widely cultivated owing to their aromatic qualities, their ease of cultivation, for their edible leaves, and for their decorative foliages (Venkateshappa and Sreenath, 2013).

Many species of plants belonging to the family *Lamiaceae* are highly aromatic, due to the presence of external glandular structures that produce volatile oils (Venkateshappa and Sreenath, 2013), terpenoids, iridoid glycosides, flavonoids, and other phenolic compounds (Leitão *et al.,* 2009; Derakhshani *et al.,* 2012).

This characteristic of plants belonging to the family *Lamiaceae* makes them to be used as culinary herbs, folk medicines, fragrant scents, in pharmaceutical industries, as flavorings, cosmetics, perfumery and medical preparations (Özdemir and Senel, 1999; Boz *et al.,* 2009; Gairola *et al.,* 2009; Conti *et al.,* 2010). That is why they were widely utilized by human culture for centauries (Quinn *et al.,* 2007). The complex mixtures of components in their EOs are responsible for their diverse bioactivities. Many biologically active EO components have been isolated from various members of the family *Lamiaceae* (Said-Al Ahl and Abdou, 2009) like thyme and oreganum (Ro¨sch *et al.,* 1999).

The major genera under the *Lamiaceae* are: Salvia (900 species), Scutellaria (360 species), Coleus (325 species), Plectranthus (300 species), Hyptis (280 species), Teucrium (250 species), *Thymus* (220 species) and Nepeta (200 species) (Venkateshappa and Sreenath, 2013). Among the aromatic plants belonging to the family *Lamiaceae*, the genus *Thymus* is noteworthy for the numerous species and varieties of wild-growing plants (Boz *et al.,* 2009) and it is discussed in the coming section.

2.1.2. The genus *Thymus*

The genus *Thymus* is one of the genera under family *Lamiaceae* and Subfamily *Nepetoidae* (Sonboli *et al.,* 2013). This genus is known for its numerous species and varieties of wild-growing plants (Boz *et al.,* 2009; Küçükbay *et al.,* 2014). However, the classification of species under this genus remains difficult due to interspecific hybridization, polyploidy (2n = 28, 30, 56, 60), and morphological similarities among species (Ali *et al.,* 2010). This genus is estimated to contain around 400 species (De Martino *et al.,* 2009) with many subspecies, varieties and forms. Species of the genus *Thymus* are native to the Mediterranean region and are found distributed in Europe, Asia, North Africa, and the Canary Islands (Boz *et al.,* 2009; Leal *et al.,* 2012).

They got their name (*Thymus*) probably from the Latin "thymus" which means "perfumed", or from Greek "thymos" which means "courage" or "strength" (Leal *et al.,* 2012). That was why the Roman soldiers bathed in thyme, as this was believed to provide vigor (Basch *et al.,* 2004). Morphologically, the species under the genus *Thymus* are characterized by: stems (woody, narrow or wiry); leaves (evergreen, oppositely arranged pairs, oval, entire, small, 4–20 mm long, and usually aromatic); flowers (dense terminal heads, with an uneven calyx, with the upper lip three-lobed, yellow, white or purple); and heights ranging from 20 to 50 cm (De Martino *et al.,* 2009; Leal *et al.,* 2012; Giweli, 2013).

The genus has been subdivided into eight sections: *Hyphodromi, Mastichina, Micantes, Piperella, Pseudothymbra, Serpyllum, Thymus* and *Teucrioides* (Nascimento *et al.,* 2000;

Leal *et al.*, 2012; Giweli, 2013). One of the subsections, subsection *Kotschiani* under the oldest section *Serpyllum* is represented in Ethiopia by two *Thymus* species; *T. serrulatus* and *T. schimperi* both of which are endemic to the country (Jaafari e*t al.*, 2007). *T. schimperi* Ronninger and *T. serrulatus* Hotsch.ex Benth locally called *Tosign* (Amharic) and *Tesni/Thasne* (Tigrigna) respectively are endemic to Ethiopian highlands (Sebsebe Demissew and Nigist Asfaw, 1994) and are restricted to the afromontane and afroalpine regions of the country (Nigist Asfaw *et al.*, 2000; IBC, 2007; IBC, 2008). Both indigenous Ethiopian species are perennial herbs, woody at the base and 5-40 cm high (Sebsebe Demissew and Nigist Asfaw, 1994; Mekonnen Biru, 2003; Abate Zewdie, 2007; Behailu Etana, 2010; Nurya Abdurhman, 2010; Tinsae Bahru *et al.*, 2010). The inflorescence of both species is commonly crowded into globose and possesses oblong heads with pink corollas (Sebsebe Demissew and Nigist Asfaw, 1994).

Thyme species in different parts of the world are used in different forms and for different purposes. They are used in the forms of essential oils, oleoresins, fresh and dried herbs (De Martino *et al.*, 2009; Giweli, 2013; Moradi *et al.*, 2014) as culinary herbs, beverage (tea), food flavors (spices and condiments), confectionery products, as well as in perfumery for the scenting of soaps and lotions (De Martino *et al.*, 2009; Arzani *et al.*, 2013; Giweli, 2013). They too are used as herbal medicines (De Martino *et al.*, 2009; Arzani *et al.*, 2013; Giweli, 2013; Sonboli *et al.*, 2013; Moradi *et al.*, 2014) due to their carminative, food digestive, antispasmodic, antitussive, expectorant, sedative, antiseptic, antiphlogistic, antimicrobial (antibacterial, antiviral, antifungal) and antioxidant

properties (De Martino *et al.,* 2009; Arzani *et al.,* 2013; Giweli, 2013; Sonboli *et al.,* 2013; Moradi *et al.,* 2014).

The different *Thymus* species are known for various biological activities. This is due to the fact that they posses complex mixtures of active constituents. The majority (nearly 90% of total oil) of *Thymus* EOs are characterized by a considerable amount of monoterpenes. Thymol and carvacrol (phenolic monoterpenes) always accompanied by the p-cymene/γ-terpinene are the four monoterpenes which are considered biogenetically. Linalool, 1, 8-cineole and borneol are the other oxygenated monoterpenes, in order of importance singled out in *Thymus* EOs (Tabrizi *et al.,* 2010; Giweli, 2013). More than 20 EO chemotypes were noticed in different species of *Thymus* genus. Thus, most aspects of medicinal use of *Thymus* spp. are related to their essential oil composition, which shows various levels of thymol and/or carvacrol, phenolic derivatives with strong and wide-spectrum antimicrobial activities (De Martino *et al.,* 2009). Essential oils are parts of secondary metabolites. Thus, it is important to discuss about primary and secondary metabolites. These metabolites are described in the following section.

2.2. Plant metabolites: primary vs secondary

Primary metabolites are those which are produced to perform essential functions like growth and development and therefore exist in all plant types. But secondary metabolites have specific functions, such as pollinator attraction or defense against herbivory and hence they are differently distributed in the plant kingdom. Secondary metabolites are often colored, fragrant, or flavorful compounds and they typically mediate the interaction of plants with other organisms like plant-pollinators, plant-pathogens, and plant-herbivores (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

2.2.1. Primary metabolites

Primary metabolites contain many different types of organic compounds such as carbohydrates, lipids, proteins, and nucleic acids. They are synthesized through metabolic pathways or cycles namely glycolysis, the Krebs cycle, and the Calvin cycle. Thus, they are found universally in the plant kingdom. They play essential roles for plants like (i) energy sources (e.g. sucrose, starch), (ii) structural components (e.g. cellulose), (iii) informational molecules (e.g. DNA and RNA), and (iv) pigments (e.g.chlorophyll). In addition to having fundamental roles in plant growth and development, some primary metabolites serve as starting materials for the synthesis of secondary metabolites (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

2.2.2. Secondary metabolites (SMs)

SMs were firstly detected in 1891 (Edreva *et al.*, 2008). It is only since the late twentieth century that SMs have been clearly recognized as having important functions in plants. It is expected to isolate at least a million different compounds from all plant species (Gunatilaka, 2012). Till now, many thousands of SMs have been isolated from plants, and many of them have powerful physiological effects in humans and are used as medicines (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html). Some of these SMs from plants are tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides and volatile oils (Banso and Adeyemo, 2006).

Since SMs do not directly involve in plant growth and development, plants can survive without particular secondary metabolites especially when grown under protection in

green houses (Wink, 2004). Rather, plants use SMs for survival through modifying their ecological interactions with other organisms namely herbivores, microorganisms, and competing plants (Wink, 2004; Gunatilaka, 2012). Other SMs too serve as UV-protection or as signal compounds (Wink, 2004). The SMs are synthesized from primary metabolites through various metabolic pathways like acetate, mevalonate, and shikimate pathways (Gunatilaka, 2012).

2.3. Classes of secondary metabolites

2.3.1. **Alkaloids**

Alkaloids are a group of nitrogen-containing compounds found in about 20 percent of flowering plants. They are synthesized from primary metabolites like amino acids such as tryptophan, tyrosine, and lysine. A great number of the alkaloids act as intense nerve poisons, enzyme inhibitors, or membrane transport inhibitors. Their bitter or generally bad taste also makes them as detterrents to animals which are able to learn to avoid eating such plants (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

Medicinal Alkaloids: Alkaloids can be used as medicines if their doses are carefully regulated. Morphine, codeine and cocaine are alkaloids which act on the nervous system and serve as pain killers. Atropine is another alkaloid which acts on the nervous system and is used in anesthesia and ophthalmology. Vincristine and vinblastine are alkaloids with anticancer activities that inhibit cell division and are used to treat cancers of the blood and lymphatic systems (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

Quinine is used as an antimalarial compound due to its toxicity to the *Plasmodium* parasite. Other alkaloids like caffeine, components of tea and cola plants, and nicotine are used as stimulants. Nicotine is also a very potent insecticide. But, its use as an insecticide is not accepted in recent years due to its toxicity to humans and the environment (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

2.3.2. Terpenoids

Terpenes, also known as terpenoids or isoprenoids when they contain oxygen, are the largest group of natural compounds, with over 30, 000 known structures. They got the name 'terpene' after isolating the first described members of this class from turpentine, the monoterpene-rich liquid obtained from the resin of various *Pinus* spp in the 1850s (Croteau *et al.,* 2000; Carson and Hammer, 2011). Terpenoids are derived from acetyl coenzyme A or from intermediates in glycolysis. They are classified by the number of five-carbon isoprenoid units they contain.

Isoprene Rule and Terpene Biosynthesis: Traditionally, terpenes have been regarded as polymers of isoprene (C_5H_8) joined together in a repetitive head-to-tail manner. This is called the isoprene rule which first was proposed by the German chemist Otto Wallach. However, combinations of isoprene subunits other than head-to-tail such as head-to-head, tail-to-tail and head-to-middle are known to occur (Figure 1) (Carson and Hammer, 2011).

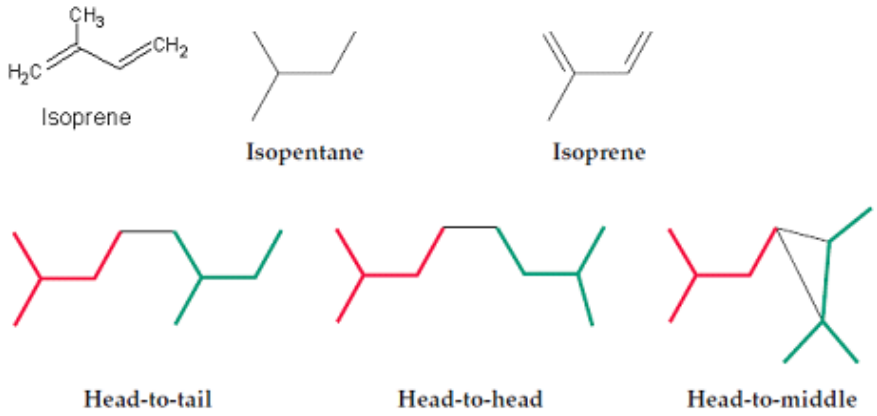


Figure 1: Biosynthesis of terpenoids from C_5 units (Source: Croteau *et al.*, 2000).

The upper panel (Figure 1) shows the structures of the isopentane skeleton and isoprene gas. The lower panel shows how different patterns of isoprene unit assembly yield a variety of different structures.

Classification of terpenes

Most natural terpenes hydrocarbons have the general formula $(C_5H_8)_n$. They can be classified on the basis of value of n or number of carbon atoms present in the structure (Table 1). Each class can further be subdivided as acyclic, or as mono-, bi-, tri-, and tetracyclic based on the numbers of rings present in its structure (Yadav *et al.*, 2014).

Table 1: Classes of terpenes based on the number of isoprene subunits they contain

Number of carbon atoms	Value of n	Class
5	1	Hemiterpenes(C_5H_8)
10	2	Monoterpenes ($C_{10}H_{16}$)
15	3	Sesquiterpenes ($C_{15}H_{24}$)
20	4	Diterpenes ($C_{20}H_{32}$)
25	5	Sesterpenes ($C_{25}H_{40}$)
30	6	Triterpenes ($C_{30}H_{48}$)
40	8	Tetraterpenes ($C_{40}H_{64}$)
>40	>8	Polyterpenes $(C_5H_8)_n$

(Source: Yadav *et al.,* 2014)

2.3.2.1. Hemiterpenes

Hemiterpenes (half-terpenes) are the smallest terpenes containing a single isoprene unit. The best known hemiterpene is isoprene itself, a volatile product released from photosynthetically active tissues. It is produced by the enzyme isoprene synthase which is present in the leaf plastids of numerous C_3 plant species (Croteau *et al.,* 2000). There are many hemiterpines other than isoprene like α-angelicalactone, angelic acid, and isovaleric acid (http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_4_1_1).

2.3.2.2. Monoterpenes

These are terpenes made up of ten carbon atoms or two isoprene units. They are best known as components of the volatile essences of flowers and of the EOs of herbs and spices, in which they make up as much as 5% of plant dry weight. Monoterpenes are isolated by either distillation or extraction and are used in flavor and perfume industries (Croteau *et al.,* 2000). They fatally affect the nervous systems of insects while being biodegradable and nontoxic to mammals including humans (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

Monoterpenes exist in acyclic or cyclic forms examples of which are presented in Figures 2-4.

A. Acylic Monoterpenes

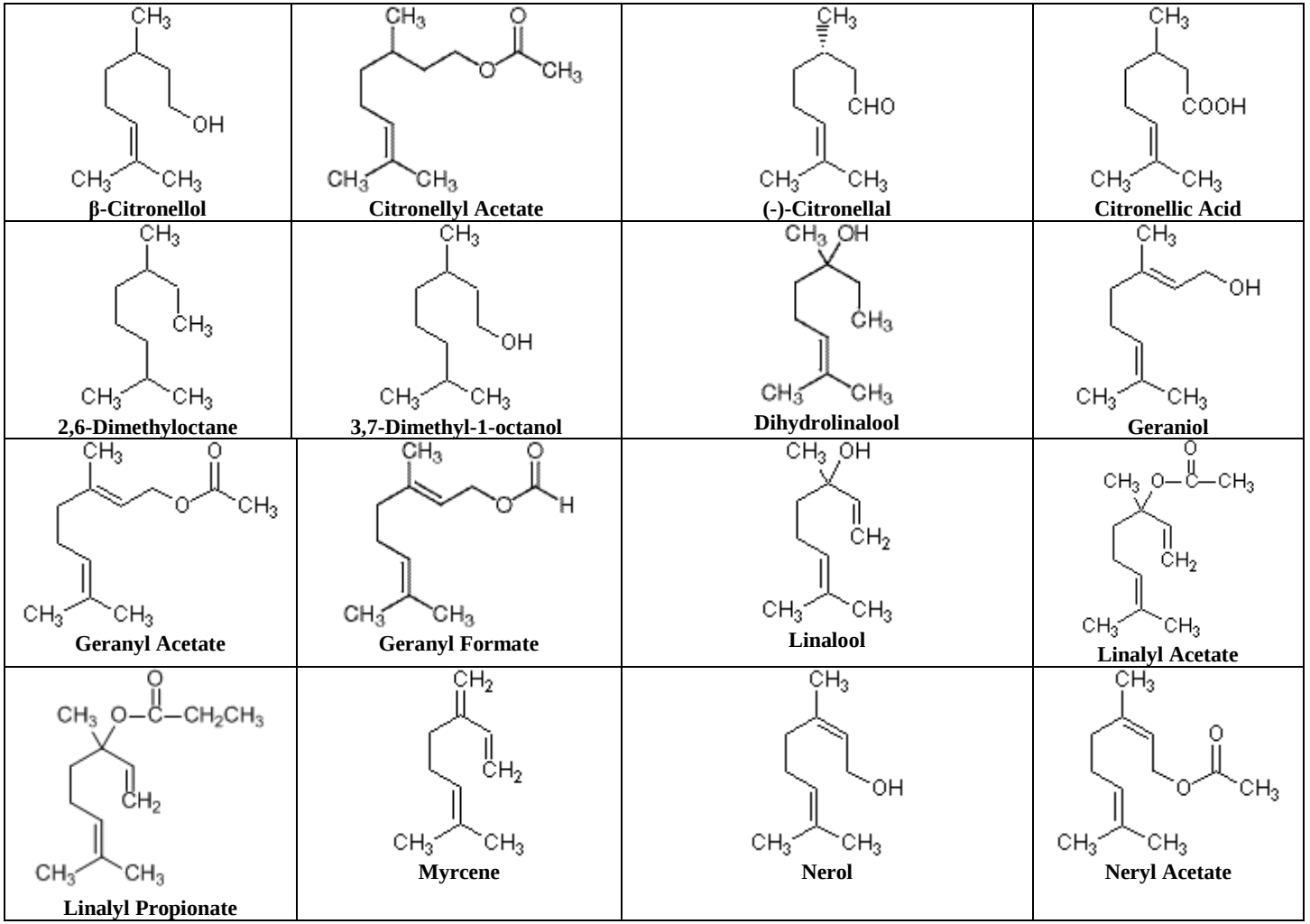


Figure 2: List of acyclic monoterpenes (Source: http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_4_1_1)

B. Monocyclic Monoterpenes

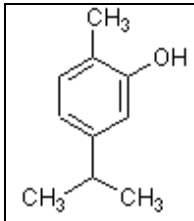
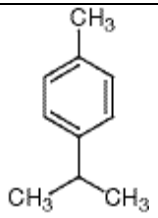
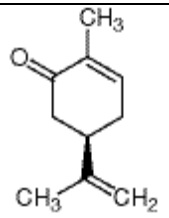
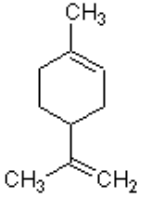
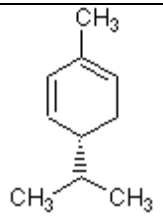
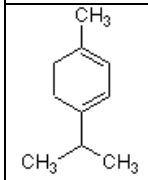
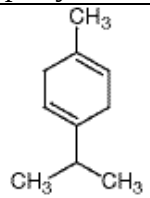
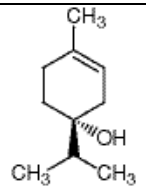
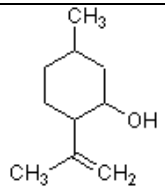
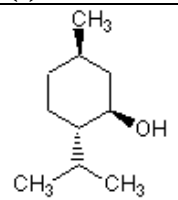
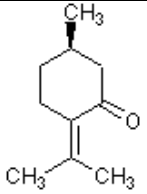
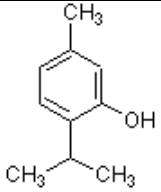
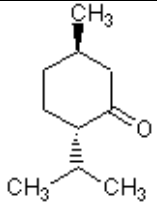
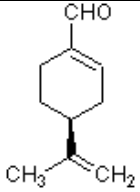
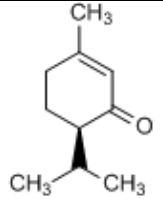
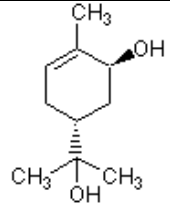
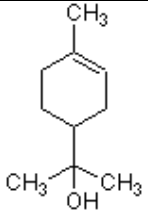
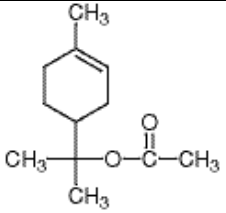
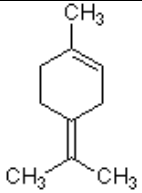
 Carvacrol	 *p*-Cymene	 Carvone	 (±)-Limonene	 (-)-α-Phellandrene
 α-Terpinene	 γ-Terpinene	 (-)-Terpinen-4-ol	 Isopulegol	 (±)-Menthol
 (+)-Pulegone	 Thymol	 (-)-Menthone	 (-)-Perillaldehyde	 (-)-Piperitone
 *trans*-Sobrerol	 α-Terpineol	 Terpinyl Acetate	 Terpinolene	

Figure 3: List of Monocyclic monoterpenes (Source: http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_4_1_1)

C. Bicyclic Monoterpenes

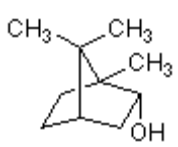
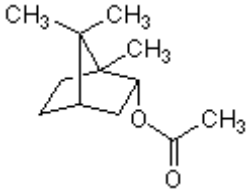
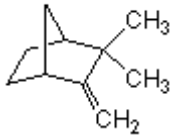
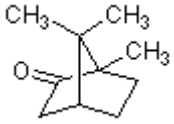
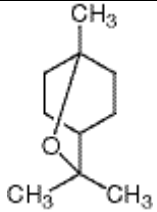
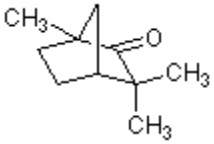
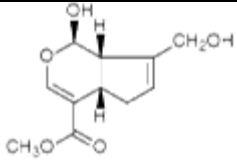
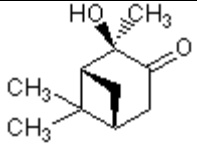
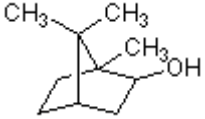
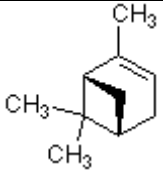
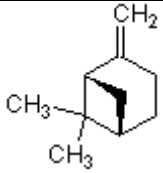
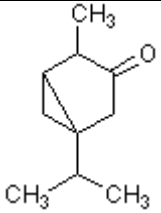
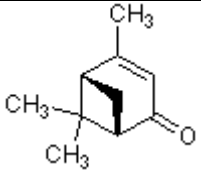
 Borneol	 Bornyl acetate	 (±)-Camphene	 (±)-Camphor	 1,8-Cineole
 (+)-Fenchone	 Genipin	 (1*R*,2*R*,5*R*)-(+)-2-Hydroxy-3-pinanone	 (±)-Isoborneol	 α-Pinene
 β-Pinene	 Thujone (α- and β-)	 (-)-Verbenone		

Figure 4: List of Bicyclic monoterpenes (Source: http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_4_1_1)

2.3.2.3. Sesquiterpenes

Sesquiterpenes are terpenoids derived from three isoprene units and containing 15 carbon atoms (i.e. made up of one and one-half terpenes). Like monoterpenes, many sesquiterpenes are found in essential oils. In addition, numerous sesquiterpenoids act as phytoalexins, antibiotic compounds produced by plants in response to microbial challenge, and as antifeedants that discourage opportunistic herbivory (Croteau *et al.,* 2000). Among the many known sesquiterpenes, some examples are presented in Figure 5.

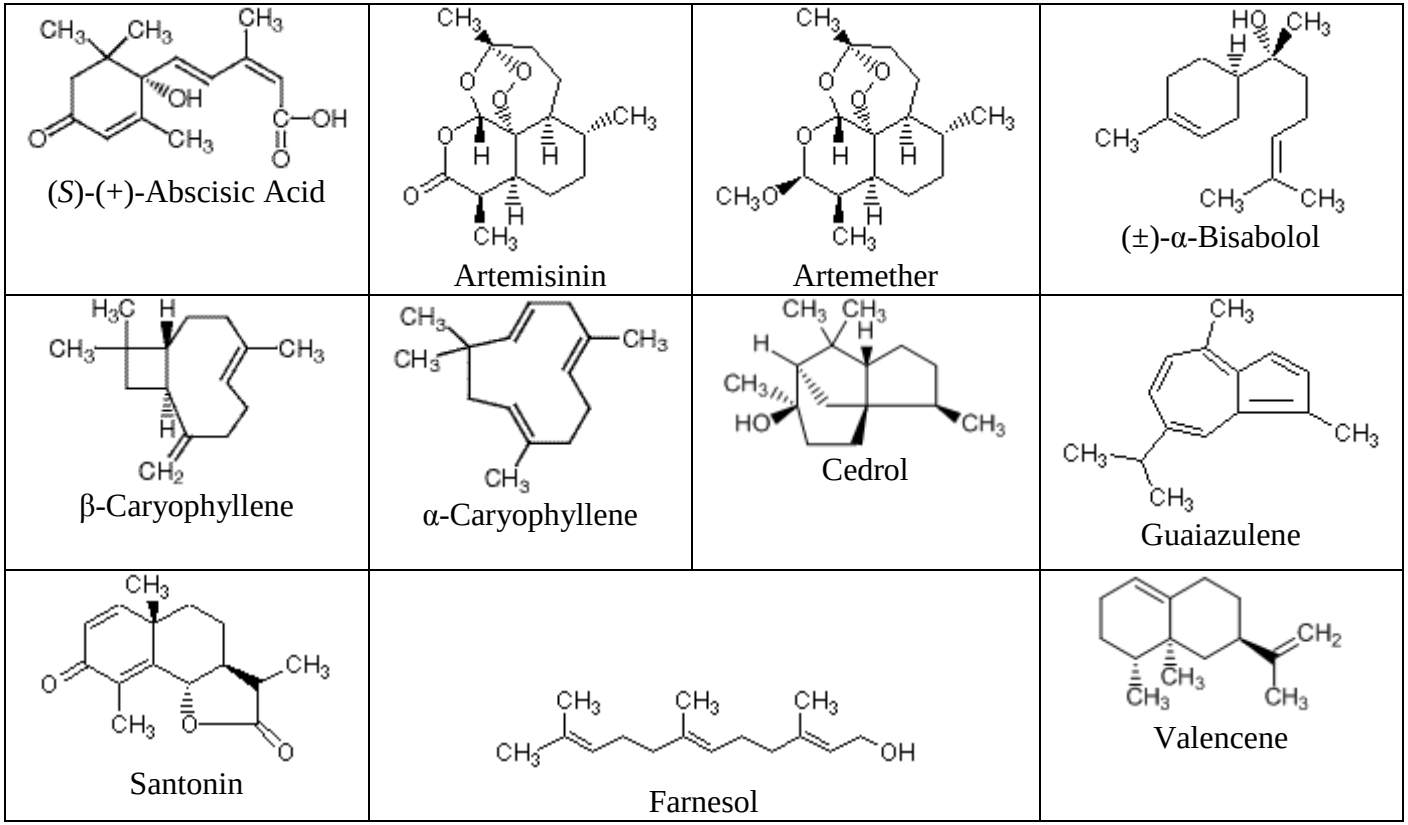


Figure 5: List of sesquiterpenes in natural compounds.

(Source: http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_4_1_1)

2.3.2.4. Diterpenes

The diterpenes, which contain 20 carbons (four C_5 units), include phytol (the hydrophobic side chain of chlorophyll), the gibberellin hormones, the resin acids of conifer and legume species, phytoalexins, and a host of pharmacologically important metabolites, including taxol, an anticancer agent found at very low concentrations (0.01% dry weight) in yew bark, and forskolin, a compound used to treat glaucoma. Some gibberellins have only 19 carbon atoms and are considered norditerpenoids since they have lost 1 carbon through a metabolic cleavage reaction (Croteau *et al.,* 2000). Paclitaxel (commonly known by the brand name Taxol), a diterpene found in bark of the Pacific yew tree, is a potent inhibitor of cell division in animals. At the end of the twentieth century, paclitaxel was developed as a powerful new chemotherapeutic treatment for people with solid tumors, such as ovarian cancer patients (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html). See Figure 6 for more examples of diterpenes.

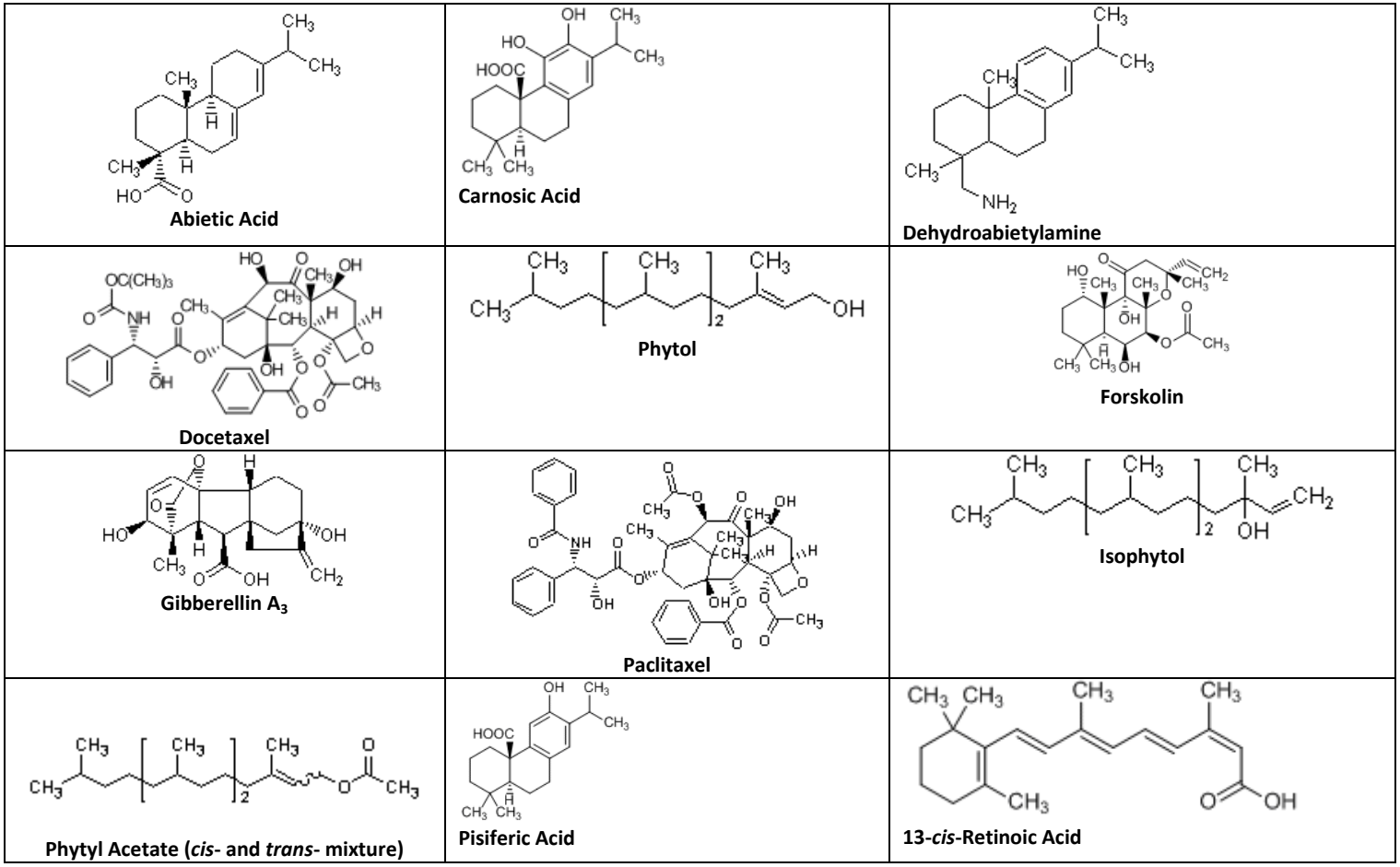


Figure 6: List of diterpenes in natural compounds (Source:

http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_4_1_1)

2.3.2.5. Triterpenes

The triterpenes, which contain 30 carbon atoms (formed from six C_5 units), are generated by the head-to-head joining of two C_{15} chains, each of which constitutes three isoprene units joined head-to-tail. This large class of molecules includes the brassinosteroids, the phytosterol membrane components, certain phytoalexins, various toxins and feeding deterrents, and components of surface waxes, such as oleanolic acid of grapes (Croteau *et al.*, 2000).

They too comprise of the plant steroids, some of which act as plant hormones. These also can protect plants from insect attack, though their mode of action is quite different from that of the pyrethroids. For example, the phytoecdysones are a group of plant sterols that resemble insect molting hormones. When ingested in excess, phytoecdysones can disrupt the normal molting cycle with often lethal consequences to the insect (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

Additional examples can be seen from Figure 7.

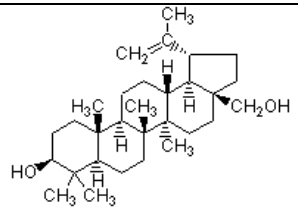
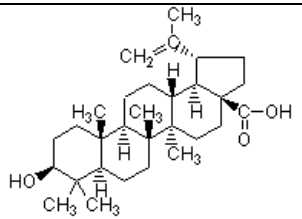
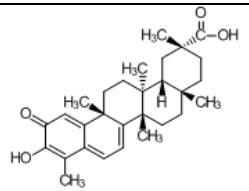
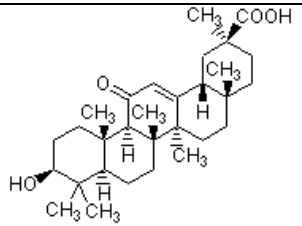
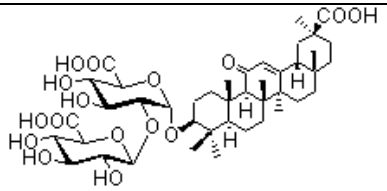
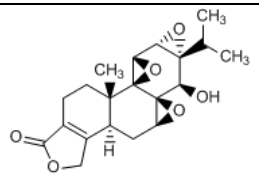
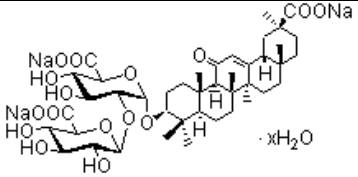
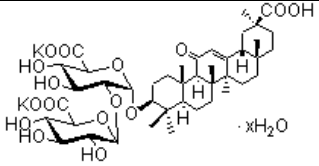
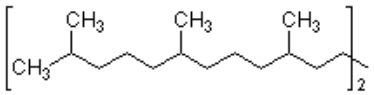
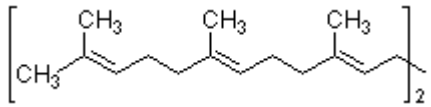
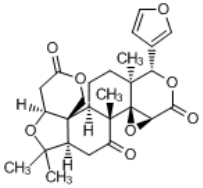
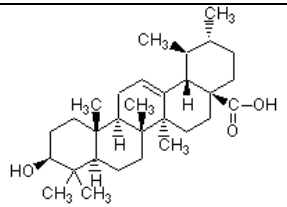
 Betulinol	 Betulinic Acid	 Celastrol
 Glycyrrhetic Acid	 Glycyrrhizin	 Triptolide
 Glycyrrhizin Trisodium Salt Hydrate	 Glycyrrhizin Dipotassium Salt Hydrate	 Squalane
 Squalene	 Limonin	 Ursolic Acid

Figure 7: List of triterpenes in natural compounds (Source:

http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_4_1_1)

2.3.2.6. Tetraterpenes and polyterpenes

The most prevalent tetraterpenes (40 carbons, eight isoprene units) are the carotenoid accessory pigments which perform essential functions in photosynthesis (Croteau *et al.*, 2000). Rather than functioning in plant defense, the colored pigments that accumulate in ripening fruits can serve as attractants to animals, which actually aid the plant in seed dispersal (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html). The polyterpenes, those containing more than eight isoprene units, include the prenylated quinone electron carriers (plastoquinone and ubiquinone), long-chain polyprenols involved in sugar transfer reactions (e.g., dolichol), and enormously long polymers such as rubber (Croteau *et al.,* 2000). The polyterpenes are polymers that may contain several thousand isoprenoid units. Rubber, a polyterpene in the latex of rubber trees that probably aids in wound healing in the plant, is also very important for the manufacture of tires and other products (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html). Look at Figure 8 for more examples of tetraterpenes and polyterpenes.

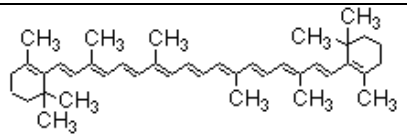
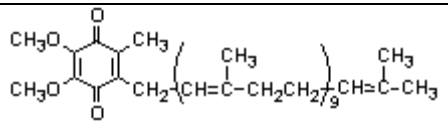
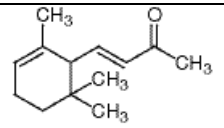
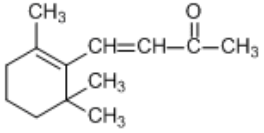
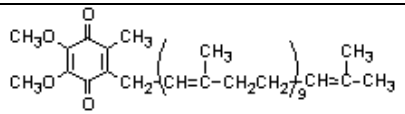
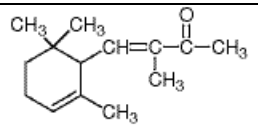
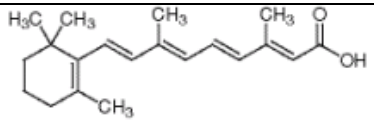
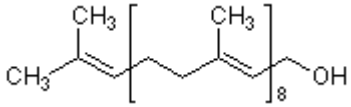
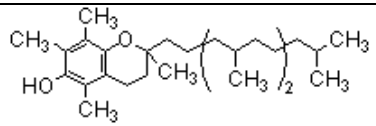
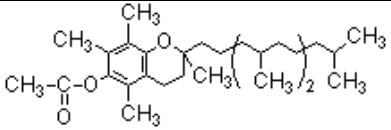
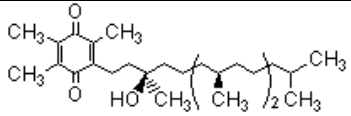
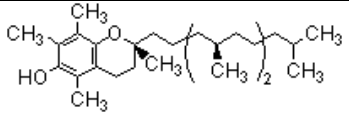
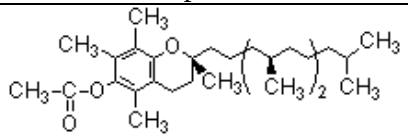
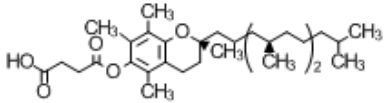
 β-Carotene	 Coenzyme Q_{10}	 α-Ionone
 β-Ionone	 Methylionone (mixture of α- and β-, predominantly α-*n*-isomer)	 α-*iso*-Methylionone
 Retinoic Acid	 Solanesol	 DL-α-Tocopherol
 DL-α-Tocopherol Acetate	 D-α-Tocopherylquinone	 D-α-Tocopherol
 D-α-Tocopherol Acetate	 D-α-Tocopherol Succinate	

Figure 8: Some representative examples of tetraterpenes and polyterpenes in natural compounds (Source:

http://www.tcichemicals.com/eshop/en/us/category_index/00306/#innerlink_12_1_1)

2.3.3. Phenolic compounds

Phenolic compounds are defined by the presence of one or more aromatic rings bearing a hydroxyl functional group. Many are synthesized from the amino acid phenylalanine. One of such phenolic acids is salicylic acid which is important in defense against fungal pathogens. That is why its concentration increases in the leaves of certain plants in response to fungal attack. Aspirin, a derivative of salicylic acid, is used by humans to reduce inflammation, pain, and fever. Isoflavones are other phenolic compunds that are synthesized rapidly by the legume family in response to bacterial or fungal attack and have strong antimicrobial activity (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

Another complex phenolic molecule is lignin which is laid down in plant secondary cell walls so that it is the main component of wood. It is an important structural molecule common to all woody plants and allows them to achieve height, girth, and longevity. In addition to being a structural component, lignin is important for plant defense. It makes plants less palatable for insects and other animals and less digestible by fungal enzymes (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

Phenolic pigmens which are parts of flowers and fruits act as attractants for pollinators and seed dispersers. Anthocyanins and anthocyanidins are among such phenolic pigments that give pink and purple colors to flowers and fruits (http://lifeofplant.blogspot.de/2011/03/metabolites-primary-vs-secondary.html).

2.4. Essential oils

2.4.2. Description

EOs are very complex mixtures of several aroma chemicals, mostly monoterpenes, sesquiterpenes and their oxygenated derivatives. It is estimated that there are more than 1000 monoterpene and 3000 sesquiterpene structures. Oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters, ethers, ketones, phenols, oxides and superoxides (Singh *et al.,* 2011). In certain plants, such as basil and thyme one main constituent may predominate. However, the odour, flavour and possibly the biological activity of oil can be influenced by the presence of trace components, even those yet unidentified (Svoboda and Hampson, 1999).

