UNIVERSITY OF ZULULAND

ESSENTIAL OIL COMPOSITION AND SOME BIOLOGICAL ACTIVITIES OF *TETRADENIA RIPARIA*

A dissertation submitted to the Department of Chemistry, Faculty of Science and Agriculture in partial fulfilment of the requirement for Masters Degree in Chemistry

by

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DECLARATION

I hereby declare that this dissertation is my own work and effort, and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been referenced and acknowledged.

Signature: …….

Date: ……

DEDICATION

This dissertation is dedicated to my family, who offered me unconditional love and support throughout the course of this study.

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In the first place, I would to thank Almighty God for all things that He has done for me, I am so grateful for His unconditional love that He has given me.

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CONTRIBUTION TO KNOWLEDGE

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2. **J.K KHUZWAYO**, A.O. EYODEJI, & F.B. LEWU; The chemical analysis of *Tetradenia riparia* and its traditional uses (Oral presentation); Ipuf conference (medicinal plants); 28 June - 1 July 2010; Upington.
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GLOSSARY OF TERMS AND ABBREVIATIONS

WHO	World Health Organisation
%	Percentage
g	gram
m	minutes
hr	hour
Nm	nanometer
µl/mg	micro litre per milligram
S	seconds
Rpm	revolutions per minute
T. riparia	*Tetradenia riparia*
KI	Kovets Index
Rt	Retention time
mm	millimetre
mg/ml	milligram per milli litre
meq.I	miller equivalent
µg/ml	micro gram per mili litre
i.p	intraperitoneol

CO_2	Carbon dioxide
GC-MS	Combined Gas Chromatography and Mass Spectrometer
ºC	Degree celsius
Dpph	diphenylpicrylhydrazyl
mg/kg	milligram per kilogram
KZN	KwaZulu-Natal
mL	millilitre
HCl	Hydrochloric acid
(v/v)	volume per volume
NaOH	Sodium hydroxide
M	molar mass
$FeCl_3$	Iron (III) chloride
α	alpha
β	beta
γ	gamma
δ	delta
BHT	Butylated hydroxytoluene

ABSTRACT

This dissertation has a brief historical overview of medicinal plants, *T. riparia* and their traditional uses. The choice of the medicinal plant studied was based on the evidence in literature that oils of the species contain phenolic compounds and the herbs belonging to the family *Lamiaceae* was implicated in the management of chronic and infectious diseases. The study was designed to determine the secondary metabolites present in the plant through phytochemical screening of the plant material, establish the chemical profile of the isolated oils using GC-MS and evaluate the biological potential of both the crude extracts and the isolated oils which included; antioxidant, antibacterial and anti-ulcer activities.

The essential oils were extracted from the dry stem and fresh leaves of *T. riparia*. Farnesol, caryophyllene oxide, α-fenchyl acetate, and guaiol (12.3 - 7.1%) were the most prominent compounds identified in the dry stem oil collected from the botanic garden, Botany Department, University of Zululand (UZ). The analysis of the fresh leaf oil from the botanic garden collection had hexane as the major compound (60.9%), while α-fenchyl acetate (6.7%), caryophyllene oxide (4.1%) and farnesol (2.5%) were other prominent components present in the oil. The dry stem oil from Maphumulo had farnesol (27.4%), humulene oxide (11.5%), α-eudesmol (8.9%), caryophyllene oxide (3.8%) and terpinene-4-ol (3.35) as the main components, while the fresh leaf oil composition from Maphumulo was similar to that of botanic garden with hexane accounting for over 50% of the oil composition. Other prominent compounds identified in the GC-MS analysis of the fresh leaf oil were farnesol (6.3%) and a-thujone (3.6%).

The chemical profiles of the *T. riparia* plant samples collected from two difference location within the KwaZulu-Natal suggest two distinct chemotypes of the plant species. Nonetheless, farnesol was found to be the major constituents of all the oils parts isolated. To the best of my literature knowledge and search, this is the first time that the chemical composition of the essential oils isolated from the leaf and stem of *T. riparia* is been reported.

The phytochemical screening of the plant reveals saponins (23.3%), flavoinoids (3.7-4.9%) and tannins (4.1%) to be prominent secondary metabolites presence. These compounds are known to exhibit anti-ulcer activities. Indeed, a 65% inhibition of indomethacin-induced gastric ulceration was observed for the plants essential oil. The antibacterial activity studies indicated that the oil had moderate inhibitory effect on most of the bacteria. Zones of inhibition, from the disc diffusion assay of the essential oil, ranged from 6.3- 19.3 mm. The minimum inhibitory concentration (MIC) value of 0.157 mg/ml was recorded for the dry stem oil extracts from Maphumulo.

The results from the biological assays showed that there is good correlation with the traditional usage of *T. riparia.* The results are explained fully in the body of the dissertation.

The limitation for this work is that the isolation of essential oil compounds using Preparative GC was not possible as the equipment though available was not functional due to some technical unresolved issues.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Medicinal plants

The use of plants, plant extracts or plant derived chemicals to treat diseases (topical, subcutaneous and systemic) has stood the test of time (Oladunmoye, 2006). Plants used for medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. Traditional healers claim that their medicine is cheaper and more effective than modern medicine. In developing countries, low-income people such as resource poor farmers, people of small isolated villages and native communities use folk medicine for the treatment of common infections (Rojas *et al*., 2006). According to World Health Organization (WHO, 2002), traditional medicine is estimated to be used by up to 80% of the population of most developing countries. Herbal medicines have been reported to be safe and with little adverse side effect, especially when compared with synthetic drugs (Iniaghe *et al*., 2009). Also, there has been little or no report of any form of microbial resistance during the use and administration of herbal preparations.

A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants are still of great importance (Diallo *et al.,* 1999). For thousands of years, Africans have relied on medicinal plants and knowledge thereof, to treat ailments. The use of medicinal plants predates the introduction of antibiotics and other modern drugs into the African continent. Africans have been able to cure a lot of diseases by using concoctions made from different plants and these have been passed from generation to generation (van Wyk *et al*., 1997 and Veeramuthu *et al*., 2006).

In recent years, there has been a gradual revival of interest in the use of medicinal plants from all sectors. These plant-based medicines are used for primary health care needs (WHO, 2002). Between 25-50% of modern drugs are derived from plants. Traditional medicine of late is viewed by the pharmaceutical industry as a source of "qualified leads" in the identification of bioactive agents for use in the production of synthetic modern drugs (de Silva, 1997).

1.2 *Tetradenia riparia*

Tetradenia riparia (Hochstetter) Codd is commonly known as misty plume bush, ginger bush (English); gemmerbos, watersalie (Afrikaans); gilinyathi, liBota (Siswati); iboza, ibozane (Zulu). The ginger bush plant belongs to the great lamiaceae or salvia family and has the characteristic aromatic leaves of this family. *T. riparia* is a soft wooded deciduous shrub, which flowers in late autumn and winter; it grows best in frost-free areas (Sheat *et al*., 2008). The species *T. riparia* is a widespread herbaceous shrub occurring throughout the wooded hillsides and stream banks of coastal KwaZulu-Natal, Mpumalanga and the Northern Province of South Africa, northern part of Namibia, Angola, Botswana and east tropical Africa. It grows rapidly to a height of 2m (6ft) and it's slightly succulent and has an irregular branch pattern (Van der Spuy, 1971). The leaves are bright green soft in texture and heart-shaped, with serrated edges **(Figure 1**). The tiny mauve flowers are carried in long graceful spray giving the whole plant a delightful misty appearance in late winter. The flowers of *Tetradenia* species range from white to lilac and some are with pink flowers. The stems are brown and smooth, except for the younger portions which are covered with glandular hairs and have a ruby tinge. The glandular hairs also cover both surfaces of the leaves and make them slightly sticky when touched.

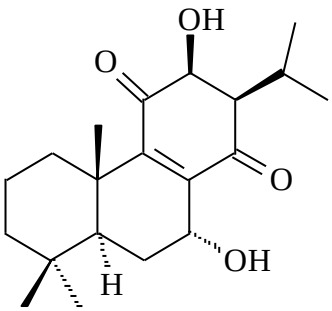
Figure 1: *Tetradenia riparia* (*Iboza riparia*)

The genus *Tetradenia* Benth, and *T. fruticosa* Benth. from the central highlands of Madagascar, were first described in 1830 (Benthnam, 1830). *T. riparia* was regarded as restricted to Madagascar only, until after about 150 years, the seven species of the genus was discovered in other places in Africa (Codd, 1983). All the seven species share many distinctive features with the Malagasy plants and it was concluded that the African and Malagasy plants are congeneric. However, Codd (1983) accepted only three of the species of *T. riparia* recognised at that time, reducing the other four to synonymy. This new combinations were made for *T. barberae* (N.E.Br) collected from the Eastern Cape of South Africa, *T. brevispicata* (N.E.Br) Codd from Botswana, South–west Zimbabwe and neighboring parts of South Africa, and *T. riparia* (Hochst.). Codd (1983) interpreted as a widespread and highly variable species extending from South Africa to Ethiopia, and west to Angola and Namibia. A fourth African species, *T. kaokoensis* was identified by van Jarrrsv. & A.E. van Wyk, which was endemic to the Otjihipa Mountains of North-western Namibia. *Tetradenia* was revised for Madagascar by Phillipson and Hedge (1998). In the field *Tetradenia* plants are often quite conspicuous in their preferred rocky habitats, and they are relatively well- represented in the relevant herbarium collections.

However, the inflorescences in most species are produced during the dry season when the plants may be leafless, and the genus is unusual in *Lamiaceae* in that most of species are deciduous (Johnson and Phillipson, 1999).

