CHEMICAL COMPOSITION AND BIOLOGICAL POTENTIAL OF THE VOLATILE AND NON VOLATILE CONSTITUENTS OF *TARCHONANTHUS CAMPHORATUS* AND *TARCHONANTHUS TRILOBUS VAR GALPINNI* OF KWAZULU – NATAL PROVINCE.

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ABSTRACT

The chemical composition and the biological activities of the volatile extracts (essential oils) and non-volatile (solvent extracts) of the aerial parts *Tarchonanthus camphoratus* and *Tarchonanthus trilobus var galpinni* collected from Sangoyana and Ubombo respectively, in the Kwa-Zulu Natal province were investigated in this study. The essential oils were separately extracted from the fresh leaves, dry leaves and dry stem by hydro-distillation and their chemical constituents determined by Gas chromatography/mass spectrometry (GC/MS). The essential oils investigated were of the dry leaf of *T. camphoratus* harvested in 2009 and of the fresh leaf, dry leaf and dry stem of both *T. camphoratus* leaf and *T. trilobus var galpinni* harvested in 2010 and 2011. The compounds present in all the essential oils were categorised as monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated monoterpenes, oxygenated sesquiterpenes and others but they differed in the type and percentage composition. Sesquiterpene hydrocarbons dominated all the essential oils with β–caryophyllene and *allo*-aromadendrene present in all the essential oils either as minor or major compounds. The oxygenated monoterpene 1,8-cineole, which is suspected to be responsible for the camphor smell of *T. camphoratus*, was present in all the essential oils of the leaves and stem of *T. camphoratus*. δ-Cadinene, a sesquiterpene hydrocarbon, featured in all the essential oils except of the fresh and dry leaf of *T. camphoratus* harvested in 2011. The chemical constituents in the non volatile extracts were determined by Pyrolysis-Gas chromatography/mass spectrometry (Py-GC/MS). A variety of compounds categorised as hydrocarbons, tetraterpenes, triterpenoids, phenols, fatty acids, fatty alcohols, steroids and sterols among others were identified in the extracts. Phenols were identified in the aqueous, methanol, dichloromethane leaf extracts and the methanol bark extract of *T. camphoratus* and in the dichloromethane leaf and ethyl acetate leaf extracts of *T. trilobus*

var galpinni. Steroids were identified in the bark extracts of *T. camphoratus* and in the methanol leaf extract of *T. trilobus var galpinni.* Hydrocarbons were identified in all the solvent/non volatile extracts but were more dominant in the dichloromethane bark extracts of both *T. camphoratus* and *T. trilobus var galpinni*. Triterpenoids were identified in the dichloromethane leaf extracts, ethyl acetate bark extracts of both species, in the methanol leaf of *T. trilobus var galpinni* and in the methanol bark extract of *T. camphoratus*. The preliminary phytochemical screening of the extracts also revealed the presence of phenols, flavonoids, saponins and tannins in some of the extracts. The volatile and non volatile extracts were screened for their antibacterial activity, antioxidant activity and cytotoxic activities and the volatile extracts were further screened for their insecticidal activities. The extracts were screened for antibacterial activity against 4 Gram positive and 5 Gram negative bacteria. The zones of inhibition of the fresh leaves, dry leaves and dry stem essential oils of *T. camphoratus* ranged from 7.3 – 14.0, 7.3 – 16.7 and 13.5 – 20.4 mm respectively and the zones of inhibition of the fresh leaf oil of *T. trilobus var galpinni* ranged from 8.2 – 21.8 mm. The volatile extracts exhibited higher percent inhibition than the non volatile extracts and the dry stem essential oil of *T. camphoratus* showed the highest antibacterial activity of all the extracts. The non volatile extracts exhibited significant antioxidant potential when tested by DPPH and $ABTS^{\bullet+}$ radical scavenging assays plus reducing power assay but showed poor nitric oxide inhibition and iron chelating ability. The volatile extracts generally showed poor antioxidant activity in all the antioxidant assays. The essential oils of both the dry leaf of *T. camphoratus* and fresh leaf of *T. trilobus var galpinni* showed the highest larval mortality of 100% which was observed at a concentration of 300 ppm after 24h whereas the lowest mortality after 24h was 20% at 25 ppm for *T. camphoratus* and 20% at 50 ppm for *T. trilobus var galpinni*. Insecticidal activity of the

volatile extracts against stored product pests, *S. zeamais* and *S. oryzae* revealed a lack of contact and fumigation toxicity by essential oils of both plant species. The essential oil of the dry leaf of *T. camphoratus*, however, showed repellent activity of over 50% after 24h at all the concentrations used on both *S. zeamais* and *S. oryzae*. Cytotoxic investigation of the volatile and non volatile extracts revealed that the extracts generally had low toxicity.

This study is the first to provide the chemical profiles and biological activities of the volatile and non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* from Kwa-Zulu Natal. The volatile extracts could be considered as potential alternatives, whether alone or in combination, to synthetic antibiotics, larvicides and repellents. The non volatile extracts on the other hand, could be potential sources of antioxidants that could have great importance as therapeutic agents.

CONTRIBUTIONS TO KNOWLEDGE

1. PAPERS PUBLISHED (PEER REVIEWED)

i) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2012. Chemical Composition and Larvicidal Activity of the Essential Oil of *Tarchonanthus camphoratus* Against *Anopheles arabiensis* Mosquito Larvae. ***Journal of Essential Oil Bearing Plants.*** 15 (2): 288-295.

ii) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2013. Variation in chemical composition and antibacterial activity of the essential oil of fresh and dry leaves and dry stem of *Tarchonanthus camphoratus*. ***Journal of Medicinal Plants Research***. 7 (8): 442-447.

iii) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA., Moganavelli S. 2013. Chemical composition, antioxidant activity and cytotoxicity of the essential oils of the leaves and stem of *Tarchonanthus camphoratus*. ***African Journal of Pharmacy and Pharmacology.*** 7(7): 360-367.

iv) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA., Singh M., Opeoluwa O. 2013. Antioxidant activity and cytotoxocity of the leaf and bark extracts of *Tarchonanthus camphoratus*. ***Tropical Journal of Pharmaceutical Research***. 12(3): 377-383.

2. PAPERS IN PRESS

i) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2013. Insecticidal activities and chemical composition of the essential oil from *Tarchonanthus camphoratus* (L.), leaves against *Sitophilus zeamais* Motschulsky, and *Sitophilus oryzae* (L.). ***African Journal of Agricultural Research***.

3. PAPERS SUBMITTED FOR EDITORIAL CONSIDERATION

i) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2013. The chemical composition, larvicidal and antibacterial activities of the essential oil of *Tarchonanthus trilobus var galpinii.* ***Journal of Essential Oil Bearing Plants.***

4. PAPERS PRESENTED AT CONFERENCES / SYMPOSIUM

i) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2010. Chemical composition and some biological properties of the essential oil of *Tarchonanthus camphoratus***.** A paper presented at the 13th Indigenous Plant Use Forum (IPUF) Annual conference. Keimoes City Hall, Nothern Cape, South Africa. 28th June – 1st July.

ii) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2010. Chemical composition and larvicidal activity of the essential oil of *Tarchonanthus camphoratus*. A paper presented at the Annual Faculty of Science and Agriculture Research Symposium, University of Zululand, KwaDlangezwa, South Africa.

iii) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2011. Chemical composition of the essential oil and insecticidal activities of the essential oil and solvent extracts of *Tarchonanthus camphoratus*. A paper presented at the Walter Sisulu University 2011 Joint International Research Conference. East London, South Africa.

iv) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2011. Antioxidant Activity of the Essential Oil and Various Extracts of *Tarchonanthus camphoratus* from Kwa-Zulu Natal.A paper presented at the Annual Faculty of Science and Agriculture Research Symposium, University of Zululand, KwaDlangezwa, South Africa. 27th October.

v) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2011. Antioxidant Activity and Cytotoxicity of the Essential Oil and Various Extracts of *Tarchonanthus camphoratus* from Kwa-Zulu Natal. A poster presented at the International Conference of Natural Products 2011. (ICNP2011). Universiti Putra Malaysia. 14-16 November.

vi) **Nanyonga SK**., Opoku A., Lewu FB., Oyedeji OA. 2012. The chemical composition, larvicidal and anti bacterial activities of the essential oil of *Tarchonanthus trilobus* var *galpinii*. A poster presented at the III International Symposium for Medicinal and Nutraceutical Plants. Aracaju Convention Center. Brazil. 14^{th} -19^{th} October.

AWARD

Best poster presenter. International Conference of Natural Products 2011. (ICNP2011). Universiti Putra Malaysia.

EPILOGUE

TO GOD BE THE GLORY

DEDICATION

To my father Mr. Jenkins Kiwanuka

and

my mother Ms Catherine Nakalema

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CERTIFICATION

We certify that Ms Nanyonga Sarah Kiwanuka (Student Number: 200901240) in the Department of Chemistry, University of Zululand, KwaDlangezwa, South Africa carried out this research work under the supervision of

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ABBREVIATIONS

Aq LF	Aqueous leaf
MET LF	Methanol leaf
DCM LF	Dichloromethane leaf
Et Ac LF	Ethyl acetate leaf
MET BK	Methanol bark
DCM BK	Dichloromethane bark
Et Ac BK	Ethyl acetate bark
Aq LF/1	Water leaf extract of *T.camphoratus*
MET LF/1	Methanol leaf extract of *T.camphoratus*
DCM LF/1	Dichloromethane leaf extract of *T.camphoratus*
MET BK/1	Methanol bark extract of *T.camphoratus*
DCM BK/1	Dichloromethane bark extract of *T.camphoratus*
Et Ac BK/1	Ethyl acetate bark extract of *T.camphoratus*

MET LF/2	Methanol leaf extract of *T.trilobus var galpinni*
DCM LF/2	Dichloromethane leaf extract of *T.trilobus var galpinni*
Et Ac LF/2	Ethyl acetate leaf extract of *T.trilobus var galpinni*
DCM BK/2	Dichloromethane bark extract of *T.trilobus var galpinni*
Et Ac BK/2	Ethyl acetate bark extract of *T.trilobus var galpinni*

CHAPTER 1

1.0 Introduction

1.0.1 Traditional medicine

The World Health Organisation[1] defines traditional medicine as "the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being." Traditional medicine comprises of therapeutic practices that were in existence before the development of modern scientific medicine and are still in use today without any documented evidence of adverse effects. Different societies in every country of the world have relied upon traditional medicines to support, promote, retain and regain human health for millennia[2,3]. Between 70 and 95% of the citizens in a majority of developing countries, especially those in Asia, Africa, Latin America and the Middle East use traditional medicines for the management of health and as primary health care to address their health-care needs and concerns[4]. In China, the traditional medicine system runs parallel to allopathic medicine and has been successfully used to diagnose, treat and prevent illness for over 2500 years[5]**.**In some industrialized nations traditional medicine is administered under the titles “complementary”, “alternative”, or “nonconventional” medicine[6,7]. Ethnobotanical studies carried out throughout Africa confirm that native plants are the main constituents of traditional African medicines[8]. In South Africa, more than 60% of the population use indigenous medicinal plants in their health care needs and approximately 3,000 species are used by an estimated 200,000 indigenous traditional healers[9].

1.0.2 Medicinal plants

Medicinal plants are plants which contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs[10]. Plant species on earth are estimated to be between 250,000 to 500,000[11], of which a small percentage (1-10 %) is used as food by both man and other animals and a higher percentage is used for medicinal purposes[12]. Plant medicine is one of the oldest practised by mankind[13,10]. Hippocrates, (460-375 BC), tackled the plague epidemic in Athens by fumigating the whole city with aromatic essences of plant oils[14]. Plant use as medicines is also cited in the bible, myrrh and frankincense being the most noteworthy[15]. Plants still remain an important source of medicine in the world to date. For example, *Aloe vera*, is widely used in the traditional herbal medicines of China, Japan, Russia, South Africa, the United States, Jamaica, Latin America and India for several external and internal medical conditions[16,17]. *Morinda citrifolia* (noni) is used in the Pacific and Caribbean islands for the treatment of inflammation and pain[18]and has also shown anti cancer activity[19]. Rooibos (*Aspalathus linearis*) has been traditionally used for skin ailments, allergies, asthma and colic in infants[20]. South Africa has remarkable biodiversity and rich cultural traditions of plant use. The country boasts of a rich diversified flora of about 30 000 species which are mostly endemic and an estimated 3 000 of these are regularly used in traditional medicine [21-26]. Considering the importance of plants in medicines, scientists began to research them a long time ago.

1.1 Motivation for the Present Study

Plants have an incredible diversity of compounds whose chemical structures are often unlikely to be synthesized in laboratories. These compounds, produced mainly for defence of the host plant have varied biological/medicinal potentials. The medicinal properties of

plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals in them [27,28]. The efficacy of medicinal plants has encouraged chemists and pharmacists to carry out rigorous analysis on the plants and to establish a relationship between chemical composition and therapeutic activities. Although it has been acknowledged that plant based traditional medicine is one of the surest means of achieving total health care coverage of the world's population, many plant species have not been studied for biologically active secondary products and lack scientific proof of their effectiveness and safety[29,30]. One of the species under investigation, *Tarchonanthus camphoratus*, has been reported to have many medicinal applications in traditional healing[31-34]. However, to the best knowledge of this researcher, there is no documented information about the chemical profiles of the volatile and non volatile constituents; the toxicity and therefore safety of *T. camphoratus* and of the other *Tarchonanthus* species of Kwa-Zulu Natal.

In South Africa malaria is categorised as seasonal and unstable, with the mosquitoes *Anopheles arabiensis* and *Anopheles funestus* being the major malaria vectors[35]. In the year 2005 the annual number of reported malaria cases was approximately 7755 while in 2006 it was 12,098[36]. The growing resistance of mosquitoes to insecticides is a potentially serious challenge to malaria vector control[37]. In South Africa, resistance to pyrethroids and DDT has been reported and the potential for carbamate resistance has been detected in *Anopheles arabiensis*[37]. This increased resistance, has paved the way for the investigation of botanical extracts for use in malaria vector control. Larval source reduction by habitat modification or use of larval control agents could be a less costly and a more effective strategy for eliminating mosquito larvae populations.

Methyl bromide and phosphine are the most commonly used fumigants for the control of stored product pests but methyl bromide depletes the ozone layer and was phased out completely by most countries in the early 2000s[38]. Cases of resistance to phosphine by some stored product insects have been reported[39]. Globally, the excessive use of synthetic insecticides in homes, croplands, urban environments and water bodies to get rid of noxious insects has resulted in an increased risk of insecticide resistance, enhanced pest resurgence, toxicological implications to human health and increased environmental pollution. Combating environmental pollution and its ill-effects on life and life-support systems is one of the most serious challenges before the present day world. As an alternative, botanical pesticides are of great interest because they are natural pesticides and are safe to humans and the environment. *T. camphoratus*, one of the species under investigation, has been reported to have insecticidal properties mainly as a repellent[40,41]. The volatile and non volatile constituents of *T. camphoratus* and other *Tarchonanthus* species could contain compounds that are toxic to mosquito larvae and to stored grain pests or could repel insect pests.

Since the discovery of penicillin by Alexander Fleming in 1929, many new classes of antibiotics have become available for the treatment of bacterial infections, but due to the excessive and often unnecessary use of antibiotics in humans and animals, bacterial resistance has now been reported against every currently available antibiotic[42,43]. Plants could be a source of antibacterial agents to manage some of the emerging resistant microbial species. Viruses and bacteria do not become resistant to plant extracts as they do to modern day synthetic antibiotic drugs. It is not possible for the microrganisms to mutate and adapt to the many chemical constituents found, for example, in essential oils

but can easily mutate and adapt to drugs because drugs are made by isolating one or two constituents to which a virus or bacteria can adopt[44].Although the antibacterial activity of the volatile and non volatile extracts of *T. camphoratus* from different localities have been reported in literature[30,45-49], no report has been cited on the antibacterial activity of *T. camphoratus* andother *Tarchonanthus* species from Kwa-Zulu Natal.

Free radicals have been implicated as causative agents of many diseases and illnesses[50]. Many medicinal plants have been reported to have antioxidant activity. No research has so far been done on the antioxidant activity of the volatile and non volatile constituents of the *Tarchonanthus* species from Kwa-Zulu Natal. These species may possess potent bioactive compounds capable of preventing and treating most oxidative related diseases and may also serve as leads for the development of novel drugs.

Several plants used in traditional medicine can cause damage to genetic material and therefore have the potential to cause long-term damage in patients when administered as medical preparations[51]. There is recorded information about the toxic symptoms caused by plants when ingested by animals but very little is known about the toxicity of herbal remedies in man[30]. There is no recorded information about the poisoning activity of *Tarchonanthus* species but acute toxicity due to the use of traditional medicines is common in South Africa[52]. Approximately 43% of poisoning cases recorded in the forensic database in Johannesburg from 1991 to 1995 were caused by traditional plant medicines[53]. Therefore it is necessary that cytotoxicity screening of *Tarchonanthus* species is carried out alongside evaluation of their biological and chemical properties.

Like other medicinal plants, the *Tarchonanthus* species is not only a potential solution to human predicaments associated with the use of synthetic chemicals, but their volatile and non volatile constituents may contain compounds that could be used as drugs or act as leads in drug development.

1.2 Aim and Objectives of the Study

The principal aim of the present study was to

- To determine the chemical constituents and evaluate the biological activities of the volatile and non volatile constituents of the *Tarchonanthus* speciesof Kwa-Zulu Natal.

1.2.1 Specific Objectives of the Study

- To extract the volatile constituents of the*Tarchonanthus* species by means of hydro distillation.
- To extract the non volatile constituents from the leaves and bark of the *Tarchonanthus* species by using solvents of different polarities.
- To investigate the chemical composition of the volatile and non volatile constituents of the*Tarchonanthus* species.
- To determine the antimicrobial activity of the volatile and non volatile constituents ofthe *Tarchonanthus* species and compare it with that of conventional antibiotics.
- To determine the antioxidant potential of the volatile and non volatile constituents of the *Tarchonanthus* species and compare it with that of known antioxidants.
- To determine the insecticidal activity of the volatile constituents of the *Tarchonanthus* species.

- To determine the larvicidal activity of the volatile extracts of the *Tarchonanthus* species against *Anopheles arabiensis* mosquito larvae.
- To determine the cytotoxicity of the volatile and non volatile extracts of the *Tarchonanthus* species using the Brine shrimp lethality assay and the MTT assay.

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CHAPTER 2

LITERATURE REVIEW

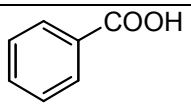
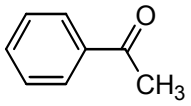
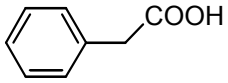
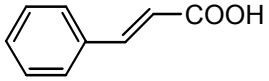
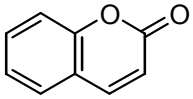
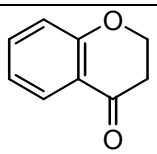
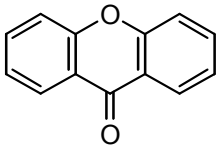
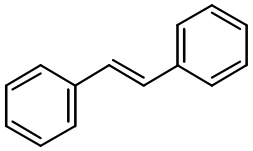
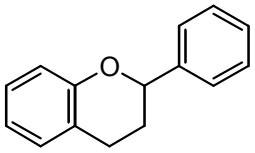
2.1 Plant secondary metabolites

Plant secondary metabolites, also known as phytochemicals, represent a diverse group of natural products which do not play a role in the primary metabolic processes essential for the maintenance of life of an individual plant but are absolutely essential to the survival of the species as a whole in a given natural habitat[1,2]. They are known to be responsible for various functions in the plant environment relationship[3]. These functions include: protection against environmental stresses such as drought and excessive light radiation; inhibiting herbivores and other pathogen attacks; influence allellopathy and act as an attractant to pollinators[4-6]. Several of these metabolites have therapeutic properties and their concentration in the plant tissues is considered as the main factor to evaluate the therapeutic value and quality of a given herb[7]. Based on their biosynthetic origins, plant secondary metabolites are divided into three major groups: phenolics, alkaloids and terpenoids[8].

2.1.1 Phenolic Compounds in Plants

Phenolic compounds make up the major families of secondary plant metabolites in plants and represent a diverse group of compounds. Structurally, they comprise of an aromatic ring, bearing one or more hydroxyl substituents and range from simple phenolic molecules to highly polymerised compounds[9]. They are categorised into several classes based on the number of carbon atoms in the basic carbon skeleton. Table 2.0 shows some examples of the different classes of phenols with their basic carbon skeleton and the number of carbon atoms in the skeleton.

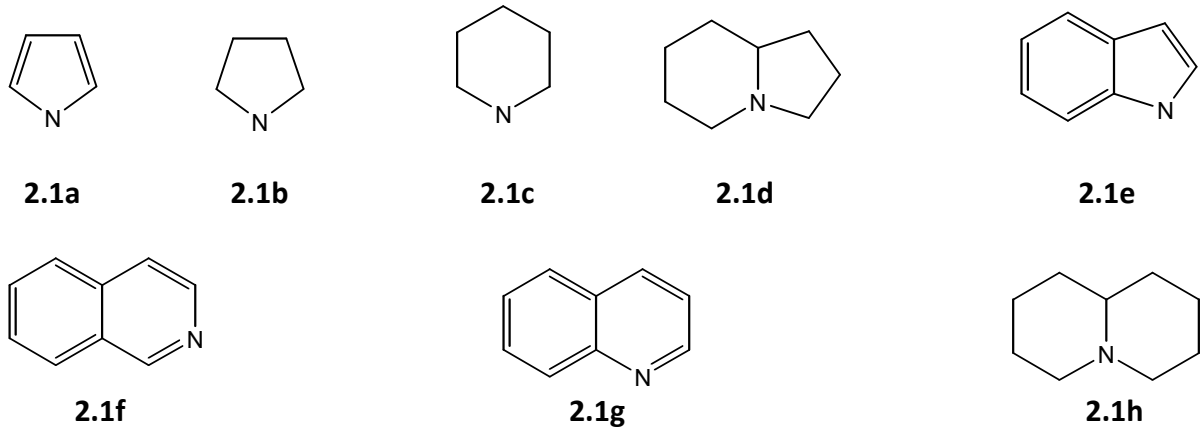
Table 2.0: Examples of different classes of phenolic compounds.[10]

Number of C-atoms	Skeleton	Classification	Basic structure
7	C_6-C_1	Phenolic acids	
8	C_6-C_2	Acetophenones	
8	C_6-C_2	Phenyl acetic acids	
9	C_6-C_3	Hydroxycinnamic acids	
9	C_6-C_3	Coumarins	
10	C_6-C_4	Napthoquinones	
13	C_6-C_1-C_6	Xanthones	
14	C_6-C_2-C_6	Stilbenes	
15	C_6-C_3-C_6	Flavonoids	

In addition to providing protection against pathogens and predators, phenolic compounds contribute towards the colour and sensory characteristics of fruits and vegetables and promote growth and reproduction in plants[9,11]. The compounds also show a broad spectrum of pharmacological activity[12-18].

2.1.2 Alkaloids

Alkaloids are a structurally diverse group of over 12,000 cyclic nitrogen-containing compounds, biosynthesized from amino acids, and are found in over 20% of plant species[19]. They are believed to function as defensive elements against predators, especially mammals, because of their general toxicity and deterrence capability[20]. They are characterised by a great structural diversity and the basic structures which are classified according to the ring system include pyrrole **2.1a**, pyrrolidine **2.1b**, piperidine **2.1c**, pyrolizidine **2.1d**, indole **2.1e**, isoquinoline **2.1f**, quinoline **2.1g** and quinolizidine **2.1h** (Figure **2.1**).



2.1a **2.1b** **2.1c** **2.1d** **2.1e**

2.1f **2.1g** **2.1h**

Figure 2.1. Alkaloids

Alkaloids rank among the most efficient and therapeutically significant plant substances[21] and theirpharmacological effects are used as medications, recreational drugs, or in entheogenic rituals. Examples of alkaloid drugs include the local anaesthetic and stimulant cocaine from *Erythroxylum* coca**;** the stimulant caffeine found in (*Coffea arabica*),

Camellia sinensis, and in *Theobroma cacao*; nicotine produced in the roots of *Nicotiana tabacum*; the analgesicmorphine from *Papaver somniferum*; the antibacterial berberine found in *Berberisvulgaris*; the anticancer compound vincristine from *Catharanthus roseus* among others[22-25].

2.1.3 Terpenoids

Terpenoids are the most numerous and structurally diverse plant metabolites[8] found in almost all plant species. It is approximated that at least 40 000 different terpenoids (isoprenoids) exist in nature, many of which are of plant origin[26]. Many terpenoids are essential for plant growth, development and general metabolism[8]. Their physiological, metabolic and structural roles include, among others, those of light-harvesting pigments in photosynthesis or the regulatory activities of the many terpenoid plant hormones. In addition, a large number of structurally diverse plant terpenoids are known or assumed to have specialized functions associated with interactions of sessile plants with other organisms in the context of reproduction, defence or symbiosis[27]. It has been shown that flowers can emit terpenoids to attract pollinating insects[28]and interestingly, terpenoids have been reported to attract beneficial mites which feed on herbivorous insects[29]. Terpenoids can be described as modified terpenes, where methyl groups are moved or removed, or oxygen atoms are added. Inversely, some authors use the term "terpenes" more broadly, to include terpenoids. A simple unifying feature of all terpenoids is that they are derived from a simple process of assembly of a C_5 unit, the isoprene unit C_5H_8[27], (Figure **2.2a**).

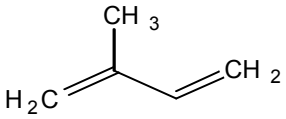


Figure 2.2a. The isoprene unit

The isoprene units are linked together in a head to tail fashion(Figure **2.2b**)and the terpenoids are classified according to the number of isoprene unit incorporated in the molecular skeleton, Table 2.1.

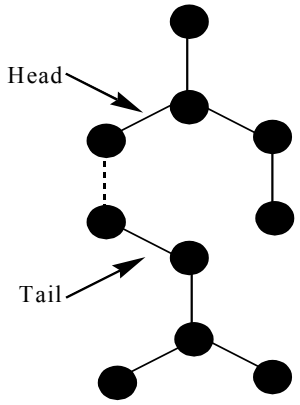


Figure 2.2b.

Table 2.1: Examples of different classes of terpenoids.[30]

No. of isoprene units	No of C-atoms	Terpenes	Example
1	5	Hemiterpenes	Prenol
2	10	Monoterpenes	Geraniol, Limonene
3	15	Sesquiterpenes	Farnesol, Farnesenes
4	20	Diterpenes	Cafestrol, Kahweol
5	25	Sesterterpenes	Geranylfarnesol
6	30	Triterpenes	Squalene
8	40	Sesquarterpenes	Ferrugicadiol
> 100	> 500	Tetraterpenes	Rubber

2.1.3.1 Biosynthesis of terpenoids

Terpenoids biogenetically, arise from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which are biosynthesized from three acetylcoenzyme A moeties through mevalonic acid (MVA) via the mevalonate pathway[31] (Figure **2.3**).

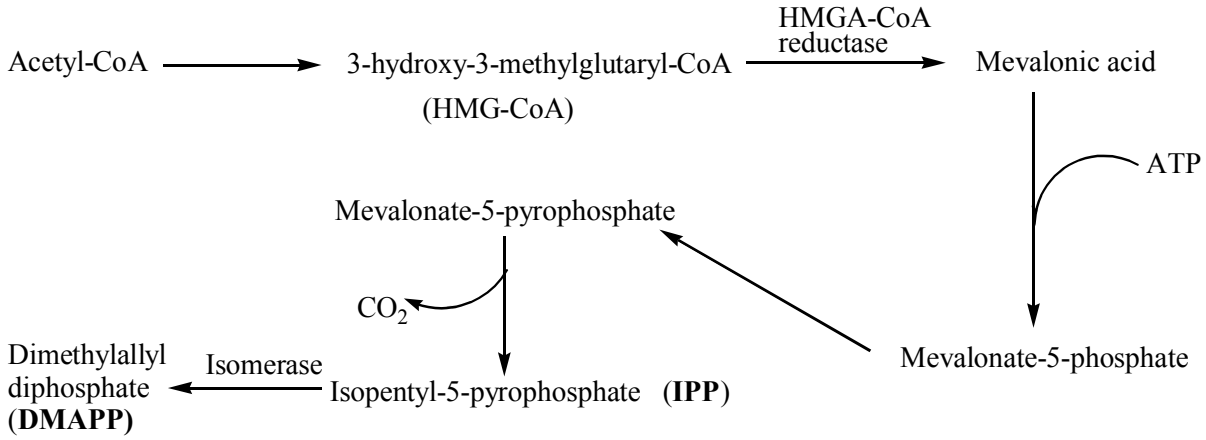


Figure 2.3. The mevalonate pathway

The IPP and DMAPP undergo condensation to geranylpyrophosphate (GPP) which on addition of another IPP unit forms farnesylpyrophospahte (FPP), (Figure **2.4**). GPP and FPP are precursors of monoterpenes and sesquiterpenes respectively[32]. Terpenoids are not only numerous but extremely variable in structure exhibiting hundreds of different carbon skeletons and a large assortment of functional groups. Their structural diversity and large numbers offer much potential for industrial and medicinal applications[8]. The lower molecular representatives of the terpenoid family, the monoterpenes and sesquiterpenes and their corresponding oxidized products: the aldehydes, ketones, alcohols, esters and phenols, are the major constituents of essential oils.

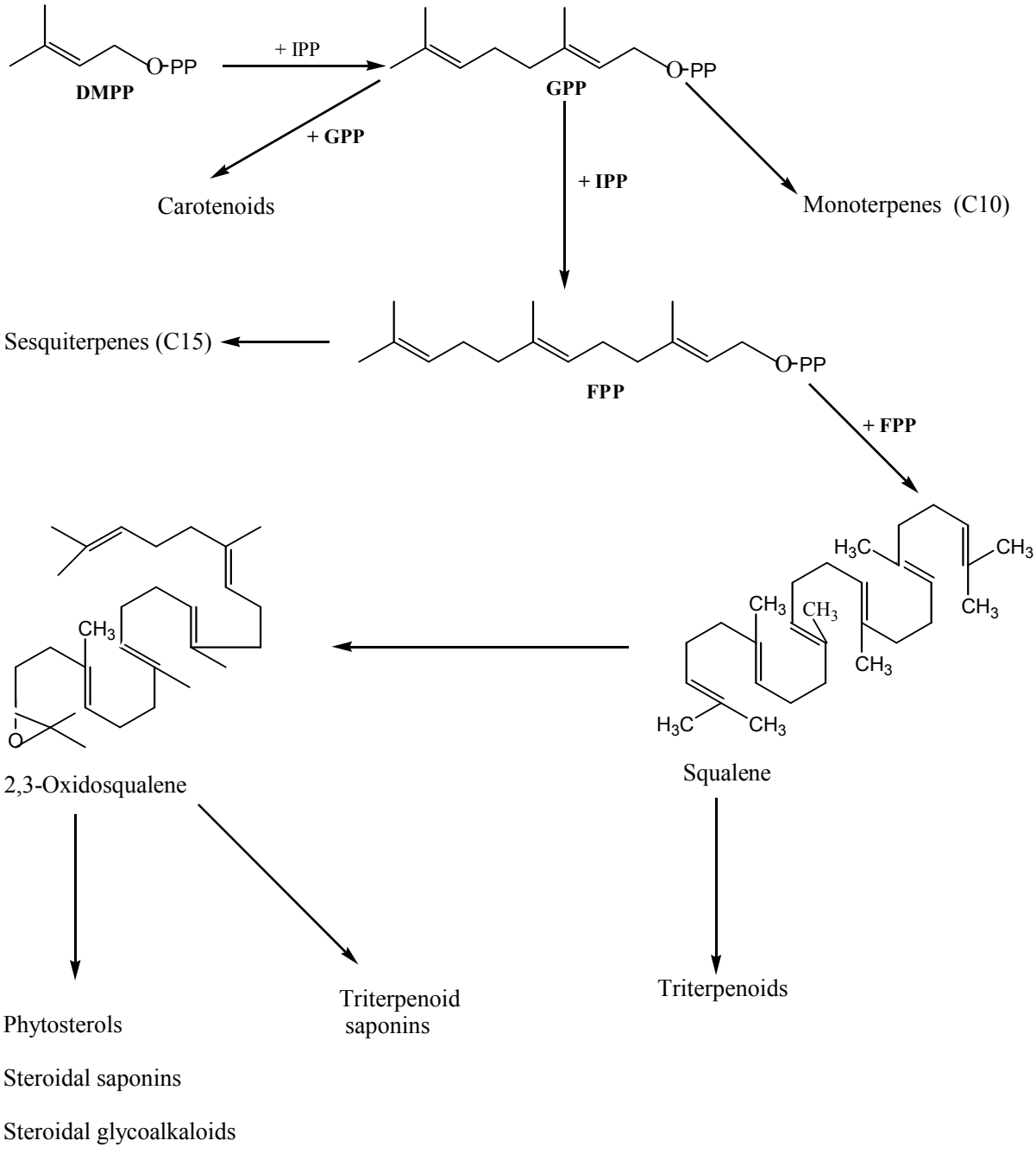
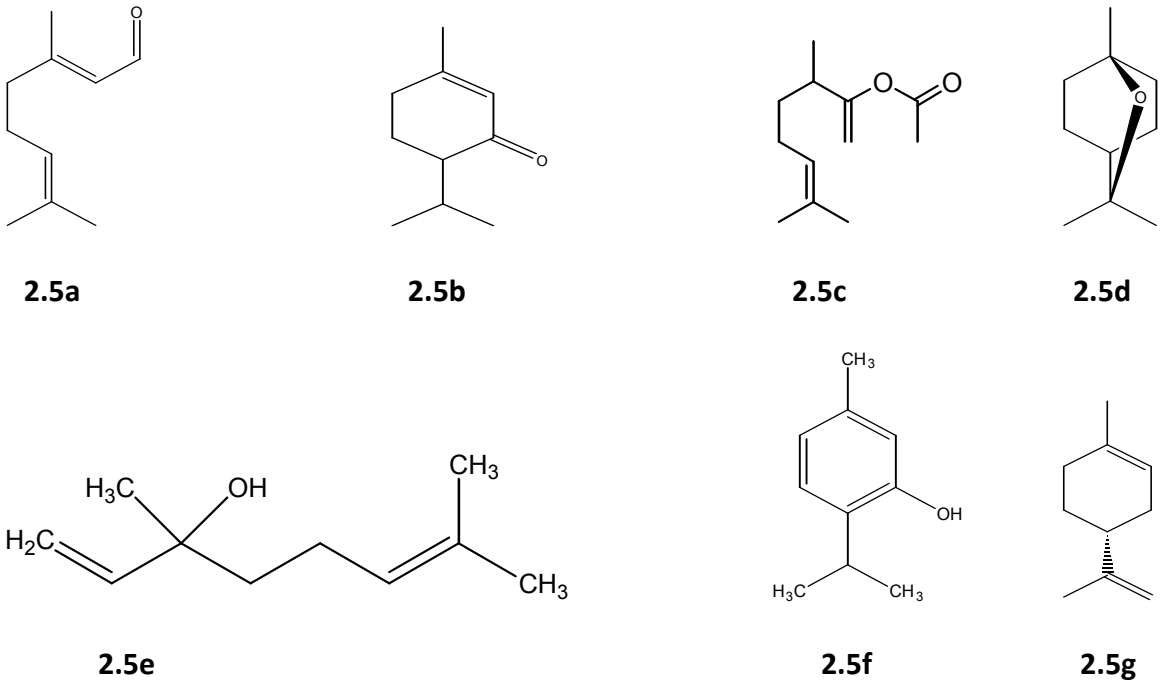


Figure 2.4. Formation of terpenoids via the mevalonate pathway

2.1.3.2 Essential oils

Essential oils are odiferous bodies of an oily nature, obtained almost exclusively from vegetable organs: flowers, leaves, bark, wood, roots, rhizomes, fruits and seeds[33,34]. Essential oils are generally liquid, and their pleasant odour and essence is responsible for the strong characteristic smell or fragrance of aromatic plants[35]. Essential oils are very

complex natural mixtures. An essential oil can contain about 20–60 components with two or three major components at fairly high concentrations (20–70%) compared to other components present in trace amounts. Essential oil components include two groups of distinct biosynthetical origin; the terpenoid group which is the main group and the non-terpenoid group which may contain short-chain aliphatic substances, aromatic substances, nitrogenated substances, and substances with sulphur[8,36]. The non-terpenoid group is less important in terms of uses and applications. In essential oils, the two terpenoid groups, the monoterpenes and sesquiterpenes allow for a great variety of structures as shown in Figure **2.5**. Depending on the functional group attached they can be: aldehydes like geranial **2.5a**, ketones such as piperitone **2.5b**, esters for example linalyl acetate **2.5c**, oxides like 1,8-cineole **2.5d**, alcohols such as linalool **2.5e**, phenols for example thymol **2.5f** and hydrocarbons like limonene **2.5g**.



2.5a **2.5b** **2.5c** **2.5d**

2.5e **2.5f** **2.5g**

Figure 2.5. Some of the monoterpene and sesquiterpene structures found in essential oils

Essential oils have a long history of use as medicines. Records have it that essential oils from the *Cedrus* (Cedar) and *Commiphora* (Myrrh) species were used for coughs,colds, inflammation[37] and in addition, Myrrh was used in embalming because of its effectiveness in preventing bacterial growth[38]. Wounds of World War II soldiers responded better to essential oils than to the conventional antibiotics of the time[39]. Essential oils exhibit a wide spectrum of pharmacological activities such as infection control, wound healing, pain relief, nausea, inflammation and anxiety[40,41]. Traditional medicines containing essential oils have been scientifically proven to be effective in treating various ailments like malaria and others of microbial origin[42-44]. It has been observed that different essential oils overlap in their actions although they may differ in their chemical composition[43]. The biological activities of essential oils have been attributed to the composition or specific essential oil constituent, for example: aldehydes in lemon grass (*Cymbopogon citrates*) have been reported to have anti-inflammatory properties[45]. Ketones in sweet fennel (*Foeniculum vulgare*) have been found to aid in wound healing and dissolve mucus and fats[43]. Alcohols in tea tree (*Melaleuca alternifolia*), true lavender (*Lavandula angustifolia*) and baboon wood (*Virola surinamensis*) have anti-microbial and anti-malarial properties[42,46]. Esters in clarry sage (*Salvia sclarea*) have anti-cholinesterase properties[47]. Phenols in thyme (*Thymus vulgaris*) and in clove (*Eugenia caryophyllata*) have antimicrobial properties and can be used as food preservatives[43]. Overall essential oils are pertinent to pharmaceutical, cosmetic and food research and are widely viewed as templates for structure optimization programs with a goal of creating new drugs[48].

2.1.3.3 Triterpenoids

Triterpenoids are a large group of phytochemicals found mainly in plant surfaces such as stem bark or leaf and fruit waxes[49,50]. Triterpenoids have a carbon skeleton based on six isoprene units and are biosynthetically derived from squalene, (Figure **2.6**), a C_{30} triterpene. Squalene is formed by head-to-head condensation of two farnesyldiphosphate (FPP) units.

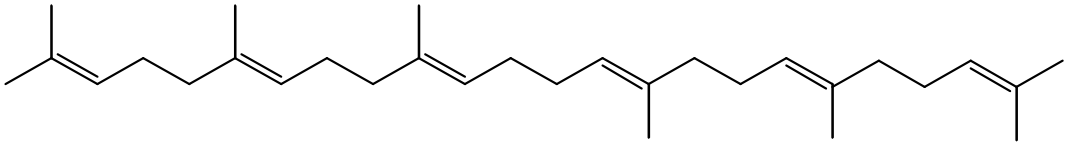


Figure 2.6

Triterpenoids are generally divided into four main groups: the true triterpenes, steroids, saponins plus the cardiac glycosides[51] and they have been reported to exhibit anti-inflammatory, antipyretic, sedative analgesic, cardiotonic and tonic effects in addition to antioxidant, antimicrobial, antiviral, antiallergic, antiangiogenic, anticancer and spasmolytic activities[52-55].

2.1.4 Lipids

Lipids constitute a group of naturally occurring molecules that include sterols, waxes, fats and fat soluble vitamins such as vitamins A, D, E and K. They form about 6% of plant biomass and play structural, defence, immunity, metabolic and regulatory roles in plants and form an energy storage in about 80% of the plant species [56]. Lipids are biosynthesized from two distinct building blocks, the isoprene and ketoacyl groups and are hence categorised according to the nature of their subunits. Sterols and fat soluble vitamins are isoprene-based lipids whereas fatty acids and waxes arise from the condensation of the ketoacyl group[56].In plants fatty acids usually occur free or in esterified form and more than 200

different fatty acids have been identified[57]. Fatty acids exhibit antibacterial and antifungal activities[58-60].

2.2 Production of secondary metabolites by plants

The amounts of secondary metabolites within a certain plant are not static but depend on a lot of factors which include the type of the plant species, season of plant harvesting, plant part to be studied, time of plant development and other environmental factors[61,62]. Any kind of stress condition has a major impact on the metabolic pathways responsible for the synthesis and accumulation of the secondary plant metabolites[63]. Drought is one of the most important environmental stresses that can depress the growth and alter the biochemical properties of plants[64]. It has been observed that plants exposed to drought stress produce a greater amount of secondary plant products such as phenols, terpenes as well as N and S containing substances such as alkaloids, cyanogenic glucosides or glucosinolates[65,66]. Accumulation of secondary metabolites can also be influenced by light intensity, nutrient deficiency, UV light and high temperature[67-69]. It was revealed that organic crops show higher amounts of antioxidants than conventionally grown crops[70]. This is because organic crops are vulnerable to attack by herbicides and hence produce higher concentrations of protective molecules to maintain themselves.The quantity of a particular compound may also vary according to the maturity of the plant. The concentration of taxol was found to change during seed maturation reaching a maximum during the middle stage of the seed development and then decreasing with further maturation[71]. A particular plant harvested from different ecological settings may show variation in the concentration of some of the chemical constituents. Robert et al. (1996) reported variation in the concentration of α-pinene, β-phellandrene and β-caryophyllene in *Juniperus phoenicea*

essential oil harvested from countries which had vastly different ecological elements[72] . The biosynthesis of secondary metabolites, although controlled genetically, is strongly affected by environmental influences. Plants do not just 'randomly' produce all kinds of constituents. These metabolites play a vital role in the survival strategy of the plant and that is the reason why the same plant may have different chemotypes.

2.3 *Tarchonanthus* species

Tarchonanthus belongs to the *Asteraceae* family which is also known as the daisy or sunflower family. It is a small genus of a few species that occur in the tropics and in southern Africa. In South Africa two species, *Tarchonanthus camphoratus* and *Tarchonanthus trilobus* are dominant.

2.3.1 *Tarchonanthus camphoratus*

Common names

(Afrikaans): kanferbos, vaalbos

(English) : camphor bush, wild camphor tree, wild cotton, wild sage wood

(Swahili): mkalambati, Leleshua

(Zulu) : amathola, igqeba-elimhlophe

T. campharatus (Figure **2.7**) is a strongly scented tree or shrub[73,74] . The name "anthos" from Tarcon- "anthus" is derived from the Greek word meaning flower, whereas the name "camphoratus" refers to the strong camphor odour, which the leaves impart[73]. The plant is a shrub or a small tree of rarely more than six meters in height, with a greyish appearance and occurs in a wide range of habitats[73]. It prefers deep soils and can withstand seasonal water logging, however, it is commonly found on stony soils. *T. campharatus* needs no special care and does well even when used for the most challenging landscape applications.

The seed takes about 8 weeks to germinate but it can also be successfully propagated from soft wood cuttings. It grows at a moderate rate of 600mm – 800mm per year. The tree is usually much-branched with a narrow crown and a trunk of diameter to 40 cm. The bark which is brown or grey is rough, longitudinally fissured and exfoliating in long strips. The leaves are dark green on the upper side and pale velvety- grey on the underside. They are up to 15cm long, most often lance-shaped to oblong, frequently with a small, thorn like point, the base narrowed, the upper surface is smooth or often finely pitted. Small creamy-white flowers are produced usually from December to May which give rise to woolly fruits that are strongly scented[73].

Figure 2.7.*Tarchonanthus camphoratus*

Herman (2002) revised the South African *T. camphoratus* complex by recognising five different species: *T. camphoratus,T. obovatus, T. littoralis, T. minor, and T. parvicapitulatus,* which had previously been lumped under *T. camphoratus*[75]. These species differ in inflorescences, flowering times, leaf shape, leaf margin and also intheir distribution[75].*T. obovatus* occurs in the same region with *T. camphoratus* but it is distinguished by its obovate leaf shape. The leaf is also enveloped by white, cottony hairs contrary to *T.*

camphoratus, which is enveloped by yellowish hairs[75] .*T. minor* is mainly distinguished by the small, narrowly elliptic entire leaves and mainly occurs in the Free State, Lesotho, Northern, Western and Eastern Cape on hillslopes, mountainsides, rocky ridges and hills[75].*T.littoralis* (Figure 2.8) leaves are strongly aromatic and leathery but the colours of the upper and lower surfaces of the leaves are distinctly different. The upper surface is dark green, hairy when young, but becoming hairless with time; the lower surface is white-grey and covered in a dense mat of velvet hairs.This species mainly occurs in the Cape Peninsula in the Western Cape along the coast, on hillsides, dunes and river banks, through the Eastern Cape to KwaZulu-Natal[75].

Figure 2.8.*T. littoralis*

T. parvicapitulatus (Figure 2.9) is recognized by the oblanceolate, obtuse-mucronate leaves mostly with denticulate margins towards the apex[75]. It is widely distributed in the Northern Province, North-West, Gauteng, Mpumalanga, Swaziland, KwaZulu-Natal and Eastern Cape[75].

Figure 2.9.*T. parvicapitulatus*

2.3.1.1 Traditional medicinal uses of *Tarchonanthus camphoratus*

In Southern Africa *T. camphoratus* has varied traditional medicinal uses in different societies. The leaf infusion is used by the Bushmen (South Africa), Herero and Damara (Namibia) against cough, asthma and bronchitis[76]. The Damara chew the leaves for fever, cough and breast illnesses and the Herero sniff powdered leaves for sinuses[76]. The fresh leaves were chewed by the Malay (South Africa) and Herero for a slight narcotic effect and for a similar purpose the dried leaves were smoked by Hottentot (South Africa) and Bushmen[74,77]. The Sotho (South Africa), inhale smoked green branches for headaches[78]. In South Africa, the leaves are chewed to alleviate toothache[79]. The Tswana and Venda (South Africa) use the woolly seeds to stuff pillows, which are considered to be excellent for headaches and sleeplessness[74]. In Botswana root decoctions are taken for asthma and whooping cough[76]. Smoke from the green branches and seeds is inhaled for rheumatism, asthma, insomnia and is used for fumigation during funeral rituals[73,80]. The Masai inhale the smoke from the leaves for headache, use the dry leaf infusion for tapeworm, use the leaves

to protect from bed bugs, put the leaves underarm as perfume and to prevent tiredness[81] . Several African tribes use the plant in the treatment of bronchitis, chest ailments, chilblains, tired legs and sore feet[82]. Many wild animals that live in the areas where *T. camphoratus* grows, particularly Cape buffaloes and black rhinoceroses, have been observed rubbing themselves against the tree. Upon examination, it has been revealed that those animals which have crushed the leaves onto their skin are relatively free of ticks. The plant also seems to discourage tsetse flies, a pathogenic agent of trypanosomiasis[81].

2.3.1.2 Non-traditional medicinal uses of *T. camphoratus*

T. camphoratus has many other uses beside the medicinal applications. The leaves are used to prepare a beverage, smoked as tobacco or inhaled as snuff. The leaves decompose slowly and improve soil fertility. The leaves do not appear to be toxic as they are eaten by domestic stock, such as cattle, sheep and goats as well as game such as giraffe, gemsbok, eland, kudu, sable, black wildebeest, nyala, impala, springbok and grey duiker[74,83].The wood of *T. camphoratus* is heavy and fairly hard, tough, termite resistant and is used for hut-building, making of hunting weaponry and fence poles which can stand for 30 years. The tree is wind firm and can act as a windbreak for low winds. Its resistance to fire is remarkable, little mortality is seen in *T. camphoratus* even after three burnings, making it ideal for firebreaks. Itis a popular indigenous ornamental in South Africa, especially suited for bonsai with its aggressive root system. However, the wood is said to be poisonous and capable of causing a septic sore on the skin that heals with difficulty[84].

2.3.2 *Tarchonanthus trilobus*

T. trilobus is a shrub or small tree 4 to 8m high with a clean trunk and a dense spreading crown of decorative, bi-coloured leaves. The bark is dark grey, vertically fissured and cracked, flaking off in narrow strips. The leaves are strongly scented, dark green and wrinkled above, densely velvety-white underneath but with veins and midrib still clearly visible, variable in shape but typically narrow at the base with broad three-lobed tips. Young growth is woolly and cream-white. The species name trilobus means three-lobed in Latin, referring to the typically 3-lobed leaves. *T. trilobus* is divided into two varieties: var. trilobus and var. galpinii. *T. trilobus* var *trilobus* (Figure 2.10) has leaves with broad three-lobed tips and in South Africa, it has a more southerly distribution from the Eastern Cape to KZN.

Figure 2.10.*Tarchonanthus trilobus* var *trilobus*

T. trilobus var *galpinii* (Figure 2.11) also known as the broad-leaved camphor bush, breëblaarkanferbos, has oblong, oval or lance-shaped leaves with rounded or pointed tips and it has a more northerly distribution from Kwa-Zulu Natal into Swaziland, Mpumalanga and Limpopo.

Figure 2.11.*Tarchonanthus trilobus* var *galpinii*

No reference was found to *T. trilobus* var. *trilobus* being used medicinally, but *T. trilobus* var. *galpinii* and other *Tarchonanthus* species are used and it is likely that this species is used in a similar way. *T. trilobus* var. *galpinii* roots and bark are used to improve libido and for vomiting[85]. The trunk is used for construction, including the building of the King's kraal[86].

2.3.3 Previous work done on *Tarchonanthus* species

2.3.3.1 *Tarchonanthus camphoratus*

Phytochemical analysis by Scott and Springfield (2005) indicated the presence of tannins, saponins and reducing sugars[87] whereas flavanones, sesquiterpine lactone and alkaloids were identified from Egyptian collections[87]. Earlier on, Van Wyk et al. (1997) had also identified a flavanone in *T. camphoratus*[73]. The volatile extracts of *T. camphoratus* have been shown to be very complex and variable at different locations[88-91]. All the oils had 1,8-cineoleas one of the major compounds but in different amounts. An alcohol, α-fenchol, featured as a major compound in the oils of *T. camphoratus* harvested from Kenya and

Arabia but was not present at all in the *T. camphoratus* oil from Eastern Cape, South Africa[88-91]. Despite the camphor-like smell, the plant was found to contain only very small amounts of camphor[90]. Various biological studies have been done on both the volatile and non volatile constituents of *T. camphoratus* from different ecosystems. Van Vuuren and Viljoen (2009) reported significant antibacterial activity of the essential oils of *T. camphoratus* with MIC values in the range of 1.5 - 16.0 mg/ml for the pathogens studied[92]. Also the essential oil of *T. camphoratus* from Kenya showed marked antibacterial activity in the disc diffusion assay and conflicting poor activity in the MIC assay with MIC values as high as 900 mg/ml for the pathogens tested[88]. In another study the essential oil of *T. camphoratus* was found to be relatively toxic to *Anopheles gambiae* and had an $LD_{50}$of 3.8 x 10^{-3} mg/ml[93]. Investigation of the repellent effect of the essential oil of *T. camphoratus* from Kenya revealed that the essential oil was less potent than N,N diethyl-m-toluamide (DEET), a synthetic repellent[93]. Antibacterial studies done on the non volatile extracts of *T. camphoratus* showed conflicting results. According to Watt and Breyer-Brandwijk, 1962, the water extracts of the leaves had no antibacterial activity[74]. McGaw *et al*., 2000, also reported no antibacterial activity of the aqueous, ethanolic and hexane extracts of the dried leaf of *T. camphoratus*[94].Later on, however, Braithwaite et al., 2008 reported good antimicrobial activity of the methanol extract of *T. camphoratus* against *S. aureus*[95] . Also according to Vermaak *et al*., 2009, the crude methanol and water extracts of *T. camphoratus* showed good to poor antimicrobial activity against the test micro-organisms[96]. Other studies revealed that the aqueous extracts of the leaves of *T. camphoratus* demonstrated *in vivo* analgesic and antipyretic activity[97]. In another study, it was reported that feeding sand fly larvae on the plant powder and crude extracts of *T. camphoratus* recorded no mortality[98].

Derivatives and formulations of *T. camphoratus* have been reported to have medicinal uses. A spray lotion containing 3% *T. camphoratus* essential oil was shown to be protective against mosquitoes[99]. The efficacy of the *T. camphoratus* derivatives has also been found on some irritation and inflammatory conditions for example eczema, ache, reddening, swelling and on genital and mouth mucosa[100]. However, there have been no reported studies on the chemical composition and biological activity of the volatile and non volatile constituents of *T. camphoratus* species in Kwa-Zulu Natal.

2.3.3.2 *Tarchonanthus trilobus*

Not much has been reported onthe chemical composition and biological activity of the volatile and non volatile constituents of *T. trilobus* species from any ecosystem.

2.4 Extraction of medicinal plants

Medicinal plant extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures. When the solid plant material comes into contact with the solvent, there is mass transfer of the soluble active principle from the plant to the solvent. Mass transfer stops when equilibrium is reached between the solid material and the solvent. Replacing the solvent at equilibrium with a fresh solvent, enables more active principles to be extracted from the plant material. Heating the solvent also increases the solubility of the active principles and enhances mass transfer. Before plant material can be subjected to solvent extraction, some preparation has to be done and this may include drying and reducing the size of the plant material. Drying is necessary, if the plant material is not required fresh and to avoid microbial contamination during storage. To prevent decomposition of thermo labile constituents and avoid chemical transformations resulting from exposure of UV radiation, drying is usually done at temperatures below 30^{o}C

in the absence of direct sunlight. It's also necessary for air to flow around and through the plant material to prevent the build up of moisture and heat. In order to maximise the surface area of the plant material which in turn enhances the mass transfer of active principle from plant material to the solvent, the plant material is ground to a powder using an electric mill or in a mortar and pestle. Care should be taken not to reduce the size to very small particles which become slimy and prevent the solvent from flowing around them easily during extraction. Plant size of between 30 and 40 mesh is optimal[101]. Various technologies are used in the extraction of active components from medicinal plants.

2.4.1 Infusion

Infusions are dilute solutions containing the readily soluble constituents of the plant material. The plant material which is usually dried herbs, flowers or berries is steeped in solvents like water, oil or alcohol to extract the desired chemical compounds or flavours. The solvent at an appropriate temperature is poured over the plant material and allowed to steep in the liquid for a period of time which depends on the purpose for which the infusion is being prepared. Quantities of the herb and the liquid used will vary according to the herb or how strong the infusion is required to be.