In addition to the monoterpenes and sesquiterpenes, EOs possess phenylpropenes and specific compounds containing sulphur or nitrogen which are characterized by strong odor, usually lower density than water, and used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phytotherapy, spices and nutrition (Tripathi *et al.,* 2009).

Production of EOs by plants is believed to be primarily as a defence mechanism. Currently, the interest to use EOs and their components is increasing because of their relatively safe status, wide acceptance by consumers and their multifunctions (Afolayan and Ashafa, 2009). The following section presents the bioactivities of EOs and their components.

2.4.3. Bioactivities of EOs

An EO is a natural, 100% pure oil extract of aromatic plants (flowers, buds, leaves, peel, bark, roots, or seeds) or organs of certain animals that secrete aromatic components. They are extracted using distillation or other methods (www.jetro.go.jp/ext_images/mexico/mercadeo/2Eessential.pdf).

Their mixed functional groups and complex structures make EOs efficient antimicrobial drugs against drug resistant microbes. So they are used as alternative medicines owing to their least residual effect in the body in addition to their high lethality against pathogens (Upadhyay, 2010). People take EOs in three ways; (i) diffuse them aromatically, (ii) apply them topically, or (iii) take internally as dietary supplements (http://www.sd43.bc.ca/elementary/birchland/Parents/PAC/General%20Information%20%20Notices/doTerra%20newsletter%20pg%201%20of%202.pdf).

EOs are naturally safe and have few, if any, undesirable side effects. But cautions should be taken since they are powerfully concentrated. One should never use them in the eyes or inside the ear canals. If redness or irritation occurs when using EOs topically, simply apply a vegetable oil such as fractionated coconut oil to the affected area—water will not dilute them (www.doterratools.com/documents/ProductCatalog.pdf). Vegetable oils on the other hand can solubilize the EOs and remove them from the irritated body parts.

2.4.3.1. Chemical components of *Thymus* EOs and their determinant factors

Each constituent of EOs contributes to the beneficial or adverse effects of these oils. Therefore, intimate knowledge of EO composition allows for a better and specially directed application. Considering all the aforementioned differences in EO composition, it is clear that only a detailed knowledge of the constituents of an EO will lead to a proper use in cosmetics by perfumers and cosmetic chemists. However, such a detailed knowledge can only be obtained by means of carefully performed capillary-GC experiments (Lahlou, 2004).

2.4.3.1.1. Overview of the Chemical Composition of *Thymus* EOs

Various chromatographic techniques have been applied to identify the different components of *Thymus* EOs and revealed fluctuations in the composition of the volatile oils depending on the chemotype under consideration (WHO, 1999). Reports recently published about the composition and biological properties of *Thymus* EOs emphasize the existence of marked chemical differences among oils extracted from different species or varieties (De Martino *et al.,* 2009). Moreover, most aspects of medicinal use of *Thymus* spp. are related to their EO composition, which shows various levels of thymol and/or carvacrol, phenolic derivatives with strong and wide-spectrum antimicrobial activity. The principal components of *Thymus* EOs are thymol and carvacrol (up to 64% of oils) (Iraj, 2005; Alekseeva, 2009), along with linalool, *p*-cymol, cymene, thymene, α-pinene and many others (WHO, 1999). Overall, more than 20 EO chemotypes were noticed in different species of *Thymus* genus. These differences in chemotypes can be noticed in species grown in the same habitat which makes the study of this genus interesting (De

Martino *et al.,* 2009). This chemical diversity indirectly can influence the biological activity of the oils and it is generally a function of three factors: genetic, physiological and environmental conditions (De Martino *et al.,* 2009).

2.4.3.1.2. Major Chemical Components of *Thymus* Species

The chemical composition of different *Thymus* species is determined by a lot of factors. Thus differences appear even within the same species. *Thymus* species can be called as different chemotypes based on their most prominent chemical components. Differences or similarities in chemotypes can happen either within the same or different *Thymus* species. For example different research works on the chemical composition of *T. vulgaris* showed different chemotypes: Thymol type (Sharafzadeh *et al.,* 2010), Camphor type (Imelouane *et al.,* 2009), and terpinen-4-ol type (Viuda-Martos *et al.,* 2007). Similarly, *T.daenensis* was found to be thymol (Leila *et al.,* 2008) and carvacrol (Sfaei-Ghomi *et al.,* 2009) chemotypes. On the other hand, different *Thymus* species can be found to have similar chemotypes. For example *T.daenensis, T.persicus, T.satureoides* and *T.transcaspicus* were carvacrol chemotypes (Jaafari *et al.,* 2007; Sfaei-Ghomi *et al.,* 2009; Tabrizi *et al.,* 2010), *T.vulgaris, T.eriocalyx, T.zygis,* and *T.daenensis* were thymol chemotypes (Jaafari *et al.,* 2007; Leila *et al.,* 2008; Sfaei-Ghomi *et al.,* 2009; Grigore *et al.,* 2010).

2.4.3.1.3. Determinants of the Yield, Chemical Diversity and Bioactivities of the EOs in *Thymus* Species

EOs contain numerous aromatic chemicals, the relative proportions of which are usually characteristic of a given genus but may vary significantly depending on the plant species, its geographical source and the environmental conditions associated with its growth, harvesting and predistillation handling (Singh *et al.,* 2011). In addition, all biological raw materials show qualitative and quantitative differences in their chemical composition on account of annual variations, different soils, solar radiation, rain and fertilizing. Furthermore, each species consists of various subspecies or hybrids with a specific chemical composition (Rosch *et al.,* 1999).

Generally, the yield and composition of EOs in plants can be influenced by various factors. These fctors may include; climatic, seasonal conditions, geographic conditions, harvest period, distillation technique, plant maturity level at the time of oil production, chemotypic differences, and others in addition to genetic factors (Lahlou, 2004).

These factors affect the biosynthesis of volatile oils (Khadhri *et al.,* 2011). Thus secondary metabolites (volatile oils) in *Thymus* species have ecological advantages and can be associated with adaptations to the environment and interactive competitions with other plants. They also serve as chemical defense mechanisms against herbivors and plant pathogens. There is evidence indicating the importance of the diversity of monoterpenes as an adaptation strategy to different environments (Tabrizi *et al.,* 2010). Determinants of secondary metabolite (EO) yield and composition are indirectly detrminants of the

biological activities of these EOs. Those factors affecting the yield and composition of EOs are treated separately in the following sections.

A. Factors Affecting EO yield and Composition

EO yield and composition of medicinal plants varies based on different plant growing, cultivating, drying, and extracting conditions next to their genetic makeup. The impact of each of these factors is discussed in the coming pages.

Genetics: Genetics is the first factor which determines EO yield and composition. This is evidenced by the test of Echeverrigaray *et al.* (2001). They found correlations between oil compositions and Randomized Amplified Polymorphic DNA (RAPD) analysis of genetic makeup among six commercial thyme (*T. vulgaris* L.) cultivars. EOs as parts of SMs are variously distributed in the plant kingdom and are specific to the plant group where they are found (Gunatilaka, 2012) which in turn is influenced by their genetic make up.

Altitude where plants grow: Elevation where the plants grow also determines yield, concentration, and bioactivity of EOs from plants. For example in the work by Martínez *et al.,* (2005), volatile components of *T.hyemalis* L. obtained from high altitude cultivation area contained a volatile profile rich in low molecular weight components and phenolic compounds when compared to those obtained from low altitude cultivation areas (Zuzarte *et al.,* 2011b). This is due to the reason that low molecular weight components with low boiling points are found more at lower temperature areas than higher temperature areas. When plants grow at high altitude, temperature decreases and consequently the low molecular weight components of EOs increases.On the other hand

when such plants grow at low altitude, temperature is relatively higher and evaporates off the low boiling compounds and leaves the ones with high mol wt and thus high boiling ones.

Habit of plant growth: The habit of the plant whether it is wild or cultivated may also affect the composition and biological activities of EOs as was shown by the wok of Tabrizi *et al.* (2010) where thyme from natural habitats contained high EO yield and composition than the cultivated ones.In the study mentioned, the sum of thymol and carvacrol from the wild and the cultivated thyme were 58.1% and 51.8 % respectively.

Nutrients in the soil and fertilization: This may also have influence on yield and composition of *Thymus* EOs. For example thymol concentration was found to be positively correlated with humus and Na, K, Mg, and Cd contents of the soil (Tabrizi *et al.,* 2010). A research work on the influence of nitrogen fertilizers on the yield and composition of thyme (*T.vulgaris*) showed that nitrogen fertilizers increased thyme crop, but differences in the yield of EO were not remarkable and only slight changes in the percentage composition were detected after drying (Baranauskiene *et al.,* 2003).

However, it was speculated by Duke (2009) that fertilizing plants would lower their percentage of secondary metabolites and increase their percentage of primary metabolites. As can be seen from reseach works, organic fertilizers may result in better yields of EOs than inorganic (NPK= 20-20-20) fertilizers (Janmohammadi *et al.*, 2014). On the other hand, application of biofertilizers (nitrogen fixing bacteria) on Ajwan (*Carum copticum*) resulted in increased biological yield, seed yield, EO content and EO

yield (Ghilavizadeh *et al.*, 2013). The addition of phosphorus fertilizers was also found to affect EO content of basil (*Ocimum basilicum* L.) (Ramezani *et al.,* 2009).

The trend of irrigation or watering: This plays roles on the yield and composition of EOs. In a study by Pirzad *et al.,* (2006), differences in EO percentage were seen in German Chamomile (*Matricaria chamomilla* L.) at different irrigation schemes (55, 70, 85, and 100% field capacities) and the highest amount of EO percentage was obtained at 85% field capacity and lowest at 55 and 100% field capacities. Water stress could affect the terpenoid level in the tissue of *Thymus* species. For example, during dry periods of the year, the phenolic monoterpenes thymol and carvacrol were found to be the main components of the EOs *of T. serrulatus;* however, during wet periods, an acyclic monoterpene, linalool, was the main component of the EO (Inderjit and Moral, 1997).

Stress: Plants lack motility and immune system so that they need alternative defense stratagies against changing environments and stressing constraints (Edreva *et al.*, 2008). Thus they produce secondary metabolites to adapt to both biotic and abiotic stress situations and servive (Edreva *et al.,* 2008; Duke, 2009). Biosynthesis and yield of EOs increases with increases in stressing situations like; salt stress (Khadhri *et al.,* 2011), pruning (Manhães *et al.,* 2012), water stress (Khalid, 2006), plant density (Ghilavizadeh *et al.,* 2013), wounding or herbivore and microbial attack, plant-plant competetion, UV-radiation (Edreva *et al.,* 2008) and others. Drought stress can be a major factor in increasing concentration of secondary plant products (Moradi *et al.,* 2014).

Plant part used for EO extraction: EOs from different parts of plants may have different compositions and so different bioactivities. For example, the EOs obtained from

the fruits and leaves of *Citrus aurantium* var. *amara* Link (Rutaceae) showed different mollucicidal activities against *B. truncatus* snails (Lahlou, 2004). In the same way, the leaf extracts of *T. capitatus* L. (*Lamiaceae*) showed stronger antibacterial activities than their stem extracts (Qaralleh *et al.*, 2009).

Differences in bioactivities of the EOs extracted from different organs of the same plant were also seen in EOs from *Juniperus oxycedrus* subsp. *oxycedrus* where EOs from leaves were more effective than those from berries; *Cupressus lusitana* oils obtained from leaves were significantly more active than those from fruits; and leaf oils of *Vitex-agnus castus* showed a higher antifungal activity than flower and fruit oils (Zuzarte *et al.*, 2011b). In the same way, oil obtained from leaves of *Citrus aurantium* var. *amara* Link (Rutaceae) against *B. truncatus* snails at tested concentrations exhibited less potent molluscicidal activity than the fruit oil (Lahlou, 2004).

In a study on *T. vulgaris*, the EO yield from the young leaves was higher than that from the old leaves. At the same time, the components of the EOs from the young leaves were different from EOs from the old leaves. The major components from the young leaves were thymol, carvacrol, p-cymene, terpenolene, β-caryophyllene, γ-terpinene, and linalool in decreasing order and EOs from the old leaves were thymol, p-cymene, γ-terpinene, carvacrol, terpenolene, linalool and β-caryophyllene in decreasing order (Sharafzadeh *et al.*, 2011). These differences in EO compositions of the different parts of plants have impacts on the bioactivities of the EOs. Moreover, the leaf/stem proportions in the plants to be extracted may affect the yield, composition and bioactivities of EOs (Németh and Bkáe, 2005).

Plant Maturity Level during Harvesting: The developmental stage of the plants during harvesting affects EO yield and composition in plants. For example in a work by Arraiza *et al.* (2009) the average EO yield of *T. vulgaris* L. was the lowest during its vegetative stand (VS, no flowering plants), increased during the initial flowering period (IF, plants start to bloom), reached its highest value during full flowering period (FF, more than 50% flowered plants), and decreased again after flowering (AF, 50% plants have lost their flowers). To summarize, the highest yield of EO was obtained in the period of full flowering and the highest concentration of thymol in the period of initial flowering (Arraiza *et al.,* 2009).In addition, this work revealed variations in the percentage of each major constituent in the oil during all the phenological periods. In another study by Senatore (1996) on *T.pulegioides* L., the best time of harvesting for both EO yield and phenol content, was found to be during or immediately after the full bloom**.** This is indirectly related to plant life time.

In another study, an inverse relationship existed between plant life time and the total amount of oil yield (Nezhadali *et al.,* 2014). Changes in EO accumulation and composition during ontogenesis are characteristic for each taxon. However, in a general pattern, oil accumulation is highest at the beginning of blossoming in some species like peppermint and at full flowering stage in many plant species. In addition, in fruiting species, EO accumulation is highest in ripen fruits compared with the green ones. In *Lamiaceae* species, metabolization of synthesized compounds seems to be responsible for changes in oil composition (Németh and Bkáe, 2005). These ontogenically changes of the

composition may also be affected by other factors like weather, age, and condition of plantation (Németh and Bkáe, 2005).

Furthermore, in the same individual (the same genotype), significant differences in the chemical compositions of EOs occur during one growing season. For example, in a research done on *Salvia officinalis* (*Lamiaceae*), EOs from leaves were three types; young leaves ("yl-oils" = α-humulene type), early old leaves "early-ol-oils" and late old leaves "late-ol-oils" (Lakušić *et al.,* 2013).

A study by Golparvar (2011) recommends 50% blooming as the best harvesting time to gain the highest essence and thymol yield and dry herbage in *T. vulgaris* L. Not only these but also EO yield and composition can be affected by harvesting hours of a single day. In a research work on the yield and chemical composition of the EOs of *T.vulgaris*, maximum oil yield was obtained by harvesting at the early hours of the day which was superior to the oil yield corresponding to the late hours of the day. At the same time, the best harvesting hours of *T. vulgaris* for higher thymol concentrations were between 6:00 Am and 11:00 Am (Kaya *et al.,* 2013).

Seasonal variation during harvesting: Seasonal variation during harvesting is another factor that determines EO composition and yield. The season when plants are harvested affects their yield. For example in the study by Abu-Lafi *el al.,* (2007), the yield of thyme EOs collected during April was lower than that collected during may. The EOs of *T. vulgaris* were found to be richer with oxygenated compounds in spring, followed by summer, autumn and winter (Atti-Santos *et al.,* 2004). This is directly related with plant

maturity level particularly for wild plants. But seasonal variation can be manipulated by humans in the cultivated ones.

Post harvest drying: Post harvest drying also plays major roles on the EO yield and composition of EOs. With increases in time of dryness, yield of EOs generally decreases due to evaporative losses of the EOs. At the same time drying in open air yields better EOs than oven drying. Loss of oil by oven drying increases with increases in temperature and 40°c is the best temperature with minimum oil loss (Mejdoub and Katsiotis, 1998). In another study by Fathi and Sefidkon (2009), the yield of EOs was high after shade drying (3.39%) followed by oven drying at 40°c (2.92%), sun drying (2.66%), oven drying at 30°c (2.59%), and oven drying at 50°c (2.30%) in decreasing order.

EO extraction technique: Different techniques are used to extract EOs. Thus, it is better to understand the influence of each extraction technique on the yield and composition of the EOs. Comparison between classical hydrodistillation (CHD) and Microwave hydrodistillation (MHD) showed that the yield of EOs by MHD is better than that of CHD. In addition, MHD consumes less solvent than CHD (Rmili *et al.*, 2014). In another study, EO yield by hydro-distillation, water and steam distillation and steam distillation were compared by Fathi and Sefidkon (2009) and the EO yield of water distillation (2.39%) was found to be higher than water and steam distillation (2.89%) which in turn was higher than that of steam distillation (1.35%).

Distillation time (DT): is another factor that determines EO yield and composition. Generally, with an increase in duration of time, there is an increase in oil yield. In a study

by Mejdoub and Katsiotis (1998), EO yields of *Eucalyptus citriodora* after 2hrs and 3hrs of hydrodistillation were substantially higher than the yield after 1hr distillation. During hydrodistillation, polar (oxygenated) components of the EOs are distilled first followed by the hydrocarbons and the sesquiterpenes and trace elements at delayed duration of hydrodistillation. At the same time, during increased duration of hydrodistillation, the oxygenated components, which are soluble in water, will be dissolved in the distillation water so that the hydrocarbon and sesquiterpene components of the EO will increase and the oxygenated components will decrease (Mejdoub and Katsiotis, 1998). Different EOs may have their own distillation times (DT) for maximum yield. For example, the maximum yields of peppermint, lemongrass, and palmarosa EOs were at DT between 20 and 40 minutes. However, increases of DT may result in yield decrease. At DT 2F40 min, the yields of lemongrass and palmarosa oil were lower than those from 20 to 160 min. Over-distillation in these oils led to 25 to 40 percent reductions in yield. In the same way differences in EO compositions are also evident with variations in DTs. As a result, knowing the appropriate DTs may help us to extract the needed composition of EOs to meet a specific targeted aroma. For instance, Menthofuran, a component of peppermint, is considered undesirable for some peppermint oil uses, and in that case shorter DTs should be considered to avoid it (Cannon *et al.,* 2013). This is because its concentration can be minimized by extracting for short period of time as it is a high boiling component it needs time to accumulate. Therefore, DT may be used to modify the chemical profile of EOs and to obtain EOs with different chemical profiles from the same plant (Zheljazkov *et al.,* 2013). This may be due to the reason that heating for long period of time may bring about decomposition, oxidation, and hydloysis of the different essential oil components.

B. Factors affecting the biological activities of EOs

The biological activities of EOs are determined majorly by their chemical composition which indirectly is determined by different factors mentioned under 2.4.3.1.3.1 above. In addition to their chemical compostions, the bioactivities of EOs are dependent on various factors like dosage, solvents used to dissolve the EOs before usage, presence or absence of toxic components in the EOs, the techniques of applications of EOs, the biological stages of target organisms and others. Look at the influences of each of these factors on the bioactivities of EOs below.

Interaction between the components of EOs: The bioactivities of EOs may be resulted from the bioactivities of their major components or the interactions of all their components (Zengin and Baysal, 2014). For example in a review of studies by Bassolé and Juliani (2012), 50% of thymol/carvacrol interactions showed additive, more than 33% synergistic, and only 17% antagonistic activities against pathogenic bacteria. The interaction between thymol and eugenol was synergistic against *E.coli*. The interaction between carvacrol and eugenol was synergistic in 50% of the studies and antagonism in other 50% of the studies. Other interactions observed were; synergism of carvacrol and linalool against *Listeria monocytogene*; synergism of Menthol and Geraniol and menthol and thymol against *S. aureus* and *B. cereus.* On the other hand, the interaction between cinnamaldehyde and carvacrol was found to be additive against *E.coli* and *S. typhinurium* and synergistic against *S. typhinurium.* In the same way, the interaction of cinnamaldehyde and thymol was synergism against *E.coli* and *S. typhinurium* against *S.*

typhinurium. Carvacrol and thymol also displayed additive effects against *E.coli* (Burt *et al.,* 2005).

Chemical composition: Bioactivities of EOs are directly related to their chemical compositions. That is the biological activities of EOs may be both due to the major and minor components they contain. Thus all the components may act synergistically to contribute to their total action (Lahlou, 2004). However, the overall activity of the plants is determined by their main compounds: carvacrol and thymol. For example, carvacrol and thymol were found to be responsible for the antifungal activity of *T.pulegioides* and *T.* x *viciosoi* EOs (Zuzarte *et al.,* 2011b). But the activity of minor compounds can not be forgotten. For example δ-3-carene, an exclusive compound of *Juniperus oxycedrus* subsp. *oxycedrus* leaf oils was proved to be fundamental for the higher antifungal activity, although it occurred in low quantities (Zuzarte *et al.,* 2011b).

Dosage: It is important to determine the doses which are effective biologically and less toxic to humans and other nontarget organisms. All substances are poisons so that the right dose differentiates a poison and a remedy (Paracelsus cited in Lahlou, 2004). So concentrations should be choosen in limits already documented in the literature. If an EO is found to be toxic to biological organisms at a certain concentration, other appropriate lower concentrations should be prepared and performed in order to find the minimal toxic concentration (Lahlou, 2004). Since EOs are highly concentrated fluid substances, they are rarely used in an undiluted form. Therefore, they should be blended with carrier oils before application to; (i) dilute them to a safer dose, (ii) slow down the rate of

evaporation, (iii) spread them evenly and (iv) increase their eventual absorption into the skin (Lahlou, 2004).

Solvents used to dissolve EOs: The most useful solvents in laboratory are toxic to biological organisms and consequently interfere with the activity being studied (Lahlou, 2004). Thus the solvents (detergents) like tween 80 (T-80) and dimethylsulfoxide (DMSO) should be used at their lowest solubilizing doses to the EOs (Lahlou, 2004).

Presence of toxic components: The presence/absence of toxic substances in the EOs affects their bioactivities. For example, EOs with thujone components like sage and *Salvia officinalis* are known for their hepatotoxicity and neurotoxicity. In the same way, EOs with pulegone such as *Mentha cervina* and *Calamintha nepeta* are toxic against the liver (Zuzarte *et al.,* 2011b).

The techniques of application of EOs: The techniques of application of EOs on target organisms like microatmoshphere versus direct application may have impacts on their biological activities. For example, the microatmosphere application of the EO of *T.broussonettii* (*Lamiaceae*) was found to be more effective against *Pediculus humanus capitis* (head lice) than its direct application. However, the reverse happened when EOs of *Lavandula stoechas* (*Lamiaceae*), *Chrysanthemum viscidehirtum* (*Asteraceae*), and *Cedrus atlantica* (*Pinaceae*)] were applied to the same parasite (Lahlou, 2004).

The biological stage of target organisms: The biological stage of organisms to which EOs are applied is also a factor that affects their susceptibility to EOs. For example, EOs of *Mentha pulegium* and *Thymus broussonettii* (*Lamiaceae*), *Chenopodium ambrosioides*

(*Chenopodiaceae*), and *Ruta chalepensis* (*Rutaceae*) resulted in a powerful licicidal and niticidal activities towards head lice, *Pediculus humanus capitis* and the EO of *Origanum compactum* (*Lamiaceae*) was found to be active only against nits (Lahlou, 2004).

2.4.3.2. Antibacterial activity of *Thymus* EOs

The increasing occurrence of antimicrobial resistance and adverse side effects of classic antimicrobial drugs results in a growing interest in the antimicrobial screening of extracts and EOs from plants in order to discover new antimicrobial agents. The *Lamiaceae* is one of the most diverse and widespread plant families in terms of ethnomedicine and its medicinal value is mainly based on its volatile oils concentration. It is well documented that some plants belonging to this family possess antimicrobial properties (Niculae *et al.,* 2009).

The genus *Thymus* is one of the plant groups under *Lamiaceae* and is represented by *T. serrulatus* and *T. schimperi* which are endemic to Ethiopia (Fichtl and Adi, 1994; Woldemedhin Zebene, 2011). They are used by people in Ethiopia to treat different illnesses (Chewaka Tura, 2009; Mohammed Nasir, 2010; Pagiotti *et al.,* 2010; Shewaye Lakew, 2011).

These biological activities of thyme EOs is owing to the lipophilic character of the hydrocarbon skeleton of their constituents as well as the hydrophilic character of their functional groups. Antimicrobial activity of EO constituents based on their functional groups can be ranked in decreasing order as: phenols, aldehydes, ketones, alcohols, esters and hydrocarbons (Figueiredo *et al.,* 2008).

Possessing these different functional groups in combination thyme EOs demonstrated antibacterial activities (Kaloustian *et al.,* 2005) towards both Gram-positive and Gram-negative bacteria. For example EO from *T. vulgaris* had antibacterial activities against *Staphylococcus aureus* (Gram-positive), *Salmonella enteritidis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative bacteria) (Niculae *et al.,* 2009). Thyme (herb) and Turmeric (spice) were found to be most effective against *E. coli* (Amrita *et al.,* 2009). Extracts of *T. vulgaris* inhibited *Helicobacter pylori* and *Streptococcus mutans* (Zarchil and Babaei, 2006; Azuma *et al.,* 2003).

Among the constituents of thyme EOs, those with phenolic components like thymol and carvacrol have major antimicrobial activities than non-phenolic oxygenated monoterpenes- or even sesquiterpene hydrocarbons (Figueiredo *et al.,* 2008). The Ethiopian thyme species are known to possess carvacrol and thymol as their major components. For example in the EOs of *T. schimperi* collected from Dinsho, thymol, γ-terpinene, carvacrol, and *p*-cymene were the dominant components in descending order. In EOs of *T. schimperi* collected from Addis Ababa, carvacrol and γ-terpinene were major components (Ermias Dagne *et al.,* 1998). In the same way, in Nigist Asfaw *et al.'s* work, the EOs of *T. schimperi* from Bale contained carvacrol, *p*-cymene, γ-terpinene, and thymol as major components and *T. schimperi* EO from Shewa constituted thymol, p-cymene, carvacrol, and γ-terpinene as its major components in descending order (Nigist Asfaw *et al.,* 2000). Similarly, the components of *T. schimperi* from Gondar were thymol, *p*-cymene, γ-terpinene, and carvacrol in descending order and *T. schimperi* from Wello had thymol, *p*-cymene, γ-terpinene, and carvacrol. The same study also revealed

that thymol, γ-terpinene, *p*-cymene, and carvacrol as major components of *T. serrulatus* from Tigray (Nigist Asfaw *et al.,* 2000).

2.4.3.2.1. Dental plaque bacteria: plaque development and control

Over 750 species of bacteria inhabit the oral cavity. However, mutans *Streptococci* and *Lactobacilli* are characterized by marked acidogenicity and aciduricity. They metabolize sucrose to organic acids (mainly lactic acid) that dissolve the calcium phosphate in teeth, causing decalcification and eventual decay or caries (Palombo, 2011). Dental caries is a chronic endogenic multifactorial bacterial infection with gram-positive mutans *Streptococci* and *Lactobacilli* long being recognized as the primary cariogenic organisms (Loesche, 1986).

In particular, *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* are important for the initiation of dental caries and *Lactobacilli* are implicated with caries progression (Loesche, 1986). Key organisms which cause dental caries include *Streptococcus* species (*S. sanguinis, S. mitis* and *S. crista*), *Lactobacillus* species (*L. gasseri, L. fermentum* and *L. salivarius*), *Fusobacterium*, *Bacteroides*, *Porphyromonas*, *Prevotella, Neisseria, Veillonella, Corynebacterium, Actimomyces* and *Treponema* species (Sinkiewicz, 2010).

Humans are using various drugs to avoid the infestation of teeth by these bacteria. In advance of the unpleasant side effects of artificial drugs, the number of drug resistant microorganisms is increasing year after year. So researchers are trying to pay more attention to herbal drugs. Research centers and World Health Organization prepare lots of programs to make use of plant extracts (Dalirsani *et al.,* 2011).

Mouthwashes for example, chlorhexidine, have several adverse effects despite their good plaque control and antimicrobial effects. Finding plants that have antimicrobial effects and using them as mouthwashes have advantages, such as, a decrease of side effects and also they are more economical. *Thymus* species are practiced as additives to tooth pastes in different parts of the world (Figueiredo *et al.,* 2008). For instance, thymol-based mouthwashes are known for their abilities to rapidly-kill plaque bacteria and have plaque-permeating capacities (Nostro *et al.,* 2007) a good example is Listerine.

Listerine antiseptic mouthrinse contains a combination of EOs, including eucalyptol, menthol, thymol, and methyl salicylate. It has antibacterial activity when used as a mouthwash to reduce oral bacteria. It demonstrates broad-spectrum antibiotic properties against *Streptococcus mutans*, herpes simplex virus, and influenza A virus. It is reported to be efficacious in the treatment of supragingival plaque and gingivitis because of its ability to penetrate dental plaque biofilm and kill Gram-positive organisms interproximally (Basch *et al.,* 2004).

Research works too show that the volatile components of different thyme species have antibacterial activities against those cariogenic bacteria. Example: components of *T.vulgaris*, α-pinene, γ-terpinene, ρ-cymene, linalool, thymol and carvacrol inhibited *S. mutans* with respective MICs of 0.5mM, 0.25mM, 0.25mM, 4.00mM, 1.00mM, and 1.00mM (Azuma *et al.,*2003). All of these volatile components are also found in Ethiopian *Thymus* species (*T. serrulatus* and *T. schimperi*) with Thymol and Carvacrol as major components (Nigist Asfaw *et al.,* 2000). Thus the present study is intended to test

the plaque protective capacity of essential oils of these species by *in vitro* testing of their antibacterial activities against *S. mutans* and Lactic acid bacteria from oral isolates.

2.4.3.2.2. Mode of action of EOs against bacteria

The mode of action of EOs depends mainly on the composition and concentration of their active components (Lüüs, 2009) and the mechanism of action of EOs is very specific to the chemical structures of their components (Lüüs, 2009). Due to the presence of large numbers of constituents in them, EOs seem to lack specific cellular targets (Tripathi *et al.,* 2013; Abdul-Hafeez *et al.*, 2014). Rather, they use multiple targets of action which makes them better than the use of conventional antimicrobials in that this situation prevents the adaptability of the microorganisms against them (Barkat and Bouguerra, 2012).

EOs are lipophilic or hydrophobic compounds (Lüüs, 2009; Szumacher-Strabel and Cieślak, 2012; Tripathi *et al.,* 2013) due to their possession of phenolic rings (Lüüs, 2009). Owing to this property, they are able to pass through the cell walls and cell membranes (Tripathi *et al.,* 2013). EO constituents possessing alcohols, phenols, and aldehydes in their functional groups are powerful cytotoxic ones against cariogenic bacteria (Tripathi *et al.,* 2013). Thymol and carvacrol are examples of phenolic terpenes in thyme EOs with antimicrobial activities (Lüüs, 2009).

These EO constituents may act on cariogenic bacteria by affecting (i) the structures and functions of cell walls and membranes, (ii) enzyme activities, (iii) energy translocation (ATP synthesis), (iv) cytoplasm coagulation and (v) others (Figure 9).

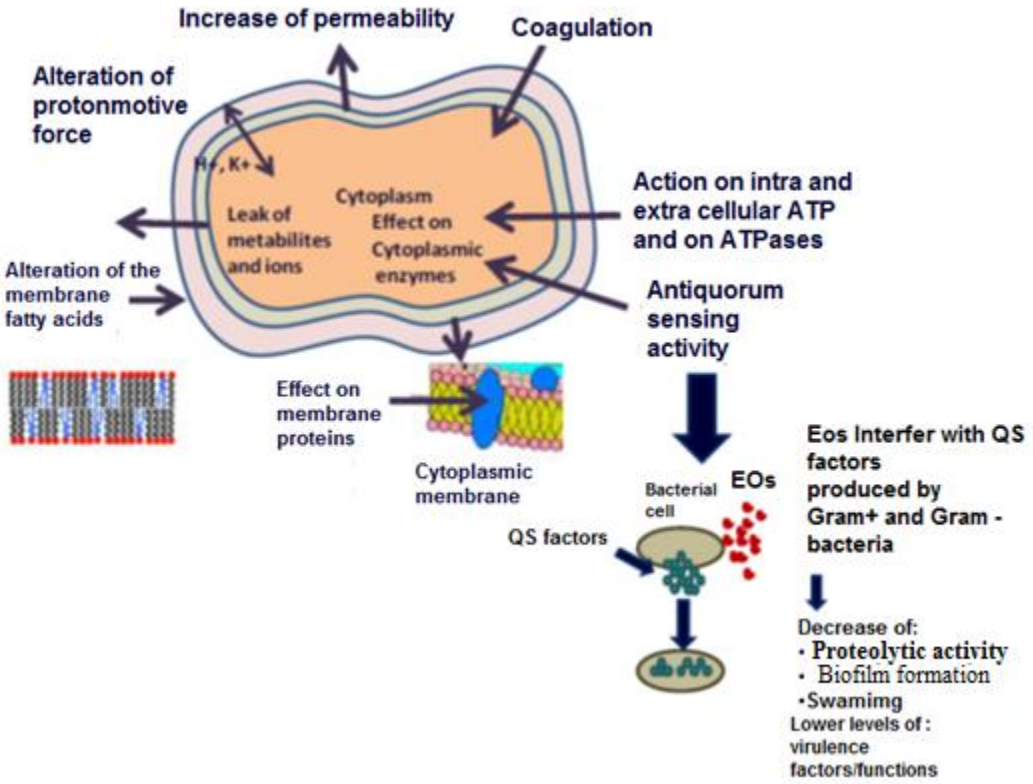


Figure 9: Mechanism of action and target sites of the EOs on microbial cells (Nazzaro *et al.*, 2013)

Action on cell walls and cell membranes: The entrance of EOs across the cell walls and cell membranes of bacteria disrupts polysaccharides, fatty acids and phospholipids in different layers of these structures (Tripathi *et al.*, 2013; Abdul-Hafeez *et al.*, 2014). This causes deleterious effects on cellular membranes and cell walls permeabilizing them. Consequently, there may result in increased: leakage of substances such as ions like K^+, reduced membrane potential, collapsed proton pump, depleted ATP pool, affected chemiosmotic cell control and increased leakage of macromolecules like ATP, nucleic acids, amino acids (e.g. glutamate), and lysis (Sharma *et al.*, 2010; Montanari *et al.*,

2011; Tripathi *et al.*, 2013). Thus, EOs result in cytoplasmic membrane disruption and cell death (Zuzarte *et al.,* 2011a). In addition, coagulation of the cytoplasm and damage of lipids and proteins are common in EO treated microorganisms (Tripathi *et al.*, 2013). This mode of action of EOs is similar to that of others broad-spectrum membrane-active disinfectants and preservatives, such as phenol derivatives, chlorhexidine and parabenzoic acid derivatives (Montanari *et al.*, 2011).

Owing to their possession of additional inner membrane made of complex structure of proteins, phospholipids, and liposaccharides (ProSafeBeef, 2007; Szumacher-Strabel and Cieślak, 2012); Gram-negative bacteria seem to be less sensitive to the antimicrobial action of EOs than Gram-positive bacteria (ProSafeBeef, 2007). But, EO components like thymol and carvacrol inhibit growth in gram-negative bacteria by affecting the proper function of the inner membrane. This is because; EOs are capable of getting access into the periplasm of Gram-negative bacteria through the porin proteins (ProSafeBeef, 2007; Szumacher-Strabel and Cieślak, 2012).Thus, both Gram-positive and Gram-negative bacteria can be controlled using EOs with thymol and carvacrol constituents (Szumacher-Strabel and Cieślak, 2012).

EOs and their phenolic components like thymol, carvacrol and eugenol result in reduced chain length and decreased abundance of UFAs. Rather, they result in increased amount of saturated fatty acids (SFAs) in the membrane lipid bilayer which leads to loss of membrane fluidity and consequently increases membrane rigidity. This icreases the overall permeability of the outer membrane so that material exchange with their surrounding environment is affected (Nazzaro *et al.*, 2013).

Action on enzymes: EOs and/or their components affect either intracellular or extracellular enzymes or membrane-embedded enzymes (Cox *et al.,* 2001; Trajano *et al.,* 2010**).** The effect of EOs on enzymes indirectly affects cellular functions such as genetic material functionality, energy production, and structural components synthesis (Celikel and Kavas, 2008). Specific examples are (i) fatty acid synthesis – they lead to increased amounts of SFAs in membrane lipid bilayers (Nazzaro *et al.,* 2013).(ii) They affect the enzymes and cofactors which are directly involved in the respiratory electron transport chain span of cytoplasmic membranes of bacteria so that energy production by bacteria treated with EOs is prohibited (Cox *et al.,* 2001).Terpenoids are among those lipid-soluble agents that affect the activities of membrane-catalyzed enzymes (Mendonça-Filho, 2006). Among these terpenoids, the oxygenated terpenoids such as thymol, eugenol, or carvacrol may inactivate the essential enzymes responsible for cell membrane activity, genetic material functionality, energy production, and structural components synthesis (Celikel and Kavas, 2008).

Action on proteins: Effect of EOs and their components on structural and functional proteins in bacteria may affect cellular functions such as cell division, material synthesis, material exchange, defense mechanisms, and other activities (Nazzaro *et al.*, 2013).

Action on energy transduction (ATP biosynthesis) and ATPase: Certain components of EOs can interfere with proton translocation over a membrane vesicle and subsequently interrupt ADP phosphorylation. For example, specific terpenoids with functional groups, such as phenolic alcohols or aldehydes,interfere with membrane-integrated enzyme proteins which are involved in ATP synthesis (Mendonça-Filho, 2006).The antimicrobial

action of EOs with carvacrol and thymol as their main constituents uses this mechanism of action (Mendonça-Filho, 2006).

Carvacrol applied on bacteria for example affects the cytoplasmic membrane, acts upon 'proton exchanger', results in reduction of pH gradient across the cytoplasmic membrane, and leads to ATP depletion and eventually to cell death. Further cell damage may also be related to nutrient uptake, nucleic acid synthesis and ATPase activity. Several reports have demonstrated that most EOs impair the respiratory activity of different bacteria (ProSafeBeef, 2007).

EOs disrupt the cell membrane and alter the intracellular and external ATP balance through; loss of ATP across the disturbed membrane, loss of inorganic phosphate via the permeable membrane, or due to the disruption of the proton motive force and changes in the balance of some essential ions, such as K+ and H+. Besides, treatment of bacteria with EO components like eugenol, cinnamaldehyde and carvacrol inhibit the ATPase activity of bacterial cells (Nazzaro *et al.,* 2013).

EOs of *Mentha* species, *Thyme,* and Oregano containing the active components menthol, thymol, and carvacrol caused total inhibition of oxidative phosphorylation and consequently demonstrated strong antifungal activity. But carvacrol was the best of the three (Soković *et al.,* 2013). The desired bioactivity of these compounds is due to the presence of the hydroxyl group in them. The delocalized electron system in carvacrol and thymol facilitates the dissociation of H^+ from the OH group. This, in turn, would allow them to shuttle H^+ and monovalent cations, such as K^+, across membranes, dissipating pH and K^+ gradients across cell membranes. Through this mechanism, EOs cause into

depolarized bacterial cell membranes which further result in increased membrane permeability (Rao *et al.,* 2010).

2.4.3.3. Hepatoprotective activity of *Thymus* essential oils

2.4.3.3.1. The liver: structure, functions, threatening factors and treatments

Structure of the Liver: The liver is made up of many structural and functional units called hepatic lobules (Figure 10). In a lobule, many hepatic cells are arranged in longitudinal groups that radiate out from a central vein. Groups of cells in a lobule are separated from each other using sinusoids. Attached to the lining of hepatic sinosoids exist fixed phagocytic macrophages called *Kupffer cells* whose function is to remove pathogens and debris that may have entered the hepatic portal vein (Mader, 2011).

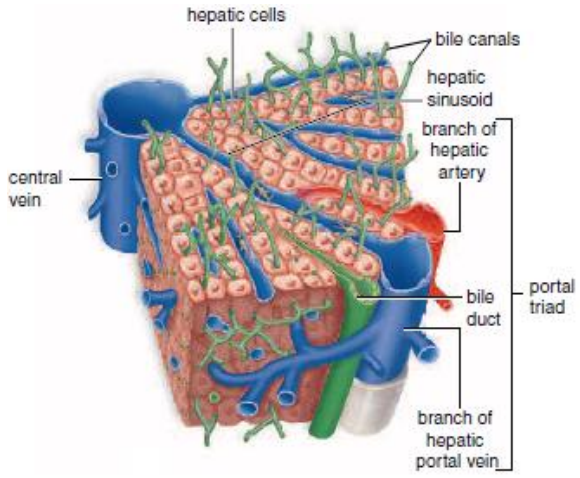


Figure 10: Cross section of a hepatic lobule, illustrating microscopic structure (Source Mader, 2011).