These characteristics make studies on *Tetradenia* rather challenging because herbarium specimens often lack leaves, and representative male and female plants have only rarely been collected together. In Rwanda *Tetradenia riparia* is used for antimicrobial and antispasmodic activities (van Puyvelde and de Kampe, 1987). Moderate anti-malarial activity of the leaf essential oil against two strains of *Plasmodium falciparum* (Campbell *et al*., 1997) has been reported.

Previous phytochemical studies of *T. riparia* revealed the isolations of diterpenoids ibozol (**1**) (**Zelnik *et al*., 1978)**, 7α-hydroxyroyleanone (**2)** and **8**(14), 15-sandaracopimaradiene-7α, 18-diol (**3**) (van Puyvelde *et al.*, 1987), *α*-pyrones umuravumbolide (**4**) (Davies-Coleman and Rivett, 1995), and tetradenolide (**5**) (van Puyvelde and de Kimpe, 1998). The diterpenediol (**3**) was found to display good antimicrobial, antispasmodic and antiparasitic activity. The diterpenediol (**3**) has also been shown to possess papaverine-like antispasmodic activity on methacholine, histamine and barium chloride-induced contractions of guinea pig ileum as well as on noradrenaline-induced contractions of rabbit aorta (van Puyvelde and de Kimpe, 1987). deacetylumuravumbolide (**6**), deacetlyboronolide (**7**) and 1'2'- dideacetyl-boronolide (**8**) were also isolated from *T. riparia* (Hakizamungu *et al*., 1988).



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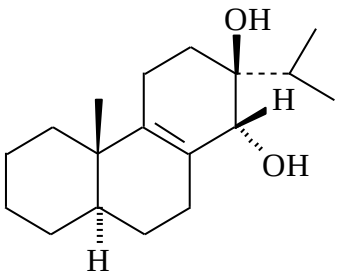

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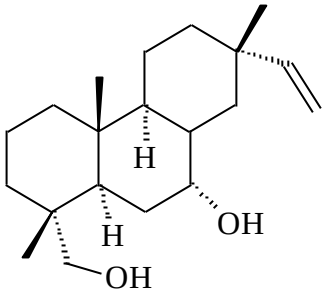

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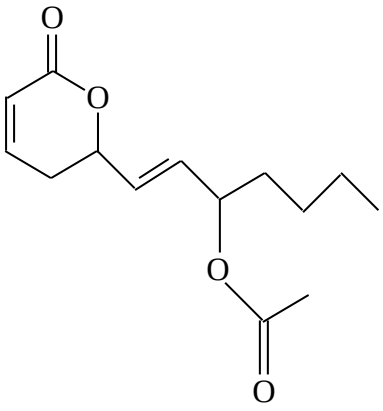

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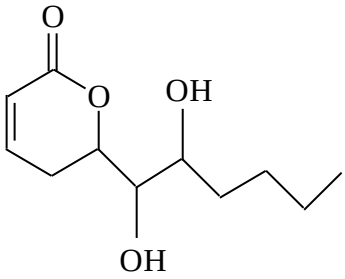

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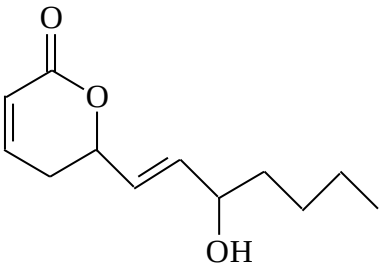

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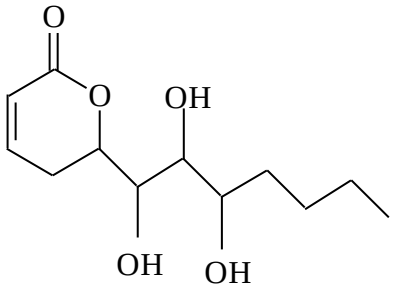

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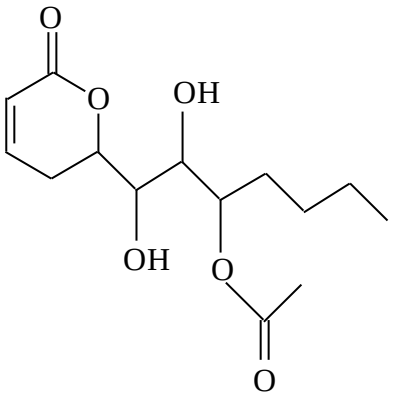

(**8**)

Leaf extracts of *T. riparia* (80% ethanol), tested for antimicrobial and antiviral activity, inhibited the growth of *Staphylococcus aureus, Candida albicans, Mycobacterium smegmatis, Microsporum canis, Trichophyton mentagrophytes* and *Bacillus subtilis.* No antiviral activity against Coxsackie virus, poliovirus (unspecified), measles virus and Semliki-Forest virus was demonstrated in these studies (Vlietinck, 1995). Some of the observed antibacterial activity of *T. riparia* has been attributed to the presence of diterpenes (van Puyvelde *et al*., 1986 and 1994). Research has shown that *Tetradenia riparia* has antibacterial and anti-fungal effects and some anti-malarial activity (Pieters and Vlietinckvan 2005).

1.3 Medicinal usage of *Tetradenia riparia* in Zulu and other African cultures

Leaf decoctions and infusions are widely taken for coughs and sore throats. Cold water infusion of ground leaves may also be taken for chronic coughs, followed by sufficient warm water to induce vomiting **(**Bryant, 1996). Leaf infusion are reported to be effective against malaria (Watt and Breyer-Brandwijk, 1962**),** also pieces of the leaf are chewed for dengue fever. The Twana people use leaf or shoot infusion for fever and also for gallsickness in cattle **(**Roberts, 1990**)**. Leaves are used in unspecified areas of Southern

Africa for diarrhoea and haemoptysis **(**Watt and Breyer-Brandwijk, 1962). Unspecified parts are also used for boils and mumps in Kenya (Githinji and Kokwaro, 1993**)**. Leaves blended with bananas and castor oil are useful as cattle medicine to treat insects (van Puyvelde *et al*., 1987).

In Rwanda, water macerates of leaves with those of *Vernonia amygdalina* Del. and *Markhamia utea* Schum are used for malaria (Hakizamungu *et al.*, 1992). *Tetradenia* plant is also used for the treatment of stomach ache, mouth ulcers, toothache, influenza, and swollen legs. Inhaling the scent of the crushed leaves apparently also relieves headaches. The plant is also used as a hallucinogenic herb (like tobacco). The strongly aromatic leaf has been reported to produce drowsiness (Watt and Breyer-Brandwijk, 1962). Two cases of suspected human poisoning from self-administered over dosage of hot water extract have been reported (Bodenstein, 1977).

1.4 Essential oil and its uses

An essential oil is a concentrated, hydrophobic liquid containing volatile aromatic compounds from plants. They are also known as volatile or ethereal oils, or simply as the "oil of" the plant material from which they were extracted, such as *oil of clove*. An oil is "essential" in the sense that it carries a distinctive scent, or essence, of the plant. They are not to be confused with essential fatty acids. The oil bears the name of the plant from which it is derived (Gwinn, 1985).

They are used in perfumes, cosmetics and bath products, for flavoring food and drinks, and household cleaning products and those used for therapeutic purposes.

1.4.1 Methods of extraction and isolation of essential oil

There are different methods employed in the extraction of essential oil which include **steam distillation, cold pressing, solvent extraction, carbon dioxide, vacuum distillation and hydrodistillation** (Anisman-Reiner, 1989).

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Steam distillation uses an outside source of steam which pipes the steam into the distillation unit, sometimes at high pressure. The steam passes through the aromatic material, and exits into the condenser. The gaseous oil then condenses as oily drops which accumulate on top of water forming two layers.

Cold-pressed expression or scarification is used to obtain citrus fruit oils such as bergamot, grapefruit, lemon, lime, mandarin, orange, and tangerine oils. In this process, fruits are rolled over a trough with sharp projections that generate the peel. This pierces the tiny pouches containing the essential oil after which the whole fruit is pressed in order to squeeze the juice from the pulp and to release the essential oil from the pouches. The essential oil rises to the surface of the juice and is separated from the juice by centrifugation (Anisman-Reiner, 1989).

Solvent extraction method is used on delicate plants, which yields a higher amount of essential oil at a lower cost. In this process, a chemical solvent such as hexane is used to saturate the plant material and pull out the aromatic compounds. This renders a substance called a concrete. The concrete can then be dissolved in alcohol to remove the solvent. When the alcohol evaporates, an absolute remains. Solvent extraction has disadvantages: Residues of the solvent may remain in the absolute and can cause side effects. While absolutes or concretes may be fine for fragrances or perfumes, they are not especially

desirable for skin care applications. Some trees, such as benzoin, frankincense, and myrrh, exude aromatic 'tears', or sap that is too thick to use easily in aromatherapy. In these cases, a resin or essential oil can be extracted from the tears with alcohol or a solvent such as hexane. This renders a resin or an essential oil that is easier to use. However, only those oils or resin extracted with alcohol should be used for aromatherapy purposes (Anisman-Reiner, 1989).

Turbo or vacuum distillation is suitable for hard-to-extract or coarse plant material, such as bark, roots, and seeds. In this process, the plant is soaked in water and pressurized steam is circulated through the plant and water mixture. Throughout the entire process, the same water is continually recycled through the plant material. This method allows faster extraction of essential oils from hard-to-extract plant materials (Anisman-Reiner, 1989).