2.4.2 Decoction

Decoction is a liquid preparation made by boiling the herbs with water for a specified period of time or until a certain volume is achieved. Decoctions are usually the method of choice when working with tougher and more fibrous plants, barks and roots which have water soluble compounds. More heat is needed in making decoctions than infusions and this heat tends to destroy some of the herb's volatile constituents.

2.4.3 Maceration

Maceration is a simple and widely used procedure which involves soaking the pulverised plant material into a suitable solvent in a closed container at room temperature. The mixture is stirred occasionally or constantly using mechanical shakers or mixers to ensure homogenous mixing and to increase the speed of the extraction. The extraction stops when equilibrium is reached between the concentration of the metabolites in the extract and that in the plant material. After extraction the residual plant material (marc) is separated from the solvent usually by decanting followed by filtration. To ensure exhaustive extraction, fresh solvent is added to the marc and all the filtrates pooled together. The method can be used for both small and bulk extractions and since extraction is done at room temperature, degradation of the thermo labile metabolites is not likely to occur. However, the method is time consuming taking from a few hours to several weeks, and some compounds may not be efficiently extracted if they are poorly soluble at room temperature and exhaustive maceration requires large volumes of solvents.

2.4.4 Ultra sound assisted solvent extraction

Ultra sound assisted solvent extraction is a modified maceration method where the extraction is facilitated by the use of ultra sound. The plant powder in a vial is placed in an ultrasonic bath. The ultrasound allows the solvent to penetrate the cell walls and the bubbles produced by acoustic cavitations aids in the disruption of the cell wall, which increases the solubilisation of the active ingredients in the solvent. It's a highly efficient method which is less solvent- and time-consuming. It has been applied in the extraction of many chemical constituents from different plant materials[102,103].

2.4.5 Percolation

Plant material is soaked in a solvent in a percolator which is a cylindrical container with a tap at the bottom. Additional solvent is then poured on top of the plant material and allowed to percolate slowly out of the bottom of the percolator. The extractable substances in the plant material are washed out by movement of the solvent through the plant material. Successive percolations can be performed to extract the plant material exhaustively by refilling the percolator with fresh solvent and pooling the extracts together. Percolation done without heating the extracting solvent is referred to as cold percolation. Since no heat is involved, there are no losses of thermo labile constituents. A variety of compounds like the diterpenoids, ellagitannins, coumarins and flavones have been obtained by cold percolation methods[104,105]. Hot percolation, on the other hand, involves increasing the temperature of the extracting solvent to increase the solubility of the active principle, provided the active principle is not heat sensitive.

2.4.6 Microwave-assisted extraction

Microwave-assisted extraction is a process of applying the microwave energy to a liquid-solid system and partition compounds of interest from the solid sample into the surrounding solvents. The process allows for the direct extraction of dried as well as fresh plant material. It is based on the use of extraction solvents that are relatively transparent to microwaves as compared to the target material to be extracted, thereby serving at the same time as a coolant as well as a solvent. Plant cells contain minute microscopic traces of moisture that serves as the target for microwave heating. The moisture when heated up inside the plant cell, due to microwave effect, evaporates and generates tremendous pressure on the cell wall due to swelling of the plant cell[106]. The pressure pushes the cell

wall from inside, stretching and ultimately rupturing it, which facilitates leaching out of the active constituents from the ruptured cells to the surrounding solvent thus improving the yield of phyto constituents. The advantages of this process over the conventional methods include shorter extraction times, less energy and solvent usage with minimum generation of waste[107].

2.4.7 Distillation

Distillation is mainly used for obtaining aromatic compounds from plants. There are different processes used but in all of them, steam is generated either in a boiler or in a distillation tank and is allowed to pass through the aromatic material to rupture the oil glands. The steam and essential oil vapours are then cooled in a condenser and the resulting distillate collected. The essential oil will normally float on top of the distilled water component/hydrosol and can easily be separated. The essential oil obtained called 'primary' or 'decanted' or 'main' essential oil, is filtered, and dried over anhydrous sodium sulphate before its storage. However, during the process of distilling aromatic plant materials, hydrophilic components of the essential oil dissolve in the distillation water and the product is known as 'distillate water' or 'hydrosol' or 'hydrolate' is also highly aromatic.

2.4.7.1 Hydro or water distillation

This is the simplest and usually cheapest distillation method. The plant material is immersed in water and boiled. The steam and oil vapour is condensed and the oil is separated from the water. In the laboratory hydro distillation is done using a Clevenger-type apparatus, shown in Figure 2.12. The method is cheap, easy to construct and suitable for plant material like flower blossoms that have a tendency to agglomerate or to agglutinate into an impenetrable mass when steam is passed through. However, the method is slow and hence

consumes a lot of fuel since the distillation time is longer. Besides, it's not easy to control the quantity of heat and hence has a variable rate of distillation and the prolonged action of hot water can cause hydrolysis of some constituents of the essential oils such as esters. It's also not a suitable method for large scale distillations and for distillation of high boiling woody plant materials.

Figure 2.12.Clevenger-type apparatus

2.4.7.2 Water and Steam Distillation

The method which is also referred to as wet steam distillation was developed to overcome the drawbacks of hydro distillation. Direct contact of plant material with a hot furnace bottom is thus avoided. The plant material is supported on a cage / perforated grid below which water is boiled. Steam rises through the plant material vaporizing the essential oil with it and is condensed usually in a coil condenser by cooling water. The method is suitable for distilling leafy materials but does not work well for woods, roots and seeds. The distillation units are cheap, easy to operate and are extremely popular with essential oil

producers in developing countries. The method, however, is time consuming, gives poor quality oil yields and oil separation is not complete.

2.4.7.3 Direct steam distillation

The boiling point of most essential oil components exceeds that of water and generally lies between 150-300^{0}C, however, in the presence of steam they are volatilised at a temperature close to 100^{0}C.The principle behind steam distillation is that two immiscible liquids, when mixed, each exerts a vapour pressure, as if each liquid were pure[108]. The total vapour pressure of the boiling mixture is therefore equal to the sum of the partial pressures exerted by each of the individual components of the mixture. When the total vapour pressure reaches atmospheric pressure, the mixture starts to boil. The plant material is placed in a still and steam prepared in a separate chamber is forced over it. The temperature of the steam is carefully controlled so as not to burn the plant material or the essential oil. The rate of distillation and yield of the oil are high and the oil obtained is of good quality. However, partial loss of more polar constituents of the oil, due to their affinity for water, may occur[109,110]. The method is quite expensive and only bigger producers can afford to own the distillation unit, hence it is much popular for the isolation of essential oils on commercial scale[110].

2.4.7.4 Hydro diffusion

Hydro diffusion is a type of steam distillation where steam is fed in from the top onto the botanical material. The process uses the principle of osmotic pressure to diffuse oil from the oil glands. The system is connected and low pressure steam is passed into the plant material from a boiler from the top.The oil and water are collected below the condenser in a typical oil separator. The various components of the essential oils are liberated based on their

solubility in the boiling water rather than the order of their boiling points[111]. The main advantage of this method over steam distillation is that less steam is used hence a shorter processing time and therefore higher oil yield.

2.4.8 Liquid Carbon Dioxide Extraction Method

The liquid carbon dioxide extraction method though commonly used in the extraction of essential oils can also be used in the extraction of other active principles from plants. Extraction is carried out in a specially designed high-pressure soxhlet apparatus with supercritical/liquid carbon dioxide as the extracting solvent. The plant materials are put into the extraction columns, which are under high pressure (55–58 bar) and the liquid CO_2 flows through the extraction columns until it is saturated with the essential oil. At the end of the extraction the column is taken and liquid carbon dioxide is drained from it. Extraction is carried out at a low temperature and this allows for maximum preservation of all healthful substances in the extract like the aroma, taste, vitamins and enzymes. The essential oils obtained by this method have been found to be superior in quality and flavour as compared with the conventional steam distilled essential oils[112]. However, the method is expensive in terms of plant and, in some cases, results in an unusual balance of extracted oil components.

2.4.9 Expression

Expression is a method of fragrance extraction where raw materials are pressed, squeezed or compressed and the oils are collected. The method is suitable for plant material with naturally high oil content and is often applied to peels of fruits in the citrus family[113]. There is no heat which may decompose the aromatic compounds and hence damage the oil. Essential oils obtained by this method have superior natural fragrance characteristics. The

most ancient way of extracting essential oils by expression is the sponge extraction procedure and was done by hand. The fruit pulp was removed, with the rind and pith then soaked in warm water and after the fruit has absorbed the water and become more elastic, it was squeezed to release the volatile oil, which was then collected directly into the sponge. Upon saturation, the sponge was squeezed and the essential oil collected in a vessel and then decanted. A less labour intensive method, the Écuelle à piquer extraction method, involved putting the fruit in a device rotated with spikes, which puncture the oil cells in the skin of the fruit. The oil is removed from the water-based parts of the mixture and decanted. Expression can also be done by machine abrasion where a machine strips off the outer peel of the citrus fruit and the peel is carried in a stream of water into a centrifugal separator where the essential oil is separated from other components. Although the centrifugal separation is done extremely fast, the essential oil is combined with other cell contents for some time and some alteration may occur in the oil due to enzymatic action[114].

2.5 Identification of natural compounds

Natural compounds can be identified by using both physical and chemical methods. In the past, a class of compounds was usually obtained from its response to colour tests, its solubility, retention factor (R_f) properties and its UV spectral characteristics[115]. Other characteristics like melting the point for solids, boiling point for liquids and optical rotation for optically active compounds could be verified for confirmation. Today, equally informative data on plant substances are its spectral characteristics.

2.5.1 Mass spectrometry(MS)

Mass spectrometry is an analytical technique that has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. The sample is first introduced into the ionisation source, where the molecule is given a positive electrical charge, either by removing an electron or by adding a proton. The positively charged fragments and any remaining unfragmented molecular ions are extracted into the analyser region where they are separated according to their mass to-charge ratios (m/z).The separated ions are detected and this signal sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of anm/z spectrum.Interpretation of mass spectra can be done by using mass spectra interpretation software where the mass spectrum revealed is compared with the mass spectra in a database and the agent with a spectrum that best matches that of the unknown analyte is considered.

2.5.2 Gas chromatography (GC)

Gas chromatography is a special form of general chromatography technique for the identification and quantification of complex mixtures of substances including essential oils[116,117]. It can also be used for preparative work. The gas chromatograph comprises an injector, oven, column detector and data analysis unit. The sample to be analysed (about 0.5 – 1.0 µL of a 10% of the sample solution in n-hexane, diethyl ether or dichloromethane) is injected by a micro syringe into a stream of an inert gas (hydrogen or helium) and carried down the column. The column is placed in an oven where isocratic and temperature programmed conditions can be achieved. The vaporized sample is swept through the liquid

stationary phase, which is held on an inert support in the column and the separated analytes flow through a detector, whose response is displayed on a computer or recorder. The detector senses and measures the small amounts of the separated components present in the carrier gas stream leaving the column. The output from the detector is fed to a recorder, which produces a pen-trace called a chromatogram.

2.5.3 Gas chromatography / Mass spectrometry (GC/MS)

Gas chromatography / Mass spectrometry (GC/MS), (Figure 2.13), uses a combination of chromatographic and spectroscopic techniques for separation, detection and identification of organic compounds in complex mixtures[118,119]. The GC/MS has become an integral part of in the analysis of chemical constituents of plant extracts.

Figure 2.13.Gas chromatography / Mass spectrometry (GC/MS) apparatus

In principal, the gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g 5% phenyl polysiloxane) to vaporise and separate the sample into different components.

The components take different times (retention times) to elute from the gas chromatograph and this allows the mass spectrometer to capture, ionize, accelerate, deflect and detect the ionised molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio[120]. The mass spectrum of each compound detected can be instantaneously taken and searched through thousands of mass spectra contained in the gas chromatograph / mass spectrometry computerized libraries for identification. The integrators calculate the retention time, as well as the relative percentages of each peak.

2.5.4 Pyrolysis –Gas Chromatography/Mass Spectrometry (Py-GC/MS)

Pyrolysis is a technique mainly used in the analyses of natural and artificial polymers and macromolecules. A sample is heated in an inert atmosphere usually to decomposition and the fragments are carried by a carrier gas usually helium or nitrogen to the next instrument for characterization. The fragments which are of a lower molecular weight and more volatile than the macromolecule are specific units of a particular macromolecule. Although polymer analysis and characterization remain the chief applications, pyrolysis has found application in less common domains. The pyrolysis technique can introduce many volatile or non volatile organic compounds into a GC, GC/MS or any other analytical instrument[121]. Pyrolysis coupled to gas chromatography/mass spectrometry (Py-GC/MS) has a wide range of applicability as illustrated by its use inthe forensic field for the differentiation of paints, analysis of whole organisms in bacterial identification and classification and analysis of vegetable material[122-127]. The use of this technique in the analysis of herbal products has been very limited[128]but its potential is very promising. Ithas been possible to discriminate samples of *Eucalyptus camaldulensis* grown from seeds of different origin based on the

pyrolysis data using a micro-furnace pyrolyser at 450^{o}C[129]. Pyr-GC/MS has displayed the huge applicability to identify components of plant extracts with its advantages to rapidity, high sensitivity, high accuracy and small sample mass in micrograms [130]. Many non-polymeric organic compounds that are somewhat volatile at elevated temperatures do not fragment upon pyrolysis[131]. Volatiles and additives vaporise at lower temperatures while pyrolysis fragments from polymers are formed at higher temperatures 600-800^{o}C together with evaporation of metallic components.

2.6 Biological activities of plant extracts

2.6.1 Antioxidant activity

An antioxidant is a substance that, when present at a low concentration compared with that of an oxidisable substrate, inhibits oxidation of the substrate[132]. There are many types of free radicals but those of most concern in biological systems are derived from oxygen, and are collectively known as reactive oxygen species (ROS). Reactive oxygen species (ROS) such as singlet oxygen ($^{1}O_2$), superoxide anion (O_2^{-}) and hydroxyl ($^{\cdot}OH$) radical and hydrogen peroxide (H_2O_2) are often generated as by-products of biological reactions or are from exogenous factors[133]. Chemically, free radicals are molecules that are missing an electron and this makes them highly reactive as oxidants. In the act of desperately snatching an electron from any other molecule, ROS exert oxidative damaging effects to molecules found in living cells including DNA[134]. Damage to DNA, if not reversed by DNA repair mechanisms, can cause mutations and possibly cancer[135]. There is an increased evidence for the participation of free radicals in the etiology of various diseases like cancer, diabetes, liver injury, atherosclerosis, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases and aging[136,137]. Antioxidants may act as reducing agents that prevent oxidative

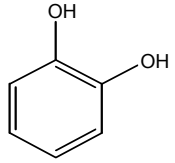
reactions, often by scavengingreactive oxygen species before they can damage cells[138]. Some compounds contribute to antioxidant defence by inhibiting generation of oxidants for example by chelating transition metals and preventing them from catalysing the production of free radicals in cells. Antioxidants may also act by inducing the production of antioxidants within the body. Antioxidants from both natural and synthetic origin have been investigated for use in the treatment of various human diseases[139]. Synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylhydroquinone are commonly used in processed foods. However, it has been reported that these compounds have toxic effects like liver damage and mutagenesis and hence their use has been restricted[140]. This has necessitated finding alternatives to synthetic antioxidants for use in foods or medicinal materials. Many compounds, naturally occurring in plants have been identified as antioxidants[141]. Flavonoids are considered to be one of the most powerful antioxidant groups of carbon-based phenolics synthesized by plants[142]. Therapeutic properties of medicinal species used in folkloric medicine are often associated with their antioxidant properties due to the presence of various types of flavonoids[143]. The antioxidant potential of phenolic compounds is the result of their redox properties, which permits them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators[144]. It is known that plants accumulate antioxidant chemicals as secondary metabolites through evolution as a natural means of surviving in a hostile environment[145]. Other secondary plant metabolites reported to have antioxidant activity include terpenoids, cardiac glycosides, steroids and tannins[146,147]. There is a growing interest all over the world for discovering the untapped reservoir of medicinal plants. The presence of structurally diverse antioxidant compounds in plants suggests that further study of medicinal plants for antioxidant potential is necessary.

Different *in-vitro* antioxidant assays are used to determine the antioxidant potential of a plant sample. The most popular and frequently used for the determination of antioxidant activity of volatile and non volatile plant extracts is the DPPH radical scavenging assay[148]. Related to DPPH, is the 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging assay. Bleaching of β-carotene in linoleic acid system is also a simple, reproducible and time efficient method for rapid evaluation of antioxidant properties and has been employed in many studies for evaluating the antioxidant potential of essential oils and plant extracts[148]. Other methods include ferric reducing antioxidant power (FRAP), nitric oxide (NO) radical inhibition, chelating effect of ferrous ions, hydrogen peroxide scavenging activity and superoxide anion scavenging activity[149].

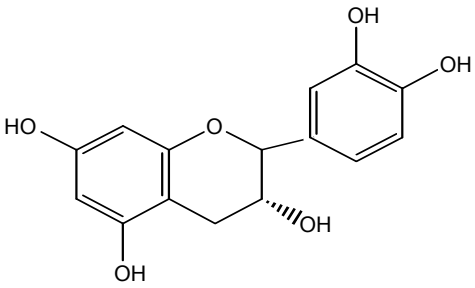
2.6.2 Antibacterial activity

The use of plants as antibiotics is an ancient practice. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century[150]. Different assays like the disc diffusion assay, well diffusion assay, micro dilution assay and measurement of minimum inhibitory concentration are often used for measuring the antimicrobial activity of essential oils and plant based constituents[151]. However, factors such as volume of the inoculums, growth phase, culture medium used, pH of the media, incubation time and temperature have made comparison of published data complicated[152,153]. Despite the differences in the methods of assessment, it is apparent that many plant species contain anti-microbial compounds. A relationship between chemical structure of the volatile and non-volatile plant constituents and antimicrobial activity has been reported[154]. Examples of antibacterial compounds in plant non volatile extracts include phenolic compounds like catechol **2.14a** and epicatechin **2.14b**, alkaloids like berberine

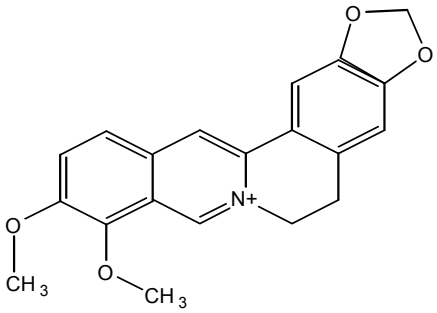
2.14c and piperine **2.14d**, hydrolysable tannins **2.14e**, flavonoids like chrysin **2.14f**, flavonols like totarol **2.14g** and coumarins like warfarin **2.14h** [155-160], (Figure **2.14**).



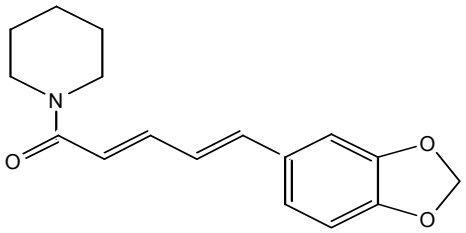
2.14a



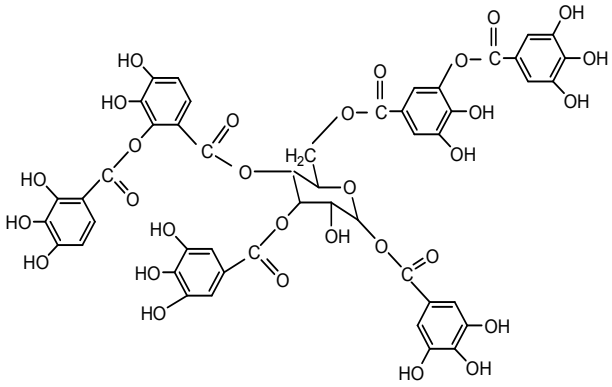
2.14b



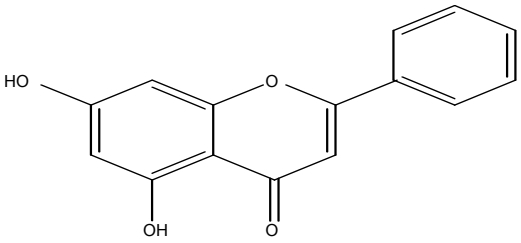
2.14c



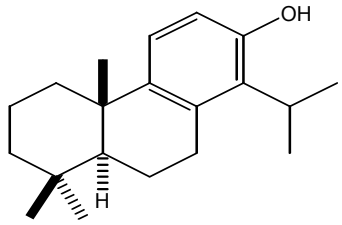
2.14d



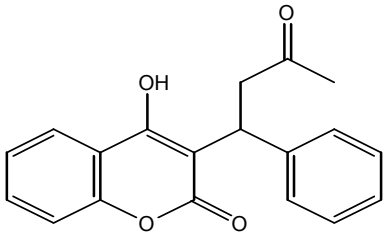
2.14e



2.14f



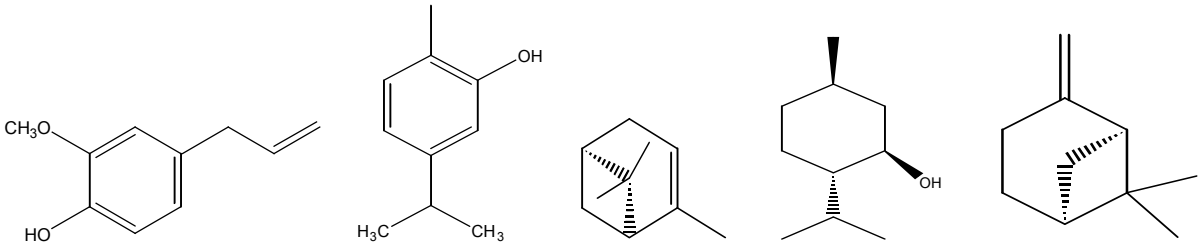
2.14g



2.14h

Figure 2.14. Examples of antibacterial compounds found in non volatile extracts

In addition, essential oil constituents reported to have antimicrobial activity include 1,8-cineole **2.5d**, linalool **2.5e**, thymol **2.5f**, (Figure 2.5) and eugenol **2.15a**, carvacrol **2.15b**, α-pinene **2.15c**,menthol **2.15d,** β-pinene **2.15e**[161,162], (Figure**2.15**).



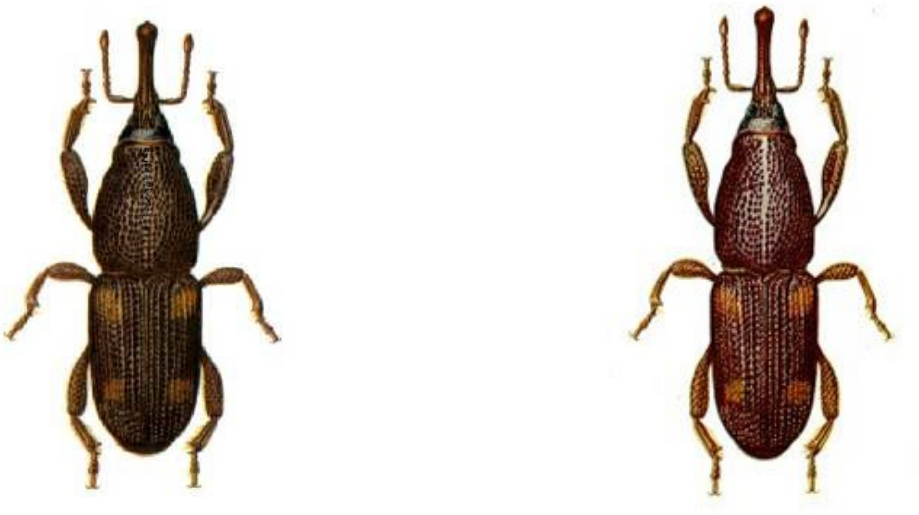
2.15a **2.15b** **2.15c** **2.15d** **2.15e**

Figure 2.15.Examples of some of the essential oil constituents with antibacterial activity.

2.6.3 Insecticidal activity

2.6.3.1 Stored-grain pests (*Sitophilus zeamais*and *Sitophilus oryzae* L.)

*Sitophilus zeamais*and *Sitophilus oryzae* L. have been reported as some of the severe pests of stored grains and their products[163-165]. *Sitophilus zeamais*, although associated primarily with maize is capable of developing in all cereal grains and their products[164]. *Sitophilus oryzae* can also develop in different types of grains but mainly prefers soft varieties of wheat grains[165]. *Sitophilus zeamais* (Figure 2.16a) and *Sitophilus oryzae*,(Figure 2.16b),are similar in appearance. Their colours vary from dull red-brown to nearly black and are usually marked on the back with four light reddish or yellowish spots. They have fully developed wings beneath the wing covers; the maize weevil has more distinct coloured spots on the forewings, it is slightly larger in size and is a stronger flier. Their habits and life cycle are similar with a minimum life cycle of 28 days. The egg, larva and pupa stages occur in the grain kernels. The adults live an average of 4 to 5 months, and each female lays 300 to 400 eggs during this period.



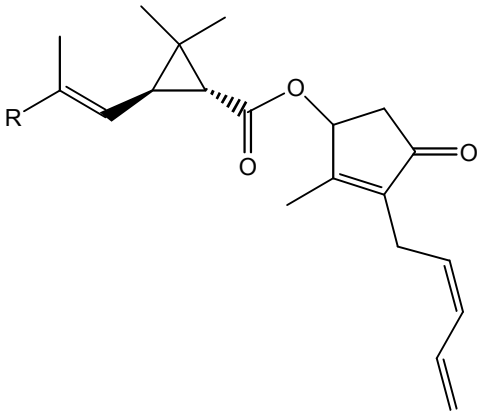
(a) **(b)**

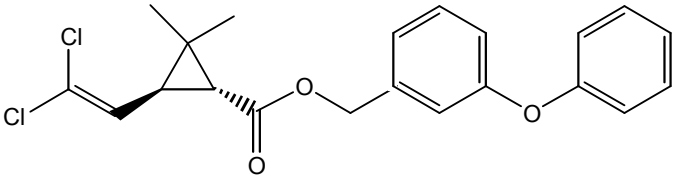
Figure 2.16.*Sitophilus zeamais* and *Sitophilus oryzae.*

The weevils can survive in extreme cold and hot temperatures hence they are found all over the world. The maize and rice weevil causes similar damage to the grains they infest. The larvae feed within the kernel and consumes the endosperm. The adults leave a large, ragged exit hole in the kernel and feed on damaged kernels. The adult weevil gathers and reproduces in stored grains. This produces heat and moisture which can lead to mould development and invasion by other insect species. The grain can also be tainted with white, dusty excreta which contaminate the product as well as render it unpalatable. Infestations can result in reduction of weight and quality of grain as a result of the larvae and adult weevils feeding on the endosperm. Heating of grain also occurs which accelerates the development of the insects making the commodity liable to caking, moulding and even germination. Stored grain pests can be controlled physical treatments which include: mechanical impact, physical removal and physical barriers to prevent the entrance of insects, abrasive and inert dusts, ionizing radiation, light and sound[166]. Stored grain pests can also be controlled thermally by altering the temperature. The optimal temperature for

stored-product insects is between 25-33^{o}C[167]. Fluidized beds, spouted beds, pneumatic conveyors, a counter-flow heat exchanger, high frequency waves, microwaves, infra waves and solar radiation have been used to satisfactorily disinfect grains using high temperatures but high costs are involved[167]. Stored grain pests can be controlled biologically by the use of living organisms, parasitoids and predators[168,169]. Laboratory tests have demonstrated that *Anisopteromalus calandrae*, a wasp, has the potential to control the maize weevil in corn and the rice weevil in wheat[170]. Although parasitoids and predators are potential stored grain pest control agents and even some of them have been sold commercially, their use may be limited by many factors[171]. Such factors include increased costs which make the method expensive; releasing enough parasitoids in large amounts of the grain may be difficult and hence the efficiency of the parasitoids in such situations may be poor and the presence of the parasitoids or predators in the end products will not be accepted in retail trade. Stored grain pests are mainly controlled by fumigation, a method where gaseous pesticides completely fill an area to suffocate or poison the pests within[172]. Phosphine and methyl bromide are the most common fumigants used for stored product protection[173]. However, insect resistance to phosphine and hence control failures have been reported in some countries[174]. In addition, methyl bromide was declared to be ozone-depleting and was phased out completely by most countries in the early 2000s[175]. In view of the problems with the current fumigants, there is a global interest in alternative strategies including the development of chemical substitutes, exploitation of controlled atmospheres and integration of physical methods[176]. Active research is being undertaken to exploit ozone as a potential quarantine treatment for controlling stored-product pests[177]. Ozone offers several safety advantages over conventional post-harvest pesticides. It can be easily generated at the treatment site using only electricity and air and hence there are no stores of toxic

chemicals, no chemical mixing hazards and no disposal of left over insecticides or containers[178]. In addition, ozone has a short half-life, 20-50 minutes, it reverts back to oxygen leaving no residue on the product and if necessary ozone can be neutralized by thermal activated charcoal[178]. The major disadvantage of ozone is that it is corrosive towards most metals[179]. Plants may provide potential alternatives to currently used insect-control agents because they constitute a rich source of bioactive chemicals. The use of plants, plant material or crude plant extracts (botanical insecticides) for the protection of crops and stored products from insect pests is probably as old as crop protection itself[180]. Before the development of synthetic insecticides, botanical insecticides were major weapons in the farmer's arsenal against crop pests and are still used to date in some societies especially in Africa[181]. For example, in Uganda and Kenya, farmers use the whole plant of *Tagetes minuta* to protect maize stores [182] while in Malawi, crushed tobacco leaves are mixed with grains during storage[183]. Natural compounds from plants have also been used as insecticides and as templates for commercial pesticides. Various monoterpenoids from plant essential oils have attracted attention in recent years as potential pest control agents due to their insecticidal, repellent and antifeedant properties[184-187]. Pyrethrins I and II, **2.17a**, related esters from *Chrysanthemum cinerariaefolium* (pyrethrum), are neurotoxins that attack the nervous system of all insects[188]. When present in amounts not fatal to insects, they have an insect repellent effect[189]. They are the compounds upon which pyrethroid insecticides like permethrin **2.17b** were designed and their actions are similar, (Figure **2.17**).





2.17a. **2.17b**

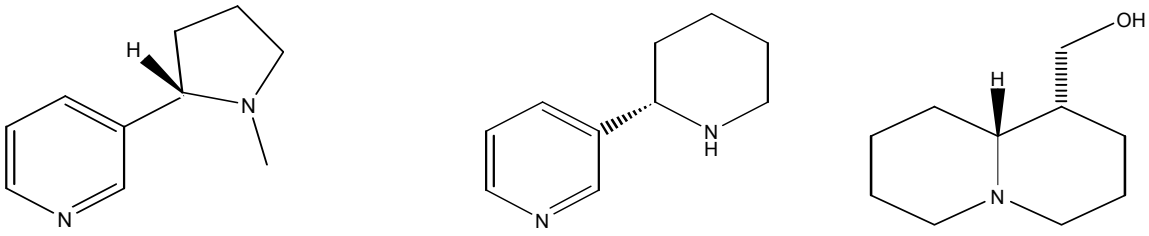
Pyrethrin I, R = CH_3
Pyrethrin II, R = CO_2CH_3

Figure 2.17.Pyrethrins andpermethrin

2.6.3.2 *Anopheles arabiensis* mosquito larvae

The mosquito is the principal vector of many of the vector-borne diseases affecting human beings and other animals. The principal mosquito borne diseases are the viral diseases yellow fever and dengue fever, transmitted mostly by the *Aedes aegypti*, and the parasitic disease malaria carried by the genus *Anopheles.* Malaria infects 350-500 million people each year, killing 1 million, mostly children in Africa where as dengue fever infects around 220 million people worldwide annually with 12,500 to 25,000 deaths[190,191]. The WHO estimates that yellow fever causes 200,000 illnesses and 30,000 deaths every year in unvaccinated populations and around 90% of the infections occur in Africa[192]. All mosquitoes must have water in which to complete their life cycle and the larval stage is the most vulnerable stage to attack mosquitoes as they are concentrated in smaller areas. Thus, one of the approaches for control of mosquito transmitted diseases is by interrupting the mosquito life cycle at

larval stage. This can be by changing the mosquito larvae habitat through drainage, levelling and filling of the mosquito breeding sites[193]. Mosquito larvae can also be controlled biologically, for example by using fishes to feed on the larvae and chemically, by the use of chemicals to kill the mosquito larvae[194]. Chemical larvae control worldwide depends primarily on continued applications of organophosphates such as temephos, fenthion and insect growth regulators such as diflubenzuron and methoprene[195]. Besides disruption of natural biological control systems, outbreaks of other insect species, widespread development of resistance, undesirable effects on non-target organisms, and repeated use of chemicals in controlling mosquito larvae involves high costs[195]. These problems have highlighted the need for new strategies of mosquito larval control. It has been found that herbal extracts are a safer alternative method of control, especially the extracts of certain medicinal plants. One of the earliest reports on the use of plant extracts against mosquito larvae was that of Campbell et al.(1933), where it was reported that plant alkaloids nicotine **2.18a**, anabasine **2.18b**, methyl anabasine and lupinene **2.18c**, (Figure **2.18**), extracted from the Russian weed, *Anabasis aphylla*, killed the larvae of *Culex* sp[196].

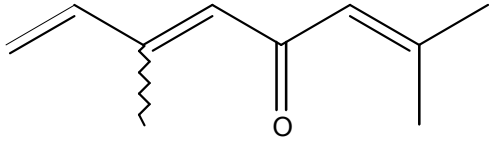


2.18a **2.18b** **2.18c**

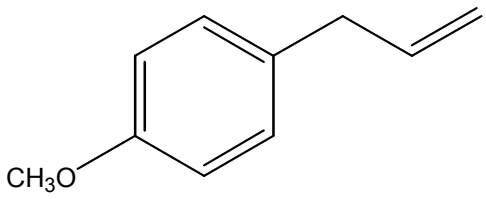
Figure 2.18. Alkaloids with larvicidal activity against mosquito larvae

In recent times, many plants have been reported to contain toxic principles that can play a useful role in the control of mosquito larvae[197-202]. Essential oils derived from plants have also received much interest as potential bioactive agents against mosquito vectors. Studies

of essential oils obtained from the plants, *Cymbopogan citrates*, *Tagetus minuta*, *Ocimum basilicum*, *Thymus capitatus*, *Lippia sidoides, Hyptis martiusii* and many other plants have demonstrated promising larvicidal activities against mosquito vectors[203-209]. Some of the essential oil compounds reported to have larvicidal activity against mosquito larvae include ocimenone **2.19a**, methylchavicol **2.19b**, **(**Figure 2.20), and thymol**2.5f**[204-206].



2.19a



2.19b

Figure 2.19. Some essential oil compounds with larvicidal activity

Nature possesses many useful medicinal plants which may be useful for the control of vector borne diseases. Not only can medicinal plant extracts be effective they may also greatly reduce the risk of adverse ecological effects and they do not induce pesticide resistance in mosquitoes. Since these chemicals are taken from medicinal plants, they are expected to have low human toxicity and a high degree of biodegradation[210].

2.6.4 Cytotoxicity

The active ingredients of plant extracts are chemicals that are similar to those in purified medications, and they have the same potential to cause serious adverse effects. Whilst the literature documents severe toxicity resulting from the use of herbs, on many occasions the potential toxicity of herbs and herbal products has not been recognized[211]. The potential toxicity of plants is an important consideration when studying the traditional use of plants and also when identifying their potential for other biological properties.

2.6.4.1 The brine shrimp lethality assay (BSLA)

The brine shrimp assay, proposed by Michael et al. (1956) and later developed by Vanhaecke et al. (1981) and Sleet and Brendel, (1983), is a general bioassay for the preliminary assessment of toxicity[212-214]. This method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the micro well scale. One indicator of the toxicity of a substance is LC_{50} which is the lethal dose/concentration of a substance that kills 50% of the test organisms. According to Meyer et al. (1982) and Alanis-Garza et al. (2007), crude extracts and pure substances are considered toxic if their LC_{50} value is less than 1000 µg/ml and non-toxic if their LC_{50} value is greater than 1000 µg/ml[215,216]. It has often been stated that pharmacology is toxicity at a lower dose and toxicity is pharmacology at a higher dose, hence extracts derived from natural products with $LC_{50} \leq 1000$ µg/ml using BSLA contain physiological active principles[217]. The evaluation of plant extracts by using brine shrimp cytotoxicity assay not only describes their cytotoxicity but also depicts trends of other biological activities like anticancer, antiviral, insecticidal and pesticide potential of the plant extracts[218]. Some published data has suggested a good correlation between the toxic activity in the brine shrimp assay and the cytotoxicity against some tumor cell lines and hepatotoxic activity[219,220]. According to the American National Cancer Institute, the LC_{50} limit to consider for a crude extract promising further purification in order to isolate potent anticancer compounds is lower than 30µg/ml[221]. As mentioned previously, the BSLA is useful to screen for the toxicity of plant extracts as a preliminary step since it is easy, fast and economical. But the results obtained using the BSLA are not conclusive about the toxicity of a plant extract. It has been found that some plants known to be toxic to livestock have displayed non toxicity to brine shrimps[222]. Hence other

cytotoxicity tests are carried out alongside the BSLA to establish the cytotoxicity of plant extracts.

2.6.4.2 The MTT cytotoxicity assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay is the most widely used in vitro cytotoxicity assay. This is because it is rapid, sensitive, versatile, quantitative and highly reproducible. The assay detects the reduction of MTT to an insoluble and coloured formazan. When MTT enters a living cell, the mitochondria succinate dehydrogenase enzyme in the cell cleave to the tetrazolium rings of the pale yellow MTT and form dark blue/purple crystals called formazan crystals which are largely impermeable to cell membranes, thus resulting in their accumulation in healthy cells. Formazan is solubilised with dimethyl sulfoxide(DMSO)[223]. The intensity of the dark blue/purple colour of formazan is quantified spectrophotometrically at 570nm and is proportional to the number of live cells present. Mitochondria are essential organelles that play an important role in cell metabolism, and the MTT assay represents an important method to evaluate mitochondrial damage[224].

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CHAPTER THREE

MATERIALS AND METHODS

3.0 Materials and methods

3.1 Plant collection

Fresh plant materials of the leaves, stem and bark of *T. camphoratus* and *T. trilobus var*. *galpinii* were collected from naturally growing plants from Sangoyana and Ubombo respectively. *T. camphoratus* was collected at three different times, in the months of November 2009, April 2010 and April 2011. *T. trilobus var. galpinii* was collected twice in the months of April 2010 and April 2011. Both Sangoyana and Ubombo are found in the northern part of Kwa-Zulu Natal. The plants were identified by the local people during the time of collection and further identified by Mrs N.R Ntuli in the Department of Botany, University of Zululand. Voucher specimens (NSKN 1), for *T. camphoratus* and (NSKN 2) for *T. trilobus var.galpinii* harvested in 2010 were deposited at the University of Zululand herbarium.

3.2 Preparation of the plant material

The leaves which were required fresh were used immediately after harvesting and other leaves were kept for drying. The bark was chopped into pieces, the leaves were removed from the stem and they were air dried at room temperature in the chemistry laboratory away from direct sunlight. To avoid formation of moulds the plant material was dried in open air and turned over periodically for about 30 days. After drying, the bark was ground to a fine powder using a Warring commercial blender (Model 32BL79, Dynamics Corporation

USA) and the powdered plant material was stored in tightly closed glass bottles in the dark at room temperature.

3.3 Extraction of the plant materials

3.3.1 Extraction of the volatile extracts

The volatile extract of the dry leaf of *T. camphoratus* harvested in November 2009 and the volatile extractsof the fresh leaf, dry leaf and dry stem of *T. camphoratus* and T. trilobus var galpinni harvested in April 2010 and April 2011 were extracted separately byhydro distillation using a Clevenger-type apparatus[1-3].Plant materials (400 – 700 g) of each sample were added to 1.5 litres of distilled water in a round bottomed flask and heated to boiling. The volatile extracts were collected 4 h after boiling, weighed and kept at 4^{o}C for further analysis.

3.3.2 Extraction of the non volatile extracts

Ground bark (400g) and 1500 g of the dried leaves of *T.camphoratus* and 300g of the ground bark and 1200 g of the dried leaves of *T.trilobus var galpinni* were sequentially macerated in 4 litres for the bark and 6 litres for the leaves of hexane, followed by dichloromethane, ethyl acetate and methanol each for 5 days with occasional stirring. After maceration in a particular solvent, the extract was filtered through a cotton plug and finally through a Whatman No. 1 filter paper after which the extract was concentrated under reduced pressure below 50^{o}C through a rotator vacuum evaporator. The concentrated extracts were collected in a beaker and allowed to air dry for complete evaporation of the extracting solvents. Maceration in a particular solvent was done three times before transferring the extract to another solvent. The aqueous extract for *T. camphoratus* was done by maceration

of 600 g of the dried leaves in 600 ml of water for 3 days with vigorous shaking. After filtration, the aqueous extract was concentrated to a brown powder in a freeze dryer, FD—Bench top K, VirTis model. The concentrated extracts were then kept in a refrigerator pending further use.

3.4. Determination of the physicochemical properties of the volatile extracts/essential oils

The physicochemical properties of the volatile extracts were determined using the British Pharmacopoeia methods[4,5]

3.4.1. Determination of the refractive index of the essential oils

The refractive index of the volatile extractswas determined using the Abbey refractometer. Water (refractive index 1.333) followed by hexane (refractive index 1.3749) was used to calibrate the refractometer. One drop of each volatile extract was applied separately on the refractometer and the instrument was allowed to come to equilibrium with the sample before the reading was taken. Once the proper illumination was secured, the alidade was moved forward and backward until the sharp dividing line that fell on the point of intersection was obtained. The refractive index was measured with the ATAGO IT, Japan.

3.5. Determination of the chemical constituents in the extracts

3.5.1. Determination of the chemical composition of the volatile extracts/essential oils

3.5.1.1. Gas Chromatography / Mass Spectrometer (GC/MS) analysis

The GC-MS analysis of the volatile extractswas carried out using an Agilent 6890 GC with an Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC was equipped with a fused silica capillary HP-5 MS column of an internal

diameter of 0.25mm, film thickness 0.25 µm and a length of 30 m. The initial temperature of the column was 70 ^{o}C and was heated to 240 ^{o}C at a rate of 5 ^{o}C/min. Helium was used as the carrier gas at a flow rate of 1ml/min. The split ratio was 1:25. Scan time was 50 min with a scanning range of 35 to 450 amu. A 1%, v/v, solution of the samples in hexane was prepared and 1µL was injected using a split less injection technique.

3.5.1.2 Identification of the volatile extracts/essential oil components

The identification of the volatile extracts constituents was based on their retention indices determined by reference to a homologous series of *n*-alkanes (C_8-C_{30}), and by comparison of their mass spectral fragmentation patterns with those reported in the literature[6,7] and stored in the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA version C.00.01.080)]. The percentages of each component are reported as raw percentages based on the total ion current without standardization.

3.5.2 Determination of the chemical composition of the non volatile/ solvent extracts

3.5.2.1 Phytochemical screening of the non volatile extracts

Phytochemical screening was performed on the non volatile extracts using standard procedures to identify chemical constituents as described by Trease and Evans (1989), Harborne (1973) and Sofowora (1993)[8-10].

Screening for alkaloids

The extract (0.5g) was stirred in 5ml of 1% HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1ml of the filtrate was treated with a few drops of Wagner's reagent. A reddish brown precipitate indicates the presence of alkaloids.

Screening for flavonoids

Two millilitres of dilute sodium hydroxide was added to 2ml of the extract. The appearance of a yellow colour indicates the presence of flavonoids.

Screening for phenols

Equal volumes (1 ml) of extract and Iron (III) chloride were mixed. A deep bluish green solution gave an indication of the presence of phenols.

Screening for tannins

A portion of the extract was dissolved in water, after which the solution was clarified by filtration. Ferric chloride solution (10%) was then added to the resulting filtrate. The appearance of a bluish black colour indicates the presence of tannins.

Screening for saponins

One millilitre of distilled water was added to 1 ml of the extract and shaken vigorously. A stable persistent froth indicated the presence of saponins.

Screening for cardiac glycosides

The extract (0.5g) was dissolved in 2ml glacial acetic acid containing one drop of ferric chloride solution. This was under layered with 2 ml of concentrated sulphuric acid. A brown ring formation at the inter phase indicates the presence of deoxy sugar characteristics of cardiac glycosides.

Screening for terpenoids

Acetic anhydride (0.5 ml) was mixed with 1 ml of the sample extract and a few drops of concentrated sulphuric acid. A bluish green precipitate indicates the presence of terpenes.

Screening for cardenolides

Benzene (2 ml) was added to 1 ml of the sample extract. The formation of a turbid brown colour is an indication of the presence of cardenolides.

3.5.2.2 Determination of the chemical constituents of the non volatile extracts by Pyrolysis Gas Chromatography / Mass Spectrometer (Pyr-GC/MS) analysis

The Py-GC/MS analysis was performed on a pyrolysis injector (PY-2020iD, Frontier Lab, Japan) interfaced directly with a GC/MS (Shimadzu GCMS-QP2010). During analysis 3mg of the sample was loaded into a quartz tube which was introduced into a micro-furnace pyrolyzer which was then heated from 250^{o}C to 650^{o}C at 5^{o}C /ms and held at 650^{o}C for 20s. Helium was the carrier gas used to flush the pyrolytic products into the GC/MS. A split mode of 1:25 v/v was applied at the GC inlet for the introduction of products from the pyrolysis chamber into the GC column. The column temperature was initially set at 50^{o}C for 2 min then increased at 8^{o}C/min to 310^{o}C and held for 0.5 min. The pyrolysis products were directly analysed on a DB5-HT column with an internal diameter of 0.25mm, film thickness 0.10 and a length of 30 m. The mass spectrometer consisted of a source temperature of 220^{o}C, a filament emission current of 3.7µA, an ionizing voltage of 70eV and a scan range from m/z 25 to m/z 1200 with scan time of 0.2s. Identification of the chemical constituents was done via the NIST database.

3.6 Determination of the biological activities of the volatile and non volatile extracts

The volatile extracts/essential oils were tested for their antibacterial, antioxidant, insecticidal and cytotoxic activities. The essential oils that were investigated for antibacterial, antioxidant and cytotoxic activities were of the fresh leaf, dry leaf and dry stem of *T. camphoratus* and of the fresh leaf of *T. trilobus var galpinni* harvested in April 2010 whereas the essential oil of the dry leaf of *T. camphoratus* harvested in November 2009 and the essential oil of the fresh leaf of *T. trilobus var galpinni* harvested in April 2010 were investigated for their larvicidal activity against *Anophelese arabiensis* mosquito larvae. The essential oils of the dry leaf of *T. camphoratus* and of the fresh leaf of *T. trilobus var galpinni* harvested in 2010, were

investigated for their insecticidal activities against *S. zeamais* and *S. oryzae*. The non volatile extracts of the leaf and bark of *T. camphoratus* and of *T. trilobus var galpinni* harvested in 2010, were tested for their antibacterial, antioxidant and cytotoxic activities.

3.6.1 Determination of the antibacterial activity

In vitro antibacterial activity was evaluated against four gram-positive bacteria (*Staphylococcus aureus* ATCC 6538*, Enterococcus faecalis* KZN*, Bacillus cereus* ATCC 10702*, Staphylococcus epidermidis* KZN) and five gram-negative bacteria (*Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 7700*, Klebsiella pneumonia* ATCC 4352*, Shigella flexineri* KZN and *Salmonella spp* KZN) for both the volatile and non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni*. Six of the nine strains of bacteria were of the American Type Culture Collection (ATCC) and three were of the Kwa-Zulu Natal (KZN) collection. The micro-organisms were obtained from the Department of Biochemistry & Microbiology, University of Fort Hare, Alice and were maintained on nutrient agar. They were revived for bioassay by sub-culturing on nutrient agar and were grown and maintained on Nutrient Broth medium at 37^{o}C for 24 h before use.

3.6.1.1 Agar disc diffusion method

The agar disc diffusion method was employed for the determination of antibacterial activities of the volatile extracts and non volatile extracts[11,12]. The nutrient agar plates were seeded with a suspension of approximately10^{5} CFU mL^{-1} of the bacteria strains. For the volatile extracts, the empty sterilized Whatmann No.1 filter paper disc (6mm) was impregnated with 10µL of the essential oil dissolved in DMSO giving a final concentration of 10% (v/v) whereas for the non volatile extracts, the paper discs were saturated with 5mg/ml of the extracts. The paper discs were dried and placed aseptically on seeded plates with the

help of sterile forceps. The standard antibiotics, streptomycin, ampicillin and chloramphenical 2µg/disc, were used as positive controls while the DMSO solvent was used as a negative control. The plates were incubated at 37^{o}C for 24 h after which the diameters of inhibition zones were measured in millimetres. Tests were carried out in triplicate and the diameters of inhibition were expressed as means ± standard deviation. The data was analysed using one-way analysis of variance (ANOVA) and the effect of the difference among group means were considered significant when the P value was ≤ 0.05.

3.6.1.2 Minimal Inhibitory Concentration (MIC) assay

The MIC assay was used to determine the minimum concentration of the volatile and non volatile extracts that would inhibit bacterial growth. The volatile extracts/essential oils were dissolved in DMSO and diluted to the highest concentration of 500 µg/ml and then serial 2-fold dilutions were made in order to obtain a concentration range from 7.8 to 500 µg/ml. The highest concentration used for the non volatile plant extracts was 5mg/ml and serial 2-fold dilutions were made in order to obtain a concentration range from 0.078 to 5 mg/ml. The inocula of the bacterial strains were prepared from 12 h broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. This was used to inoculate 96-well micro titre plates containing serial two-fold dilutions of the volatile and non volatile extracts under aseptic condition. The plates were incubated under aerobic conditions at 37^{0}C and examined after 24 h. Fourty micro litres (40µL) of 0.2mg/ml p-Iodonitrotetrazolium (INT) were added to each well and incubated for 30min at 37^{0}C. The colourless p-Iodonitrotetrazolium indicator was reduced by the biologically active micro-organisms to red coloured formazan. Where bacterial growth was inhibited, the solution remained clear after incubation with INT[13,14]. Each treatment was done in triplicate.

3.6.2 Determination of the antioxidant activity

The volatile extracts' antioxidant activities were investigated by the DPPH, $ABTS^{\bullet+}$, Nitric oxide radical scavenging assay and total reducing power assay. The antioxidant activities of the non volatile extracts were investigated by all the assays outlined for the non volatile extracts plus the iron chelating activity assay. The concentrations used for both volatile and non volatile extracts in all the antioxidant assays were in the range of 25 -250 µg/ml.

3.6.2.1. DPPH (1,1-Diphenyl-2-picryl-hydrazil) radical scavenging assay

The DPPH radical scavenging activity of both the volatile and non volatile extracts was performed according to the modified method of Mensor et al[15]. One millilitre of 0.3mM DPPH methanol solution was added to 2.5ml solution of the extract or standard (25 -250 µg/ml) and allowed to react at room temperature in the dark for 30 min. The absorbance was then measured against a corresponding blank at 517nm using a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Methanol (1.0 ml) plus extract solution (2.5ml) was used as the blank. Synthetic antioxidant, BHT was used as a positive control and all measurements were done in triplicate.

Inhibition percentage of free radical DPPH was calculated in the following way:

% DPPH radical scavenging = [(control absorbance – sample absorbance)/control absorbance)] x 100

3.6.2.2. The 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) scavenging assay

The $ABTS^{\bullet+}$ radical scavenging activity of both the volatile and non volatile extracts was determined according to the procedure described by Re et al. (1999)[16]. The pre-formed

radical mono cation of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was generated by oxidation of ABTS solution (7mM) with 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand for 16 hours in the dark at room temperature before use. One millilitre of ABTS was added to 1 millilitre of different extract concentrations (25-250, µg/ml) and after 6 min decolourization of $ABTS^{\bullet+}$ was measured spectrophotometrically against a corresponding blank at 734nm. BHT was used as the positive control and all measurements were done in triplicate. Percent inhibition of $ABTS^{\bullet+}$ was calculated as follows:

% inhibition = [(control absorbance – sample absorbance)/control absorbance)] x 100

3.6.2.3. Nitric oxide (NO) radical inhibition assay

Nitric oxide inhibition was determined by the use of Griess Illosvoy reaction principle[17]. The reaction mixture containing 2 ml sodium nitroprusside (10 mM), 0.5 ml phosphate buffer saline (pH 7.4) and 0.5 ml of different concentrations of both the volatile and non volatile extracts (25-250 µg/ml) or standard solution (ascorbic acid, 0.5ml) was incubated at 25^0C for 150 min. After incubation, 1 ml samples of reaction mixtures were removed and diluted with 1 ml Greiss reagent. The absorbance of these solutions was measured spectrophotometrically at 540 nm against the corresponding blank solution. The same reaction mixture without the extract but with an equivalent amount of water served as the control.

% Nitric oxide scavenged = [(control absorbance – sample absorbance)/control absorbance)] x 100

3.6.2.4.Chelating effects of ferrous ions

The chelating effect of the ferrous ions by the non volatile plant extracts was determined according to the method of Dinis et al. (1994)[18]. Two millilitres of various concentrations (25-250 µg/ml) of the extracts in methanol was added to 0.05ml of 2 mM $FeCl_2$. This was followed by addition of 0.2ml of 5Mm ferrozine and the mixture was then shaken vigorously and left at room temperature for 10 min. Ferrozine reacted with the ferrous ion to form a stable magenta Fe^{2+}- Ferrozine complex whose absorbance was measured spectrophotometrically at 562nm using a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Percent metal chelating effect was determined from the equation:

Metal chelating effect (%) = [(control absorbance – sample absorbance)/control absorbance)] x 100

The control was the blank without the extract and Na_2EDTA was used as a positive control.

3.6.2.5.Total reducing power

The reducing power of the volatile and non volatile extracts was determined according to the method of Oyaizu (1986)[19]. Each extract (25-250 µg/ml) in methanol or water (2.5 ml) was mixed with 2.5 ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide and the mixture was incubated at 50 ^{O}C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 1000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5ml of deionised water and 0.5 ml of 0.1 % ferric chloride and its absorbance measured at 700 nm against a blank. Ascorbic acid was used as the reference standard.

3.6.2.6.Statistical analysis of antioxidant results

The results obtained were expressed as mean ± standard error of mean (SEM) of three determinations. The data were evaluated through regression analysis using QED statistics program, PISCES software (PISCES conservation Ltd, 2007, England). The LC_{50} values, where applicable, were determined by linear regression. Means between treatments were compared by Tukey's Studentized Range Test using one-way analysis of variance (ANOVA).