Functions of the liver: The liver is the largest glandular organ in the body (Bhawna and Kumar, 2010; Al-Fartosi *et al.,* 2011; Mowsumi *et al.,* 2013) and has more functions than any other human organ. A person's entire blood supply passes through the liver several times a day (Bhawna and Kumar, 2010) so that the liver plays a crucial role in metabolism (Ebenyi *et al.,* 2012) and excretion of waste products (Roy *et al.,* 2012; Sindhu *et al.,* 2012).

The liver metabolizes nutrients (carbohydrates, proteins and lipids) (Roy *et al.,* 2012) as well as various drugs, xenobiotics and toxins (Dar *et al.,* 2012). Consequently it plays surprising roles in the maintenance and regulation of body homeostasis (Al-Fartosi *et al.,* 2011) and detoxifies the body from a variety of drugs, xenobiotics, environmental pollutants and chemotherapeutic agents (Mowsumi *et al.,* 2013). Moreover, it is a site of biochemical pathways that are crucial for growth, fight against disease, nutrient supply, energy provision, and reproduction (Roy *et al.,* 2012). Apart from its role in detoxification and elimination of toxic metabolites, the liver synthesizes and stores useful principles (Mowsumi *et al.,* 2013) such as bile (Roy *et al.,* 2012), blood clotting proteins (prothrombin and fibrinogen), and heparin, the substance that prevents blood clotting within the circulatory system (Bhawna and Kumar, 2010). The bile secreted by the liver plays an important role in fat digestion (Roy *et al.,* 2012). Hence, maintenance of a healthy liver is essential for the overall wellbeing of an individual (Kumar *et al.,* 2012).

2.4.3.3.2. Causes of liver diseases

The liver is continuously exposed to xenobiotics which result in a variety of serious liver disorders (El-Banna *et al.*, 2013). Furthermore, excessive free radicals generated during metabolism may cause liver damage (Dar *et al.*, 2012). Since the liver is the center of drug metabolism, over dose of drugs can lead to oxidative stress and toxicity (Ebenyi *et al.*, 2012). Liver injury caused by such toxic chemicals and certain drugs is one of the very common ailments resulting into serious incapacities ranging from severe metabolic disorders to mortality (Kanchana and Sadiq, 2011).

Liver diseases are among the major causes of morbidity and mortality all over the world (Pinho *et al.*, 2014) and are still serious health problems affecting more people world wide at an alarming rate (Mowsumi *et al.*, 2013). Liver diseases pose a serious challenge to international public health (Al-Fartosi *et al.*, 2011). Annually, about 20,000 deaths happen due to liver disorders (Sindhu *et al.*, 2012). Liver diseases are among the most serious ailments which can be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatosis (non-inflammatory diseases) and cirrhosis (degenerative disorders resulting in fibrosis of the liver) (Kumar *et al.*, 2012).

In general, liver diseases can result from toxic chemicals such as peroxidized oil, carbon tetrachloride, chlorinated hydrocarbons, drugs like paracetamol, isoniazide, certain antibiotics, chemotherapeutics; excess alcohol consumption; due to viruses,bacteria, mushrooms, parasites like amoebas or giardiasis (Bhawna and Kumar, 2010) and autoimmune challenges (Mowsumi *et al.*, 2013). Most of the chemical agents and drugs result in cellular as well as metabolic liver damage when used on a routine basis

(Wankhade *et al.,* 2011). In addition, enhanced lipid peroxidation produced from ethanol microsomal metabolism results in hepatitis and cirrhosis (Kumar et al., 2012; Roy *et al.,* 2012).

2.4.3.3.3. Paracetamol as a liver disease causing agent

Paracetamol Paracetamolis a drug commonly used for the treatment of mild pain (Ebenyi *et al.,* 2012). It is a widely used anti-pyretic and analgesic drug (Al-Fartosi *et al.,* 2011) which is safe at therapeutic doses (Ebenyi *et al.,* 2012). However, an overdose of paracetamol causes severe hepatotoxicity and necrosis in humans and experimental animals (Galal *et al.,* 2012). Hepatic damage and death following a paracetamol overdose was first reported from Britain in 1966 and since that time there has been a rapid increase in the use of this drug in self-poisoning (Gazzard *et al.,* 1974).

At therapeutic levels, around 95% of paracetamol is primarily metabolized in the liver by glucuronidation and sulfation. However, a small proportion (5%) undergoes cytochrome P450 (CYP450)-mediated bioactivation to N-acetyl-p-benzoquinoimine (NAPQI) (Wankhade *et al.,* 2011), which further is rapidly reduced by reduced glutathione (GSH) and excreted through urine (Figure 11) (Grespan *et al.,* 2014). But, when paracetamol intake far exceeds therapeutic doses, the glucuronidation and sulfation pathways get saturated and the cytochrome P450 pathway becomes increasingly important (Ebenyi *et al.,* 2012). That is, the formation of NAPQI exceeds the rate of detoxification by GSH and there exists dramatic depletion of cellular glutathione levels in the liver (Balamurugan, 2007). This in turn results in a rapid accumulation of the reactive and toxic metabolite (NAPQI), a causative agent for hepatotoxicity (Ebenyi *et al.,* 2012).

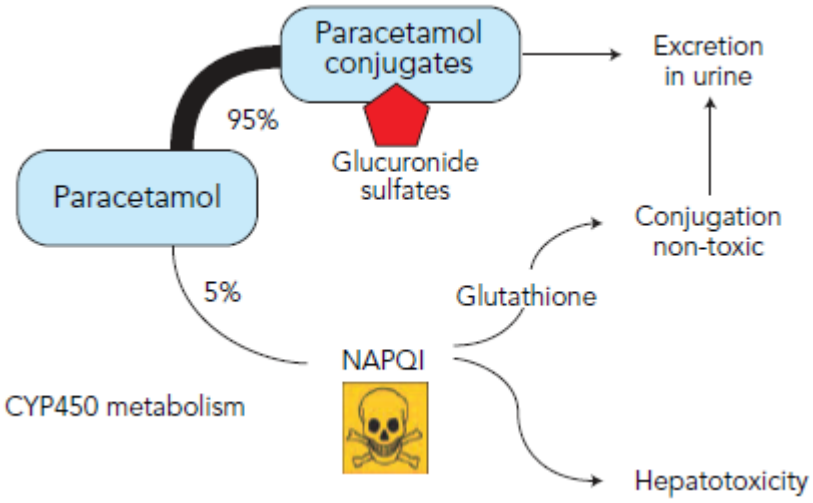


Figure 11: Metabolism and hepatotoxicity of paracetamol (Source: Lubel *et al.*, 2007)

The mechanism of action of excess NAPQI is through: (1) covalently binding with cellular macromolecules (Balamurugan, 2007) such as proteins or lipids resulting into cellular injury; (2) producing toxic free radicals which cause or initiate hepatocellular injury (Balamurugan, 2007; Ebenyi *et al.*, 2012); and (3) through glutathione (GSH) depletion and lipid peroxidation (Galal *et al.*, 2012).

2.4.3.3.4. Importance of GSH and its biosynthesis

GSH is an antioxidant in the body which is made up of the amino acids glutamate, cysteine and glycine. It has multiple functions in the body such as: detoxification of certain drugs and toxic environmental chemicals; protection of the body from lipid peroxidation and electrophiles; has antiviral effects, and is involved in the biosynthesis of DNA, proteins, and leukotrienes. Besides, it plays roles in cell proliferation, apoptosis,

neurotransmission, and neuromodulation. Therefore, decreased levels of GSH may manifest several diseases such as liver cirrhosis, pulmonary disease, gastrointestinal or pancreatic inflammation, diabetes, and neurodegenerative diseases (Shaw, 2013).

In the normal physiological state (Figure 12), GSH is produced by the condensation of the amino acids glutamate and cysteine to form the dipeptide γ-glutamylcysteine, which is catalyzed by the enzyme, γ-glutamyl cysteine synthetase. The dipeptide then condenses with the amino acid glycine to form the tripeptide GSH which is catalyzed by the enzyme GSH synthetase.The end product, GSH, exhibits negative feedback inhibition of γ-glutamyl synthetase to prevent the overproduction of GSH. If the amino acid glycine is present at high concentrations, γ-glutamylcysteine is converted in small amounts to pyroglutamic acid that can be recycled to form glutamate (Shaw, 2013).

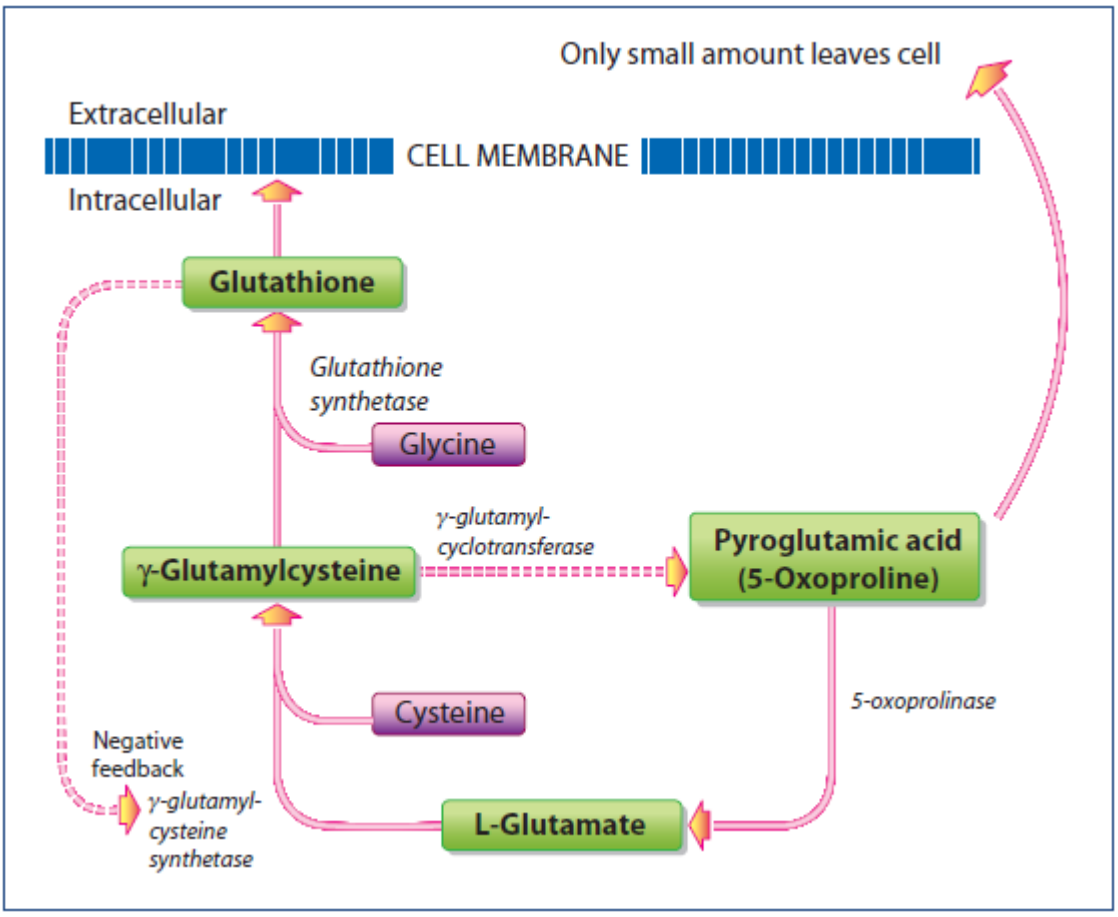


Figure 12: Metabolism of GSH in the absence of a toxic paracetamol load (Source: Shaw, 2013).

Severe depletion of GSH resulted from depletion by NAPQI (Figure 13) results in lack of negative feedback of GSH on γ-glutamyl cysteine synthetase.This in turn leads to the synthesis of large amounts of γ-glutamylcysteine, which further is converted to pyroglutamate, leading to metabolic acidosis.In addition, NAPQI deactivates some of the enzymes that recycle GSH (e.g. GSH peroxidase). As a whole, the depletion of GSH by moderate increases in NAPQI diminishes the ability of the body to detoxify toxic chemicals and heavy metals (Shaw, 2013).

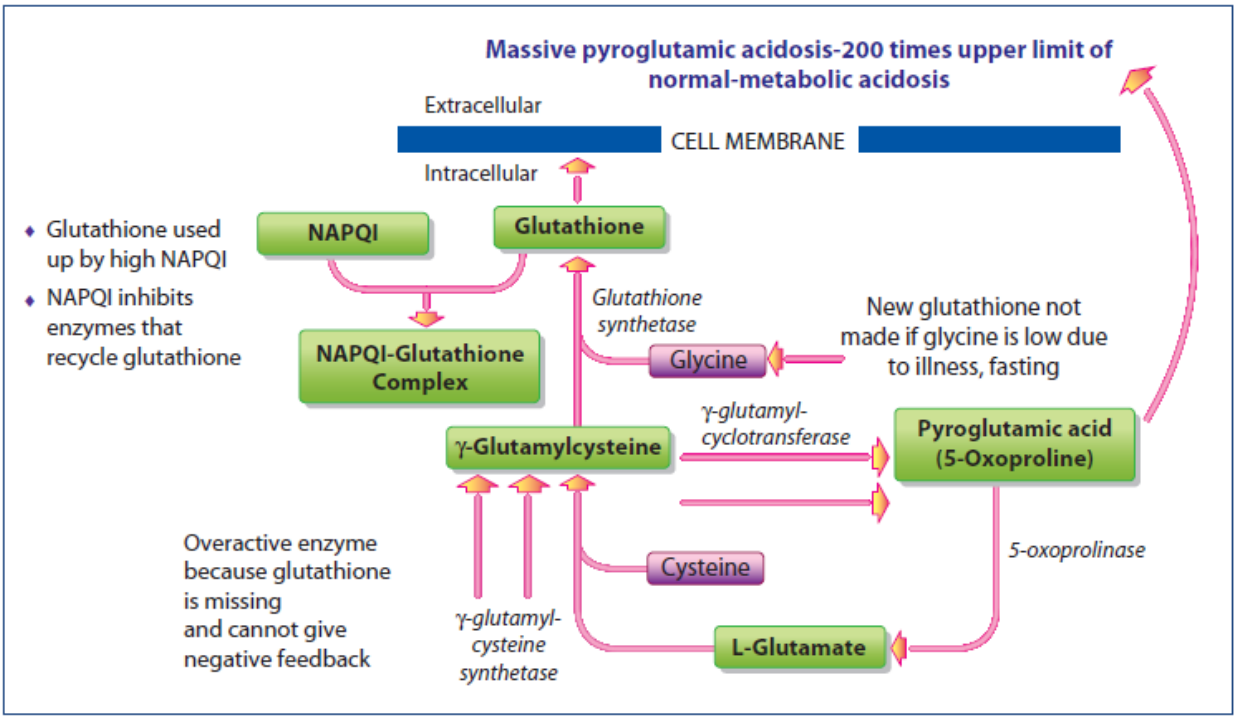


Figure 13: Metabolism of GSH after exposure to high doses of paracetamol (Source: Shaw, 2013).

Paracetamol toxicity is also associated with a wide range of other disorders in humans and/or experimental animals including cancer, birth defects, asthma, allergies, and brain toxicity (Shaw, 2013).

Hepatocellular degeneration and necrosis are associated with elevated enzyme markers, such as serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) that indicate hepatotoxicity (Grespan *et al.*, 2014). Paracetamol injury results in abnormal blood levels of sodium, potassium, urea, creatinine, albumin, bilirubin, AST, ALT, and ALP (www.fda.gov/downloads/ICECI/Inspections/IOM/UCM135835.pdf).

2.4.3.3.5. Treatment and prevention of liver diseases

Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for the treatment of liver disorders (Al-Fartosi *et al.,* 2011). Conventional and complementary medicines are used to treat these illnesses. However, many of these conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects (Manokaran *et al.,* 2008; Kader and Mhamed, 2012; Singh, 2013).

Hence, there is an ever increasing need for safe hepatoprotective agents (Kumar *et al.,* 2012) and there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity (Manokaran *et al.,* 2008). Liver protective herbal drugs contain a variety of chemical constituents like phenols, coumarins, lignans, EOs, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes most of which are antioxidants (Sindhu *et al.,* 2012) indicating the importance of natural products in the treatment of liver diseases (Mowsumi *et al.,* 2013; Alamgeer *et al.,* 2014).

The use of natural remedies for the treatment of liver diseases has a long history and medicinal plants and their derivatives are still in use all over the world in one form or another (Bhawna and Kumar, 2010). Now-a-day's due to inadequacy of liver protective agents, researchers and traditional medicine practioners concentrate in herbal based remedies for various liver disorders (Singh, 2013). Therefore, many plant products have been evaluated for their possible antioxidant and hepatoprotective effects which might make them suitable for treatment of chemical-induced liver damage in experimental

animals (El-Banna *et al.*, 2013). Plant derived natural products such as flavonoids, terpenoids and steroids have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and hepatoprotective activity (Kanchana and Sadiq, 2011).

Since the damaging effect of paracetamol is thought to be mainly due to the free radical interaction, it could be worth looking for antioxidants capable of scavenging these reactive species (Sindhu *et al.*, 2012). Plants are able to be functioning in this capacity because they have many important chemical substances found all over their various parts such as alkaloids, carbon compounds, nitrogen, glycosides, essential oils, fatty oils, resins, mucilage, tannins, gums and others (Ebenyi *et al.,* 2012). But management of liver disorders by a simple and precise herbal drug is still an intriguing problem (Kumar *et al.*, 2012). Hence, there is an ever increasing need for safe hepatoprotective agents (Roy *et al.,* 2012).

Despite the significant popularity of several herbal medicines in general, and for liver diseases in particular, they are still unacceptable treatment modalities for liver diseases. The limiting factors that contribute to this eventuality are: (i) lack of standardization of the hepatoprotective herbal drugs; (ii) lack of identification of active ingredients(s)/principles(s) involved in hepatoprotection; (iii) lack of randomized controlled clinical trials (RCTs) about hepatoprotective herbal drugs; and (iv) lack of toxicological evaluation of hepatoprotective herbs (Roy *et al.,* 2012). The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver disease models by carefully synergizing the strengths of the traditional systems of medicine with

that of the modern concept of evidence-based medicinal evaluation, standardization and randomized placebo controlled clinical trials to support clinical efficacy (Roy *et al.,* 2012).

T. schimperi and *T. serrulatus* both of which are endemic to Ethiopia (Fichtl and Adi, 1994, Woldemedhin Zebene, 2011) are used by local people in Ethiopia to treat liver diseases (Kunert, 2000).

Therapeutic functions of thyme are owing to their classes of compounds like flavonoids, phenols, saponins, luteolin and tetramethoxylated flavones and due to their specific components such as thymol, carvacrol, and eugenol. The hepatoprotective activity of these plants is due to the antioxidant activities of their components. Different research works reveal the hepatoprotective activity of extracts and EOs of different *Thymus* species. For example, the hepato- and reno-protective potential of *T. vulgaris* after oral co-administration with paracetamol was revealed by significantly decreased levels of liver enzymes (ALT, AST and ALP), total bilirubin, total protein, blood urea and creatinine. It also resulted in significantly increased levels of CAT, SOD and GSH in both liver and kidneys (Kader and Mhamed, 2012).

Similar hepatoprotective activity against paracetamol-induced hepatotoxicity was seen in rats pretreated orally with EOs of *T. capitatus* (El-Banna *et al.,* 2013). The hepatoprotective activity of aqueous and ether extracts of *T. linearis* evaluated against carbon tetrachloride- and paracetamol-induced hepatic damage in mice too resulted in a dose dependent reduction in serum levels of AST, ALT, and ALP when compared to carbon tetrachloride- and paracetamol treated groups (Alamgeer *et al.,* 2014). Grespan *et*

al. (2014) also found reduced levels of serum marker enzymes AST, ALT, ALP and myeloperoxidase (MPO) against paracetamol-induced hepatic damage in mice after treating with *T. vulgaris* EO (Grespan *et al.,* 2014).

As observed by Alam *et al.* (1999), oral administration of one of these components, thymol demonstrated hepatoprtective activities against carbon tetrachloride (CCl_4)-induced hepatotoxicity in male Swiss albino mice. Thymol also inhibited lipid peroxidation induced by CCl_4 *in vivo*. This implies thymol protects the liver against CCl_4-induced toxicity may be through its ability to inhibit lipid peroxidation (Alam *et al.,* 1999). Therefore, *T. serrulatus* and *T. schimperi* whose EOs are rich in thymol (Nigist Asfaw *et al.,* 2000) may have hepatoprotective activities. As a result the present study was focused on the investigation of the hepatoprotective activities of *T. serrulatus* and *T. schimperi* EOs against paracetamol-induced hepatotoxicity in rats.

2.4.3.4. Mosquitocidal activity of *Thymus* EOs

Vector-borne diseases: Vectors are organisms that transmit pathogens and parasites from one infected person (or animal) to another (WHO, 2014a). Vector-borne diseases commonly found in tropical and sub-tropical countries account for 17% of the estimated global burden of all infectious diseases (Abiy Andemo *et al.,* 2014; WHO, 2014a). Mosquitoes are among these deadly vectors which impacted human health since time immemorial and they are ranked as man's most important insect pests (Manimaran *et al.,* 2012). They transmit diseases like malaria, filariasis, dengue fever, yellow fever, Japanese encephalitis, chikungunya fever, and other viral infections (Conti *et al.,* 2010; Dua *et al.,* 2010; Abiy Andemo *et al.,* 2014). They too cause allergic responses in

humans that include local skin and systemic reactions such as angioedema (Govindarajan *et al.,* 2012). History shows that mosquito-transmitted diseases continue to be major sources of illness and death (Aarthi and Murugan, 2010).

Malaria, the most deadly mosquito-borne diseases is known to be transmitted by mosquito species within the *Anopheles gambiae* (*An. gambiae*) complex and the *Anopheles funestus* (*An. funestus*) group in Africa. The *An. gambiae* complex contains excellent and efficient vectors of malaria [*An. gambiae S.S.* and *Anopheles arabiensis* (*An. arabiensis*)], as well as minor vectors (*Anopheles merus*) and non-vectors (*Anopheles quadriannulatus* species A and B). In Ethiopia *An. arabiensis* serves as the major vector, while *An. funestus, Anopheles pharoensis* and *Anopheles nili* act as secondary vectors (Karunamoorthi *et al.,* 2014).

Malaria kills an estimated amount of 627, 000 people every year, mainly children under 5 years of age in sub-Saharan Africa. In every 60 seconds, a poor African child is killed by this disease (Karunamoorthi *et al.,* 2014). In 2013, malaria occurred in 97 countries and the annual global malaria cases surpassed 200 million (WHO, 2014b). In the years 2000 through 2011, the trend of confirmed malaria cases in Africa region showed increment. By the year 2010 one percent of the deaths in south and Sub-Saharan countries were due to malaria and Ethiopia was one of these victim countries (AFRO, 2014).

Living in malaria-endemic regions places an economic burden on households through out-of-pocket payment and person-days lost (Wakgari Deressa *et al.,* 2007). That is malaria infection has negative effects on productivity, increases private expenditure, and has a positive effect on mortality rate (Abdullateef and Oluwatoyin, 2011). A study in

Ethiopia by Wakgari Deressa *et al.* (2007) revealed the economic burden posed by malaria on rural households and individuals as follows: every patient paid an average treatment cost of Birr 24.00 ($2.76) at private clinics and Birr 12.50 ($1.44) at public facilities. Similarly, the average direct cost of malaria per patient was estimated to be Birr 14.00 ($1.60) and the average indirect cost, Birr 35.26 ($4.08) (Wakgari Deressa *et al.*, 2007).

According to the Ministry of Health of Ethiopia, the economic burdons of malaria include: '1) reduced production activities due to the high morbidity and mortality rate in the adult population; 2) occurrence of malaria in resource-rich areas like river valleys prevents people to settle there. Rather, people are densely populated in less productive malaria-free areas which further results in a massive environmental and ecological degradation and loss of productivity, exposing a large population of the country to repeated droughts, famine and overall horrible poverty; 3) the increased school absenteeism during malaria epidemics significantly reduces learning capacity of students; 4) coping up with malaria epidemics overwhelms the capacity of the health services in Ethiopia, and thus substantially increases public health expenditures' (Senay and Verdin, 2005). Thus, malaria in Ethiopia, is not only a health issue, it is also a food-security, developmental, economic and environmental issue. Neverthhhless to the facts mentioned in this paragraph, unlimited intervention by the Ethiopian government against malaria has changed the situation.

Control of mosquito-borne diseases: Malaria can be controlled by the control of the causative agent (plasmodium), the vector (mosquito) or a combination of both. However,

the emergence of resistant strains of *Plasmodium falciparum* and lack of effective malaria vaccine causes vector control to be considered as a cornerstone to interrupt the cycle of disease transmission (Karunamoorthi *et al.,* 2014). Synthetic insecticides such as organochlorine and organophosphate compounds are major tools in mosquito control (Govindarajan *et al.,* 2012). But the use of synthetic chemical insecticides made mosquito control difficult since they exert adverse impacts on the environment, man, animals, and disturb ecological balance (Govindarajan *et al.,* 2012).

As a result, use of many of the former synthetic insecticides in mosquito control programme has been limited. Furthermore, issues such as lack of novel insecticides, high cost of synthetic insecticides, concern for environmental sustainability, harmful effect on human health and other non-target populations, their non-biodegradable nature, higher rate of biological magnification through ecosystem, and increasing insecticide resistance on a global scale made synthetic insecticides not good choices for mosquito control (Aarthi and Murugan, 2010; Abiy Andemo *et al.*, 2014; WHO, 2014b).

In addition, the increased use of these insecticides may enter into the food chain and thus may irreversibly damage the vital organs such as the liver and kidneys and may result in mutation of genes. Knowing the non-selective nature and harmful effects of chemical insecticides on other organisms in the environment, there is a rise in search of alternative vector control strategies such as herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable (Ghosh *et al.,* 2012; Govindarajan *et al.,* 2012).

One of the most effective alternative approaches under the biological control programme is to explore the floral biodiversity and enter the field of using safer insecticides of botanical origin as a simple and sustainable method of mosquito control. Further, unlike conventional insecticides which are based on a single active ingredient, plant derived insecticides comprise botanical blends of chemical compounds which act concertedly on both behavioral and physiological processes. Thus there is little chance of pests developing resistance to such substances. Identifying bio-insecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management. Botanicals have widespread insecticidal properties and will obviously work as a new weapon in the arsenal of synthetic insecticides and in the future may act as suitable alternative products to fight against mosquito borne diseases. *Thymus* species are among the plants with mosquitocidal activities (Ghosh *et al.,* 2012).

Insecticide resistance is also a challenge to control vectors in Ethiopia. For example, *An. arabiensis*, the most significant malaria vector in the country, is strongly resistant to DDT and pyrethroids. Thus, alternative tools or approaches should be followed to control this malaria vector (Abiy Andemo *et al.,* 2014). For instance, there is a need (1) to search new highly selective and biodegradable insecticides and (2) to study the environmentally friendly insecticides. Natural products are excellent preferences to synthetic insecticides and are safe and ecofriendly (Koul *et al.,* 2008). They too are safer, more cost effective, and biodegradable and are target specific against mosquitoes (Gokulakrishnan *et al.,* 2013).

Plants and plant extracts are reported to be effective against mosquitoes at various stages of development due to their possession of complex phytochemicals with unique biological activities. These phytochemicals can act as larvicides, insect growth regulators, repellents, and oviposition attractants or repellents (Aarthi and Murugan, 2010; Gokulakrishnan *et al.,* 2013).

Therefore, plants as rich sources of bioactive chemicals may be used as alternative sources of mosquito control agents (Aarthi and Murugan, 2010). At present, environmental protection agencies have banned or placed severe restrictions on the use of many pesticides which were formerly used in mosquito control programmes (Aarthi and Murugan, 2010). As a result the use of scientifically proven non-chemical methods and limited use of drugs is being considered safe to the environment and human health (Gokulakrishnan *et al.,* 2013).

Among the efforts made to investigate the efficacy of different natural products against a wide range of arthropod pests, plant EOs are reported to be toxic to Culicidae, acting as adulticidal, larvicidal, ovicidal, oviposition deterrents, growth and/or reproduction inhibitors and/or adult repellents (Conti *et al.,* 2014). Besides, the development of resistance by malaria vectors against plant derived bioactive molecules has not been reported so far. Moreover, extracts and pure compounds from plant families are found to show promising results in the control of mosquitoes and other insect pests and are known to be effective against both immatures and adult stages (Abiy Andemo *et al.,* 2014).

In recent years, plant-based EOs are given attention as potent bioactive compounds against various mosquito species. Certainly, mosquito vector control targeting the larval

stage of mosquitoes with potential larvicides is one of the most appropriate malaria vector control strategies as the target is exceptionally specific unlike adult control (Karunamoorthi *et al.*, 2014). EOs are among such larvicides which are potentially suitable for mosquito larval control since they are rich in bioactive molecules that are effective and naturally biodegradable into non-toxic products (Manimaran *et al.*, 2012). This opportunity has spurred the development of EO-based insecticides. Thyme oil as well as its active ingredients is among such isecticides (Isman, 2006).

Experimental findings in many of the *Thymus* species showed the insecticidal activities of their EOs and their components on different developmental stages of the insects. For example, EOs of *T.serpyllum* were effective against larvae of *Aedes, Anopheles* and *Culex* mosquitoes (Fekadu Massebo *et al.*, 2009). Belaqziz *et al.* (2010) found effective larvicidal effects of EOs of two thyme species (*T. broussonetti* and *T. maroccanus*) against larvae of the mosquito *C. pipiens.* From the dose of 0.125ppm, the EOs activities were significantly higher in comparison to the negative control (1mL of Ethanol). The lethal concentrations for 50% of insect population (LC_{50}), during exposure of the EOs at 24h, were 0.23 and 0.31 respectively for *T. broussonetti* and *T. maroccanus*. Carvacrol and thymol may play the role of effective toxicity on the larvae of *C. pipiens.*

Similarly, Owayss and Abd-Elgayed, (2007)'s work showed the larvicidal effects of the volatile oils of *T. vulgaris* L. against Greater Wax Moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) GWM larvae. In a study of the larvicidal activities of pure components, thymol, 1, 8-Cineole, major components of thyme EOs, were identified as

active components for the larvicidal action against *Aedes aegypti*, causing 100% larval mortality at lowest tested concentration of 0.017% (w/v) (Carvalho *et al.*, 2003).

Being a country around the tropics, Ethiopia harbors a lot of insect borne diseases like malaria and trypanosome. Thus finding environmentally friendly insecticides is very crucial. Fortunately, we have two endemic species of *Thymus* (*T. serrulatus* and *T. schimperi*) which can be good sources of mosquitocidal chemicals. Thus this project had embarked on evaluating the mosquitocidal activities of the EOs of these species.

Mode of action of EOs against insects: EOs are lipophilic in nature and interfere with basic metabolic, biochemical and physiological and behavioural functions of insects (Tripathi *et al.*, 2009).The mechanism of action of EOs in the control of insects like head lice may be through blocking the "breathing" spiracles (Barker and Altman, 2010). The insect repellent activity of EOs may be by interacting with the female mosquito olfactory receptors thereby blocking the sense of smell which therefore comes as a hurdle in the recognition of host by the mosquitoes. Studies have indicated that terpenoids containing two functional groups are biologically active as mosquito repellents (Kalita *et al.*, 2013). In addition, recent investigations revealed that, the mechanism of action of EOs against insects is by interference of their components with the octopaminergic nervous system in insects. As this target site is not shared with mammals, most EO chemicals are relatively non-toxic to mammals and fish in toxicological tests, and meet the criteria for "reduced risk" pesticides (Koul *et al.*, 2008).

Plant volatile oils have long been known to affect the behavioural responses of pests, with the monoterpenoid components appearing most useful as insecticides or

antifeedants. Low-molecular-weight (LMW) terpenoids may be too lipophilic to be soluble in the haemolymph after crossing the cuticle, and proposed a route of entry through the tracheae. Most insecticides bind to receptor proteins in the insect and, in doing so; they interrupt normal neurotransmission, which lead to paralysis and subsequently death. Recent evidence suggests that low-molecular-weight (LMW) terpenoids may also bind to target sites on receptors that modulate nervous activity. Ionotropic, γ-aminobutyric acid, GABA receptors, the targets of organochlorine insecticides lindane and dieldrin, are modulated by LMW terpenoids with vastly different structures. Some EOs have larvicidal effect and the capacity to delayed development and suppress adult emergences and induce abnormalities during development of insects of medical and veterinary importance (Khater, 2012).

2.4.3.5. Toxicity of thyme EOs

The use of medicinal plant as a therapy for various disease conditions is an age long practice. In regions with rich diversity of flora, it forms an important component of their natural wealth. Herbs and herbal formulations for the treatment of ailments have continued to receive increased attention because of the strong belief that these products are safe (Farnsworth and Soejarto, 1985; Said *et al*., 2002). This assumption to a large extent may have influenced the indiscriminate use of these formulations by many, particularly amongst people living in rural areas. The incidence of adverse effects and sometimes life-threatening conditions that potentially emanate from these herbal medicines has been reported among various ethnic groups (Elvin-Lewis, 2001; Chan,

2003). Consequently, it has become vital to ascertain the toxicity profile of these medicinal herbs.

Research work by Oyewole *et al.,* (2010) on *T. vulgaris* leaf extracts in rats showed no significant signs of toxicity at doses of 100 mg and 200 mg/kg body. At the same time, findings of Tarawneh *et al.,* (2011), showed no acute oral toxicity of Ivy-*Thyme* syrup in rats at a dose level of 3, 6 and 12 ml/kg. In the same way, this research work was focussing on acute oral toxicity tests of EOs of *T. serrulatus* and *T. schimperi* in mice since both the plants are commonly used as food additives and as traditional medicines.

Acute oral toxicity: Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours (EPA, 2002).

The levels of acute toxicities can be categorized using Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (UN, 2011). Their LD_{50} value categories vary based on their exposure routes; oral, dermal, gases, vapours, and dusts/mists. Based on GHS, acute oral toxicity of EOs is classified in to categories one to five. Category classes of the oral, dermal, gases, vapours, and dusts/mists are presented in Table 2.

Table 2: Conversion from experimentally obtained acute toxicity range values (or acute toxicity hazard categories) to acute toxicity point estimates for use in the formulas for the classification of mixtures

Exposure routes	Classification category or experimentally obtained acute toxicity (LD_{50}) range estimate	Converted acute toxicity point (LD_{50}) estimate
Oral	0 < Category 1 ≤ 5	0.5
(mg/kg bodyweight)	5 < Category 2 ≤ 50	5
	50 < Category 3 ≤ 300	100
	300 < Category 4 ≤ 2000	500
	2000 < Category 5 ≤ 5000	2500
Dermal	0 < Category 1 ≤ 50	5
(mg/kg bodyweight)	50 < Category 2 ≤ 200	50
	200 < Category 3 ≤ 1000	300
	1000 < Category 4 ≤ 2000	1100
	2000 < Category 5 ≤ 5000	2500
Gases	0 < Category 1 ≤ 100	10
(PpmV)	100 < Category 2 ≤ 500	100
	500 < Category 3 ≤ 2500	700
	2500 < Category 4 ≤ 20000	4500
	Category 5	
Vapours	0 < Category 1 ≤ 0.5	0.05
(µL/L)	0.5 < Category 2 ≤ 2.0	0.5
	2.0 < Category 3 ≤ 10.0	3
	10.0 < Category 4 ≤ 20.0	11
	Category 5	
Dust/Mist	0 < Category 1 ≤ 0.05	0.005
(µL/L)	0.05 < Category 2 ≤ 0.5	0.05
	0.5 < Category 3 ≤ 1.0	0.5
	1.0 < Category 4 ≤ 5.0	1.5
	Category 5	

Source (UN, 2011 page 113)

2.5. Plant material extraction techniques

Extraction is the technique for separation of medicinally active portions of plant (and animal) tissues using selective solvents and standard procedures. The products of extraction are complex mixtures of plant metabolites, in liquid or semisolid state or in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts.

These products all contain complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans. To use as modern drugs, the extracts can be further treated through numerous techniques of fractionation to isolate individual chemical entities like vincristine, vinblastine, hyoscyamine, hyoscine, pilocarpine, forskolin and codeine (ICS-UNIDO, 2008). The quality of extracts can be controlled by understanding and using appropriate extraction methods, plant part used, and extraction solvents (Abu Khalaf, 2011).

The techniques which generally are used for medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents) (ICS-UNIDO, 2008; Abu Khalaf, 2011).

The microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE) are new methods that are used to reduce or minimize the use of organic solvents and improve the extraction process (Abu Khalaf, 2011). Aromatic plants may be extracted using hydrodistillation techniques such as water distillation, steam distillation, water and steam distillation, hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) (ICS-UNIDO, 2008).

Extraction of plant materials involves four major steps. First, size reduction; their sizes should be reduced into smaller pieces. Following size reduction, the plants should be extracted using various extraction techniques. Extraction on its turn is followed by filtration and then the extracts should be concentrated and finally dried.

2.5.3. Essential oil extraction

EO extraction involves classical and modern techniques. The classical techniques are still in use and play great roles in commercial production of EOs. The major EO techniques include hydrodistillation, hydraulic maceration distillation, extraction using expression, extraction using cold fat (enfleurage) and many other modern techniques of EO extraction which are discussed in the coming pages. Methods for producing EOs from plant materials are summarized in Figure 14.

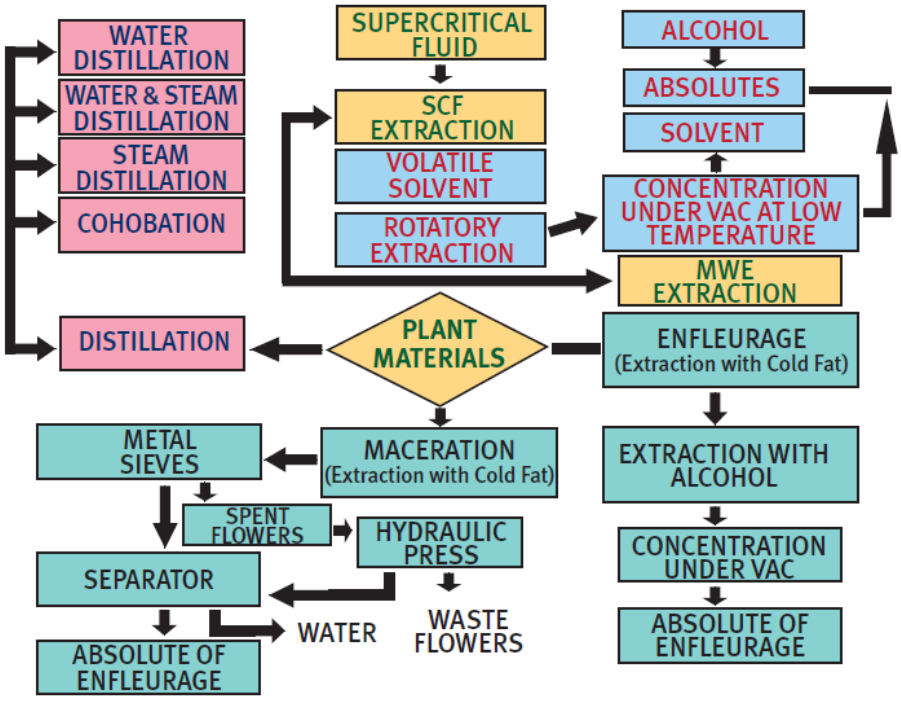


Figure 14: Methods of extraction of EOs from plant materials (Source: Handa, 2008)

2.5.3.1. Hydrodistillation

Hydrodistillation is the process of extracting essential oils by either boiling them in water or by passing stem through herbal preparations. The heat from hot water and steam force the release of the EO from the oil glands in the plant tissue. Finally, the vapor mixture of water and oil is condensed by indirect cooling with water and the distillate flows into a separator where oil separates automatically from the distillate water (Handa, 2008). There are three types of hydrodistillation for isolating EOs from plant materials namely water distillation, water and steam distillation, and direct steam distillation.

2.5.3.1.1. Water distillation

In this procedure, the plant material is fully immersed in water and boiled by heat from direct fire, steam jacket, closed steam jacket, closed steam coil or open steam coil. The

plant material is in direct contact with the boiling water. The Clevenger type apparatus is mostly used for this distillation (Figure 15). Water distillation has its own advantages such as: (i) it permits processing of finely powdered material or plant parts that, by contact with live steam, would otherwise form lumps, through which the steam cannot penetrate; and (ii) the stills are inexpensive, easy to construct, portable, and suitable for field operation (Handa, 2008).