When carbon dioxide (CO_2) is subjected to high pressure, the gas turns into liquid. This liquid CO_2 can be used as a very inert, safe, "liquid solvent" which will extract the aromatic molecules in a process similar to the solvent extraction. The advantage of this method is that no solvent residue remains, since at normal pressure and temperature, the CO_2 simply reverts to a gas and evaporates (Anisman-Reiner, 1989).

Hydrodistillation is one of most ancient method of distillation and the most versatile. Hydrodistillation seems to work best for powders (i.e., spice powders, ground wood, etc.) and very tough materials like roots, wood, or nuts (Anisman-Reiner, 1989).

1.4.2 Analysis of the Essential Oil Mixture

Essential oils are usually very complex mixture of chemical compounds. Essential oils although complex in composition, are constituted of mainly hydrocarbon and oxygenated terpenoids with 10, 15 and sometimes 20 carbon atoms such as monototerpenes, sesquiterpenes and diterpenes. Traces or great amounts of fats can be present in plant extracts, depending on the method of isolation and the origin (plant) of the oil.

Although GC and GC-MS are the best methods in use for quantitative and qualitative analysis of essential oils, many factors should be considered when using GC for the separation of essential oils. Since essential oils are mixtures of products with a wide range of chemical and physical properties, it is usually reasonable to perform some prefractionations before using gas chromatography. Complete essential oils are often chromatographed without any preliminary separation (Sonwa, 2000).

1.4.2.1 Combined Gas Chromatography and Mass Spectrometer

Owing to the fact that some essential oil components have similar or close retention times, the possibility of overcrowding of peaks do occur. GC-MS helps in resolving this issue as the compounds separated by the GC component are sent into the Mass spectrometer which further analyze each compound. The MS gives the molecular ion and the fragmentation pattern for each constituent of the mixture analyzed.

1.5 Research gap

In the course of literature survey the following research gaps were identified on *T. riparia*.

- Lack of chemical information on the South African *T. riparia* species.
- Inadequate information on the biological potential of South African *T. riparia*.
- *T. riparia* is used traditionally for healing mouth ulcers, a scientific investigation is necessary as a new source of medicine.
- Development of new antibiotic as the emergence of drug-resistance pathogen is increasing.
- The fear of diseases and death has made cultural groups to respond by developing the medical system and making use of natural product to cure various ailments, without adequate scientific backing.

1.6 Hypothesis

The leaves and stem of *T. riparia* contain essential oils of different composition. Essential oils of *T. riparia* collected from various locations differ in chemical compositions.

1.7 Aim and Research Objectives

The aim of this project was to **investigate the essential oil composition of *Tetradenia riparia* and elucidate some biological assays of the species.**

Specifically, the objectives of the study were:

1. **to isolate essential oils from the leaves and stem of *T. riparia***

2. analyze **the chemical composition of the essential oils using** Combined Gas Chromatography and Mass Spectrometer (GC-MS)**.**
3. **determine the** qualitative and quantitative **phytochemical screening of the secondary metabolites present in *T. riparia,* and**
4. **investigate the biological assay of *T. riparia* which includes, antibacterial, antioxidant and anti-ulcer assays.**

CHAPTER 2

MATERIALS AND METHODOLOGY

2.1 Plant collection

The plant materials were collected from the botanical garden, Botany Department, University of Zululand, and along stream banks in Kwa-Maphumulo area. The two collecting areas are within KwaZulu-Natal Province, South Africa. The authenticated voucher specimens were prepared and stored in the University Herbarium. Voucher numbers JKK 01 and JKK 02 were assigned to the plants collected from Botanic garden, UZ and Maphumulo village, respectively. Some of the plant materials were used fresh while some were air dried at room temperature for various experiments as indicated below.

2.2 Isolation of Essential Oil from Plant Sample

Hydro distillation method was used to obtain the oil using Clevenger apparatus and the British pharmacopeia method (British, 1998).

2.3 Analysis of the Essential Oil Mixtures

Qualitative and quantitative analyses of the oil mixtures were achieved by using GC-MS equipment following (Oyedeji *et al*., 2009) method. GC-MS analyses of the oils were performed using a Hewlett Packard Gas Chromatography HP 6890 equipped with a HP 5-MS capillary column (30m x 0.25 mm id, film thickness 0.25 µm) interfaced with Hewlett Packard 5973 mass spectrometer system. The oven temperature was programed from 70 ^{o}- 240 ^{o}C at the rate of 5 ^{o}C/min. The ion source was set at 240 ^{o}C and electron ionization at 70eV. Helium was used as the carrier gas at a flow rate of 1 ml/min, with

split ratio of 1:25. Scanning range was 35 to 425 amu. 1.0 µl of diluted oil in hexane was manually injected into the GC/MS.

2.4 Phytochemical Screening

Qualitative and quantitative phytochemical screening of plants extract is vital especially if there are some ethnomedicinal claims on the plant. Since *T. riparia* has been reportedly used by traditional healers in treating sores and wound, a need, therefore, arose, to test for the presence of certain secondary metabolites: alkaloids, saponins, anthraquinones, tannins, flavonoids and anthocyanosides (Mdlolo *et al*., 2008; Edeoga *et al*., 2005 and Harbone, 1973) that are known to enhance the healing process of some of these illness or infection.

2.4.1 Qualitative Screening

Plant filtrates were prepared by boiling 20 g of the fresh plant material in distilled water. The solution was filtered using a vacuum pump. The filtrates were used for the phytochemical screening of flavonoids, tannins, saponins, alkaloids, reducing sugars, anthraquinones and anthocyanosides.

Alkaloids

One millilitres of the plant filtrate was mixed with 2 ml of Dragendoff's reagent; a turbid orange color indicated the presence of alkaloids. The confirmation test was done using Mayer's reagent; a yellow precipitate indicated the presence of the alkaloids.

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Anthraquinones

About 1 ml of the plant extract to be tested was shaken with 10 ml of benzene and then filtered. Five millilitres of the 10% ammonia solution was then added to the filtrate and thereafter the shaken. Appearance of a pink, red or violet color in the ammoniacal (lower) phase was taken as proof of the presence of free anthraquinones.

Anthocyanosides

I m1 of the plant filtrate was mixed with 5 m1 of dilute HCI; a pale pink color indicated the positive test.

Flavonoids

One millilitre of the plant extract was dissolved in water and filtered; to this 2 ml of the 10% aqueous sodium hydroxide was later added to produce a yellow colouration. A change in color from yellow to colourless on addition of dilute hydrochloric acid was an indication for the presence of flavonoids.

Saponins

Two millilitres of distilled water was added to 1 ml of the extract. The mixture was vigorosly shaken and left to stand for 10 min. A stable persistent froth indicated the presence of saponins.

Tannins

A portion of the extract was dissolved in water, after which the solution was clarified by filtration. 10 % ferric chloride solution was then added to the resulting filtrate. The appearance of a bluish black color indicated the presence of tannis.

Reducing Sugars

One millitre of the plant filtrate was mixed with Fehling A and Fehling B separately; a brown color with Fehling B and a green color with Fehling A indicated the presence of reducing sugars.

2.4.2 Quantitative Screening

Alkaloids

5 g of the plant sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, the reaction mixture was covered and allowed to stand for 4 h. This was later filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide and then filtered; the residue being the alkaloid, which was dried and weighed to a constant mass.

Flavonoids

About 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol, at room temperature. The whole solution was filtered through Whatman filter paper No 42. The filtrate was later transferred into a crucible and evaporated into dryness over a water bath; the dry content was weighed to a constant weight.

Saponins

About 20 g each of dried plant samples were ground and, put into a conical flask after which 100 ml of 20% aqueous ethanol was added. The samples were heated over a hot water bath, at about 55 °C, for 4 h with continuous stirring. The mixture was filtered and the residue re-extracted with a further 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and then shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated three times and 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight; the saponins content was calculated as percentage of the starting material.

Tannins

About 500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h on a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the marked level. Then, 5 ml of the filtrate was transferred into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 minutes and the results were observed.

2.5 Biological Assay

2.5.1 Antioxidant Assay

Anti-oxidant is a substance that when present at low concentration compared to that of an oxidizing substrate prevents or delays oxidation of that substrate (Demo *et al*., 1998).

DPPH assay

The hydrogen atom or electron donation ability of the corresponding extract and some pure compounds were measured from the bleaching of purple colored methanol solution of DPPH. This spectrophotometer assay uses stable radical diphenylpicrylhydrazyl (DPPH) as a reagent (Burits and Bucar, 2000; Cuendet *et al*., 1997).

Aliquots (50µl) of various concentrations of the oil extract in methanol were added to 5ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517nm. Inhibition free radical DPPH in percent (I %) was calculated in the following way:

$$\mathbf{I\ \% = (A_{blank} - A_{sample}/A_{blank}) \times 100}$$

Where A_{blank} was the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} was the absorbance of the test compounds. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plotted graph of inhibition percentage against extract concentration. Synthetic antioxidant reagent hyroxytoluene (BHT) was used as the positive control and all tests were then carried out in triplicate.

Total reducing power

To determine reducing power, different concentrations of essential oils (5-250, µg/ml) in methanol were mixed with 2.5 ml of 200 mM sodium phosphate (pH 6.6) and (2.5ml of 1 % potassium ferrocyanide). The mixture was incubated at 50 ºC for 20 min followed by the addition of 2.5 ml of 10 % Trichloroacetic acid (TCA) and centrifuged at 1000rpm for 10 min. 2.5 ml of the mixture was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1 % ferric chloride and its absorbance measured at 700 nm. Ascorbic acid was used as the reference standard. Higher absorbances of the reaction mixture indicate greater reducing powers (Duh *et al*., 2001).