3.6.3. Insecticidal activity

3.6.3.1.Larvicidal activity of the volatile extracts against *Anopheles arabiensis* mosquito larvae

The fresh leaf essential oil of *T. trilobus var galpinni* harvested in 2010 and the dry leaf essential oil *T. camphoratus* harvested in 2009 were tested for their larvicidal activity against *Anopheles arabiensis* mosquito larvae. The larvae of *Anopheles arabiensis* were collected from storage water tanks in the premises of the Department of Zoology, University of Zululand. The mosquito species were identified by an entomologist in the Department of Zoology. The essential oils were dissolved in degassed distilled water using Tween 20 (20%) and concentrations of 25, 50, 100, 150, 200 and 300 ppm of dissolved oil were prepared. The oil-Tween 20-water solution was properly mixed to get a homogeneous mixture. Ten, (10), of fourth-instar mosquito larvae were placed in 25 ml of each test solution and four replicates of each concentration were run at a time. A negative control containing a mixture of distilled water and Tween 20 (20%) was also run. Mortality was checked after 1, 6 and 24h exposure during which no food was given to the larvae. All experiments were conducted at 26±2^{o}C and 60±10% relative humidity. Dead larvae were identified when they failed to move after probing with a needle. Data were evaluated through regression

analysis using QED statistics program, PISCES software (PISCES conservation Ltd, 2007, England), and from the linear equation, the LC_{50} values representing the lethal concentration for 50% larval mortality for *Anopheles arabiensis* were calculated.

3.6.3.2. Insecticidal activity of the volatile extracts against *S.zeamais* and *S.oryazae*

The contact toxicity, fumigation activity and repellent activity against stored product pests, *S. zeamais* and *S. oryzae* of the essential oils of the dry leaf of *T. camphoratus* harvested in 2010 and the essential oil of the fresh leaf of *T. trilobus var galpinni* harvested in 2010 were also determined.

Rearing of test insects

Adults of *S. zeamais* and *S. oryzae* were obtained from a colony maintained by the Plant Protection Research Institute, Pretoria. South Africa. These were mass reared on whole maize grains in 5-L glass jars in a controlled chamber, at 28 ± 20^0C and 56-65% Relative humidity in the Department of Agriculture, University of Zululand. Newly emerged, one week old insects were used in the bioassay[20].

Contact toxicity

The contact effect of the essential oil of *T. camphoratus* on the adults of *S.zeamais* and *S. oryzae* was investigated[21]. Maize grains (40g) in 500 ml glass jars were each treated with 0, 25, 50, 100, 200 and 300 µl of essential oil in 1 ml hexane and the corresponding concentrations were 0, 0.625, 1.25, 2.5, 5.0 and 7.5 µl/g of maize grain respectively. These were thoroughly stirred to allow for homogeneity of the oil on the treated grains. Treated samples were air dried for an hour in order to get rid of the solvent. The grains were then infested with twenty, one-week old, *S. zeamais* or *S. oryzae* adults per jar and each jar was

covered with a cotton mesh held in place by cover rims, Figure **3.1**. There were four replicates per treatment. Insect mortality was checked daily for 28 days.

Figure 3.1.Contact toxicity experiment set up

Fumigation toxicity

The fumigation chambers consisted of 500 ml glass jars with screw-on lids. For the bioassay, solutions of 0, 5, 10, 20, 30 and 40µl of the oil were each diluted with 1ml hexane to correspond to concentrations of 0, 10, 20, 40, 60 and 80 µl/l air. One ml of each concentration was then separately applied to 7 mm discs of Whatman No.1 filter paper, air-dried for 10 min and placed at the bottom of the jars. Twenty, one-week old, adult insects were placed on muslin cloths (21 x 29 mm) each with 40g whole maize grains. The cloths were tied closed with rubber bands and hung at the centre of the jars, which were then

sealed with air-tight lids, Figure **3.2**. There were four replicates for each concentration. Fumigation was carried out for 24 hours after which the insects were transferred from the fumigationchambersonto clean maize, and mortality was checked daily for 28 days [21]

Figure 3.2. Fumigation experiment set up

Repellent activity

The repellent effect of *T. camphoratus* essential oil against *S. zeamais* and *S. oryzae* was studied using a modified area preference method[21]. The test area consisted of a 9 cm Whatman No.1 filter paper cut into two halves. Different oil concentrations were prepared by diluting 10, 20, 30 and 40 µl of the oil in 1ml hexane and these ccrresponded to concentrations of 0.314, 0.628, 0.943 and 1.257 µl of oil/cm^2 of the filter paper respectively. The other half was treated with 0.5ml hexane alone and this served as a control. Both

essential oil treated and hexane treated filter paper halves were air-dried under a fan to evaporate the solvent completely. With the aid of a clear adhesive tape, both halves were later joined together into full discs and placed in 9 cm glass Petri dishes. Twenty one-week old, unsexed adult insects were released at the centre of the rejoined filter paper disc and the Petri dish was covered, Figure **3.3**. Each treatment was replicated four times for each *S. zeamais* and *S. oryzae* .

Figure 3.3. Repellency experiment set up

The numbers of insects present on the control and on the treated areas of the filter paper was recorded after 1, 2, 6, 4 and 24 hours.

Percentage repellency (PR) was calculated as follows (Nerio et al., 2009):

$$PR = ((Nc - Nt)/(Nc + Nt)) \times 100$$

Nc was the number of insects on the untreated area after the exposure interval and Nt was the number of insects on the treated area after the exposure interval. The mean number of

insects on the treated portion of the filter paper was compared with the number on the untreated portion. Results were presented as the mean of percentage repellency ± the standard error.

Statistical analysis

Data was analysed using the QED statistics software, PISCES software (PISCES conservation Ltd, 2007, England). Means for percentage repellency for both insects at the four concentrations at a particular time interval were compared using one way ANOVA. The median repellent dose (RD_{50}) was determined from the linear regression equation through regression analysis.

3.6.4. Cytotoxicity screening of both the volatile and non volatile extracts

Cytotoxicity screening of the extracts was done by the brine shrimp cytotoxicity assay and by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay.

3.6.4.1. Brine shrimp cytotoxicity assay

The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method[22]. The eggs of the brine shrimp were collected from a local fish farm and hatched in artificial seawater (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. For each extract, 2 mg was dissolved in 1 ml of 10% DMSO. From this solution, 2.5, 1.25, 0.25 and 0.025 ml were transferred into test tubes and made up to 5 ml using sea water. The corresponding concentrations were 1000, 500, 100 and 10 µg/ml, respectively. Ten (10) brine shrimps (nauplii) were transferred into each of the test tubes using a Pasteur pipette. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration. Potassium dichromate was used as a positive control and each test was done in triplicate.

3.6.4.2. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay

Human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells were all grown to confluency in 25 cm^3 flasks. This was then trypsinized and plated in 48 well plates at specific seeding densities. The cells were incubated overnight at 37 ^oC, the medium removed, and fresh Minimal Essential Medium (MEM) + Glutmax + antibiotics (100 µg/ml penicillin and 100µg/ml streptomycin sulphate) were added. Two hundred microliters (200 µl) of the extract (50 - 350 µg/ml) was then added and incubated for 4 h. Thereafter, the medium was removed and replaced with complete medium (MEM + Glutmax + antibiotics +10 % fetal bovine serum). After 48 h, the cells were evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The medium was removed from the cells and 200 µl of 5 mg/ml MTT in phosphate buffered saline (PBS, pH 7.4), as well as 200 µl of medium were added to each well containing cells. The multiwell plate was incubated for 4 h and thereafter the medium and MTT were removed and 200 µl of DMSO added to each well, and incubated at 37 ^oC for 10 min. The control consisted of 200 µl of the medium only. All determinations were in triplicate. The absorbances of the dissolved solutions were read using a Mindray Plate Reader at 570 nm. The percent cell survival was evaluated by the equation:

Percent cell survival = {Ab/Ao} 100

Where Ab is the absorbance of the sample and Ao is the absorbance of the control.

Statistical analysis

The results obtained were expressed as mean ± standard error of mean (SEM) of three determinations. The data were evaluated through regression analysis using the QED statistics program, PISCES software (PISCES conservation Ltd, 2007, England). The LC_{50}

values, where applicable, were determined by linear regression. Means between treatments were compared by Tukey's Studentized Range Test using one-way analysis of variance (ANOVA).

3.7. References

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CHAPTER FOUR

RESULTS

4.1. Volatile extracts / essential oils

The volatile extracts/essential oils of the fresh leaf (FL), dry leaf (DL) and dry stem (DS) of both *T. camphoratus* and *T. trilobus var galpinni* collected from Sangoyana and Ubombo respectively, at different times were examined by GC/MS to determine their chemical constituents. The physicochemical properties of the essential oils were determined. Their oils were also investigated for their antimicrobial, antioxidant, insecticidal and cytotoxicity activities.

4.1.1. Physicochemical properties of the essential oils

The physicochemical properties of the essential oils are shown in Table 4.0. The characteristic colours of the oils of *T. camphoratus* were yellow whereas those of *T. trilobus var galpinni* ranged from colourless to pale green. A strong camphor smell was pronounced in all the essential oils of *T. camphoratus* whereas the odour of the *T. trilobus var galpinni* oils was aromatic. The refractive index ranged between 1.4807- 1.5028 for *T. camphoratus* and 1.3796-1.4114 for *T. trilobus var galpinni* essential oils.

Table 4.0 Physicochemical properties of the essential oils of *T. camphoratus* and *T. trilobus var galpinni*.

	Sample	% yield (w/w)	Colour	Odour	Refractive index
Tarchonanthus camphoratus					
	DL 2009	0.23	Yellowish-green	Strong camphor	1.4968
	FL 2010	0.14	Yellowish-green	Strong camphor	1.4826
	DL 2010	0.09	Yellowish-green	Strong camphor	1.4807
	DS 2010	0.03	Yellowish-green	Camphor	1.5028
	FL 2011	0.16	Yellowish-green	Strong camphor	1.4996
	DL 2011	0.10	Yellowish-green	Strong camphor	1.4973
	DS 2011	0.04	Yellowish-green	Camphor	1.4928
Tarchonanthus trilobus var galpinni					
	FL 2010	0.04	Faint greeen	Aromatic	1.4032
	DL 2010	0.02	Faint green	Aromatic	1.3987
	DS 2010	0.01	Colourless	Aromatic	1.3796
	FL 2011	0.06	Faint green	Aromatic	1.4276
	DL 2011	0.03	Faint green	Aromatic	1.4114
	DS 2011	0.01	Colourless	Aromatic	1.3872

FL = Fresh leaf DL = Dry leaf DS = Dry stem

4.1.2 Chemical composition of the volatile extracts

4.1.2.1 Chemical composition of the essential oil of the dry leaf of *T. camphoratus* harvested in November 2009.

A total of 47 compounds were identified which amounted to 72.8 % of the total oil composition. The oil was mainly dominated by sesquiterpene hydrocarbons (30.0 %) whereas oxygenated monoterpenes (19.7 %) and monoterpene hydrocarbons (9.9 %) and oxygenated sesquiterpenes (5.8%) were also present (Table 4.1**)**. The major compounds in the essential oil were 1,8-cineole (5.4 %), linalool (4.5 %), α-copaene (3.8 %), hexadecanoic acid (3.5 %), neoalloocimene (3.5 %), (-)-globulol (3.3 %) and δ-cadinene (3.1 %). The GC/MS chromatogram is displayed in Figure 4.1 (Appendix 1).

Table 4.1: Chemical constituents of the essential oil of the dry leaf of *T. camphoratus* from Kwa Zulu Natal, South Africa, harvested in November 2009.

Compound	KI	Percentage composition	Compound	KI	Percentage composition
Monoterpene hydrocarbons			**Oxygenated monoterpenes**		
α-Pinene	938	1.6	1,8 cineole	1033	5.4
Camphene	952	0.8	Linalool	1101	4.5
β-Pinene	978	0.6	1-Terpineol	1120	0.3
α-Terpinene	998	2.6	α-Fenchyl alcohol	1122	0.1
γ-Terpinene	1062	0.2	Camphor	1143	0.9
Terpinolene	1089	0.3	(-)-Borneol	1172	0.7
trans- Pinene hydrate	1137	0.3	Terpinen-4-ol	1177	1.2
Neoalloocimene	1138	3.5	(-)-α-Terpineol	1191	2.1
			Carvomenthone	1234	1.1
			Nerol	1593	0.4
			Geraniol	1609	0.2
			Carvacrol	1612	0.1
Sesquiterpene hydrocarbons			**Oxygenated sesquiterpenes**		
α-Copaene	1379	3.8	Palustrol	1555	0.6
Isoledene	1382	2.1	Epiglobulol	1564	0.8
iso-Caryophyllene	1400	0.7	(-)- Globulol	1584	3.3
β-Caryophylene	1421	2.6	Vulgarol	1605	0.3
Aristolene	1423	2.1	Valerenol	1729	0.8
γ-Elemene	1429	1.3			
Calarene	1437	1.1	**Others**		
Aromadendrene	1443	1.8	n-Hexanol	864	2.7
α-Humulene	1455	1.0	Tetradecanoic acid	1763	0.9
allo-Aromadendrene	1464	0.8	Phytol	1945	0.5
Isobicyclogermacrene	1477	1.6	Hexadecanoic acid	1996	3.5
α-Amorphene	1479	2.7	Linoleic acid	2078	2.5
β-Selinene	1486	2.6			
allo-Aromadendr-9-ene	1489	1.0			
γ-Cadinene	1507	0.9			
δ-Cadinene	1524	3.1			
α-Calacorene	1536	0.8			

Total number of compounds	47	Oxygenated monoterpenes	19.7%
Total percentage composition	72.8%	Monoterpene hydrocarbons	9.9%
Oxygenated monoterpenes	19.7%	Sesquiterpene hydrocarbons	30.0%
Oxygenated sesquiterpenes	5.8%	Others	7.4%

4.1.2.2 Chemical composition of the volatile extracts of the fresh leaf, dry leaf and dry stem of *T.camphoratus* harvested in April 2010.

From the essential oils a total of 33, 27 and 25 compounds were identified representing 85.9, 73.01 and 72.66% of the total oil composition from the fresh leaves, dry leaves and dry stem respectively (Table 4.2) and the GC/MS chromatograms are displayed in Figure 4.2-4.4 (Appendix 1). The compounds that were most predominant in the essential oil of the fresh leaves were α-cadinol (9.40%), 1,8-cineole (9.19%), δ-cadinene (6.89%), butanal (6.10%) and caryophyllene oxide (4.21%) whereas β-guaiene (10.7%), γ-cadinene (9.09%), δ-cadinene (6.80%), aromandrene (6.12%), β-caryophyllene (5.48%) and γ-muurolene (5.13%) featured as major compounds in the dry leaf oil. On the other hand, butanal (35.77%), t-muurolol (10.33%) and δ-cadinene (5.54%) were the major components in the dry stem oil. Sesquiterpene hydrocarbons dominated the oils of the fresh and dry leaves and in all of the three essential oils investigated, δ-cadinene featured as a major compound. One of the major compounds in the fresh leaf oil, 1,8-cineole, featured as a minor compound in both the dry leaf oil and the dry stem oil while α-cadinol and caryophyllene oxide, major compounds in the fresh leaf oil, were not found at all in the dry leaf oil. The oxygenated sesquiterpene, t-murolol, whose concentration has been reported to increase with the age of a plant[1], featured as a major compound in the oil of the dry stem but was absent in the oils of both the fresh and dry leaves. Compounds that were present in all the essential oils, but as minor components included; α-copaene, isoledene, α-humulene, eremophilene, linalool, terpinen-4-ol and α-terpineol. The dry stem oil contained the lowest number of identified compounds and the dry leaf oil contained a lower number of compounds than the fresh leaf oil. No monoterpene hydrocarbons were found in the dry stem essential oil.

Table 4.2: Chemical constituents of the essential oil of the fresh leaf, dry leaf and dry stem of *T. camphoratus* harvested in April 2010

Name of compound	KI	Percentage composition		
		FL	DL	DS
Monoterpene hydrocarbons				
α- Pinene	938	2.52	0.45	
Camphene	952	2.15	0.33	
β- Pinene	978	0.65		
α-Terpinene	1017	2.61	0.65	
p-Cymene	1026	1.64	0.18	
Sesquiterpene hydrocarbons				
α-Copaene	1378	1.45	2.33	1.21
Isocomene	1392			1.35
α-Elemene	1393		2.98	
α-gurjunene	1411			0.13
Isoledene	1419	1.22	2.72	0.63
Beta-caryophyllene	1427	2.73	**5.48**	1.05
α-Guaiene	1439		2.73	0.86
α-humulene	1458	0.64	0.97	0.29
γ-gurjunene	1472	1.25	0.43	
γ-Muurolene	1477	**3.19**	**5.13**	2.11
Alloaromandrene	1478	2.05	**6.12**	0.16
Ledene	1482	0.93		
Germacrene D	1484			0.69
Eremophilene	1486	1.25	0.10	1.04
α-Selinene	1488	1.08		
β-Guaiene	1490	1.20	**10.70**	
Valencene	1491	2.39		
Calarene	1494	2.02	**3.60**	
α-Muurolene	1499	**3.50**		1.35
γ-Cadinene	1513	1.50	**9.09**	2.13
cis-calamenene	1520	**3.17**		
δ-Cadinene	1526	**6.89**	**6.80**	**5.54**

Table 4.2 Cont:

Name of compound	K1	Percentage composition		
		FL	DL	DS
Oxygenated monoterpenes				
Linalool	1101	1.42	1.77	0.33
1,8-Cineole	1033	**9.19**	1.94	0.15
Camphor	1143		0.62	
(-)-Borneol	1172			0.07
Terpinen-4-ol	1180	0.56	0.43	0.08
α-Terpineol	1190	0.62	0.82	0.16
Carvacrol	1295		0.68	
Oxygenated sesquiterpenes				
Elemol	1549	**3.5**	2.76	
Spathulenol	1583		0.43	
Caryophyllene oxide	1586	**4.21**		2.90
Hinesol	1632	2.39		
t-Muurolol	1641			**10.33**
t-Cadinol	1648			1.03
α-cadinol	1662	**9.40**		
OTHERS				
Butanal	620	**6.10**	2.77	**35.77**
Isoaromadendrene epoxide	1579	1.5		
α-Costol	1801	0.98		
Hexadecanoic acid	2117			**3.30**
Total number of compounds		33	27	25
Total percentage composition		85.90%	73.01%	72.66%
Monoterpene hydrocarbons		9.57%	1.61%	0.00%
Oxygenated monoterpenes		11.79%	6.26%	0.79%
Sesquiterpene hydrocarbons		36.46%	59.18%	18.54%
Oxygenated sesquiterpenes		19.50%	3.19%	14.26%
Others		8.58%	2.77%	39.07%

4.1.2.3 Chemical composition of the volatile extracts of the fresh leaf, dry leaf and dry stem of *T. camphoratus* harvested in April 2011.

A total of 41, 32 and 36 compounds were identified representing 96.80, 86.89 and 85.39 % of the total essential oil composition from the fresh leaves, dry leaves and dry stem respectively (Table 4.3). The GC/MS chromatograms of the essential oils are displayed in Figure 4.5-4.7 (Appendix 1). Sesquiterpene hydrocarbons were the most dominant compounds in the essential oils of the fresh leaves (44.91%), dry leaves (64.75%) and dry stem (41.81%). The sesquiterpene hydrocarbons present in all of the three essential oils as major compounds included; γ-elemene (3.17%, 3.93% and 3.49%); *allo*-aromadendrene (3.51%, 6.83% and 4.88%); γ-muurolene (3.86%, 8.77% and 4.04%) in the fresh leaf, dry leaf and dry stem essential oils respectively. Also β-caryophyllene featured as a major compound in both the fresh leaf (5.05%) and dry leaf (16.32%) essential oils but as a minor compound in the dry stem oil (2.47%). Other sesquiterpene hydrocarbons present in either one or two essential oils as major components included; epizonarene present as a major compound in the fresh leaf oil (6.72%) and as a minor component in the dry leaf oil (2.63%) but was not present in the dry stem oil. Sativene and *cis*-calamenene were present in the fresh leaf oil as major compounds but were not present in the oils of the dry leaf and dry stem. Eremophilene featured as a major compound in the dry stem oil but was not present in the fresh leaf and dry leaf oils whereas in the dry leaf oil, γ-patchoulene was a major component but a minor component in the fresh leaf and dry stem oils. Compounds like α-copaene and α-caryophyllene were present in all the three oils but as minor components. The monoterpenes; α-pinene featured in all the three oils but as a major compound in only the fresh leaf oil whereas 3-carene was present in the fresh leaf oil as a major component and in

the dry stem oil as a minor component but was not found in the dry leaf oil. Camphene was present in all the oils but as a minor component. The oxygenated sesquiterpenes, t-cadinol, featured as a major compound in all the three essential oils of the fresh leaf (5.11%), dry leaf (5.10%) and dry stem (11.33%) whereas t-muurolol was present only in the dry leaf and dry stem oils but as a major component. Juniper camphor was present in all the three oils as a minor component whereas 1,8-cineole featured as a major component in the fresh leaf oil (7.96%) and the dry leaf oil (3.61%) but as a minor compound in the dry stem oil (2.45%). The essential oil of the dry leaf contained a lower number of compounds than that of the fresh leaf.

Table 4.3: Chemical composition of the essential oils of the fresh leaf, dry leaf and dry stem of *T.camphoratus* harvested in April 2011.

Name of compound	KI	Percentage composition		
		FL	DL	DS
Monoterpene hydrocarbons				
α- Pinene	938	4.20	1.23	1.31
Camphene	952	2.34	0.72	1.05
α-Fenchene	958		2.04	
β- Pinene	978	0.88		0.36
α-Phellandrene	1005	0.56		
3-Carene	1009	3.52		1.06
o-Cymene	1020	2.22		
p-Cymene	1026		0.63	0.62
Limonene	1030	0.52		
γ-Terpinene	1062		0.70	0.58
Terpinolene	1089		0.99	
α-Terpinolene			0.49	
Sesquiterpene hydrocarbons				
α-Cubenene	1352		1.22	
α-Copaene	1379	1.98	1.43	0.55
β-Cubenene	1380	0.60	0.96	
Sativene	1389	3.11		
β-Panasinsene	1390		0.71	
α-Elemene	1398		1.93	
Longifolene	1401		0.86	
α-Cedrene	1409		1.93	
α-Caryophyllene	1419	1.18	2.53	0.68
β-Caryophyllene	1421	5.05	16.32	2.47
Aromadendrene	1422		3.65	
γ-Elemene	1429	3.17	3.93	3.49
γ-Neo clovene	1454		1.25	
α-Panasinsene	1458		0.73	
allo-Aromadendrene	1464	3.51	6.83	4.88
γ-Gurjunene	1472	0.52		0.76
α-Armophene	1479	1.69		2.06
γ-Muurolene	1480	3.86	8.77	4.04
γ-Himachalene	1481		2.89	
Germacrene D	1482	0.91		
Epizonarene	1485	6.72	2.63	
Eremophilene	1486			4.97
α-Selinene	1488	0.46		0.65

Table 4.3 Cont:

Name of compound	KI	Percentage composition		
		FL	DL	DS
Sesquiterpene hydrocarbons				
Valencene	1491	1.02		1.90
(-)-Aristolene	1495	1.74	0.76	
α-Muurolene	1498	1.39	3.85	1.46
γ-Patchoulene	1503	2.49		
β-Selinene	1515			2.38
β-Guaiene	1516		1.70	
cis-calamenene	1520	4.64		
δ-Cadinene	1524			11.1
α-Calacorene	1536	0.87	0.63	
Cadalene	1673			0.42
Oxygenated monoterpenes				
1,8-Cineole	1033	7.96	3.61	2.45
Linalool	1098	3.37		
Terpinen-4-ol	1177	0.79		
α-Terpineol	1190	0.94		0.59
Geraniol	1255	0.33		
Oxygenated sesquiterpenes				
Spathulenol	1578	0.80		2.76
t-Cadinol	1640	5.11	5.76	11.33
t-Muurolol	1642		5.33	13.30
δ-Cadinol	1646			1.94
Cis-Z-alpha Bisabolene epoxide	1680	0.43		
Juniper camphor	1691	0.76	1.10	0.93
OTHERS				
Hexanol	868	2.05		
Phenol	989	0.77		
2-Hydroxybenzaldehyde	1049	5.18		
Nonanal	1106	0.34		
Lilial	1522	6.16		
1,4-Cadinadiene	1532	2.66		
n- Hexadecanoic acid	1996		0.71	
Total number of compounds		41	32	36
Total percentage composition		96.80%	86.89%	85.39%
Monoterpene hydrocarbons		14.24%	6.8%	4.98%
Oxygenated monoterpenes		13.39%	3.61%	3.04%
Sesquiterpene hydrocarbons		44.91%	62.48%	41.81%
Oxygenated sesquiterpenes		7.1%	12.19%	30.26%

4.1.2.4 Comparison of the chemical constituents of the essential oils of the fresh leaf, dry leaf and dry stem of *T. camphoratus* harvested April 2010 and in April 2011.

With the exception of the dry stem essential oil of *T. camphoratus* harvested in April 2010, the rest of the essential oils harvested in the two years were mostly dominated by sesquiterpene hydrocarbons. Comparison of the essential oil constituents is shown in Figure **4.8**. The percentage composition of the monoterpene hydrocarbons and sesquiterpene hydrocarbons was higher in the essential oils of *T. camphoratus* harvested in 2011 whereas oxygenated sesquiterpenes were more dominant in the essential oils of the fresh leaf of 2010 and in the dry stem of both 2010 and 2011. Compounds that were present in all the essentials of the fresh leaf, dry leaf and dry stem of both years but in different percentage compositions were; γ-muurolene which was a major compound in all the essential oils except in the dry stem oil of 2010, β-caryophyllene featured as a major component in the dry leaf oil of 2010 and in the fresh leaf and dry leaf oils of 2011 and in the other oils as a minor compound, *allo*-aromadendrene was a major component in the dry leaf oil of 2010 and in all the three oils of 2011, 1,8-cineole was dominant in the fresh leaf of 2010 and in the fresh leaf and dry leaf of 2011 and in the rest of the oils as a minor component. The compound, δ-cadinene was present as a major compound in all the essential oils of both years except in the fresh leaf and dry leaf oils of 2011 whereas α-copaene featured in all the oils of both years but as a minor component. There were also differences and similarities in the chemical composition of similar essential oils. Both the essential oils of the fresh leaf of *T. camphoratus* harvested in 2010 and 2011 contained 1,8-cineole, γ-muurolene and *cis*-calamenene as major compounds and the minor compounds common to both oils included; α-pinene, camphene, β-pinene, α-copaene, α-selinene, valencene, linalool, terpinen-4-ol

and α-terpineol. In the dry leaf oils of both years, β-caryophyllene, γ-muurolene and *allo*-aromadendrene featured as major compounds and among the minor components were α-pinene, camphene, *p*-cymene and α-copaene. Compounds that featured as major components in the dry leaf essential oil of 2010 but were not present in the dry leaf essential oil of 2011 were calarene, γ-cadinene and δ-cadinene. Likewise γ-elemene and aromadendrene were present in the dry leaf oil of 2011 as major components but were not present in the dry leaf oil of 2010. A sesquiterpene hydrocarbon, β-guaiene was a major compound in the dry leaf oil of 2010 (10.70%) but was a minor component in the dry leaf oil of 2011 (1.70%) whereas the oxygenated sesquiterpenes, t-cadinol and t-muurolol featured prominently in the dry leaf oil of 2011, 5.76% and 5.33% respectively, but were absent in the dry leaf oil of 2010. Likewise, the dry stem essential oils of 2010 and 2011 showed similarities and differences in the major and minor constituents. Both oils had δ-cadinene and t-muurolol as major components and α-copaene, β-caryophyllene, α-muurolene, 1,8-cineole and α-terpineol as minor constituents. The oxygenated sesquiterpene, t-cadinol was dominant in the dry stem oil of 2011 (5.76%) but existed as a minor component in the dry stem oil of 2010 (1.03%). The essential oils of the fresh leaf, dry leaf and dry stem of *T. camphoratus* harvested in 2010 and 2011 showed variations and similarities in their minor and major chemical constituents.

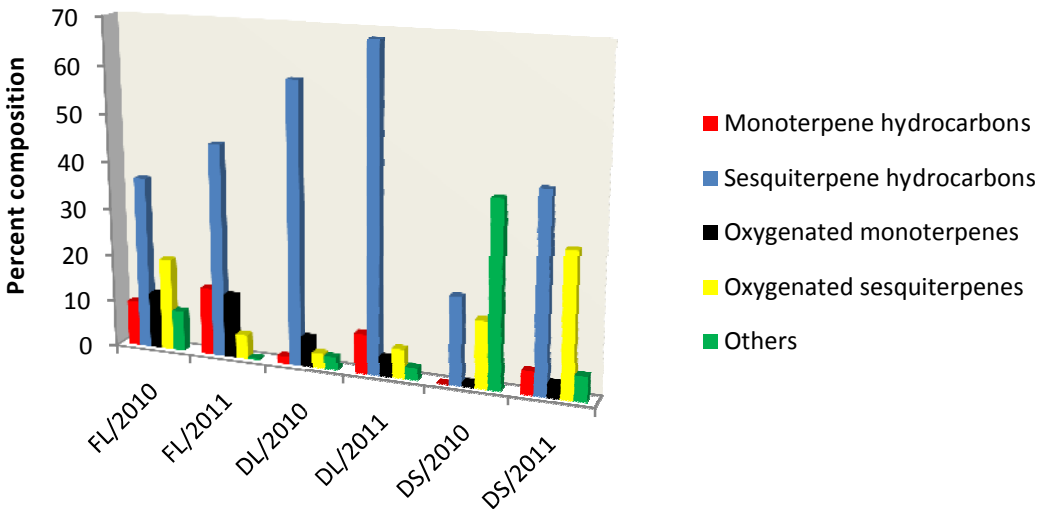


Figure 4.8. Comparison of the essential oil constituents of *T. camphoratus* harvested in 2010 and 2011.

4.1.2.5 Chemical composition of the essential oils of the fresh leaf, dry leaf and dry stem of *T. trilobus* var *galpinni* harvested in April 2010.

The oils gave a total of 26, 23 and 20 identified compounds representing 95.74, 93.59 and 90.49 % of the total oil composition from the fresh leaves, dry leaves and dry stem respectively. The essential oils were dominated by sesquiterpene hydrocarbons: 69.91%, 60.51% and 45.24% in the fresh leaf, dry leaf and dry stem respectively (Table 4.4) and the GC/MS chromatograms of the essential oils are displayed in Figure 4.9-4.11 (Appendix 2). The sesquiterpene hydrocarbons present in all the three essential oils as major constituents were; β-caryophyllene (27.54%, 18.88% and 6.30%), δ-cadinene (4.67%, 8.93% and 6.72%), γ-muurolene (5.32%, 3.71% and 6.25%) in the essential oils of the fresh leaf, dry leaf and dry stem respectively. In addition, the oxygenated monoterpene, 1,8-cineole (4.33%, 7.19% and 10.76%), the oxygenated sesquiterpenes, t-muurolol (4.19%, 3.98% and 11.66%) and t-cadinol (3.58%, 3.87% and 10.21%) were major constituents in the fresh leaf, dry leaf and

dry stem essential oils respectively. Among the compounds that featured in all the three oils, in some as minor and in others as major, included α-pinene which was a major component in both the fresh leaf (3.01%) and dry leaf (3.06%) oils and a minor component in the dry stem (1.56%) oil; Camphene was a major component of the dry leaf oil (3.22%) and a minor constituent of the fresh leaf (1.47%) and dry stem (2.18%) oils; *allo*-aromadendrene was present in the fresh leaf oil (9.26%) as a major component but as a minor constituent in the dry leaf (2.15%) and dry stem (1.36%) oils; α-armophene was a major component in both the dry leaf (10.24%) and dry stem (3.34%) oils but a minor constituent in the fresh leaf oil (0.63%). The oxygenated monoterpene, α-terpineol featured in all of the oils but as a minor constituent. Other compounds were among the major compounds in one or two essential oils and were present as minor components or absent in the other oils. Longifolene featured as a major compound in the dry stem oil (8.22%) and as a minor compound in the dry leaf oil (2.56%) but was absent in the fresh leaf oil whereas α-caryophyllene was dominant in the fresh leaf oil (4.07%) and was a minor component in the dry leaf oil (2.88%) and was not present in the dry stem oil. Likewise, epizonarene was a major constituent in the fresh leaf oil (4.15%) and absent in the dry leaf and dry stem oils, calamine was a major component in the dry stem oil (3.83%) and was not found in the fresh leaf and dry leaf oils whereas β-cadinene featured as a major component in the fresh leaf (3.19%) and dry stem (4.31%) oils but was absent in the dry leaf oil.

Table 4.4: Chemical composition of the essential oil of the fresh leaf, dry leaf and dry stem of *T. trilobus* var *galpinii* harvested in April 2010.

COMPOUND	**KI**	**Percentage composition**		
		FL	**DL**	**DS**
Monoterpene hydrocarbons				
α-Pinene	938	3.01	3.06	1.56
Camphene	952	1.47	3.22	2.18
β-Pinene	978		1.06	
(*Z*)-β-Ocimene	1039		1.13	1.36
γ-Terpinene	1058			1.38
Sesquiterpene hydrocarbons				
Ylangene	1368		1.60	2.60
Copaene	1378		1.59	
Longifolene	1408		2.56	8.22
α-Cedrene	1409	0.98		
α-Caryophyllene	1419	4.07	2.88	
Aromadendrene	1422	1.27		
Beta-caryophyllene	1427	27.54	18.88	6.30
γ-Elemene	1429		2.13	
α-Gurjunene	1436	2.93		
α-Guaiene	1439		1.29	
allo-Aromadendrene	1460	9.26	2.15	1.36
γ-Gurjunene	1472	2.34		2.34
γ-Muurolene	1480	5.32	3.71	6.25
α-Armophene	1485	0.63	10.24	3.34
β-Selinene	1487	1.65		
α-Selinene	1489		1.99	
Epizonarene	1490	4.15		
Valencene	1491	1.91		
α-Muurolene	1496		2.56	
β-Cadinene	1519	3.19		4.31
Calamenene	1520			3.83
δ-Cadinene	1526	4.67	8.93	6.72
Oxygenated monoterpenes				
1,8-Cineole	1033	4.33	7.19	10.76
Linalool	1098	1.00		
α-Terpineol	1190	1.43	0.71	1.68

Table 4.4: Cont

Oxygenated sesquiterpenes				
4-Carvomenthenol	1572	0.78		
Spathulenol	1578	2.37		
Beta-caryophyllene oxide	1581		4.58	
t-Cadinol	1640	3.58	3.87	10.21
t-Muurolol	1642	4.19	3.98	11.66
β-Eudesmol	1649	2.69		2.78
Others				
Cyclopentane	716			1.65
Adamantane	1128	0.98		
4-Methoxycinnamaldehyde	1572		4.28	

Total number of compounds	26	23	20
Total percentage composition	95.74%	93.59%	90.49%
Monoterpene hydrocarbons	4.48	8.47	6.48
Oxygenated monoterpenes	6.76	7.90	12.44
Sesquiterpene hydrocarbons	69.91	60.51	45.27
Oxygenated sesquiterpenes	13.61	12.43	24.65
Others	0.98	4.28	1.65

4.1.2.6 Chemical composition of the essential oils of the fresh leaf, dry leaf and dry stem of *T. trilobus var galpinni* harvested in April 2011.

A total of 37, 26 and 22 compounds were identified representing 95.73, 95.56 and 97.79 % of the total oil composition from the fresh leaf, dry leaf and dry stem respectively. Monoterpene hydrocarbons were not present in the essential oil of the dry leaf and were found in relatively low percentage concentrations of 4.49% and 0.64% in the essential oils of the fresh leaf and dry stem respectively, Table 4.5. Oxygenated sesquiterpenes were found only in the essential oil of the fresh leaf (1.85%) whereas oxygenated sesquiterpenes were present only in the dry stem oil (3.64%). All of the three essential oils were dominated by sesquiterpene hydrocarbons, 88.49%, 95.56% and 92.32% in the fresh leaf, dry leaf and dry stem oils respectively. The major components in the oils were also sesquiterpene hydrocarbons and those that were present in the three oils were β-caryophyllene (30.40%, 23.97% and 38.38%) and δ-cadinene (7.96%, 8.53% and 4.32%) in the fresh leaf, dry leaf and dry stem essential oils respectively. Some compounds were present in all of the three essential oils but as major components in some oils and as minor components in others. In the essential oil of the dry leaf, copaene, α-guaiene and β-cadinene were present as major components in compositions of 5.60%, 10.69% and 10.94% respectively and were also present in the fresh leaf and dry stem oils but as minor components. The sesquiterpene hydrocarbons, β-guaiene, calamenene and α-muurolene were present as minor constituents in the fresh leaf and dry leaf oils but were among the major constituents in the dry stem essential oil. Longifolene also featured as a major compound in the fresh leaf oil (5.64%) but as a minor component in the dry leaf and dry stem oils. Compounds present in all of the essential oils but as minor constituents included α-cedrene and *allo*-aromadendrene. Some

compounds were dominant in two of the essential oils and these included α-armophene, which was a minor compound in the dry stem oil but was a major component in both the fresh leaf (4.01%) and dry leaf (6.83%) oils, and γ-cadinene which was dominant in the fresh leaf (4.55%) and dry stem (4.33%) oils but was absent in the dry leaf essential oil. Included in compounds that featured as major compounds in some essential oils and were absent in other oils was α-cubebene a major and minor constituent in the dry leaf and fresh leaf oils respectively but absent in the dry stem oil, γ-neoclovene a major constituent of the fresh leaf oil and a minor compound in the dry leaf oil but absent in the dry stem oil and γ-Muurolene, present as a major and minor constituent in the fresh leaf and dry stem oils respectively but was not present in the dry leaf oil. Other compounds featured as major compounds in one essential oil and were absent in the other essential oils. For example, (+)-calarene and epizonarene were present only in the fresh leaf oil (3.89%) and (4.15%) respectively whereas (-)-isoledene featured only in the dry leaf oil (5.29%). Similar to other essential oils investigated, many qualitative and quantitative variations in the chemical constituents were observed in the essential oil of the fresh leaf, dry leaf and dry stem of *T. trilobus var galpinni* harvested in 2011. The GC/MS chromatograms of the essential oils are displayed in Figure 4.12-4.14 (Appendix 2).

Table 4.5: Chemical composition of the essential oil of the fresh leaf, dry leaf and dry stem of *T. trilobus* var *galpinii*harvested in 2011.

COMPOUND	KI	FL	DL	DS
Monoterpene hydrocarbons				
α-Pinene	938	2.55		
Camphene	952	1.37		
β-Pinene	978	0.30		
(*Z*)-β-Ocimene	1039	0.46		
Terpinolene	1086			0.64
Sabinene hydrate	1098	0.31		
Sesquiterpene hydrocarbons				
α-Cubebene	1351	0.07	6.63	
Ylangene	1368	0.09	1.17	
β-Patchoulene	1377	0.31		
Copaene	1378	0.26	5.60	0.75
β-Cubebene	1386		0.20	
β-Elemene	1391		0.50	
α-Elemene	1393	0.94		3.91
(+)-Calarene	1403	3.89		
Longifolene	1408	5.64	0.77	1.26
α-Cedrene	1409	0.08	1.63	1.28
α-Caryophyllene	1419	2.62		2.76
Isoledene	1421		5.29	
β-Caryophyllene	1427	30.40	23.97	38.38
Aristolene	1428	0.22	1.93	
(+)-Epi-bicyclosesquiphellandrene	1438	0.24		
α-Guaiene	1439	0.58	10.69	0.53
γ-Neoclovene	1447	5.75	0.80	
allo-Aromadendrene	1460	0.95	0.33	2.87
α-Humulene	1461		2.79	
γ-Muurolene	1480	5.80		0.63
α-Armophene	1485	4.01	6.83	1.07
Eremophilene	1486	1.68		
β-Selinene	1487	0.24		
δ-Selinene	1488	2.99		
Epizonarene	1490	4.15		
Valencene	1491	2.14		
α-Muurolene	1496	0.53	0.59	7.97
β-Guaiene	1500	0.18	1.72	13.06
γ-Cadinene	1509	4.55		4.33

Table 4.5 Cont.

α-Farnesene	1516		0.22	0.82
β-Cadinene	1519	0.77	10.94	2.21
Sesquiterpene hydrocarbons				
Calamenene	1520	0.96	0.07	3.83
Selina-3,7 (II)-diene	1522			1.61
δ-Cadinene	1526	7.96	8.53	4.32
α-Calacorene	1536			0.73
α-Maaliene	1541		0.32	
Cadalene	1673	0.49	0.96	
Oxygenated monoterpenes				
1,8 Cineole	1033	1.85		
Oxygenated sesquiterpenes				
δ-Cadinol	1646			3.64
Others				
3,5-Octadien-2-one	1081	0.27		
(*E)*-Geranyl acetone	1453			1.19
Tridecanoic acid	1663	0.13		
Tetradecanoic acid	1763		0.30	
Phytol	1949		0.18	
2-Propenoic acid			2.60	

Total number of compounds	37	26	22
Total percentage composition	95.73%	95.56%	97.79%
Monoterpene hydrocarbons	4.99	0.00	0.64
Oxygenated monoterpenes	1.85	0.00	0.00
Sesquiterpene hydrocarbons	88.49	95.56	92.32
Oxygenated sesquiterpenes	0.00	0.00	3.64

4.1.2.7 Comparison of the essential oil constituents of the fresh leaf, dry leaf and dry stem of *T. trilobus var galpinni* harvested April 2010 and in April 2011.

The essential oils of the fresh leaf, dry leaf and dry stem of *T.trilobus var galpinni* harvested in April 2010 and April 2011 showed some similarities and differences in their chemical composition. The sesquiterpene hydrocarbons, which were the most dominant compounds of all the essential oils of 2010 and 2011, were in a higher percentage composition in the essential oils of 2011 as illustrated in Figure **4.15**. Oxygenated monoterpenes and oxygenated sesquiterpenes were more prevalent in the essential oils of 2010 which was the same case with monoterpene hydrocarbons except for the fresh leaf of 2011. β-caryophyllene and δ-Cadinene featured as major components in all of the essential oils of both years although in different percentage compositions. Some compounds like t-cadinol and t-muurolol featured as major compounds in the fresh leaf, dry leaf and dry stem oils of 2010 but were not found at all in the essential oils of 2011. Likewise, 1,8-cineole was present as a major component in all of the essential oils of 2010 but featured only in the fresh leaf oil of 2011 as a minor component. In the dry leaf oils of both years α-armophene was present as a major compound and was also present in the fresh leaf and dry stem oils but as a minor constituent. α-pinene was present in all of the essential oils of 2010 but among the essential oils of 2011, it was found only in the fresh leaves. Comparing the chemical composition of the fresh leaf oils of both years, the fresh leaf oil of 2010 had α-pinene (3.01%), 1,8-cineole (4.33%), α-caryophyllene (4.07%), *allo*-aromadendrene (9.26%) and β-cadinene featuring as major compounds but also present in the fresh leaf oil of 2011 as minor compounds. Compounds that featured as major compounds in the fresh leaf oils of both years were β-caryophyllene (27.54% and 30.40%), γ-muurolene (5.32% and 5.80%),

epizonarene (4.15% and 4.15%) and δ-cadinene (4.67% and 7.96%) in the leaf oils of 2010 and 2011 respectively. In the dry leaf oils of both years, β-caryophyllene (18.88%), 23.97%), α-armophene (10.24% and 6.83%) and δ-cadinene (8.93% and 8.53%) were present as major compounds in the dry leaf oils of 2010 and 2011 respectively. In the dry leaf oil of 2011, α-copaene (5.60%) and α-guaiene (10.69%) were present as major compounds but featured as a minor constituent in the dry leaf oil of 2010. Compounds that were present in the dry leaf oils of both years but as minor constituents were ylangene, longifolene, α-cedrene, *allo*-aromadendrene and α-muurolene. Other compounds like 1,8-cineole and γ-muurolene, major components in the dry leaf oil of 2010 were not present in the dry leaf oil of 2011. Likewise, isoledene and β-cadinene, major constituents of the dry leaf oil of 2011 were not present in the dry leaf oil of 2010. The dry stem essential oils of both years also had variations and similarities in their chemical composition. The sesquiterpene hydrocarbons; β-caryophyllene (6.30% and 38.38%), β-cadinene (4.31%, 10.94%), calamenene (3.83% and 3.83%) and δ-cadinene (6.72% and 4.32%) featured as major components in the dry stem oils of 2010 and 2011 respectively. Compounds like, longifolene, γ-muurolene and α-armophene were major components in the dry stem oil of 2010 whereas they were minor components in the dry stem oil of 2011. One compound, *allo*-aromadendrene, featured in the dry stem oils of both years but as a minor constituent. Present as major constituents of the dry stem oil of 2010 but absent in the dry stem oil of 2011 were, 1,8-cineole (10.76%), t-cadinol (10.21%) and t-muurolol (11.66%). Likewise the dry stem essential oil of 2011 contained β-elemene (3.91%), α-muurolene (7.97%), β-guaiene (13.06%) and γ-cadinene as major constituents which were absent in the dry stem oil of 2010.

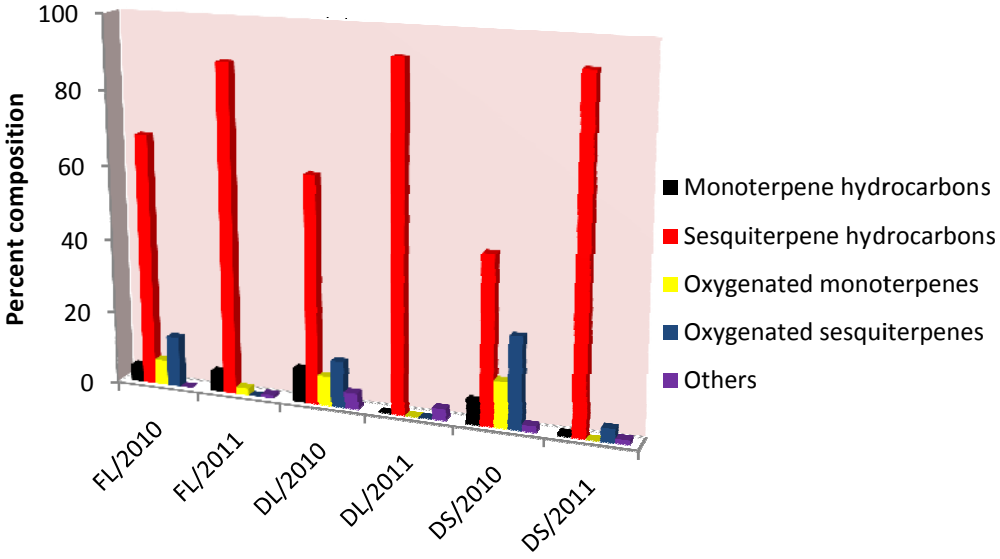


Figure 4.15. Comparison of the essential oil constituents of *T. trilobus var galpinni* harvested in 2010 and 2011

4.1.3 Antibacterial activities of the volatile extracts

4.1.3.1 Antibacterial activity of the volatile extracts of the fresh leaf, dry leaf and dry stem of *T. camphoratus* using the agar diffusion method

The essential oils of the fresh leaf, dry leaf and dry stem of *T.camphoratus* harvested in April 2010 were investigated for their antibacterial activities. The mean diameters of the zones of inhibition of the fresh leaf, dry leaf and dry stem of *T. camphoratus* are shown in Table 4.6. The fresh leaf and dry leaf essential oils showed similar trends in their antibacterial activity and their diameters of the zones of inhibition were less than those of the standard, streptomycin. The diameters of the zones of inhibition of the fresh and dry leaf essential oils against *Staphylococcus epidermidis*, *Bacillus cereus* and *Klebsiella pneumonia* were in close range and both essential oils showed no activity against *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Shigella flexiner* (Table 4.6).The dry stem essential oil inhibited the growth of

all the tested micro-organisms with diameters of the zones of inhibition bigger than those of both the fresh and dry leaf essential oils. The dry stem essential oil also had wider inhibition zones for *Staphylococcus aureus* and *Escherichia coli* than the standard drugs. All the essential oils showed a wider spectrum of antibacterial activity than the standard antibiotic, chloramphenicol.

Table 4.6. The antibacterial activities of the volatile extracts of the leaves and bark of *T. Camphoratus* using the agar disc diffusion method.

	FL	**DL**	**DS**	**S**	**A**	**C**
	5 mg/disc			2µg/disc		
Micro-organism						
Gram- positive						
Staphylococcus aureus	11.70 ± 1.20	15.00 ± 0.50	19.80 ± 0.20	18.00 ± 1.26	12.25± 0.5	0.00
Staphylococcus epidermidis	7.30 ± 0.50	7.30 ± 0.50	16.60 ± 6.90	21.00 ± 0.96	10.00± 1.2	16.33 ± 0.5
Bacillus cereus	10.30 ± 0.5	10.00 ± 0.80	20.00 ± 1.70	22.00 ± 1.71	9.25 ± 1.3	0.00
Enterococcus faecalis	0.00	0.00	13.50 ± 2.10	20.00 ± 1.29	11.0 ± 0.8	0.00
Gram-negative						
Escherichia coli	12.30 ± 1.40	16.70 ± 1.40	20.40 ± 1.90	17.5 ± 1.29	12.5 ± 0.6	10.3 ± 0.5
Pseudomonas aeruginosa	0.00	0.00	20.00 ± 3.30	21.0 ± 1.71	12.0 ± 0.9	0.00
Klebsiella pneumonia	14.00 ± 0.80	13.70 ± 0.90	19.30 ± 1.20	20.0 ± 1.71	14.25 ± 0.9	13.0 ± 1.4
Shigella flexineri	0.00	0.00	17.90 ± 5.40	21.0 ± 0.82	10.0 ± 1.0	11.7 ± 1.2
Salmonella spp.	13.00 ± 1.40	10.00 ± 0.50	19.20 ± 1.60	21.0 ± 1.29	9.0 ± 0.86	17.5 ± 0.9

FL = Fresh leaves DL= Dry leaves DS = Dry stem S = Streptomycin A = Ampicillin

C = Chloramphenicol

The diameters of zones of inhibition are expressed as mean ± S.D. (n = 3);

Disc diameter = 6mm

4.1.3.2 Antibacterial activity of the volatile extracts of the leaves and stem of *T. camphoratus* using the minimum inhibition concentration (MIC) method

The MIC results, (Table 4.7), revealed that most of the bacteria showed similar susceptibility to the essential oils of the fresh leaves, dry leaves and dry stem of *T. camphoratus* with an MIC value of 250 µg/ml. The least sensitive bacteria to the oils of the fresh leaves, dry leaves and dry stem was *Staphylococcus aureus* with an MIC value of 500 µg/ml and the lowest MIC value was shown by the dry leaf oil against *Salmonella spp.*

Table 4.7.Minimum inhibitory concentrations for the volatile extracts of *T. camphoratus*.

	FL	DL	DS
Micro-organism			
Gram- positive			
Staphylococcus aureus	500	500	500
Staphylococcus epidermidis	250	250	250
Bacillus cereus	250	250	250
Enterococcus faecalis	ND	ND	250
Gram-negative			
Escherichia coli	250	250	250
Pseudomonas aeruginosa	ND	ND	250
Klebsiella pneumonia	250	250	250
Shigella flexineri	ND	ND	250
Salmonella spp.	250	125	250

FL = Fresh leaves DL= Dry leaves DS = Dry stem

4.1.3.3 Antibacterial activity of the volatile extract of the fresh leaf of *T.trilobus* var *galpinni*using the agar disc diffusion and minimum inhibition concentration (MIC) method.

The results for the diameters of the zones of inhibition and the minimum inhibitory concentration (MIC) of the essential oil of the fresh leaf of *T. trilobus var galpinni* against the tested micro-organisms are shown in Table 4.8. The essential oil did not show antibacterial

activity against *Enterococcus faecalis* and *Pseudomonas aeruginosa* whereas it showed higher inhibition against *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus cereus* and *Shigella flexineri* than the reference drugs, chloramphenical and ampicillin. The MIC values ranged between 31.25 and 250 µg/ml, the most sensitive bacteria with the lowest MIC value of 31.25 µg/ml was *Klebsiella pneumonia* and the least sensitive bacteria was *Staphylococcus epidermidis* with an MIC value of 250 µg/ml.

Table 4.8. The mean diameters in mm of the zones of inhibition and the MIC value of the volatile extract of the fresh leaf of *T.trilobus var galpinni.*

	Zone of inhibition				**MIC (µg/ml)**
	FL	**S**	**A**	**C**	**FL**
Micro-organism					
Gram- positive					
Staphylococcus aureus	9.13 ± 0.85	18.00 ± 1.26	12.25 ± 0.5	0.00	125
Staphylococcus epidermidis	8.23 ± 0.22	21.00 ± 0.96	10.0 ± 1.2	16.33 ± 0.5	250
Bacillus cereus	19.00 ± 2.58	22.00 ± 1.71	9.25 ± 1.3	0.00	ND
Enterococcus faecalis	0.00	20.00 ± 1.29	11.0 ± 0.8	0.00	ND
Gram-negative					
Escherichia coli	19.50 ± 1.96	17.5 ± 1.29	12.5 ± 0.6	10.3 ± 0.5	125
Pseudomonas aeruginosa	0.00	21.0 ± 1.71	12.0 ± 0.9	0.00	ND
Klebsiella pneumonia	14.50 ± 2.87	20.0 ± 1.71	14.25 ± 0.9	13.0 ± 1.4	31.25
Shigella flexineri	21.75 ± 1.71	21.0 ± 0.82	10.0 ± 1.0	11.7 ± 1.2	62.5
Salmonella spp.	7.88 ± 0.62	21.0 ± 1.29	9.0 ± 0.86	17.5 ± 0.9	125

FL = Fresh leaf A = Ampicillin C = Chloramphenicol S = Streptomycin

The diameters of zones of inhibition are expressed as mean ± S.D. (n = 3);

Disc diameter = 6mm

4.1.3.4 Comparison of the antibacterial activity of the fresh leaf oil of *T. trilobus var galpinni* with the essential oils of the leaf and stem of *T. camphoratus*

Comparing the diameters of the zones of inhibition, the essential oil of *T. trilobus var galpinni* showed weaker antibacterial activity against *Staphylococcus aureus* and *Salmonella spp* than all the essential oils of *T*. *camphoratus* but showed stronger activity against *Bacillus cereus, Escherichia coli* and *Klebsiella pneumonia* than the fresh and dry leaf essential oils of *T. camphoratus.* The essential oil of *T. trilobus var galpinni* showed strongest inhibition against *Shigella flexineri* and its inhibition against *Escherichia coli* and *Bacillus cereus* was comparable to that of the dry stem essential oil of *T. camphoratus*. There was no significant difference in the means of the diameters of the zones of inhibition at p = > 0.05 of the essential oils of the fresh leaf and dry leaf of *T. camphoratus,* the essential oil of *T. trilobus var galpinni*, ampicillin and chloramphenicol but there was a significant difference at p = < 0.05, in the diameters of the zones of inhibition between the fresh leaf essential oil of *T. camphoratus*, the dry leaf essential oil of *T. camphoratus,* chloramphenical and the dry stem essential oil of *T. camphoratus*. The action of the dry stem essential oil was comparable to that of the standard drugs, streptomycin and ampicillin. Considering MIC values, the essential oil of the fresh leaf of *T. trilobus var galpinni* generally showed lower MIC values hence higher activity than the essential oils of *T. camphoratus* when tested on similar micro-organisms.