Figure 15: Clevenger-type laboratory-scale hydrodistillation apparatus (Source: Handa, 2008)

On the contrary, water distillation is associated with some drwabacks. They are (i) oil components like esters are sensitive to hydrolysis while others like acyclic monoterpene hydrocarbons and aldehydes are susceptible to polymerization (since the pH of water is often reduced during distillation, hydrolytic reactions are facilitated); (ii) oxygenated components such as phenols have a tendency to dissolve in the still water, so their complete removal by distillation is not possible; (iii) it takes a long time to accumulate much oil, so good quality oil is often mixed with bad quality oil; (iv) the distillation process is treated as an art by local distillers, who rarely try to optimize both oil yield and quality; and (iv) water distillation is a slower process than either water and steam distillation or direct steam distillation (Handa, 2008).

2.5.3.1.2. Water and steam distillation

This is a method in which the plant material is not immersed directly in water. The equipment used is generally similar to that used in water distillation, but the plant material is supported above the boiling water on a perforated grid. This reduces the capacity of the still but affords a better quality of oil. If the amount of water is not sufficient to allow the completion of distillation, a cohobation tube is attached and condensate water is added back to the still manually, thereby ensuring that the water, which is being used as the steam source, will never run out (Handa, 2008).

2.5.3.1.3. Direct steam distillation

Direct steam distillation is the process of distilling plant material with steam generated outside the still in a satellite steam generator generally referred to as a boiler. The plant material is supported on a perforated grid above the steam inlet. The amount of steam can be readily controlled and the plant material is heated no higher than 100° C so that thermal degradation of the oils is minimized. For this and other reasons, steam distillation is the most widely accepted process for the production of EOs on large scale. Its obvious drawback is the much higher capital expenditure needed to build such a facility (Handa, 2008).

2.6. Chemical analysis of EOs

Chemical analysis of EOs is generally performed using GC and GC/MS. Identification of the main components is carried out by the comparison of both the GC retention times and MS data against those of the reference standards (with known source) (Lahlou, 2004). Analytical conditions and procedures used should carefully be described. These should include: apparatus of oil analysis (make and model number of the equipment); column type and dimensions; carrier gas flow rate; and the temperature programming conditions including injector temperature, detector and column temperatures; in addition to mass spectra (electronic impact).

Sometimes identification by GC/MS must be confirmed by retention indices (Kovats Indices) on two columns of different polarity, or on the same column, but at a different temperature; and claims for the identification of new constituents should be supported by co-injection with authentic compounds (Lahlou, 2004).

On the other hand, compounds which are not easily separated by GC and molecules structurally similar like stereo-isomeric compounds of EOs are analysed by either proton or ^{13}C NMR. This technique is also applied to the study of the chemical intraspecific variation and could also be used in the quality control of volatile oils (Lahlou, 2004).

Chapter 3 - Materials and Methods

Chapter 3: Materials and methods

3.1. Study area

Samples of *Thymus* species, ethnobotanical information, socioeconomic data, and threatening factors were collected from six localities in Ethiopia (Figure 16). These localities were Ofla and Alamata woredas (districts) of South Tigray (Tigray Region); Yilmana Densa district of West Gojjam (Amhara Region); Mojana district of North Shewa (Amhara Region); Meskena Mareko district of Gurage (Southern Nations, Nationalities and Peoples Region); and Sinana Dinsho district(Oromia Region). These areas were randomly selected based on literature data.

The specific areas for plant collection were: (1) Menkere (Ofla, South Tigray) 625 Kms North of Addis Ababa (Capital city of Ethiopia); Akojira (Alamata, Southern Tigray) 605 Kms North of Addis Ababa; Bir Adama Mountain (Yilmana Densa, West Gojjam) 443 Kms North west of Addis Ababa; Tarma Ber (Mojana district , North Shewa) 190 Kms North east of Addis Ababa; Zebidar Mountain (Meskena Mareko district , Southern Nations Nationalities and Peoples Region) 135 Kms South west of Addis Ababa; and Dinsho (Bale Mountains National park, Oromia Region) which is 370 Kms away from Addis Ababa through Assela (Figure 16).

Ofla districthas an altitudinal range of 1800–2440 m. Its mean annual rainfall is between 700–800 mm with mean daily temperatures ranging from 10–22 ºC. Rainfall is bimodal; a short rainy season “belg” between February and May, and a long rainy season “meher” between June and September (Girmay Tesfay *et al.,* 2014).

Alamata district has an altitudinal range of 1178 to 3148 m a.s.l of which 75% is low land (1500 m a.s.l or below) and 25% intermediate highlands (between 1500 and 3148 m a.s.l). Eutric Vertisols, Lithic Leptosols (Cambic) and Lithic Leptosols (Orthic) are the soil types covering nearly 100% of the land in the district (IPMS, 2005). The annual temperature ranges between 14.6 ^{o}c and 29.7 ^{o}c. Alamata has bimodal rainfall patterns; the belg (short rains) (from January to February) and the Meher (long rains) (from July to August). The mean annual rainfall of the area is around 963.5 mm (IPMS, 2005; Dawit Gebregziabher, 2010).

Yilmana Densa district is one of the fifteen districts of West Gojjam Administrative zone. Its capital town, Adet is found 42 km. from Bahir Dar on the south and 443 km from Addis Ababa through Mota. The average annual temperature ranges between 8.8°c-25.2°c, and the average annual rainfall ranges between 1100-1270 mms. The district has three types of soil: red (65%), brown (15%) and black (20%) (Solomon Abie, 2011).

Mojana district (Tarmaber) is located 190 km away from Addis Ababa and covers about 54,000 ha of lands. Its altitude is ranging from 1500 to 3100 meter above sea level. The average annual temperature and the mean monthly rain fall are about 15.5 °C and 1200 mm respectively (http://edaethiopia.org/index.php/where-we-work/amhara/tarma-ber).

Meskana Mareko is a district located in the Gurage zone, Southern Nations, Nationalities, and Peoples' Regional state (SNNPR) 135 km south of Addis Ababa. The district lies at an average altitude of 1900 m above sea level, ranging from 1750 m a.s.l in the lowlands to 3400 m a.s.l in the mountains. Annual rainfall in Meskana Mareko area ranges between 700-1870 mm. Although the main rainy season is from June to September, light

rains are common around March and April. The warmest months are between January and June with a maximum temperature of 30.4 °C in March during the last ten years. During the last decade the annual mean maximum temperature was 26.3 °C and minimum mean temperature was 11.1 °C (Solomon Tesfaye *et al.*, 2012).

Dinsho District is one of the districts in Oromia Region. Dinsho is located at the northern edge of the Bale Mountains National Park (BMNP) 370 Kms South East from Addis Ababa. It has altitudinal ranges from 2441-3600 m a.s.l. (Haile Yineger *et al.*, 2008). Its mean annual temperature and rainfall are 10.26^{o}C and 1218.64 mm respectively (Haile Yineger *et al.*, 2008). The trend of the rainfall distribution is bimodal namely "belg**"** **(**small rains occurring from February to May) and "kiremt**"** **(**big rains occurring from August to October) (Luizza *et al.*, 2013). Dinsho's loamy, fairly fertile, and low-density Mollic Andosol soils are results of extended weathering of lava outflows stemming from the Oligocene Epoch (33.9 - 23 million years B.P.) (Luizza *et al.*, 2013). It has a typical vegetation type of undifferentiated Afromontane forests (Haile Yineger *et al.*, 2008).

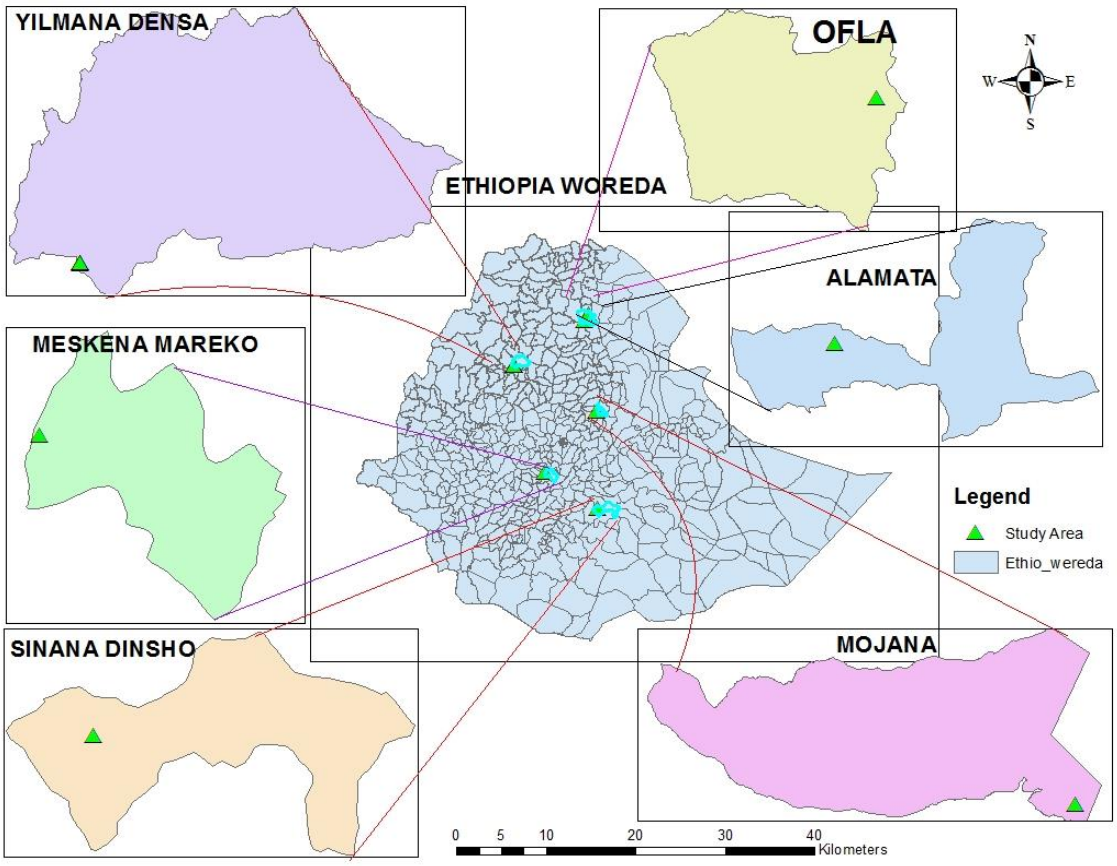


Figure 16: Collection sites of *Thymus* species for the present study

3.2. Data collection

This information about *T. serrulatus* and *T. schimperi* species were collected from informants using semi-structured questionnaires (Appendix 3). The development agents (DAs) in each study site involved in the informant selection and data collection processes. The informants in each study site were farmers who had meetings with the DAs. A total of 140 male informants were randomly selected and asked about the medicinal value, economic advantage, current status, and threatening factors of *Thymus*

species in their localities. Data collection took place from 28th July through 28th September 2013.

3.3. Plant material collection and identification

Aerial parts of *T. serrulatus* and *T. shimperi* were collected from 28th July through 28th September from Ofla (South Tigray), Alamata (South Tigray), Yilmana Densa (West Gojjam), Tarmaber (North Shewa), Butajira (Gurage zone), and Dinsho (Bale zone) in Ethiopia. Plant materials were collected up to the third week of August 2013 and the Ethnobotanical data till 28th September 2013. The collected specimens were pressed and taken to National Herbarium of Addis Ababa University for authentication. They were identified by an expert in the National Herbarium of Addis Ababa University. After identification, voucher specimens were deposited in the National Herbarium of Addis Ababa University with voucher numbers Ofl-2013, Ala-2013, But-2013, Yil-2013, Tar-2013, But-2013 and Bal-2013 respectively.

3.4. Plant material preparation

Plants were first washed by tap water and then by distilled water to remove dirt and debris. The collected pant materials were shade dried at room temperature in the biomedical laboratory of Addis Ababa University. The dried parts were then reduced to powder by an electric mill in Ecophysiology laboratory in order to rupture maximum cell walls of oil glands (Ahmad *et al.*, 2006) until the particles sizes were able to pass through 0.6 mm sieve.

3.5. Extraction and storage of EOs

The fine powder (200 g) of each plant were added to 2 L of distilled water (with vegetal material/ extraction solvent rate = 1/10 (w/v) in a 4L round bottom glass flask and subjected to water distillation for 3 h using Clevenger type apparatus (Figure 17) in Insect Science Laboratory of Zoological Science Department of Addis Ababa University. Then the volume of each oil was quantified in milliliters (mL), dried over anhydrous sodium sulphate and stored in dark glass (Appendix 4: h) at 4°C until used (Imelouane *et al.*, 2009). The end percentage yield (v/w) of volatile oils from each plant based on dry weight was calculated using the formula:

$$\text{Yield (\%)} = v / w \times 100$$

Where "V" is the volume of oil in milliliters (mL) and "w" the weight in grams (g) of milled *Thymus* plant parts (Grigore *et al.*, 2010).

Figure 17: Water distillation of *Thymus* powder (dry weight) using clevenger type apparatus

3.6. Chemical analysis

GC-MS analysis

Individual components of the EOs were analyzed in the laboratory of the Institute of Bioorganic Chemistry, Hohenheim University, Germany. The instruments used were TRACE GC ULTRA Gas Chromatograph (Thermo Electron Corporation) coupled with the mass spectrometer POLARIC Q (Thermo Electron Corporation). For GC-MS analysis the EOs from different regions were prepared as follows: One micro liter of each EO was dissolved in 1mL of dichloromethane (Sigma Aldrich) and 1 µL of this solution was injected. Pure compounds (references) were dissolved and injected in to the GCMS with exactly the same conditions as EOs. The GC analysis of the oil was performed on a GC apparatus, equipped with nonpolar SGE capillary column (30 m × 0.25 mm, 0.25µm film thickness). The carrier gas for the process was helium with constant flow rate of 1.2 mL/min. The column temperature was programmed from 40-300°C at 5°C /min and maintained at 300°C for 10 minutes. The inlet temperature was programmed to be 250°C. The ion source temperature was set to 220°C and the ionization voltage 70 eV. The samples were injected in split mode with a ratio of 1:20 and a split flow rate of 24. Finally, the mass spectra were fully scanned in a range of 50 to 650 (m/z).

NMR analysis

Materials: Six thyme EOs from Ethiopia, deuterated chloroform ($CDCl_3$) (Chloroform d_1 99.8% -Sigma Aldrich) and 5 mm NMR tubes obtained from the Laboratory of the Institute of Bioorganic Chemistry, University of Hohenheim.

Proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR) data for pure compounds and EO components was collected. The ^{1}H NMR spectra (at=5s) were used to get the relative intensities of thymol and carvacrol and carvacrol methyl ether and thymol methyl ether in the EOs. These components of EOs were selected due to the good separation of their signals. In this process, carvacrol was set to one (1) and thymol was integrated with respect to carvacrol. At the same time, carvacrol methyl ether was set to one (1) and thymol methyl ether was integrated with respect to carvacrol methyl ether.

Sample preparation: 20 µL of each EO was diluted in 700 µL of deuterated chloroform ($CDCl_3$) in 5 mm NMR tubes and submitted to the NMR expert.

^{1}H NMR conditions: Spectra were recorded on a 500 MHz spectrometer (Varian ^{Unity}INOVA) equipped with a 5 mm-triple resonance inverse probe. 128 scans were acquired with a spectral width of 1000 Hz, an acquisition time of 5 s and a delay of 1 s. The NMR spectra were processed by SpinWorks software. All ^{1}H NMR spectra were referenced to the residual $CDCl_3$ peak (7.26 ppm).

Identification of compounds

Identification of the compounds in the EOs followed different steps. At the beginning, retention times of the different components of the EOs were compared with retention times of known pure compounds processed with exactly the same GC-MS conditions as EOs. Next, Mass Spectra (MS) of the EO components were compared with that of the pure compounds. MS of EO components were also compared with MS from literature and NIST online library. Finally, the Kovats indexes of the different components of the EOs were calculated and compared with pure compounds and references from literature.

To determine the Kovats indexes, saturated alkanes (C7-C30, SUPELCO®, Sigma Aldrich) were run under exactly the same conditions as for running the EOs. The retention times of the alkanes were recorded and the Kovats retention indexes were calculated using the equation for temperature programmed conditions (H´erent *et al.*, 2007).

$$KI = 100\,[\frac{tx - tn}{t(n+1) - tn} + n]$$

Where KI is the temperature-programmed retention index; t_n, t_{n+1} and t_x the retention time (in minute) of the two *n*-alkanes containing *n* and *n* + 1 carbons and of the compound of interest, respectively. The Kovats indexes were used to get hints about the components. At the end, % peak areas of the components of each EO were taken using Xcalibur software tool and % peak areas ≤ 0.04 were rejected because of their low MS signal intensity (bad signal/noise).

Determination of concentration of components and chemotypes

The percent peak areas under each curve of the chromatograms were used to guess the relative abundances of each component of the EOs. The relative intensities of carvacrol to thymol and carvacrol methyl ether to thymol methyl ether were also integrated by setting percent peak areas of carvacrol and carvacrol methyl ether to one (1). Thus the relative intensities from percent peak areas and NMR results together with their percent peak areas were used to identify the chemotypes of *T. serrulatus* and *T. schimperi*.

3.7. Antibacterial testing of *T. serrulatus* and *T. schimperi* EOs against *S. mutans* and *Lactobacilli*

3.7.1. Collection of unstimulated whole saliva and bacterial isolation

Unstimulated whole saliva and cotton swabs after brushing of the teeth were collected from five male and five female third year biology students of Addis Ababa University Department of Biology according to the guidelines for saliva collection of the University of Southern California School of Dentistry (Navazesh and Kumar, 2008).

Students with age ranges of 20 to 25 years and with a DMFT (Decayed, Missing and Filled Teeth) score of zero were included as subjects to donate saliva and swab samples. On the other hand students who took antibiotics or corticoids in the last 20 days, who have the habit of smoking, with mucosal inflammatory lesions, and who took orthodontic treatment were excluded.

The students were given full explanation about the purpose of the research and signed letters of consent which was approved by the Research Ethics Review Committee of the College of Natural Sciences of Addis Ababa University. Saliva was collected by a trained laboratory technologist in the biomedical laboratory.

The subjects were advised to refrain from intake of any food or beverage one hour before the test session. Smoking, chewing gum and intake of coffee were also prohibited during this hour. They were advised to rinse their mouth several times with distilled water and then to relax for five minutes. They were then told to swallow to void the mouth of

saliva. Then they were told to lean their heads forward over the test tube. They were told to slightly open their mouths and allow saliva to drain into the tubes. At the end of the collection period, they were asked to collect any remaining saliva in their mouths and spit it into the test tubes. In addition, the subjects were asked to brush their teeth using sterile swabs and put them into the test tubes containing their saliva. The method was adapted from Navazesh and Kumar (2008).

The collected saliva and swabs were transferred to the laboratory and placed in a refrigerator until used. The saliva and swabs were vortex mixed and diluted one hundred times in phosphate buffer solution (Murray *et al.,* 2003). After homogenization of the collected saliva, one milliliter of the diluted mixture was spread on petri-dishes containing Mitis Salivarius Agar and MRS (de Man, Rogosa and Sharpe) agar for selectively growing *Streptococcus mutans* and *Lactobacilli* respectively.

3.7.2. Culture method

Vortex mixed saliva and content from swabs (0.5 mL) was diluted with 9.5 mL of sterile water and ten fold serial dilutions of saliva were made up to 10^{-6} before plating. Mitis Salivarius agar (MSA) supplemented with potassium tellurite and Rogosa (MRS) was used as culture media for isolation of total *Streptococci* and *Lactobacilli* respectively. Appropriate amounts of semi solid media were poured on to petri-plates and were allowed to solidify at room temperature. To each plate, 100 µL of diluted sample was spread evenly. The plates were collected and sealed in an anaerobic jar (Figure 18).

Anaerobic atmosphere in the anaerobic jar was created using candle light. Then the plates in the anaerobic jar were incubated at 37 °c for 48 hours.

Figure 18: Anaerobic jar where *S. mutans* on MitisSalivarius agar (MSA) and *Lactobacilli* on Rogosa (MRS) agar were incubated.

3.7.3. Identification and conformation of salivary *Streptococci* and *Lactobacilli*

To isolate salivary streptococci especially *Streptococcus mutans*, 2 bacitracin disks which contain 5 µg bacitracin were placed 2cm apart on the inoculated agar. Bacterial colonies were observed under a dissecting microscope and deduced on the basis of colony morphology, shape and color. Furthermore, conformation for *S. mutans* and *Lactobacilli* was done by Gram staining, catalase tests, arginine dehydrogenase test, and sugar fermentation tests (mannitol, sorbitol, inulin, lactose, raffinose and mellibiose). Aerobic growth was also tested by inserting inverted Durham tubes into test tubes containing broths where the bacteria inoculated. Presence or absence of air bubbles at the bottom of inverted Durham tubes was considered as an indicator of aerobic or anaerobic respiration respectively (www.asmcue.org/.../CarbohydrateFermentationProtocol.ASMCUE.pdf*).*

3.7.4. Biochemical tests to determine salivary *Streptococci* and *Lactobacilli*

These bacteria were allowed to grow in Tryptone Soy Broth containing 0.1% carbohydrates (raffinose, inulin, mannitol, mellibiose, lactose and sorbitol) and 0.5% arginine. In addition, 7.2 mL/L of 0.25% phenol red was used as an indicator for fermentation of the carbohydrates and arginine by the bacteria. The formation of yellow and reddish pink colors respectively after incubation for 48hrs was considered as positive and negative results of carbohydrate fermentation (MacFaddin, 2000). On the other hand, the negative and positive fermentation results towards arginine were indicated by formation of yellow or red and purple colors respectively at the end of 48hrs incubation.

3.7.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC) was determined using agar dilution method which is described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID, 2000). The following procedure was followed to determine the MIC: 20-milliliter volumes of agar were used in 9-cm Petri dishes for agar dilution. 19 mL volumes of molten agar (Mitis Salivarius Agar and MRS Agar) were added to 1 mL volumes of each EO dose to make the total volume 20 mL.

Mitis Salivarius Agar and MRS Agar prepared as recommended by the manufacturers were set to cool to 50°C in a water-bath. EOs of *thyme* were prepared into doses of 0.25

µL/mL, 0.5 µL/mL, 1 µL/mL, 2 µL/mL, and 4 µL/mL in 25-30mL containers (five containers for each selective medium). Nineteen-mL of molten agars were added to each container and mixed thoroughly, and finally poured into pre-labeled sterile Petri dishes on a level surface. The plates were allowed to dry at room temperature to avoid drops of moisture on the surface of the agar. Bacterial suspensions grown in Tryptic Soy broths supplemented with 0.2 % glucose (Mcghie *et al.,* 1977; Bokhout *et al.,* 1996) and incubated for 48hrs were inoculated on the dry plates. The inoculum spots were then allowed to dry at room temperature before inverting the plates for incubation. Finally, the plates were incubated at 37 °C in anaerobic jars for 48 h. The MIC (the lowest concentration of the extracts that completely inhibited visible growth) was judged by the naked eyes. MBC was determined by taking scratches from MIC tests and trying to grow bacteria on new agar plates. In the case of growth of colonies from scratches taken from the MIC test, the MIC was not considered as MBC and viceversa.

3.7.6. Determination of the antibacterial activities of the EOs

Susceptibility tests against *S. mutans* and *Lactobacilli* were accomplished using disk diffusion method with Mitis Salivarius agar (Phankhongsap *et al.,* 2012; Penmetsa *et al.,* 2014) and MRS agar respectively. Paper disks (6mm diameter) immersed in EO doses of 16 µL/mL, 32 µL/mL, 64 µL/mL and 128 µL/mL were allowed to dry at room temperature and placed on petri-plates inoculated with salivary *S. mutans* and *Lactobacilli*. Other disks immersed in 3 percent DMSO and 3 percent hydrogen peroxide (bought from Ambessa pharmacy) were also used as negative and positive controls respectively.

3.7.7. Ethical considerations

This study was conducted after the necessary ethical clearance was obtained from Addis Ababa University, College of Natural Sciences, Addis Ababa University, Research Ethics Review Committee (Appendix 1).

3.8. Determination of hepatoprotective activity

3.8.1. Determination of serum ALT, AST, and ALP Levels

The methods described by Pinho *et al.* (2014) and Grespan *et al.* (2014) were used for this study. Male Wistar albino rats with weight ranges of 148 to 224 grams were divided into six groups of five animals each. Each group was orally pretreated for six days as follows: Group I and II received normal saline containing 0.1% T-80 (APAP, TEO vehicle); Group III was delivered with Silybinin (100mg/kg) (Dar *et al.,* 2012; Roy *et al.,* 2012; El-Banna *et al.,* 2013). Groups IV, V, and VI received EOs of Tar, Yil, and Ala respectively at a dose of 200 mg/kg. On the seventh day, 30 minutes after treatment administration (Sindhu *et al.,* 2012), rats in groups II to VI were administered with oral APAP at a dose of 2g/kg body weight (Galal *et al.,* 2012; Sindhu *et al.,* 2012; Abd-Algader *et al.,* 2013) and group I received only APAP vehicle (T-80).

On the eighth day, the rats were anaestized with chloroform, and blood was collected using heart puncture for the determination of serum AST, ALT and ALP assays. For this purpose, blood samples were collected allowed to clot, and centrifuged at 3000 × g for 15 min. After centrifugation, serum was collected using micropipetes transferred to vials, labled and transferred to the TB-HIV laboratory of Ethiopian Public Health Institute

(EPHI). Serum AST, ALT and ALP levels were then measured using Cobas Integra 400 plus analyzer (Hoffmann-La Roche Ltd).

3.8.2. Histopathological analysis.

The livers were washed in 0.9% (w/v) sodium chloride solution and placed in 10% neutral buffered formalin for fixation till subjected to sectioning. The 10% neutral buffered formalin for fixation was prepared according to Sigma-Aldrich product information (Sigma-Aldrich, 2012) by mixing formaldehyde, 37-40% 100 ml/L; sodium phosphate, monobasic (4.0 g/L) and sodium phosphate, dibasic (anhydrous) (6.5 g/L) to 900 ml/L of distilled water. Afterwards, the livers were administered to paraffin embedded and sectioned in semiserial at a 6 μm thickness on a Leica rotary microtome (Leica Microsystems, Gladesville, New SouthWales, Australia). The sections were stained with hematoxylin and eosin to estimate tissue morphology using light microscopy (Olympus BX-41, Tokyo, Japan) (Grespan *et al.*, 2014). Finally, tissue sections were observed by a pathologist from Bahir Dar University.

3.9. Determination of larvicidal, oviposition deterrent, and mosquitocidal activities of EOs against *An. arabiensis*

3.9.1. Larvicidal bioassay

Larvae of *An. arabiensis* were reared in larval rearing trays in the insectary of the Ethiopian Public Health Institute (EPHI). Larvae were supplied with larval food (brewery yeast and dog biscuit). The EOs of Ala, Yil, and Tar were made in concentrations of 12.5, 25, 50, 100, and 200 µL/L in acetone (Appendix 4: r). Ala, Yil, and Tar were selected

because they were found to be highly thymol (Ala = 65.63%), carvacrol (Yil = 80.84%), and thymol-carvacrol (Tar = 48.84%: 42.12%) chemotypes. A batch of 25, 3rd and 4th instar larvae of *An. arabiensis* were placed in 300 mL white enamel cups containing 149 mL of distilled water (Fekadu Massebo *et al.,* 2009). After 3 hours of larval acclimatization, 1 mL of desired concentration of Thyme EOs (12.5, 25, 50, 100, and 200 µL/L) were added to each beaker to make a final volume of 150 mL. Equal volume of acetone was used as a negative control. Larval mortality was recorded 24 hrs of post exposure. Larvae were confirmed dead when they failed to move after probing them with a needle (WHO, 2005).

3.9.2. Oviposition deterrent bioassay

Fifteen gravid females (8–10 days old, 4 days after blood feeding) were transferred to each mosquito cage (45×30×30 cm). Serial dilutions of EOs were made in DMSO. In the multiple concentration test, five cups, each containing 100 mL distilled water with a 9-cm piece of white filter paper for oviposition as well as EOs at a concentration of 12.5, 25, 50, 100, and 200 µL/L were placed in each cage. A sixth cup containing DMSO served as a negative control in each cage (Appendix 4: s). The positions of the plastic cups were alternated between the different replicates so as to nullify any effect of position on oviposition. Three replicates for each concentration were run with cages placed side by side for each bioassay. A sucrose solution (10%) was available at all times as food for mosquitoes. The room was set to have a temperature of 26 °C and relative humidity of 75–85%. After 24 h, the number of eggs laid in treated and control cups was counted

under a hand lens. The percent effective repellency for each concentration was calculated using the following formula (Elango *et al.,* 2009).

$$\%ER = \frac{NC-NT}{NC} \text{ X } 100$$

Where ER=effective repellency, NC=number of eggs in control, and NT=number of eggs in treatment.

The oviposition experiments were expressed as mean number of eggs and oviposition activity index (OAI) was calculated using the following formula (Elango *et al.,* 2009).

$$OAI = \frac{NT - NS}{NT + NS}$$

Where NT=total number of eggs in the test solution and NS= total number of eggs in the control solution. EO doses with oviposition active index of +0.3 and above were considered as attractants while those with −0.3 and below as repellents (Elango *et al.,* 2009).

3.9.3. Fumigation test

Adulticidal activity was tested by airtight fumigation in conical flasks, as described by Pavela *et al.* (2009). Twenty non-blood-fed female mosquitoes (2–6 days old) were placed in 250 mL conical flasks (Appendix 4: t). A 10-µl volume of the EO solution of five doses; 3.125, 6.25, 12.5, 25, and 50 µL/L in acetone were immediately dropped onto a filter paper (1×3 cm) with a micropipettee. A cork stopper was used to seal each conical flask, and filter paper was placed at the base of the cork. The controls were treated under the same conditions with pure acetone.

The conical flasks were sealed tightly, and placed in a room at a temperature of 26°C for 4 hours (WHO, 2006). Mortality was determined 24 h post exposure after being transferred to clean cages.

3.9.4. Determination of lethal dose 50% (LD_{50}) for larvicidal and fumigation tests

This was done using the arthemetical method of Karber (Pandey and Sahni, 2013). The interval mean of the number of larvae/mosquitoes that died in each group, and the difference in doses for the same interval were used. The results from the dose larger than the *'least tolerated dose'* (100% mortality, LD_{100}) and the dose smaller than *'most tolerated dose'* (0% mortality, LD_0) were not used. Generally, (i) the animals that died in each group were determined, (ii) dose differences were determined, (iii) the mean of maximum number of dead insects in two adjacent doses were calculated, (iv) the products of means and dose differences were calculated, (v) the sum of the products (probbit) was taken, and finally (vi) the sum of the products (probbits) was divided by the number of larvae/mosquitoes in a group which in turn was subtracted from the *'least tolerated dose'* to obtain the LD_{50} (Table 3).

Table 3: Template for LD_{50} calculation

Group	No. of animals in each group	Dose (µL/mL)	No. of animals dead	Dose difference (a)	Mean mortality (b)	Probbit (a*b)
1	N	Vehicle	0			
2	N	Dose 1	0			
3	N	Dose 2	n_1	Dose 2- Dose 1 = a_1	$(n_1+0)/2 = b_1$	$a_1 * b_1$
4	N	Dose 3	n_2	Dose 3- Dose 2 = a_2	$(n_2+ n_1)/2 = b_2$	$a_2 * b_2$
5	N	Dose 4	n_3	Dose 4- Dose 3 = a_3	$(n_3+ n_2)/2 = b_3$	$a_3 * b_3$
6	N	Dose 5	n_4	Dose 5- Dose 4 = a_4	$(n_4+ n_3)/2 = b_4$	$a_4 * b_4$
7	N	Dose 6	n_5	Dose 6- Dose 5 = a_5	$(n_5+ n_4)/2 = b_5$	$a_5 * b_5$
8	N	∗LTD	n_6	LTD - Dose 6 = a_6	$(n_6+ n_5)/2 = b_6$	$a_6 * b_6$
						$\sum (a*b) =$ --

∗ LTD = Least tolerated dose; Dose 1= the least dose for each test (e.g. 12.5 µL/L for larvicidal and oviposition deterrent tets and 3.125 µL/L for fumigation tests); Doses 2-6 = 2^{nd} to 6^{th} doses of each test.

$$LD_{50} = \text{Least tolerated dose} - \sum (a*b)/N$$

Where (a= the dose difference), (b= mean mortality), (N= number of animals in each group)

3.10. Acute oral toxicity testing

3.10.1. Animal handling

Animals for hepatotoxicity and toxicity experiments were handled ethically according to the guideline developed by Guide for the Care and Use of Laboratory Animals Developed by the National Academy of Sciences (2011).

Experimental Animals: Animals were selected as per the OECD guideline for testing chemicals 420. Healthy young and nulliparous, non-pregnant female mice weighing from 19 to 27 gm and with age ranges of 8 – 12 week olds were selected. Female mice were used because literature surveys of conventional LD_{50} tests show that usually there is little difference in sensitivity between the sexes, but in those cases where differences are observed, females are generally slightly more sensitive (OECD, 2001). These animals were randomly grouped, marked, and kept in cages for five days before the experiment to acclimatize to the laboratory conditions at room temperature. The sequence of lighting was 12 hours light and 12 hours dark. Unlimited conventional laboratory diet and drinking water was made available.

Doses were prepared by varying the concentration of EOs. At the same time each mouse was given the preparations in a single dose by gavage and the volume to be delivered for each mouse was in 1 mL/kg body weight calculation.

Test Procedure followed

Prior to the dosing, the mice were fasted for 4 hours from food but not from water during this time. After fasting, the weight of each mouse was determined and the dose was calculated based on the body weight. After substance administration, food but not water was withheld for a further one hour (OECD, 2001). The procedure of dosing started from 2000 mg/kg body weight and followed Annex 3 of OECD guideline 420 (Figure 19). This 2000 mg/kg body weight was selected due to the fact that *T. serrulatus* and *T. schimperi* are used as human food additives in different localities of Ethiopia (UN, 2011).

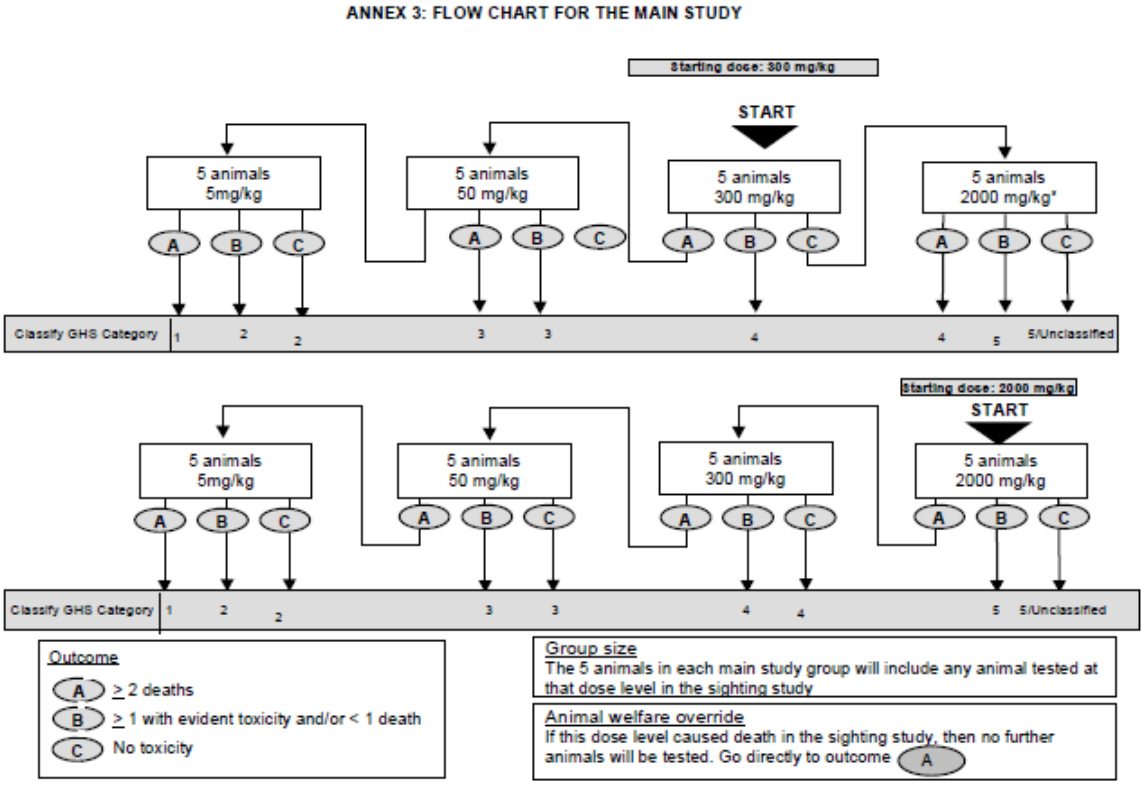


Figure 19: Procedure for acute toxicity testing (Adapted from (OECD, 2001)

Thirty-five female mice were randomly assigned in to seven groups each group containing five animals. Mice in Group I (control group) were administered with

calculated amounts of 0.1% Tween-80 in normal saline, the vehicle for EO administration (Grespan *et al.,* 2014; Pinho *et al.,* 2014), Groups II –VII were delivered with 2000 mg/Kg body weight of Ofl, Ala, Yil, Tar, Buta and Bal EOs respectively. The EO dose 2000 mg/Kg body weight of the EOs was made in a vehicle (normal saline containing 0.1% T-80 (Grespan *et al.,* 2014; Pinho *et al.,* 2014).

Observations made: Animals were observed individually after dosing once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. The toxic reactions, time of onset and length of recovery period were noted. All observations were systematically recorded, with individual records being maintained for each animal.

Individual weights of animals were determined shortly before the test substance was administered, on the 24^{th} hour, 48^{th} hour, and 7^{th} and 14^{th} days. Weight changes were calculated and recorded. At the end of the test surviving animals were weighed and then humanely killed. The hearts, kidneys, livers, brains, lungs, and spleen of the humanely killed mice were weighed so that body to organ weight ratios were calculated and compared with that of the control mice (OECD, 2001). These organs were selected according to the recommendation given by Sellers *et al.* (2007). In addition packed Cell Volumes (PCVs) of each mouse was measured.

Observations included changes in skin and fur, eyes and mucous membranes and behavioral pattern. Attention was given for observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep, coma and mortality (OECD, 2001) (Appendix 2).

Evaluation of the LD_{50} Values: The LD_{50} values of the EOs were determined based on the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (UN, 2011).

3.11. **Statistical analysis**

Data for serum levels of AST, ALTand ALP were expressed as mean ± SEM and analyzed statistically using One Way Analysis of Variance (ANOVA) followed by LSD Post Hoc Multiple Comparisons. The minimum level of significance was set at $P<0.05$. Statistics was computed using SPSS programme version 20 and SAS 9.2.

Chapter 4- Results

Chapter 4: Results

4.1. Types, medicinal values, and economic importance of *Thymus* species from six localities in Ethiopia

4.1.1. Identified thyme species collected from six localities in Ethiopia

Experts from National Herbarium used standard methods of identification to identify *Thymus* species. Morphological appearance of the leaves was one of the characteristic features to differentiate between the two species. Those with leaf blades ovate, elliptic to lanceolate and with leaf margins entire were identified as *T. schimperi* and those having leaf blades obovate to oblanceolate with leaf margin crenate in the upper ⅓ to ½ were identified as *T. serrulatus* (Hedberg *et al.*, 2006). The appearances of the leaves are presented in Figures 20 and 21. Finally, thyme specimens collected from three of the localities (Ofla, Alamata, and Yilmana Densa) were found to be *T. serrulatus* and those collected from the other three localities (Tarmaber, Butajira, and Bale) as *T. schimperi*.

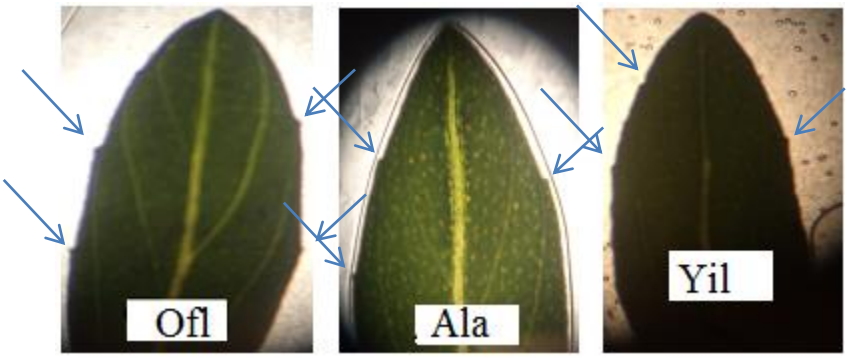


Figure 20: Morphology of of *T. serrulatus* leaves (arrows show crenate points on the leaf margines).