2.5.2 Antibacterial Assay

Twenty one microorganisms namely, *Escherichia coli ATCC* 8739, *Escherichia coli ATCC* 25922, *Escherichia coli KZN*, *Pseudomonas aeruginosa* (ATCC 19582), *Enterobacter cloacae ATCC* 13047, *Klebsiella pneumonia ATCC* 10031, *Klebsiella pneumonia ATCC* 4352, *Klebsiella pneumonia* KZN, *Proteus vulgaris ATCC* 6850, *Proteus vulgaris KZN*, *Serratia marscens ATCC* 9986, *Shigella flexineri KZN*, *Pseudomonas aeruginosa KZN*, *Salmonella spp. KZN*, *Acinetobacter calcaoceuticus anitratus CSIR*, *Streptococcus faecalis ATCC* 29212, *Bacillus cereus* ATCC10702, *Bacillus pumilus ATCC* 14884, *Enterococcus faecalis KZN*, *A15 Micrococcus kristinae*, *A20 Micrococcus luteus*, were obtained from the department of Biochemistry and Microbiology of the University of Fort Hare, Alice and University of Zululand. Agar disc diffusion method and minimum inhibition concentration were the two bioassay carried out on the essential oils.

Agar disc diffusion assay of the essential oils

The antimicrobial activity of the essential oils isolated from leaves and stem part of *T. riparia* from Botanical garden, University of Zululand and Maphumulo village, were evaluated using the agar diffusion method. One ml of initially prepared bacterial culture at a concentration of 10^6cfu/ml (colony forming unit/ml) was diluted in 20ml of molten Muller Hinton agar, and poured into sterile petri-dishes to solidify before wells were made with heat sterilized cork borer (Piddock, 1990). The size of the well was 6mm. The weight for each essential oil for the antibacterial assay was determined by diluting certain ml (w/w) in 1ml of dimethyl sulfoxide (DMSO). The equivalent weight was calculated and used in the assay. Two drops of each essential oil which was equated to 5 mg/ml concentration was dispensed into labelled wells in agar, allowed to stand for 1 h before being incubated at 37^oC for 24 h. After the incubation period, inhibition zones were measured with transparent meter rule. Two standard antibiotics were taken as positive experimental control while DMSO was considered a negative control. For stock antibiotic solutions, 10mg/ml concentrations of chloramphenicol and ampicillin antibiotics were prepared. (Oyedeji *et al*., 1999; Afolayan *et al*., 2004 and Oyedeji *et al*., 2010).

Minimum Inhibitory Concentration (MIC)

The 96 well microtitre plates method was used (Ellof, 1998) to determine the MIC value of the promising inhibiting bacteria from Agar diffusion assay result. Distilled water, nutrient broth and pipette tips were autoclaved and the cultures of bacteria were prepared and left for 24 h to grow. Dilute concentrations (50:50) of bacterial strains were used. A stock solution of each plant extracts was prepared by dissolving 0.4 g of the extract in 10 ml of the solvent; 10 mg/ml of chloramphenicol and ampicillin was used as a positive control. 100 ml of sterile water was added to the wells, then a stock solution was added

and serial dilutions of the solution was done down the column. 100 µl of bacteria culture was then added to each well. The microplates were covered and incubated overnight at 37 °C. Iiodonitrotetrazolium chloride (INT) solution (0.2 mg/ml) was prepared and 40 µl of the prepared solution was added to all the wells after 24 hr and was further incubated for 30 min; the red-colored of wells was noticed indicating the reduction of INT by the mitochondrial dehydrogenase which is an evidence of living bacteria. This was done in triplicate.

2.5.3 Anti-ulcer Assay

This study was carried out after the approval from the ethics committee on animal use and care of the University of Zululand (See appendix).

The essential oil extracts were evaporated under vacuum to dryness and the residue were re-dissolved in 2% Tween 20 for administration to animals. The anti-ulcer activity was determined using modified methods of (Elegbe, 1978 and Njar *et al.*, 1994).

Five groups (A- E) of rats (4 per group) of approximately the same age and weight were used for test purposes. Group A received 2% Tween 20, B received propranolol and from C to E each received different concentrations of the plant extract (see Figure 2). Food and water were withdrawn 24hrs and 2hrs respectively before the start of the experiment. All animals received their drugs orally expect the group that received propranolol (i.p). Ulcers were induced in all five groups. Ulcers were induced 1 hr after administration of propranolol and 2hrs after extract. This was done by the intraperitoneol (i.p) administration of indomethacin. After 4hrs, all animals were sacrificed and their stomachs were opened along the greater curvature.

Intragastric pH

The stomach contents were washed into measuring cylinder with 10ml distilled water and gastric contents- water mixtures were centrifuged at 2500rpm for 10 minutes. The pH of the supernatant was then measured using the pH meter.

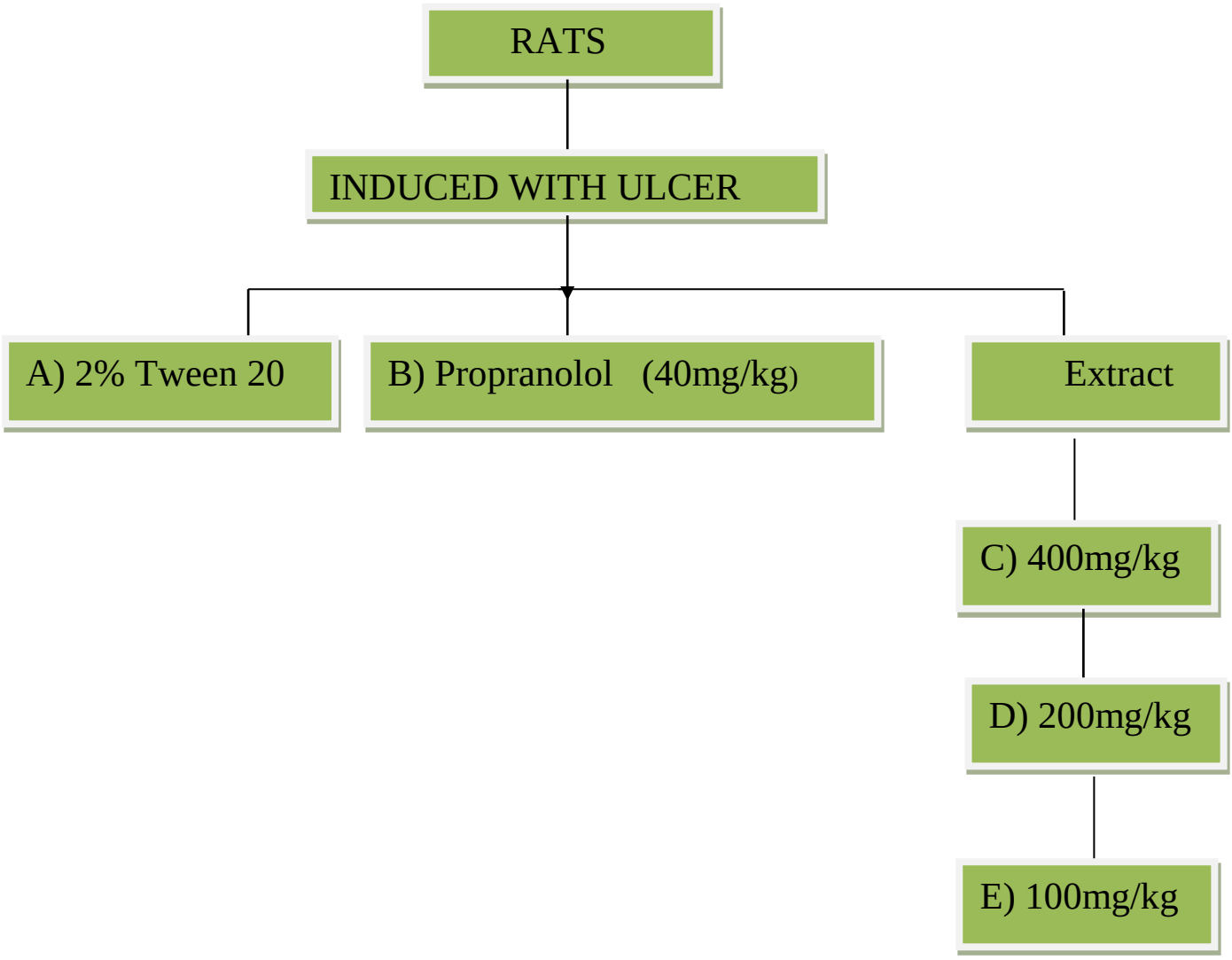


Figure 2. Diagrammatic presentation of the rats groups for anti-ulcer assay

Macroscopic examinations of the stomach

The stomach was washed with saline and macroscopic examination was carried out using the hand lens with × 2 magnifications, the criteria according to Elegbe (1978) were used for ulcer scoring (Table 1).

Table 1: Total score of ulcers found in stomach of the rats

0	Normal stomach
0.5	**Punctuate haemorrhage or pin point ulcers**
1	**Two or more small haemorrhagic ulcers**
2	**Ulcers greater than 3mm in diameter**

Histological sections of the stomach were made and stained with haematoxylin and eosin and mounted in Canada balsam. Microscopic examination of the section was then carried out under a light microscope.

Ulcer index was calculated using the formula;

Ulcer index = (mean degree of ulceration × % group of ulcerated) / 100

Inhibition of ulceration was calculated using the formula;

% inhibition of ulceration = (ulcer index in control – ulcer index in test) / ulcer index in control) × 100

CHAPTER 3

RESULTS, DISCUSSION AND CONCLUSION

The results of the experiment described in chapter two are presented below along with the analysis of each result (discussion).