4.1.4 Antioxidant activity of the volatile extracts

The antioxidant activity of *T. camphoratus* non volatile extracts was determined by DPPH radical scavenging assay, $ABTS^{\bullet+}$ radical scavenging assay, nitric oxide inhibition assay and total reducing power assay whereas only two antioxidant assays, DPPH radical scavenging

and total reducing power were done for the essential oil of the fresh leaf of *T. trilobus var galpinni.* Thiswas because the oil yield was so low and a small amount of the essential oil was available.

4.1.4.1 DPPH radical scavenging of the leaves and stem of *T. camphoratus*

The results for the percentage DPPH radical inhibition for the essential oils of the fresh leaf, dry leaf and dry stem of *T. camphoratus* is shown in Table 4.9.The essential oils showed a relatively weak dose dependent inhibition of DPPH activity, with high LC_{50} of 12578.89, 9942.08 and 7010.03 µg/mL for fresh leaves, dry leaves and dry stem respectively. The LC_{50} values of the oils were not comparable to that of the standard BHT at $p \leq 0.05$.

Table 4.9: Percentage DPPH radical scavenging of the essential oils of the leaves and stem of *T. camphoratus*.

Percentage DPPH radical scavenging activity				
Concentration (µg/ml)	[a]FL	[b]DL	[c]DS	[d]BHT
25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	57.47 ± 4.78
50	0.00 ± 0.00	3.13 ± 0.09	4.87 ± 1.45	82.03 ± 1.11
100	7.37 ± 0.05	9.00 ± 0.05	7.50 ± 0.10	92.87 ± 1.23
150	11.13 ± 0.45	13.47 ± 0.01	10.85 ± 0.35	94.80 ± 0.14
200	15.90 ± 0.17	16.67 ± 0.40	14.20 ± 0.40	96.80 ± 0.02
250	18.47 ± 0.99	19.57 ± 0.06	16.56 ± 0.05	99.10 ± 0.12

FL = Flesh leaf DL = Dry leaf DS = Dry stem

a. Linear equation: y = 19.57x – 30.23 LC_{50} = 12578.89[a]

b. Linear equation: y = 19.82x – 29.23 LC_{50} = 9942.08[b]

c. Linear equation: y = 21.26x– 31.76 LC_{50} = 7010.03[c]

d. Linear equation: y = 38.77x + 9.81 LC_{50} = 10.88[d]

4.1.4.2: $ABTS^{\bullet+}$ radical scavenging of the essential oils of the leaves and stem of *T. camphoratus*.

The results for percentage $ABTS^{\bullet+}$ radical scavenging at different concentrations and LC_{50} values for the essential oils are shown in Table 4.10. There was a significant difference in the means of the oils and that of BHT at $p \leq 0.05$. The high LC_{50} values of the essential oils suggest poor $ABTS^{\bullet+}$ radical scavenging activity.

Table 4.10: Percentage $ABTS^{\bullet+}$ radical scavenging for the essential oils of the leaf and stem of *T.camphoratus*.

	Percentage $ABTS^{\bullet+}$ radical scavenging activity			
Concentration (µg/ml)	[a]FL	[b]DL	[c]DS	[d]BHT
25	0.00 ± 0.00	3.10 ± 0.26	5.60 ± 0.45	48.0 ± 0.03
50	3.80 ± 0.74	14.90 ± 1.40	18.70 ± 0.61	65.0 ± 3.83
100	7.60 ± 2.99	20.20 ± 0.84	23.70 ± 0.74	80.0 ± 0.98
150	10.30 ± 5.42	23.60 ± 0.21	25.40 ± 0.47	82.0 ± 0.24
200	13.80 ± 5.58	27.10 ± 1.90	29.40 ± 1.62	85.0 ± 1.72
250	17.60 ± 5.63	29.60 ± 0.73	32.70 ± 0.90	88.2 ± 1.20

FL = Flesh leaf DL = Dry leaf DS = Dry stem

a. Linear equation: $y = 18.81x - 29.16$ $LC_{50} = 16158.45^{a}$

b. Linear equation: $y = 20.87x - 21.05$ $LC_{50} = 2539.42^{b}$

c. Linear equation: $y = 19.01x - 14.23$ $LC_{50} = 2391.93_{c}$

d. Linear equation: $y = 31.55x + 13.32$ $LC_{50} = 14.54_{d}$

4.1.4.3: Nitric oxide inhibition of the essential oils of the leaves and stem of *T. camphoratus*.

The percentage inhibition of nitric oxide generation by the essential oils and the standard, ascorbic acid is shown inTable 4.11. From the LC_{50} values, it was deduced that the essential oils showed very poor nitric oxide inhibition. The activity of the standard ascorbic acid was more pronounced with LC_{50} value of 210.50 µg/ml when compared to LC_{50} values of the essential oils at $p \leq 0.05$.

Table 4.11: Percentage nitric oxide inhibition of the essential oils of the leaves and stem of *T. camphoratus*.

	Percentage nitric oxide inhibition				
Concentration (µg/ml)	[a]FL	[b]DL	[c]DS	[d]Ascorbic acid	Concentration of Ascorbic acid(µg/ml)
125	6.88 ± 1.20	5.76 ± 2.25	11.58 ± 1.27	29.8 ± 1.3	25
250	11.48 ± 2.36	5.94 ± 0.45	24.15 ± 3.89	41.2 ± 3.0	50
500	13.07 ± 2.72	6.71 ± 0.80	26.68 ± 0.85	48.7 ± 2.2	100
750	19.17 ± 4.52	6.88 ± 2.21	27.14 ± 3.54	61.3 ± 2.3	150
1000	24.74 ± 3.55	27.51 ± 1.50	28.95 ± 2.25	68.4 ± 3.3	200
1250	32.46 ± 3.53	39.42 ± 2.40	36.52 ± 6.28	77.1 ± 1.7	250

FL = Flesh leaf DL = Dry leaf DS = Dry stem

a. Linear equation: y = 22.71x – 43.22 LC_{50} = 12729.16[a]

b. Linear equation: y = 19.79x – 27.49 LC_{50} = 8735.82[b]

c. Linear equation: y = 27.77x – 59.45 LC_{50} = 8234.05[c]

d. Linear equation: y = 50.92x – 68.30 LC_{50} = 210.50[d]

4.1.4.4 Total reducing powerof the essential oils of the leaves and stem of *T. camphoratus*.

In the reducing power assay, the presence of antioxidants in the samples results in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The method evaluates the ability of plant extracts to reduce potassium ferricyanide solution which is monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. It was found that the reducing power of the essential oil of the dry stem, fresh and dry leaves of *T. camphoratus* was much lower than that of the standards, ascorbic acid and BHT. The dose-response curves for the reducing powers of the essential oils are shown in Figure **4.16**.

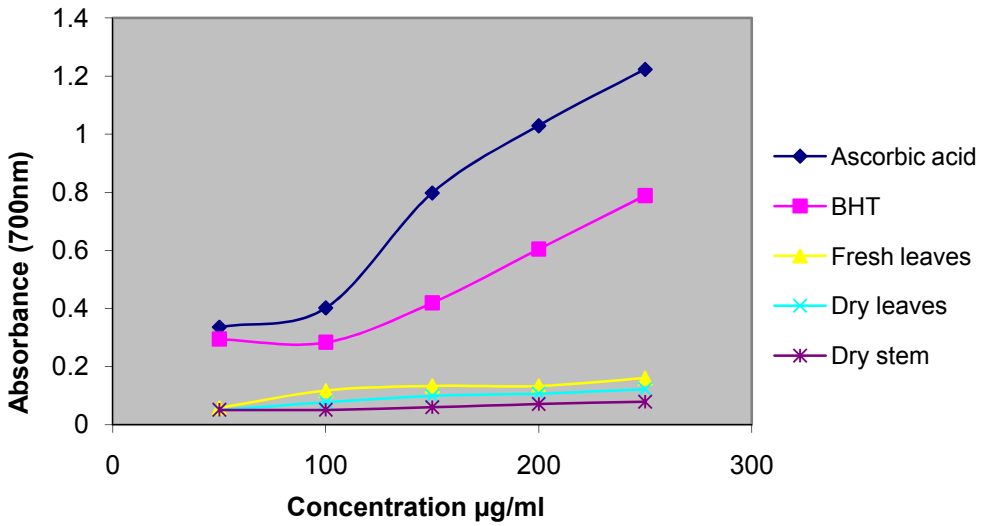


Figure 4.16.The dose-response curves for the reducing powers of the essential oils of *T.camphoratus*

4.1.4.5 DPPH radical scavenging activity of the essential oil of the fresh leaves of *T.trilobus var galpinni*

The results for the DPPH radical scavenging of the essential oil of the fresh leaf of *T.trilobus var galpinni* are shown in Table 4.12. The essential oil also showed poor DPPH radical scavenging as shown by the high LC_{50} value. There was a significant difference between the DPPH radical scavenging of the essential oil and that of the standard, BHT.

Table 4.12: DPPH radical scavenging of the essential oils of the fresh leaves *T.trilobus var galpinni*.

Concentration (µg/ml)	[a]FL	[b]BHT
25	0.00 ± 0.00	57.47 ± 4.78
50	0.00 ± 0.00	82.03 ± 1.11
100	0.00 ± 0.00	92.87 ± 1.23
150	6.24 ± 4.32	94.80 ± 0.14
200	7.98 ± 3.05	96.80 ± 0.02
250	10.72 ± 2.24	99.10 ± 0.12

a. Linear equation: y = 10.75x – 17.30 $LC_{50} = 1.82 \times 10^{6a}$

b. Linear equation: y = 38.77x + 9.81 $LC_{50} = 10.88^{b}$

4.1.4.6 Total reducing power of the essential oil of the fresh leaves of *T. trilobus var galpinni*

The essential oil showed very poor reducing power which was not comparable with that of the standard, Figure 4.17. There was a significant difference at $p \leq 0.05$ in the reducing power between the essential oil and the standards.

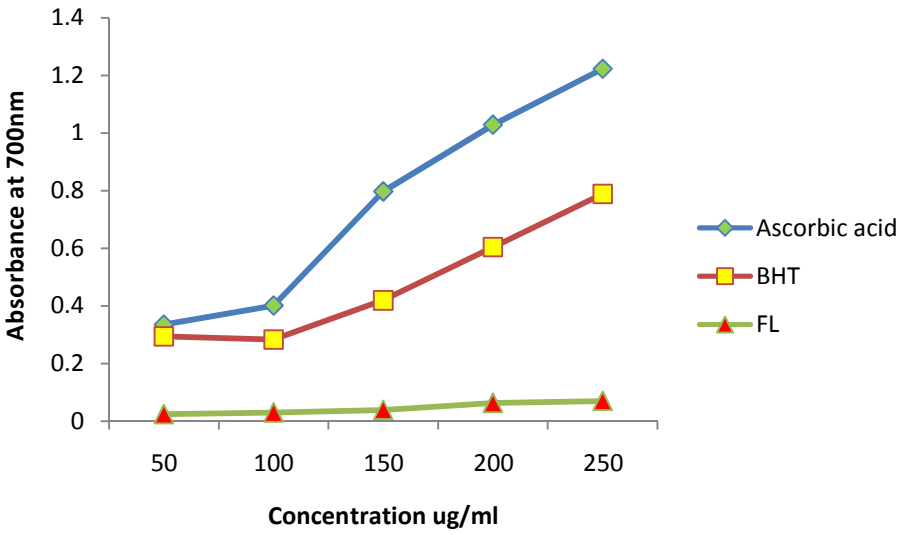


Figure 4.17**.** The plot of reducing activity of the essential oil of *T. trilobus var galpinni* compared the standards.

4.1.5 Insecticidal activity

4.1.5.1 Larvicidal activity of the essential oil of the dry leaf of *T.camphoratus*.

The larvicidal activity of the essential oil of the dry leaves of *T. camphoratus* harvested in November 2009 was investigated against *Anopheles arabiensis* mosquito larvae at 1h, 6h and 24h intervals.The result of the larvicidal study revealed that the highest larval mortality of 100 % was observed at 300 ppm concentration after 24h whereas the lowest mortality after 24h was 20 % at 25 ppm level of concentration(Table 4.13). A positive correlation was observed between the essential oil concentration and the percentage mortality, the rate of mortality being directly proportional to the concentration.Control treatments had no effect on the mosquito larvae. The lowest LC_{50} value was obtained after 24h whereas the highest was obtained after 1h.

Table 4.13: Percentage mortality of larvae of *Anopheles arabiensis* at different concentrations of the essential oil of *T. camphoratus* at different time intervals.

	Percentage mortality		
	1h	6h	24h
Concentration (ppm)			
25	0.00 ± 0.00	10.00 ± 5.00	20.00 ± 5.00
50	10.00 ± 8.66	10.00 ± 7.07	30.00 ± 10.00
100	10.00 ± 5.00	10.00 ± 0.00	50.00 ± 8.29
150	30.00 ± 10.89	50.00 ± 5.00	70.00 ± 4.33
200	30.00 ± 8.29	60.00 ± 8.29	80.00 ± 4.33
300	50.00 ± 7.07	80.00 ± 8.29	100.0 ± 5.00
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Linear equation	Y = 43.4x – 64.9	Y = 69.2x – 101.4	Y = 77.4x – 96.2
LC_{50} (ppm)	444	154	78.7

4.1.5.2 Larvicidal activity of the essential oil of the fresh leaf of *T. trilobus var galpinni*

The trend in the larvicidal activity of the essential oil of *T. trilobus var galpinni* was similar to that of *T. camphoratus*. A positive correlation was observed between the essential oil concentration and the percentage mortality, the rate of mortality being directly proportional to the concentration. The highest larval mortality of 100% was observed at 300 ppm concentration after 24h whereas the lowest mortality after the same time was 20% at 50 ppm(Table 4.14). There was a significant difference in means between the treatments at $p \leq 0.05$ at the different time intervals.

Table 4.14: Percentage mortality of *Anopheles arabiensis* larvae at different concentrations of the essential oil of *T. camphoratus* var *galpinni* at different time intervals.

	Percentage mortality		
	1h	6h	24h
Concentration (ppm)			
25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
50	10.00 ± 8.66	10.00 ± 0.00	20.00 ± 5.77
100	10.00 ± 5.00	20.00 ± 5.00	30.00 ± 10.00
200	20.00 ± 5.77	40.00 ± 5.77	70.00 ± 15.26
300	30.00 ± 5.77	60.00 ± 10.00	100.0 ± 0.00
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Linear equation	Y = 24.82x – 35.03	Y = 53.25x – 79.16	Y = 88.85x – 131.49
LC_{50} (ppm)	2666	266.4	110.32

Although the trend in the larvicidal activity of the essential oils of both *T. camphoratus* and *T. trilobus var galpinni* were similar, their activities were not the same. The essential oil of *T. camphoratus* showed slightly higher activity as evidenced by the LC_{50} values lower than those obtained for *T. trilobus var galpinni*, at different time intervals. The comparison is illustrated in Figure **4.18**.

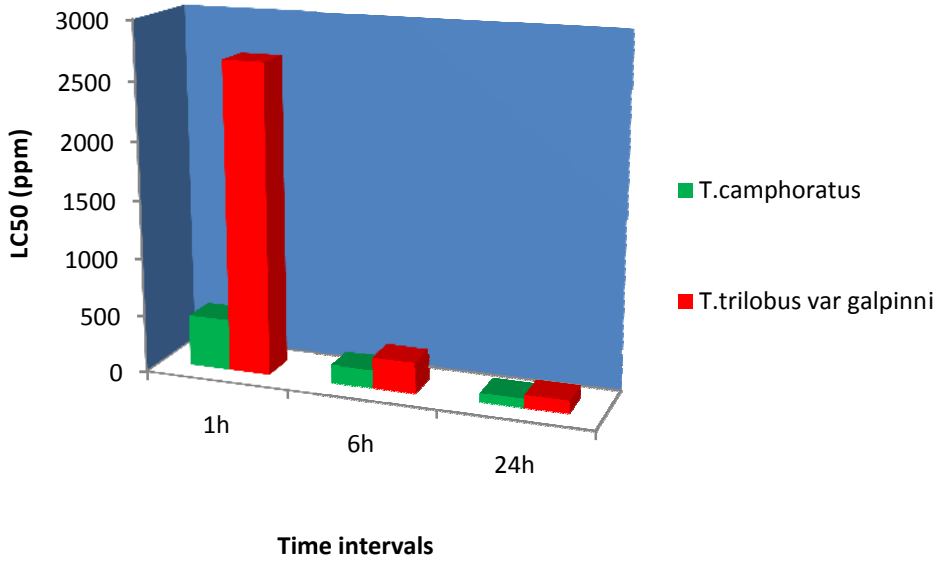


Figure 4.18.Comparison of the larvicidal activity of the essential oils of *T. camphoratus* and *T. trilobus var galpinni*.

4.1.5.3 Fumigation and Contact toxicity of the essential oil of the dry leaf of *T. camphoratus* against *S.zeamais* and *S.oryzae*.

The dried leaf essential oil of *T. camphoratus* exhibited no fumigation and contact toxicity against both *S.zeamais* and *S.oryzae* after 14 days exposure at all the concentrations used.

4.1.5.4 Repellent activity of the essential oil of the dry leaf of *T. camphoratus* against *S.zeamais* and *S.oryzae*.

The dried leaf essential oil of *T. camphoratus* exhibited repellency against both *S. zeamais* and *S.oryzae*. Repellency increased with increasing oil concentration and reached a maximum after 24h. An illustration of the repellent action of the essential oil of *T.camphoratus* against *S. zeamais* at 0.314 µl/cm^2 **after 24h is shown in Figure 4.19**.

Figure 4.19. Repellent action of the essential oil of *T.camphoratus* against *S. zeamais* at 0.314 µl/cm^2 after 24h.

The essential oil repellent activity increased with increasing time of exposure. Tables 4.15 and 4.16 show the number of *S.zeamais* and *S.oryzae* respectively, on the control side and on the treated side. The numbers on the treated side reduced with increasing exposure time suggesting increasing repellent action of the essential oil with increasing time of exposure.

Table 4.15: Average number of *S.zeamais* on the control and on the treated half of the filter paper.

Exposure time (h)	Concentration							
	0.314 µl/cm^2		0.628 µl/cm^2		0.943 µl/cm^2		1.257 µl/cm^2	
	N_c	N_t	N_c	N_t	N_c	N_t	N_c	N_t
1	10	11	10	11	11	10	13	8
2	9	12	10	11	14	7	16	5
3	9	12	16	5	16	5	17	4
6	16	5	17	4	18	3	19	2
24	18	3	20	1	20	1	21	0

N_c is the number of insects on the control and N_t is the number of insects on the treated side

Table 4.16: Average number of *S.oryzae* on the control and on the treated half of the filter paper.

Exposure time (h)	Concentration							
	0.314 µl/cm^2		0.628 µl/cm^2		0.943 µl/cm^2		1.257 µl/cm^2	
	N_c	N_t	N_c	N_t	N_c	N_t	N_c	N_t
1	7	14	7	14	14	7	15	6
2	8	13	13	8	15	6	15	6
3	12	9	14	7	15	6	17	4
6	14	7	16	5	17	4	18	3
24	17	4	20	1	20	1	21	0

N_c is the number of insects on the control and N_t is the number of insects on the treated side

The percentage repellent activities of the essential oil against S.*zeamais* and *S.oryzae* are shown in Table 4.17 and Table 4.18 respectively. A percentage repellency value greater than 50% was noted at all concentrations for both *S. zeamais* and *S.oryzae* after 24 hours of exposure.*S.oryazae* was more sensitive to the essential oil at lower concentrations and at shorter periods of exposure than *S.zeamais*. At both 0.314 µl/cm^2 and 0.628 µl/cm^2,

S.zeamais was repelled after a longer period of exposure. The mediam repellent dose was 0.945 µl/cm^2 and 0.910µl/cm^2 against *S. zeamais* and *S.oryzae* respectively.

Table 4.17: Average percentage repellency of the essential oil of *T.camphoratus* against *S.zeamais*.

Exposure time (h)	Percentage repellency			
	0.314 µl/cm^2	0.628 µl/cm^2	0.943 µl/cm^2	1.257 µl/cm^2
1	0.00	0.00	4.76	23.8
2	0.00	0.00	33.3	52.4
3	0.00	52.4	52.4	61.9
6	52.4	61.9	71.4	80.9
24	71.4	90.5	90.5	100

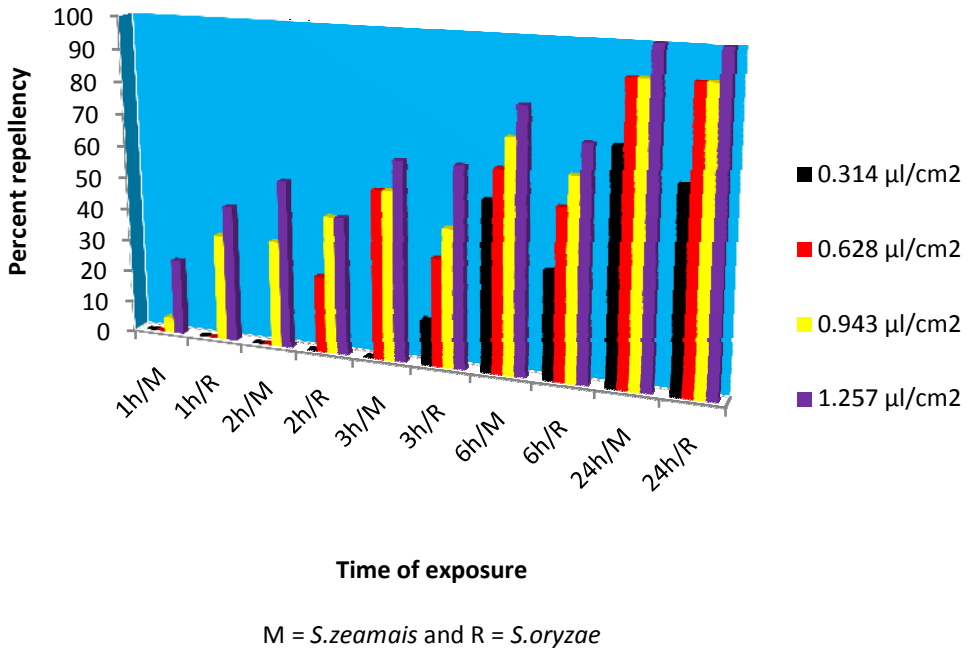
Linear equation: y = 40.20x + 12.32 RD_{50} = 0.945µl/cm^2

Table 4.18: Average percentage repellency of the essential oil of *T.camphoratus* against *S.oryzae*.

Exposure time (h)	Percentage repellency			
	0.314 µl/cm^2	0.628 µl/cm^2	0.943 µl/cm^2	1.257 µl/cm^2
1	0.00	0.00	33.3	42.8
2	0.00	23.8	42.8	42.8
3	14.0	33.3	42.8	61.9
6	33.3	52.4	61.9	71.4
24	61.9	90.5	90.5	100

Linear equation: y = 40.84x + 12.81 RD_{50} = 0.910µl/cm^2

A comparison of the percentagerepellent activity of the essential oil against both weevils is shown in Figure **4.20**. The essential oil showed slightly higher repellent activity against S. *zeamais* than against *S.oryzae* after 2h, 3h, 6h and 24h exposure but there was no significant difference between the repellency action of the essential oil against both *S. zeamais* and *S.oryzae* at $p \geq 0.05$.



M = *S.zeamais* and R = *S.oryzae*

Figure 4.20. Comparison of the repellent activity of the essential oil of *T.camphoratus* against *S. zeamais* and *S. oryzae* at different time intervals.

4.1.5.5 Fumigation, Contact toxicity and repellent activity of the essential oil of the fresh leaf of *T. trilobus var galpinni* against *S.zeamais* and *S.oryzae*.

The essential oil of the fresh leaf of *T. trilobus var galpinni* did not show any insecticidal activity against *S.zeamais* and *S. oryzae*.

4.1.6 Cytotoxicity of the volatile extracts

4.1.6.1 Cytotoxicityof the volatile extracts *T. camphoratus* against brine shrimps

The results for the cytotoxicity of the essential oils of the fresh leaf (FL), dry leaf (DL) and dry stem (DS) of *T. camphoratus* are shown in Table 4.19.The essential oils exhibited cytotoxicity against brine shrimps in a dose dependent manner and none of the essential oils exhibited mortality against brine shrimps at the lowest concentration of 10 µg/ml. The highest percentage mortality was 76.67% and it was shown by the dry stem oil at highest

concentration of 1000 µg/ml. There was a significant difference in the means of percentage mortality of the essential oils and of the standard, potassium dichromate ($p \leq 0.05$). There was no significant difference in the means of percentage mortality of the essential oils against the brine shrimps between the treatments at ($p \geq 0.05$). The LC_{50} values of the essential oils were greater than 100 µg/ml and lower than 1000 µg/ml. The dry stem oil had the lowest LC_{50} value followed by the dry leaf oil and the highest LC_{50} value was shown by the fresh leaf oil.

Table 4.19: Percentage mortality of the essential oils of the fresh leaves, dry leaves and dry stem of *T. camphoratus* against brine shrimps.

	Percentage mortality			
	[a]Fresh leaf oil	[b]Dry leaf oil	[c]Dry stem oil	[d] Potassium dichromate
Concentration (µg/ml)				
10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	93.33 ± 5.77
100	6.67 ± 5.77	10.00 ± 0.00	13.33 ± 5.77	100.00 ± 0.00
500	33.33 ± 5.77	36.67 ± 5.77	43.33 ± 5.77	100.00 ± 0.00
1000	66.67 ± 5.77	70.00 ± 0.00	76.67 ± 5.77	100.00 ± 0.00

a. Linear equation: $y = 30.14x - 38.88$ $LC_{50} = 889.0^{a}$

b. Linear equation: $y = 31.79x - 39.98$ $LC_{50} = 676.78^{b}$

c. Linear equation: $y = 35.35x - 43.55$ $LC_{50} = 442.99^{c}$

d. Linear equation: $y = 3.31x + 91.13$ $LC_{50} = 3.75 \times 10^{-12d}$

4.1.6.2 Cytotoxicityof the volatile extracts *T. trilobus var galpinni* against brine shrimps

The cytotoxicity of the essential oil of the fresh leaf of *T. trilobus var galpinni* was determined and the results are shown in Table 4.20. At the highest concentration, the oil showed 43.33% mortality against brine shrimps. The oil showed weak cytotoxicity against the brine shrimps as evidenced by its high LC_{50} value. There was a significant difference in the means of the percentage mortality of the essential oil and of the reference standard potassium dichromate.

Table 4.20: Percentage mortality of the essential oils of the fresh leaves of *T. trilobus var galpinni* against brine shrimps.

	Percentage brine shrimp mortality	
	[a]Fresh leaf oil	[b]Potassium dichromate
Concentration (µg/ml)		
10	3.33 ± 5.77	93.33 ± 5.77
100	10.00 ± 10.00	100 ± 0.00
500	30.00 ± 10.00	100 ± 0.00
1000	43.33 ± 5.77	100 ± 0.00

a. Linear equation: $y = 19.37x - 20.45$ $LC_{50} = 4335^{a}$

b. Linear equation: $y = 3.31x + 91.13$ $LC_{50} = 3.75 \times 10^{-12b}$

4.1.6.3 Comparison of the brine shrimp cytotoxicity between the essential oil of *T. camphoratus* and *T. trilobus var galpinni*.

The essential oil of *T. trilobus var galpinni* showed weaker brine shrimp cytotoxicity than the essential oils of *T. camphoratus*. The percentage brine shrimp mortality by the essential oils is compared in Figure **4.21**.

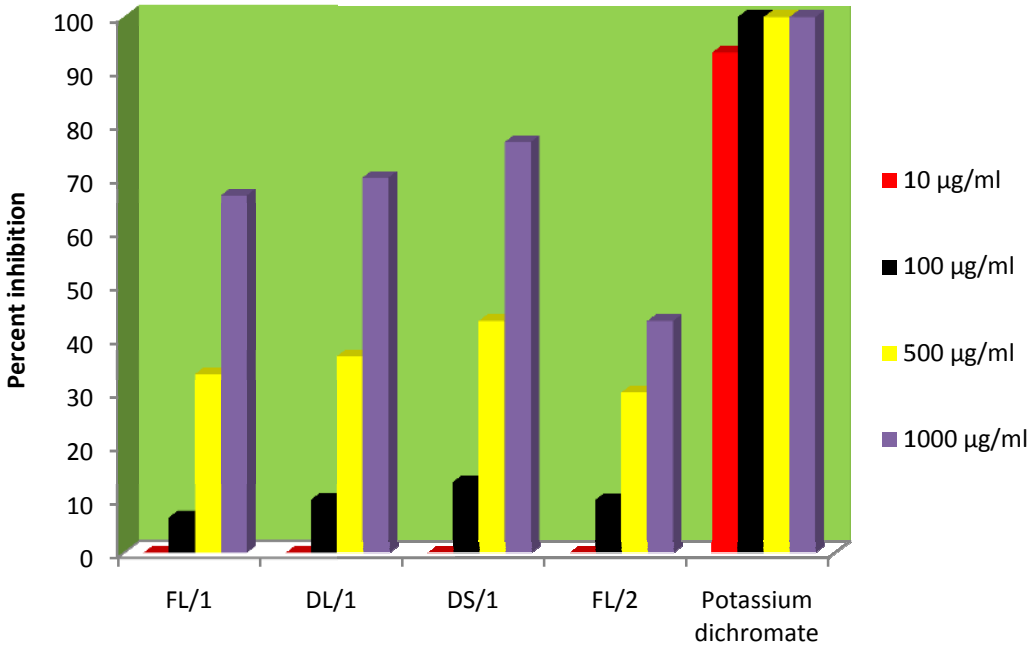


Figure 4.21.Comparison of the percentage brine shrimp mortality by the essential oils of *T.camphoratus and T. trilobus var galpinni*.

4.1.6.4 Cytotoxicityof the volatile extracts of *T. camphoratus* using the MTT assay

The percentages of the cytotoxicities of the essential oils of the fresh leaf, dry leaf and dry stem of *T. camphoratus* against human embryonic kidney cells (HEK293) and human hepatocellular carcinoma cells (HepG2) are shown in Table 4.21. At the highest concentration of 350 µg/ml, the dry stem oil showed the highest cytotoxicity of 48.3% and 52.6% against the HEK293 and HepG2 cell lines respectively. The LC_{50} values obtained for all the essential oils against both cells were between 100µg/ml and 1000 µg/ml with the exception of the LC_{50} value of fresh leaf oil against HEK293 whose LC_{50} value was above 1000 µg/ml. For both cell lines, the LC_{50} values increased in the order DS < DL < FL hence the order of decreasing cytotoxicity was DS > DL > FL. The results revealed that there was no

significant difference at $p \geq 0.05$ in the action of the essential oils of the dry stem, fresh and dry leaves on each of the human cells used.

Table 4.21: Percentage cytotoxicity of the volatile extracts of *T. camphoratus* against human embryonic kidney cells and human hepatocellular carcinoma cells.

	Percentage mortality					
	HEK293			HepG2		
	[a]FL	[b]DL	[c]DS	[d]FL	[e]DL	[f]DS
C						
50	0.5 ± 0.09	9.70 ± 0.08	10.3 ± 0.07	3.90 ± 0.06	14.1 ± 0.04	5.60 ± 0.05
100	8.9 ± 0.05	10.8 ± 0.08	18.4 ± 0.04	9.10 ± 0.02	18.5 ± 0.04	13.8 ± 0.06
150	10.1 ± 0.07	14.8 ± 0.04	20.3 ± 0.04	12.3 ± 0.02	23.9 ± 0.05	23.8 ± 0.05
200	18.2 ± 0.07	25.2 ± 0.07	31.6 ± 0.06	18.2 ± 0.03	31.4 ± 0.03	32.3 ± 0.05
250	22.5 ± 0.03	27.3 ± 0.09	41.4 ± 0.06	25.5 ± 0.06	40.1 ± 0.01	40.6 ± 0.06
300	33.7 ± 0.08	36.5 ± 0.05	43.9 ± 0.04	34.0 ± 0.05	42.1 ± 0.03	47.3 ± 0.04
350	34.1 ± 0.06	46.3 ± 0.06	48.3 ± 0.04	44.0 ± 0.05	52.6 ± 0.02	54.5 ± 0.02

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

FL = Fresh leaf DL = Dry leaf DS = Dry stem

a. Linear equation: $y = 40.15x - 71.17$ $LC_{50} = 1042.15^{a}$

b. Linear equation: $y = 41.19x - 67.40$ $LC_{50} = 708.28^{b}$

c. Linear equation: $y = 46.86x - 73.80$ $LC_{50} = 438.44^{c}$

d. Linear equation: $y = 43.86x - 76.72$ $LC_{50} = 774.80^{d}$

e. Linear equation: $y = 43.95x - 66.11$ $LC_{50} = 438.39^{e}$

f. Linear equation: $y = 58.31x - 98.78$ $LC_{50} = 356.07^{f}$

4.1.6.5 Cytotoxicityof the volatile extracts of *T. trilobus var galpinni* using the MTT assay

The essential oil of the fresh leaf of *T. trilobus var galpinni* was not cytotoxic to the HEK293 cell line (Table 4.22). The HepG2 cell line was slightly sensitive to the essential oil as shown by the low percentage mortality values and a very high LC_{50} value of 4.32×10^3 µg/ml.

Table 4.22: Percentage cytotoxicity of the essential oil of the fresh leaf of *T.trilobus var galpinni* against kidney and carcinoma cells.

	Percentage mortality	
	HEK293	[a]HepG2
Concentration (µg/ml)		
100	0	0.13± 0.03
150	0	0.97± 0.04
200	0	4.57± 0.03
250	0	6.75± 0.01
300	0	12.53± 0.03
350	0	18.80± 0.02

a. Linear equation: $y = 32.36x - 67.66$ $LC_{50} = 4.32 \times 10^{3a}$

4.2 Non volatile/solvent extracts

The water (Aq), methanol (MET) and dichloromethane (DCM) leaf (LF) extracts and the MET, DCM and ethyl acetate (Et Ac) bark (BK) extracts of *T. camphoratus* plus the MET LF, Et Ac LF, DCM LF, Et Ac BK and DCM BK extracts of *T.trilobus var galpinni* were investigated. Preliminary phytoscreening to determine the type of phyto constituents in the extracts was done and the chemical constituents were determined by using the Py-GC/MS apparatus. Some categories of compounds like alkaloids and terpenoids which were not detected by preliminary phytoscreening were detected by the Py-GC/MS. The extracts were screened for their antimicrobial, antioxidant and cytotoxic activities.

4.2.1 Chemical composition of the non volatile extracts

4.2.1.1 Phytochemical screening of the non volatile/solvent extracts

The preliminary phytochemical analysis of the extracts of *T. camphoratus* revealed the presence of flavonoids in all of the extracts; phenols were present in all of the extracts except in the DCM BK and Et Ac BK, whereas saponins were not detected in the Aq LF, DCM LF and DCM BK extracts. No tannins were found in the DCM LF and DCM BK whereasalkaloids, terpenoids, cardenolides and cardiac glycosides were absent in all of the extracts,(Table 4.23).

Table 4.23: Phytochemical constituents of the studied extracts of *T.camphoratus.*

Phytochemical component	Aq LF	MET LF	DCM LF	MET BK	DCM BK	Et Ac BK
Alkaloids	-	-	-	-	-	-
Flavonoids	+	+	+	+	+	+
Saponins	-	+	-	+	-	+
Phenols	+	+	+	+	-	-
Tannins	+	+	-	+	-	+
Terpenoids	-	-	-	-	-	-
Cardenolides	-	-	-	-		-
Cardiac glycosides	-	-	-	-	-	-

Meanwhile in the *T. trilobus var galpinni* non volatile extracts flavonoids and phenols were detected in all of the extracts except the DCM BK and Et Ac BK. Terpenoids were detected in all of the extracts except in the DCM BK and tannins were only found in the DCMBK and Et Ac BK extracts. Alkaloids, saponins, cardenolides and cardiac glycosides were not detected in all of the extracts, (Table 4.24).

Table 4.24: Phytochemical constituents of the studied extracts of *T. trilobus var galpinni.*

Phytochemical component	MET LF	DCM LF	Et Ac LF	DCM BK	Et Ac BK
Alkaloids	-	-	-	-	-
Flavonoids	+	+	+	-	-
Saponins	-	-	-	-	-
Phenols	+	+	+	-	-
Tannins	-	-	-	+	+
Terpenoids	-	+	+	-	+
Cardenolides	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-

4.2.1.2 Chemical composition of the non volatile extracts of *T. camphoratus*

A total of 19 compounds were identified in the aqueous leaf extract which were categorised as hydrocarbons, phenols, alkaloids and others(Table 4.25). Phenolic compounds comprised of 42% whereas alkaloids contributed 11% of the total number of compounds. The methanol leaf extract consisted of a total of 17 compounds which were categorised as hydrocarbons, phenols, fatty alcohols, fatty acids, fatty acid methyl esters, alkaloids and others, (Table 4.28). Of the total number of compounds, the percentage due to fatty acid methylesters was 18%, alkaloids 12%, phenols 12% and hydrocarbons 12%. Compounds present in both of the aqueous leaf extract and methanol leaf extract were 1-methyl-1H-pyrrole, 2,3-dihydrobenzofuran and hydroquinone. Among the leaf extracts, the dichloromethane leaf extract contained the highest number of compounds which were categorised as hydrocarbons, phenols, fatty alcohols, fatty acids, triterpenes, monoterpenes, diterpene alcohols and others, (Table 4.29). Of the total number of compounds, phenols comprised of 13%, triterpenes 10%, fatty acids 10% and fatty alcohols 6%. Hydrocarbons and phenols were found in all of the leaf extracts, fatty alcohols and fatty acids were present in only the methanol and dichloromethane leaf extracts and fatty acid

methyl esters were found only in the methanol extract whereas alkaloids were present only in methanol and aqueous leaf extracts. The Pyr-GC/MS chromatograms of the non volatile leaf extracts of *T. camphoratus* are shown in Figure 4.22-4.24 (Appendix 3).

Table 4.25: Chemical composition of the water leaf extract of *T. camphoratus.*

	Compound	Mr. Wt	S.I (%)
	Hydrocarbons		
1	(Z)-1,3-Pentadiene	68	86
2	Benzene	78	91
3	1,3,5-Cycloheptatriene	92	92
4	p-Xylene	106	95
5	1,3,5,7-Cyclooctatetraene	104	97
6	Tetrapentacontane	758	94
	Phenol		
7	Phenol	94	98
8	2-Methyl phenol/*o*-cresol	108	95
9	4-Methyl phenol/*p*-cresol	108	97
10	4-Ethyl phenol	122	97
11	1,2-Benzenediol/ Catechol	110	96
12	Hydroquinone	110	95
13	2-Methyl-1,4-benzenediol	124	92
14	4-Ethyl-1,3-benzenediol	138	91
	Alkaloids		
15	1-Methyl-1H-pyrrole	81	98
16	1-Ethyl-1H-pyrrole	95	79
	Others		
17	2,3-Dihydro-benzofuran	120	90
18	Cyclopropanecarboxylic acid 3-formyl-2,2-dimethyl ethyl ester	170	74
19	1-(7-Hydroxy-1,6,6-trimethyl-10-oxatri cyclo[5.2.1.0(2,4)]dec-9-yl)ethanone	238	71

Table 4.26: Chemical composition of the methanol leaf extract of *T. camphoratus.*

	Compound	Mr. Wt	S.I (%)
	Hydrocarbon		
1	1,3,5- Cycloheptatriene	92	85
2	1,3,5,7-Cyclooctatetraene	104	97
	Phenol		
3	Phenol	94	97
4	Hydroquinone	110	95
	Fatty alcohol		
5	3,7,11-Trimethyl-1-dodecanol	228	90
	Fatty acid		
6	n-Hexadecanoic acid	256	93
	Fatty acid methyl ester		
7	Hexadecanoic methyl ester	270	95
8	9-Octadecenoic methyl ester	296	91
9	Octadecanoic methyl ester	298	93
	Alkaloids		
10	1-Methyl-pyrrolidine	85	93
11	1-Methyl-1H-pyrrole	81	97
	Others		
12	2,3-dihydro benzofuran	120	84
13	Trimethylamine	59	98
14	2,3,4,5,6,7-Hexahydro-3a,6-methano-3aH-indene	134	72
15	1-Carboxy-5,5-dimethylcyclohexa-1,3-diene	152	79
16	1-methoxy-2-methoxymethyl-benzene	152	79
17	Pentadecanal	226	85

Table 4.27: Chemical composition of the dichloromethane leaf extract of *T. camphoratus.*

	Compound	Mr.Wt	S.I (%)
	Hydrocarbons		
1	1,3,6-Heptatriene	94	96
2	5,5-Dimethyl-1,3-Cyclooctatetraene	94	97
3	1- Ethyl-1,4-Cyclohexane	94	89
4	1,3,5,7-Cyclooctatetraene	104	96
5	Tetrapentacontane	758	95
6	Hexacontane	842	90
7	Dotriacontane	450	95
8	1-Bromo-triacontane	500	78
	Fatty acids		
9	β-amino-4-methoxy-benzenepropanoic acid	195	81
10	n-Hexadecanoic acid	256	93
11	Eicosanoic acid	312	84
	Phenols		
12	4-Ethyl-1,3-Benzenediol	138	91
13	3,4-Dihydro-6-hydroxy-2H-benzopyran-2-one	164	84
14	2,3-Dihydro-5,7-dihydroxy-2-phenyl-4H-1-benzopyran-4-one	256	92
15	4,7,7-Trimethyl (1.alpha., 3.alpha. 4.beta.,6.alpha) -Bicyclo [4.1.0] heptan-3-ol	154	82
	Triperpenoid		
16	3- β-D:B-Friedo-B':A'neogammacer-5-en-3-ol	426	75
17	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a, 6b,7,8,8a,9,10,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	424	87
18	4,4,6a,6b,8a,11,12,14b-Octamethyl-1,4,4a,5,6,6a, 6b,7,8,8a,9,10,11, 12, 12a, 14,14a,14b-octadeca hydro-2H-picen-3-one	424	84
	Monoterpenoid		
19	Eucalyptol	154	89
	Fatty alcohols		
20	3,7,11-Trimethyl-1-dodecanol	228	90
21	1-Heptacosanol	396	91
	Diterpene alcohol		
22	Phytol	296	96

Table 4.27: Cont

	Compound	Mr.Wt	S.I (%)
	Others		
23	4-Hepytn-2-ol	112	92
24	5,6-Dihydro-6-pentyl-2H-pyran-2-one	168	84
25	Nonadecyl heptafluorobutyrate	480	90
26	Trimethylamine	59	98
27	5-Aminoisoxazole	84	78
28	Nonadecyl trifluoroacetate	380	94
29	Pentadecanal		
30	Acetic acid, 3-hydroxy-7-isopropenyl-1, 4a-dimethyl-2,3,4,4a,5,6,7,8-octa hydro naphthalen-2-yl ester	278	81
31	1-Naphthalenepropanol, alpha.-ethyl deca hydro-alpha., 5,5,8a-tetra methyl -2-methyl ene-[1S[1alpha (S*), 4a.beta .,8a.alpha.]]	292	75

In the methanol bark extract, 34 compounds were identified of which phenols represented 21%, hydrocarbons 12%, sterols 9%, fatty alcohols 6%, fatty acids 6%, fatty acid methylester 3%, triterpene 3% and steroids 3%, (Table 4.28)**.** The dichloromethane bark extract with 41 compounds had the highest number of compounds of which hydrocarbons contributed 46%, steroids 10%, fatty alcohols 7%, fatty acids 5%, fatty acid methylesters 2%, triterpenes 2% and sesquiterpenes 2%, (Table 4.29). Among the bark extracts, ethyl acetate extract had the lowest number of compounds, (Table 4.30). Of the 28 compounds present in the ethyl acetate bark extract, 21% were hydrocarbons, 18% were steroids, 7% were fatty acid methyl esters, 7% were triterpenes and each of the fatty alcohols, sterol and tetraterpene contributed 4%. Hydrocarbons, steroids, fatty alcohols, fatty acid methylesters, triterpenes were present in all the bark extracts whereas phenols and fatty acids were present only in the dichloromethane and methanol extracts. Sterols were found in the methanol and ethyl acetate extracts only. The Pyr-GC/MS chromatograms of the non volatile bark extracts of *T. camphoratus* are shown in Figure 4.25-4.27 (Appendix 3).

Table 4.28: Chemical composition of the methanol bark extract of *T.camphoratus.*

	Compound	Mr. Wt	S.I (%)
	Hydrocarbons		
1	(Z)-1,3,5-Hexatriene	80	89
2	1,3,5-Cycloheptatriene	92	92
3	3,4,4-Trimethylcyclohexene	124	85
4	1-Decene	140	77
	Fatty alcohol		
5	3,7,11-Trimethyl-1-dodecanol	228	90
6	1-Heptacosanol	396	94
	Phenol		
7	Phenol	94	94
8	2-Methoxy-phenol	124	95
9	3,4-Dimethyl-phenol	122	93
10	1,2-Benzenediol	110	96
11	4-Methyl-1,2-benzenediol	124	82
12	4-Ethylcatechol	138	80
13	4-(1E)-3-Hydroxy-1-propenyl)-2-methoxy phenol	180	70
	Fatty acid		
14	n-Hexadecanoic acid	256	92
15	3-Hydroxy-6-methoxy-1,2,3,4-tetrahydro naphthalene-2-carboxylic acid	250	67
	Fatty acid methylester		
16	Hexadecanoic acid methylester	270	93
	Steroid		
17	4,22-Cholestadien-3-one	382	80
	Sterols		
19	Stigmasterol	412	83
20	ɣ-Sitosterol	414	76
18	Stigmast-5-en-3-ol oleate	678	79
	Triterpenoids		
21	Taraxerone	428	72

Table 4.28: Cont

	Compound	Mr. Wt	S.I (%)
	Others		
22	Trimethylamine	59	96
23	2,5-Dimethylphenyl methyl carbinol	150	68
24	1-(2-hydroxy-5-methylphenyl)-ethanone	150	87
25	1,2,4-Trimethyl-5-1methylethenyl-benzene	160	79
26	Glaucic acid	234	73
27	4,8a-Dimethyl-6-(2-methyl-oxiran-2-yl)-41, 5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	234	69
28	Isoaromadendrene epoxide	220	71
29	8S, 13-Cedran-diol	238	57
30	1-Phenanthrene carboxylic acid	314	81
31	Bis(2-ethylhexyl) phthalate	390	79
32	Nonadecyl heptafluorobutyrate	480	84
33	1,2,4,8-Tetramethylbicyclo[6.3.0]undeca-2,4-diene	204	67
34	1-isopropenyl-4,5-dimethyl-5-phenyl sulfonylmethyl-bicyclo[4.3.0] nonane	346	72

Table 4.29: Chemical composition of the dichloromethane bark extract of *T. camphoratus.*

	Compound	Mr. Wt	S.I (%)
	Hydrocarbons		
1	1,3-Pentadiene	68	91
2	2-Methyl-1,3-pentadiene	82	93
3	3,5,5-Trimethyl-cyclohexene	124	91
4	1-Decene	140	93
5	1-Undecene	154	97
6	1-Dodecene	168	95
7	7-Methylene-tridecane	196	84
8	Cyclotetradecane	196	91
9	1-Pentadecene	210	88
10	2-Methyl hexadecane	240	88
11	2-Methyl-hexadecane	240	89
12	Eicasane	282	96
13	Pentatriacontane	492	95
14	Tetrapentacontane	758	93
15	Dotriacontane	450	95
16	Hexatriacontane	506	94
17	Tetracontane	562	92
18	1,2,4,8-Tetramethylbicyclo[6.3.0]undeca-2,4-diene	204	75
19	1,4-Dimethyl-8-isopropylidenetri cyclo [5.3.0.0(4,10)]decane	204	72
	Fatty alcohol		
20	1-Tetradecanol	214	94
21	(E)-11-Tetradecen-1-ol	212	88
22	3,7,11-Trimethyl-1-dodecanol	228	91
	Fatty acid		
23	n-Hexadecanoic acid	256	93
24	(Z)-6-Octadecenoic acid	282	93
	Phenol		
25	2,3,5-Trimethyl-1,4-benzenediol	152	93
	Fatty acid methyl ester		
26	Methyl-11,14-eicosadienoate	322	74
	Triterpene		
27	Lup-20(29)-en-3-ol, acetate (3β)	468	81

Table 4.29: Cont

	Compound	Mr. Wt	S.I (%)
	Sesquiterpene		
28	α-Selinene	204	72
	Steroids		
29	5β-pregn-14-en-3-one	300	78
30	9,19-Cyclo-9-β-lanostane-3β.,25-diol	444	73
31	4,22-Cholestadien-3-one	382	83
32	Cholest-4-en-3-one	384	78
	Others		
33	Trimethylamine	59	94
34	Duroquinone	164	96
35	Durohydroquinone	166	94
36	Methyl tetrahydroionol	212	88
37	Glaucic acid	234	76
38	2-Methyl-3-vinyl-1-cyclopentne-1-carboxylic acid	152	67
39	Octatriacontyl pentafluoropropionate	696	93
40	2,6-Dihexadecanoate 1-(+)-ascorbic acid	652	69
41	Tricyclo[5.4.3.0(1,7)tetradecane-3,6-diol, 4-formyl-2,4,7,14-tetramethyl-diacetate	392	76

Table 4.30: Chemical composition of the ethyl acetate bark extract of *T. camphoratus.*

	Compound	Mr. Wt	S.I (%)
	Hydrocarbon		
1	(Z)-1,3-Pentadiene	68	89
2	2-Methyl-(E)-1,3-pentadiene	82	92
3	1,3,5-Cycloheptatriene	92	89
4	3,4,4-Trimethylcyclohexene	124	91
5	Pentatriacontane	492	93
6	Dotriacontane	450	91
	Fatty alcohol		
7	3,7,11-Trimethyl-1-dodecanol	228	91
	Fatty acid methyl ester		
8	Hexadecanoic methylester	270	89
9	Octadecanoic methylester	298	85
	Steroid		
10	Androst-5-en-3.β-ol,4,4-dimethyl acetate	344	82
11	5β-Pregn-14-en-3-one	300	79
12	3β-(17E)-Pregn-5,17(20)dien-3-ol	300	71
13	5α,6αCholesta-2,8-dien-6-ol, 14-methyl acetate	440	60
14	Cortisone	360	54
	Sterol		
15	Ergosta-4,7,22-trien-3-β-ol	396	57
	Tetraterpene		
16	Lycopene	536	60
	Triterpenoid		
17	D-Friedoolean-14-ene-3β,28-diol diacetate	526	63
18	D-Friedoolean-14-en-3-one	424	74
	Others		
19	Trimethylamine	59	91
20	3-Furan-2-ylmethyl aminomethyl-4a-hydroxy-5-meth oxy-5,8a-dimethyl-decahydronaphthol2,3-furan-2-one	377	83
21	4,4-Dimethyl-oct-5-enal	154	83
22	Methyl tetrahydoionol	212	87
23	1,2,3,4,5-Pentamethylcyclopenta-2,4-dienyl ester benzoic acid	256	72

Table 4.30: Cont

	Compound	Mr. Wt	S.I (%)
24	Farnesyl bromide	284	76
25	1,3-Dimethyl-4,6-dicyclopentyl benzene	242	53
26	Acetic acid, 3-(2,2-dimethyl-6-methylene-cyclohexy lidene)-1-methyl-butyl ester	250	62
27	1-Isopropenyl-4,5-dimethyl-5-phenyl sulfonylmethyl-bicyclo[4.3.0]nonane	346	75
28	1,2,3,8-Tetramethylbicyclo[6.3.0]undeca-2,4-diene	204	74

4.2.1.3 Chemical composition of the non volatile extracts of *T. trilobus var galpinni*

The non volatile/solvent extracts of the leaves and bark of *T. trilobus var galpinni* revealed the presence of different categories of compounds among the extracts which included hydrocarbons, phenols, fatty alcohols, fatty acids, fatty acid methylesters, triterpenes, steroids, diterpene alcohols, monoterpenes and sesquiterpenes. The dichloromethane extract with 25 compounds had the highest number of components. Hydrocarbons contributed 36%, phenols 12%, triterpenes 12%, fatty acids 8%, monoterpenes, diterpenes and sterols each contributed 4% of the total number of compounds (Table 4.31).The methanol leaf extract contained a total of 22 compounds, (Table4.32), of which 32% were hydrocarbons, 18% phenols, 18% fatty acids, 9% fatty alcohols and 5% triterpenes.The ethyl acetate leaf extract with the least number of components, (Table 4.33), consisted of hydrocarbons, steroids and triterpenes contributing 30%, 30% and 15%, respectively, of the total number of compounds. Hydrocarbons and triterpenes were present in all of the leaf extracts whereas phenols and fatty acids were not present in the methanol leaf extract. Steroids were found only in the methanol extract. The Pyr-GC/MS chromatograms of the non volatile leaf extracts of *T. trilobus var galpinni* are shown in Figure 4.28-4.30 (Appendix 4).