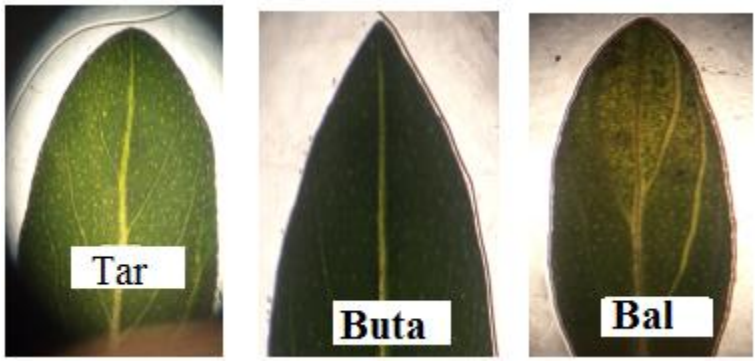


Figure 21: Morphology of *T. schimperi* leaves.

4.1.2. Medicinal values of *T. serrulatus* and *T. schimperi* in Ethiopia

The majority of the respondents [43 (30.7%)] have at least heard about the use of *Thymus* species as treatments for blood pressure although a lot of them [33 (23.5%)] have no information about the health significances of these species. On the other hand, [52 (37.1%)] of the respondents have mentioned that these plants have applications to treat general pain syndrome, influenza and abdominal pain (Table 4). Some respondents from Tigray Region mentioned the ascaricidal [4 (2.9%)] and intestinal paraciticidal [4 (2.9%)] effects of *T. serrulatus* grown in Tigray. In almost all the localities, the responses mentioned that it is the aerial parts of *T. serrulatus* and *T. schimperi* which are dried, crushed, made into tea and taken orally to treat the ailments mentioned.

Table 4: Human ailments reported to be treated by *T. serrulatus* and *T. schimperi*

Human disease	Scientific name	Family	Local name *	Voucher λ No.	Part used	Form used	Methods of preparation	Route of admn.	Respon-dents
	T. schimeri + *T. serrulatus*	*Lamiac -eae*	Tosign (Amh) Toshigne (Gur) Tosigni (Oro) Teshne (Tig)	Ofl-2013, Ala-2013, Yil-2013, Tar-2013, Buta-2013, Bal-2013,					
Blood pressure	>>	>>	>>	>>	Leaves and flowers	Dried	Crushed and drunk as tea	Oral	43 (30.7%)
General pain syndrome	>>	>>	>>	>>	Leaves and flowers	Dried	Crushed and drunk as tea	Oral	14 (10%)
Influenza	>>	>>	>>	>>	Leaves and flowers	Dried	Crushed and drunk as tea	Oral	14 (10%)
Abdominal pain	>>	>>	>>	>>	Aerial parts	Dried	Crushed and drunk as tea	Oral	14 (10%)
Ascaris	>>	>>	>>	>>	leaves	Dried	Drunk as tea	Oral	4 (2.9%)
Intestinal parasites	>>	>>	>>	>>	leaves	Dried	Drunk as tea	Oral	4 (2.9%)
I don't know	>>	>>	>>	>>	-	-	-	-	33 (23.5%)

∗Amh- Amharic; Gur- Guragigna, Tig- Tigrigna, Oro- Oromipha

λ Ofl- *T. serrulatus* from Ofla, Ala- *T. serrulatus* from Alamata, Yil- *T. serrulatus* from Yilmana Densa, Tar- *T. schimperi* from Tarmaber, But- *T. schimperi* from Butajira, Bal- *T. schimperi* from Bale.

4.1.3. Economic advantages of *T. serrulatus* and *T. schimperi* in Ethiopia

The respondents mentioned the economic uses of *Thymus* species in Ethiopia as honey bee forage [100 (71.5 %)], animal forage [100 (71.5%)], food additives (condiments) [95 (68%)], tea [95 (68%)], and washing and fumigating household utensils such as buckets for milking and dough preparation [65 (46%)] (Table 5). Such fumigation of milking jars and buckets for putting dough of injera maintain milk and injera with best flavors and without rancidity. According to the respondents, the honey from *Thymus* species has medicinal value and with special taste. Milk, yogurt, butter, and meat from animals fed with *Thymus* species have special taste and flavor. In addition, application of *Thymus* species as food additives increases the flavor and shelf-life of foods and sauces such as shiro, berbere, butter, Besso etc. Furthermore, the respondents have mentioned that *Thymus* as an animal forage is useful for fattening. Furthermore fumigating the honey bee hives attracts honey bees and eliminates honey bee diseases as was raised by the respondents from Southern Tigray. During interview with the respondents, it was clear that people from other areas of the country use *Thymus* species as food additives than people in Southern Tigray. The people in Tigray know that it can be used as tea but most of them do not use it.

Table 5: Economic uses of *T. serrulatus* and *T. schimperi* (n=140)

Scientific name	Family	Local name *	Voucher λ No.	Economic use	Respondents
T. schimeri + *T. serrulatus*	*Lamiaceae*	Tosign (Amh) Toshigne (Gur) Tosigni (Oro) Teshne (Tig)	Ofl-2013 Ala-2013 Yil-2013 Tar-2013 Buta-2013 Bal-2013		
T. schimeri + *T. serrulatus*	*Lamiaceae*	>>	>>	Bee forage	100 (71.5 %)
T. schimeri + *T. serrulatus*	*Lamiaceae*	>>	>>	Animal forage	100 (71.5%)
T. schimeri + *T. serrulatus*	*Lamiaceae*	>>	>>	Condiment (additive to shiro, berbere, butter, Besso)	95 (68%)
T. schimeri + *T. serrulatus*	*Lamiaceae*	>>	>>	Drink (tea)	95 (68%)
T. schimeri + *T. serrulatus*	*Lamiaceae*	>>	>>	Washing and fumigating jars for milking and buckets for putting paste of injera	65 (46%)

∗Amh- Amharic; Gur- Guragigna, Tig- Tigrigna, Oro- Oromipha

λ Ofl- *T. serrulatus* from Ofla, Ala- *T. serrulatus* from Alamata, Yil- *T. serrulatus* from Yilmana Densa, Tar- *T. schimperi* from Tarmaber, But- *T. schimperi* from Butajira, Bal- *T. schimperi* from Bale.

4.1.4. Current status and threatening factors of *T. serrulatus* and *T. schimperi* in Ethiopia

According to the respondents' responses, *Thymus* species endemic to Ethiopia (*T. serrulatus* and *T. schimperi*) exist as wild species and their current status is decreasing from year to year (Table 6). The major threatening factors for these species were identified to be overgrazing followed by agricultural expansion, overharvesting, uprooting during harvesting, and lack of recognition. This reduction in *Thymus* species is high in North Shewa and Gurage zone due to the mentioned threatening factors. However, the situation is better in Tigray (Alamata and Ofla), Yilmana Densa (West Gojjam) and Dinsho (Bale) since the collection sites in these areas are closed from human and animal encroachment. Uprooting during harvesting is the biggest problem in North Shewa due to the fact that the plant is an income source for inhabitants there. It is usual to see the youth and women selling the dried plant parts to travellers on the high way from Addis Ababa to North Ethiopia (Wello and Tigray).

Table 6: Current status and threatening factors of *T. serrulatus* and *T. schimperi* in Ethiopia (n =140)

Scientific name	Family	Local Name	Occurence	Current status	Threatening factors	Respondents
T. schimeri + T. serrulatus	*Lamiaceae*	Tosign (Amh) Toshigne (Gur) Tosigni (Oro) Teshne (Tig)	wild			
>>	>>	>>	>>	Decreasing	Overharvesting	68 (48.57 %)
>>	>>	>>	>>	Decreasing	Overgrazing	113 (80.7 %)
>>	>>	>>	>>	Decreasing	Lack of Recognition	19 (13.6 %)
>>	>>	>>	>>	Increasing	Due to its high seed production	2 (1.4 %)
>>	>>	>>	>>	Decreasing	Agricultural expansion	90 (64.2 %)
>>	>>	>>	>>	Decreasing	Uprooting during harvesting	20 (14.2 %)

4.2. Calculated yield of EOs from *T. serrulatus* and *T. schimperi* in Ethiopia

The EO yields of *T. serrulatus* and *T. schimperi* were 0.9% (Bal), 0.8% (Yil, Tar, and Buta) and 0.5% (Ala and Ofl) (Table 7).

Table 7: Essential oil yield of *T. schimperi* and *T. serrulatus* in Ethiopia (based on dry weight)

No	*Thymus* species collected from	Yield (%)
1	*T. serrulatus* from Ofla (Ofl)	0.5
2	*T. serrulatus* from Alamata (Ala)	0.5
3	*T. serrulatus* from Yilmana Densa (Yil)	0.8
4	*T. schimperi* from Tarmaber (Tar)	0.8
5	*T. schimperi* from Butajira (Buta)	0.8
6	*T. schimperi* from Bale (Bal)	0.9

4.3. Chemical composition of *Thymus* Species from six localities in Ethiopia

The chemical composition of EOs from *Thymus* species were seen from the retention times and peaks in Gas Chromatograms (Appendix 4: V to Z_1). In all the six EOs, the strongest peaks were thymol and carvacrol at retention times close to 20.23 and 20.46 minutes respectively.

Components of the six *Thymus* EOs identified using mass spectra (MS), Kovat's index (KI), comparison with reference (Ref) compounds, and NMR are presented in Table 8. Ala was found to be the most diverse EO containing 42 components followed by Bal (39 components), Buta (32 components), Ofl (31 components), Yil (30 components) and Tar (18 components). Of the components from each EO, some were confirmed using MS, KI, and Ref; some others using MS and KI and the rest remained unidentified using the laboratory setup we had during our work. Eight, 14, 7, 4, 6, and 12 components of Ofl, Ala, Yil, Tar, Buta and Bal respectively were not identified by the existing laboratory setup.

Identification of EOs was perfect for those identified using MS, KI and comparison with reference compounds (Ref) (Table 8). Fifteen components from Ofl, 16 components from Ala, 16 components from Yil, 10 components from Tar, 17 components from Buta and 11 components from Bal were identified in this way. From 33 reference compounds run in GCMS, 17 were confirmed to exist in EOs. The next groups of EO components were identified using MS and KI (Table 8). These include 8 components from Ofl, 12 components from Ala, 7 components from Yil, 4 components from Tar, 9 components

from Buta and 16 components from Bal. In addition, indicators for the presence of 1-octen-3-ol (exomethylene group), thymol methyl ether and carvacrol methyl ether (methoxy groups) were seen using NMR. Proton NMR also revealed the presence of thymol and carvacrol respectively in the EOs. Furthermore, signals of linalool were seen by proton NMR.

Table 8: Essential oil composition of *T. serrulatus* and *T. schimperi* collected from six localities in Ethiopia.

No	Compound	*Thymus serrulatus*				*Thymus schimperi*			Identification
		Ofl		Ala	Yil	Tar	Buta	Bal	
		KI	% Area	% Area	% Area	% Area	% Area	% Area	
1	Unidentified	986	--	--	--	0.22	--	--	
2	β-Pinene	979	--	--	0.09	--	0.09	--	MS, KI, Ref
3	1-octen-3-ol	982	0.11	0.14	0.19	--	0.19	0.11	MS, KI, Ref, (NMR)
4	Unidentified	989	--	0.05	--	--	--	0.04	
5	Octan-3-ol	996	0.04	0.09	0.27	--	0.2	0.24	MS, KI, Ref
6	α-Terpinene	1017	0.07	0.17	0.14	0.14	0.21	0.11	MS, KI, Ref
7	P-cymene	1023	3.06	4.84	3.65	1.88	3.75	3.2	MS, KI, Ref
8	Limonene	1030	--	0.07	0.06	--	0.05	0.06	MS, KI, Ref
9	Unidentified	1040	--	--	0.04	--	--	--	
10	γ–terpinene	1058	0.31	0.42	0.3	0.31	0.68	0.29	MS, KI, Ref
11	cis-sabinene hydrate	1070	0.15	0.38	0.14	0.28	0.54	0.19	MS, KI
12	Terpinolene	1086	0.04	0.07	0.05	--	0.05	0.07	MS, KI, Ref
13	α-Terpinolene	1089	--	0.04	--	--	--	--	MS, KI
14	Linalool	1097	0.42	3.29	0.25	2.97	1.3	2.6	MS, KI, Ref
15	Unidentified	1101	0.1	--	0.1	0.26	--	--	--
16	l-terpineol	1124	--	--	--	--	0.04	0.04	MS, KI
17	Unidentified	1143	--	--	--	--	--	0.04	--
18	Unidentified	1072	--	0.04	--	--	0.04	0.04	--
19	Terpinine-4-ol	1181	0.34	0.45	0.5	0.51	0.45	0.44	MS, KI

20	Para-cymen-8-ol	1185	0.07	0.1	0.1	0.13	0.14	0.12	MS, KI
21	α-terpineol	1194	0.18	0.36	0.37	0.95	0.18	0.41	MS, KI, Ref
22	Thymol methyl ether	1229	2.73	6.55	1.32	0.1	0.1	0.49	MS, KI, Ref, (NMR)
23	Carvacrol methyl ether	1239	0.89	0.82	2.71		1.32	0.29	MS, KI, Ref, (NMR)
24	Unidentified	1248	--	0.14	--	0.13	0.09	--	--
25	Unidentified	1282	0.08	0.12	--	0.21	--	0.1	--
26	Thymol	1292	49.55	65.63	6.52	48.84	15.77	53.57	MS, KI, Ref, (NMR)
27	Carvacrol	1300	36.34	6.68	80.84	42.12	71.83	34.55	MS, KI, Ref, (NMR)
28	Unidentified	1352	--	--	--	--	0.04		--
29	Unidentified	1390	--	--	0.07	--	--	0.08	--
30	cis-caryophyllene	1412	--	0.05	--	--	--	0.04	MS, KI
31	t-caryophyllene	1426	0.9	1.5	0.82	0.43	0.69	0.49	MS, KI, Ref
32	Unidentified	1437	0.06	0.06	0.1	--	0.04	0.16	--
33	(z)-β-farnesene	1446	0.17	0.14	--	--	0.1		MS, KI
34	α-Humulene	1463	--	0.14	--	--	--	0.04	MS, KI
35	allo-aromadendrene	1469	--	0.13	--	--	--	0.07	MS, KI
36	Unidentified	1481	--	0.07	0.06	--	0.07	0.07	--
37	β-Selinene	1488	--	--	0.04	--	--	--	--
38	Valencene	1500	0.15	0.18		--	0.16	0.04	MS, KI, Ref
39	γ-Cadinene	1510	1.61	1.46	0.05	--	0.04	0.05	MS, KI
40	δ-Cadinene	1520	0.07	0.08		--	0.06	0.09	MS, KI
41	α-Cadinene	1525	0.19	0.2	0.14	--	0.14	0.28	MS, KI

42	Unidentified	1543	--	--	0.05	--	--	--	--
43	Unidentified	1579	0.05	0.07	--	--	--	--	--
44	Caryophyllene oxide	1584	1.11	2.18	0.29	0.21	0.94	0.18	MS, KI, Ref
45	Viridiflorol	1590	0.89	2.18	0.69		0.62	0.98	MS, KI
46	Unidentified	1602	--	0.16	--	--	--	0.21	--
47	Unidentified	1609	--	0.13	--	--	--	--	--
48	Unidentified	1618	--	0.31	--	--	--	0.08	--
49	Unidentified	1635	--	--	--	--	--	0.04	--
50	Unidentified	1641	0.15	0.17	--	--	0.07	--	--
51	Unidentified	1647	0.07	0.05	--	--	--	0.04	--
52	Unidentified	1662	0.05	0.08	--	--	--	--	--
53	Unidentified	1677	0.05	--	0.05	--	--	0.06	--
	Total	--	100	99.79	100	99.69	99.99	100	--

The prominent essential oil components from the six *Thymus* EOs are presented in appendix Z_8. 1-Octen-3-ol, Octan-3-ol, α-terpinene, p-cymene, γ-terpinene, cis-sabinene hydrate, linalool, terpinene-4-ol, α-terpineol, thymol methyl ether, carvacrol methyl ether, thymol, carvacrol, t-caryophyllene, γ-cadinene, caryophyllene oxide, and viridiflorol were found in almost all the essential oils. Other components like β-pinene, on the other hand, are limited only to some EOs (*T. serrulatus* from Yilmana Densa and *T. schimperi* from Butajira).

These components can further be classified as hydrocarbons (1-octen-3-ol and Octan-3-ol), monoterpenes (β-pinene, α-terpinene, p-cymene, γ-terpinene, cis-sabinene hydrate, linalool, terpinene-4-ol, α-terpineol, thymol, carvacrol); monoterpene derivatives (thymol

methyl ether, carvacrol methyl ether); sesquiterpines (t-caryophyllene, γ-cadinene, valencene); and oxygenated sesquiterpenes (caryophyllene oxide, viridiflorol).

4.3.1. Chemotypes of *T. serrulatus* and *T. schimperi*

As can be seen from Table 8 above, the percent area of thymol and carvacrol is more than that of the rest components one after the other. The percent concentration of thymol to carvacrol was found as follows: 49.55 to 36.34 (*T. serrulatus* from Ofla), 65.63 to 6.68 (*T. serrulatus* from Alamata), 6.52 to 80.48 (*T. serrulatus* from Yilmana Densa), 48.84 to 42.12 (*T. schimperi* from Tarmaber), 15.77 to 71.83 (*T. schimperi* from Butajira), and 53.57 to 34.55 (*T. schimperi* from Bale). When these relative concentrations are integrated by setting percent area of carvacrol one (1), results in Table 9 were found.

The relative intensities of these two components from NMR (Table 9) had also similar ratios as that of GCMS (relative concentrations). Therefore, *T. serrulatus* from Ofla, *T. serrulatus* from Alamata, *T. schimperi* from Tarmaber and *T. schimperi* from Bale were found to be thymol chemotypes. The other two; *T. serrulatus* from Yilmana Densa and T. *schimperi* from Butajira were found to be carvacrol chemotypes. Looking at Tables 9 and 10, one can generalize that both the GCMS and NMR were good measures of component analysis since similar values of relative intensities of thymol to carvacrol as well as thymol methyl ether to carvacrol methyl ether were obtained using both methods.

Table 9: Relative concentrations and relative intensities of carvacrol to thymol integrated from GCMS and NMR results respectively.

EO	Method				Chemotype
	GCMS		NMR		
	Carvacrol	Thymol	Carvacrol	Thymol	
T. serrulatus from Ofla	1	1.36	1	1.3	Thymol
T. serrulatus from Alamata	1	9.82	1	8.1	Thymol
T. serrulatus from Yilmana Densa	1	0.08	1	0.09	Carvacrol
T. schimperi from Tarmaber	1	1.16	1	1.1	Thymol
T. schimperi from Butajira	1	0.22	1	0.25	Carvacrol
T. schimperi from Bale	1	1.55	1	1.57	Thymol

(EO) Essential Oil; (GCMS) Gas Chromatography Mass Spectrometry; (NMR) Nuclear Magnetic Resonance.

Table 10: Relative concentrations and relative intensities of carvacrol methyl ether to thymol methyl ether integrated from GCMS and NMR results respectively

EO	Method			
	GCMS		NMR	
	C.Met.et	T.Met.et	C.Met.et	T.Met.et
T. serrulatus from Ofla	1	1.3	1	1.3
T. serrulatus from Alamata	1	7.98	1	7.5
T. serrulatus from Yilmana Densa	1	0.5	1	0.5
T. schimperi from Tarmaber	1	trace	-	-
T. schimperi from Butajira	1	0.08	1	0.05
T. schimperi from Bale	1	1.68	1	1.75

(-) trace amounts; (C.Met.et) carvacrol methyl ether; (T.Met.et) thymol methyl ether; (EO) Essential Oil; (GCMS) Gass Chromatography Mass Spectrometry; (NMR) Nuclear magnetic resonance.

The relative intensities of carvacrol and thymol were integrated using first proton of carvacrol and the 6^{th} proton of thymol. They are indicated in Appendix 4 (Z_1 - Z_7**).**

4.4. Antibacterial activity of thyme EOs

4.4.1. Isolation of *S. mutans* and *Lactobacilli*

The bacteria of interest grew as separate colonies on selective media i.e. individual colonis of *S. mutans* and *Lactobacillus* grew on Mitis Salivarius agar (MS agar) and MRS agar respectively (Appendix 4: i and j). Both isolates produced yellow colors by fermenting the sugars raffinose, mannitol, melibiose, lactose, inulin and sorbitol. Both isolates grown in broths supplemented by arginine produced brick red color (Figure 22). They too were not able to release gas fom hydrogen peroxide and they were unable to generate gas from glucose (Figure 23). The gram stain of the isolates revealed gram positive spherical chains (*Streptococci*) and cylinderical cells (*Lactobacilli*) (Figure 24a and 24b respectively). Furthermore, *Streptococcus* was found to be resistant to Bacitracin discs (5 µg/disc) (Appendix 4: i).The overall confirmatory test results of salivary isolates are presented in Table 11.

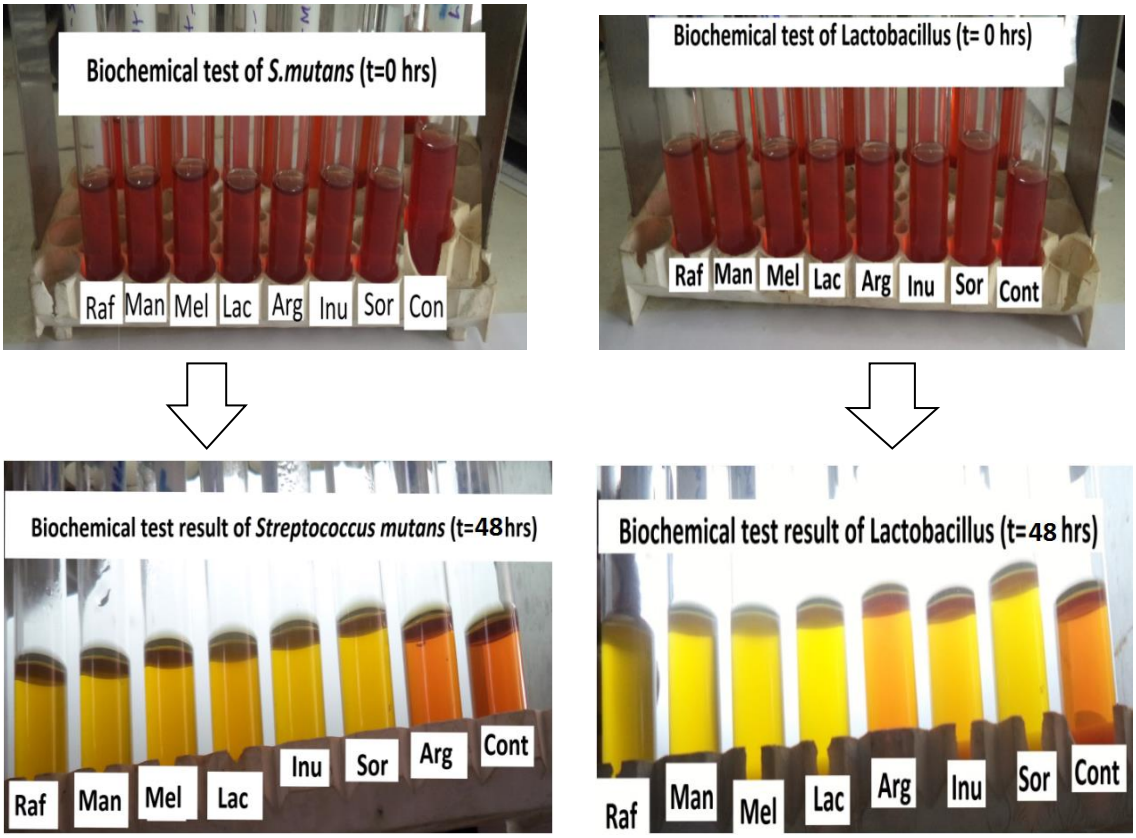


Figure 22: Sugar hydrolysis and arginine hydrolysis test results of *S. mutans* (a) and *Lactobacillus* (b)

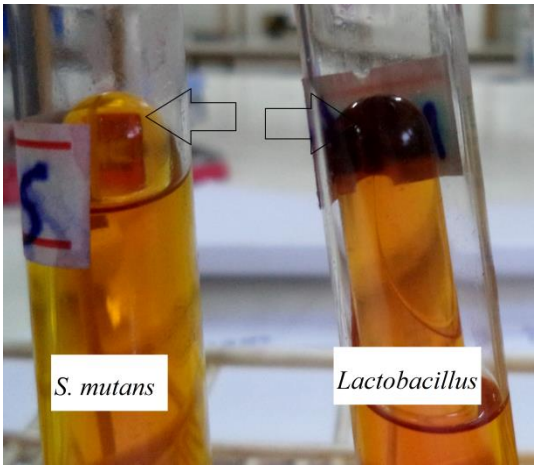


Figure 23: Aerobic growth test result of *S. mutans* and *Lactobacillus*

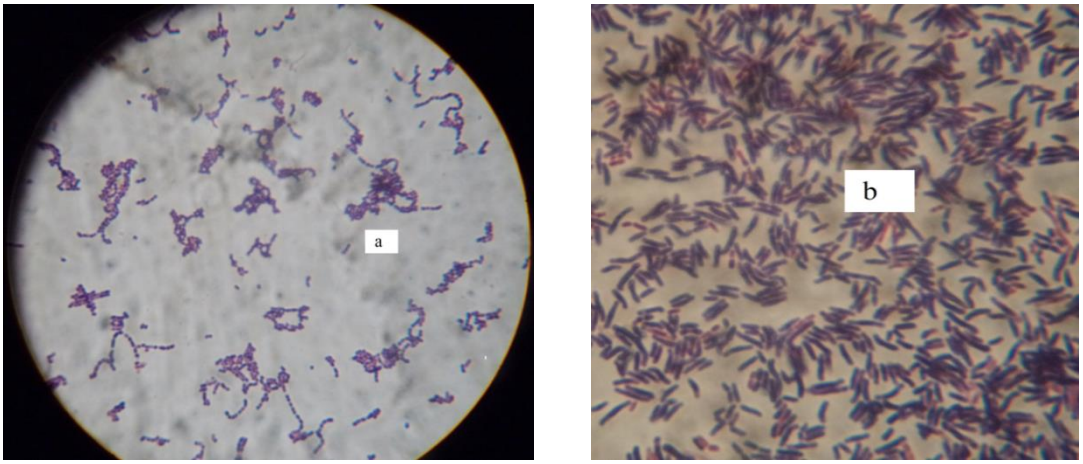


Figure 24: Results for Gram staining experiments on *S. mutans* (a) and *Lactobacillus* (b)

Table 11: Confirmatory test results against *S. mutans* and *Lactobacillus* collected from saliva

Bacterium	Sugar/Ammino Acid fermentation							Cat. test	Gas from Glu	Gram stain	Cell shape	Bacitracin test
	Raf	Man	Mel	Lac	Arg	Inu	Sor					
Streptoccoci	+	+	+	+	-	+	+	-	-	+	Spherical	-
Lactobacilli	+	+	+	+	-	+	+	-	-	+	cylinderical	+

Raf- Raffinose; Man- Mannitol; Mel- Melibiose; Arg-Arginine; Inu- Inulin; Cat-Catalase; Glu-Glucose; (+) able to ferment or breakdown, gram positive, bacitracin sensitive; (-) not able to ferment or break down or do not produce gas from glucose fermentation, gram negative, resistant to bacitracin.

4.4.2. Antibacterial tests of thyme EOs against *S. mutans* and *Lactobacilli*

The inhibition zones that resulted from the application of the different doses of *T. serrulatus* and *T. schimperi* EOs are clearily shown in Appendix 4 (Z_9 - Z_{18}) while the negative and positive controls are given in Appendix 4 (Z_{19} and Z_{20}) respectively.

Antibacterial Activity of the EOs against *Lactobacillus*: *T. serrulatus* and *T. schimperi* EOs resulted in a dose dependent inhibition of *Lactobacillus* of which Tar was the best followed by Yil, Bal, Ofl, Buta and Ala in decreasing order (Figure 25).The 16 mg/mL dose of these EOs against *Lactobacillus* resulted in inhibition zones close to 10 mm. Correspondingly, the 32 mg/mL doses except that of Buta and Ala resulted in inhibition zones higher than 10 mm. The trend of increase is also visible at 64 mg/mL doses which resulted in inhibition zones between 10 mm and 20 mm in which Buta was the least close to 10 mm and Tar was the highest close to 20mm. At the dose of 128 mg/mL, Tar and Yil resulted in inhibition zones higher than that of the positive control (3%H_2O_2) while Bal and Ofl resulted in inhibition zones closer to this control. However, inhibition zone recorded by 128 mg/mL EO of Ala was lower than that of the positive control. The EOs doses ranging from 16 mg/mL to 128 mg/mL of Ala too showed considerable inhibition to this bacterium even though their zones of inhibition were less than that of 3% H_2O_2. The negative control (3% DMSO) on the other hand did not inhibit this bacterium.

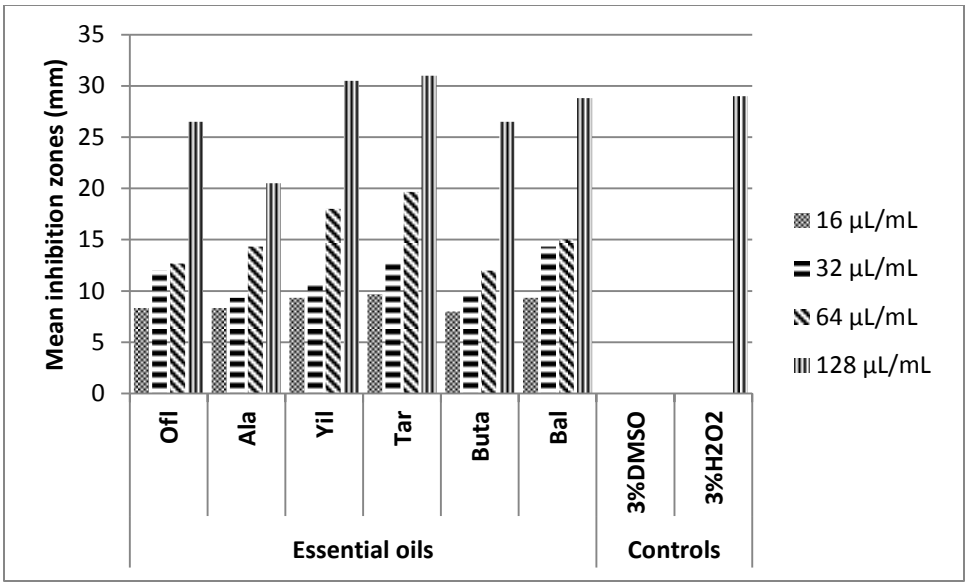


Figure 25: Inhibition of *Lactobacillus* by Essential olis from six localities with doses ranging from 16 mg/mL to 128 mg/mL in comparison with the negative and positive controls.

The differences in mean inhibition zones of *Lactobacillus* are indicated in Table 12. The highest inhibitions of *Lactobacillus* resulted by the 128 mg/mL doses of Yil and Tar which were significantly higher than similar doses of Ofl, Ala, Buta, Bal EOs and the positive control (3% H_2O_2). On the other hand, 3% H_2O_2 inhibited this bacterium with inhibition zones significantly higher than the 16, 32, and 64 mg/mL doses of all EOs and the 128 mg/mL doses of Ofl, Ala, Buta, and Bal EOs. *Lactobacillus* was not inhibited by the negative control (3% DMSO). Thus the highest and lowest significant mean inhibition zones against *Lactobacillus* resulted by 128 mg/mL EO of Tar and 16 mg/mL of Buta respectively. Generally, the different doses of Tar and Yil EOs were found to be the strongest EOs against *Lactobacillus*.

Antibacterial activity of the EOs against *S. mutans*

As can be depicted from Figure 26, *S. mutans* were inhibited by the different doses of the EOs; Ofl, Ala, Yil, Tar, Buta and Bal in a dose dependent manner. This bacterium was inhibited by 3% H_2O_2 (positive control) and was not inhibited by 3% DMSO (negative control). The smallest range of inhibition of this bacterium was due to the 16 mg/mL concentration of all the EOs which was between 8 and 10 mm. The mean minimum inhibition zones of all the test EOs at the dose of 32 mg/mL lied between 10 and 20 mm Ofl being with the least (11.67 ± 0.33mm) and Tar with the highest (18.67 ± 0.67) inhibition zones.

At 64 mg/mL concentration, *S. mutans* was inhibited with mean inhibition zones better than that resulted due to the 32 mg/mL concentration. At this dose, four of the EOs Ala, Yil, Tar, and Buta resulted in mean inhibition zones between 20 and 30mm and Tar resulted in the highest mean inhibition zone (26.00 ± 1.00) and Buta the lowest (20.33 ± 0.33). The rest two EOs Ala and Bal, however resulted in mean inhibition zones lower than 20mm. At 128 mg/mL concentration of the EOs, *S. mutans* was highly inhibited by all the EOs. At this dose level, around 67 % of the EOs (Ofl, Yil, Tar and Bal) inhibited *S. mutans* with inhibition zones higher than that of the positive control. The rest 33 % of the EOs (Ala and Buta) inhibited this bacterium with inhibition zones comparable to that of the positive control.

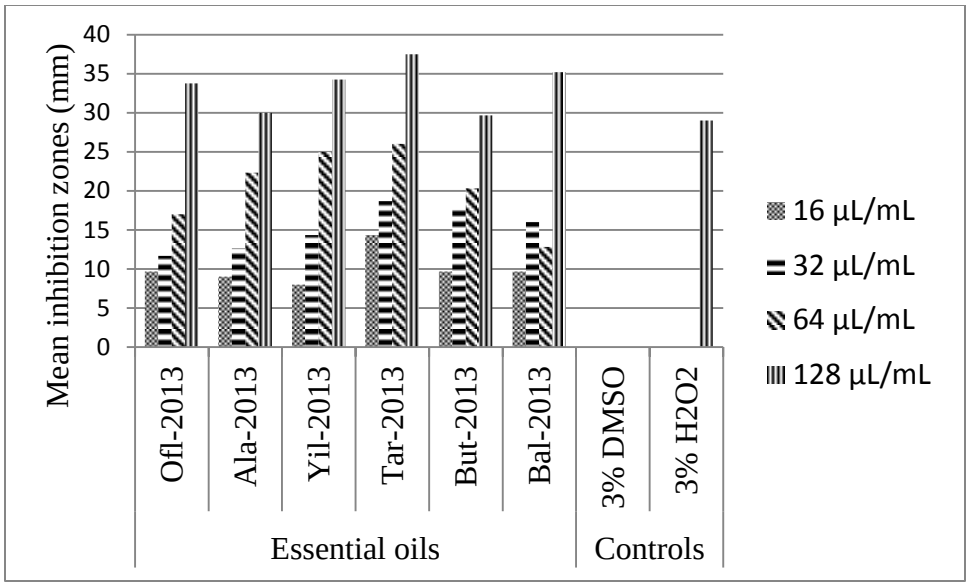


Figure 26: Inhibition of *S. mutans* by Essential Olis from six localities with doses ranging from 16 mg/mL to 128 mg/mL in comparison with the negative and positive controls.

At the dose 128 mg/mL, Tar inhibited *S. mutans* with inhibition zones significantly higher than the rest EOs and the positive control. It was followed by the 128 mg/mL dose of Bal which inturn was followed by the 128 mg/mL doses of Ofl and Yil. The least inhibition of *S. mutans* at this dose was seen in Ala and Buta EOs. At 64 mg/mL dose, significant inhibition of *S. mutans* was as follows: Yil and Tar>Ala>Buta and Bal. At the dose of 32 mg/mL, the order of inhibition of *S. mutans* was Tar and Buta> (significantly higher than) Ala>Bal>Yil>Ofl. The 16 mg/mL dose of Tar inhibited *S. mutans* with inhibition zone comparable with the 32 mg/mL of Yil and with significantly higher inhibition zone than the 32 mg/mL of Ala> 16 mg/mL of Ofl, Ala, Buta and Bal> 16 mg/mL of Yil> 3%DMSO.

Thus the Tar (128 mg/mL) and the Yil (16 mg/mL) were found to be the most and the least inhibitor doses of *S. mutans* respectively. The positive control resulted in inhibition zones higher than the 16, 32, and 64 mg/mL doses of the entire test EOs and the negative control (3%DMSO) resulted in no inhibitions. In general, Tar EO appeared to be the strongest inhibitor of *S. mutans* than the rest EOs (Table 12).

Table 12: Antibacterial activity of the EOs of Ofl, Ala, Yil, Tar, Buta and Bal using agar disc diffusion method (mean ± SEM for three repetitions)*

		Bacteria	
		S. mutans	***Lactobacillus***
Treatments	**Concentration**	**Mean ± SEM**	**Mean ± SEM**
Controls	3% DMSO	0.00 ± 0.00^{o}	0.00 ± 0.00^{m}
	3% H_2O_2	30.00 ± 1.15^{d}	29.00 ± 1.00^{b}
Ofl	16 mg/mL	9.67 ± 0.33^{m}	8.33 ± 0.67^{kl}
	32 mg/mL	11.67 ± 0.33^{l}	10.33 ± 0.33^{ij}
	64 mg/mL	17.00 ± 0.58^{ij}	12.67 ± 0.33^{h}
	128 mg/mL	33.33 ± 0.33^{c}	26.67 ± 0.33^{c}
Ala	16 mg/mL	9.00 ± 0.58^{mn}	8.33 ± 0.33^{kl}
	32 mg/mL	12.67 ± 0.67^{l}	9.33 ± 0.33^{jk}
	64 mg/mL	22.33 ± 0.33^{f}	14.33 ± 0.33^{g}
	128 mg/mL	30.00 ± 0.58^{d}	21.33 ± 0.33^{d}

Yil	16 mg/mL	8.00 ± 0.00 [n]	9.33 ± 0.33 [jk]
	32 mg/mL	14.33 ± 0.33 [k]	10.67 ± 0.33 [i]
	64 mg/mL	25.00 ± 0.00 [e]	18.00 ± 0.00 [f]
	128 mg/mL	33.67 ± 0.33 [c]	30.67 ± 0.33 [a]
Tar	16 mg/mL	14.33 ± 0.89 [k]	9.67 ± 0.33 [ij]
	32 mg/mL	18.67 ± 0.67 [h]	10.33 ± 0.33 [ij]
	64 mg/mL	26.00 ± 1.00 [e]	19.67 ± 0.88 [e]
	128 mg/mL	37.33 ± 0.33[a]	31.00 ± 0.58 [a]
Buta	16 mg/mL	9.67 ± 0.33 [m]	8.00 ± 0.00 [l]
	32 mg/mL	17.67 ± 0.33 [hi]	9.67 ± 0.33 [ij]
	64 mg/mL	20.33 ± 0.33 [g]	12.00 ± 0.58 [h]
	128 mg/mL	29.67 ± 0.33 [d]	27.00 ± 0.58 [c]
Bal	16 mg/mL	9.67 ± 0.00 [m]	9.33 ± 0.33 [jk]
	32 mg/mL	16.00 ± 0.58 [j]	12.33 ± 0.33 [h]
	64 mg/mL	20.00 ± 1.00 [g]	15.00 ± 0.00 [g]
	128 mg/mL	36.00 ± 0.58 [b]	27.67 ± 0.33 [c]

3% DMSO- negative control-did not show any activity

3% H_2O_2– Positive control

Least significant difference for *S. mutans* = 1.1861

Least significant difference for Lactobacillus = 1.0757

Means with the same letters in the same column are not significantly different ($p>0.05$).

*: The strength of activity is presented as resistant (> 7mm), intermediate (>12mm) and susceptible (> 18mm) (Upadhyay *et al.,* 2010)

4.4.3. MIC and MBC of EOs against *S. mutans* and *Lactobacillus*

The MIC and MBC of the test EOs were determined for both bacteria. The MIC and MBC of *S. mutans* were found to be 0.25 mg/mL by Bal and 0.5 mg/mL by the rest EOs (Table 13). On the other hand, the MIC and MBC against *Lactobacillus* were at the dose of 0.5 mg/mL by all the test EOs.