3.1 Physicochemical analysis of the isolated oils

Table 2: Percentage yield, color and odor of the essential oils extracts from Botanical garden and Maphumulo.

Properties	FLBG	DSBG	FLM	DSM
% yield (w/w)	0.289	0.363	0.303	0.341
Color	Light yellow	Yellow (thick)	Light yellow	Yellow (thick)
Odor	Aromatic	Burnt	Aromatic	Burnt
Refractive index	1.4998	1.50280	1.4978	1.5028

FLBG = fresh leaves from botanical garden, *DSBG* = dry leaves from botanical garden, *FLM* = fresh leaves from Maphumulo area (KZN), *DSM* = dry stems from Maphumulo area (KZN)

Physical and chemical properties such as color, odor, percentage yield and refractive index of the essential oil of *Tetradenia riparia* were observed as shown in (Table 2). Color of absolute oil was noted from physical appearance. Refractive index of the oil was determined at 25 ^{o}C using Abbe's refractometer. The essential oil of the dry stems from botanical garden DSBG) and dry stem for Maphumulo area (DSM) had the highest refractive index being 1.5028 and 1.5028 respectively, whilst the oil of fresh leaves from botanical garden (FLBG) and fresh leaves from Maphumulo area (FLM) had 1.4998 and 1.4978, respectively. These results indicated that *Tetradenia riparia* contains important

chemical constituents and is suitable for the extraction of essential oil for medicinal and perfumery purposes on commercial scale.

3.2 GC-MS analysis of isolates oils

3.2.1 Analysis of fresh leaf oil of *T. riparia* from Botanical Garden (FLBG)

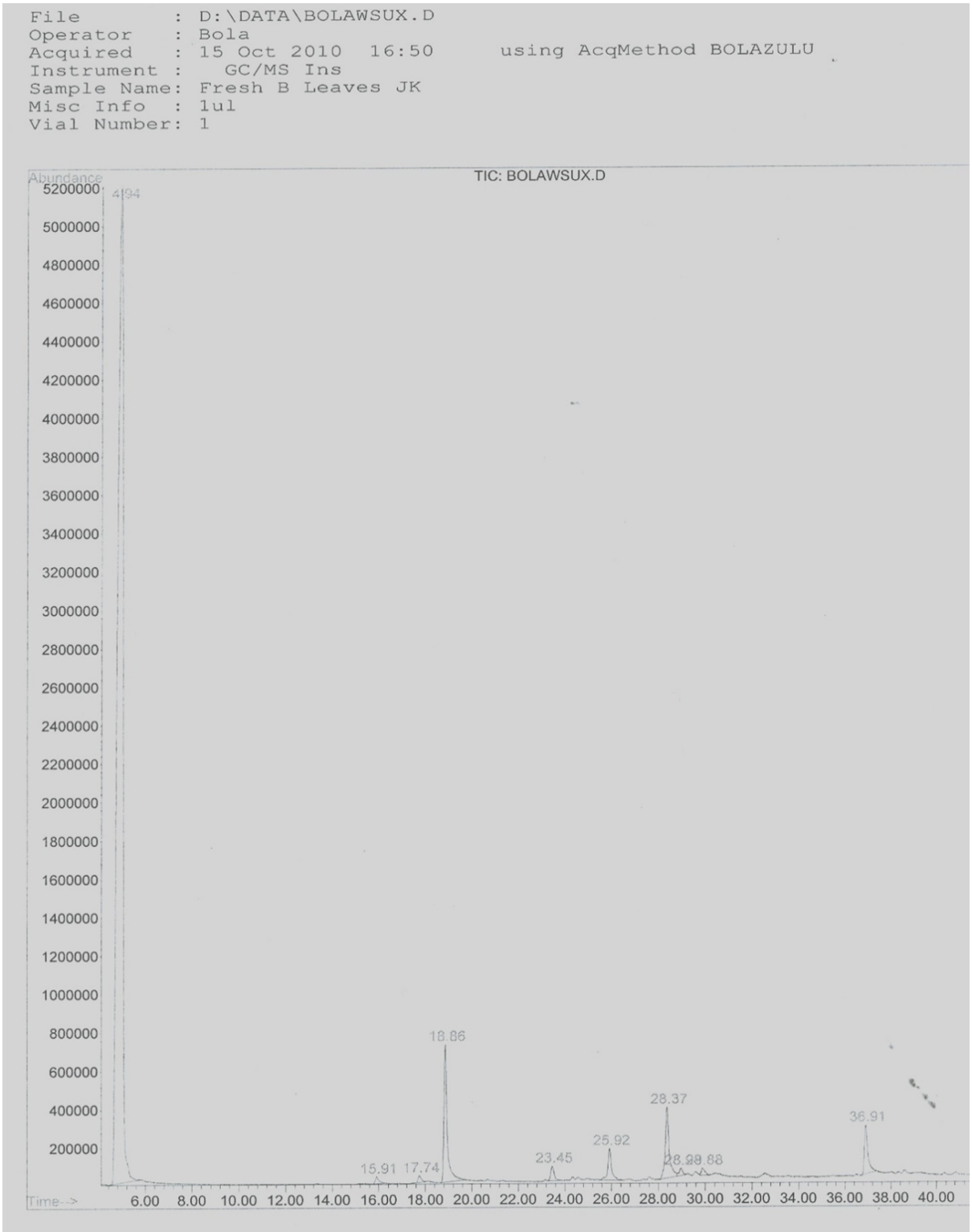


Figure 3: GC-MS Chromatogram of the fresh leaf oil of *T. riparia* from Botanic Garden (FLBG)

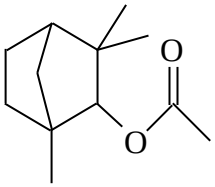
Table 3: Chemical composition of fresh leaf oil of *T. riparia* from Botanic Garden (FLBG)

Eluting Serial no.	RT	% composition	RI	Compound
1.	5.89	**60.9**		Hexane
2.	15.91	0.4	1112	Fenchol
3.	17.73	0.3	1180	4 – Terpineol
4.	18.86	**6.7**	1221	Fenchyl acetate
5.	23.45	0.7	1393	β – bourbonene
6.	25.92	1.8	1499	α -Muurolene
7.	28.37	**4.0**	1588	Caryophyllene oxide
8.	36.91	**2.5**	1719	Farnesol
	Total	**77.3%**		

Rt (min) = Retention time (as minutes); *RI* = Calculated retention indices (kovats Index)

The GC-MS chromatogram of the essential oil of the fresh leaf oil obtained from the sample collected at the botanical garden, University of Zululand is presented in Figure 3. Eight compounds were identified from the GC-MS analysis of the oil which accounted for 77.3% (Table 3). Hexane was the highest compound with 60.9% of the total oil composition. The reason for the high hexane content cannot be immediately accounted for. Nonetheless, other significant components of the oil were fenchyl acetate (**9**) (6.7%), caryophyllene oxide (**10**) (4.0%) and farnesol (**11**) (2.5%). Apart from hexane, the oil had more of oxygenated compounds. β-Bourbonene and α-murrolene were the only hydrogenated terpene. The chemical pattern of the oil can be said to contain a high percentage of volatile hexane, followed by oxygenated monoterpene (7.4%),

sesquiterpene (3.5%) and oxygenated sesquiterpenes (6.5%). Monoterpenes were not detected at all.



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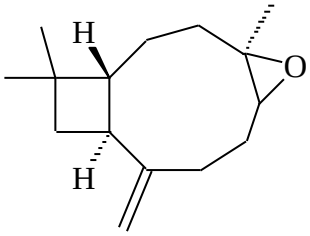

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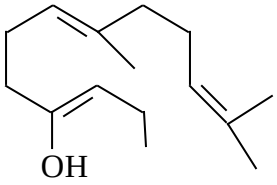

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3.2.2 Analysis of dry stem oil of *T. riparia* from Botanic Garden (DSBG)