Table 4.31: Chemical composition of the dichloromethane leaf extract of *T.trilobus var galpinni.*

	Compound	S.I (%)	Mr.Wt
	Hydrocarbon		
1	1,3-Pentadiene	97	68
2	1,3,6-Heptatriene	96	94
3	1,3-Cyclopentadiene	96	94
4	3-Methylenecyclohexene	90	94
5	1,3,5-Hexatriene	95	94
6	p-Xylene	97	106
7	1,3,5,7-Cyclooctatetraene	96	104
8	Tetrapentacontane	95	758
9	Dotriacontane	94	450
	Monoterpene		
10	D-Limonene	92	136
	Phenol		
11	Catechol	95	110
12	4-Ethyl-1,3-benzenediol	92	138
13	3,4-Dihydro-6-hydroxy-coumarin	80	164
	Fatty acid		
14	Tetradecanoic acid	87	228
15	n-Hexadecanoic acid	93	256
	Diterpene alcohol		
16	Phytol	95	296
	Triterpenoid		
17	Stigmast-5-en-3-ol, oleate	82	678
18	4,4,6a,6b, 8a,11,11,14b-Octamethyl-1,4,4a,5,6, 6a, 6b,7,8,8a,9,10,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one.	85	424
	Sterols		
19	β-Sitosterol	80	414
	Others		
20	5,6-Dihydro-6-pentyl-2H-pyran-2-one	83	168
21	2-Hydroxy-4-methyl benzaldehyde	80	136
22	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	90	340
23	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	88	296
24	(*E*)-6-Octadecen-1-ol acetate	86	310
25	1,2-Benzenedicarboxylic acid mono(2-ethylhexyl) ester	93	390

Table 4.32: Chemical composition of the methanol leaf extract of *T. trilobus var galpinni.*

	Compound	S.I (%)	Mr.Wt
	Hydrocarbon		
1	(*Z*)-1,3-Pentadiene	93	68
2	1,3,6-Heptatriene	96	94
3	5,5-Dimethyl-1,3-cyclopentadiene	96	94
4	1-Methyl-1,4-cyclohexadiene	92	94
5	Styrene	98	104
6	Dotriacontane	94	450
7	Tetrapentacontane	94	758
	Phenol		
8	Catechol	97	110
9	4-Methyl catechol	94	124
10	4-Ethyl-1,3-benzenediol	92	138
11	Coumarin	81	164
	Fatty alcohol		
12	3,7,11-Trimethyl-1-dodecanol	90	228
13	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	87	296
	Fatty acid		
14	4-Hydroxy benzenepropanoic acid	88	166
15	3,4-dihydroxy-benzenepropanoic acid	95	182
16	n-Hexadecanoic acid	93	256
17	Linoleic acid	92	280
	Triterpenoid		
18	4,4,6a,6b, 8a,11,11,14b-Octamethyl-1,4,4a,5,6, 6a, 6b,7,8,8a,9,10,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one.	84	424
	Others		
19	Ethyl silane	91	60
20	1-Methyl-4-(1-methylethenyl)-cyclohexanol acetate	95	196
21	α-(2-nitropropyl)-2-furanmethanol	75	185
22	3-Nitrobenzyl iodide	80	268

Table 4.33: Chemical composition of the ethyl acetate leaf extract of *T. trilobus var galpinni.*

	Compound	S.I (%)	Mr.Wt
	Hydrocarbon		
1	(Z)-2-Butene	90	56
2	(Z)-1,3-Pentadiene	91	68
3	2-Methyl-(E)-1,3-pentadiene	95	82
4	3-Methylene-tricyclooctane	85	120
5	3,4,4-Trimethylcyclohexene	92	124
6	Spiro[5.5]undecane	86	152
	Triterpenoid		
7	Stigmastan-3,5,22-triene	56	394
8	Lupenone	75	424
9	Friedoolean-24-en-3-one	74	424
	Steroid		
10	5β-Pregn-14-en-3-one	79	300
11	5-Pregnen-3β-ol-20-one	74	412
12	(9b)-3,23-dioxo-7,25(27)-lanostadien-26-oic acid methyl ester	66	482
13	4,4-Dimethyl-3β-cholesta-5,7-dien-3-ol	51	412
14	3β-Lanosta-8,24-dien-3-yl-oxytrimethyl silane	61	498
15	3β-9,19-Cyclolanost-24-en-3-ol	67	426
	Others		
16	2,5,5-Trimethyl-3-cyclohexen-1-one	89	138
17	Benzoic acid, 1,2,3,4,5-pentamethylcyclopenta-2,4-dienyl ester	73	256
18	Cadala-1(10)-3,8-triene	61	202
19	Acetic acid, 3-(2,2-dimethyl-6-methylene-cyclohexylidene)-1-methyl-butyl ester	67	250
20	1,2,4,8-Tetramethylbicyclo[6.3.0]undeca-2,4-diene	74	204

Among the bark extracts, the ethylacetate extract had thehighest number of constituents. Hydrocarbons contributed 20%, triterpenes 20%, fatty alcohols 9%, fatty acids 7%, sesquiterpenes 4%, monoterpenes 2% and fatty acid methylesters 2% (Table 4.34). The dichloromethane bark extract consisting of 20 compounds, (Table 4.35), was mainly dominated by hydrocarbons 54%. Also present were fatty alcohols 17% and fatty acid methylesters 8%. Both bark extracts contained fatty acids and fatty acid methylesters. The

Pyr-GC/MS chromatograms of the non volatile bark extracts of *T. trilobus var galpinni* are shown in Figure 4.31-4.32 (Appendix 4).

Table 4.34: Chemical composition of the ethyl acetate bark extract of *T.trilobus var galpinni.*

	Compound	S.I (%)	Mr.Wt
	Hydrocarbon		
1	(E)-1,3-Pentadiene	93	68
2	1,3,5-Hexatriene	86	80
3	1,3,5-Cycloheptatriene	89	92
4	p-Xylene	97	106
5	1-Decene	92	140
6	1-Undecene	95	154
7	1-Dodecene	94	168
8	1-Tridecene	86	182
9	1-Nonadecene	83	266
	Fatty alcohol		
10	2-Undecanol	73	172
11	3,7,11-Trimethyl-1-dodecanol	91	228
12	1-Heptacosanol	93	396
13	1-Hexacosanol	91	396
	Fatty acid		
14	Caprylic acid	68	144
15	n-Hexadecanoic acid	94	256
16	Petroselinic acid	92	282
	Fatty acid ethylester		
17	Docosanoic acid ethylester	78	368
	Monoterpene		
18	D-Limonene	94	136
	Sesquiterpene		
19	α-Muurolene	80	204
20	α-Cadinol	89	222
21	1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methyl ene-1-methylethyl-(1.α.,4a.β.,8a.α)-naphthalene	87	204
22	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphthalene	72	204
23	2α.4aα. 8aβ-1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-1-methylethnyl-naphthalene	79	204
24	Diepi-α-cedrene epoxide	74	220

Table 4.34 Cont.

	Compound	S.I (%)	Mr.Wt
	Triterpenoid		
25	3β-Lupeol acetate	84	468
26	Betulin	78	442
27	Taraxerone	73	424
28	Lupenone	86	424
29	A'-Neogammacer-22(29)-en-3-one	79	424
30	A'-Neogammacer-22(29)-ene	77	410
31	17-Acetyl-17-noranthiaerostan-1,5,7,9-tetraene.	69	294
32	4,4,6a,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,7, 8,8a,9,10,11,12,12a,14,14a,14b-octahydro-2H-picen-3-one.	86	424
33	3-Acetyloxy-24-methyl-9,19-cyclo-27-norlanostan -25-one	76	484
	Others		
34	Trimethylamine	94	59
35	Acetic acid	89	60
36	1-[2-hydroxy-5-methylphenyl] ethanone	88	150
37	Acetate, (2,4a,5,8a-tetramethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenyl acetate	72	250
38	Oxacycloheptadec-8-en-2-one	83	252
39	Octadecanal	90	268
40	Heptadecyl-oxirane	90	282
41	2,3,3-Trimethyl-2-3-methyl-buta-1,3-dienyl-cyclohexanone	60	206

Table 4.35: Chemical composition of the dichloromethane bark extract of *T.trilobus var galpinni.*

	Compound	S.I (%)	Mr.Wt
	Hydrocarbons		
1	1-Nonene	95	126
2	1-Decene	97	140
3	1-Undecene	97	154
4	1-Tridecene	97	182
5	1-Pentadecene	96	210
6	1-Tetradecene	95	196
7	1-Heptadecene	96	238
8	1-Nonadecene	97	266
9	Dotriacontane	96	450
10	Tetrapentacontane	95	758
11	Pentacosane	96	352
12	Hexatriacontane	95	506
13	Tetracontane	96	562
	Fatty alcohol		
14	n-Nonadecanol	96	284
15	Behenic alcohol	97	326
16	1-Heptacosanol	96	396
17	n-Tetracosanol	96	354
	Fatty acid methylester		
18	Hexadecanoic acid methyl ester	95	270
19	Octadecanoic acid methyl ester	95	298
	Others		
20	Trimethylamine	98	59

4.2.2 Biological activities of the non volatile extracts

4.2.2.1 Antibacterial activity of the non volatile extracts

Antibacterial activity of the non volatile extracts of *T. camphoratus* using the agar disc diffusion method

The extracts showed varying antibacterial activities on the bacterial strains used and had a broader spectrum of antibacterial activity. The results for the mean diameters of the zones of inhibition are shown in Table 4.36. The Aq LF, MET BK and Et Ac BK extracts showed activity on all of the tested bacteria with zones of inhibition in the range: 9.20 – 13.00, 8.00 - 11.00 and 7.30 – 14.00 respectively. The microorganisms which showed sensitivity to all the extracts were *Staphylococcus aureus*, *Enterococcus faecalis* and *Shigella flexineri*. The standard drug streptomycin showed higher inhibition than the extracts on the tested micro-organisms with diameters of zones of inhibition in the range of 17.50 – 22.00. There was a significant difference between the means of the diameters of the zones of inhibition of the extracts and of streptomycin at p =<0.05 and there was no significant difference between the means of the diameters of the zones of inhibition of the different solvent extracts and also between the extracts and the standard drugs, ampicillin and chloramphenical at p = > 0.05.

Table 4.36: The mean diameters of the zones of inhibition of the non-volatile extracts of the leaves and bark of *T. camphoratus* against different micro-organisms.

	Aq LF	**MET LF**	**DCM LF**	**MET BK**	**Et Ac BK**	**DCM BK**	**S**	**A**	**C**
Micro-organism									
Gram- positive									
Staphylococcus aureus	9.20 ± 1.30	9.25 ± 0.96	12.75 ± 1.71	8.50 ± 0.58	7.30 ± 0.5	12.50 ± 1.29	18.00 ± 1.26	12.25 ± 0.5	0.00
Staphylococcus epidermidis	10.75 ± 0.96	0.00	9.70 ± 0.5	8.00 ± 0.82	12.25 ± 2.08	13.75 ± 0.96	21.00 ± 0.96	10.00 ± 1.2	16.33 ± 0.5
Bacillus cereus	11.00 ± 0.82	10.75 ± 1.26	10.50 ± 1.29	9.25 ± 0.5	11.75 ± 0.96	0.00	22.00 ± 1.71	9.25 ± 1.3	0.00
Enterococcus faecalis	13.00 ± 1.58	11.75 ± 1.71	10.00 ± 0.82	10.50 ± 0.58	11.50 ± 1.29	12.50 ± 0.58	20.00 ± 1.29	11.0 ± 0.8	0.00
Gram-negative									
Escherichia coli	9.80 ± 0.95	8.75 ± 1.71	0.00	8.50 ± 0.58	10.00 ± 0.82	12.00 ± 0.82	17.50 ± 1.29	12.5 ± 0.6	10.3 ± 0.5
Pseudomonas aeruginosa	12.20 ± 1.30	12.00 ± 0.82	11.00 ± 0.82	10.50 ± 1.29	11.75 ± 0.96	0.00	21.00 ± 1.71	12.0 ± 0.9	0.00
Klebsiella pneumonia	12.10 ± 1.47	10.50 ± 1.29	12.50 ± 1.29	11.00 ± 0.82	9.70 ± 0.5	0.00	20.00 ± 1.71	14.25 ± 0.9	13.0 ± 1.4
Shigella flexineri	11.60 ± 1.14	10.25 ± 2.06	11.00 ± 0.82	11.00 ± 0.82	14.00 ± 1.83	10.00 ± 0.82	21.00 ± 0.82	10.0 ± 1.0	11.7 ± 1.2
Salmonella spp.	9.50 ± 0.58	0.00	9.50 ± 0.58	10.00 ± 1.83	12.75 ± 1.71	10.00 ± 0.82	21.00 ± 1.29	9.0 ± 0.86	17.5 ± 0.9

The diameters of zones of inhibition are expressed as mean ± S.D. (n = 3);

Disc diameter = 6mm; Extract dose = 5 mg/disc , Standard drug dose = 2 µg/disc

S = Streptomycin C = Chloramphenicol A = Ampicillin

Antibacterial activity of the non-volatile extracts of the leaves and bark of *T.camphoratus* using the minimum inhibition concentration (MIC) method

The results for the minimum concentration (MIC) of the extracts that would inhibit the growth of the tested micro-organisms are shown in Table 4.37. The MIC values were in the range 0.625–5.00 mg/ml and were different among the extracts and even for the same

extract against different strains of bacteria. The Aq LF extract showed the lowest MIC value of 0.625 mg/ml against *Enterococcus faecalis* and an MIC value of 1.25 mg/ml was shown by: Aq LF against *Staphylococcus epidermidis* and *Bacillus cereus*: MET LF and Et BK against *Pseudomonas aeruginosa* and *Shigella flexineri*. The highest MIC value of 5 mg/ml was shown by the MET BK and Et BK extracts against *Staphylococcus aureus*, the MET LF and MET BK extracts against *Escherichia coli*,the DCM LF and MET BK extracts against *Staphylococcus epidermidis* and the Aq LF, DCM LF, MET BK and DCM BK extracts against *Salmonella spp.*

Table 4.37: Minimum inhibitory concentrations in mg/ml for the non-volatile extracts of *T. Camphoratus*.

	Aq LF	MET LF	DCMLF	MET BK	Et AcBK	DCM BK
Micro-organism						
Gram- positive						
Staphylococcus aureus	2.5	2.5	2.5	5.0	5.0	2.5
Staphylococcus epidermidis	1.250	ND	5.0	5.0	2.5	2.5
Bacillus cereus	1.250	2.5	2.5	2.5	2.5	ND
Enterococcus faecalis	0.625	2.5	2.5	2.5	2.5	2.5
Gram-negative						
Escherichia coli	2.5	5.0	ND	5.0	2.5	2.5
Pseudomonas aeruginosa	2.5	1.25	2.5	2.5	2.5	ND
Klebsiella pneumonia	2.5	2.5	2.5	2.5	2.5	ND
Shigella flexineri	2.5	2.5	2.5	2.5	1.25	2.5
Salmonella spp.	5.0	ND	5.0	5.0	2.5	5.0

ND = Not determined

Antibacterial activity of the non-volatile extracts of the leaves and bark of *T. trilobus var galpinni* using the agar disc diffusion method

The extracts exhibited different zones of inhibition in mm in the range 8.27-9.63, 8.13-8.85, 7.75-9.60, 8.63-12.50 and 8.25-10.08 for the MET LF, DCM LF, Et Ac LF, Et Ac BK and DCM BK extracts respectively, (Table 4.38). The highest inhibition zones of 12.3 and 12.5 mm were

exhibited by Et Ac BK extract against *Staphylococcus aureus* and *Salmonella spp* respectively. *Bacillus cereus, Escherichia coli* and *Shigella flexineri* were sensitive to all of the extracts and none of the extracts showed activity against *Pseudomonas aeruginosa.* There was a significant difference between the means of the diameters of the zones of inhibition of the extracts and of streptomycin at $p =< 0.05$ and there was no significant difference between the means of the diameters of the zones of inhibition of the different solvent extracts and also between the extracts and the the standard drugs, ampicillin and chloramphenicol at p = > 0.05.

Table 4.38: The antibacterial activities of the non-volatile extracts of the leaves and bark of *T. trilobus* var *galpinni* using the agar disc diffusion method.

	MET LF	DCM LF	Et Ac LF	Et Ac BK	DCM BK	S	A	C
Micro-organism								
Gram- positive								
Staphylococcus aureus	8.27 ± 0.87	8.13 ± 0.22	7.75 ± 0.65	12.3 ± 0.16	9.10 ± 1.75	18.00 ± 1.26	12.25± 0.5	0.00
Staphylococcus epidermidis	8.87 ± 0.85	0.00	8.66 ± 1.11	8.63 ± 1.11	0.00	21.00 ± 0.96	10.00± 1.2	16.33 ± 0.5
Bacillus cereus	8.38 ± 0.52	8.85 ± 0.24	8.88 ± 0.85	8.88 ± 0.85	10.08 ± 1.06	22.00 ± 1.71	9.25 ± 1.3	0.00
Enterococcus faecalis	8.70 ± 0.93±	8.50 ± 0.49	9.60 ± 0.21	9.60 ± 0.21	9.35 ± 1.39	20.00 ± 1.29	11.0 ± 0.8	0.00
Gram-negative								
Escherichia coli	9.48 ± 0.68	8.15 ± 0.13	8.13 ± 0.22	10.3 ± 0.12	8.25 ± 0.19	17.5 ± 1.29	12.5 ± 0.6	10.3 ± 0.5
Pseudomonas aeruginosa	0.00	0.00	0.00	0.00	0.00	21.0 ± 1.71	12.0 ± 0.9	0.00
Klebsiella pneumonia	9.63 ± 1.11	8.78 ± 0.66	0.00	10.7 ± 0.15	8.45 ± 0.47	20.0 ± 1.71	14.25 ± 0.9	13.0 ± 1.4
Shigella flexineri	9.05 ± 0.60	8.50 ± 0.53	9.13 ± 0.85	8.65 ± 0.29	8.88 ± 1.15	21.0 ± 0.82	10.0 ± 1.0	11.7 ± 1.2
Salmonella spp.	8.68 ± 0.52	8.65 ± 1.39	8.3 ± 0.89	12.5 ± 0.18	0.00	21.0 ± 1.29	9.0 ± 0.86	17.5 ± 0.9

The diameters of zones of inhibition are expressed as mean ± S.D. (n = 3);

Disc diameter = 6mm; Extract dose = 5 mg/disc, Standard drug dose = 2 µg/disc

S = Streptomycin C = Chloramphenicol A = Ampicillin

Antibacterial activity of the non-volatile extracts of the leaves and bark of *T.trilobus var galpinni* using the minimum inhibition concentration (MIC)

The MIC values were in the range of 1.25–5.0 mg/ml and the lowest MIC value of 1.25 mg/ml was shown by the Et Ac BK extract against *Staphylococcus aureus* and *Salmonella spp*, (Table 4.39). Most of the extracts showed a high MIC value of 5 mg/ml against different strains of bacteria.

Table 4.39: Minimum inhibitory concentrations in mg/ml for the non-volatile extracts of *T.trilobus var galpinni*.

	MET LF	**DCM LF**	**Et Ac LF**	**Et Ac BK**	**DCM BK**
Micro-organism					
Gram- positive					
Staphylococcus aureus	5.0	5.0	5.0	1.25	2.5
Staphylococcus epidermidis	5.0	ND	5.0	5.0	ND
Bacillus cereus	5.0	5.0	5.0	5.0	5.0
Enterococcus faecalis	5.0	5.0	2.5	2.5	2.5
Gram-negative					
Escherichia coli	2.5	5.0	5.0	2.5	5.0
Pseudomonas aeruginosa	ND	ND	ND	ND	ND
Klebsiella pneumonia	2.5	5.0	ND	2.5	5.0
Shigella flexineri	5.0	5.0	5.0	5.0	5.0
Salmonella spp.	5.0	5.0	5.0	1.25	ND

ND = Not determined

Comparison of the antibacterial activities of the non volatile extracts of *T.camphoratus* and *T. trilobus var galpinni*.

Generally,*T. camphoratus* extracts had larger zones of inihibition than *T. trilobus var galpinni* extracts against the same micro-organisms but the antibacterial activities of the non volatile extacts for both species was similar. Comparing the means of the diameters of the zones of inhibition of the extracts of *T. camphoratus* and *T. trilobus var galpinni*, there was no significant difference at p = > 0.05. The MIC values for *T. trilobus var galpinni*

extracts were generally higher or equal to those of *T. camphoratus* except for the activity of the Et Ac BK extract against *Staphylococcus aureus* and *Salmonella spp*.

4.2.2.2 Antioxidant activities of the non volatile extracts

DPPH radical scavenging activity of the non volatile extracts of *T.camphoratus* and *T. trilobus var galpinni*.

The non volatile extracts of the leaf and bark of *T. camphoratus* and*T. trilobus var galpinni* were investigated for their DPPH radical scavenging activities. The results showed that the radical scavenging activities of all the extracts increased with increasing concentration with the leaf extracts showing the highest activity for both *Tarchonanthus* species, (Tables 4.40 and 4.41). The *T. camphoratus* leaf extracts showed the lowest LC_{50} values in the order DCM LF/1 < MET LF/1 < Aq LF/1 and hence the highest radical scavenging activity in the order DCM LF/1 > MET LF/1 > Aq LF/1. The LC_{50} of the DCM LF/1 extract, 17.59 µg/ml, was comparable to that of the reference standard BHT, 10.88 µg/ml. The bark extracts had very high LC_{50} values and hence very low DPPH radical scavenging activities. There was a significant difference in the means of percentage radical scavenging between the treatments at $p = < 0.05$. The DPPH radical scavenging activity of *T. trilobus var galpinni* non volatile extracts showed a similar pattern to that of*T. camphoratus* in that the leaf extracts also showed higher DPPH scavenging activity than the bark extracts. From the LC_{50} values the DPPH radical scavenging activity of *T. trilobus var galpinni* non volatile extracts,was in the order MET LF/2 > DCM LF/2 > Et Ac LF/2 > DCM BK/2 > Et Ac BK/2. There was a significant difference in the means of percentage radical scavenging between the treatments at $p = < 0.05$. For both *Tarchonanthus* species, the bark extracts showed very poor DPPH radical scavenging activities and overall the *T. camphoratus* extracts showed

higher DPPH radical scavenging, lower LC_{50} values, than the *T. trilobus var galpinni* extracts, (Figure **4.33**). There was however, no significant difference in the means of the LC_{50} values between treatments at p = > 0.05 for both *Tarchonanthus* species.

Table 4.40: Percentage DPPH radical scavenging activity of the non volatile extracts of *T. camphoratus*.

Percentage DPPH radical scavenging activity							
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK	[g]BHT
C							
25	38.70 ± 1.9	41.46 ± 0.9	52.57 ± 4.1	19.93 ± 3.7	3.25 ± 0.4	6.46 ± 0.4	57.47 ± 4.78
50	46.20 ± 0.7	51.67 ± 3.0	67.42 ± 5.5	37.24 ± 3.5	5.42 ± 0.9	9.03 ± 1.3	82.03 ± 1.11
100	64.03 ± 1.4	68.59 ± 6.5	78.00 ± 0.8	59.06 ± 3.3	7.01 ± 0.7	15.23 ± 2.4	92.87 ± 1.23
150	72.23 ± 1.9	72.06 ± 1.1	84.81 ± 0.9	74.35 ± 3.4	10.04 ± 0.8	19.57 ± 0.7	94.80 ± 0.14
200	83.89 ± 0.4	81.60 ± 0.3	85.89 ± 0.3	83.22 ± 0.3	12.74 ± 0.3	23.59 ± 3.2	96.80 ± 0.02
250	84.73 ± 0.6	83.06 ± 0.7	85.94 ± 0.7	83.97 ± 0.8	17.35 ± 1.7	28.67 ± 2.7	99.10 ± 0.12

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 49.53x - 33.86 LC_{50} = 49.33[a]

b. Linear equation: y = 43.17x - 19.74 LC_{50} = 41.25[b]

c. Linear equation: y = 34.33x + 7.25 LC_{50} = 17.59[c]

d. Linear equation: y = 68.24x - 76.54 LC_{50} = 71.50[d]

e. Linear equation: y = 12.52x - 15.69 LC_{50} = 1.7 x 10^{5}[e]

f. Linear equation: y = 21.59x - 25.99 LC_{50} = 3.31 x 10^{3}[f]

g. Linear equation: y = 38.77x + 9.81 LC_{50} = 10.88[g]

Table 4.41: Percentage DPPH radical scavenging activity of the non volatile extracts of *T. trilobus var galpinni*.

PercentageDPPH radical scavenging activity						
	[a]MET LF	[b]DCM LF	[c]Et Ac LF	[d]DCM BK	[e]Et Ac BK	[f]BHT
C						
25	12.16 ± 0.85	16.97 ± 1.51	12.42 ± 1.22	3.36 ± 0.17	3.48 ± 0.48	57.47 ± 4.78
50	21.90 ± 0.49	25.02 ± 0.79	14.48 ± 0.34	3.70 ± 0.30	5.33 ± 0.56	82.03 ± 1.11
100	29.88 ± 0.56	32.52 ± 0.93	19.19 ± 0.68	8.08 ± 1.00	7.29 ± 0.18	92.87 ± 1.23
150	34.32 ± 1.26	35.18 ± 1.15	25.89 ± 0.56	10.75 ± 0.75	7.57 ± 0.39	94.80 ± 0.14
200	38.44 ± 2.53	39.22 ± 0.40	32.96 ± 2.52	12.42 ± 1.05	8.82 ± 0.31	96.80 ± 0.02
250	40.28 ± 3.10	40.84 ± 0.66	41.54 ± 1.04	15.20 ± 0.84	12.23 ± 1.12	99.10 ± 0.12

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 28.05x – 26.47 LC_{50} = 532[a]

b. Linear equation: y = 23.72x – 15.72 LC_{50} = 589.7[b]

c. Linear equation: y = 27.09x – 29.64 LC_{50} = 870[c]

d. Linear equation: y = 11.99x – 15.02 LC_{50} = 2.6 x 10^{5}[d]

e. Linear equation: y = 7.32x - 7.15 LC_{50} = 6.4 x 10^{7}[e]

f. Linear equation: y = 31.55X + 13.32 LC_{50} = 10.88[f]

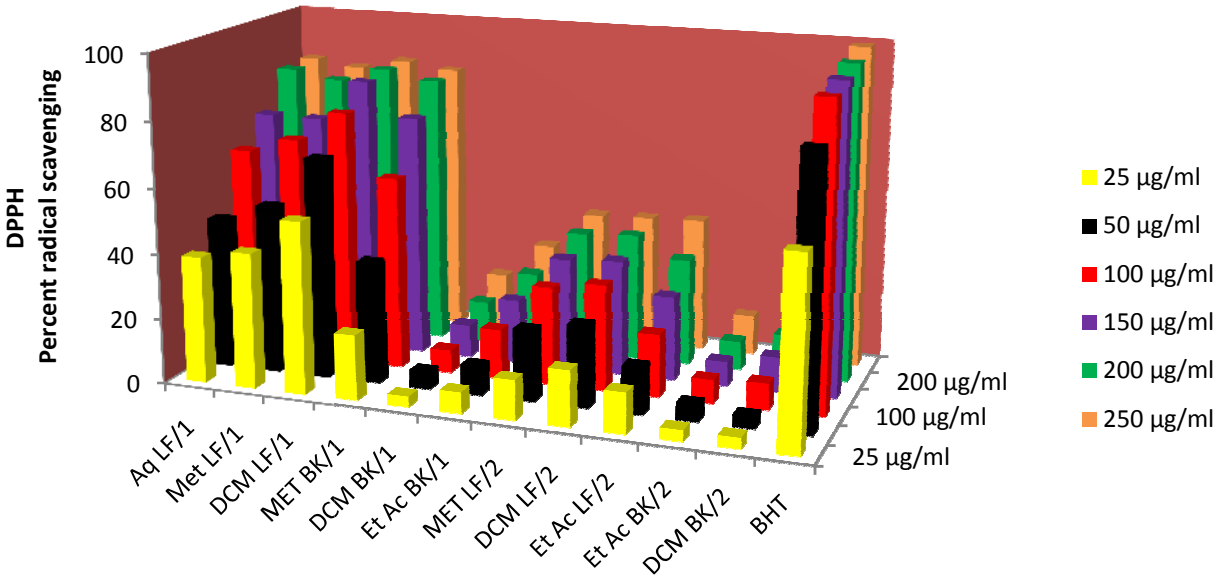


Figure 4.33. Comparison of the DPPH radical scavenging of the non volatile extracts of *T. camphoratus*and *T.trilobus var galpinni*.

ABTS$^{+\bullet}$scavenging activityof the non volatile extracts of *T.camphoratus* and *T. trilobus var galpinni*.

Tables 4.42 and 4.43 show the ABTS$^{+\bullet}$scavenging activities of the non volatile extracts of*T. camphoratus* and *T. trilobus var galpinni* respectively. The trend in the **ABTS$^{+\bullet}$**and the DPPH radical scavenging activities of non volatile extracts was similar. **The DCM LF/1 extract with** the lowest LC_{50} value (22.46 µg/ml) showed the highest ABTS$^{\bullet+}$radical scavenging activity. ABTS$^{+\bullet}$radical scavenging activity of *T. camphoratus* extracts was in the order: DCM LF/1 > Aq LF/1 > MET LF/1 > MET BK/1 > DCM BK/1 > Et Ac BK/1 while that for *T. trilobus var galpinni* extracts was in the order: MET LF/2 > DCM LF/2 > Et Ac LF/2 > DCM BK/2 > Et Ac BK/2. For each *Tarchonanthus* species, there was a significant difference in the means of the percentage radical scavenging activity among the extracts between the treatments at p = > 0.05. The leaf extracts of both species showed higher ABTS$^{\bullet+}$radical scavenging activity

than the bark extracts whose LC_{50} values were higher than 1000 µg/ml. Generally the *T. camphoratus* non volatile extracts showed higher $ABTS^{\bullet+}$radical scavenging activity than the non volatile extracts of *T. trilobus var galpinni*, (Figure **4.34**).

Table 4.42: Percentage $ABTS^{+\bullet}$scavenging activity of the non volatile extracts of *T. camphoratus*.

Percentage$ABTS^{+\bullet}$radical scavenging activity							
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK	[g]BHT
C							
25	31.10 ± 14.6	13.10 ± 5.9	42.41 ± 4.8	18.33 ± 5.6	22.83 ± 2.1	1.36 ± 0.6	48.0 ± 0.03
50	45.41 ± 2.5	45.67 ± 6.9	74.40 ± 3.8	39.95 ± 6.7	26.90 ± 2.8	13.6 ± 3.5	65.0 ± 3.83
100	74.34 ± 8.5	73.29 ± 5.6	91.30 ± 4.4	63.75 ± 3.9	32.10 ± 2.1	19.18 ± 3.0	80.0 ± 0.98
150	88.36 ± 5.2	86.89 ± 0.7	91.56 ± 0.5	74.24 ± 2.6	35.03 ± 1.4	25.97 ± 1.7	82.0 ± 0.24
200	91.18 ± 4.1	93.27 ± 1.2	91.87 ± 0.4	83.35 ± 0.9	47.04 ± 8.7	36.35 ± 3.1	85.0 ± 1.72
250	93.16 ± 1.1	94.18 ± 0.2	92.47 ± 0.3	86.27 ± 1.1	47.37 ± 6.9	36.58 ± 4.9	88.2 ± 1.20

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 67.75x – 64.61 LC_{50} = 49.16[a]

b. Linear equation: y = 83.38x – 98.65 LC_{50} = 60.64[b]

c. Linear equation: y = 47.62x –14.36 LC_{50} = 22.46[c]

d. Linear equation: y = 69.77x - 78.24 LC_{50} = 68.87[d]

e. Linear equation: y = 18.4x – 5.3 LC_{50} = 1012[e]

f. Linear equation: y = 26.17x – 30.75 LC_{50} = 1239.5[f]

g. Linear equation: y = 31.55X + 13.32 LC_{50} = 14.54[g]

Table 4.43: Percentage $ABTS^{+\bullet}$ radical scavenging activity of the non volatile extracts of *T. Trilobus var galpinni*.

Percentage $ABTS^{+\bullet}$ scavenging activity						
	[a]MET LF	[b]DCM LF	[c]Et Ac LF	[d]DCM BK	[e]Et Ac BK	[f]BHT
C						
25	21.38 ± 1.9	19.61 ± 1.5	6.47 ± 2.5	2.78 ± 1.6	5.38 ± 2.23	48.0 ± 0.03
50	26.97 ± 2.7	25.95 ± 3.4	10.50 ± 1.3	10.24 ± 6.6	7.05 ± 1.74	65.0 ± 3.83
100	33.59 ± 3.6	32.40 ± 1.6	18.22 ± 2.7	14.32 ± 3.3	9.70 ± 1.32	80.0 ± 0.98
150	40.70± 4.3	35.84 ± 3.7	25.81 ± 4.1	18.87 ± 1.6	13.00 ± 1.89	82.0 ± 0.24
200	44.29 ± 4.7	40.62 ± 1.9	33.61 ± 2.9	23.05 ± 3.1	17.72 ± 2.48	85.0 ± 1.72
250	51.75 ± 3.3	45.25 ± 2.8	39.61 ± 4.7	27.78 ± 3.2	21.76 ± 2.10	88.2 ± 1.20

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: $y = 28.82x - 21.06$ $LC_{50} = 292^{a}$

b. Linear equation: $y = 24.35x - 15.31$ $LC_{50} = 480^{b}$

c. Linear equation: $y = 32.73x - 42.95$ $LC_{50} = 691^{c}$

d. Linear equation: $y = 23.21x - 30.14$ $LC_{50} = 2836^{d}$

e. Linear equation: $y = 15.37x - 18.24$ $LC_{50} = 2.7 \times 10^{4e}$

f. Linear equation: $y = 31.55X + 13.32$ $LC_{50} = 14.54^{f}$

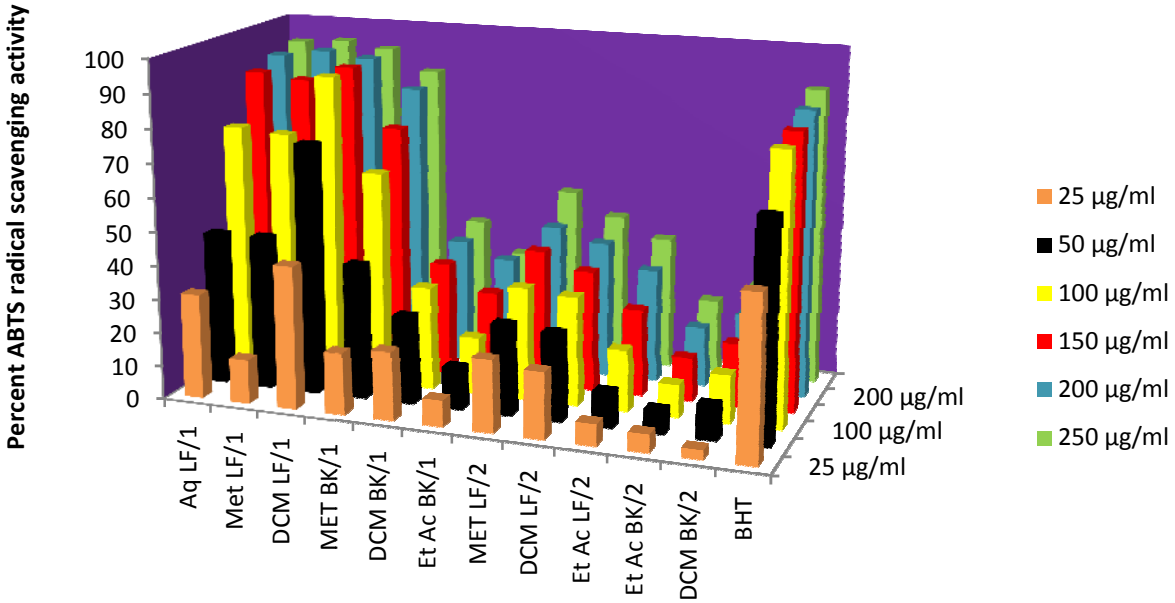


Figure 4.34. Comparison of the $ABTS^{\bullet+}$ radical scavenging og the non volatile extracts of *T. camphoratus*and *T.trilobus var galpinni*.

Total reducing powerof the non volatile extracts of *T. camphoratus* and *T.trilobus var galpinni*.

Similar to radical scavenging activity, the reducing power of the extracts increased with increasing concentration and the leaf extracts showed higher reducing abilities than the bark extracts. For *T.camphoratus*, the DCM LF extract showed a reducing ability greater than that of the standard ascorbic acid at all concentrations whereas at concentrations less than 250 µg/ml, the Aq Lf/1 and MET LF/1 showed higher reducing abilities than the reference standard, ascorbic acid, (Table 4.44). The reducing ability of the bark extracts was in the order MET BK > DCM BK >Et Ac BK. There was a significant difference in the means of the absorbance between the treatments at $p = < 0.05$. Just like in the radical scavenging activities, the reducing activities of the extracts of *T. trilobus var galpinni* were lower than

that of *T. camphoratus*. Again the leaf extracts showed higher activity when compared to the bark extracts, the MET LF/2 and DCM LF/2 having the highest reducing power, (Table 4.45). All the *T. trilobus var galpinni* non volatile extracts showed a very low reducing power as compared to that of the standard, ascorbic acid. There was a significant difference in the means of the absorbance between the treatments at p = < 0.05.

Table 4.44: Total reducing power of the non volatile extracts of *T.camphoratus.*

Absorbance at 700nm							
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK	[g]Ascorbic acid
C							
25	0.75 ± 0.1	0.73 ± 0.2	1.45 ± 0.1	0.47 ± 0.0	0.15 ± 0.1	0.13 ± 0.1	0.34 ± 0.01
50	0.88 ± 0.1	0.88 ± 0.1	1.69 ± 0.1	0. 56 ± 0.3	0.20 ± 0.0	0.14 ± 0.3	0.40 ± 0.03
100	1.02 ± 0.0	1.02 ± 0.1	1.99 ± 0.1	0.63 ± 0.0	0.21 ± 0.0	0.16 ± 0.0	0.78 ± 0.03
150	1.21 ± 0.1	1.15 ± 0.0	2.32 ± 0.2	0.71 ± 0.0	0.22 ± 0.1	0.19 ± 0.2	1.03 ± 0.01
200	1.37 ± 0.1	1.27 ± 0.1	2.5 ± 0.0	0.85 ± 0.1	0.23 ± 0.0	0.20 ± 0.1	1.19 ± 0.06
250	1.40 ± 0.0	1.36 ± 0.1	2.5 ± 0.0	0.92 ± 0.4	0.25 ± 0.1	0.21 ± 0.2	1.58 ± 0.03

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

Table 4.45:Total reducing power of the non volatile extracts of *T. Trilobus var galpinni*.

Absorbance at 700nm						
	[a]MET LF	[b]DCM LF	[c]Et Ac LF	[d]DCM BK	[e]Et Ac BK	[f]Ascorbic acid
C						
25	0.14 ± 0.004	0.106 ± 0.005	0.13 ± 0.011	0.10 ± 0.001	0.07 ± 0.06	0.34 ± 0.01
50	0.22 ± 0.014	0.128 ± 0.002	0.19 ± 0.007	0.10 ± 0.002	0.11 ± 0.004	0.40 ± 0.03
100	0.28 ± 0.011	0.140 ± 0.002	0.21 ± 0.005	0.11 ± 0.005	0.114 ± 0.003	0.78 ± 0.03
150	0.29 ± 0.005	0.144 ± 0.005	0.24 ± 0.025	0.12 ± 0.004	0.118 ± 0.002	1.03 ± 0.01
200	0.31 ± 0.007	0.177 ± 0.01	0.28 ± 0.009	0.12 ± 0.01	0.125 ± 0.003	1.19 ± 0.06
250	0.36 ± 0.032	0.214 ± 0.007	0.29 ± 0.003	0.15 ± 0.01	0.126 ± 0.003	1.58 ± 0.03

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

Nitric oxide inhibition of the non volatile extracts of *T. camphoratus* and*T.trilobus var galpinni*.

The nitric oxide inhibition by all the extracts of *T. camphoratus* and *T. trilobus var galpinni* was very poor. For *T. camphoratus* extracts, the maximum inhibition was 9.7% for the DCM LF/1 extracts at the highest concentration of 250 µg/ml and that of ascorbic acid, the standard, at the same concentration was 77%, (Table 4.46). The lowest LC_{50} value of the extracts, 1.28 x 10^{7}µg/ml, was far higher than 7.91x10^{1} µg/ml for the standard. There was a significant difference in the means of percentage inhibition between the treatments p = <0.05. Among the *T. trilobus var galpinni* extracts, the MET LF extract showed the highest nitric oxide inhibition with an LC_{5O} value of 4.74 x 10^{9} which was not comparable to that of the standard, (Table 4.47). Comparison of the nitric oxide inhibition abilities of *T. camphoratus* and *T. trilobus var galpinni* extracts is illustrated in Figure **4.35**. The *T. trilobus var galpinni* showed poorer nitric oxide inhibition than *T. camphoratus.*

Table 4.46: Percentage nitric oxide inhibition of the leaf and bark extracts of *T.camphoratus*.

Percentage nitric oxide inhibition							
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK	[g]Ascorbic acid
C							
25	0.53 ± 0.6	0.2 ± 0.4	1.23 ± 0.7	0	0.26 ± 0.3	0.2 ± 0.2	29.8 ± 1.3
50	0.93 ± 0.8	0.65 ± 0.7	2.67 ± 2.0	0. 17 ± 0.3	0.47 ± 0.4	0.27 ± 0.3	41.2 ± 3.0
100	2.03 ± 0.5	2.67 ± 0.3	3.77 ± 1.3	1.83 ± 0.3	1.53 ± 0.5	0.7 ± 0.5	48.7 ± 2.2
150	4.23 ± 1.0	3.47 ± 0.5	7.43 ± 2.8	2.17 ± 0.1	1.97 ± 0.2	1.13 ± 0.2	61.3 ± 2.3
200	6.13 ± 0.5	5.7 ± 0.3	8.6 ± 2.6	3.6 ± 0.7	3.03 ± 0.9	1.23 ± 0.1	68.4 ± 3.3
250	6.63 ± 0.5	6.26 ± 0.2	9.7 ± 0.9	4.2 ± 0.4	4.4 ± 0.7	1.5 ± 0.2	77.1 ± 1.7

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 6.45x – 9.45 $LC_{50} = 1.65 \times 10^{9a}$

b. Linear equation: y = 6.26x – 9.34 $LC_{50} = 3.01 \times 10^{9b}$

c. Linear equation: y = 8.69x – 11.77 $LC_{50} = 1.28 \times 10^{7c}$

d. Linear equation: y = 4.29x - 6.56 $LC_{50} = 1.53 \times 10^{13d}$

e. Linear equation: y = 3.77x – 5.58 $LC_{50} = 5.52 \times 10^{14e}$

f. Linear equation: y = 1.35x – 1.85 $LC_{50} = 2.55 \times 10^{38f}$

g. Linear equation: y = 45.36x – 36.09 $LC_{50} = 7.91 \times 10^{1g}$

Table 4.47: Percentage nitric oxide inhibition of the leaf and bark extracts of *T.trilobus var galpinni*.

Percentage nitric oxide inhibition						
	[a]MET LF	[b]DCM LF	[c]Et Ac LF	[d]DCM BK	[e]Et Ac BK	[f]Ascorbic acid
C						
25	0.59 ± 0.34	0.68 ± 0.19	0.16 ± 0.08	0.37 ± 0.30	0.38 ± 0.22	29.8 ± 1.3
50	1.30 ± 0.39	1.50 ± 0.26	1.81 ± 0.15	0.67 ± 0.12	0.84 ± 0.08	41.2 ± 3.0
100	1.88 ± 0.06	1.86 ± 0.08	2.13 ± 0.13	0.91 ± 0.04	1.11 ± 0.15	48.7 ± 2.2
150	4.44 ± 0.47	2.40 ± 0.07	2.65 ± 0.30	1.53 ± 0.31	1.57 ± 0.09	61.3 ± 2.3
200	4.93 ± 0.07	5.21 ± 0.54	3.78 ± 0.12	2.48 ± 0.27	1.85 ± 0.12	68.4 ± 3.3
250	7.12 ± 0.39	6.05 ± 0.07	3.99 ± 0.03	2.93 ± 0.06	2.13 ± 0.11	77.1 ± 1.7

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 6.07x – 8.73 $LC_{50} = 4.74 \times 10^{9a}$

b. Linear equation: y = 4.94x – 6.90 $LC_{50} = 3.30 \times 10^{11b}$

c. Linear equation: y = 3.57x - 4.71 $LC_{50} = 2.11 \times 10^{15c}$

d. Linear equation: y = 2.45x – 3.41 $LC_{50} = 6.31 \times 10^{21d}$

e. Linear equation: y = 1.69x – 2.05 $LC_{50} = 6.29 \times 10^{30e}$

f. Linear equation: y = 45.36x – 36.09 $LC_{50} = 7.91 \times 10^{1f}$

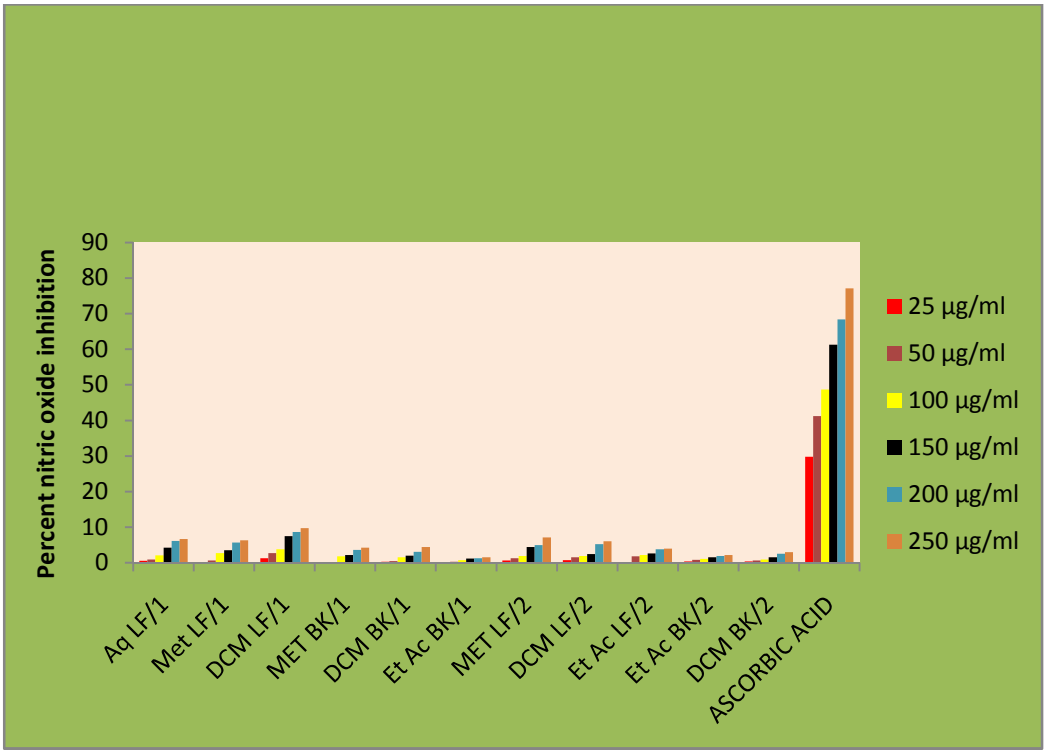


Figure 4.35.Comparison of the nitric oxide inhibition abilities of *T. camphoratus* and *T. trilobus var galpinni*non volatile extracts.

Iron chelating ability of the non volatile extracts of *T. camphoratus* and *T.trilobus var galpinni*.

The iron chelating abilities of the both *T. camphoratus* and *T. trilobus var galpinni* extracts reflected by their LC_{50} values in Table 4.48 and Table 4.49 respectively. The order of iron chelating ability among the *T. camphoratus* extracts was Et Ac BK > DCM LF > MET BK > Aq LF > MET LF > DCM BK. It is only in this assay that some of the bark extracts of *T. camphoratus* showed higher antioxidant activity than the leaf extracts. The iron chelating activities of the *T. trilobus var galpinni* were in the order MET LF > DCM LF >Et Ac LF > DCM BK > Et Ac BK. All the extracts showed lower iron chelating ability compared to EDTA as evidenced by the high LC_{50} values of the extracts, which were not comparable to that of the standard EDTA, (Figure **4.36**).

Table 4.48: Percent iron chelating activity of the leaf and bark extracts of *T.camphoratus*.

Percent iron chelating activity							
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK	[g]Na_2EDTA
C							
25	6.6 ± 2.4	5.5 ± 1.42	6.4 ± 3.7	3.8 ± 0.8	1.22 ± 0.2	8.3 ± 0.7	14.6 ± 3.4
50	9.2 ± 0.3	5.8 ± 1.2	9.3 ± 2.4	9.5 ± 0.5	3.6 ± 0.4	10.7 ± 1.8	21.1 ± 1.9
100	11.1 ± 2.9	8.2 ± 0.1	9.4 ± 2.4	12.3 ± 0.9	3.8 ± 0.1	14.6 ± 3.5	35.0 ± 0.6
150	10.8 ± 0.3	8.7 ± 0.8	11.4 ± 2.1	12.9 ± 0.5	4.3 ± 0.4	16.2 ± 1.3	39 0 ± 2.9
200	13.6 ± 0.8	9.3 ± 0.4	14.8 ± 1.4	13.8 ± 1.0	4.6 ±0.5	17.2 ± 0.8	60.2 ± 1.1
250	14.7 ± 0.2	11.8 ± 1.7	17.9 ± 3.9	14.1 ± 1.3	5.1 ± 0.2	17.6 ± 0.8	72.0 ± 0.9

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 7.38x – 3.71 $LC_{50} = 1.88 \times 10^{7a}$

b. Linear equation: y = 5.74x – 3.24 $LC_{50} = 1.88 \times 10^{9b}$

c. Linear equation: y = 9.95x – 8.34 $LC_{50} = 7.29 \times 10^{5c}$

d. Linear equation: y = 9.85x - 8.58 $LC_{50} = 8.85 \times 10^{5d}$

e. Linear equation: y = 3.32x – 2.86 $LC_{50} = 8.35 \times 10^{15e}$

f. Linear equation: y = 9.86x – 5.59 $LC_{50} = 4.34 \times 10^{5f}$

g. Linear equation: y = 54.07x – 67.57 $LC_{50} = 1.49 \times 10^{2g}$

Table 4.49: Percent iron chelating activity of the leaf and bark extracts of *T. trilobus var galpinni*.

Percent iron chelating activity						
	[a]MET LF	[b]DCM LF	[c]Et Ac LF	[d]DCM BK	[e]Et Ac BK	[f]Na_2EDTA
C						
25	1.28 ± 0.3	1.99 ± 0.1	4.76 ± 0.4	5.42 ± 1.2	1.72 ± 0.2	14.6 ± 3.4
50	3.19 ± 0.2	3.82 ± 0.5	5.76 ± 0.9	5.82 ± 0.1	1.93 ± 0.1	21.1 ± 1.9
100	5.35 ± 0.6	7.43 ± 0.9	8.44 ± 0.4	6.98 ± 0.2	2.19 ± 0.2	35.0 ± 0.6
150	9.49 ± 0.7	8.43 ± 0.6	9.65 ± 0.6	8.23 ± 0.3	2.59 ± 0.2	39 0 ± 2.9
200	11.07 ± 0.5	9.23 ± 0.5	11.63 ± 1.3	9.25 ± 0.2	3.11 ± 0.2	60.2 ± 1.1
250	12.98 ± 0.3	9.99 ± 0.1	12.05 ± 0.9	9.61 ± 0.1	3.14 ± 0.1	72.0 ± 0.9

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 11.79x – 16.31 $LC_{50} = 4.21 \times 10^{5a}$

b. Linear equation: y = 8.29x – 9.74 $LC_{50} = 1.61 \times 10^{7b}$

c. Linear equation: y = 7.69x – 6.64 $LC_{50} = 2.32 \times 10^{7c}$

d. Linear equation: y = 4.44x - 1.31 $LC_{50} = 3.60 \times 10^{11d}$

e. Linear equation: y = 1.49x – 0.53 $LC_{50} = 8.17 \times 10^{33e}$

f. Linear equation: y = 54.07x – 67.57 $LC_{50} = 1.49 \times 10^{2g}$

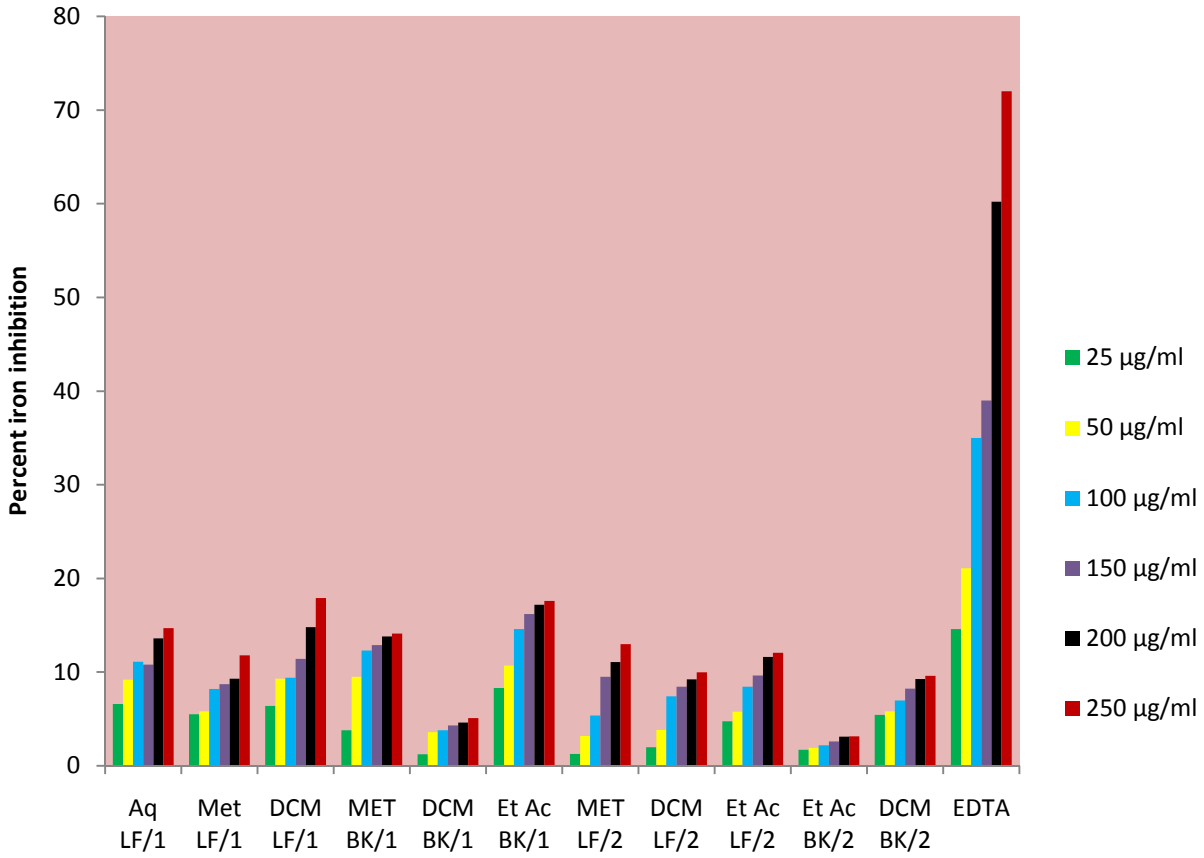


Figure 4.36.Comparison of the iron chelating ability of the non volatile extracts of of *T. camphoratus* and *T. trilobus var galpinni.*

4.2.2.3 Cytotoxicity of the non volatile extracts

Cytotoxicity of the non volatile extracts *T. camphoratus* against brine shrimps

Results of the percentage mortality of the extracts of *T. camphoratus* at different concentrations and LC_{50} values after 24 h are shown in Table 4.50. The extracts showed toxicity to brine shrimps in a dose dependent manner. At the lowest concentration of 10 µg/ml, no mortality was observed for Aq LF, MET LF, DCM BK and Et Ac BK whereas at the highest concentration of 1000 µg/ml, only the MET BK extract showed 100% mortality. The DCM BK, MET BK and standard potassium dichromate had LC_{50} values < 100 µg/ml whereas

DCM LF, Aq LF and Et Ac BK had LC_{50} values in the range of 100 - 1000 µg/ml and only the MET LF extract had an LC_{50} value > 1000 µg/ml. The LC_{50} values were in the order MET BK < DCM BK <Et Ac BK < DCM LF < Aq LF < MET LF. There was no significant difference in the means of percentage mortality of the different extracts between the treatments at p = > 0.05.

Table 4.50: Percentage mortality of the non volatile extracts of *T. camphoratus* against brine shrimps.