Table 13: Antibacterial activity (MIC and MBC) of *Thyme* EOs against *S. mutans* and *Lactobacillus*

			S. mutans		**Lactobacillus**	
S.No	**Botanical Name**	**Essential Oil**	**MIC* (mg/mL)**	**MBC** (mg/mL)**	**MIC* (mg/mL)**	**MBC** (mg/mL)**
1	*Thymus serrulatus*	Ofl	0.5	0.5	0.5	0.5
2	*Thymus serrulatus*	Ala	0.5	0.5	0.5	0.5
3	*Thymus serrulatus*	Yil	0.5	0.5	0.5	0.5
4	*Thymus schimperi*	Tar	0.5	0.5	0.5	0.5
5	*Thymus schimperi*	Buta	0.5	0.5	0.5	0.5
6	*Thymus schimperi*	Bal	0.25	0.25	0.5	0.5

*MIC: Highest dilution (minimum concentration) showing no detectable growth

**MBC: Highest dilution (minimum concentration) yielded no single colony on a solid medium.

4.5. Hepatoprotective activity

4.5.1. Serum levels of ALT, AST, and ALP

The levels of serum AST, ALT and ALP in the normal, paracetamol injured, Silybinin treated and EO treated rats are presented in Table 14. The levels of AST, ALTand ALP were increased significantly in paracetamol treated rats (Group II). Treatment with the EOs of *T. serrulatus* (Yil, Ala) and *T. schimperi* (Tar) at 200 mg/kg was found to reverse this effect i.e, reduce serum levels of AST, ALT and ALP with values close to the normal control. The ALT levels of rats treated by all the EOs, normal control and Silybinin (100 mg/kg) were comparable and significantly lower than the paracetamol treated group. In the same way, the EOs Tar and Yil reversed the serum levels of AST with efficiencies comparable with Silybinin and to the level comparable to the normal control while Ala EO was found to be the best of all with respect to reducing the level of AST. Paracetamol induced ALP too was significantly reduced by the treatment of rats with Silybinin (100 mg/kg) and the EOs (Tar, Yil, and Ala) (200 mg/kg). In all the cases, the Ala EO was the best of all.

Table 14: Levels of ALT, AST, and ALP after treatment with EOs

Groups and design of treatment	ALT	AST	ALP
	U/L		
Group I, control, tween 80	79.64 ± 13.96 [b]	243.94 ± 49.23 [b]	143.94 ± 12.25 [ab]
Group II, Par (2g/kg)	182.96 ± 66.24 [a]	486.98 ± 127.83 [a]	173.45 ± 23.89 [a]
Group III, Silybinin (100 mg/kg) + Par	75.00 ± 16.18 [b]	165.46 ± 34.99 [bc]	81.03 ± 17.35 [cd]
Group IV, Tar (200 mg/kg) + Par	98.62 ± 18.34 [b]	178.96 ± 14.88 [bc]	121.28 ± 29.86 [bc]
Group V, Yil (200 mg/kg) + Par	42.90 ± 4.23 [b]	119.72 ± 10.41 [bc]	81.91 ± 9.27 [cd]
Group VI, Ala (200 mg/kg) + Par	34.20 ± 4.98 [b]	72.73 ± 8.37 [c]	63.46 ± 9.21 [d]

Values are mean ± SEM. 5 mice in each group (n = 5), means with the same letter among columns are not significantly different ($P > 0.05$).

4.5.2. Histopathological observations

The histological observations basically supported the results obtained from serum enzyme assays. The architecture of hepatocytes in liver sections varied according to the treatments administered to them in such a way that the paracetamol treated rat liver cells showed the highest degree of necrosis when compared to the normal control, silybinin and paracetamol treated and thyme EO and paracetamol treated rats (Figure 27). In liver cells of rats treated with paracetamol vehicle only (T-80) (normal control), liver sections showed normal hepatic cells with well preserved cytoplasm and well defined nuclei (Figure 27 a). In rats treated with paracetamol only, hepatocytes were found to be extensively necropsied (Figure 27 b). In rats pretreated with Tar (200 mg/kg), Yil (200

mg/kg), and Ala (200 mg/kg) for seven days and paracetamol (2 g/kg) administered on the seventh day, the liver sections of all the rats showed normal hepatocytes except some inflammatory cell infiltrations in only single samples of (Ala + Par) and (Tar + Par) (Figure 27 d and f). In the same way, liver sections of rats pretreated with Silybinin (100 mg/kg) and then given paracetamol (2 g/kg) after seven days showed normal hepatocytes with some nuclear changes in one of the rats and congestion of blood in blood vessels in another rat (Figure 27 c).

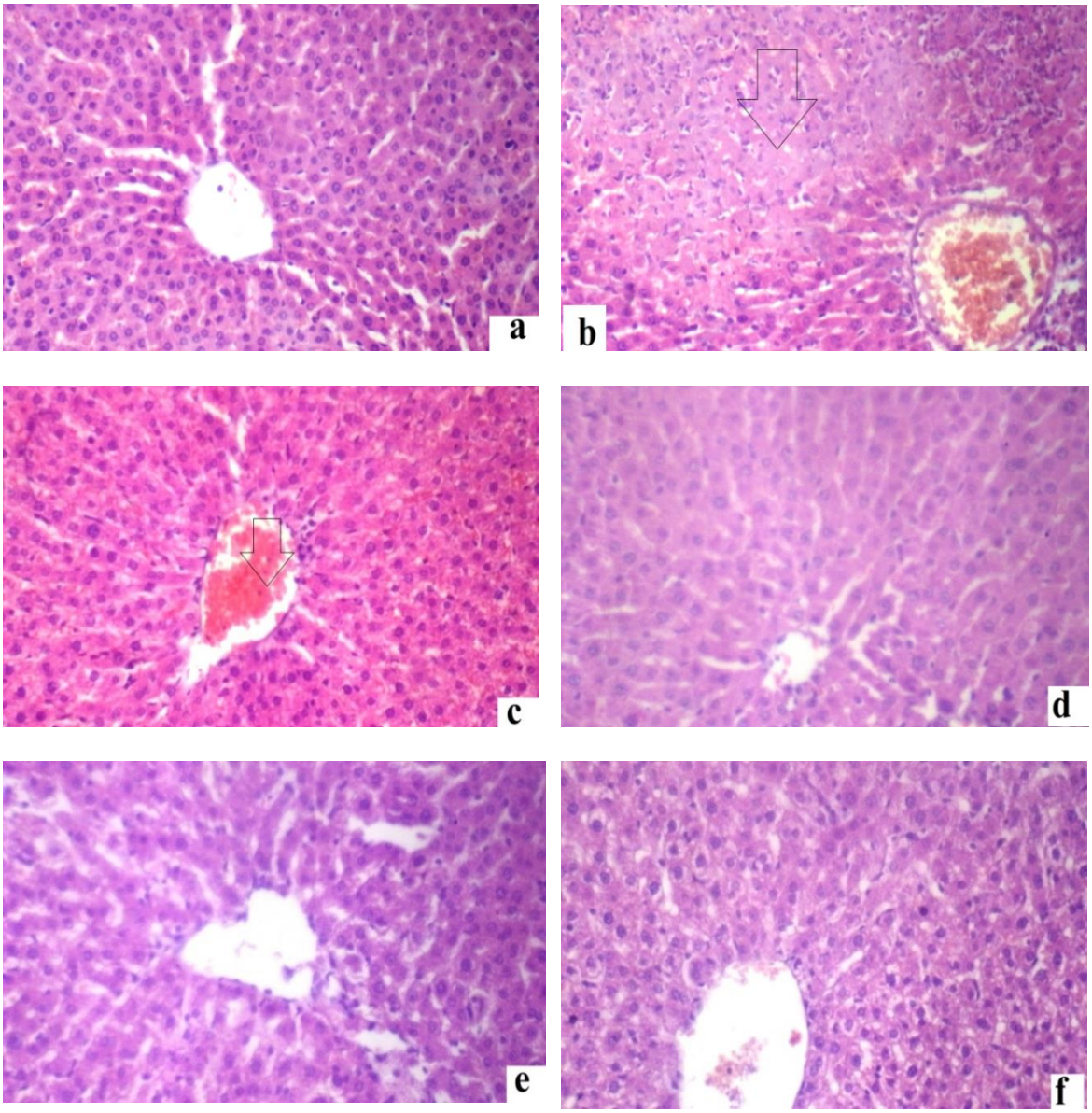


Figure 27: Histopathological appearance of liver cells (a) normal control with normal hepatocytes ; (b) liver cells of rats treated with paracetamol showed extensisve necrosis of hepatocytes and were found to be infiltrated with inflammatory cells; (c) liver cells of rats treated with silybinin and paracetamol showed normal hepatocytes except blood congestion; (d) liver cells of rats treated with Ala EO and paracetamol showed normal hepatocytes; (e) liver cells of rats treated with Yil EO and paracetamol showed normal hepatocytes; (f) liver cells of rats treated with Tar EO and paracetamol showed normal hepatocytes

4.6. Mosquitocidal activity of *T. serrulatus* and *T. schimperi* EOs

4.6.1. Larvicidal activity

The 200 and 100µL/L of the EOs of Ala, Yil, and Tar resulted in complete mortality of 3rd and 4th instar larvae of *An. arabiensis* with mean deathes significantly higher than that of the 50, 25 and 12.5µL/L doses of all these EOs (Figure 28).

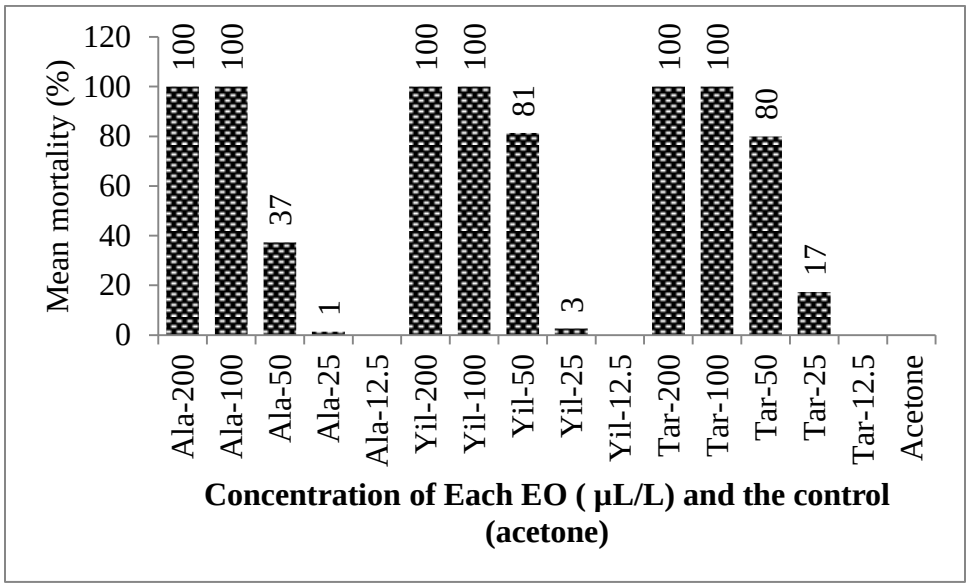


Figure 28: Percent mortality of larvae at different doses of EOs and Acetone

With a dose of 50µL/L, EOs of Yil and Tar killed larvae with death rates significantly higher than that of the Ala at the same dose. The mean number of larvae that died due to the 25 µL/L of Tar was significantly higher than that of Ala and Yil EOs. On the other hand, the 25 µL/L doses of Ala and Yil as well as the 12.5 µL/L doses of all the three EOs did not result in larvicidal activities significantly higher than that of the negative control (acetone) (Table 15). Thus the essential oil Tar was the best larvicidal followed by Yil which in turn was followed by Ala. The LD_{50} values of these EOs were 60.75

µL/L (Ala), 44 µL/L (Yil) and 41.75 µL/L (Tar) so that the larvicidal activities of these EOs can be ordered as follows: Tar > Yil >Ala

Table 15: Larvicidal test results of Ala, Yil, and Tar EOs

EO	Dose (µL/L)	Mean ± SEM	LD_{50} (µL/L)
Ala	200	25 ± 0.0[a]	60.75
	100	25 ± 0.0 [a]	
	50	9.33 ± 1.85 [c]	
	25	0.33 ± 0.33 [e]	
	12.5	0 ± 0.0 [e]	
Yil	200	25 ± 0.0[a]	44
	100	25 ± 0.0[a]	
	50	20.33 ± 3.18[b]	
	25	0.67 ± 0.67 [e]	
	12.5	0 ± 0.0 [e]	
Tar	200	25 ± 0.0[a]	41.75
	100	25 ± 0.0[a]	
	50	20 ± 0.58[b]	
	25	4.33 ± 2.6 [d]	
	12.5	0 ± 0.0 [e]	
Control	Acetone	0 ± 0.0 [e]	

Means with the same letter are not significantly different (p>0.05)

Least Significant Difference = 3.1547

4.6.2. Fumigation test

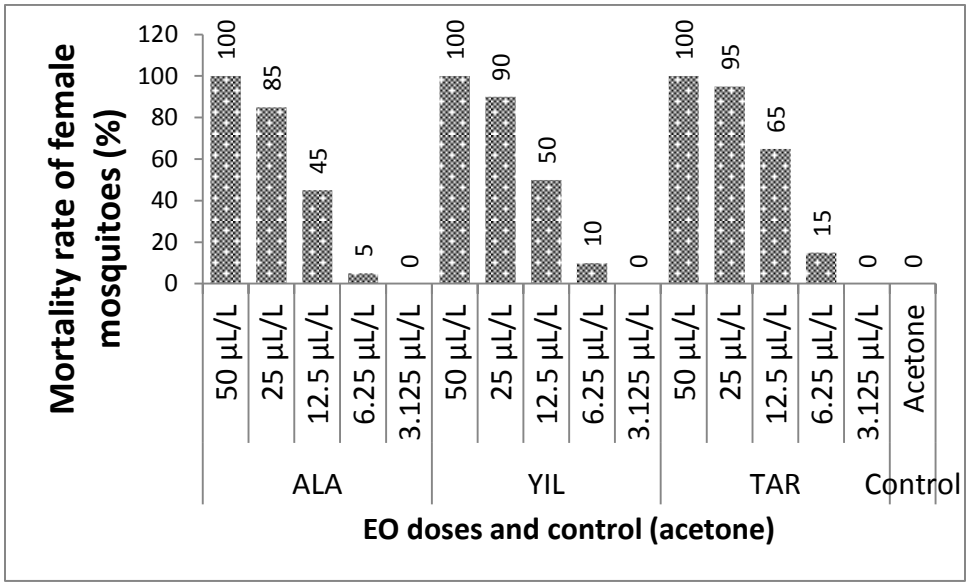


Figure 29: % Mortality of adult female mosquitoes due to the different concentrations of Ala, Yil and Tar Eos and the negative control (acetone).

The fumigation mosquitocidal activity of the three EOs showed dose dependent activities against adult female *An. arabiensis* mosquitoes (Table 16). At 50 µL/L concentration, the EOs Ala, Yil, and Tar resulted in 100% death of adult female mosquitoes (Figure 29). The dose of the EOs which were able to kill 50% of the mosquitoes (LD_{50} values) were found to be 20.47 µL/L, 19.00 µL/L, and 17.72 µL/L for Ala, Yil, and Tar respectively. This shows that the fumigation (mosquitocidal) activity of Tar > Yil > Ala (Table 16).

Table 16: Mosquitocidal test results of Ala, Yil, and Tar EOs

EO	Dose (µL/L)	Mean ± SEM	LD_{50} (µL/L)
Ala	50	20.00 ± 0.00[a]	17.19
	25	16.67 ± 0.88[b]	
	12.5	9.33 ± 0.88 [d]	
	6.25	1.33 ± 0.33 [ef]	
	3.125	0.00 ± 0.00[f]	
Yil	50	20.00 ± 0.00 [a]	14.92
	25	18.33 ± 0.67 [ab]	
	12.5	10.33 ± 1.85[d]	
	6.25	2.33 ± 0.33 [e]	
	3.125	0.00 ± 0.00[f]	
Tar	50	20.00 ± 0.00 [a]	13.20
	25	18.67 ± 0.67 [a]	
	12.5	13.00 ± 0.58[c]	
	6.25	3.00 ± 0.58 [e]	
	3.125	0.00 ± 0.00[f]	
Control	Acetone	0.00 ± 0.00[f]	

Means with the same letter are not significantly different ($p>0.05$)

Least significant difference = 1.8591

4.6.3. Oviposition deterrent activity

The results of the oviposition deterrent bioassay are presented in two ways: Oviposition Activity Index (OAI) and Effective Repellency (% ER) (Figures 30 and 31 respectively). The oviposition activity indexes of the essential oils Ala and Tar was below -0.3 at all the doses tested (200, 100, 50, 25, and 12.5 µL/L). Similar findings were observed in Yil EO at the doses of 200, 100, and 50 µL/L. This means these EOs acted as deterrents at the mentioned doses. The Effective Repellency (% ER) of Tar was the highest with its 200, 100, and 50 µL/L doses resulting in 100% repellency and its 25 and 12.5 µL/L doses resulted in 94% and 58% repellency respectively. The Ala EO was the next effective repellant whose 200 and 100 µL/L doses resulted in 100% ER. Its 50, 25, and 12.5 µL/L doses also caused ERs of 90%, 77%, and 62% respectively. Yil also resulted into a 100% ER at 200 µL/L, 98% (100 µL/L), 69% (50 µL/L), 27% (25 µL/L) and 16% (12.5 µL/L). The ER of the control (3% DMSO) on the other hand was zero which was lower than all the doses of the three EOs.

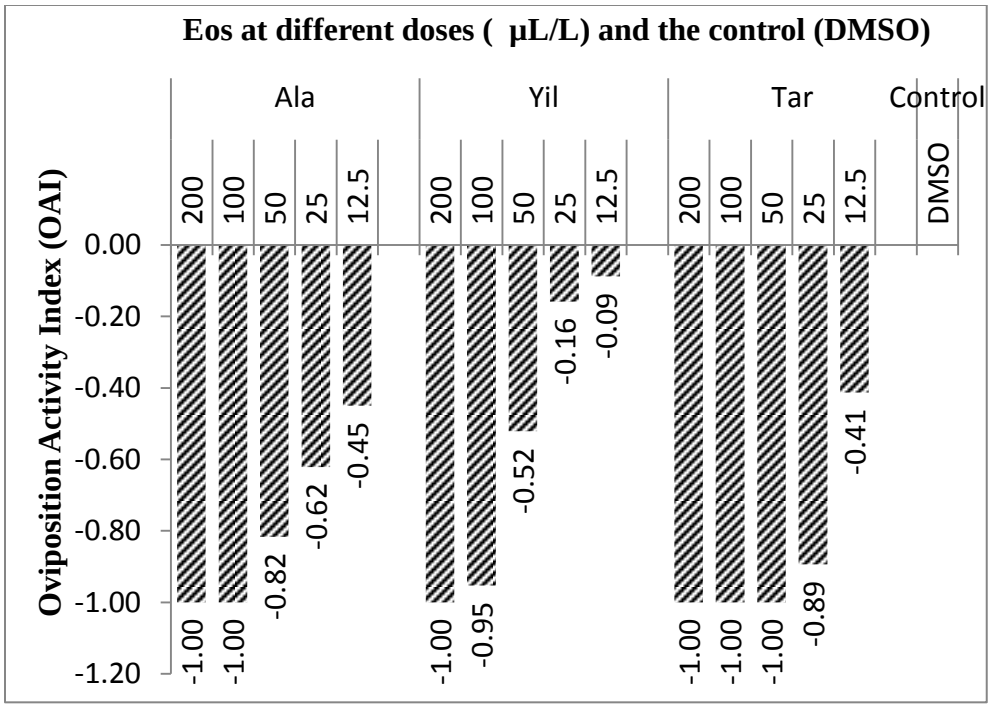


Figure 30: Oviposition activity index (OAI) values of three EOs against *An. arabiensis*

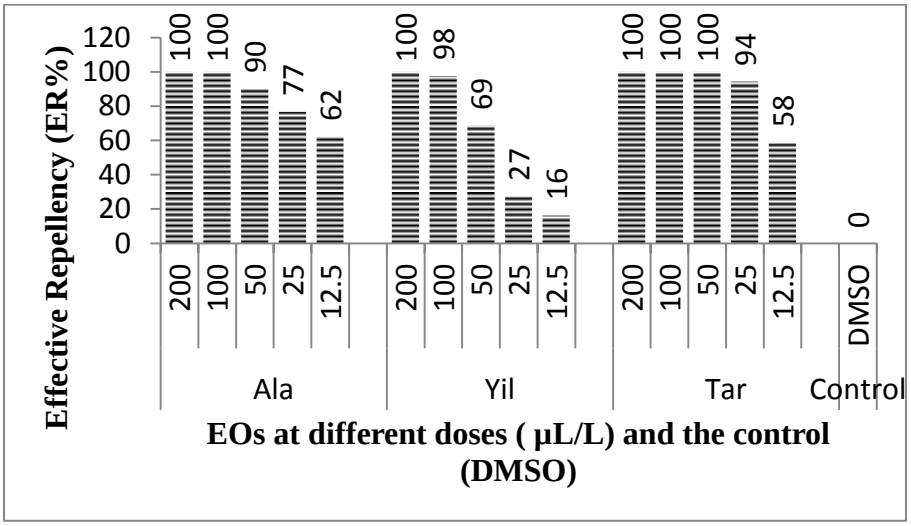


Figure 31: Effective Repellency (%ER) of three EOs against *An. arabiensis*

4.7. Acute oral toxicity of the EOs of *T. serrulatus* and *T. schimperi*

4.7.1. Observations of mice dosed with EOs at 2000 µL/Kg body weight

Observation of body weight changes: As can be seen from Figure 32, the control mice grew continuously in the duration of 14 days. On the other hand, mice treated with EOs at 2000 mg/kg body weight resulted in reduction of body weight within the first 24 to 48 hours may be due to the reason that they stopped eating food immediately after test administration. This inturn may be due to the burning sensations of the essential oils on their oropharengial tract and the stress as a result of gavage insertion in their esophagus. After that, improvement in body weight was seen except those treated with Buta. Mice given Buta however continued to waste through the first seven days after which they started to improve. This group of mice reached their initial weight by day 14. Yil was the next EO which resulted in slow growth of the treated mice.

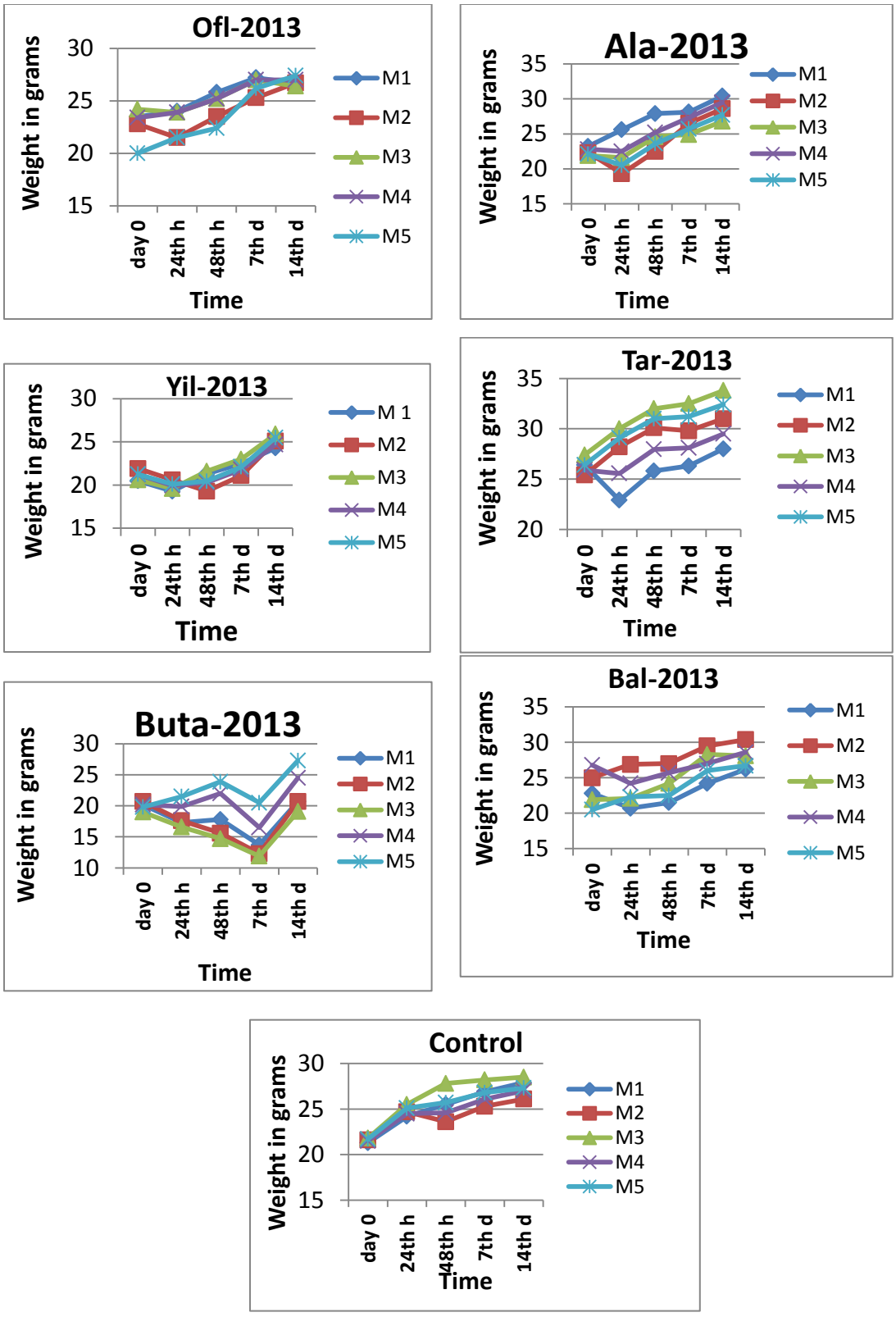


Figure 32: Body weight changes during acute oral toxicity test period (M1-M5 = Mice number)

Organ weight to body weight ratio (bw/ow): The organ weight to body weight ratios and packed cell volume (PCV) were calculated and are presented in Table 17 below. With respect to the heart, kidneys, liver, brain and lung to body weight ratios, the control group (T-80 treated group) had either significantly higher values than or comparable with that of the essential oils (2000 mg/kg) treated groups. This has an implication that the essential oils didn't result in enlargment of these organs. . On the other hand, the spleen to body weight of Yil essential oil treated mice was significantly higher than the control group. This may be due to the influence of carvacrol on spleen enlargement. The % PCV of mice treated with Ofl, Buta and Bal were significantly higher than mice treated with the control.

Table 17: Organ to body weight ratio and %PCV in mice 14 days after treatment with 2000µL/kg bw of *T. serrulatus* and *T. schimperi* EOs (n=5)

EO	% Organ to body weight ratio (Mean ± SEM)						%PCV (Mean ± SEM)
	Heart	Kidneys	Liver	Brain	Lung	Spleen	
Ofl	0.53 ± 0.03 ^{b}	1.45 ± 0.06^{a}	6.98 ± 0.31 ^{a}	1.48 ± 0.06 ^{a}	0.21 ± 0.01 ^{c}	0.58 ± 0.02 ^{b}	52.85 ± 0.98 ^{b}
Ala	0.72 ± 0.01 ^{ab}	1.27 ± 0.01^{b}	6.65 ± 0.06 ^{a}	1.50 ± 0.06 ^{a}	1.05 ± 0.14 ^{a}	0.57 ± 0.01 ^{bc}	39.86 ± 0.08 ^{c}
Yil	0.63 ± 0.08 ^{ab}	1.37 ± 0.01^{ab}	6.81 ± 0.13 ^{a}	1.66 ± 0.03 ^{a}	0.82 ± 0.03 ^{ab}	0.74 ± 0.01 ^{a}	44.29 ± 0.15 ^{c}
Tar	0.72 ± 0.05 ^{ab}	1.39 ± 0.02^{ab}	6.64 ± 0.16 ^{a}	1.45 ± 0.02 ^{a}	0.73 ± 0.01^{b}	0.56 ± 0.03 ^{bc}	43.62 ± 1.01 ^{c}
Buta	0.73 ± 0.03 ^{a}	1.27 ± 0.06^{b}	7.00 ± 0.33 ^{a}	1.71 ± 0.11 ^{a}	0.19 ± 0.01 ^{c}	0.46 ± 0.04 ^{c}	64.28 ± 4.46^{a}
Bal	0.59 ± 0.02 ^{ab}	1.26 ± 0.07^{b}	6.19 ± 0.32^{a}	1.45 ± 0.06 ^{a}	0.24 ± 0.01 ^{c}	0.54 ± 0.04 ^{bc}	54.36 ± 0.64 ^{b}
Control*	0.69 ± 0.04 $^{a\ b}$	1.34 ± 0.02^{ab}	6.01 ± 0.09^{a}	1.54 ± 0.02^{a}	1.00 ± 0.02 ^{a}	0.51 ± 0.02 ^{bc}	39.39 ± 0.19 ^{c}

a, b, c,……means with the same letters in columns are not significantly differenct ($p > 0.05$); * Control- given only 0.1 % T-80 in 0.9% normal saline; PCV= Packed cell volume.

Chapter 5 - Discussion, Conclusion and Recommendations

Chapter 5: Discussion, Conclusion and Recommendations

5.1. Discussion

5.1.1. The distribution, medicinal value, economic advantage, current status and threatening factors of *Thymus* Species (*T. serrulatus* and *T. schimperi*) from Ethiopia

Thymus specimens collected from the Northern parts of Ethiopia (Tigray and Gojjam) were found to be *T. serrulatus* and those collected from central (Shewa) and Southern (Bale and Butajira) were *T. schimperi*. This agrees with the reports of (Sebsebe Demissew and Nigist Asfaw, 1994; Nigist Asfaw *et al.*, 2000). The medicinal value of *T. serrulatus* and *T. schimperi* were identified by the respondents as; to treat blood pressure, to treat general pain syndrome, influenza, abdominal pain, and to treat intestinal parasites like ascaris. Literature supports these bioactivities of *Thymus* species. For example the work by Miloradovic *et al.* (2010) showed that thyme extract (TE- *T. serpyllum* L.) resulted in a significantly reduced level of Systolic Atrial Pressure (SAP) and Diastolic Atrial Pressure (DAP) in hypertensive rats. In addition, *T. vulgaris* was found to reduce pain in mice while applying to plate, tail flick and formalin tests (Taherian *et al.*, 2009).

So it is not surprising if *T. serrulatus* and *T. schimperi* are applied as treatments for general pain syndrome. EOs of thyme are also known for their antiviral, antibacterial, antifungal and antiworm activities owing to their active component thymol (Lezak, 2000). This may be the reason why *T. serrulatus* and *T. schimperi* are used for treating intestinal parasites and abdominal pain. Furthermore thymol is the major component of

Thymus species from Ethiopia. For example the works of (Ermias Dagne *et al.,* 1998) and (Nigist Asfaw *et al.,* 2000) revealed that the components of *T. schimperi* were by large thymol 50% and more than 30 % respectively. In addition, the abdominal pain healing capacity of these plants may be due to their abilities to kill *Helicobacter pylori* (Esmaeili *et al.,* 2012) and their anti-acidic nature.

The respondents mentioned the economic value of *T. serrulatus* and *T. schimperi* as honey bee forage, animal forage, food additives, drinks as tea and washing and fumigation of jars for milking and baking. This agrees with the work by Tewodros Eshete *et al.* (2013), showing that food supplements with thyme mix improve intake, digestibility, daily weight gain, final body weight, empty body weight, hot carcass weight, and dressing percentage compared to non-supplemented group and improves the sensory quality of sheep meat. Furthermore thyme honey has medicinal values. For example, the work by Tsiapara *et al.* (2009) showed that thyme honey reduces the viability of prostate cancer cell line (MCF-7). Thus the information from the informants is valuable.

According to the informants, the status of *T. serrulatus* and *T. schimperi* is declining mainly due to overgrazing followed by agricultural expansion, overharvesting, uprooting during harvesting, and lack of recognition. The threatening factors for these plants are similar to those identified by Kalayu Mesfin *et al.* (2013). Harvesting the whole plant including the root continuously is a dangerous practice which interferes with the life cycle of the species and results in eradication of the whole plant (Wangchuk *et al.,* 2008). These plants are restricted to limited geographical regions (Alpine and Afroalpine) so that

degradation of these areas may result in degradation of the whole species. Overgrazing of *T serrulatus* and *T. schimperi* is a big challenge owing to their suitability as forages for cattle, sheep and goats as well as the beliefs of inhabitants that animals which feed these plants give tasty meat, mutton, and milk.

The other problem is commercialization of these wild species without conserving them. This may be due to the reason that wild species are communal and no one cares about them as private (cultivated) species. Their multiple use as condiments, forage, medicinal value, and tea increased the harvesting pressure on these plants and they are continuously declining. In the same way all these pressures may be indirectly caused by human population expansion and increased level of unemployment rate (Wangchuk *et al.*, 2008). In addition, there is continuous and bulk requirement of these herbs. To supply such large quantity of the herbs, large scale cultivation would be required, which in turn will generate good business opportunities and human resource development (Tripathi *et al.*, 2009).

5.1.2. Chemical composition of *Thymus* species from six localities in Ethiopia

The yields of EOs were 0.5% (Ofl and Ala), 0.8% (Yil, Tar, and Buta), and 0.9% (Bal). These yields agree with the fact that EOs constitute only a minor proportion of the wet weight of plant material, usually about 1% or less (Carson and Hammer, 2011). The yield for EOs when computed per Kg plant material were 5 mL/kg (Ofl and Ala), 8 mL/kg (Yil, Tar, and Buta), and 9 mL/kg (Bal). These yields show conformity with European Pharmacopoeia standard (at least 3 mL/kg) (Raal *et al.*, 2004).

In Ofl, the major components were thymol (49.55%), carvacrol (36.34%), p-cymene (3.06%), thymol methyl ether (2.73%), γ-terpinene (1.61%) and caryophyllene oxide (1.11%). Ala's major components were thymol (65.63%), carvacrol (6.68%), thymol methyl ether (6.55%), p-cymene (4.84%), linalool (3.29%), caryophyllene oxide (2.18%), viridiflorol (2.18%), t-caryophyllene (1.50%), and γ-cadinene (1.49%). Yil is majorly composed of carvacrol (80.84%), thymol (6.52%), p-cymene (3.65%), and carvacrol methyl ether (1.32%). In Tar, thymol (48.84%), carvacrol (42.12%), linalool (2.97%) and p-cymene (1.88%) make the biggest composition. Carvacrol (71.83%), thymol (15.77%), p-cymene (3.75%), carvacrol methyl ether (1.32%) and linalool (1.30%) were the prominent components of Buta EO. In case of Bal, EO components with the biggest share include thymol (53.57%), carvacrol (34.55%), p-cymene (3.20%) and linalool (2.60%).

These major components in the six Ethiopian *Thymus* EOs agree with literature data (WHO, 1999). For example the major components *Thymus vulgaris* L. EO grown in Cuba were thymol (34.60%), γ-terpinene (17.61%) and p-cymene (17.65%) (Pino *et al.*, 1997).

Major components of the EOs from *Thymus bmussonetti* Boiss.were carvacrol (72.5%), γ-terpinene (14.2%), p-cymene (6.9%), pinene (3.5%), β-caryophyllene (1.8%), and α-thujene (1.1%) (Charai *et al.,* 1999). Similarly, the main components of the EOs of the Albanian thyme (*Thymus vulgaris* L.) identified were: p-cymene: (7.76-43.75%), γ-terpinene (4.20-27.62%), thymol (21.38-60.15%), carvacrol (1.15-3.04%) and p-caryophyllene (1.30-3.07%) (Asllani and Toska, 2003).

In all the six EOs, thymol and carvacrol which are isomers and with the boiling points of 232 ^{o}C, 237 ^{o}C respectively (Abu-Lafi *el al.,* 2007) were found to be the predominant compounds one following the other. The percent peak areas calculated from GCMS results revealed this situation. Of the total composition of the EOs, 85.89% of Ofl, 72.31% of Ala, 87.36% of Yil, 90.96% of Tar, 87.6% of Buta, and 88.12% of Bal EOs was made of combinations of only thymol and carvacrol. The combinations of thymol and carvacrol in this work were found to be a little bit higher than that of the works of Ermias Dagne *et al.,* (1998) and Nigist Asfaw *et al.,* (2000). This difference may be due to the differences in the times of collection. The former researchers collected *Thymus* plants during May and the later during flowering time (most probably March to June) (Fichtl and Adi, 1994). Both collection times differ from the collection times used by the present study (the first three weeks of August).

Out of the six EOs studied, four (Ofl, Ala, Tar, and Bal) were found to contain thymol as their major components (thymol chemotypes) with respective percentages of 49.55%, 65.63%, 48.84%, and 53.57%. The other two EOs (Yil and Buta) on the other hand were found to have carvacrol as their major components with percent compositions of 80.84%

and 71.83% respectively. The next major compound for these EOs was found to be thymol implying the importance of both components for their biological activities. Generally speaking, most *Thymus* species are known to be either thymol or carvacrol chemotyoes. Examples of carvacrol chemotypes include *T. daenensis, T. persicus, T. serpyllium* (Sfaei-Ghomi *et al.,* 2009); *T. satureoides, T. broussonettii, T. maroccanus, T. leptobotris, T. algeriensis* (Jaafari *et al.,* 2007). Thymol chemotypes are also represented by *T. pallidus, T. zygis* (Jaafari *et al.,* 2007); *Thymus numidicus* (Saidj *et al.,* 2008); *T. vulgaris* (Grigore *et al.,* 2010; Sharafzadeh *et al.,* 2010); *T. eriocalyx* (Sfaei-Ghomi *et al.,* 2009) and many others.

In any ways, there existed variations in compositions among the EOs Ofl, Ala, Yil, Tar, Buta and Bal. These variations may be due to: genetic differences (Echeverrigaray *et al.,* 2001), elevation where the plants grow (Martínez *et al.,* (2005) (Ofl = 2675m asl., Ala = 2600m asl., Yil =3315m asl., Tar = 3222m asl., Buta=2678m asl., and Bal = 3079m asl.). Since all the *Thymus* species were collected from wild, the effect of their growth habit on their chemical composition differences is assumed to be minimal (Tabrizi *et al.,* 2010).

The plants were collected from geographically separate areas. Thus differences in their chemical compositions may be resulted from differences in the types of nutrients in the soils where they grow (Tabrizi *et al.,* 2010). The altitudinal and geographical variations in the collection sites may also result in moisture variations which indirectly may result in variation of EO composition among the *Thymus* EOs (Jordan *et al.,* 2003). Stress may be another variable that resulted in variation of components among the *Thymus* EOs (Khalid,

2006; Edreva *et al.,* 2008; Khadhri *et al.,* 2011; Manhães *et al.,* 2012; Ghilavizadeh *et al.,* 2013; Moradi *et al.,* 2014).

EO components may also be affected by plant parts used as EO sources (Lahlou, 2004), maturity levels of plants used (Arraiza *et al.,* 2009), differences in harvesting seasons (Abu-Lafi *el al.,* 2007), differences in post-harvest drying conditions (Mejdoub and Katsiotis, 1998), differences in EO extraction techniques (Tepe *et al.,* 2004), and variations in distillation time (DT) (Mejdoub and Katsiotis, 1998). However, these conditions were seriously controlled in this study. Aerial parts with similar maturity levels were collected, parts were harvested during a rainy season common to the six collection sites, all the collected plant materials were shade dried under similar conditions, essential oils from the plants were extracted using water distillation under similar conditions and similar distillation times.

5.1.3. Antibacterial tests of thyme EOs against *S. mutans* and *Lactobacilli*

5.1.3.1. Isolation of *S. mutans* and *Lactobacilli*

Tooth decay bacteria isolated using selective media MS agar and NMR agar produced yellow color while grown in nutrient broths containing the sugars (Raffinose, Mannitol, Mellibiose, Lactose, Inulin, and Sorbitol) and phenol red indicator. This is a positive result for their ability of hydrolyzing these sugars (Hendriksen, 2003). On the other hand they produced brick-red color in broths containing arginine implying that they were unable to hydrolyze this amino acid (Hendriksen, 2003). They too were not able to break

down hydrogen peroxide (negative catalase test) and they were unable to generate gas from glucose fermentation (were anaerobic). The gram stain of the isolates revealed gram positive spherical chains (those from MS agar = *Streptococci*) and cylinderical cells (those from MRS agar = *Lactobacilli*). Further more, those from MS agar were found to be resistant to Bacitracin discs (5 µg/disc).