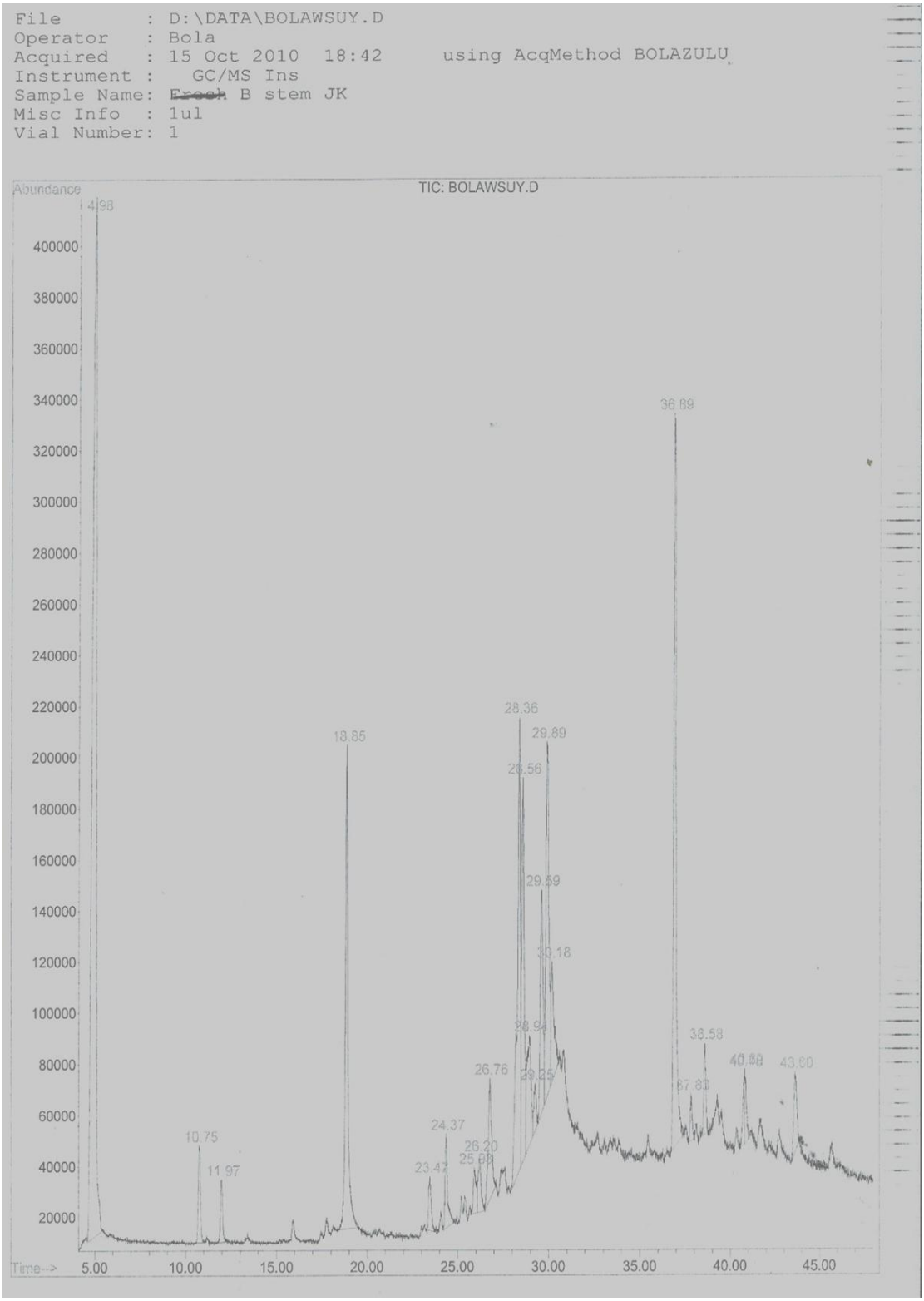


Figure 4: GC-MS chromatogram of the dry stem oil of *T. riparia* from Botanic Garden (DSBG)

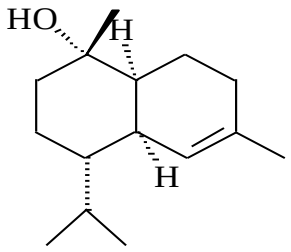
Table 4: Chemical composition of the dry stem oil of *T. riparia* from Botanic Garden (DSBG)

Eluting Serial no.	R_t(min)	% composition	KI	Component
1.	4.99	6.8		Hexane
2.	10.75	1.5	937	α – Pinene
3.	11.98	1.0	974	β - Pinene
4.	18.85	**9.0**	1220	α – Fenchyl acetate
5.	24.37	1.6	1415	β –Caryophellene
6.	25.93	0.9	1495	α - Amorphene
7.	26.16	1.2	1510	γ – Cadinene
8.	26.76	2.9	1520	δ – Cadinene
9.	28.36	**9.2**	1577	Caryophyllene oxide
10.	28.56	**7.1**	1589	Guaiol
11.	28.94	3.2	1590	Viridiflorol
12.	29.25	0.2	1591	Humulene epoxide
13.	29.59	4.2	1638	Torreyol
14.	29.89	**7.3**	1642	T-Muurolol
15.	30.18	2.5	1647	α- Eudesmol
16.	36.89	**12.3**	1719	Farnesol
	Total	**70.9%**		

Rt (min) = Retention time (mins), KI = Kovats Index

The GC-MS chromatogram of the essential oil of the dry stem from the botanical garden plant collected is presented in Figure 4. The oil analysis reveals the presence of 16

compounds. Two were monoterpene (α- and β-pinene) accounting for only 2.5% of the total oil composition. Fenchyl acetate (**9**) (9.0%) was the only oxygenated monoterpene identified in the dry stem oil. The presence of the volatile hexane component was, however, reduced in percentage amount when compared to the fresh leaves oil. This could be to the fact that the hexane component could have evaporated during the drying process. Four sesquiterpenes: β-caryophyllene, α- amorphene, γ-and δ-cadinene, (6.6% in total) were present in the oil. It was noted from the analysis that the dry stem bark was very rich in oxygenated sesquiterpenoid (45.9%) with farnesol (**11**) accounting for 12.3%, caryophyllene oxide (**10**) 9.2%, T-muurolol (**12**) 7.3% and guaiol (**13**) 7.1% (Table 4).



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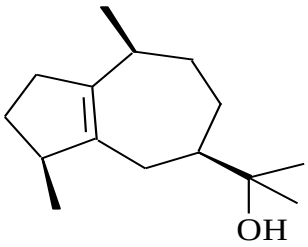

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3.2.3 Analysis of fresh leaf oil of *T. riparia* from Maphumulo (FLM)

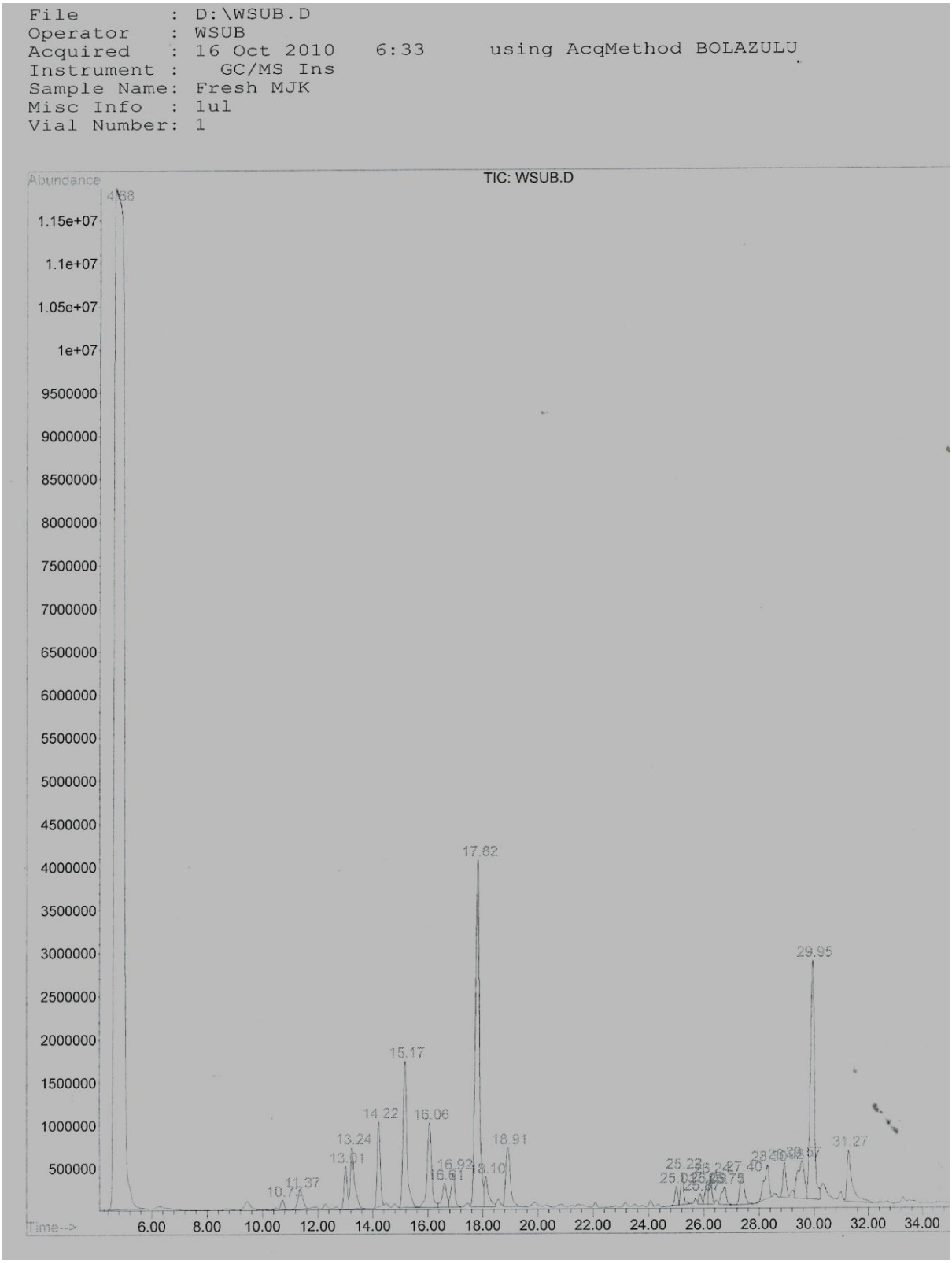


Figure 5: GC-MS chromatogram of the fresh leaf oil of *T. riparia* from Maphumulo (FLM)

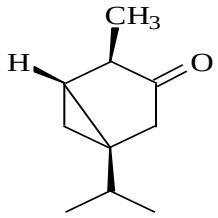
Table 5: Chemical composition of fresh leaf oil of *T. riparia* from Maphumulo (FLM)

Serial no. of elution	RT	% composition	KI	Compound
1.	4.49	60.4		Hexane
2.	10.73	0.2	940	α – Pinene
3.	11.37	0.6	1087	Fenchone
4.	13.01	0.8	1089	Terpinolene
5.	13.24	1.6	1089	para Cymenene
6.	15.17	**3.6**	1117	α- Thujone
7.	16.61	0.7	1145	Terpenene-1-ol
8.	25.00	0.3	1461	α- Humulene
9.	25.87	0.2	1484	Germacrene –D
10.	26.24	0.5	1495	Germacrene B
11.	26.75	0.5	1523	B – Cadinene
12.	27.40	0.9	1549	Elemol
13.	28.30	1.1	1588	Caryophyllene oxide
14.	29.95	**6.3**	1719	Farnesol
	Total	**77.7%**		

Rt (min) = Retention time (as minutes)KI = kovats Index

Fourteen compounds were identified in the Maphumulo fresh leaf oil from GC-MS chromatogram (Figure 5) which accounts for 77.7% of the total oil composition isolated from the leaves. It was noted that hexane constituted more than 50% of the total oil composition.