% mortality of brine shrimps							
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK	[g] $K_2Cr_2O_7$
C							
10	0.00 ± 0.00	0.00 ± 0.00	13.33 ± 5.77	6.67 ± 5.77	0.00 ± 0.00	0.00 ± 0.00	93.33 ± 5.77
100	10.0 ± 10.00	20.00 ± 0.00	16.67 ± 5.77	56.67 ± 15.27	53.33 ± 15.27	10.0 ± 10.00	100 ± 0.00
500	23.33 ± 5.77	36.67 ± 5.77	63.33 ± 11.55	96.67 ± 5.77	90.00 ± 17.32	86.67 ± 11.54	100 ± 0.00
1000	86.67 ± 5.77	53.33 ± 5.77	86.67 ± 0.5	100.00 ± 0.00	96.67 ± 5.77	96.67 ± 5.77	100 ± 0.00

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 34.65x – 45.36 LC_{50} = 565.06[a]

b. Linear equation: y = 25.24x – 27.40 LC_{50} = 1165.63[b]

c. Linear equation: y = 36.41x – 34.17 LC_{50} = 204.98[c]

d. Linear equation: y = 48.79x - 41.11 LC_{50} = 73.67[d]

e. Linear equation: y = 49.71x – 48.11 LC_{50} = 94.11[e]

f. Linear equation: y = 52.17x – 65.13 LC_{50} = 160.99[f]

g. Linear equation: y = 3.31x + 91.13 $LC_{50} = 3.75 \times 10^{-12}$[g]

Cytotoxicity by the non volatile extracts *T. trilobus var galpinni* against brine shrimps

The brine shrimp percentage mortality results at different concentrations and the LC_{50} values of the non volatile extracts of *T. trilobus var galpinni* are shown in Table 4.51. No

mortality was observed at the lowest concentration which was10 µg/ml in all the extracts and at all the concentrations used no extract showed 100% mortality on the brine shrimps. All the extracts showed a percentage mortality of 50% and above except the DCM BK extract. The MET LF and DCM LF exhibited the highest cytotoxicity with LC_{50} values of 517 µg/ml and 659 µg/ml respectively. The Et Ac LF, DCM BK and Et Ac BK had LC_{50} values > 1000 µg/ml. There was no significant difference in the means of percentage mortality between the treatments at p = > 0.05.

Table 4.51: Percentage mortality of the non volatile extracts of *T. trilobus var galpinni* against brine shrimps.

Percentage mortality of brine shrimps						
	[a]MET LF	[b]Et Ac LF	[c]DCM LF	[d]DCM BK	[e]Et Ac BK	[f]$K_2Cr_2O_7$
C						
10	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	93.33 ± 5.77
100	30.00 ± 10.00	13.33 ± 5.78	30.00 ± 10.0	16.67 ± 5.77	23.33 ± 0.58	100.0 ± 0.00
500	43.33 ± 15.27	43.33 ± 5.78	43.33 ± 5.77	30.00 ± 10.0	40.00 ± 1.00	100.0 ± 0.00
1000	63.33 ± 5.77	56.67 ± 5.78	56.67 ± 5.77	46.67 ± 11.55	50.00 ± 1.00	100.0 ± 0.00

C = Concentration (µg): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 29.47x – 29.92 $LC_{50} = 5.17 \times 10^{2a}$

b. Linear equation: y = 27.38x – 33.38 $LC_{50} = 1.11 \times 10^{3b}$

c. Linear equation: y = 27.15x – 26.54 $LC_{50} = 6.59 \times 10^{2c}$

d. Linear equation: y = 21.69x – 23.84 $LC_{50} = 1.02 \times 10^{3d}$

e. Linear equation: y = 24.58x – 25.11 $LC_{50} = 1.14 \times 10^{3e}$

f. Linear equation: y = 3.31x + 91.13 $LC_{50} = 3.75 \times 10^{-12f}$

Comparison of the brine shrimp mortality of the non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni*

In Figure **4.37**, the percentage brine shrimp mortalities by the extracts of both *T. camphoratus* and *T. trilobus var galpinni* are compared. Generally, the non volatile extracts of *T. camphoratus* exhibited higher cytotoxicity against brine shrimps than the *T. trilobus var galpinni* non volatile extracts.

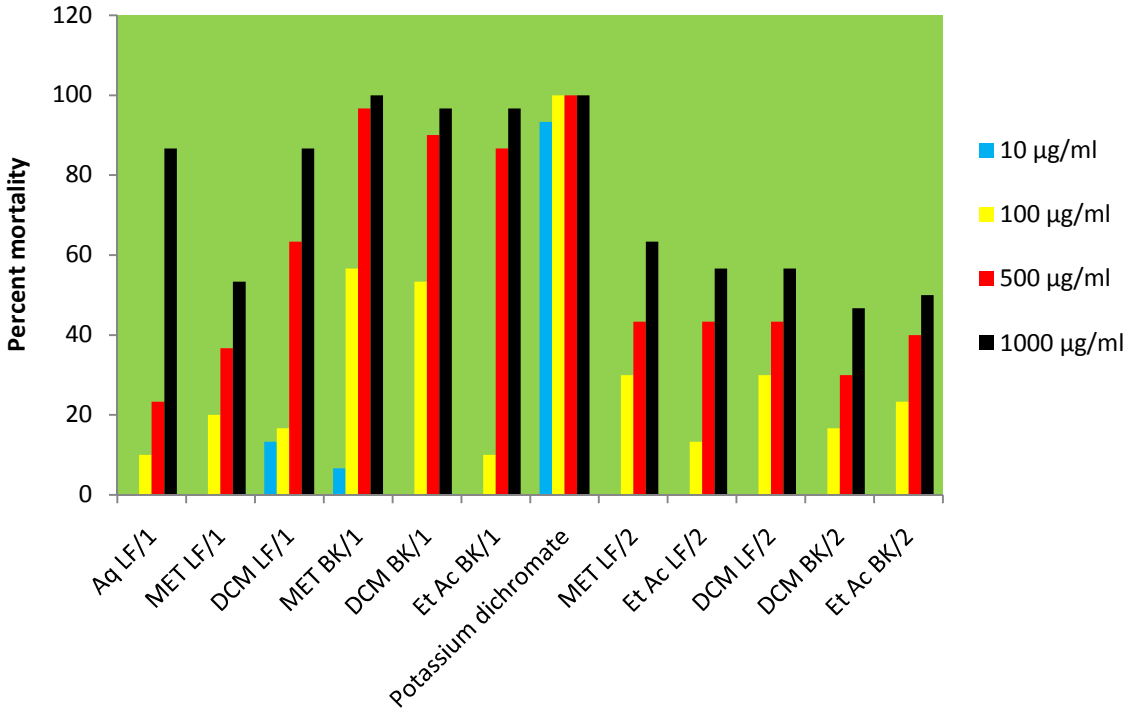


Figure 4.37.Comparison of the percentagebrine shrimp mortalities by the extracts of *T. camphoratus* (1) and *T. trilobus var galpinni* (2).

Cytotoxicity by the non volatile extracts of *T. camphoratus* using the MTT assay

The percentages of the cytotoxicity of the non volatile extracts of *T. camphoratus* against human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells are summarised in Table 4.52 and 4.53 respectively. The extracts showed toxicity towards both

the cell lines in a dose dependent manner. At the highest concentration used the Aq LF extract and the DCM BK extract showed the lowest percentage cytotoxicity against HEK293 and HepG2 respectively. The MET LF extract showed the highest percentage cytotoxicity against both cell lines at the highest concentration used. The DCM LF and the MET LF extract had the lowest LC_{50} value against the HEK293 and HepG2 cell lines respectivelywhereas the Aq LF and DCM BK had the highest LC_{50} value against HEK293 and HepG2 cell lines respectively. The order of increasing cytotoxicity of the extracts against the HEK293 cell line was Aq LF < DCM BK < MET BK <Et Ac BK < MET LF < DCM LF where as against HepG2 cell line it was DCM BK < MET BK < DCM LF < Aq LF < Et Ac BK < MET LF.

Table 4.52: Percentage cytotoxicity of the non volatile extracts of *T. camphoratus* against human embryonic kidney cells.

Percentage cytotoxicity						
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK
C						
50	15.4 ± 0.02	31.6 ± 0.01	36.3 ± 0.01	32.1 ± 0.03	38.9 ± 0.02	13.3 ± 0.05
100	21.5 ± 0.02	35.1 ± 0.03	39.7 ± 0.03	37.1 ± 0.02	42.6 ± 0.02	22.3 ± 0.05
150	30.4 ± 0.03	39.1 ± 0.02	44.0 ± 0.02	39.8 ± 0.01	42.4 ± 0.01	29.7 ± 0.02
200	31.6 ± 0.05	41.1 ± 0.02	44.6 ± 0.03	42.6 ± 0.01	43.7 ± 0.01	35.7 ± 0.06
250	31.3 ± 0.02	45.8 ± 0.03	47.4 ± 0.02	45.1 ± 0.01	44.9 ± 0.01	43.7 ± 0.05
300	35.4 ± 0.02	50.5 ± 0.04	51.6 ± 0.02	46.7 ± 0.01	47.3 ± 0.01	47.4 ± 0.02
350	41.4 ± 0.05	55.2 ± 0.02	53.5 ± 0.01	50.1 ± 0.02	49.6 ± 0.02	47.8 ± 0.02

C = Concentration (µg): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 28.13x – 33.09 LC_{50} = 899.05[a]

b. Linear equation: y = 26.56x – 16.54 LC_{50} = 320.09[b]

c. Linear equation: y = 19.92x + 0.918 LC_{50} = 291.04[c]

d. Linear equation: y = 20.29x - 3.28 LC_{50} = 422.6[d]

e. Linear equation: y = 10.97x – 19.95 LC_{50} = 572.16[e]

f. Linear equation: y = 43.92x – 63.59 LC_{50} = 385.73[f]

Table 4.53: Percentage cytotoxicity of the non volatile extracts of *T. camphoratus* against human hepatocellular carcinoma cells.

Percentage cytotoxicity						
	[a]Aq LF	[b]MET LF	[c]DCM LF	[d]MET BK	[e]DCM BK	[f]Et Ac BK
C						
50	12.40 ± 0.04	13.08 ± 0.03	11.09 ± 0.01	7.70 ± 0.02	10.02 ± 0.04	12.49 ± 0.02
100	15.88 ± 0.01	19.66 ± 0.08	18.49 ± 0.02	12.43 ± 0.02	17.93 ± 0.04	17.48 ± 0.03
150	19.51 ± 0.01	23.25 ± 0.04	22.15 ± 0.03	17.45 ± 0.04	20.25 ± 0.03	21.89 ± 0.03
200	22.66 ± 0.04	32.65 ± 0.07	25.22 ± 0.04	19.66 ± 0.05	26.05 ± 0.04	26.64 ± 0.03
250	27.39 ± 0.02	34.49 ± 0.09	32.68 ± 0.04	25.78 ± 0.05	30.60 ± 0.03	31.82 ± 0.04
300	37.50 ± 0.07	42.76 ± 0.05	36.16 ± 0.02	33.57 ± 0.05	31.58 ± 0.03	38.69 ± 0.02
350	49.48 ± 0.05	51.68 ± 0.06	39.58 ± 0.03	39.10 ± 0.03	38.24 ± 0.02	47.67 ± 0.03

C = Concentration (µg) Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 38.27x – 58.90 LC_{50} = 700.76[a]

b. Linear equation: y = 42.67x – 63.99 LC_{50} = 469.28[b]

c. Linear equation: y = 33.36x – 47.84 LC_{50} = 856.75[c]

d. Linear equation: y = 35.31x - 56.43 LC_{50} = 1033.14[d]

e. Linear equation: y = 31.45x – 44.88 LC_{50} = 1039.57[e]

f. Linear equation: y = 38.35x – 57.33 LC_{50} = 629.07[f]

Comparison of the cytotoxicity of the extracts of *T. camphoratus* against the HEK293 and HepG2 cell lines.

The extracts showed a different pattern of activity against the two cell lines. The DCM LF extract showed the highest cytotoxicity against HEK293 cell line whereas MET LF showed the highest cytotoxicity against HepG2 cell line. Generally the non volatile extracts of *T. camphoratus* exhibited higher toxicity against HEK293 than HepG2, (Figure **4.38**). However, all the extracts showed low toxicity against human embryonic kidney cells and human hepatocellular carcinoma cells with high LC_{50} values (> 30 µg/ml). There was no significant

difference between the LC_{50} values of various the treatments in both cytotoxic assays ($p \geq 0.05$).

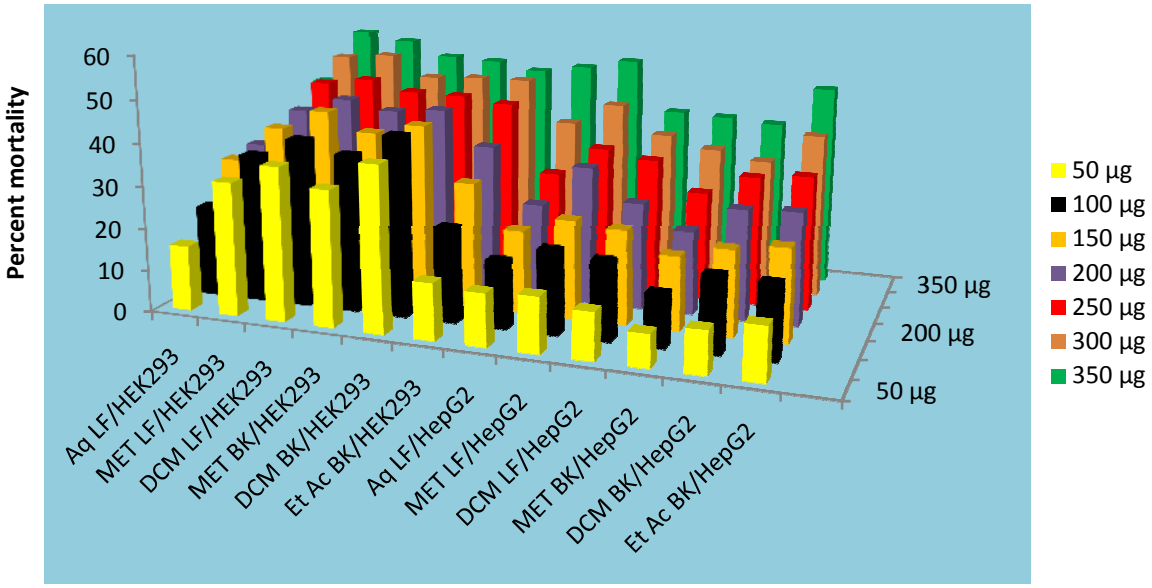


Figure 4.38.Comparison of the cytotoxicity of the extracts of *T. camphoratus* against the HEK293 and HepG2 cell lines

Cytotoxicity by the volatile extracts of *T. trilobus var galpinni* using the MTT assay

The results of the percentage cytotoxicity of extracts revealed that all the extracts except MET LF, showed cytotoxicity against human embryonic kidney, HEK293, cells at higher concentrations, (Table 4.54). The DCM LF extract showed no cytotoxicity at all the concentrations except at the highest concentration of 350 µg/ml. The Et Ac LF extract did not show cytotoxicity at the first three concentrations and even exhibited a low percentage toxicity of 4.19% at the highest concentration. The DCM BK and Et Ac LF showed cytotoxicity only at the highest two concentrations but with low percentage cytotoxicities of 7.21% and 9.86% respectively. The MET LF extract showed toxicity to the HEK239 cells at all the concentrations used and it exhibited the highest cytotoxicity among the extracts as

evidenced by its lowest LC_{50} value. All the extracts except the MET LF had LC_{50} value above 1000 µg/ml. There was no significant difference among the percentage mortality means of Et Ac LF, DCM LF, DCM BK and LF Et Ac BK between the treatments at $p \geq 0.05$ but there was a significant difference in the means of MET LF and the other extracts between the treatments at $p \leq 0.05$. Cytotoxicity of the extracts against human hepatocellular carcinoma cells, HepG2 was also exhibited in a dose dependent manner, (Table 4.55). The highest percentage cytotoxicity of 27.50% was shown by the DCM BK extract at the highest concentration of 350 µg/ml whereas the lowest percentage cytotoxicity at the same concentration was 17.87% by the MET LF extract. There was no significant difference in the percentage cytotoxicities of MET LF, Et Ac LF, DCM LF and Et Ac BK between the treatments at $p \geq 0.05$ whereas there is a significant difference in the percentage cytotoxicities of MET LF and DCM BK between the treatments at $p \leq 0.05$. It is evident from the high LC_{50} values, that the extracts exhibited low cytotoxicity against the human hepatocellular carcinoma cells in the order DCM LF > DCM BK >Et Ac BK > Et Ac BK > MET LF.

Table 4.54: Percentage cytotoxicity of the non volatile extracts of *T. trilobus var galpinni*against human embryonic kidney cells.

Percentage mortality					
	[a]MET LF	[b]Et Ac LF	[c]DCM LF	[d]DCM BK	[e]Et Ac BK
C					
100	28.60 ± 0.03	0	0	0	0
150	30.27 ± 0.04	0	0	0	0
200	36.42 ± 0.03	0	0	0	0
250	37.49 ± 0.03	2.79 ± 0.05	0	0	0
300	42.32 ± 0.02	1.88 ± 0.03	0	3.59 ± 0.04	0.64 ± 0.03
350	46.90 ± 0.03	4.19 ± 0.03	1.28 ± 0.05	7.21 ± 0.03	9.86 ± 0.01

C = Concentration (µg/ml): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 33.07x – 39.64 LC_{50} = 513.58[a]

b. Linear equation: y = 7.27x – 15.37 LC_{50} = 9.8 x 10^{8b}

c. Linear equation: y = 1.43x – 3.09 LC_{50} = 1.34 x 10^{37c}

d. Linear equation: y = 10.87x -23.38 LC_{50} = 5.63 x 10^{6d}

e. Linear equation: y = 11.50x – 24.90 LC_{50} = 3.25 x 10^{6e}

Table 4.55: Percentage cytotoxicity of the non volatile extracts of *T. trilobus var galpinni* against human hepatocellular carcinoma cells.

Percentage mortality					
	[a]MET LF	[b]Et Ac LF	[c]DCM LF	[d]DCM BK	[e]Et Ac BK
C					
100	2.0 ± 0.02	5.15 ± 0.02	4.0 ± 0.01	16.70 ± 0.04	4.44 ± 0.03
150	4.13 ± 0.01	5.46 ± 0.01	4.35 ± 0.02	16.90 ± 0.02	6.30 ± 0.02
200	6.40 ± 0.01	7.42 ± 0.02	5.91 ± 0.01	19.30 ± 0.02	10.67 ± 0.03
250	10.71 ± 0.02	14.22 ± 0.02	13.73 ± 0.04	21.40 ± 0.02	13.25 ± 0.02
300	12.40 ± 0.02	16.45 ± 0.01	14.93 ± 0.01	26.90 ± 0.04	16.31 ± 0.02
350	17.87 ± 0.01	18.14 ± 0.01	20.76 ± 0.02	27.50 ± 0.02	23.07 ±0.02

C = Concentration (µg): Data are mean ± SD values of triplicate determinations.

a. Linear equation: y = 27.61x - 55.04 $LC_{50} = 6.37 \times 10^{3a}$

b. Linear equation: y = 26.66x – 50.60 $LC_{50} = 5.94 \times 10^{3b}$

c. Linear equation: y = 30.83x – 60.80 $LC_{50} = 3.93 \times 10^{3c}$

d. Linear equation: y = 21.64x – 28.68 $LC_{50} = 4.32 \times 10^{3d}$

e. Linear equation: y = 31.94x – 61.64 $LC_{50} = 4.68 \times 10^{3e}$

Comparison of the cytotoxicity of the extracts of *T.trilobus var galpinni* against the HEK293 and HepG2 cell lines.

Generally, the non volatile extracts of *T. trilobus var galpinni* showed low cytotoxicity against HEK293 and HepG2 cell lines but the activity towards the HepG2 cell line was higher than against HEK293 cell line, (Figure **4.39**). Similar to *T. camphoratus* non volatile extracts, the non volatile extracts did not show the same trend in cytotoxicity against both cell lines. The MET LF extract which had the highest cytotoxicity against HEK293 had the lowest cytotoxicity against HepG2.

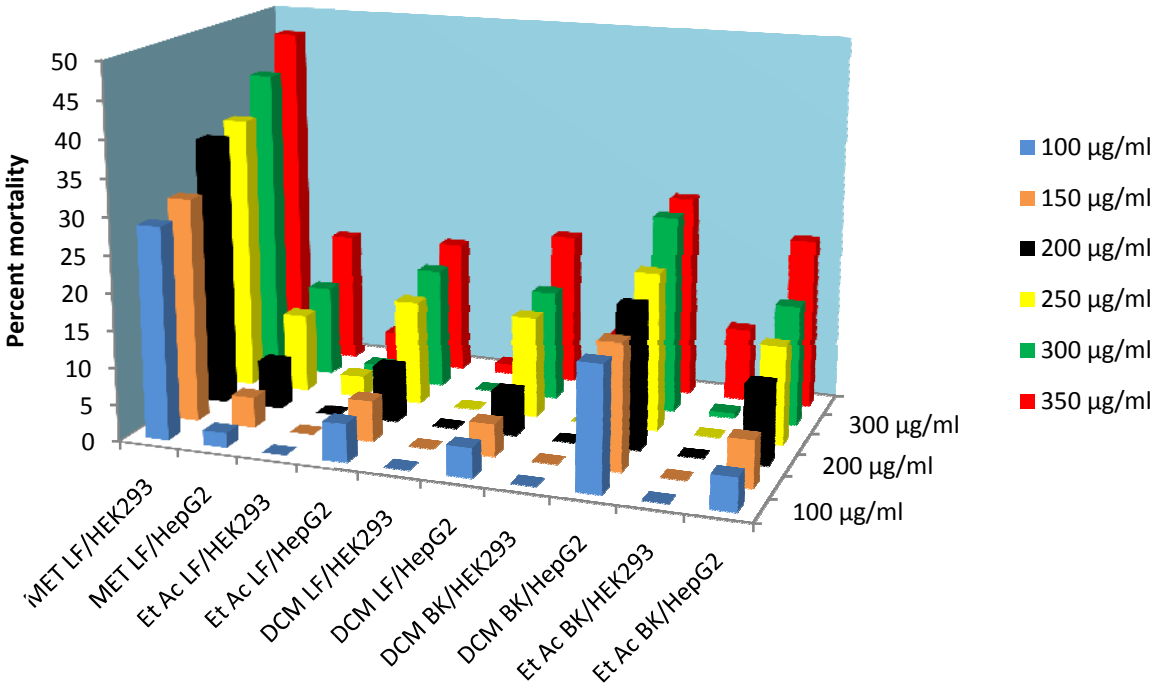


Figure 4.39.Comparison of the cytotoxicity of the extracts of *T. trilobus var galpinni* against the HEK293 and HepG2 cell lines.

4.3 References

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CHAPTER 5

DISCUSSION

5.0 Introduction

In this chapter the results in chapter 4 are discussed. These include chemical composition of the volatile and non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* plus results for the biological activities of the extracts.

5.1 Volatile extracts/essential oils

5.1.1 Chemical composition of the volatile extracts/essential oils of *T.camphoratus* and *T. trilobus var galpinni*

The chemical profiles of the essential oils of two *Tarchonanthus* species were determined by investigating the chemical composition of the essential oils in different categories. Among the different categories was the investigation of the chemical composition of the different plant parts of the leaf and stem of the same species, and identification of the chemical constituents of essential oils of the fresh and dried plant parts of the leaf of the same species. In addition, the essential oil composition of the same plant parts of leaf and stem of the same species harvested at different times was investigated plus the chemical profile of similar plant parts of different species. Quantitative and qualitative similarities and variations were observed for each category investigated. The differences in the chemical composition and yield of the essential oils depend on factors such as geographical origin, genetic variation, growth stages, plant parts utilised, post harvest drying, storage, climate and soil conditions[1-4] and these factors may be difficult to segregate in some instances, since they are interdependent and influence one another[5]. In this study, the essential oils of the

stem and leaves of both species showed variations and similarities in their chemical composition. This variation in chemical constituents, in different plant parts of the same plant, has been reported by other researchers[6,7]and it may be due to the presence of different chemotypes, which depend on the way a plant adapts to its surrounding environment, and also on its stage of development. Differences in the oil composition between glands even of a single leaf have been observed[8]. Oil composition in the glands of any part of a plant is related to the gland age[8] hence different growth stages of a plant creates variations in the oil composition within the same organ or different organs of the same plant[9] and this may partly explain the variation in essential oil chemical constituents and percentage yields in different plant parts. Also noted in this study, were differences inthe chemical profiles of the essential oils of the fresh and dry leaves of the same species harvested at the same time. A similar observation had been made by Demir et al. (2004) and Capecka et al. (2005) who reported significant differences in the chemical composition of the fresh and dry leaves of bay and lemon balm essential oils respectively[10,11]. In both *Tarchonanthus* species, the essential oil of the dry leaves had less chemical compounds than the essential oil of the fresh leaves. In their earlier studies, Fatemeh et al. (2006) and Raghavan et al. (1997) observed that there could be a 50-fold reduction in essential oil chemical composition when plant materials were dried[12,13] and according to Moyler (1994), the essential oils components that are lost in the dried leaves are those stored on or near the leaf surfaces[14]. However, Ibanez et al. (1999) observed no difference in the essential oil composition of fresh and dry rosemary plant[15]. The changes in the nature of the essential oil constituents during drying have been reported to depend on factors such as the drying method, the species of the plant and the drying period[16-18]. Variation in the chemical profiles of essential oils of the same *Tarchonanthus* species, harvested from the same place

at around the same time but in different years, was also observed in this study. Ecological factors like light and temperature influence the production of essential oils[2,19] and since such factors vary greatly throughout the year, it is not surprising therefore, that the essential oil compositions of the same plant harvested from the same locality will vary from time to time. Each of the *Tarchonanthus* species investigated in this study was obtained from the same location but at different times hence no comparison of essential oil components was made based on the differences in the geographical locations. However, essential oil composition of the fresh leaves of *T. camphoratus* from different ecosystems has been reported in literature and the results revealed quantitative and qualitative variations in the chemical constituents[20-22]. The reported major constituents were: 1,8 cineole (16.5%) and α-fenchol alcohol (29.1%)[20], 1,8-cineole (14.3%), α-terpineol (13.2%) and α-fenchol (15.9%)[21], β-caryophyllene (13.4%) and 1,8-cineole (9.3%)[22]. Many reports in the literature show variation in the yield and chemical composition of essential oils of the same plant from different geographical regions[3,23-25] and such variation is mainly attributed to ecological factors. The vernacular name, camphor bush, of *T. camphoratus* suggests the presence of camphor which was also suspected to be responsible for the camphor smell of the essential oils of *T. camphoratus* [20]. Surprisingly, camphor was not present in all the investigated essential oils of *T. camphoratus*[20-22] and it featured in minor quantities in the essential oil investigated by Mwangi et al. (1994)[20] and in the essential oils of 2011 investigated in this study. The oxygenated monoterpene, 1,8-cineole, present either as a major compound or a minor compound in all the investigated essential oils of *T. camphoratus* has a fresh camphor-like smell[26] and it is likely to be the compound responsible for the strong camphor smell of *T. camphoratus.* To the best of our knowledge, there are no earlier reports on the chemical composition of the essential oil of any part of *T.*

trilobus var galpinni. Genetic composition of a particular plant greatly influences the chemical composition of the essential oils produced by the plant. The essential oils of *T. camphoratus* and *T. trilobus var galpinni* showed differences in the chemical constituents for similar plant parts irrespective of the time of harvest and this may be to the differences of the genetic makeup between the species. Variation of the essential oil composition among plants of the same species is not uncommon. A significant difference in the essential oil composition has been observed within plants of the same species [27-33]. Genetic makeup of a plant is one of the significant contributors to its essential oil composition and variations in the essential oil composition of plants within the same species suggest the presence of different chemotypes within the species[34-38]. In addition to genetic makeup, edaphic and climatic factors have been reported to influence the essential oil composition in different plant species[39-43]. The variations in the chemical constituents of the essential oils of *T. camphoratus* and *T. trilobus var galpinni* could be explained by the same factors.

5.1.2 Antibacterial activities of the volatile extracts/essential oils

The volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* were tested for antibacterial activity against four gram-positive and five gram-negative multi drug resistant bacteria known as causative agents for various infectious diseases. *Staphlococcus aureus* which is one of the major causes of hospital-acquired infections, pneumonia, and staphylococcal meningitis and it has been reported to be resistant to ciprofloxacin, erythromycin, clindamycin, gentamicin, trimethoprim /sulphamethoxazole, linezolid and vancomycin[44]. *Bacillus cereus* a causative agent for food-borne illnesses, meningitis, pneumonia and chronic skin infections[45-47] has been reported to be resisitant to erythromycin and tetracyclines[48]. *Klebsiella pneumoniae* is a common hospital-acquired

pathogen, causing urinary tract infections, nosocomial pneumonia, and intra abdominal infections[49]. Resistance to ampicillin, amoxylline, carbenicillin and to ceftazidime by *Klebsiella pneumoniae* has been reported[50]. *Pseudomonas aeruginosa*, which is a major cause of infectious-related mortality among the critically ill patients, is resistant to a large number of antibiotics[51,52] and hence carries the highest case fatality rate of all gram-negative infections[53]. Another pathogen causing nosocomial infections, *Staphylococcus epidermidis*, has been cited as resistant to many common antibiotics such as methicillin, novobiocin, clindamycin, and benzyl penicillin[54]. *Enterococcus faecalis* and *Escherichia coli* also cause life threatening infections especially in the hospital environment and are resistant to commonly usedantimicrobial agents *like* trimethoprim-sulfame-thoxazole[55,56]. *Shigella flexneri* is a human intestinal pathogen, causing dysentery by invading the epithelium of the colon and is responsible, worldwide, for an estimated 165 million episodes of shigellosis and 1.5 million deaths per year[57]. Resistance of *Shigella flexneri* to antimicrobial agents has also been reported[58]. *Salmonella* is a genus of bacteria that cause typhoid fever which is a major health problem especially in developing countries[59,60] and multidrug-resistant strains of *Salmonella* have been encountered[61,62].

5.1.2.1.Antibacterial activity of the volatile extracts of fresh leaf, dry leaf and dry stem of *T. camphoratus*

The essential oils investigated showed varying zones of inhibition and MIC values. The large diameters of zones of inhibition did not correlate with low MIC values and vice versa although such correlation has been reported for other essential oils[63]. Some researchers have also reported that Gram (+) bacteria are more susceptible to essential oils than Gram (−) bacteria[64] but in this study the essential oils did not show any preferential activity against

Gram positive versus Gram negative micro-organisms. The oils however, showed good antibacterial activity with MIC values less than 1000 µg/ml. The antibacterial activity of essential oils is significant from a clinical perspective if the MIC values are less than 1000 µg/ml[65]. The antimicrobial activity of an essential oil is linked to its chemical composition and at times to the major chemical constituents[66,67]. The major compounds, T-muurolol (10.33%) in the dry stem oil, 1,8-cineole (9.19%), α-cadinol (9.40%), caryophllene oxide (4.21%), in the fresh leaves oil, globulol (10.70%) and β-caryophyllene (5.48%) in the dry leaves oil have been reported to show antibacterial activity[68-71]. These major compounds may have been responsible for the antibacterial activity of the essential oils. However, it has been observed that the antibacterial effects of whole essential oils are stronger than their major components when tested individually[72] and this suggests contribution of other components other than the major compounds to the antibacterial activity of the essential oils. Some researchers have reported that a synergistic effect between the minor and major components in essential oils contributes to the antibacterial activity[73-76]. In *T.camphoratus* essential oils, minor components like caryophyllene oxide, hexadecanoic acid, β-caryophyllene, in the dry stem oil; linalool, β-caryophyllene, α-pinene, germacrene D, p-cymene, terpinen-4-ol and α-terpineol in the fresh leaf oil; 1,8 cineole, linalool and terpinen-4-ol in the dry leaf oil have been reported to show anti-bacterial activity[68,69,77-83]. The different chemical compounds produced by plants may act synergistically during defence against attack by pathogens or during ecological adaptation to a specific habitat[84,85]. Earlier antibacterial studies on the essential oil of the fresh leaves of *T. camphoratus* from different ecosystems revealed that the oils had good antibacterial activity but the species from Eastern Cape, South Africa, lacked activity on *E. faecalis* and *K. pneumonia*[86]and the species from Kenya lacked activity on *P. aeruginosa*[87]. There is no previous report about the

antibacterial activity of the dry stem of *T. camphoratus* from any ecosystem. The oil of the dry stem inhibited the growth of all the tested bacteria which as previously cited have developed resistance to different antibiotics. Of special interest was *P. aeruginosa,* a well known antibiotic resistant Gram-negative bacterium which is generally less sensitive to the actions of plants essential oils[88] but was sensitive to the essential oil of the dry stem. Essential oils are potential sources ofnovel antimicrobial compounds especially against bacterialpathogens[89]. Chances of development of resistance by bacteria to the essential oil as they do to modern synthetic antibiotic drugs are insignificant. Micro-organisms cannot easily mutate and adapt to the many chemical constituents in the essential oils as they can to drugs which contain one or two constituents to which the bacteria can mutate and adapt. The essential oils of *T. camphoratus* could be considered for incorporation in synthetic antibiotics.

5.1.2.2 Antibacterial activity of the volatile extracts of *T.trilobus var galpinni*

The results obtained from the agar disc diffusion method for the essential oil revealed *Escherichia coli* to be the most sensitive micro-organism with the largest inhibition zone (19.5 mm), while *Pseudomonas aeruginosa*, which is generally less sensitive to the action of plant essential oils [88], was also not sensitive to the essential oil of *T. trilobus var galpinni*. The essential oil of the fresh leaves of *T. trilobus var galpinni* was dominated by sesquiterpene hydrocarbons (84.51%) that could be considered responsible for the antibacterial activity. One of the major components in the oil, β-caryophyllene (30.40%), a sesquiterpene, has been reported to exhibit antimicrobial activity[90]. Other minor components like camphene (1.37%), α-pinene (2.55%) and β-pinene (0.30%) present as minor compounds in the oil have also been reported to have antibacterial activity[91]. As

previously mentioned the antibacterial activity shown by an essential oil may be due to compounds acting individually or synergistically. The essential oil of the fresh leaf of *T. trilobus var galpinni* showed strong antibacterial activity as evidenced by the low MIC values. No previous work has been reported on the antibacterial activity of the essential oil of *T. trilobus var galpinni* and the results from this study suggests that the essential oil has potential in the treatment of infectious diseases especially those that are caused by *Klebsiella pneumonia* and *Shigella flexineri*.

5.1.3 Antioxidant activity of the volatile extracts

The antioxidant activity of the plant extracts were determined by the DPPH radical scavenging method, the $ABTS^{+\bullet}$ radical scavenging assay, reducing power activity, nitric oxide scavenging activity and chelating effect of ferrous ions. DPPH is a stable free radical that is dissolved in methanol and its purple colour shows a characteristic absorption at 517 nm. Antioxidant molecules scavenge the free radical by hydrogen or electron donation and the colour from the DPPH assay solution becomes light yellow resulting in a decrease in absorbance. Substances which are able to perform this reduction can be considered as antioxidants and therefore radical scavengers[92]. Similar to DPPH, is the $ABTS^{+\bullet}$ a stable radical cation which is produced by a reaction between ABTS and potassium persulfate. The blue coloured $ABTS^{+\bullet}$ radical has a characteristic absorption at 734 nm. In the presence of an antioxidant/reductant, this pre-formed radical is decolourised. The degree of discoloration in both the DPPH and the $ABTS^{+\bullet}$ radical scavenging assays is monitored spectrophotometrically and it reflects the antioxidant capacities of the test samples to donate electrons or hydrogen atoms. In the reducing power assay also the ability of a sample to donate electrons is measured. The presence of reducers/antioxidants causes the

reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) hence yellow colour of the test solution is changed to blue/green and increasing absorbance at 700 nm indicates an increase in reductive ability of the extracts. Nitric oxide is a diffusible free radical which plays an important role as an effector molecule in diverse biological systems including neuronal messenger, antimicrobial and antitumor activities[93]. However, its over production is linked with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis[94]. Oxygen reacts with the excess of NO to generate nitrite and peroxynitrite anions, which act as free radicals[95]. Removal of excess nitric oxide would have beneficial effects. Iron is also significant in various biological reactions but in excess, its ferrous state accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to the highly reactive hydroxyl radical ($OH^{\bullet}$) which causes severe damage to membranes, proteins and DNA[96].

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + OH^{\bullet}$$

Chelation of iron is significant in reducing its concentration and slowing down or completely stopping the catalysation of lipid peroxidation by the metal.

5.1.3.1 Antioxidant activity of the volatile extracts of *T. camphoratus* and *T.trilobus var galpinni*

Investigation of the antioxidant activity of the essential oils/volatile extracts of the dry leaves, fresh leaves, and dry stem of *T. camphoratus* and of the fresh leaves of *T. tirlobus var galpinni* was done using the DPPH radical scavenging method, the $ABTS^{\bullet+}$ radical scavenging assay, reducing power activity assay and nitric oxide inhibition assay. The oils showed poor radical scavenging activities in both the DPPH and $ABTS^{+\bullet}$ radical scavenging assays and also poor total reducing power. This suggested absence of compounds in the essential oils with

electron donating ability to react with the free radicals, hence making the essential oils studied ineffective primary antioxidants. To study a wider range of possible applications, the essential oils were also investigated for their nitric oxide inhibition. The results revealed that the oils exhibited very poor nitric oxide inhibition. A good correlation between the phenolic content in plants and their antioxidant activityhas been reported[97]. Phenolic compounds like thymol and carvacrol found in some plant essential oils have been reported to have antioxidant activity[98]. Also essential oils rich in monoterpene hydrocarbons have been reported to have high antioxidant activity[99]. Ruberto and Baratta investigated the antioxidant activity of 98 pure essential oil components, which represent the main classes of typical compounds of essential oils and found out that sesquiterpene hydrocarbons exerted a low, if any, antioxidant effect[100].The essential oils in this study were mainly dominated by sesquiterpene hydrocarbons: 36.46%, 59.18% and 18.54% for the fresh leaf, dry leaf and dry stem of *T. camphoratus* respectively and 84.51% for the fresh leaf of *T. tirlobus var galpinni.* The poor antioxidant activity of these essential oils, probably, is due to their lack of phenolic compounds and low concentrations of monoterpene hydrocarbons. However, it has been observed that correlation of the antioxidant activities of essential oils and their chemical compositions is often very complicated[101]. Essential oil constituents acting individually or synergistically may contribute to the antioxidant activity of the oil[102]. From the results obtained, it is unlikely to gain any antioxidant benefit from the essential oils of *T. camphoratus* and *T. tirlobus var galpinni.* The therapeutic potentials of plants have been linked with their antioxidant activities[103]. However, according to Makari et al, low levels of phenolics and other phytochemicals plus low value of antioxidant indices in plants do not translate to poor medical properties[104].

5.1.4 Insecticidal activity of the volatile extracts of *T. camphoratus*and *T.tirlobus var galpinni*

The insecticidal activities of the essential oils were investigated against *Anopheles arabiensis* mosquito larvaeand stored product pests, *S. zeamais* and *S. oryzae*. The essential oils investigated were of the dry leaf of *T. camphoratus* harvested November 2009 and of the fresh leaf of *T.trilobus var galpinni* harvested 2010.

5.1.4.1 Larvicidal activity of the volatile extracts of *T. camphoratus* and *T.trilobus var galpinni.*

A positive correlation was observed between the essential oil concentration and the percentage mortality, the rate of mortality being directly proportional to the concentration. According to an earlier report by Cheng et al. (2003), essential oils with LC_{50} values > 100 ppm are considered not active, whereas those with LC_{50} values < 50 ppm are regarded as highly active[105]. Considering the LC_{50} limits set by Cheng et al. (2003), the essential oil of *T. camphoratus*, with an LC_{50} value of 78.7 ppm after 24h,had moderate larvicidal activity whereas that of *T. trilobus var galpinni*, with an LC_{50} value of 110.32 ppm after 24h,had low larvicidal activity against the larvae of *Anopheles arabiensis*. Biological properties of essential oils have been attributed to their chemical constituents[106]. The observed larvicidal activity of *T. camphoratus* may be attributed to the presence of hexadecanoic acid (3.5%), which has previously been reported to exhibit larvicidal activity on other mosquito species like, the *Plasmodium falciparum* 22, *Culex quinquefasciatus, Anopheles stephensi* and *A. aegypti*[107]. Other larvicidal compounds in the essential oil included 1,8-cineole (5.4 %), which was reported to show larval mortality against *A. aegypti*[108],α-terpinene (2.6%), γ-terpinene (0.2%) and terpinolene (0.3%) which when tested singly showed larvicidal

activities against *A. aegypti* and *A. albopictus* with LC_{50}< 50 ppm[109]. Also α-pinene and β-pinene have been reported to have larvicidal activity[110]. The larvicidal activity of the oil may be a result of a synergistic effect of some or many of its constituents rather than a single major constituent. According to Isman (2000), some essential oils have a broad spectrum of biological activities, and the action of these is caused, possibly, by the compound(s) that occur(s) in greater quantity in the oil and/or synergy between compounds[106]. It is also possible that the activity of the main components is modulated by other minor compounds[111].This study revealed that the essential oil of the dry leaf of *T. camphoratus* has larvicidal potential and could be considered in the development of plant based larvicides. Although the essential oil may show much less activity than the synthetic larvicides like Temephos, it is recommended for use as a larvicide because it is more biodegradable with lower non-target toxicity and is environmentally friendly. In addition, plant derived insecticides comprise of a variety of components with different mechanisms of action and the chances of insects developing resistance to such products seem very low[112]. The low larvicidal activity of the essential oil of the leaf of *T. trilobus var galpinni* could be attributed to the nature of its chemical constituents. The oil was dominated by sesquiterpene hydrocarbons (88.49%) and generally essential oils rich in sesquiterpene hydrocarbons have been noted to have low larvicidal activity compared to oils rich in oxygenated sesquiterpenes[113]. Compounds like α-pinene and β-pinene with known larvicidal activities[114] were present in the oil in compositions of 2.55% and 0.30% respectively and probably their concentrations were not high enough to cause significant larvicidal activity. According to some researchers, however, high LC_{50} values for plant extracts are expected and are acceptable[115], considering that the extracts are generally more biodegradable, have lower non-target toxicity and are environmentally friendly.

5.1.4.2 Insecticidal activity of the volatile extracts of *T. camphoratus* and *T.trilobus var galpinni* against *S. zeamais* and *S. oryzae*.

The essential oil of the dry leaves of *T. camphoratus* showed no contact and fumigation toxicity on both *S. zeamais* and *S. oryzae* at the concentrations used. Previous studies have shown that the toxicity of essential oils obtained from aromatic plants against storage pests is related to the oil's main components[116]. Among the essential oil components monoterpenoids have drawn the greatest attention for insecticidal activity against stored product pests[106,117]. Various monoterpenes like 1,8-cineole, linalool, α-pinene, terpinen-4-ol, and α-terpinene have been reported to show contact and fumigation toxicity to stored product pests[118,119]. During fumigation the entrance of the bioactive compounds is through the cuticle whereas in contact toxicity stomach poisoning occurs while the weevils feed on the whole grains. In both cases the weevils have to pick up the lethal dose of treatment from the essential oil to cause toxicity. Although the essential oil contained monoterpenoids with reported contact and fumigation toxicity against stored product pests, the oil showed poor contact and fumigation toxicity against both *S. zeamais* and *S. oryzae*. Volatile and non volatile plant extracts have been found to be potent against adult weevils *S. zeamais* and *S. oryzae* in a concentration dependent manner[120]. The percentage composition of the monoterpenoids in the essential oil was low and the poor toxicity of the essential oils may be due to the fact that, the concentrations used could not provide the lethal dose of the toxic compounds to cause mortality. The essential oil, however, showed good repellent action against both *S. zeamais* and *S. oryzae*. Some of the major and minor compounds present in the essential oil have been reported to have insecticidal activity. Linalool, 1,8-cineole, γ-terpinene and terpinen-4-ol present in the oil as minor components have been reported to have repellent activity against stored product pests[118,119] whereas δ-cadinene,

one of the major compounds has been reported to have repellent activity against some anthropods[121]. However, there is a possibility of synergistic action between major and minor components to effect the repellent action of the oil. Plant essential oils are mixtures of different major and minor components and their biological activity is generally determined by their major components or synergism/antagonism among different components[122,123]. The repellent activity of the essential oil could be used to prevent insect infestations of stored grains by incorporating the appropriate amount into the packaging material and by increasing the repellent activity in appropriate formulations. Although this may require large scale trials to determine the application method for the repellence of these oils against stored product insect pests, the essential oil could present a possible alternative to chemical insecticides against stored product pests.

5.1.5 Cytotoxicity of the volatile extracts

5.1.5.1 Cytotoxocity of the volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* against brine shrimps

The lethal concentration, LC_{50}, of the brine shrimps is one of the indicators of the toxicity of a substance. Earlier reports indicate that crude extracts and pure substances with LC_{50} values less than 1000 µg/ml are toxic whereas other reports consider cytotoxiciy to be significant for an LC_{50} value of less than 30 µg/ml[124]. When investigating the brine shrimp toxicity of some plants used in Tanzanian traditional medicine, Moshi et al.(2010) provided circumstantial evidence that plant extracts with LC_{50} values below 20 µg/ml contained anticancer compounds[125]. According to Moshi et al. (2010) an extract with LC_{50} values between 1.0 and 10.0 µg/ml was toxic, one with LC_{50} values in the range of 10.0 to 30.0 µg/ml was moderately toxic whereas an extract with LC_{50}values greater than 30 but less than 100 µg/ml was mildly toxic and the one with LC_{50} above 100 µg/ml was not toxic. In

this study, the LC_{50} values obtained in the BSLA for the essential oil of the fresh leaf, dry leaf and dry stem of *T. camphoratus* were 889 µg/ml, 676 µg/ml and 442 µg/ml respectively and 4335 µg/ml for the fresh leaf essential oil of *T. trilobus var galpinni*. Considering the LC_{50} limits for toxicity as suggested by Moshi et al. (2010) all the essential oils investigated in this study were not toxic. The biological properties of essential oils are mainly determined by their major compounds acting individually or in synergy with other various components[126]. The major compounds in the essential oil of the fresh leaf of *T. camphoratus* were α-cadinol (9.40%), 1,8-cineole (9.19%), δ-cadinene (6.89%) and caryophyllene oxide (4.21%) and in the dry leaf were β-guaiene (10.70%), γ-cadinene (9.09%), δ-cadinene (6.80%), Aromadendrene (6.12%), β-caryophyllene (5.48%), γ-muurolene (5.13%) whereas butanal (35.77%) and t-muurolol (10.33%) were the major compounds in the dry stem essential oil. Longifolene (5.64%), γ-muurolene (5.80%) and β-caryophyllene (30.40%), were the major compounds in the essential oil of the fresh leaf of *T. trilobus var galpinni*. Generally cytotoxicity of essential oils has been reported to be mostly due to alcohols, phenols and aldehydes[127].The essential oils of the fresh leaf, dry leaf and dry stem of *T. camphoratus* were mainly dominated by sesquiterpenes and lack of cytotoxicity by the oils may be explained by the total absence of toxic compounds or their presence in trace amounts. However, considering the LC_{50} limit of 30 µg/ml, the essential oil of the fresh leaf, dry leaf and dry stem of *T. camphoratus* can be considered as having some physiologically active principles. The results from the BSLA are not conclusive about the cytotoxicity of plant extracts. Some plants, known to be toxic to livestock have been found to be non-toxic to brine shrimps[128].

5.1.5.2 Cytotoxicity of the volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* using the MTT assay

The MTT assay, which is a well established method to assess mitochondrial competence,[129] was carried out alongside brine shrimp assay in the present work for better assessment of cytotoxicity of the plant extracts. The essential oils of the fresh leaf, dry leaf and dry stem of *T. camphoratus* plus the essential oil of the fresh leaf of *T. camphoratus var galpinni* were tested for their activity against two cell lines, the human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2). Although the essential oils contained compounds like α-cadinol, β-caryophyllene, caryophyllene oxide and α-pinene[130-135] that have been reported to be cytotoxic against different cell lines, the oils did not show significant cytotoxicity as evidenced by their high LC_{50} values above 100 µg/ml. Alpha-cadinol present in the fresh leaf oil of *T. camphoratus* as a major compound has been reported to be active against tumour cell lines[130,131]. Present in the dry leaf essential oil of *T.camphoratus* and in the fresh leaf oil of *T. camphoratus var galpinni* as a major compound was β-caryophyllene whose cytotoxicity reports are controversial. Reports from some studies indicate that β-caryophyllene has no cytotoxic activity against human cells[136] whereas other studies report its cytotoxic activities against breast carcinoma cell-lines[132]. Likewise, the cytotoxicity results for caryophyllene oxide, which is one of the major compounds in the essential oil of the fresh leaf of *T. camphoratus*, are contradictory. Some investigators have reported caryophyllene oxide to be inactive against tumor/cancer cell lines[137] whereas others reported modest to excellent activity against human cancer cell lines[133,134]. However, different findings have revealed differences in sensitivities by different cell lines to the same plant extract or natural compound[135]. For example, α-pinene at a concentration of 200 µg/ml is not toxic to monkey kidney and human colon adenocarcinoma cells whereas at the

same concentration it exhibits some degree of cytotoxicity against human cervical carcinoma and human lung carcinoma cell lines[135]. The different sensitivities of different cell lines to the same plant extract or compound may be one of the explanations why the essential oils investigated in this study displayed low toxicity yet they contained some of the compounds known to be cytotoxic. The HEK293 and HepG2 cell lines were slightly more sensitive to the essential oils of *T. camphoratus* than to the essential oil of *T.trilobus var galpinni*as shown by the higher LC_{50} values of the latter. As earlier stated, major compounds generally determine the biological properties but this effect can be influenced by the chemical composition and synergism between the various components[126]. Results from the MTT assay further confirmed that the essential oils of the fresh leaf, dry leaf and dry stem of *T. camphoratus* and of the fresh leaf of *T. trilobus var galpinni* did not have anti-cancer agents and were not toxic.

5.2 Non volatile/solvent extracts

5.2.1 Chemical composition of the non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni*

Phytochemical screening of the solvent extracts of both *T. camphoratus* and *T. trilobus var galpinni* revealed the presence of phenols, flavonoids, saponins, tannins and terpenoids which are known to have different biological activities. Further analysis of the extracts by Py-GC/MS revealed a variety of compounds which were categorised as hydrocarbons, phenols, fatty acids, fatty acid methylesters, fatty alcohols, alkaloids, triterpenoids, diterpenoids and tetraterpenoids, (Tables 4.28-4.38). These groups of compounds are also known to have a variety of biological activities and some of the compounds present in the

extracts have been reported to have significant biological activities especially in the pharmaceutical field.

5.2.2 Biological activities of the non volatile extracts of *T.camphoratus* and *T. trilobus var galpinni*

5.2.2.1 Antibacterial activity

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and phenolics which have been found to have antimicrobial properties[138-143]. Phytochemical screening of the extracts of the leaves and bark of *T. camphoratus* and *T. trilobus var galpinni* revealed the presence of different phytochemicals in different extracts with flavonoids and phenols present in all the *T. camphoratus* extracts and in all the leaf extracts of *T. trilobus var galpinni*. A number of compounds identified in the extracts by the Py-GC/MS have also been reported to exhibit antibacterial activity.

Antibacterial activity of the non volatile extracts of *T. camphoratus*and *T.trilobus var galpinni*

The extracts showed antibacterial activity against the tested micro-organisms with varying mean diameters of inhibition and with MIC values above 1mg/ml. According to Gibbons (2004), plant compounds with MIC values greater than 1mg/ml are of no relevance from a clinical perspective[144]. Tegos et al. (2000), observed that plant compounds that show little *in vitro* antibacterial activity are regulatory compounds protecting the plant from microbial infections[145]. The non volatile extracts of both *Tarchonanthus* species showed little *in vitro* antibacterial activity with *T. trilobus var galpinni* having lower antibacterial activity than *T. camphoratus*. Some of the compounds present in the *T. camphoratus* extracts which have been reported to show antibacterial activity included: 2-methyl phenol, catechol,1-methyl-

1H-pyrrole and 1-ethyl-1H- pyrrole in the Aq Lf extract (Table 4.28); n-hexadecanoic acid, hexadecanoic methyl ester, 9-octadecenoic methyl ester and octadecanoic methyl ester in the MET LF extract (Table 4.29); phytol and n-Hexadecanoic acid in the DCM LF extract (Table 4.30); taraxerone and 1-heptacosanol in the MET BK extract (Table 4.31); n-hexadecanoic acid and 6-octadecenoic acid in the DCM BK extract (Table 4.32); ergosta-4,7,22-trien-3β-ol in the Et Ac BK extract (Table 4.33)[146-157]. In the *T. trilobus var galpinni* non volatile extracts: catechol, n-hexadecanioc acid, phytol and β-sitosterol in the DCM LF extract (Table 4.34); n-hexadecanoic acid in the MET LF extract (Table 4.35); taraxerone in the Et LF extract (Table 4.36); taraxerone, n-hexadecanoic acid, 1-heptacosanol, caprylic acid and α-cadinol in the ET BK extract (Table 4.37) have also been reported to possess antibacterial activity[147,149-151,153-156, 158-164]. These compounds, acting singly or in synergy with other phytochemicals may have contributed to the observed antibacterial activity of the extracts. Different solvent extracts may show different pharmacological properties[165] since the extracts contain different chemical constituents due to the differences in polarities of the extracting solvents. In this study, although different solvent extracts showed varying antibacterial activities the differences in the activities among the extracts were not significant. No previous work has been reported on the antibacterial activity of the non volatile extracts of the bark of *T. camphoratus* and of the leaf and bark of *T. trilobus var galpinni*. Earlier antibacterial studies done on the non volatile leaf extracts of *T. camphoratus* showed conflicting results. According to Watt and Breyer-Brandwijk, 1962, the aqueous extracts of the leaves had no antibacterial activity[166]. McGaw et al. 2000 also reported no antibacterial activity of the aqueous, ethanolic and hexane extracts of the dried leaf of *T. camphoratus*[167].Later on, however, Braithwaite et al. 2008 reported good antimicrobial activity of the methanol extract of *T. camphoratus* against *S. aureus*[168]. Also according to Vermaak et al. 2009 the

crude methanol and aqueous extracts of *T. camphoratus* showed good to poor antimicrobial activity against the test micro-organisms[169]. In this study, the aqueous leaf extract showed antibacterial activity against all the tested micro-organisms. The differences in the antibacterial activities may be due to the differences in the bacterial strains investigated beside the differences in the chemical composition of the extracts collected from different eco-systems.

Comparison of the antibacterial activity of the volatile and non volatile extract of *T. camphoratus* and *T. trilobus var galpinni*

From the diameters of the zones of inhibition for the volatile and non volatile extracts, Tables 4.6 and 4.7 for the volatile extracts and Tables 4.39 and 4.42 for the non volatile extracts, it was observed that the volatile extracts exhibited stronger antibacterial activity than the non volatile extracts. Medicinal plants are often administered through inhalation to treat infections of the respiratory tract[67,170]. This suggests that the volatile constituents may be responsible for the antimicrobial activity. Van Vuuren and Viljoen, (2009), when investigating the interaction between the volatile and non-volatile fractions on the antimicrobial activity of South Africa *T. camphoratus* revealed that the volatile constituents contributed to the total efficacy of the plant[86]. Antibacterial action of *T. camphoratus* is implicated in its traditional uses like the treatment of; respiratory ailments such as bronchitis and asthma, whooping cough, fever, toothache, and fumigation during funeral rites. The inhibitory action of the plant extracts against *Bacillus cereus, Pseudomonas aeruginosa* and *Klebsiella pneumonia*, pathogens associated with respiratory problems justifies the traditional use of the plant for the treatment of respiratory ailments. The results also showed that the extracts exhibited antibacterial activity against pathogens like

Escherichia coli, *Staphylococcus aureus, Enterococcus faecalis*, *Bacillus cereus* which are implicated in opportunistic infections and this also justifies the use of the plant as a fumigant during funeral rites.Furthermore, the demonstration of antibacterial activity by the water extract provides the scientific basis for the use of the plant in traditional medicine, where water is the solvent in which the decoctions are prepared.