As a whole, the biochemical and physical examinations of the bacterial isolates depicted that, the *Streptococci*i solated from MS agar had the characteristics of *S. mutans* (Coykendall, 1989) and the roads isolated from MRS agar had the characteristics of *Lactobacillus* (Wheater, 1995).

5.1.3.2. Antibacterial tests of thyme EOs against *S. mutans* and *Lactobacillus*

According to different findings, oxygenated monoterpenes like thymol and carvacrol exhibit strong antimicrobial activity and the hydrocarbon derivatives possess lower antimicrobial properties. This is because the low water solubility of hydrocarbons limits their diffusion through the medium. In the work by Soković *et al.* (2008) for example, the trend of antibacterial activity of EO components were in the order of ; linalyl acetate < limonene < β-pinene < α-pinene < camphor < linalool < 1,8-cineole < menthol <thymol < carvacrol against *Proteus mirabilis* (human isolate) *Pseudomonas aeruginosa*(ATCC 27853), *Staphylococcus aureus*(ATCC 25923), *Staphylococcus epidermidis*(ATCC 12228), *Micrococcus flavus*(ATCC 9341), *Bacillus subtilis*(ATCC 10707), *Escherichia coli* (ATCC 0157:H7), *Enterobacter cloacae* (human isolate), *Salmonella enteritidis*(ATCC 13076), *Salmonella typhimurium* (ATCC 13311).

The EOs of thyme in the present study were found to contain components with high antibacterial activities (thymol and carvacrol). The respective percentages of thymol and carvacrol in the test EOs were as follows: Ofla (49.55%, 36.34%), Alamata (65.63%, 6.68%), Yilmana Densa (6.52%, 80.84%), Tarmaber (48.84%, 42.12%), Butajira (15.77%, 71.83%), and Bale (53.57%, 34.55%). Therefore, it is possible to deduce from this finding and the findings from Soković *et al.* (2008) that the EOs of Yilmana Densa, Butajira, and Tarmaber to show the highest antibacterial activity against *S. mutans* and *Lactobacillus* followed by that of Bale, Ofla, and Alamata respectively. However, minor deviations were seen in the real result.

It was the Tarmaber EO that inhibited best both *S. mutans* and *L. acidophilus*. This may be due to the synergistic/additive activities of thymol and carvacrol components since the two are found in close percentages 48.84% and42.12% respectively. On the other hand, the least effective was the EO from Alamata since it is majorly thymol (65.63%) and carvacrol (6.68%) (Soković *et al.*, 2008).

Lippia sidoides EO (LSEO) and its components thymol and carvacrol showed similar trends of inhibition against *S. mutans* with respective doses and inhibition zones of (217.5 mg/mL, 18.7mm), (50 mg/mL, 7.8mm) and (50 mg/mL, 8.0mm) (Botelho *et al.*,2007). This implies carvacrol has more antibacterial activity against *S. mutans* than thymol which agrees with the antibacterial activities of the chemotypes of EOs in the present study. The MIC/MBC values of thymol and carvacrol against *S. mutans* were 5 mg/mL/10 mg/mL and 2.5 mg/mL/5 mg/mL respectively (Botelho *et al.*, 2007). In the present study, the MIC/ MBC doses were: Ofl, Ala, Yil and Buta EOs (0.5/0.5 mg/mL)

against both *S. mutans* and *L. acidophilus* and Bal EO (0.25/0.25) mg/mL against *S. mutans* and 0.5/0.5 mg/mL against *L. acidophilus*.

The present study shows that the EOs of *T. serrulatus* from Ala, Ofl, and Yil and *T. schimperi* from Tar, Buta, and Bal showed good antimicrobial activity against cariogenic bacteria namely *S. mutans* and *Lactobacillus*. *S. mutans* and *Lactobacillus* have been tested for antimicrobial susceptibility to other EOs (Botelho *et al.,* 2007). As far as known, this is the first report on the antibacterial activities of the EOs of *T. serrulatus* and *T. schimperi* against oral pathogenic bacteria.

Thyme species EOs have been known to possess antibacterial properties (Kaloustian *et al.,* 2005) for long, and the Gram-positive bacteria are generally susceptible than the Gram-negative ones (Niculae *et al.,* 2009). *S. mutans* and *L. acidophilus* both of which are Gram-positive bacteria thus were highly inhibited by the EOs of *T. serrulatus* and *T. schimperi*. The major components of thyme EOs result in antibacterial activities against cariogenic pathogens (Botelho *et al.,* 2007). *T. serrulates* and *T. schimperi* found to be thymol (Ofl, Ala, Bal and Tar) and carvacrol (Yil and Buta) chemotypes in this study thus inhibited *S. mutans* and *L. acidophilus*. This agrees with other thyme EOs which have antiseptic effects when applied externally or taken internally (Kaloustian *et al.,* 2005). This may be due to the lipophilic character of the hydrocarbon skeletons of the EO constituents as well as the hydrophilic character of their functional groups. The rank of activity EO components is: phenols > aldehydes > ketones > alcohols > esters > hydrocarbons (Figueiredo *et al.,* 2008).

The findings in this research show that the major components of the EOs were phenols (thymol or carvacrol). Thus they had effective antibacterial activities against cariogenic bacteria. The antibacterial activity of these EOs may be resulted from the bioactivities of their major components or the interactions of all their components (Zengin and Baysal, 2014). For example in a review of studies made by Bassolé and Juliani (2012), thymol and carvacrol interactions majorly showed synergistic activities against pathogenic bacteria. Synergistic activities were also seen between thymol and eugenol, carvacrol and eugenol, carvacrol and linalool, menthol and geraniol, and menthol and thymol against different bacteria. The total antibacterial activities of EOs in general are due to their synergistic, additive, and antagonistic activities of their components.

The mechanism of action of the EOs of *T. serrulatus* and *T. schimperi against S. mutans* and *Lactobacillus* may be owing to their action on multiple targets of action (Barkat and Bouguerra, 2012). Such sites of action include ability to inhibit respiration (Cox *et al.,* 2000; Nazzaro *et al.,* 2013), increase permeability of cytoplasmic and plasma membranes (Tripathi *et al.,* 2013; Nazzaro *et al.,* 2013), increased potassium ion leakage, increased disruption of the permeability barrier of cell membrane structures, as well as loss of chemiosmotic control (Cox *et al.,* 2000; Nazzaro *et al.,* 2013); disruption of polysaccharides, fatty acids and phospholipids in different layers of these structures (Tripathi *et al.*, 2013; Abdul-Hafeez *et al.*, 2014).

In addition, EOs may cause coagulation of the cytoplasm and damage of lipids and proteins of microorganisms (Tripathi *et al.,* 2013). To sum up, the mode of action of EOs against these bacteria may be in the same way as broad-spectrum membrane-active

disinfectants and preservatives, such as phenol derivatives, chlorhexidine and parabenzoic acid derivatives (Montanari *et al.,* 2011).*S. mutans* and *Lactobacillus* were found to be sensitive to 3% H_2O_2. This is because of their incapacity to produce the enzyme catalase.Catalase converts H_2O_2 into harmless molecules, H_2O and molecular O_2 (Mates *et al.,* 1999).

5.1.4. Hepatoprotective activities of thyme EOs against paracetamol induced hepatotoxicity in wister male rats

In the present study, paracetamol administration (2 g/kg body weight) to Wister male rats resulted in substantial increase in serum levels of AST, ALT and ALP. Several research reports show elevated serum amounts of these enzymes after administration of toxic doses of paracetamol in rats (Alamgeer *et al.,* 2014; Al-Fartosi *et al.,* 2011; Dar *et al.,* 2012; Gupta *et al.,* 2013; Grespan *et al.,* 2014). During hepatocyte toxication, the membranes of hepatocytes become leaky to these enzymes so that their elevated concentration in the serum makes them sensitive indicators of necrotic lission in the liver (Ebenyi *et al., 2012;* Galal *et al.,* 2012). Thus the marked release of AST, ALT and ALP into the circulation is an indicator of severe injury to hepatic tissue membranes during paracetamol intoxication (Galal *et al.,* 2012; Grespan *et al.,* 2014)

The observed significant decrease of serum AST, ALT and ALP levels in groups of rats treated with Silybinin and the EOs of thyme collected from Ala, Yil, and Tar demonstrated their hepatoprotective activities against paracetamol induced liver damage. Similar results have been reported by other researchers about the hepatoprotective

activities of the EOs of other thyme species and plants with common chemical components (Galal *et al.,* 2012; Grespan *et al.,* 2014; Pinho *et al.,* 2014). The liver protective activity of *T. serrulatus* and *T. schimperi* may be due to their chemical constituents particularly phenols thymol and carvacrol (Sindhu *et al.,* 2012).

Thymus species from Ethiopia are rich sources of the monoterpenoid phenols, which contain important constituents such as thymol and carvacrol (Ermias Dagne *et al.,* 1998; Nigist Asfaw *et al.,* 2000). The present study too revealed thymol and carvacrol as the major constituents of *T. serrulatus* and *T. schimperi*. For example, *T. serrulatus* collected from Ala was found to contain thymol (65.65%) and carvacrol (6.68%) and that from Yil contained thymol (6.52%) and carvacrol (80.84%). In the same way, *T. schimperi* collected from Tar was found to contain thymol (48.84%) and carvacrol (42.12%).

The possible hepatoprotective activities of these EOs on paracetamol induced hepatotoxicity may be through functional improvement of hepatocytes, which may be caused by an acceleration of regeneration of parenchyma cells or by inhibiting cytochrome p-450 activity, or by stabilizing the hepathocellular membrane, or by inhancing the antioxidant activity or all (Al-Fartosi *et al.,*2011). The radical scavenging tests against thyme EOs and their components (thymol and carvacrol) has revealed the strong antioxidant activities of these EOs and their phenolic components (Alamgeer *et al.,* 2014; Kulisic *et al.,* 2006; Gramza-Michałowska *et al.,* 2008; Salem *et al.,* 2010; Abd-Algader *et al.,* 2013).

Phenols are important plant components with free radical scavenging activities and antioxidant actions owing to their hydroxyl groups (Ebenyi *et al.,* 2012). Plant

preparations like *T. serrulatus* and *T. schimperi* EOs containing phenolic compounds in significant amounts therefore retain antioxidant activities. On the other hand, the EOs tested may increase natural antioxidants in the body like GSH, superoxide dismutase (SOD), and catalase (CAT) which in turn may protect the liver from paracetamol induced hepatotoxicity. This is supported by the work of El-Banna *et al.* (2013) where the EOs of *Thymus* and salvia resulted in increased levels of GSH and SOD in paracetamol intoxicated rats. Similarly, it was reported in the work by Ebenyi *et al.* (2012) that pretreatment of rats with ethanolic extract of *Alliums sativum* increased the levels of GSH, SOD, and CAT.

The antioxidant activity of these EOs may be due to their ability to donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step (Pinho *et al.*, 2014). The other mechanism of tissue injury is an inflammatory process and the mobilization of polymorphonuclear leukocytes to the site of acute inflammation is one of the key processes in the host response to tissue injury (Pinho *et al.*, 2014). *Thyme* EOs are known to have anti-inflammatory activity (Alviano and Alviano , 2009; Belaqziz *et al.,* 2010; Meshkatalsadat *et al.,* 2010). Thus the other mechanism of hepatoprotection of these EOs may be by their anti-inflammatory activities.

Thus, EOs of *T. serrulatus* and *T. schimperi* protect the liver from paracetamol overdose, suggesting that the hepatoprotective effect can be considered an expression of the functional improvement of hepatocytes that results from accelerated cellular regeneration (Pinho *et al.*, 2014). Furthermore, previous studies have also shown that thyme EOs have properties of scavenging free radicals and serve as antioxidants and hepatoprotectives

(Alamgeer *et al.*, 2014; Kulisic *et al.*, 2006; Gramza-Michałowska *et al.*, 2008; Salem *et al.*, 2010; Abd-Algader *et al.*, 2013).The EOs of *T. serrulatus* and *T. schimperi* could interact directly with components of the cell membrane to prevent abnormalities in the content of the lipid fraction which is responsible for maintaining normal fluidity of this lipid fraction (Pinho *et al.*, 2014).

5.1.5. mosquitocidal activity

In the larvicidal, fumigation and oviposition detterent tests, all the EOs under test (Ala, Yil, and Tar) resulted in mosquitocidal activities especially at higher doses. These mosquitocidal activities could be associated to the presence of EO components; mainly thymol and carvacrol (Amri *et al.*, 2014). The percentages of thymol and carvacrol were; 65.63% and 6.68% (Ala), 6.52% and 80.84% (Yil), and 48.84% and 42.12% (Tar). Generally, Tar with (thymol + carvacrol = 90.96%) was the most effective EO followed by that of Yil (thymol + carvacrol = 87.36%) which in turn was followed by Ala (thymol + carvacrol = 72.31%). This difference is an indicative of the importance of these phenolic monoterpenes as insecticides. Furthermore, the EO Tar was almost with equal proportions of thymol/carvacrol (1.16/1), Ala was much of thymol type (thymol/carvacrol = 9.8/1), and Yil was more of carvacrol type (thymol/carvacrol = 1/12.4). Thus the mosquitocidal activities of Tar were due to either the synergistic or additive activities of the two monoterpenes. Whereas, the activities of Ala were more due to thymol and that of Yil was more due to carvacrol. But insecticidal activities of other components like sesquiterpenes should be considered.

Thymol and carvacrol are known larvicidals against various insects. For example, they were found to be strong larvicidals against lesser mealworm (*A. diaperinus*) where the pure components had strong activities when compared with the oil containing them. That is 1% thyme oil, thymol and carvacrol, caused mortality of 50.0, 86.67 and 85%, respectively (Reddy *et al.,* 2014). These substances act in many ways on various insects namely as neurotoxins, growth regulators, antifeedants, repellents (Szczepanik *et al.,* 2012). Thymol affects the central nervous system, the longitudinal flight muscles and neuromuscular junctions. It acts on GABA-sensitive sites *in vivo*, either by mimicking or facilitating the effects of this inhibitory neurotransmitter (Waliwitiya *et al.,* 2009). Thymol also plays a repellent action against mosquitoes (Wahyuni, 2012).

Other volatile oil components which act as insecticides include; camphor, cineole, methyl eugenol, limonene, myrcene which act both as repellents and a larvicidals against mosquitoes. d-limonene, *α*-myrcene, *α*-terpineol, linalool and pulegone are neurotoxic of which linalool was identified as an inhibitor of acetylcholinesterase (Wahyuni, 2012). The sesquiterpene (*Z*)-caryophyllene inhibits the activity of several enzymes such as acetylcholinesterase, glutathione s-transferase, carboxyl esterase, which are important detoxification enzymes of insect body, involved in the detoxification of antifeedants, fumigants, and pesticides (Amri *et al.*, 2014).

Thus, it is not surprising if the EO Tar containing thymol (48.84%), carvacrol (42.12%), linalool (2.97%), α-terpineol (0.95%), and t-caryophyllene (0.43%) is a strong mosquitocidal. Similarly, Yil possessed carvacrol (80.84%), thymol (6.52%), linalool (0.25%) and t-caryophyllene (0.82%) all of which have insecticide properties as

mentioned above. The EO Ala also has thymol (65.63%), carvacrol (6.68%), linalool (3.29%), and t-caryophyllene (1.5%) all of which are mosquitocidals. Thus the three EOs (Tar, Yil, and Ala) have components with mosquitocidal activities.

Carvacrol has broad insecticidal and acaricidal activity against agriculture, stored product and medical pests, and it acts as a fumigant. Therefore, it is found that carvacrol has a stronger insecticidal activity than thymol (Szczepanik *et al.*, 2012; Popović *et al.*, 2013), and that in combination with thymol its insecticidal potential decreases. This indicates the antagonism of the two phenols when used as an insecticide (Popović *et al.,* 2013). The results from Yil and Ala agree with these statements in that the carvacrol type Yil is highly effective than the thymol type Ala. However, the presence of thymol and carvacrol in almost equal proportions in Tar had the best result contradicting with the findings of Popović *et al.* (2013) which showed reduced impact of carvacrol in the presence of thymol. This difference may be due to the synergistic activities of these EOs with other components of Tar EO.

5.1.6. Acute oral toxicity of the EOs of *T. serrulatus* and *T. schimperi*

Observation immediately after EO administration: Mice given single doses of thyme EOs (2000 mg/Kg body weight (bw) showed immediate responses after administration. Such responses included burning sensations in their oral cavities. Some mice tried to cool themselves by climbing on the water teat, eating straw bedding, and some others tried to hide under the straw bedding and produced pain sounds. This may be due to the irritating ability of the phenolic monoterpenes thymol and carvacrol (Soni, 2012). The irritating

symptoms were more pronounced in Ala EO than the rest EOs. This may probably be due to the irritating effect of thymol in this EO (Fachini-Queiroz *et al.*, 2012). It contained over 65% thymol which is much higher than that of Ofl (49.55%), Tar (48.84%), Bal (53.57%) Buta (15.77%) and Yil (6.5%).

Observation at 30 minutes after dosage: At this time, most of the mice showed no interest of feeding and drinking. Only few mice showed interest of feeding after this time. However, some mice, for example, those delivered with Yil EO remained dormant for the next two hours after dosage. Furthermore, in the first 30 minutes after dosing, all the mice exhibited symptoms of toxicity like; convulsions, tremors, morbidity, pilo-erection and depression. Some mice for instance two given Bal and one given Ofl EOs had hind legs in spasm. They were unable to walk during this time and the spasm level decreased through time. This agrees with the finding of Elhabazi *et al.* (2012) where mice treated with *T.broussonetii* and *T.leptobotrys* EOs remained immobilized for some time. This is also supported by another report which explains that a concentrated ethanol extract of *T. vulgaris* produced decreased locomotor activity and slight slowing down of respiration in mice in an acute toxicity test (EMA/HMPC, 2013).

Observation at 4^{th} hour after dosing: Convulsion, pilo-erection and depression continued to the 4^{th} hour after dosing in all mice while signs of morbidity continued in nearly 80% of the mice by the 4^{th} hour of observation. On the other hand, the control mice (delivered with 0.1% T- 80 in normal saline) remained normal starting from the

time of delivery except some symptoms of discomfort due to the gavage inserted in their throats.

Unlike mice in the control group, those treated with EOs at 2000 mg/kg body weight resulted in reduction of body weight within the first 24 to 48 hours. After this time, there was a progressive increase in body weight of mice treated with EOs from Ofl, Ala, Tar, and Bal. This suggests that these EOs did not affect normal physiological functioning implying that they did not affect general growth and body weight.

Mice treated with Buta were exceptional in that their body weights decreased till day seven and showed improvements afterwards. At the same time the trend of growth in mice treated with Yil EO was slower throughout the 14 days. Thus it is possible to conclude that mice were more sensitive to the EOs of Yil and Buta (carvacrol chomotypes) than to the rest four. The EOs of Yil and Buta possessed carvacrol with respective percentages of 80.84% and 71.83%. Where as the EOs Ofl, Ala, Tar and Bal contained predominantly thymol 49.55%, 65.63%, 48.84% and 53.57% respectively. It can be supposed that this component is mainly responsible for this toxic effect. Similar trends of toxicity were seen in two Moroccan endemic species: *T.broussonetii* and *T.leptobotrys* (Elhabazi *et al.,* 2012).

The heart to body weight ratio, kidneys to body weight ratio, liver to body weight ratio and brain to body weight ratio in the mice tretead with all the EOs showed no statistically significant difference from the control group. Verifying that all the EOs had no negtive effect on the mentioned organs. On the other hand, statistically significant differences

were observed among groups with respect to the lung to body weight ratio, spleen to body weight ratio and PCV.

Mice treated with Ofl, Buta and Bal had lung to body weight ratios significantly lower than those treated with Ala, Yil, Tar, and the control group. The mice treated with Yil had spleen to body weight ratios significantly higher than that of the rest groups including the control. Thus, spleen enlargement in Yil treated group may be resulted from the toxic effects of carvacrol. In all the cases, none of the EOs was found to be responsible for organ inflammation. Anatomical observtion of these organs from each group also showed no signs of toxicity.

When we see the trend of %PCV, mice treated with 2000mg/kg bw EOs (Ala, Yil, and Tar) had %PCVs which were not significantly different from that of the control group. The %PCVs of mice treated by 2000 mg/kg bws of Ofl, Buta, and Bal EOs, however, were higher than the control group. Generally, it is possible to argue that all the EOs had no toxicity effects on red blood cells since this finding contradicts with cases of toxicities where the %PCVs decrease (Husna *et al.,* 2013).

Furthermore, eventhough some of these mice treated at 2000 mg/kg body weight oral toxicity some symptoms of toxicity like pilo-errection, some sort of weight loss (Buta), and irritability during administration, none of them died at the end of the 14^{th} day. Thus the EOs can be categorized as category 5 according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (OECD, 2001; UN, 2011). Thus all the six EOs tested are relatively of low acute toxicity hazards but which under certain circumstances may present a danger to vulnerable populations (UN, 2011). It is generally

belevied that all substances are poisons but the right dose differentiates a poison and a remedy (Paracelsus cited in Lahlou, 2004). Thus it is necessary to determine the median lethal doses of substances to be taken as medicaments.

The EOs in this study have an oral LD_{50} values in a range of 2000-5000 mg/kg bodyweight and converted acute toxicity point (LD_{50}) estimate of 2500 mg/kg body weight (bw) (UN, 2011). The acute oral toxicity was done using EOs and EOs are known to be highly concentrated substances. The yield of EOs from dry materials was found to be 0.5% (Ofl and Ala), 0.8% (Yil, Tar, and Buta) and 0.9% (Bal). Thus the LD_{50} values of the dry weights of thyme were approximated to be around 278g /kg bw (Bal), 313g /kg bw (Yil, Tar, and Buta) and 500g /kg bw (Ofl and Ala).

This extrapolation is very important since people in these localities use the dry materials in the form of tea or food additives, not the EOs. Thus, the high LD_{50} values of *T. serrulatus* and *T. schimperi* suggest that these plants are relatively safe and nontoxic. The results from this work agree with the saying that "thyme is known to be a nonpoisonous plant" (Yürüktümen *et al.,* 2011). Reports show that pure components of thyme EOs like thymol had LD_{50} value much lower than that of the EOs tested. It had been reported by EPA that the LD_{50} values of thymol were 980mg/kg and 800mg/kg in rats and guineapigs respectively. However, the phenols in thymol are considered to be as GRAS (generally considered as safe) (EPA, 1983).

5.2. Conclusions

The specimens of *Thymus* collected from Southern Tigray (Alamata and Ofla) as well as from West Gojjam (Yilmana Densa) were found to be *T. serrulatus* while those collected from Tarmaber, Butajira, and Bale were *T. schimperi*. Many of the informants agreed about the importance of these species to treat blood pressure followed by general pain syndrome, influenza, abdominal pain, and intestinal parasites like ascaris in descending order. However great number of them especially those from Tigray do not know about the medicinal uses of these plants. Economically, these plants are important sources of animal and honey bee forage, serve as condiments, tea, and fumigants. Despite their medicinal and economic uses, these plants are highly diminishing due to overharvesting, overgrazing, agricultural expansion, whole plant harvesting including the roots, and lack of recognition.

To identify the components of the six EOs, 33 reference compounds were run by GCMS, 17 of which were confirmed to be found in the EOs. Ala was found to be the richest and Tar the poorest EOs in their number of components with 42 and 18 components respectively. In All the EOs, thymol and carvacrol were found to be the predominant compounds one following the other. Four of the EOs (Ofl, Ala, Tar, and Bal) were found to be thymol chemotypes and the other two EOs (Yil and Buta) carvacrol chemotypes. NMR data from Ofl showed the presence of indicators like methoxy, and exomethylene groups, which may be indicators of the presence of carvacrol methyl ether and thymol methyl ether and 1-octene-3-ol respectively. NMR data also showed structural elements which served as indicators for the presence of linalool.

The EOs of *T. serrulatus* and *T. schimperi* showed high level of antibacterial activity against tooth decay bacteria, *S. mutans* and *Lactobacillus* in a dose dependent manner. The trend of antibacterial activity of these EOs was mainly dependent on the major components of the EOs. EO with close proportions of thymol and carvacrol (Tar) was the most effective EO followed by the carvacrol dominant EO (Yil) and thymol dominant EOs with relatively lower antibacterial activity. The MIC/MBC of these EOs was almost the same (0.5mg/mL/0.5mg/mL) against these bacteria except the EO from Bale which had stronger effects (0.25mg/mL/0.25mg/mL) against *S. mutans*. At 128 mg/mL these EOs had antibacterial effects comparable to or higher than the pharmaceutical mouth rinse in Ethiopia (3% H_2O_2).

The results from this study suggest that EOs of *T. serrulatus*and *T. schimperi* pretreatment minimized hepatotoxicity in rats against paracetamol-induced damage. The Ala EO (predominantly thymol type) was the best hepatoprotective EO followed by Yil (predominantly carvacrol chemotype) and then by Tar (thymol chemotype but with close concentrations of thymol and carvacrol). This hepatoprotective activity of the EOs was seen by finding reduced level of indicator enzymes (ALP, ALT, and AST) and lack of necrosis in liver sections.

The EOs of *T. serrulatus* (from Ala and Yil) as well as *T. schimperi* (from Tar) acted as larvicidal, fumigant, and oviposition detterent against *An. arabiensis* 3rd and 4th instar larvae, non-gravid females and gravid females respectively. Tar was the best mosquitocidal EO followed by Yil which inturn was followed by Ala. The LD_{50} concentrations for larvae were: (60.75 µL/L, Ala); (44 µL/L, Yil); and (41.75 µL/L, Tar).

The LD_{50} values for the fumigation test on the other hand were: (17.19 µL/L, Ala); (14.92 µL/L, Yil); and (13.20 µL/L, Tar). The 200 µL/L, 100 µL/L, and 50 µL/L doses of Tar, the 200 µL/L and 100 µL/L doses of Ala and the 200 µL/L dose of Yil resulted in complete oviposition detterent activity. All the tested doses of Ala and Tar and doses of Yil (200, 100, and 50 µL/L) acted as repellents. However, the the lower doeses of Yil that is; 25 and 12.5 µL/L acted neither as ovipossition detterents nor as attractants.

The entire EOs tested for acute oral toxicity showed burning sensations in mice with Ala (thymol chemotype) being the most irritant than the rest EOs. The carvacrol chemotypes (Yil and Buta) resulted in reduced growth of mice than the thymol chemotypes (Ofl, Ala, Tar, and Bal). The LD_{50} of the EOs are in the range of 2000 mg/kg to 5000 mg/kg body weight of the test mice. Since the aerial parts of thyme are used in the form of tea and food additives and not the EOs and since the EO yield of thyme ranged from 0.5 to 0.9% (v/w), consumtion of a big load of these herbs can only be poisonous. However, they may present a danger to vulnerable populations and cautions should be taken.

To sum up, close combinations of carvacrol and thymol (Tar) resulted in effective antibacterial activities followed by carvacrol dominant chemotype (Yil) and thymol type resulted in lowest antibacterial activities. With regard to hepatoprotection against paracetamol induced hepatotoxicity, thymol dominant EOs (Ala) were more effective than the carvacrol dominant ones. The carvacrol-thymol dominant EOs were more effective mosquitocidals and larvicidals when compared to the thymol chemotypes. However, the thymol chemotypes were better oviposition deterrents than the carvacrol chemotypes. On the other hand, the carvacrol chemotypes were more oral acute toxicity

than the thymol chemotypes. As a whole, the different chemotypes of *T. serrulatus* and *T. shimperi* EOs resulted in antibacterial activities, hepatoprotective activities, mosquitocidal activities and were not toxic.

5.3. Recommendations

- Awareness creation to the public about the economic and medicinal importance of *T. serrulatus* and *T. shimperi*;
- Organizing youth and women assosiations that may protect, conserve, and sustainably use these plant resources;
- Enhancing domestication and cultivation of these plants in agro ecological zones suitable for them since this is one alternative for conservation and poverty alleviation;
- Enhancing in situ conservation of these plants;
- Expanding research works on the toxicity and biological activities of these species and generation of products for market use;
- Closing the area where these species are found from human and animal encroachment;
- Strong quarantine services to avoid illegal and over-exploitive trade;
- Studying and deciding the harvesting times throughout the year;
- The frequency of harvesting *T. schimperi* and *T. serrulatus* should be with harvesting interval of 2 years (of the same plants) and the technique of harvesting should be to cut no lower than 5 cm from the ground, or 35% of the plant foliage.
- Encouraging the use of *T. serrulatus* and *T. schimperi* EOs in mouth rinse formulations is useful in the prevention and treatment of oral conditions and to avoid

dental caries. This too is an income source and may reduce unemployment by creating employment opportunities.

- Further detailed studies are required to; (i) investigate the mechanisms of hepatoprotection of these EOs against paracetamol induced hepatotoxicity, and (ii) identify the specific constituents of these EOs that are responsible for hepatoprotection.
- Use of the EOs of *T. serrulatus* and *T. schimperi*as larvicides, fumigants, and oviposition detterents could be a nice way to control mosquitoes. There is also a need to further test other mosquitocidal tests both at laboratory and field setups.
- It is better not to give thyme EOs to vulnerable groups like babies, children and pregnant women.
- Care should be taken while using thyme EOs since they are irritants to cutaneous membranes. Furthermore it is better to apply vegetable oils not water on irritated body parts to remove EOs when someone feels burning sensations.

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7. Appendixes

Appendix 1: Ethical Clearance for Saliva Collection

COLLEGE OF NATURAL SCIENCES
Addis Ababa University
OFFICE OF THE DEAN
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የተፈጥሮ ሣይንስ ኮሌጅ
አዲስ አበባ ዩኒቨርሲቲ

Ref: ቁጥር: CNSDO/215/07/15
Date: ቀን: January 13, 2015

To Whom It May Concern

The Ethical Committee of the College of Natural Sciences in its meeting held on 22/12/2014 (Minutes No.12) has examined the project entitled **"Bioactivity of Essential Oils of Thymus serrulatus and Thymus schimperi from Ethiopia: Hepatoprotective, Dental Caries Protective, Mosquitocidal and Toxicity"** by Destie Damtew (Department of Microbial, Cellular and Molecular Biology) for ethical approval.

The Proposal is approved for implementation. The decision will remain valid until December 21, 2015.

With regards,

Negussie Retta, (Professor)
Dean, College of Natural Sciences

Encl:

- RERC Minutes

Appendix 2: Checklist for recording acute oral toxicity signs and symptoms

Observation	30 Min.		4 Hrs.		24 Hrs.		48 Hrs.		1 Week		2 Weeks	
	C	EO	C	EO	C	EO	C	EO	C	EO	C	EO
Skin and Fur												
Eyes												
Mucous Membrane												
Salivation												
Lethargy												
Sleep												
Coma												
Convulsion												
Tremors												
Diarrhea												
Morbidity												
Mortality												
Pilo-erection												
Muscle spasm												
Depression												
Lacrimation												
Arching and rolling												

C = Control; EO = Essential Oil

Appendix 3: Questionnaire for collecting Ethnomedical data

1.

Family	Botanical name (Scientific name)	Local name	Habitat/ source	Part used	Health problems cured	Methods of preparation	Administration route	Voucher number

2. Current status of the plant: (a) increasing (b) decreasing

3. If the current status of the plant is increasing mention the reasons for its increment

_____

_____

4. If the current status of the plant is decreasing mention the reasons why it is decreasing

_____

_____

5. For what purposes do the public use this plant in addition to its medicinal value?

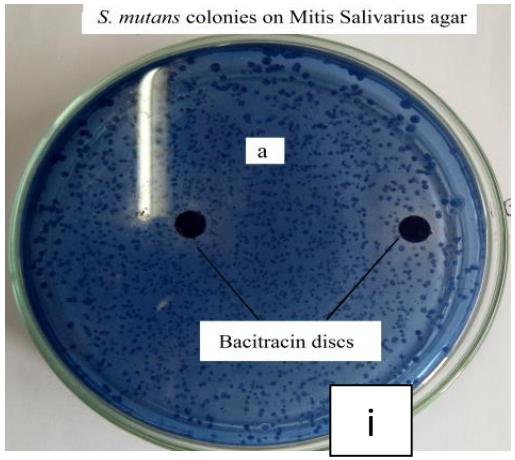
_____

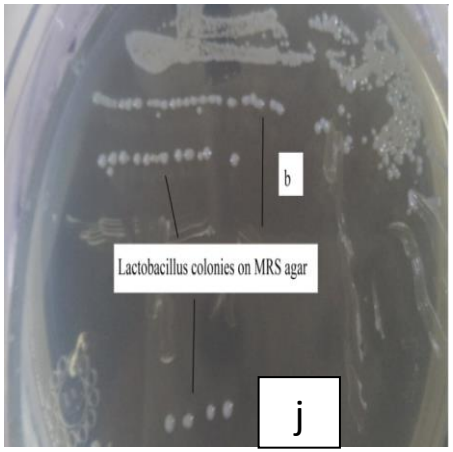
_____

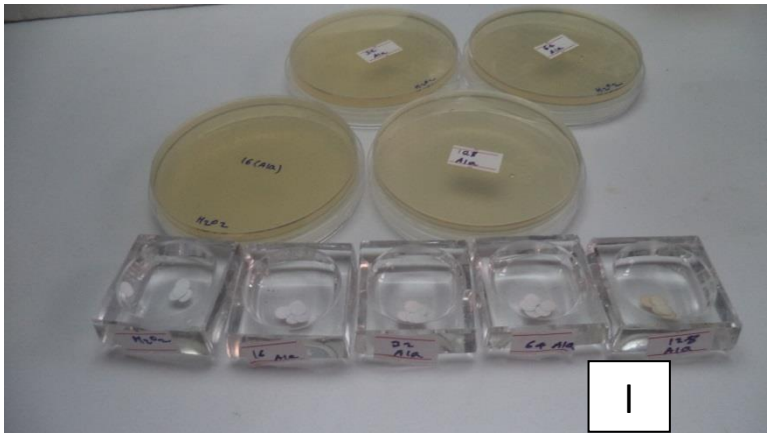
Appendix 4: Sample photographs showing the study process.

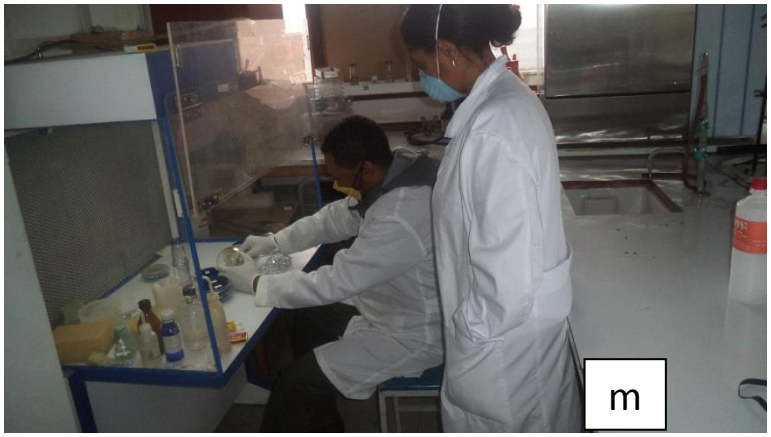
Collection of *T. serrulatus*and *T. schimperi* (a) Tarmaber; (b) Ofla (Menkere near lake Ashengie); (c) North Wollo on the way to Bahir Dar; (d) Checheho (South Gondar).