This pattern was similar to the fresh leaf and stem of the plant sample collected from the University of Zululand Botanical garden. α-Thujone (**14**) (3.6%) and farnesol (**11**) (6.3%) were the other prominent compounds of the oil (Table 5).



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3.2.4 Analysis of dry stem oil of *T. riparia* from Maphumulo (DSM)

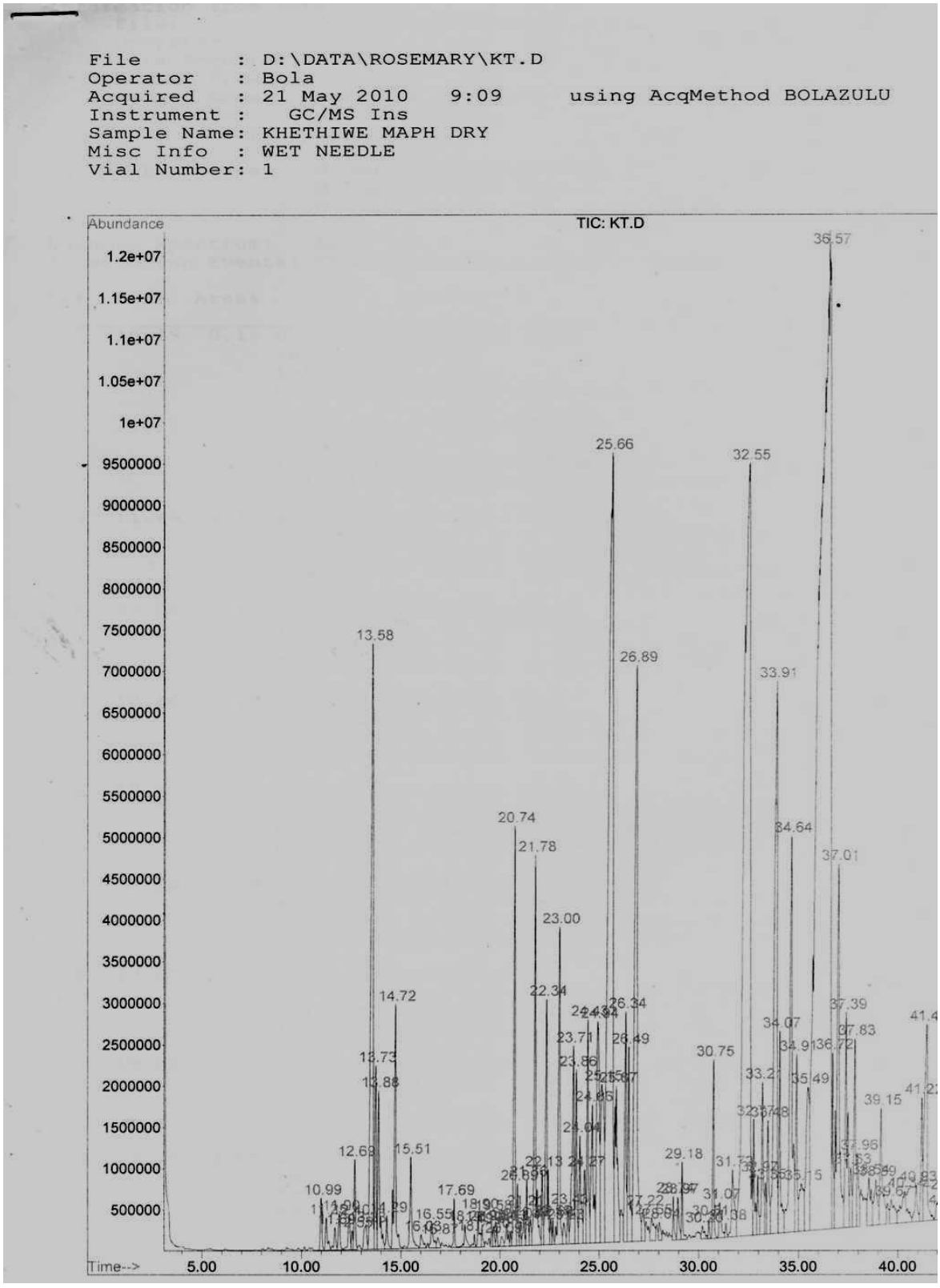


Figure 6: The GC-MS analysis of the dry stem of *T. riparia* from Maphumulo (DSM)

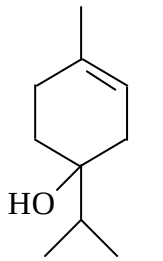
Table 6: Chemical composition of dry stem oil of *T. riparia* from Maphumulo (DSM)

Serial no. of elution Pk#	RT	KI	% Composition	Compound
1.	10.99	1087	0.2	Fenchone
2.	11.25	1097	0.2	Linalool
3.	11.69	1120	0.1	α- Fenchyl alcohol
4.	11.90	1141	0.2	1-Terpineol
5	12.55	1149	0.1	Camphor
6.	13.58	1179	**3.3**	Terpinene-4-ol
7.	13.74	1181	0.7	*p*-Cymen-8-ol
8.	13.88	1187	0.7	α- Terpineol
9.	14.29	1193	0.1	cis- Piperitol
10.	16.56	1298	0.2	Thymol
11.	17.69	1492	0.1	γ-Elemene
12.	18.23	1495	0.1	Eugenol
13.	19.84	1588	**3.8**	Caryophyllene oxide
14.	20.74	1591	**1.4**	α- Humulene
15.	23.85	1633	0.1	Globulol
16.	24.66	1647	0.8	γ-Eudesmol
17.	26.67	1617	**8.9**	α- Eudesmol
18.	32.54	1621	**11.6**	Humulene epoxide
19.	36.57	1719	**27.4**	Farnesol
20.	44.81	2300	1.0	Tricosane
21.	45.97	2500	0.1	Pentacosane
		Total	60.8%	

Rt (min) = Retention time (as minutes, KI = kovats Index

Although more peaks could be seen from the chromatogram of the Maphumulo dry stem oil, only 21 compounds (60.8%) that were identified, while the remaining compounds were unknown (Figure 6). It was noted from the oil analysis of the dry leaf oil that while hexane

was in large amount in the University of Zululand (UZ) botanical garden samples, it was found to be in trace amount in the Maphumulo oil sample. Further analysis of the GC-MS result of the oil revealed that no monoterpene was present and only 5.9% oxygenated monoterpenes with terpinene-4-ol (**15**) (3.3%) as the major monterpenoid constituent.A similar pattern was also noticed for the sesqiterpenoid constituents. β-Elemene and α-humulene were the only sesquiterpene (1.5%) identified in this oil sample. Farnesol (**11**) (27,4%), humulene expoxide (**16**) (11.6%), α-eudesmol (**17**) (8.9%) and caryophyllene oxide (**10**) (3.8%) were the main components of the oil and oxygenated sesquiterpene group present in the oil. Two fatty acids tricosane and pentacosane were identified in the GC-MS analysis of the oil, (Table 6).



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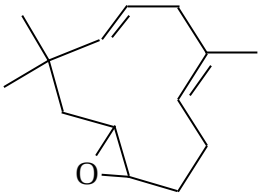

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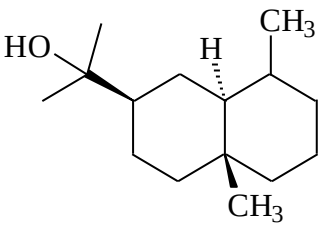

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In general, the high concentration of hexane content in three of the oil samples isolated cannot be easily over-looked as this is an indication that hexane is a major component of the volatile fraction of the oil constitution. This happens to be the first time in which the hexane will be reported as a major constituent of plant volatile oil. Farnesol (**11**) was found to be the main oxygenated sesquiterpene component present in all the oil samples. Farnesol dominancy in the *T. riparia* oil samples was in the following this order; DSM (27.4%) > DSBG(12.3%) > FLM (6.3%) > FLBG (2.5%). Farnesol (**11**) is reported to have flavoring property and it is an ingredient for cigarettes; its anti-bacterial properties has also been reported (Hemmer *et al.*, 2000). Caryophyllene oxide (**10**) reported to display anti-inflammatory, anti-carcinogenic and skin enhancing properties (Opdycke &

Letizia, 1983) was also present in all the oil samples in significant amounts. Traditional uses of *T. riparia* have good correlation with the major compounds found in the plant.