5.2.2.2 Antioxidant activity

Antioxidant activity of the non volatile extracts of the *T. camphoratus* and *T.trilobus var galpinni*

The extracts showed DPPH and $ABTS^{\bullet+}$ radical scavenging activities and also total reducing abilities which showed the presence of compounds with electron donating abilities in the extracts. Phenolic compounds are a diverse group of plant secondary metabolites with known antioxidant activity. They are classified as simple phenols or polyphenols[171-173] and among the polyphenols,the flavonoid class is the most prominent and the most important antioxidant group[174]. Flavonoids are reported to exhibit antioxidant activity in a variety of ways which include radical scavenging by hydrogen or electron donation, direct reaction with the radicals to form less reactive products and by metal chelation[175,176]. Phytochemical screening of plant extracts of both *T. camphoratus* and *T. trilobus var galpinni* revealed the presence of phenols and flavonoids in all the extracts except the DCM BK and Et Ac BK extracts of both species which did not contain phenols. Phenols were also identified in the extracts by Py-GC/MS analysis. The percentage composition of phenols in the Aq LF, MET LF, DCM LF, Et Ac BK, MET BK and DCM BK in the *T. camphoratus* extracts were 42%, 12%, 13%, 0%, 21% and 0% respectively. Mean while in the *T. trilobus var galpinni* extracts, phenols contributed 0%, 18%, 12%, 0% and 0% in the Et Ac LF, MET LF, DCM LF, DCM BK and Et Ac BK

respectively, to the total compound composition. Plant extracts are very complex mixtures of many different compounds with distinct activities[177,178]. Beside the phenol and the flavonoids, other phytochemicals and pigments acting individually or synergestically may contribute to the high antioxidant activity[179,180]. Compounds present in the *T. camphoratus* non volatile extracts with reported antioxidant activity included: catechol in the Aq LF extract (Table 4.28); phytol in the DCM LF extract (Table 4.30); stigmasterol and 1-heptacosanol in the MET BK extract (Table 4.31) and lycopene in the Et Bk extract (Table 4.33)[181-185]. In the *T. trilobus var galpinni* extracts were: phytol in the DCM LF extract (Table 4.34); 4-methylcatechol, 3,4-dihydroxyhydrocinnamic acid and coumarin in the MET LF extract (Table 4.35); betulin and α-cadinol in the Et Ac BK extract (Table 4.37)[182,186-190]. Nitric oxide inhibition was very poor and iron chelation was low which suggested that nitric oxide inhibition and metal chelation played a very little role in the antioxidant properties of the extracts. Variations in the antioxidant activities of the extracts may be due to the diversity in the basic chemical structures of their phytoconstituents which possess different degrees of antioxidant activity. The results revealed, however, that the extracts could be used to treat radical-related pathological damages, especially at higher concentrations and the extracts contained compounds with electron donating abilities whose chemical nature and mechanism of action required further investigation.

5.2.2.3 Cytotoxicity

Cytotoxocity of the non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* against brine shrimps

Results of the cytotoxicity of the non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* against brine shrimps revealed that the extracts of *T. camphoratus* had higher

cytotoxicity against brine shrimps than the extracts of *T. trilobus var galpinni*. Among the *T. camphoratus* non volatile extracts, the bark extracts exhibited the highest cytotoxicity as shown by their low LC_{50} values of 73.7, 94.1 and 161 µg/ml for the MET BK, DCM BK and Et Ac BK respectively. Low LC_{50} values less than 100 µg/ml indicate high pharmacological action by the extract[191]. This result therefore suggested higher pharmacological potential in the bark than in the leaves of *T. camphoratus*. Surprisingly, the bark is not known for any medicinal purposes rather it is the leaves that have wide medicinal application in traditional healing. The Aq LF, DCM LF and Et Ac BK of *T. camphoratus* and the MET LF and DCM LF extracts of *T. trilobus var galpinni* had LC_{50} values in between 100 and 1000 µg/ml and according to the LC_{50} limits set by Meyer et al[192], the extracts contained some physiologically active principles. Secondary metabolites are responsible for the various biological activities of plant extracts among which is cytotoxicity. Plant secondary metabolites known to be cytotoxic include saponins, cardiac glycosides, flavonoids, polyphenols, anthraquinones, coumarins, alkaloids plus tannins and they have also been identified as antitumor agents[192-196]. Phytochemical screening of the non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* revealed the presence of flavonoids, saponins, phenols and tannins but the cytotoxicities of the extracts were not directly proportional to the number of the different cytotoxic phytochemicals present in the extracts. The MET BK, Et Ac BK and the MET LF extracts of *T. camphoratus* contained flavonoids, saponins, phenols and tannins but the MET LF extracts had very weak cytotoxicity shown by its high LC_{50} value. The DCM BK extract with a low LC_{50} value of 94.1µg/ml hence high toxicity contained only flavonoids. TheMET LF,Et Ac LF and DCM LF of *T. trilobus var galpinni* contained the same type of phytochemicals but did not show the same cytotoxicity. The variation in BSLA results

may be due to the difference in the amount and kind of cytotoxic substances that were present in the different extracts.

Cytotoxicity of the non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* using the MTT assay

Generally all the extracts of *T. camphoratus* and *T. trilobus var galpinni* showed poor cytotoxicity against HEK293 and HepG2 with LC_{50} values greater than 100 µg/ml. All the extracts showed weak cytotoxicities against the two cell line as evidenced by their high LC_{50} values but the differences in the LC_{50} values showed varying levels of weakness in cytotoxicity. Considering the non volatile extracts of *T. trilobus var galpinni*, the HEK293 were more sensitive to the MET LF extracts than to the Et Ac LF, DCM LF, DCM BK and Et Ac BK whereas the HepG2 was sensitive to all the extracts with LC_{50} values in close range. Both cell lines were weakly sensitive to the non volatile extracts of *T. camphoratus* but the HEK293 was more sensitive than the HepG2 as shown by the LC_{50} values. As mentioned earlier, differences in sensitivities of different cell lines to the different extracts of the same plant have been revealed in the findings of other researchers[197]. Comparing the sensitivities of the cell lines to the extracts of both *T. camphoratus* and *T. trilobus var galpinni*, the cell lines were generally more sensitive to the extracts of *T. camphoratus.* The differences in sensitivities of the cell lines to different extracts of the same plant, or of different plants, may be attributed to the differences in nature and amount of bioactive compounds present in the extracts. The absence of significant cytotoxic activity especially in the leaves of *T. camphoratus* which are widely used in traditional medicine warrants the plant's safety for human use.

5.3 References

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The set objectives of the study were explored and from the obtained results the following conclusions can be drawn.

The main constituents of the volatile extracts/essential oils of *T. camphoratus* and *T. trilobus var galpinni* are sesquiterperne hydrocarbons although variations in the chemical constituents existed between different species and between the same species but harvested at different times and also between different plant parts.

The analytical result of pyrolysis-GC/MS revealed that the non volatile/solvent extracts of both *Tarchonanthus* species investigated, were rich in bioactive compounds which were generally categorised as phenols, triterpenoids, fatty acids and fatty alcohols. There is scanty information about the traditional medicinal use of *Tarchonanthus trilobus var galpinni* but this study revealed the presence of many biologically significant compounds in the leaves and bark of the plant.

This study provided for the first time the insecticidal activities of the essential oils of *T. camphoratus* and *T. trilobus var galpinni* against *Anopheles arabiensis* mosquito larvae and stored product pests. The essential oils of both species could be utilised in the development of envoromentally friendly compounds for the control of larvae of *Anopheles arabiensis.* The results from the study also indicated that the essential oil of *T.trilobus var galpinni* could not be utilised in the control of stored grain pests whereas the essential oil of *T.camphoratus* although not found to be toxic to *S. zeamais* and *S. oryzae* could be considered a potential in the control of stored product pests as a repellent.

The volatile and non volatile extracts showed antibacterial activity against the tested micro-organisms but their effectiveness varied. While the non volatile extracts showed moderate inhibitory activity, the volatile extracts exhibited considerable antibacterial activities and of special interest was the essential oil of the dry stem of *T. camphoratus* which showed antibacterial activity against all the tested micro-organisms. This result not only provided credence of the ethnopharmacological use of *T. camphoratus* in the treatment of diseases caused by micro-orgarnisms but also suggests that *T.trilobus var galpinni* can be used in a similar way as *T. camphoratus* in traditional healing. Furthermore the essential oils of *T.camphoratus* and *T. trilobus var galpinni* are potential candidates for use as antibacterial agents in new drugs for therapy of infectious diseases.

From the antioxidant results obtained in this study, it was established that it was unlikely to gain any antioxidant benefit from the volatile extracts of *T. camphoratus* and *T. tirlobus var galpinni.* However, it was apparent that the non volatile extracts of both *Tarchonanthus* species investigated, contained compounds that could quench free radicals and convert them into stable compounds.

The volatile and non volatile extracts of both *Tarchonanthus* species did not exhibit significant cytotoxicity. Since pharmacology is toxicity at a lower dose, the study revealed the potential of *T. camphoratus* and *T. tirlobus var galpinni* as pharmaceutical agents.

6.2 Recommendations

Intelligent use of drugs begins with the understanding of their mechanism of action. Further studies are need to investigate the mode of activity of the volatile and non volatile extracts of *T. camphoratus* and *T. trilobus var galpinni* as insecticidal, antibacterial and antioxidant agents.

APPENDIX 1

GC/MS CHROMATOGRAMS OF THE VOLATILE EXTRACTS OF *TARCHONANTHUS CAMPHORATUS*

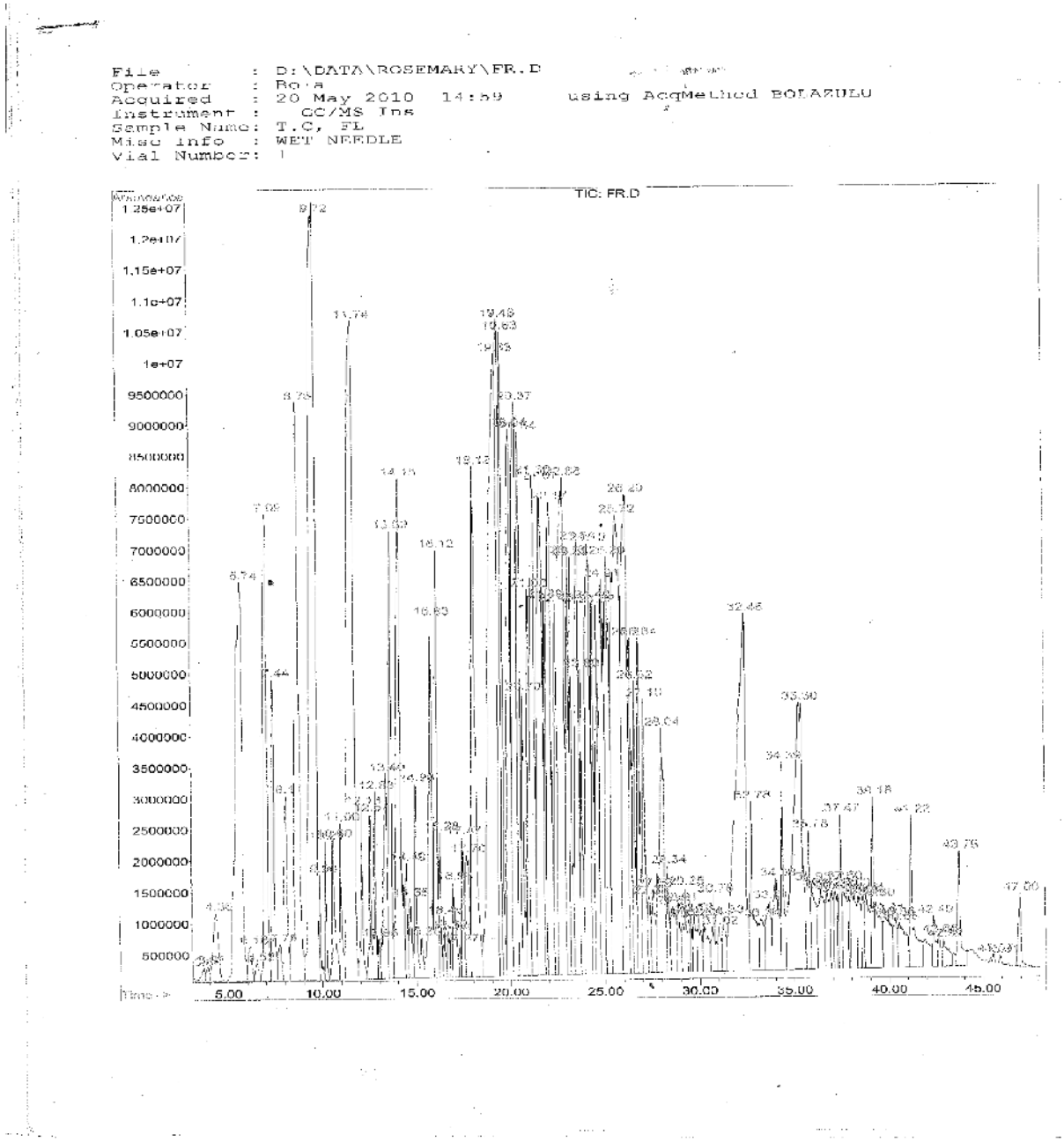

Figure 4.1: GC/MS Chromatogram of the essential oil of the fresh leaf of *T. camphoratus* harvested in November 2009.

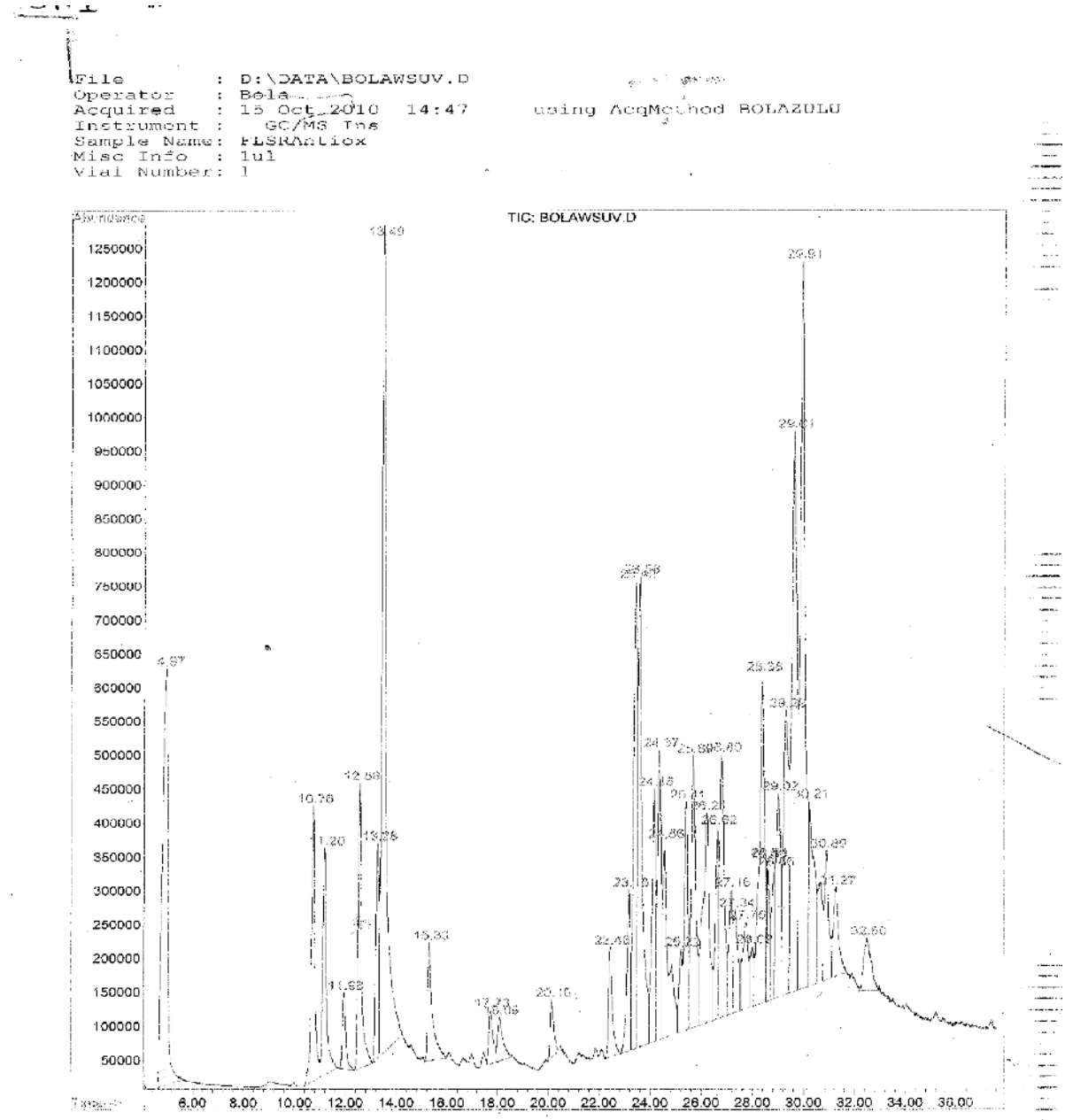

Figure 4.2: GC/MS Chromatogram of the essential oil of the fresh leaf of *T. camphoratus* harvested in April 2010.

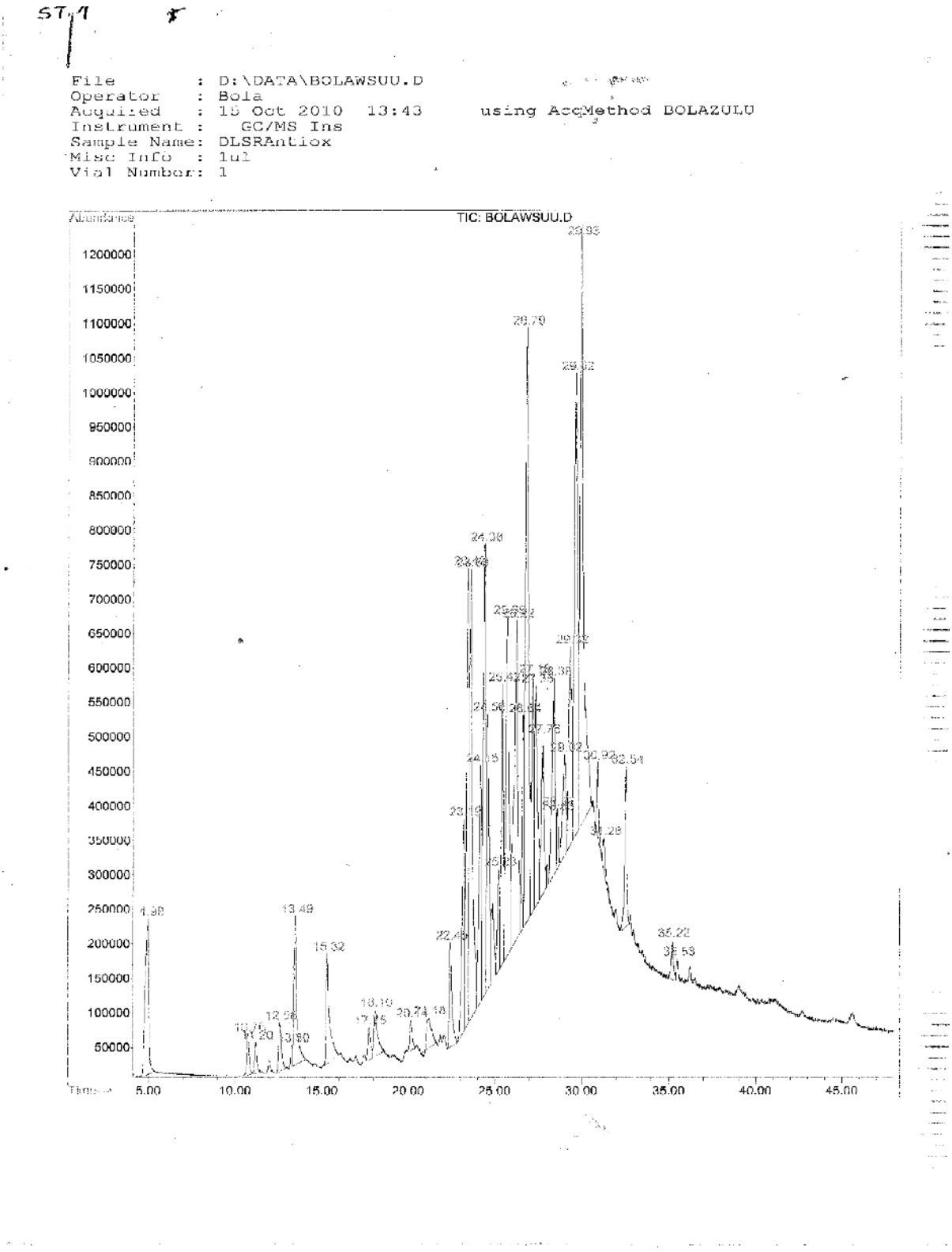

Figure 4.3: GC/MS Chromatogram of the essential oil of the dry leaf of *T. camphoratus* harvested in April 2010.

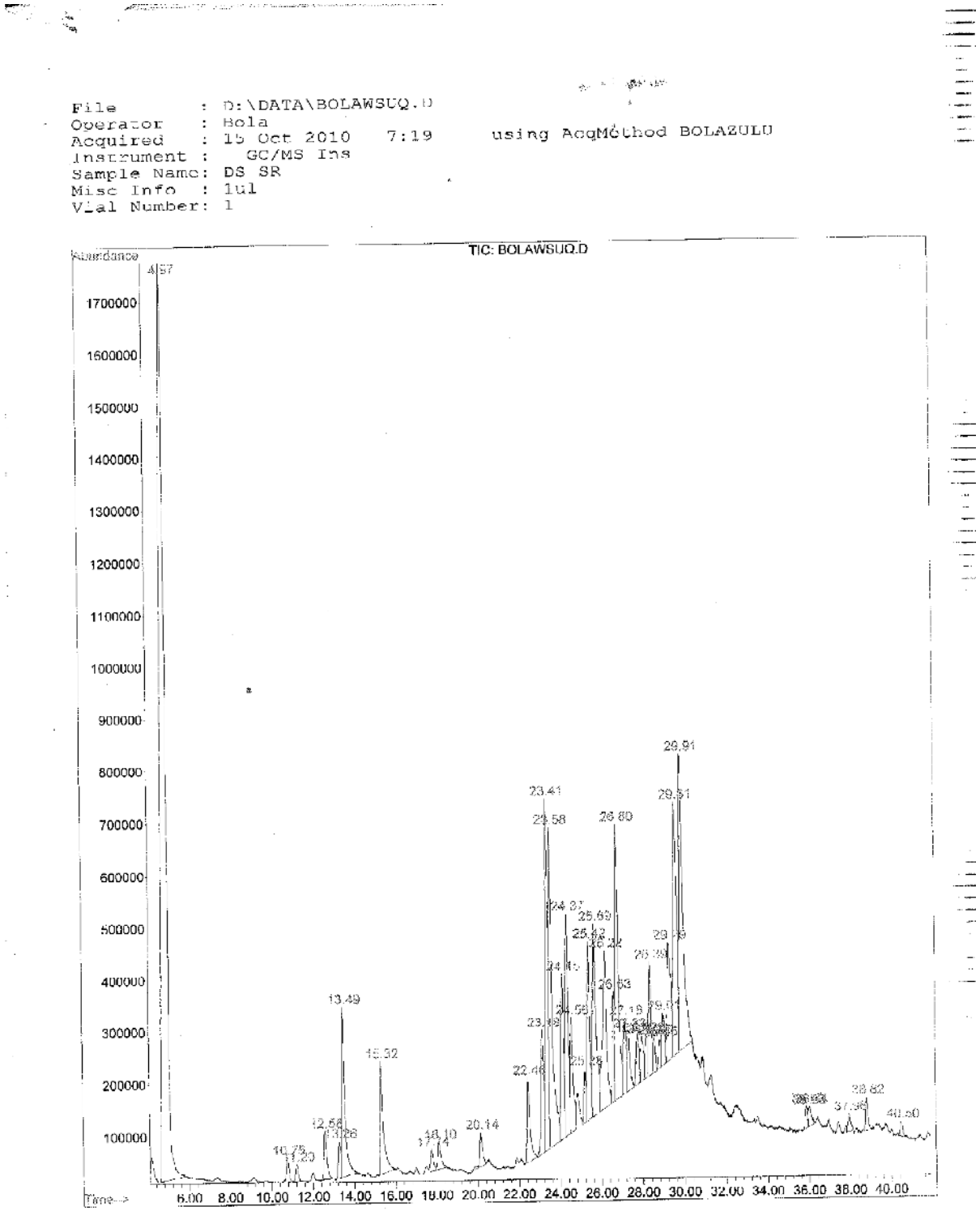

Figure 4.4: GC/MS Chromatogram of the essential oil of the dry stem of *T. camphoratus* harvested in April 2010**.**

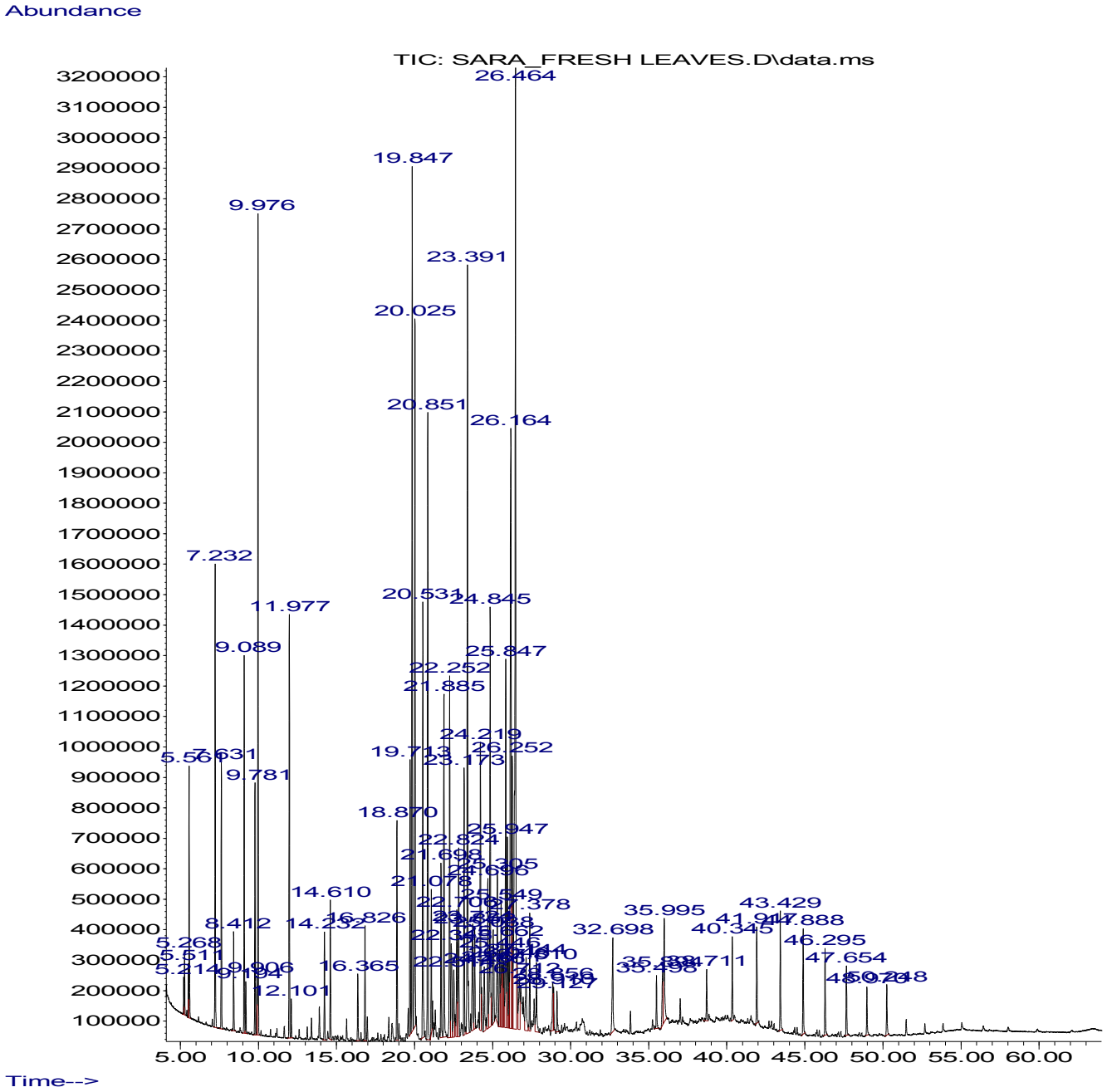

Figure 4.5: GC/MS Chromatogram of the essential oil of the fresh leaf of *T. camphoratus* harvested in 2011.

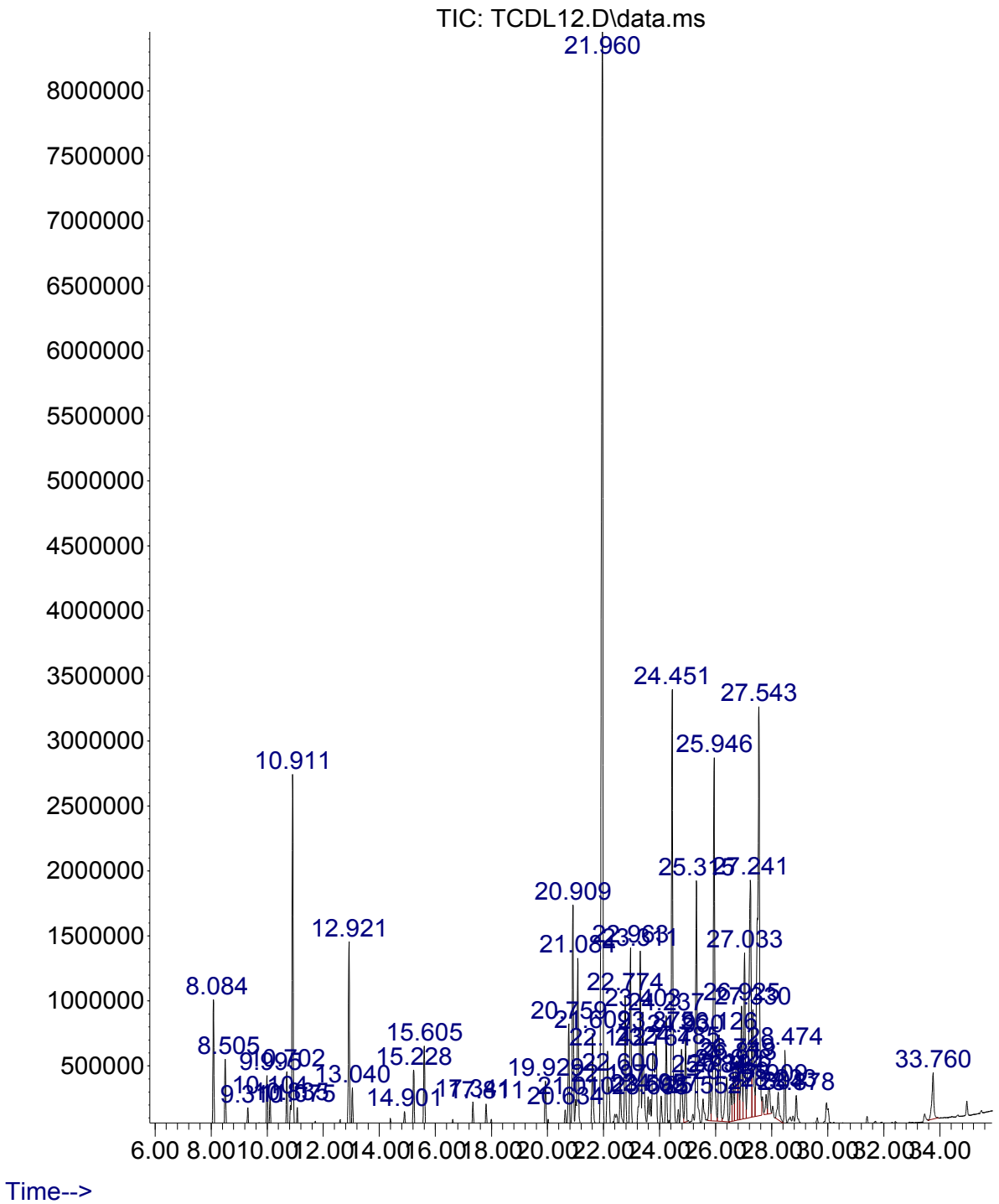

Figure 4.6: GC/MS Chromatogram of the essential oil of the dry leaf of *T. camphoratus* harvested in 2011.

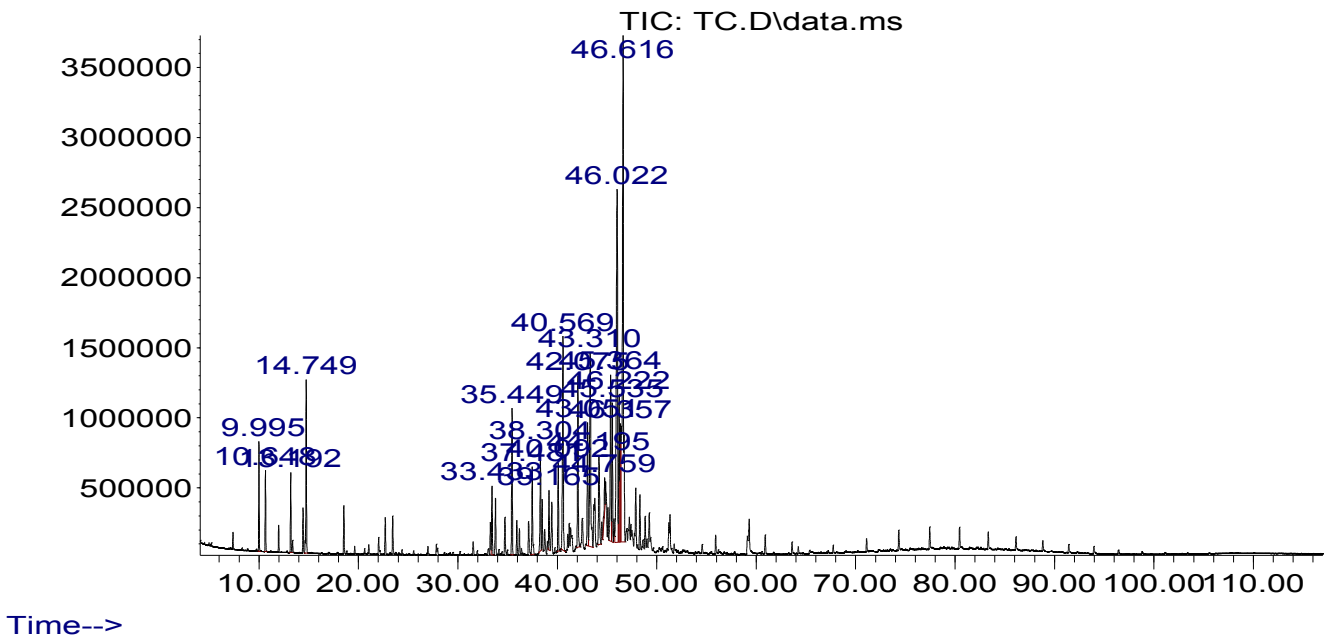

Figure 4.7: GC/MS Chromatogram of the essential oil of the dry stem of *T. camphoratus* harvested in 2011.

APPENDIX 2

GC/MS CHROMATOGRAMS OF THE VOLATILE EXTRACTS OF *TARCHONANTHUS TROLOBUS VAR GALPINNI*

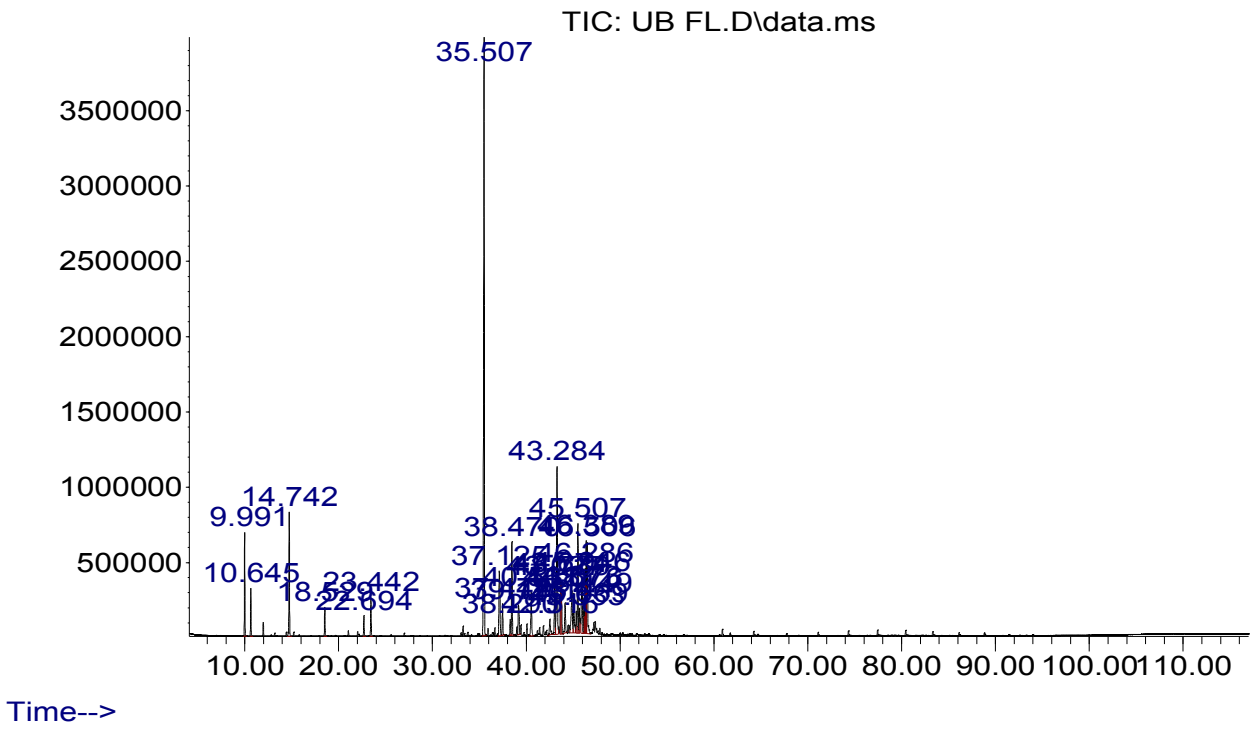

Figure 4.9: GC/MS Chromatogram of the essential oil of fresh leaf of *T. trilobus var galpinni* harvested in April 2010.

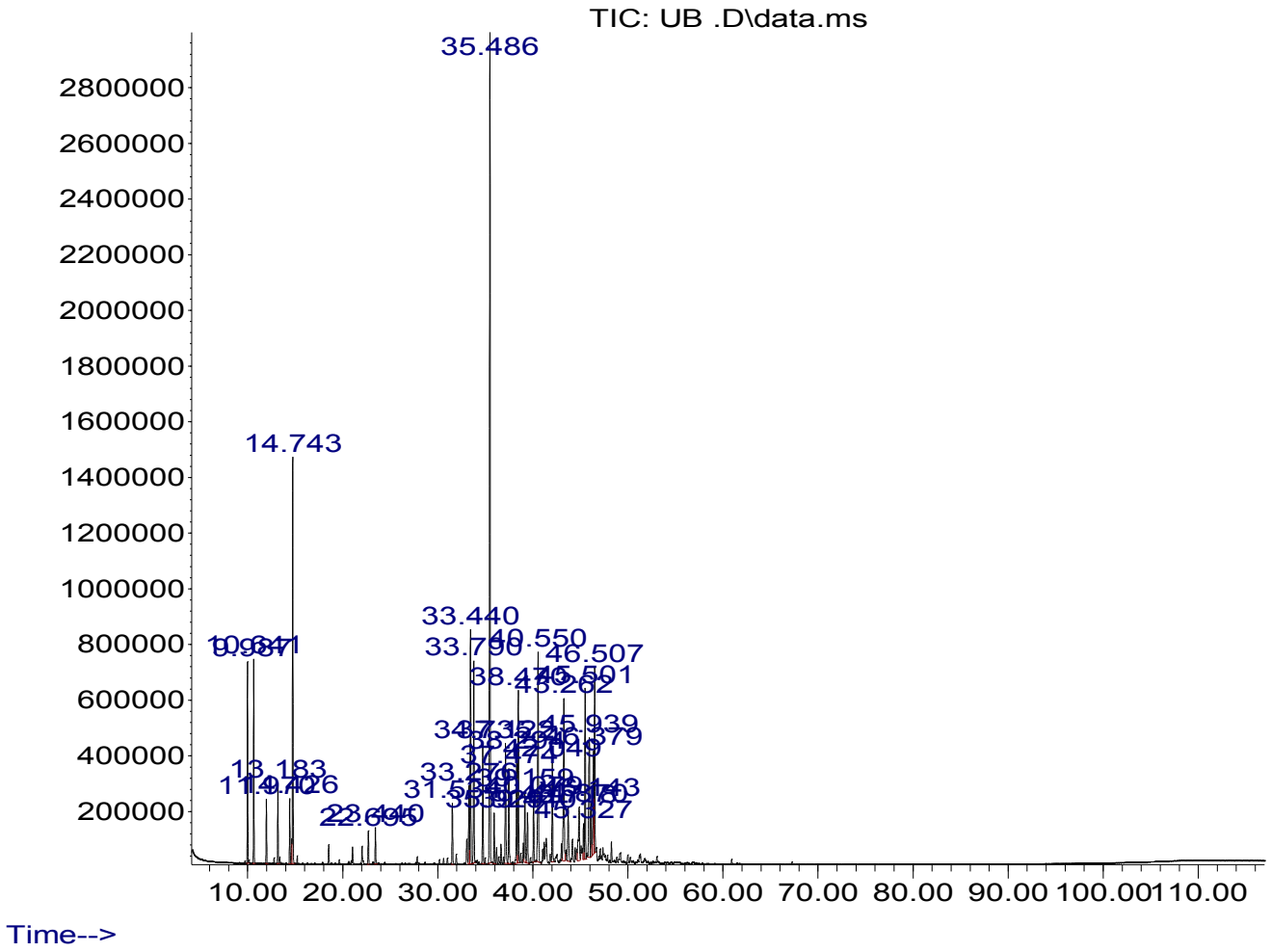

Figure 4.10: GC/MS Chromatogram of the essential oil of dry leaf of *T. trilobus var galpinni* harvested in April 2010.

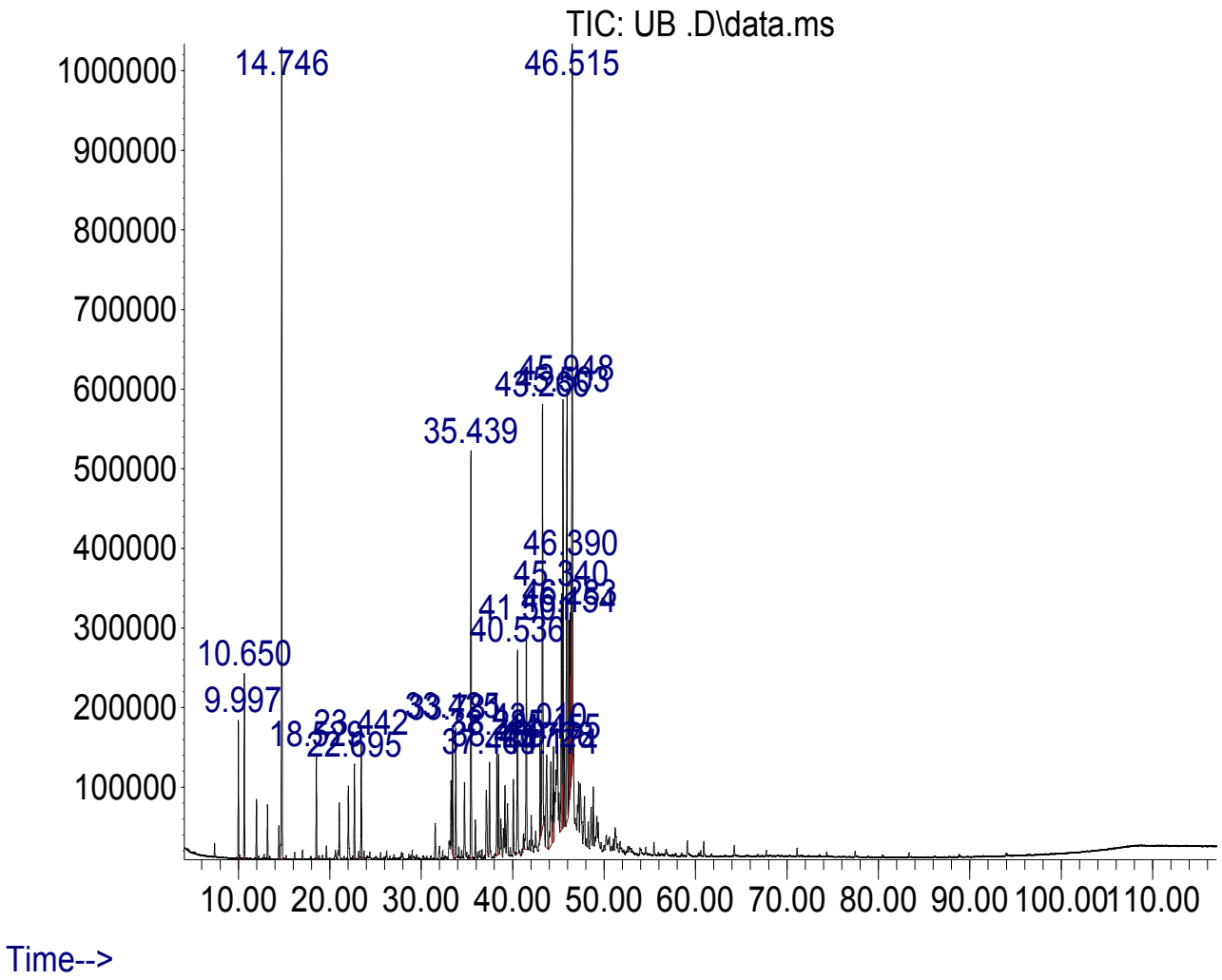

Figure 4.11: GC/MS Chromatogram of the essential oil of dry stem of *T. trilobus var galpinni* harvested in April 2010.

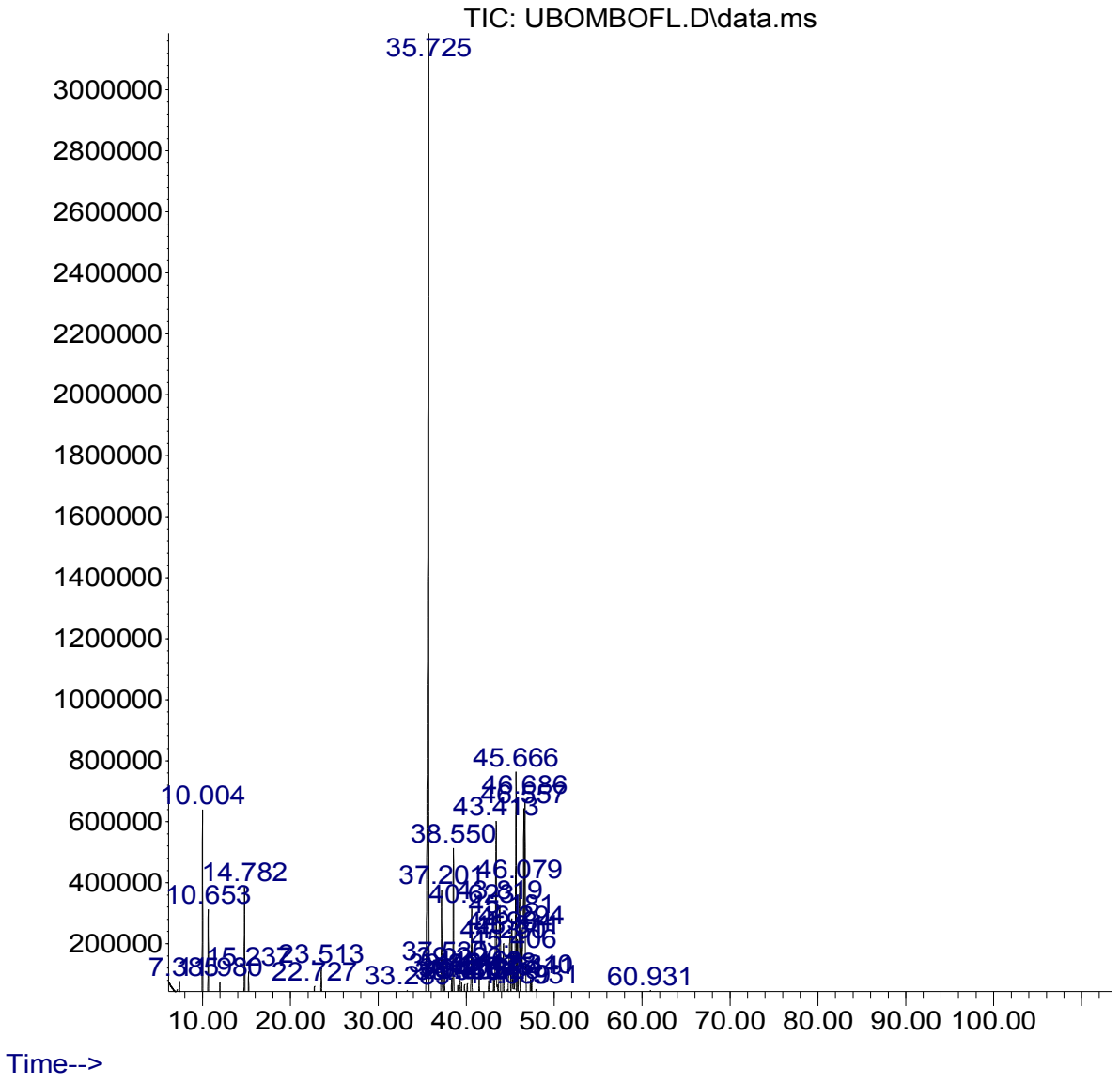

Figure 4.12: GC/MS Chromatogram of the essential oil of the fresh leaf of *T. trilobus var galpinni* harvested in 2011.

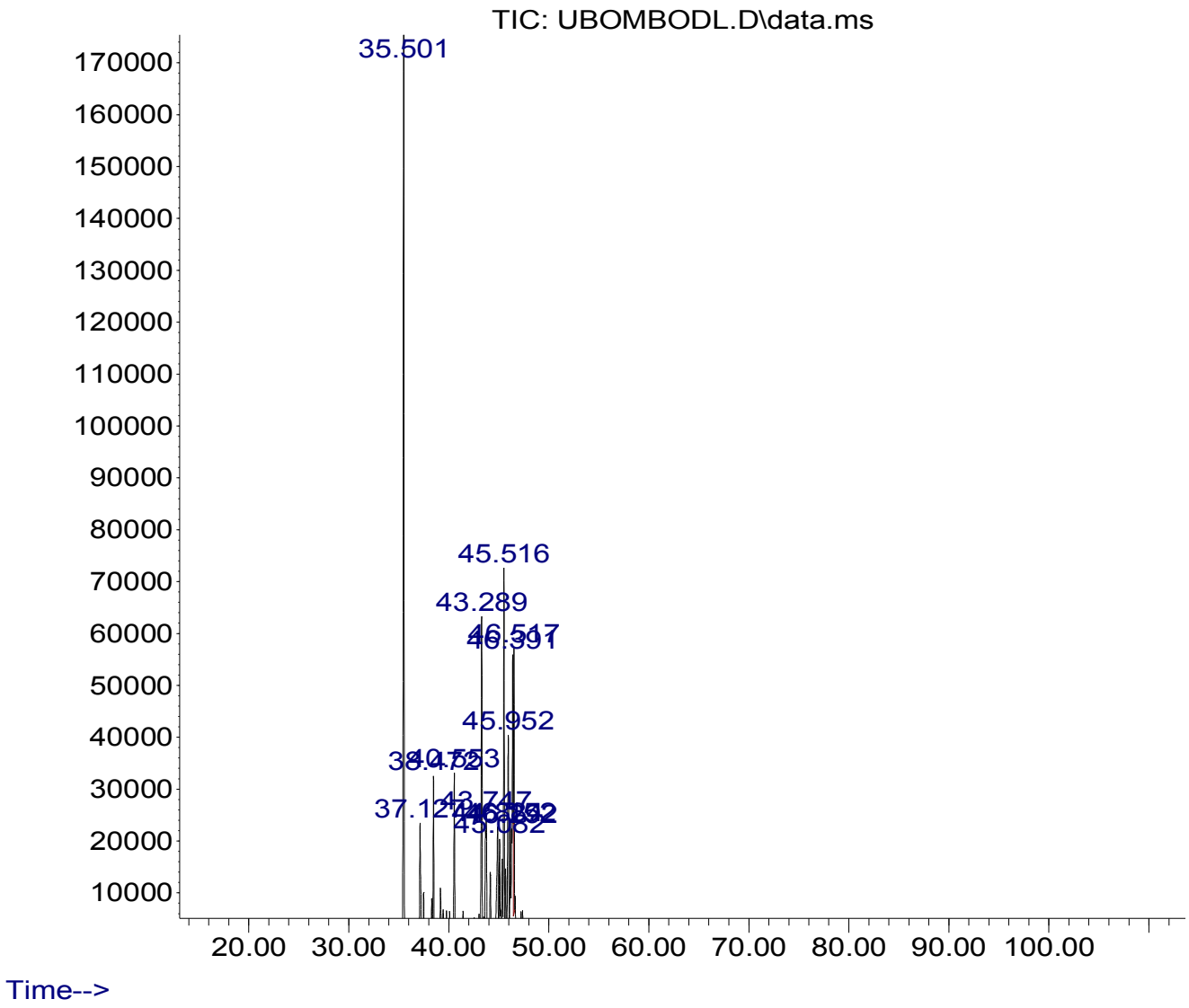

Figure 4.13: GC/MS Chromatogram of the essential oil of the dry leaf of *T. trilobus var galpinni* harvested in 2011.