(e) drying; (f) grinding; (g) extraction; (h) storage of EOs

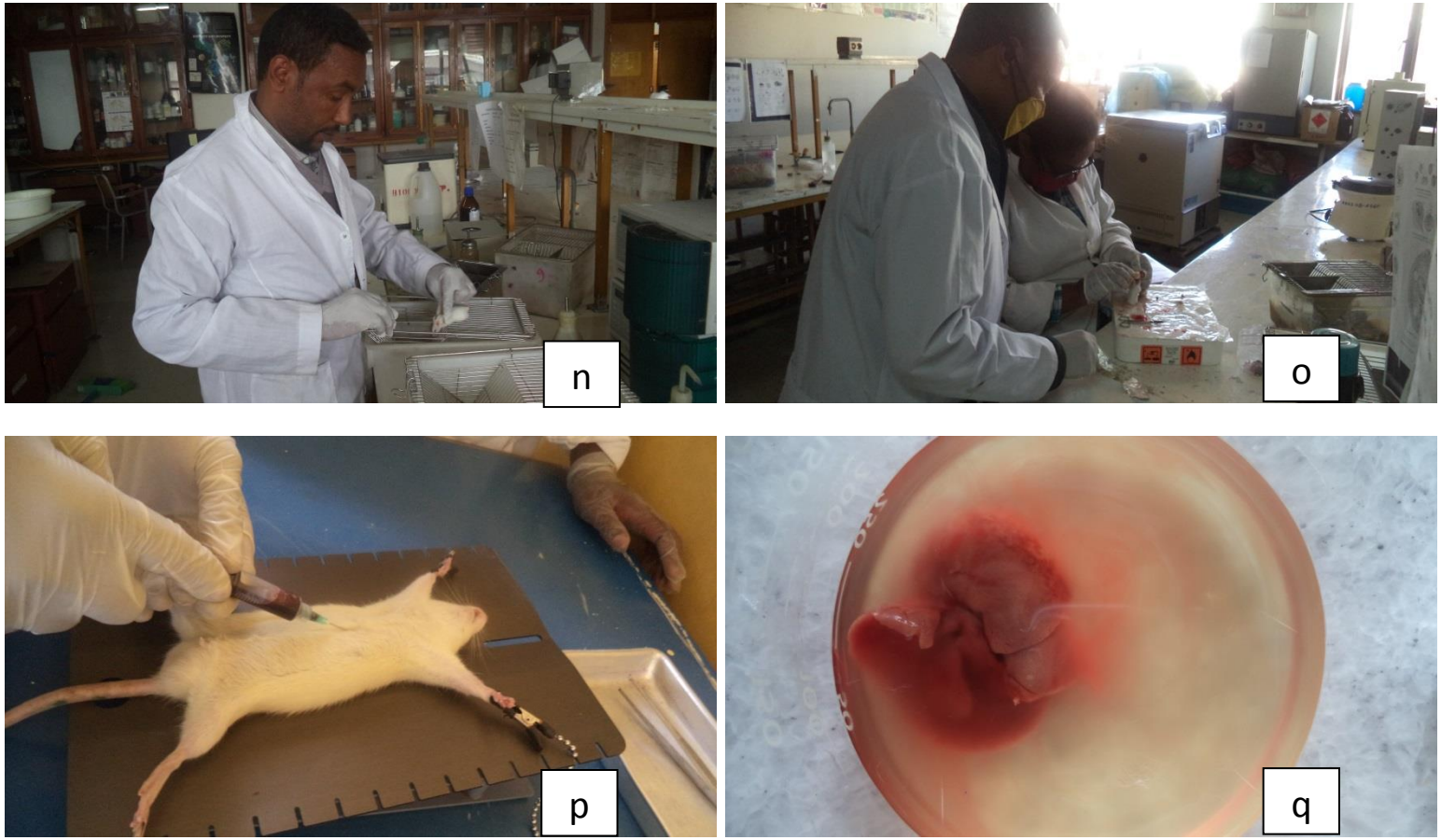






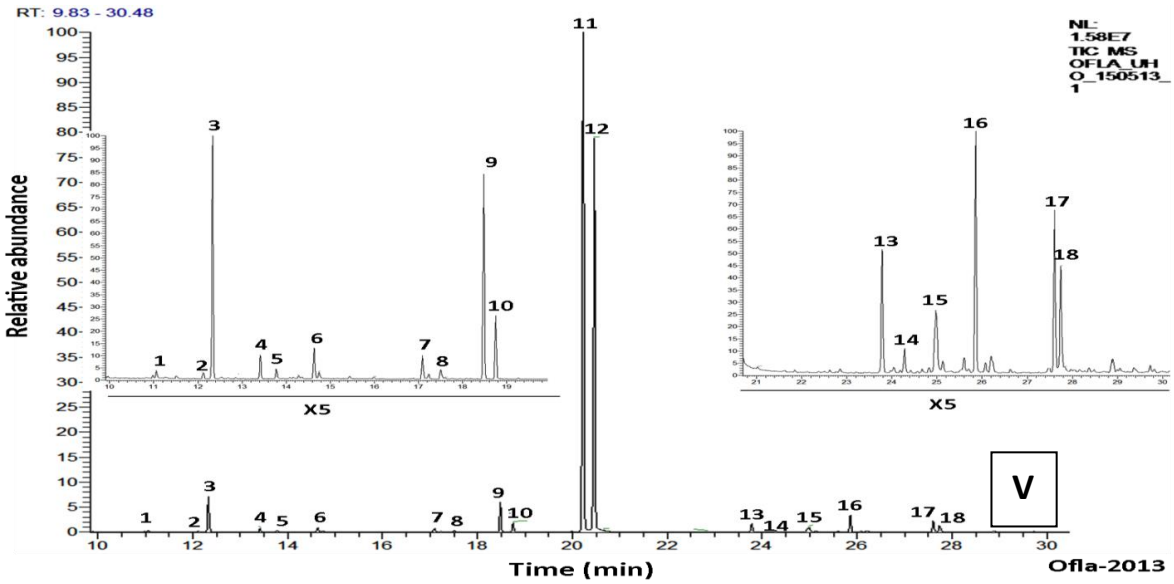


(I &j) growing onthogenic bacteria on selective media; (k) preparation of media for biochemical testing of the selected bacteria; (l) dose preparation for the antibacterial tests; (m) striking bacteria on selective media

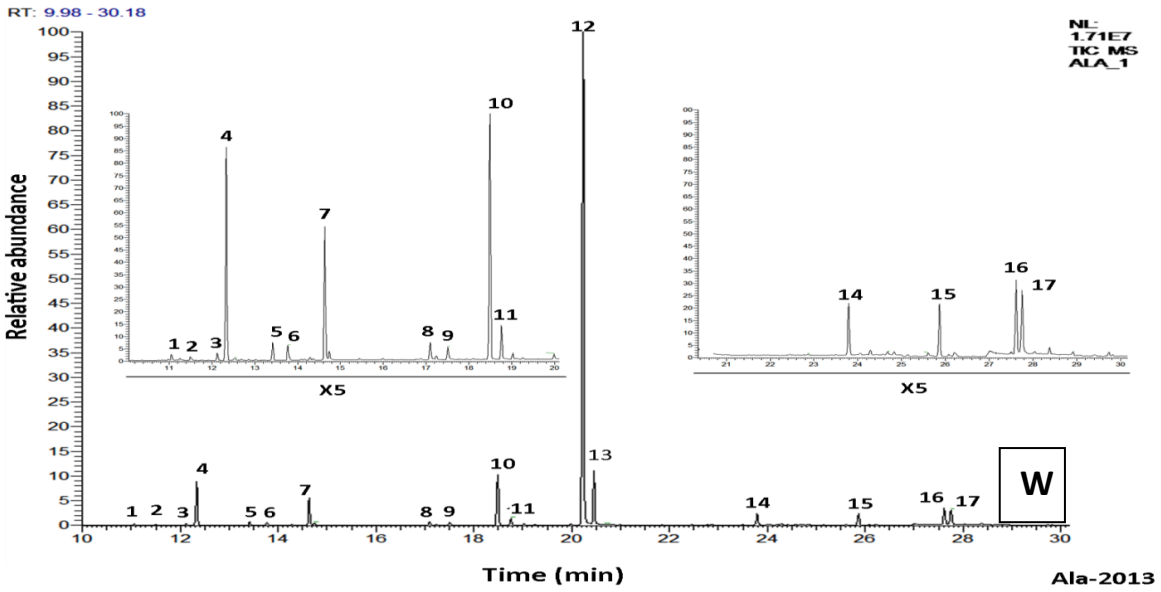


(n) working on a mouce for acute oral toxicity tests; (o) dissecting mice at the 14th day to measure organ to body weight ratios; (p) collecting a rats blood for hepatoprotective biochemical test; (q) preparing a rats liver for sectioning after hepatoprotective test

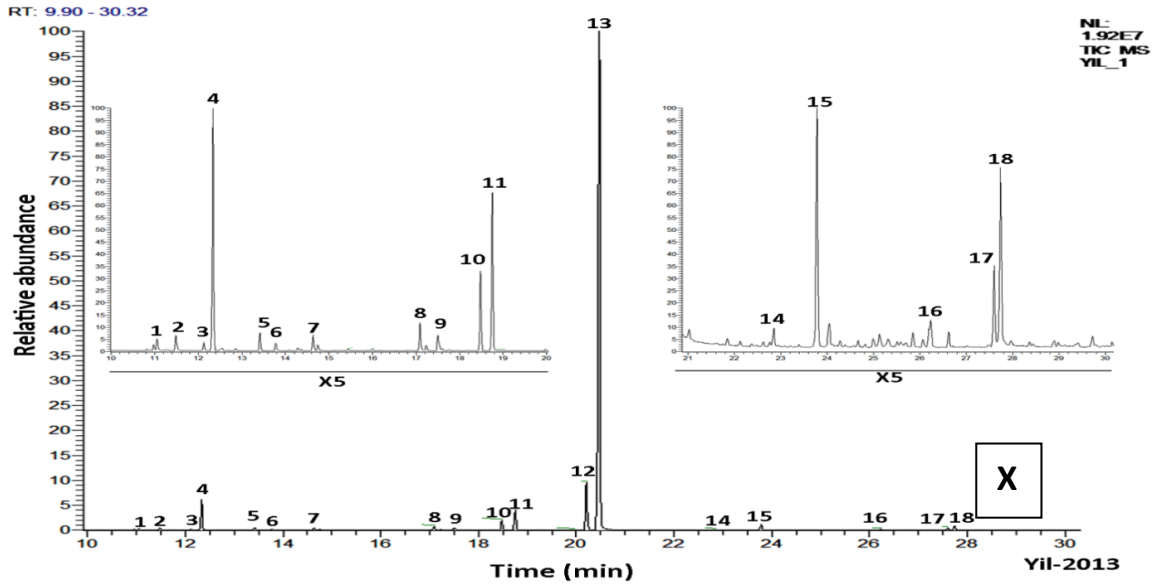
(r) larvicidal activity test; (s) fumigation test; (t) oviposition deterrent test; (u) photo with the staff of bioorganic chemistry institute of Hohenheim University, Germany.



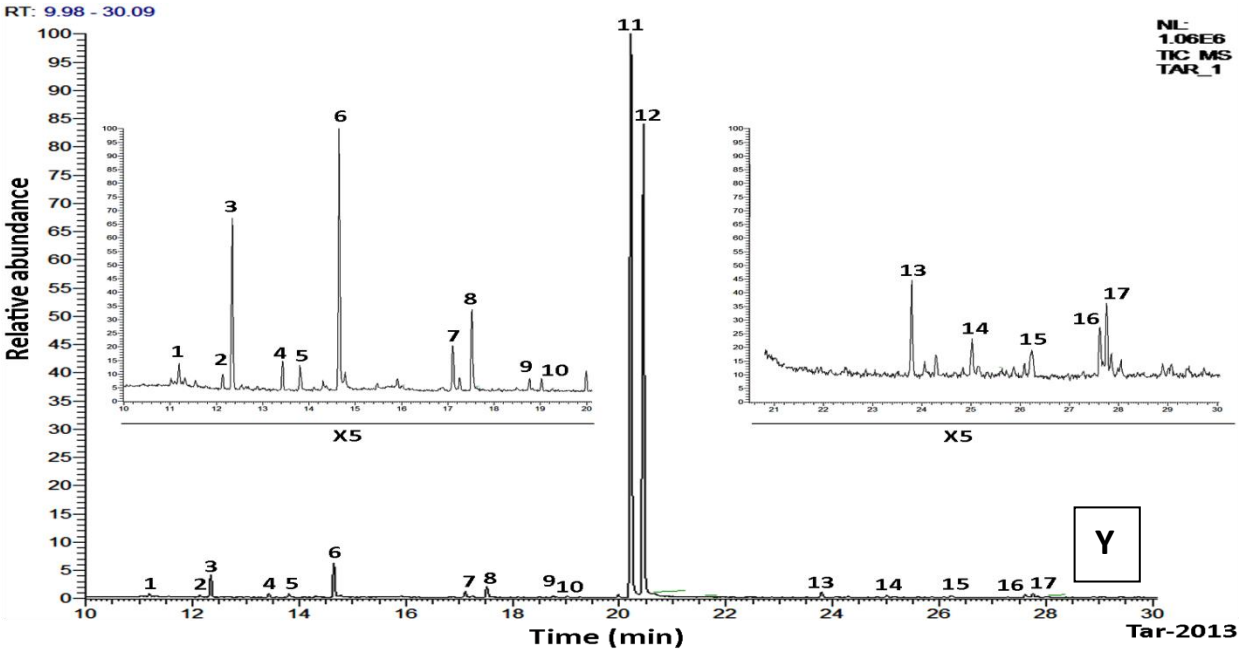
V: Gas chromatogram of Ofl (1: 1-octene-3-ol; 2: α-terpinene; 3: p-cymene; 4: γ-terpinene; 5: Cis-sabinene hydrate; 6: Linalool; 7: Terpinene-4-ol; 8: α-terpineol; 9: Thymol methyl ether; 10:Carvacrol methyl ether; 11: Thymol; 12: Carvacrol; 13: t-caryophyllene; 14: unidentified; 15: unidentified: 16: γ-cadinene; 17: Caryophyllene oxide; 18: Viridiflorol).



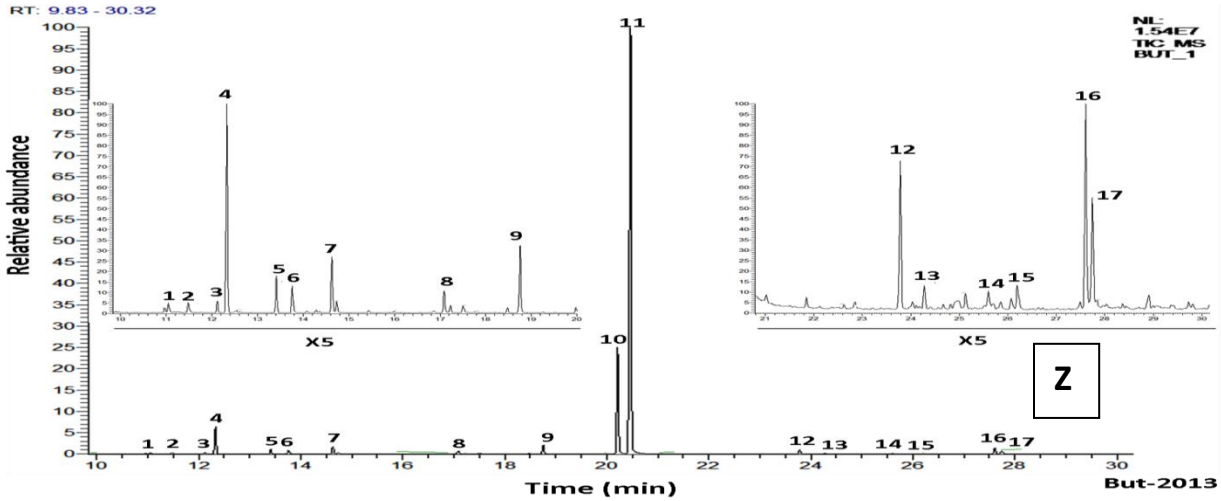
W: Gas chromatograms of Ala (1: 1-octene-3-ol; 2: Octan-3-ol; 3: α-terpinene; 4: p-cymene; 5: γ-terpinene; 6: Cis-sabinene hydrate; 7: Linalool; 8: Terpinene-4-ol; 9: α-terpineol; 10: Thymol methyl ether; 11: Carvacrol methyl ether; 12: Thymol; 13: Carvacrol; 14: t-caryophyllene; 15: γ-cadinene; 16: Caryophyllene oxide; 17: Viridiflorol).



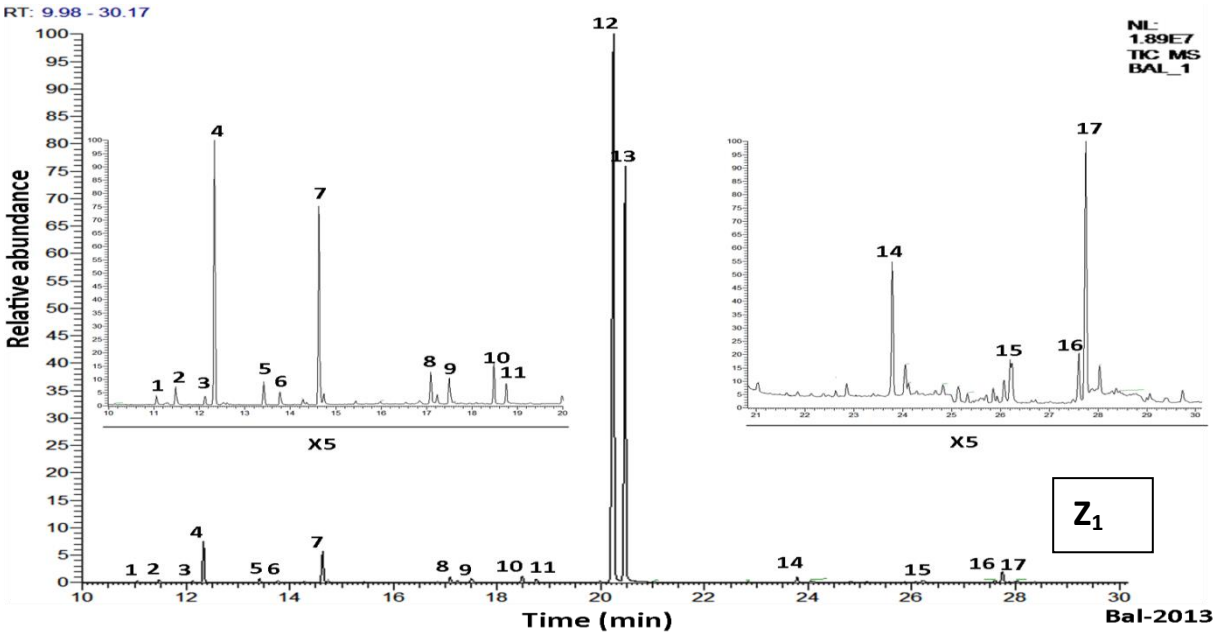
X: Gas chromatograms of Yil (1: 1-octen-3-ol; 2: Octan-3-ol; 3: α-terpinene; 4: p-cymene; 5: γ-terpinene; 6: Cis-sabinene hydrate; 7: Linalool; 8: Terpinene-4-ol; 9:α-terpineol; 10: Thymol methyl ether; 11: Carvacrol methyl ether; 12: Thymol; 13: Carvacrol; 14: unidentified; 15: t-caryophyllene; 16: α-cadinene; 17: Caryophyllene oxide; 18: Viridiflorol).



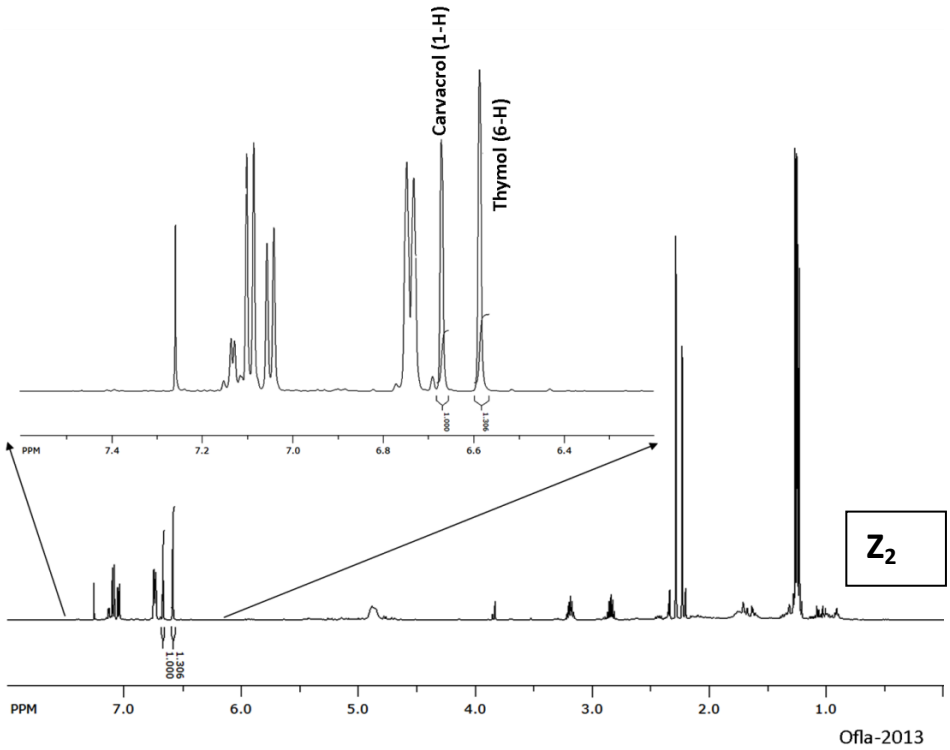
Y: Gas chromatograms of Tar (1: Unidentified; 2: α-terpinene; 3: p-cymene; 4: γ-terpinene; 5: Cis-sabinene hydrate; 6: Linalool; 7: Terpinene-4-ol; 8: α-terpineol; 9: Carvacrol methyl ether; 10: unidentified; 11: Thymol; 12: Carvacrol; 13: t-caryophyllene; 14: multiple peaks; 15: Multiple peaks; 16: Caryophyllene oxide; 17: Viridiflorol).



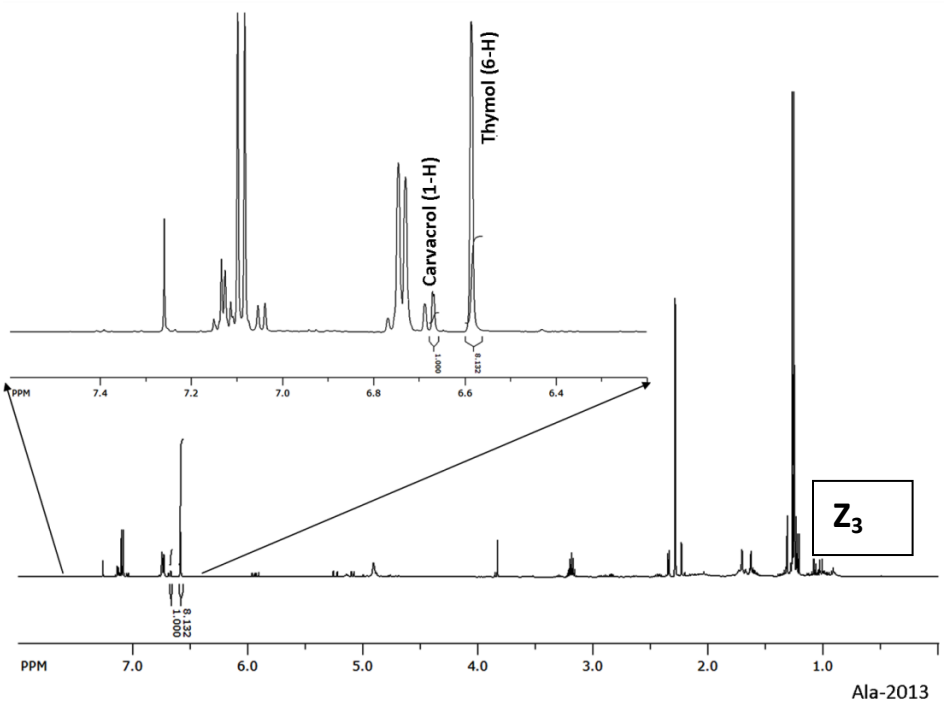
Z: Gas chromatograms of Buta (1: 1-octene-3-ol; 2: Octan-3-ol; 3: α-terpinene; 4: p-cymene; 5: γ-terpinene; 6: Cis-sabinene hydrate; 7: Linalool; 8: Terpinene-4-ol; 9: Carvacrol methyl ether; 10: Thymol; 11: Carvacrol; 12: t-caryophyllene; 13: unidentified; 14: Valencene; 15: α-cadinene; 16: Caryophyllene oxide; 17: Viridiflorol).



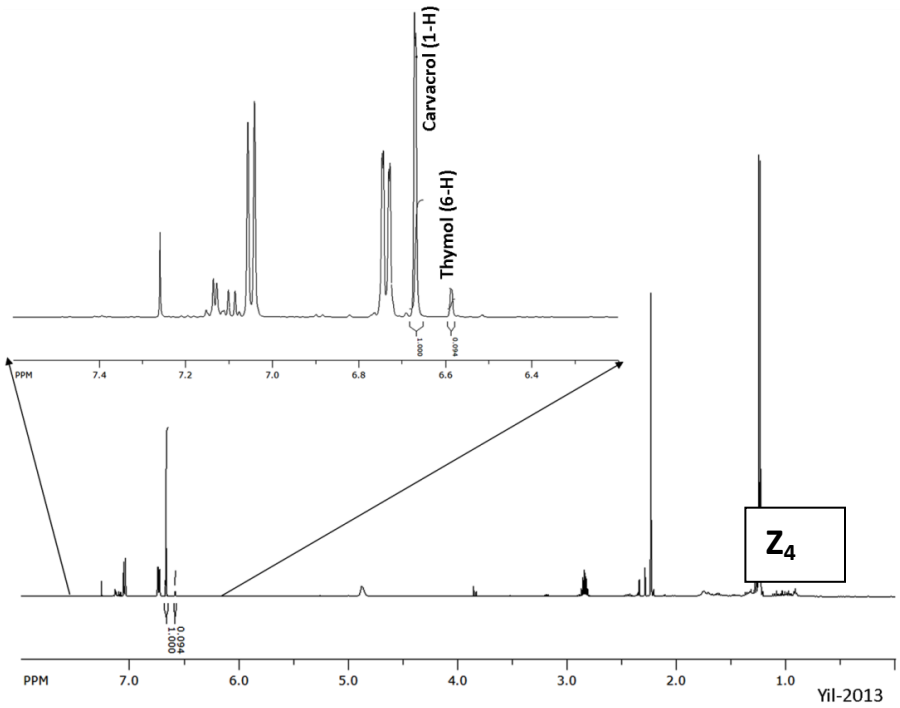
Z_1: Gas chromatograms of Bal (1: 1-octene-3-ol; 2: Octan-3-ol; 3: α-terpinene; 4: p-cymene; 5: γ-terpinene; 6: Cis-sabinene hydrate; 7: Linalool; 8: Terpinene-4-ol; 9:α-terpineol; 10: Thymol methyl ether; 11: Carvacrol methyl ether; 12: Thymol; 13: Carvacrol; 14: t-caryophyllene; 15: α-cadinene; 16: Caryophyllene oxide; 17: Viridiflorol).



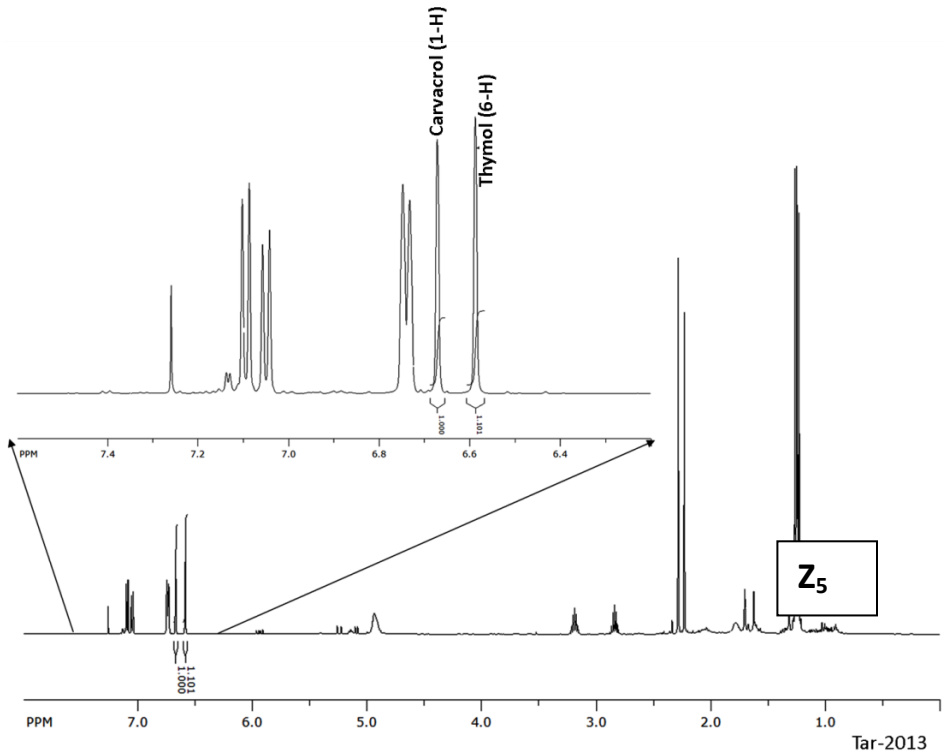
Z_2: Relative intensity of carvacrol to thymol in Ofla-2013 EO.



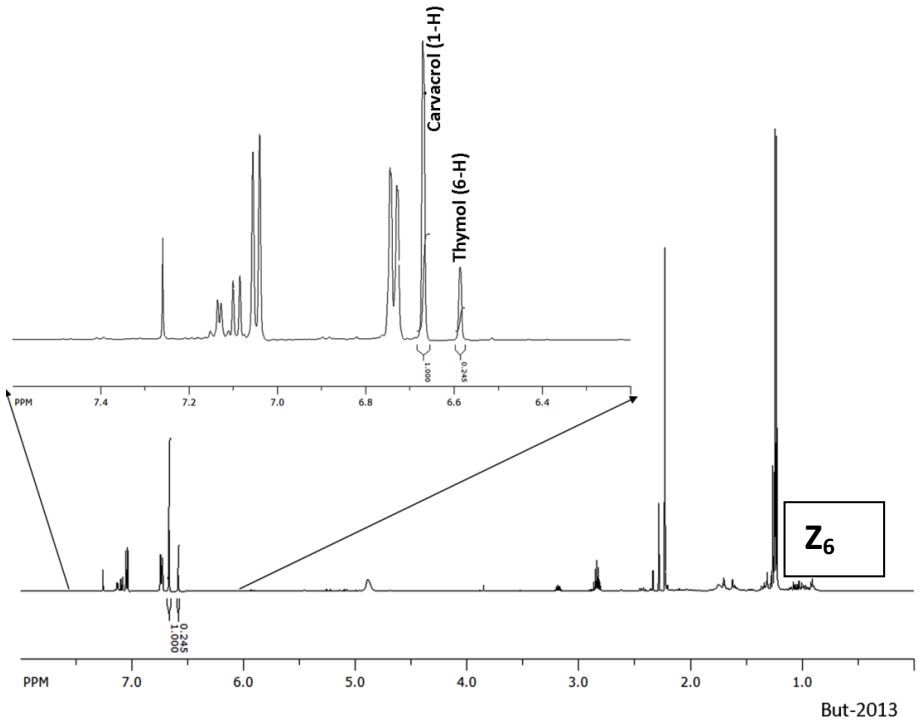
Z_3: Relative intensity of carvacrol to thymol in Ala-2013 EO.



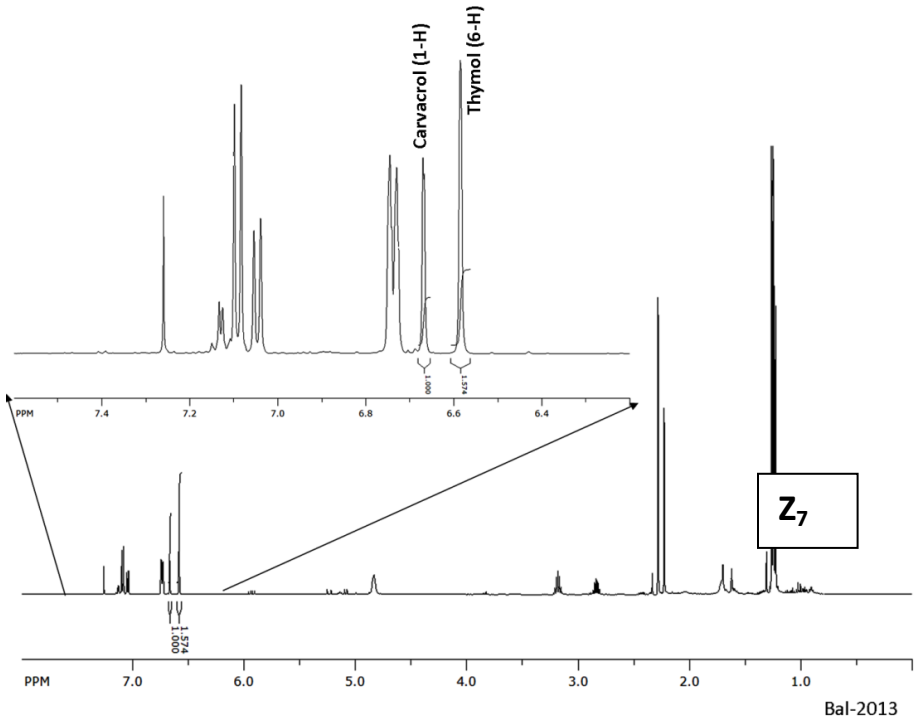
Z_4: Relative intensity of carvacrol to thymol in Yil EO.



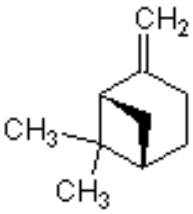
Z_5: Relative intensity of carvacrol to thymol Tar EO.



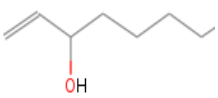
Z_6: Relative intensity of carvacrol to thymol in Buta EO.



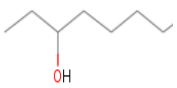
Z_7: Relative intensity of carvacrol to thymol in Bal EO.



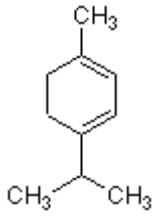
β-Pinene



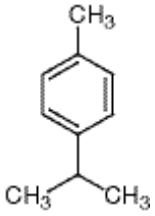
1-Octen-3-ol



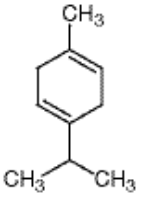
Octan-3-ol



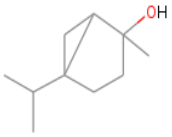
α-Terpinene



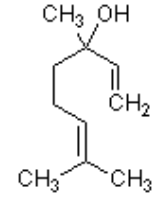
p-Cymene



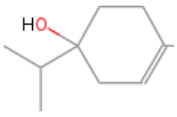
γ-Terpinene



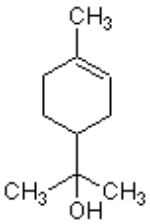
cis-sabinene hydrate



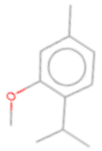
Linalool



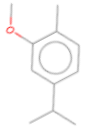
Terpinene-4-ol



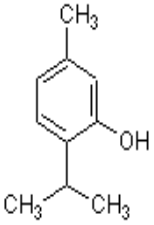
α-Terpineol



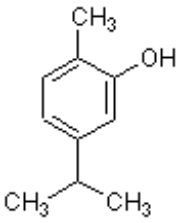
Thymol methyl ether



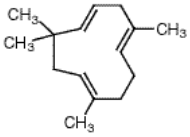
Carvacrol methyl ether



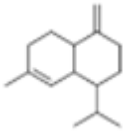
Thymol

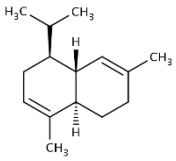


Carvacol

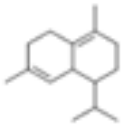


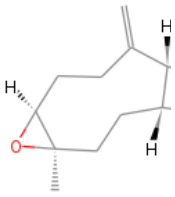
t-caryophyllene


γ-Cadinene

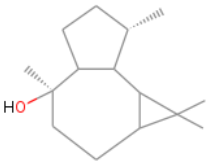


α-cadinene


δ-Cadinene

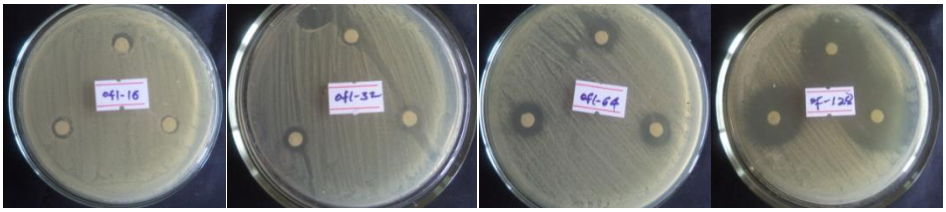


Caryophyllene oxide

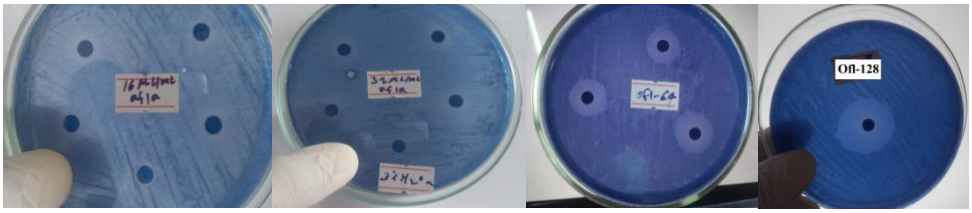


Viridiflorol

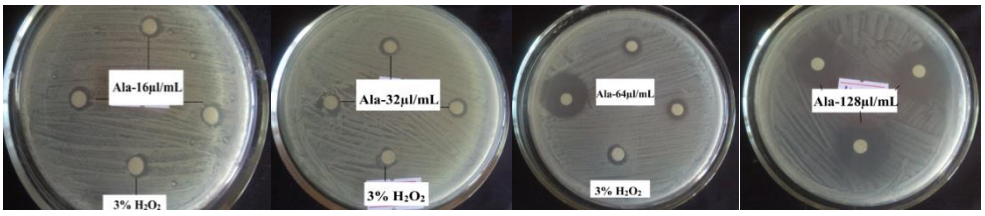
Z_8: Major compounds commonly identified from *Thymus* EOs from the six localities.



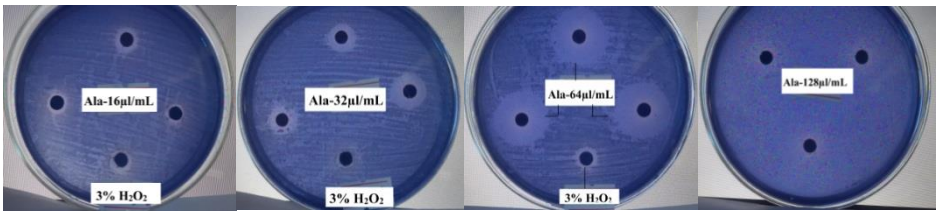
Z_9: Zones of inhibition of *Lactobacillus* by Ofl EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



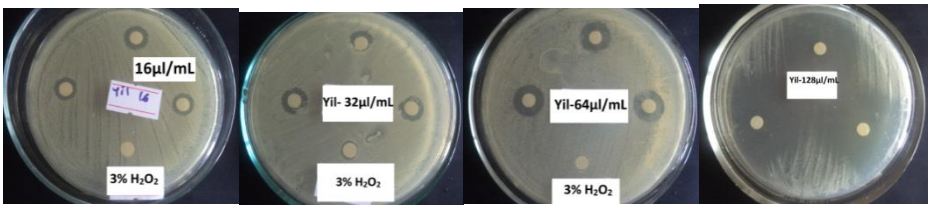
Z_{10}: Zones of inhibition of *S. mutans* by Ofl EO(doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



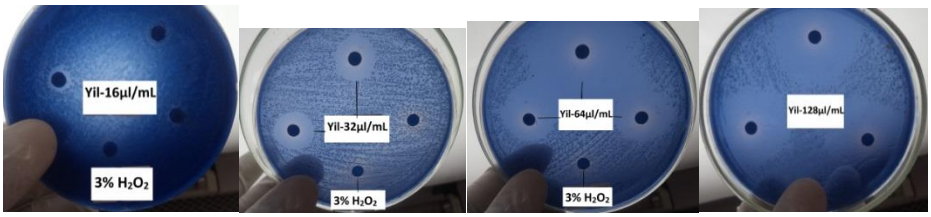
Z_{11}: Zones of inhibition of *Lactobacillus* by Ala EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



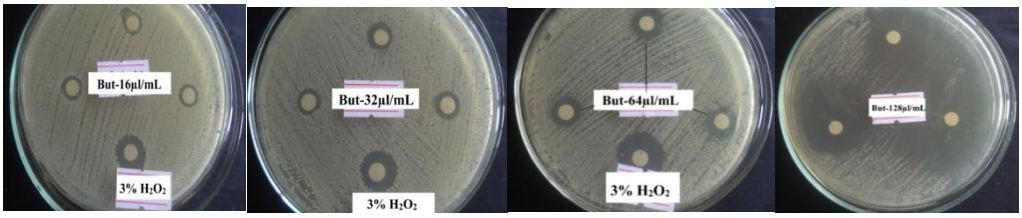
Z_{12}: Zones of inhibition of *S. mutans* by Ala EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



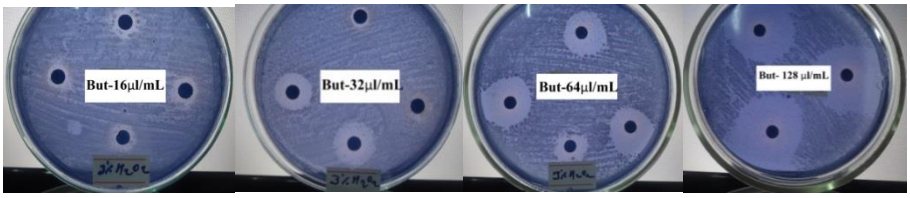
Z_{13}: Zones of inhibition of *Lactobacillus* by Yil EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



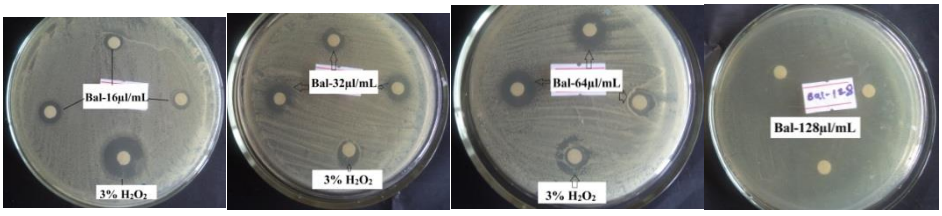
Z_{14}: Zones of inhibition of *S. mutans* by Yil EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



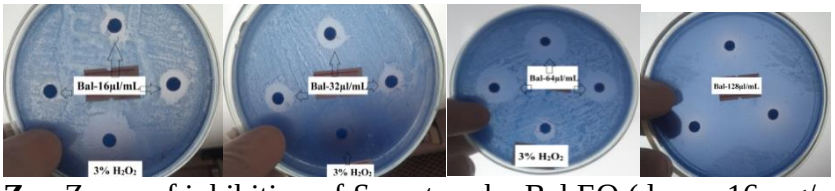
Z_{15}: Zones of inhibition of *Lactobacillus* by Buta EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



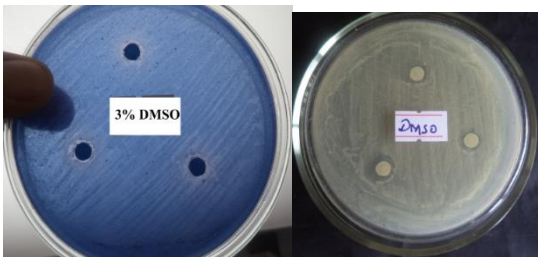
Z_{16}: Zones of inhibition of *S. mutans* by Buta EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



Z_{17}: Zones of inhibition of *Lactobacillus* by Bal EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)

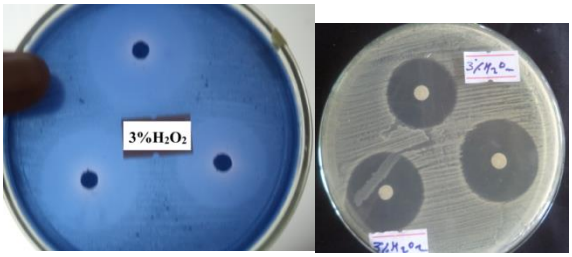


Z_{18}: Zones of inhibition of *S. mutans* by Bal EO (doses: 16 mg/mL, 32mg/mL, 64mg/mL, and 128 mg/mL respectively)



a b

Z_{19}: Zones of inhibition of *S. mutans* (a) and *Lactobacillus* (b) by 3%DMSO



a b

Z_{20}: Zones of inhibition of *S. mutans* (a) and *Lactobacillus* (b) by 3%H_2O_2

Declaration

I, the undersigned declare that this Dissertation is my original work and it has not been presented in other universities, colleges or institutes for a degree or other purpose. All sources of the materials used have been duly acknowledged.

Name: Destaw Damtie Signature_____Date_____

This work has been done under my supervision

Name_____ signature_____ Date_____

Name_____ signature_____ Date_____

Prepared based on AAU's "Thesis Writing, Examination and Grading Guidelines