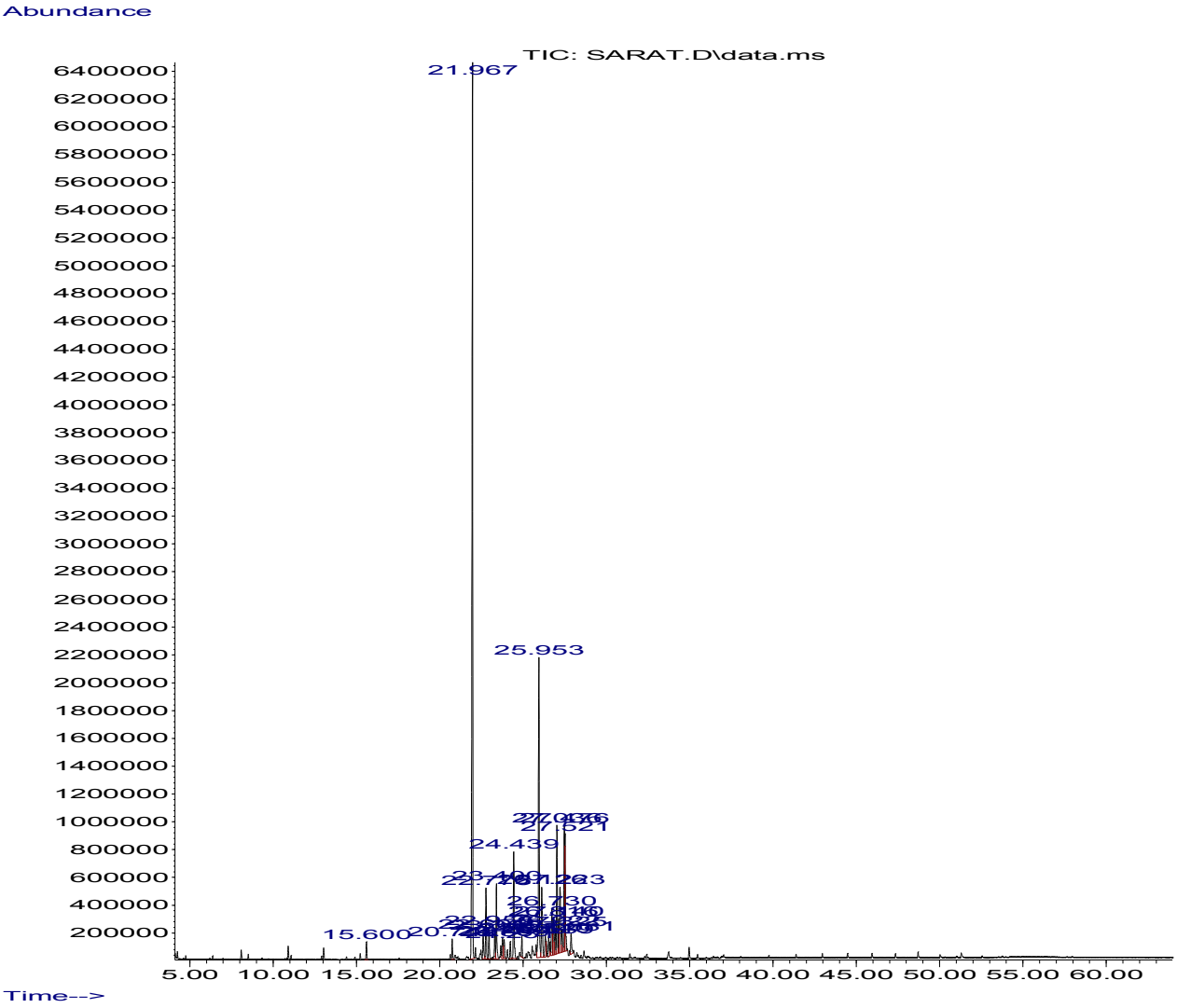

Figure 4.14: GC/MS Chromatogram of the essential oil of the dry stem of *T. trilobus var galpinni* harvested in 2011.

APPENDIX 3

Pyr-GC/MS CHROMATOGRAMS OF THE SOLVENT EXTRACTS OF *TARCHONANTHUS CAMPHORATUS*

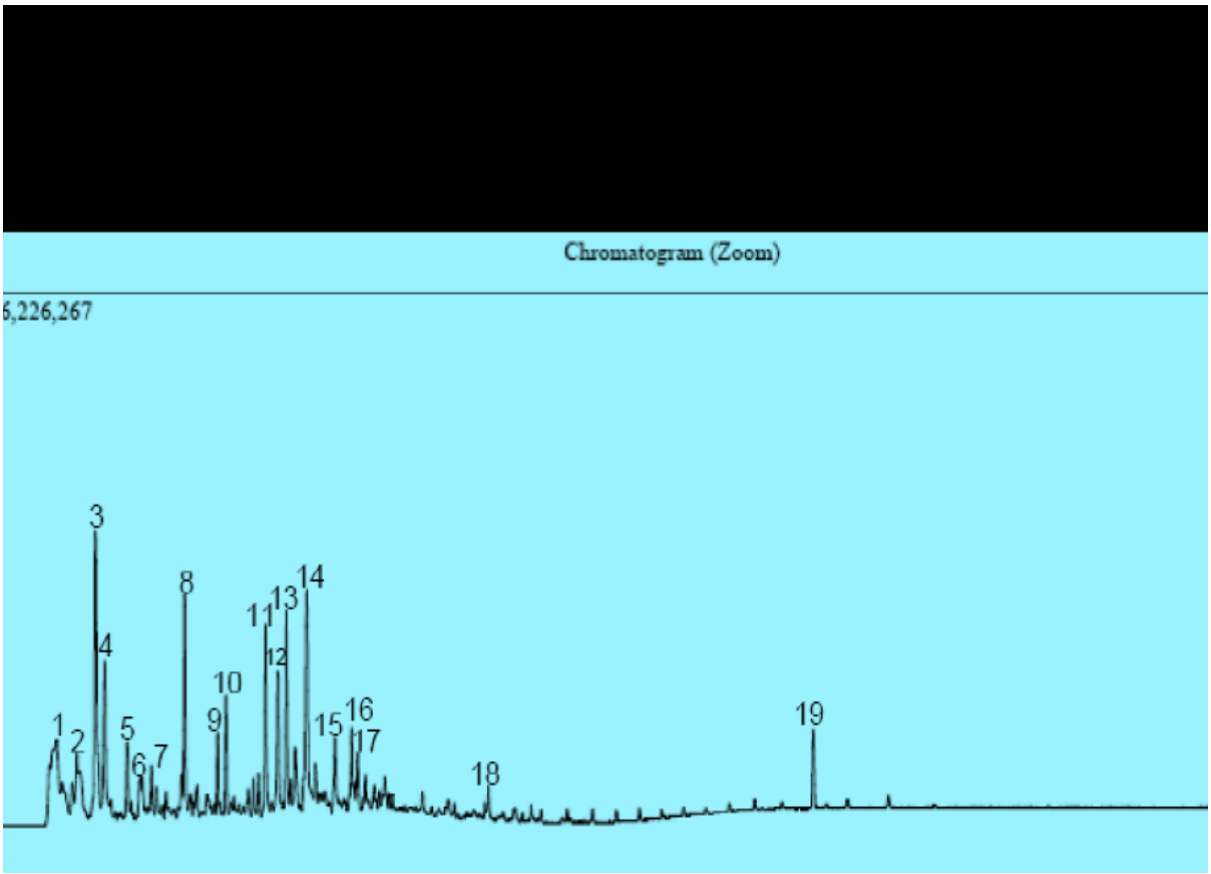

Figure 4.22: Pyr-GC/MS Chromatogram of the water leaf extract.

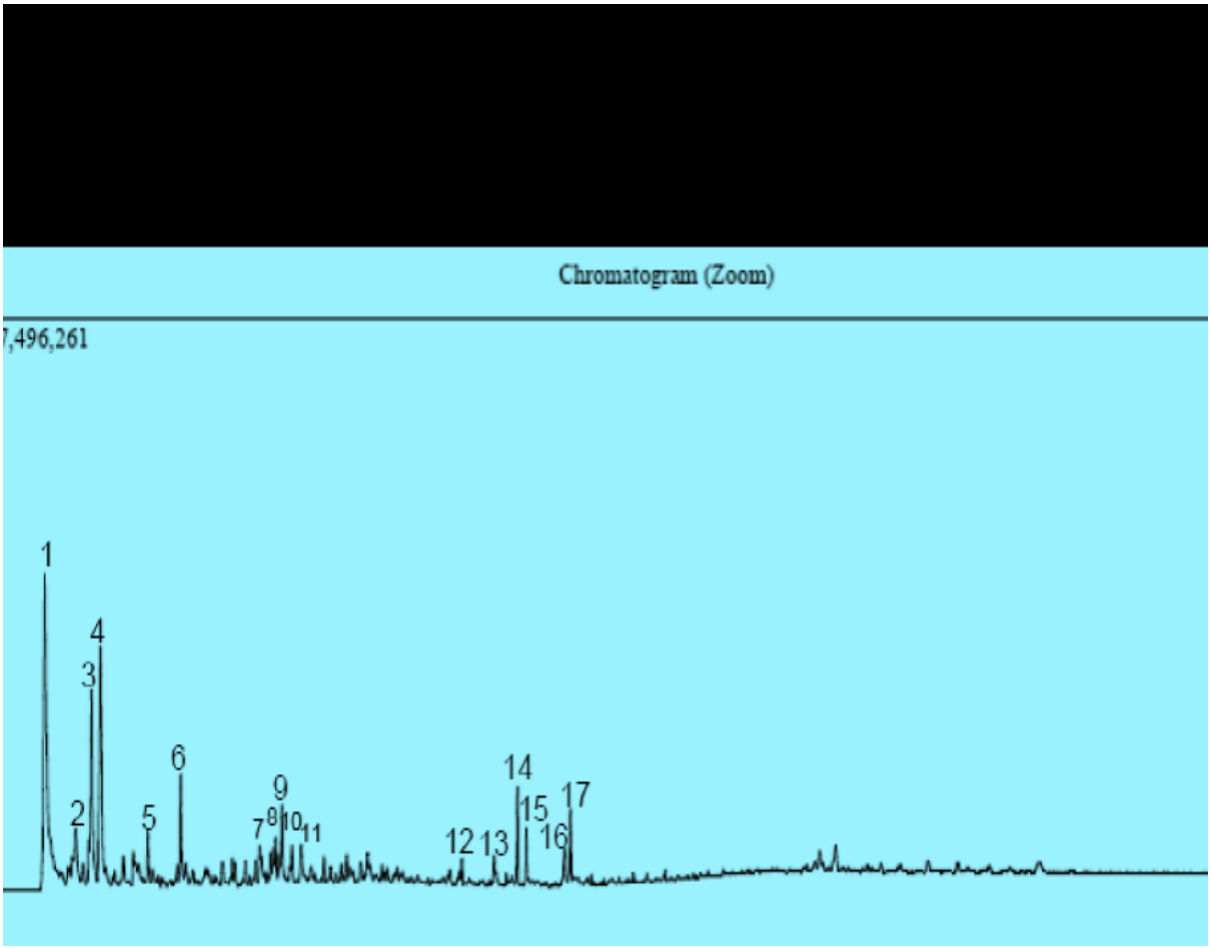

Figure 4.23: Pyr-GC/MS Chromatogram of the methanol leaf extract.

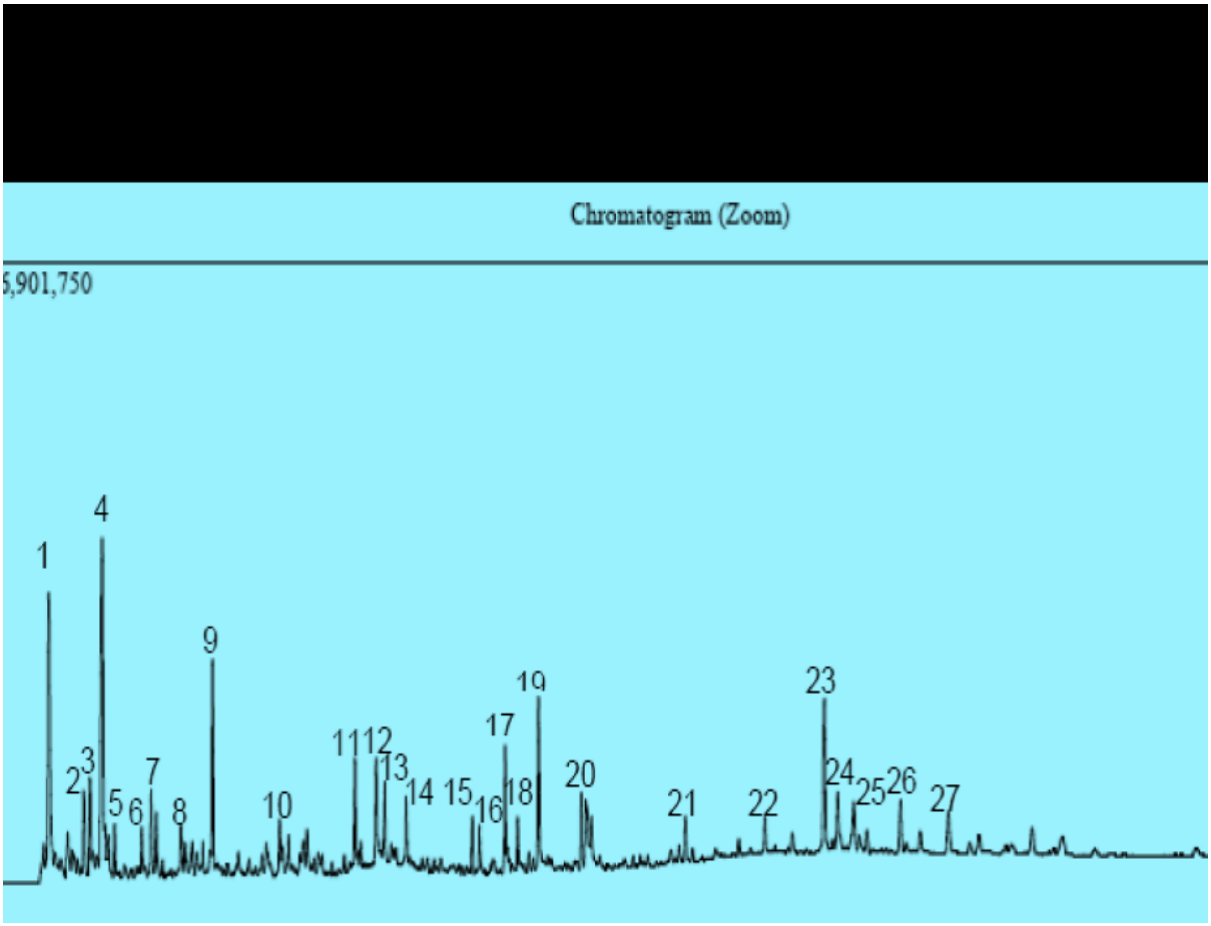

Figure 4.24: Pyr-GC/MS Chromatogram of the dichloromethane leaf extract.

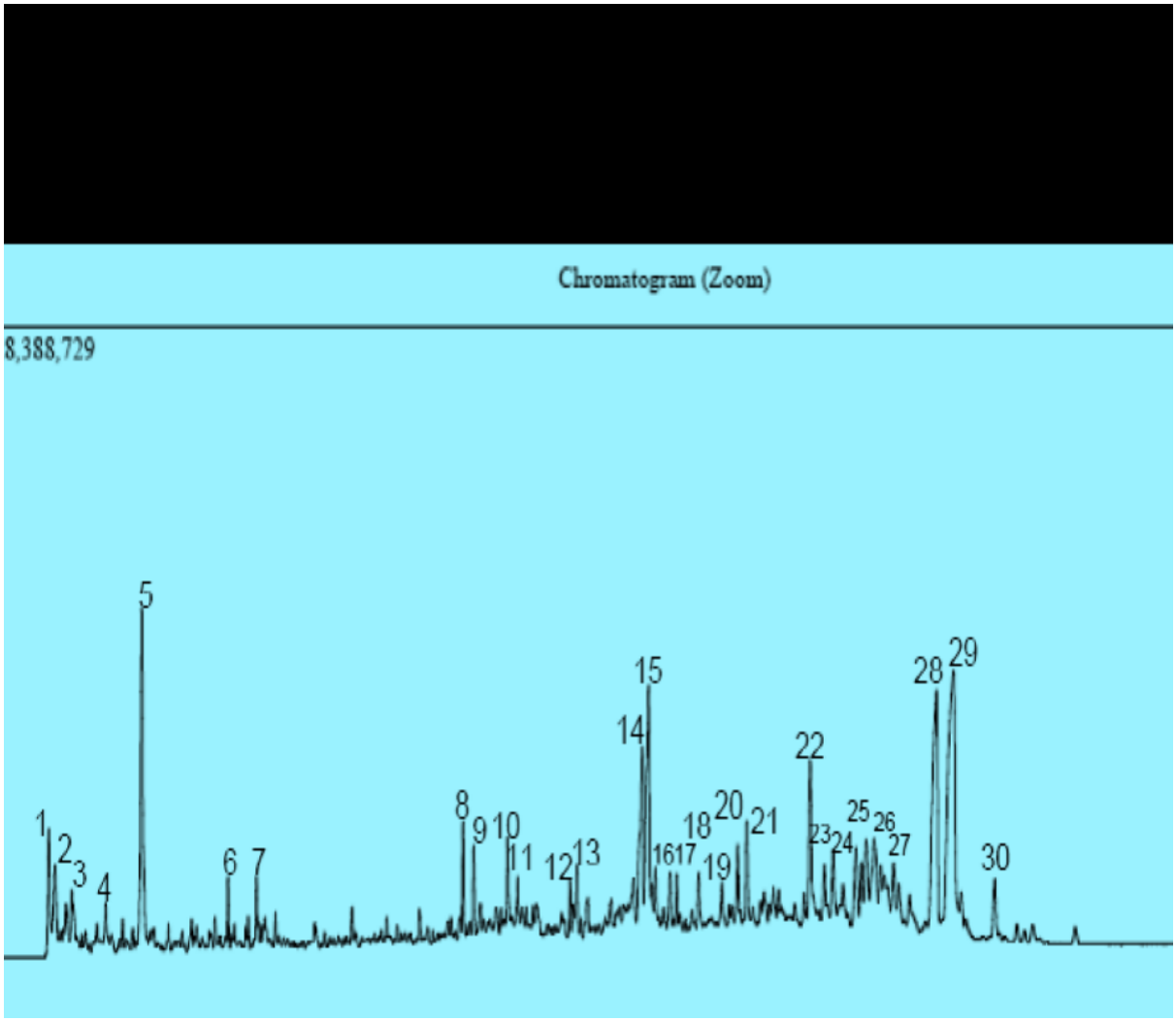

Figure 4.25: Pyr-GC/MS Chromatogram of the ethyl acetate bark extract of *T.camphoratus*

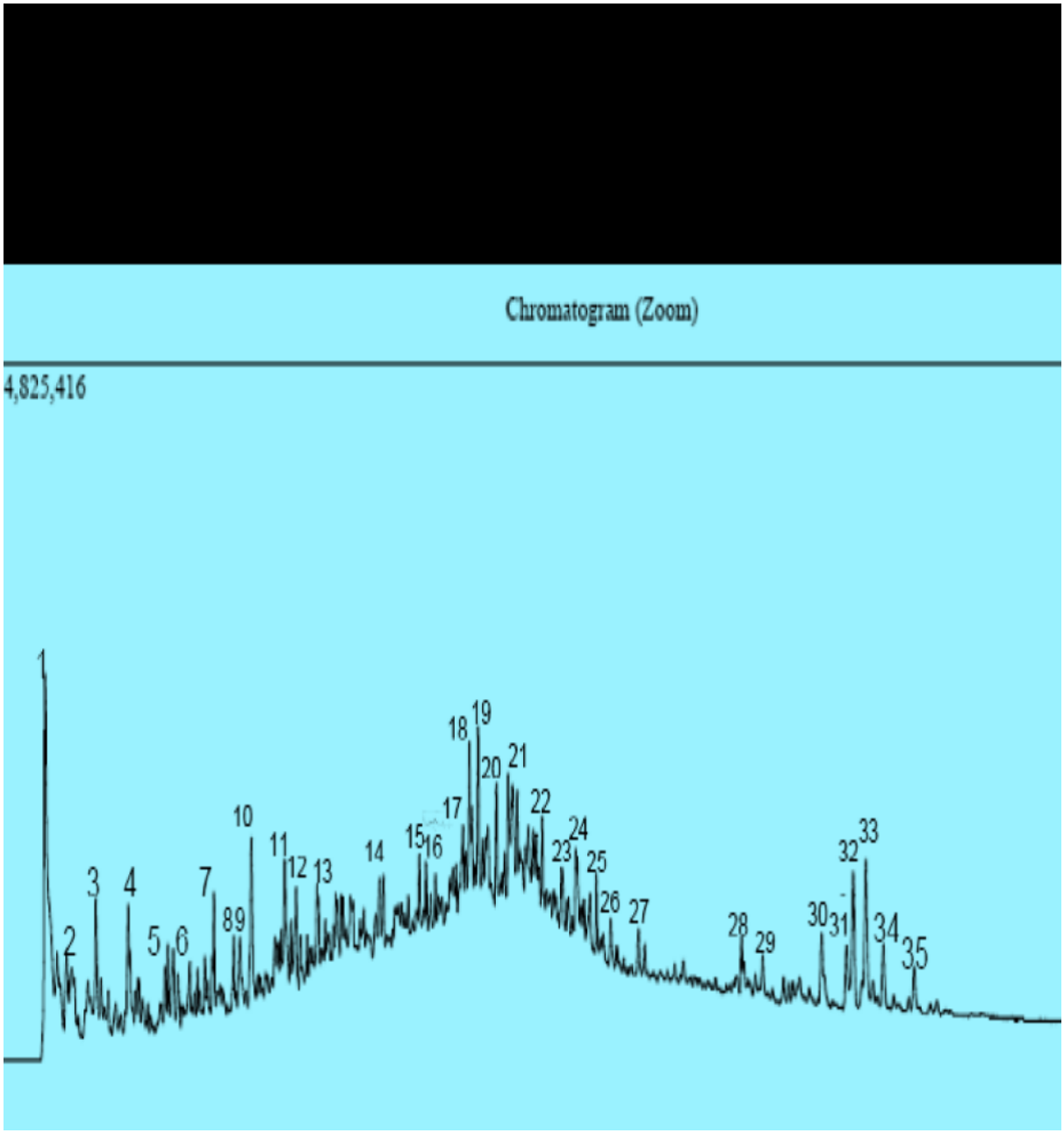

Figure 4.26: Pyr-**GC/MS Chromatogram of the methanol bark extract of** *T. camphoratus*

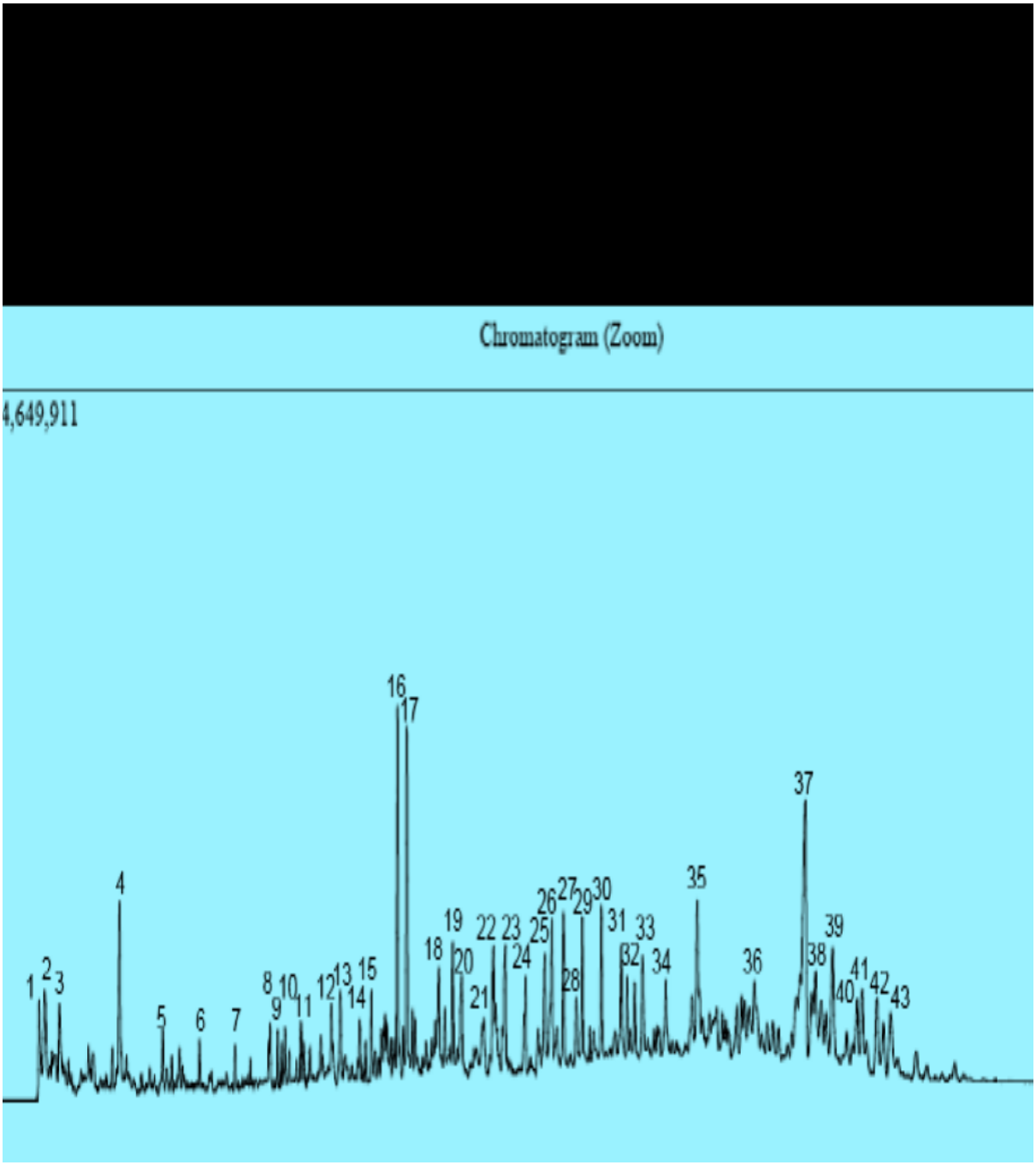

Figure 4.27: Pyr-**GC/MS Chromatogram of the dichloromethane bark extract of** *T.* *camphoratus*

APPENDIX 4

Pyr-GC/MS CHROMATOGRAMS OF THE SOLVENT EXTRACTS OF *TARCHONANTHUS TRILOBUS VAR GALPINNI*

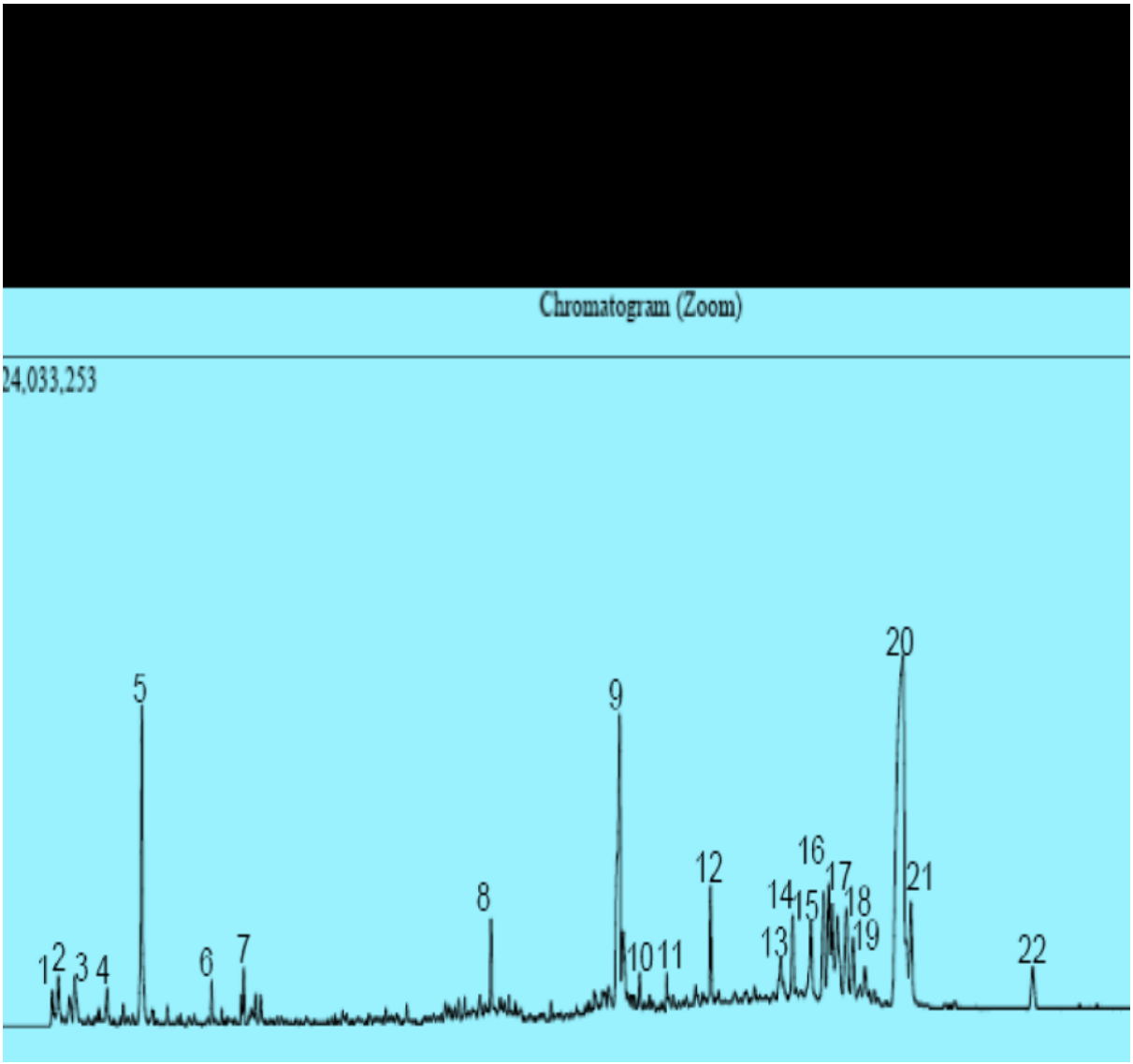

Figure 4.28: Pyr-GC/MS Chromatography of ethyl acetate leaf extract of *T. trilobus var galpinni*

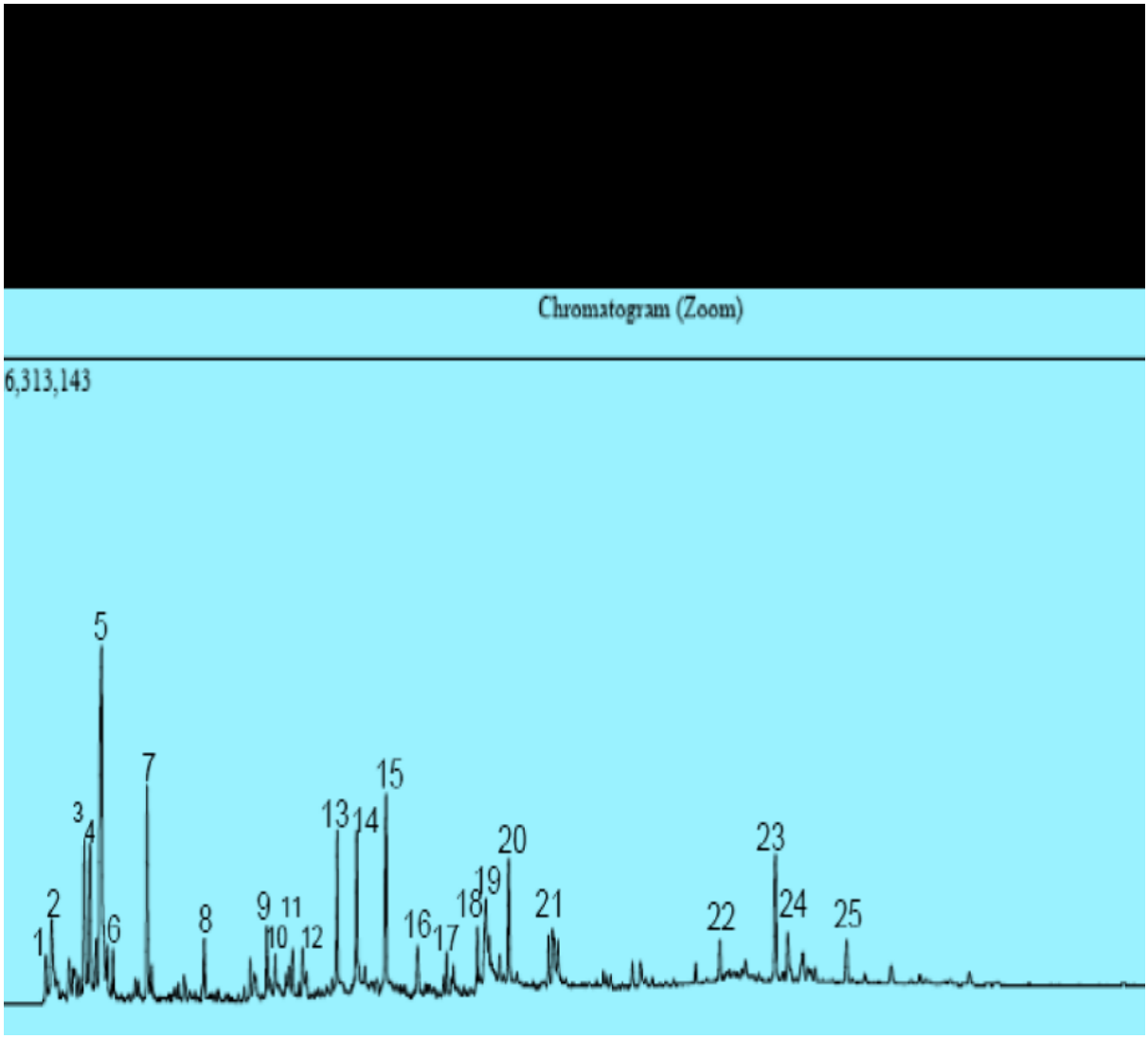

Figure 4.29: Pyr-GC/MS Chromatography of methanol leaf extract of *T. trilobus var galpinni*

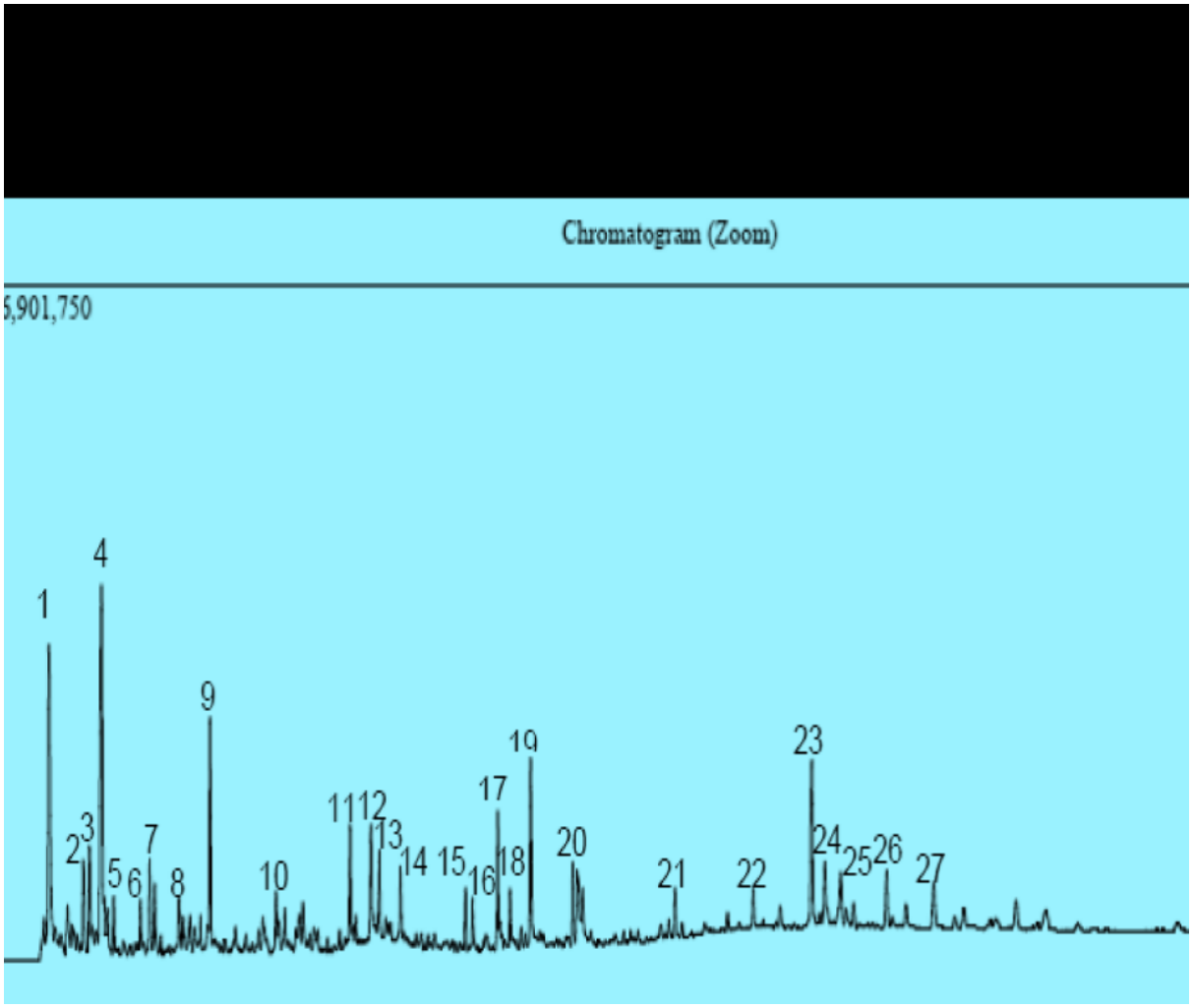

Figure 4.30: Pyr-GC/MS Chromatography of dichloromethane leaf extract of *T. trilobus var galpinni*

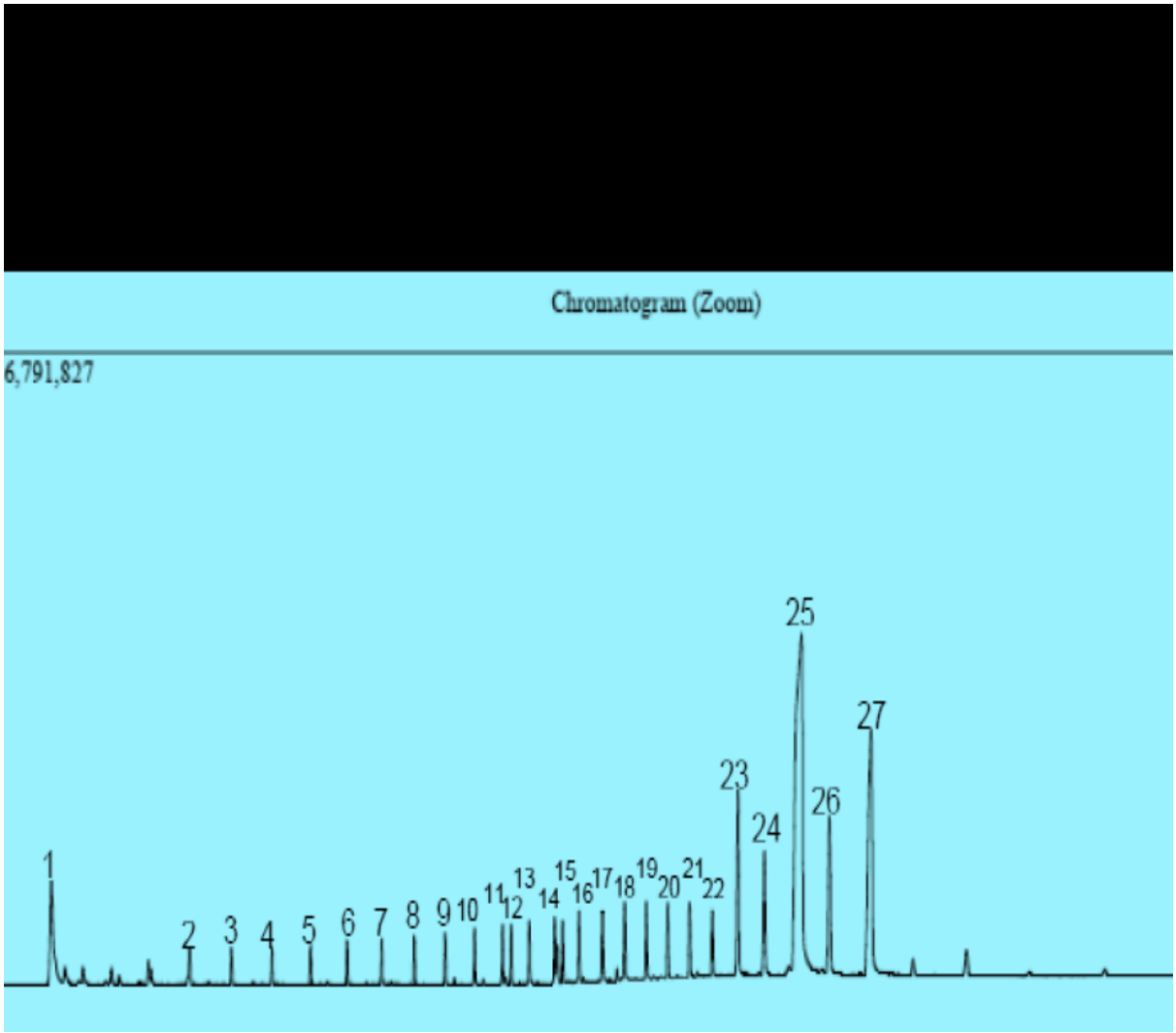

Figure 4.31: Pyr-GC/MS Chromatography of dichloromethane bark extract

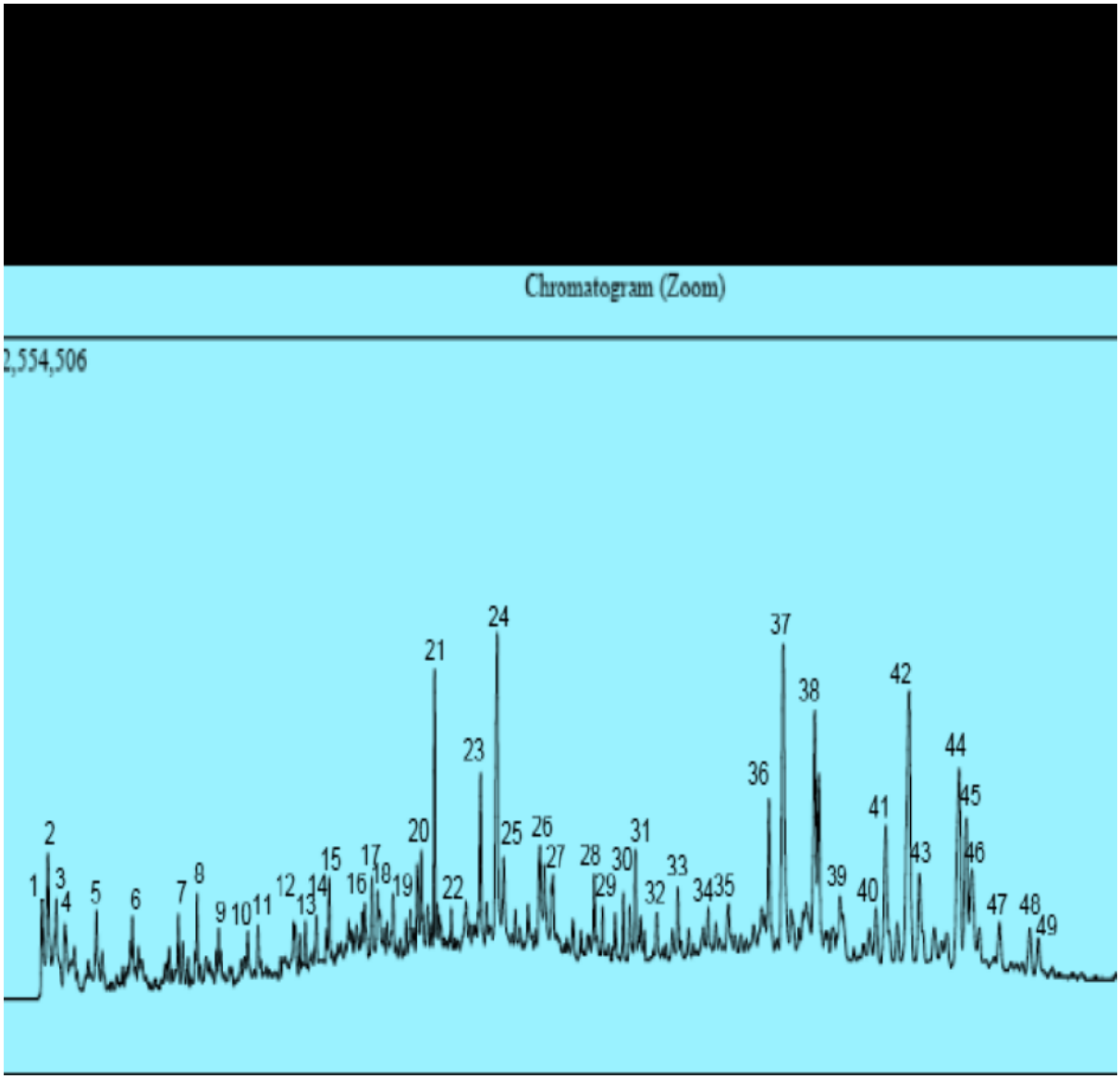

Figure 4.32: Pyr-GC/MS Chromatography of ethyl acetate bark extract of *T. trilobus var galpinni*

APPENDIX 5

PAPER PUBLICATIONS

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Chemical Composition and Larvicidal Activity of the Essential Oil of *Tarchonanthus camphoratus* Against *Anopheles arabiensis* Mosquito Larvae

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Abstract: The chemical composition of the essential oil of *Tarchonanthus camphoratus* was analysed using GC and GC-MS instruments. The major monoterpene was 1,8-cineole (5.4 %), while α-copaene (3.8 %) and δ-cadinene (3.1 %) were major sesquiterpenes. Major oxygenated monoterpenes and sesquiterpenes identified in the oil were 1,6-octadien-3-ol (4.5 %) and (-)-globulol (3.3 %) respectively. Hexadecanoic acid (3.5 %) was the major fatty acid present. The larvicidal assay of the essential oil against larvae of *Anopheles arabiensis* mosquito revealed that at a concentration of 300 ppm, the oil caused 100 % mortality after 24 hr exposure with an LC_{50} value of 78.7 %.

Key words: Essential oils, *Tarchonanthus camphoratus*, larvicidal activity, *Anopheles arabiensis*.

Introduction

Mosquito-borne diseases cause havoc in developing countries, both in urban and rural populations, and loss in terms of human lives is enormous [28]. Mosquitoes not only cause nuisance by their bites, but also transmit deadly diseases like malaria, filariasis, yellow fever, dengue and contribute significantly to poverty and social debility in tropical countries [41]. Among these diseases, malaria remains the most serious vector-borne disease affecting some 300 - 500 million people and 1.4 to 2.6 million deaths annually throughout the world. More than 40 % of the world's populations live in malaria infested areas [29]. Mosquito-borne diseases contribute significantly to disease burden, death, poverty, and social debility all over the world, particularly in tropical countries.

It is envisioned that the only successful method of reducing mosquito densities to an appreciable level for which malaria epidemics can be controlled is by attacking the larval breeding places through the use of larvicides [35]. Larviciding largely depends on the use of synthetic chemical insecticides like temephos and fenthion. Although effective, their

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Full Length Research Paper

Chemical composition, antioxidant activity and cytotoxicity of the essential oils of the leaves and stem of *Tarchonanthus camphoratus*

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Accepted 4 January, 2013

The aim of this study was to investigate the chemical composition, antioxidant potential and cytotoxicity of the essential oil of the fresh leaf, dry leaf and dry stem of *Tarchonanthus campharatus*. The antioxidant activity of the oils were examined by the 1,1-Diphenyl-2-picryl-hydrazil (DPPH), 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), nitric oxide radical scavenging and reducing power methods. The brine shrimp lethality test and the MTT cytotoxicity test were used to investigate the cytotoxicity of the oils. Sesquiterpene hydrocarbons are the major components in the essential oil of the fresh leaves (36.46%) and of the dry leaves (59.18) whereas an aldehyde, butanal (35.77%) is the major component in the essential oil of the dry stem. The oils did not show significant antioxidant activity as evidenced by their high LC_{50} values in all the antioxidant assays. The cytotoxicity results indicated that the oils had low toxicity with LC_{50} values ranging from 400 to 900 µg/ml and 400 to 1100 µg/ml for the brine shrimp lethality test and MTT cytotoxicity assay respectively.

Key words: *Tarchonanthus camphoratus*, essential oil, antioxidant activity, cytotoxicity.

INTRODUCTION

In living systems, free radicals are constantly generated and when in excess they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis (Halliwell and Gutteridge, 1998). The interest in antioxidants has been increasing because of their high capacity in scavenging free radicals which are related to various diseases (Silva et al., 2007). The most commonly used synthetic antioxidants; butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and test butylatedhydroquinone have been reported to cause liver damage and carcinogenesis (Sherwin et al., 1990). There is growing interest in natural antioxidants present in medicinal plants that might help attenuate oxidative damage (Silva et al., 2005; Muhammad et al., 2012). The health promoting effects of plants were found to be due to bioactive substances such as essential oils, flavonoids and phenolic compounds which have antioxidant activity (Liu, 2003; Komal et al., 2012).

Tarchonanthus campharatus L., (family Asteraceae) is a shrub of rarely more than six meters in height with a greyish appearance and occurs in a wide range of habitats (van Wyk et al., 1997). The strongly scented tree of *T. campharatus* has many medicinal applications in

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Full Length Research Paper

Variation in chemical composition and antibacterial activity of the essential oil of fresh and dry leaves and dry stem of *Tarchonanthus camphorates*

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Accepted 29 October, 2012

The essential oils of the fresh leaves, dry leaves and dry stem of *Tarchonanthus camphoratus* from Kwa-Zulu Natal, South Africa, were obtained by hydro-distillation and analyzed by gas chromatography mass spectrometry (GC-MS). The essential oils were evaluated for their antibacterial activity against 4 Gram positive and 5 Gram negative bacteria using the disc diffusion method and the minimum inhibitory concentration (MIC) method. A total of 33, 27 and 25 compounds were identified representing 85.9, 73.01 and 72.66% of the total oil composition from the fresh leaves, dry leaves and dry stem, respectively. Sesquiterpene hydrocarbons dominated the essential oil of the fresh leaves (36.46%) and of the dry leaves (59.18) whereas an aldehyde, butanal (35.77%) dominated the essential oil of the dry stem. The oils showed a broad spectrum of antibacterial activity. The zones of inhibition of the fresh leaves, dry leaves, dry stem and chloramphenicol ranged from 7.3 to 14.0, 7.3 to 16.7, 13.5 to 20.4 and 0.00 to 17.5 mm, respectively. The lowest MIC value for the oils was 125 µg/ml.

Key words: *Tarchonanthus camphoratus*, essential oil composition, antibacterial activity.

INTRODUCTION

Tarchonanthus camphoratus L. (family Asteraceae) is a shrub of rarely more than 6 m in height with a greyish appearance and occurs in a wide range of habitats (van Wyk et al., 1997). The strongly scented tree of *T. camphoratus* has many medicinal applications in traditional healing, mainly by smoking from burning leaves or by drinking infusions or decoctions. Traditionally, infusions and tinctures of the leaves are used for stomach trouble, headache, toothache, asthma, bronchitis, inflammation, rheumatism, venereal diseases, indigestion, heartburn, coughs, paralysis and cerebral hemorrhage (Hutchings and Van Staden, 1994; Anthony, 1999). The plant also shows powerful insect repellent action (Omolo et al., 2004) and wild animals that live in the areas where *T. camphoratus* grows, particularly Cape buffaloes and black rhinoceroses, rub themselves against the leaves to get rid of mosquitoes and flies. The plant also seems to drive away tsetse fly, a pathogenic agent of trypanosomiasis (Bekalo et al., 1996).

T. camphoratus has been reported to contain tannins, saponins and reducing sugars (van Wyk et al., 1997; Scott and Springfield, 2005). Leaves of the plant contain various flavanones such as pinocembrin, luteolin, apigenin, nepetin, and hispidulin; a sesquiterpine lactone, parthenolide; a quaternary alkaloid, tarchonanthine (Scott and Springfield, 2005). The essential oil of the leaves of *T. camphoratus* has been reported to be very complex and variable at different locations (Mwangi et al., 1994; Matasyoh et al., 2007; van Vuuren and Sandra, 2008). Antibacterial activity of the essential oil of the leaves of *T. camphoratus* from other ecosystems; Kenya and Eastern Cape, South Africa, has been reported (Matasyoh et al.,

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Original Research Article

Antioxidant Activity and Cytotoxicity of the Leaf and Bark Extracts of *Tarchonanthus camphorates*

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Abstract

Purpose: To investigate the antioxidant potential and cytotoxicity of the leaf and bark extracts of *Tarchonanathus campharatus*..

Methods: The antioxidant activity of the aqueous leaf extract (Aq LF), methanol leaf extract (MET LF), dichloromethane leaf extract (DCM LF), methanol bark extract (MET BK), dichloromethane bark extract (DCM BK), and ethyl acetate bark extract (Et Ac BK) were examined by 1,1-Diphenyl-2-picryl-hydrazil (DPPH), 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$), nitric oxide radical scavenging, reducing power and iron chelating activity methods. Brine shrimp lethality and MTT cytotoxicity tests were used to investigate the cytotoxicity of the extracts.

Results: The Aq LF, DCM LF, MET LF and MET BK showed good DPPH, ABTS radical scavenging and total reducing power activities. Total reducing power was high and in the rank order of DCM LF > Aq LF > MET LF > MET BK. All the extracts, however, showed weak nitric oxide scavenging activity as well as weak iron chelating ability. Flavonoids, phenols, tannins and saponins were present in some of the extracts, but alkaloids, terpenoids, cardenolides and cardiac glycosides were absent in all the extracts. All the extracts did not show significant cytotoxic properties ($p \geq 0.05$) with 50% inhibitory concentration IC_{50} values > 30 µg/ml in both cytotoxicity assays.

Conclusion: The antioxidant activity and low cytotoxicity of Tarchonanthus camphoratus probably justify its use in folk medicine.

Keywords: Tarchonanthus camphoratus, Antioxidant activity, Cytotoxicity

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INTRODUCTION

In the living system, free radicals are constantly generated and can cause extensive damage to tissues and biomolecules, creating various degenerative diseases [1]. Due to the adverse side effects of synthetic drugs, natural antioxidants from plant origin have drawn much attention in recent years. Plants are known to possess potent bioactive compounds capable of preventing and treating most oxidative-related diseases and these compounds might act as leads in drug development [2].

Tarchonanathus campharatus L,.(family Asteraceae) has many medicinal applications in traditional healing where infusions and tinctures of the leaves are used for stomach trouble,