3.3 Phytochemical screening

Table 7: **Result of the Qualitative analysis of *Tetradenia riparia***

Phytochemical components	Observed/Expected Confirmatory test	FLBG	DSBG	DLM	FLM
Alkaloids	Yellow precipitate	+	+	+	+
Tannins	Dark green colour	+	+	+	+
Saponins	Foamy suspension on the solution	+	+	+	+
Antraquinones	Development of a pinkish solution	-	-	-	-
Anthocynosides	Development of a pale pinkish solution	-	-	-	-
Flavonoids	Golden yellow colouration	+	+	+	+
Phenolic Flavonoids	Brown precipitate	+	+	+	+
Reducing sugar Fehlings A	Brown color solution	+	+	+	+
Reducing sugar Fehlings B	Green color solution	+	+	+	+

Note: + = Present; - = Absence

Table 8: Result of the Quantitative analysis of *Tetradenia riparia*

Name	% yield (FLM /FLB)
Saponins	23/25
Flavoinoids	4.9/3.7
Alkaloids	0.4/0.3
Tannins	4.1/3

FLM = Fresh leaves from Maphumulo, University of Zululand; FLBG = Fresh leaves from Botanic Garden,

To particularize the research gap in literature on this plant of study, a routine check of the phytochemical screening of the *T. riparia* was done. The results shown in (Figure & Table 7) revealed the presence of alkaloids, tannins, saponins, flavonoids and reducing sugar; anthraquinones were however, absent.

The quantitative analysis of four secondary metabolites (tannin, saponin, flavonoids and alkaloid) was carried out as a proof to the botanical usage of the plant in folklore medicine. Saponins had the highest percentage composition in both plant sample. This was closely followed by flavonoids (Table 8). Saponins and flavonoids are known to be anti-inflammatory (Just *et al*., 1998) and antiviral, and are major ingredients in traditional Chinese medicine (Liu and Henkel, 2002). Flavonoids serve as health promoting compound as a results of its anion radicals (Hausteen, 1983). These observations support the usefulness of this plant in the treatment of mouth sores (Mathekga, 2001; Lourens *et al*., 2004; Fergusion, 2001; Grierson and Afolayan, 1999). Saponins and flavonoids tend to justify that South African *T. riparia* can be used traditionally for such related diseases. Alkaloids are also known to possess a lot of pharmacological properties. They are mostly used as antidepressant (morphine), stimulants (caffine), anaesthetic (cocaine), antitumor

(vinblastine) antimalaria (quinine), antibacterial (berberine) and amoebicide (emetine), (Cowan, 1999). The percentage composition was low in both plant samples.

In view of the quantitative and qualitative analysis above, one would not expect to be considerable variation in the biological application of these plant samples, even though they were collected at difference location and time.

Figure 7: The appearance of extracts after phytochemical screening

3.4 Antibacterial assay

3.4.1 *Agar Disc - diffusion assay*

Table 9: Antibacterial activity of an essential oil of the *T. riparia* using disc-diffusion method.

Microorganism	Zone of Inhibition (mm)					
	FLBG 5mg/ml	DSBG 5mg/ml	FLM 5mg/ml	DSM 5mg/ml	Amp. 10mg/ml	Chlo 10mg/ml
Gram –ve						
Escherichia coli ATCC 8739	7.0±2.12	6.5 ±1.49	0.00±0.00	6.5±0.02	6.5±2.12	22.5±3.54
Escherichia coli ATCC 25922	6.5±0.70	6 ±1.49	0.00±0.00	6.3±1.70	8.5±0.70	28±1.07
Escherichia coli KZN	18.5±0.73	17.9±2.12	11±1.41	19.3±1.70	8±2.83	29.5±2.12
Pseudomonas aeruginosa ATCC 19582	10.0±0.75	7.5 ±0.71	12±0.01	12.8±0.70	10.5±2.12	22±2.83
Enterobacter cloacae ATCC 13047	15±0.70	13.0±4.24	8±1.41	7.5±0.70	11±2.82	25±1.41
Klebsiella pneumonia ATCC 10031	15.0±2.89	10.0±2.83	13.5±2.12	12.3±1.95	6.5±0.707	28.5±4.95
Klebsiella pneumonia ATCC 4352	8.5±0.20	7.5±2.12	9.5±0.71	8.6±0.70	10±0.00	18.5±2.12
Klebsiella pneumoniae KZN	7.0±0.13	6.5±0.71	11±1.41	11.6±0.70	7±1.41	27.5±0.36
Proteus vulgaris ATCC 6850	8.7±0.80	9±1.41	7.5±2.12	7.3±0.92	5.5±0.70	28±2.83
Proteus vulgaris KZN	10.9±0.71	9±1.41	10.5±3.58	11.1±0.45	9±2.83	26±1.66
Serratia marscens ATCC 9986	8.2±0.50	8±0.00	9±4.24	7.9±0.63	11.5±1.41	24.5±0.70
Shigella flexineri KZN	11.3±0.70	10.5±1.36	9±0.00	10.9±0.10	7±1.41	19±4.24
Pseudomonas aeruginosa KZN	13.5±2.53	11.5±1.95	16.5±0.71	14.2±0.71	26.5±0.71	22±0.00
Salmonella spp. KZN	14.9±1.56	16.5±1.33	12.5±0.54	18.1±2.78	12±5.66	17.5±1.70
Acinetobacter calcaoceuticus anitratus CSIR	7.3±0.42	12.5±1.54	7.5±0.61	8.0±0.70	9±1.41	27±1.49
Gram +ve						
Streptococcus faecalis ATCC 29212	10.2±2.70	10 .0 ± 2.21	8.5±2.12	9.5±3.40	7.5±2.12	21.5±2.12
Bacillus cereus ATCC10702	13.5±1.17	11.0±7.07	9.5±2.12	9.4±1.70	9.5±2.12	20.5±0.70
Bacillus pumilus ATCC 14884	8.3±0.30	7.0±1.41	13.5±0.95	16.8±1.40	6.5±2.12	21±1.41
Enterococcus faecalis KZN	12.7±0.26	11.5±2.02	8±2.82	8.9±0.30	8.5±3.54	20.5±0.70
A15 Micrococcus kristinae	6.9±0.73	7±2.83	8.5±0.71	7.3±0.71	6±1.41	20.5±0.70
A20 Micrococcus luteus	7.3±0.70	8.5±0.71	11±2.82	13.8±0.40	8.5±2.12	30±0.00

FLM= Fresh leaves of T. riparia from Maphumulo area, DSM= dry stem of T. riparia from Maphumulo area, FLBG=Fresh leaves Botanical Garden, DSBG=Dry Stem Botanical Garden, KZN, CSIR, A20 and A20 are typed organisms; are clinical isolates. Amp. =Ampicillin, Chlo =Chloramphenicol, Values represented as mean ± SD (n = 3)

The antibacterial activities of *T. riparia* fresh leaves, and dry stem were investigated against both fifteen Gram negative and six Gram positive bacteria and the results are presented in Table 9. All the four oil extracts had a broad spectrum of antibacterial

activity, although the activity appears to be a moderate inhibitory effect. However, there are some strong inhibitions that were noticed by all four oil extracts against the growth of *Pseudomonas aeruginosa KZN* (11.5-16.5 mm), *Escherichia coli KZN* (11.1-19.3 mm), *Klebsiella pneumonia ATCC* 10031(10.0 – 15.0 mm), *Salmonella spp. KZN* (11.5 – 16.5 mm) bacteria. The essential oils of DLM and DSM from Maphumulo showed moderate to strong inhibition against *Bacillus pumilus ATCC* 14884 (13.5 - 16.8 mm), *Pseudomonas aeruginosa* ATCC 19582 (12.0 – 12.8mm) and *Klebsiella pneumonia KZN* (11.1 – 11.6mm) when compared to the activity of the other oils extracts from the Botanical garden samples. In general, it was also noticed from the result in Table 9, that the essential oil extracts from Maphumulo (FLM and DSM) area had weaker potential for antibacterial activity than the oil extracts from Botanical garden (FLBG and DSBD). The oil was, however, more active against the Gram-positive bacteria than the Gram-negative ones.

The inhibitory activities of the oils extracts, nonetheless, compares favorably with the standard antibiotic used as control in this study.

3.4.2 *Minimum Inhibitory Concentration (MIC)*

Twelve bacteria which had high inhibition value was used in the MIC evaluation. The MIC results (Table 10) showed that the dry stem oil from Maphumulo (DSM) had more low promising activity when compared to the other three remaining oil extracts. This was followed closely by the fresh leaf oil from Maphumulo (FLM) and dry stem from the botanical garden (DSBG). The four oil extract showed good inhibitory effect at low concentration against *Bacillus pumilus* ATCC 14884 (1.250 -0.313 mg/ml) and *Proteus vulgaris* KZN (1.250 – 0.625 mg/ml).

Table 10: Minimal inhibitory concentration (MIC) of essential oil of *T. riparia* with antibacterial activity

Microorganism	Minimal inhibitory concentration *of T. riparia* oil (mg/ml)					
	FLBG	DSBG	FLM	DSM	Chl.	Amp.
Shigella flexineri KZN	5.000	5.000	2.500	2.500	5.000	2.500
Escherichia coli KZN	2.500	1.250	0.625	0.157	1.250	1.250
Micrococcus luteus A20	1.250	2.500	2.500	1.250	2.500	0.625
Bcillus pumilus ATCC 14884	1.250	1.250	0.625	0.313	1.250	0.313
Acinetobacter cacaoceuticus CSIR	5.000	2.500	5.000	5.000	2.500	5.000
Proteus vulgaris KZN	0.625	0.625	1.250	1.250	1.250	1.250
Pseudomonas aeruginosa KZN	2.500	2.500	5.000	5.000	1.250	5.000
Klebsiella pneumonia ATCC 10031	0.625	1.250	0.625	0.625	0.625	1.250
Enterococcus faecalis KZN	1.250	1.250	2.500	1.250	0.625	2.500
Pseudomonas aeruginosa ATCC 19582	1.250	0.625	1.250	1.250	1.250	0.078
Streptococcus faecalis ATCC 29212	1.250	1.250	1.250	1.250	0.625	1.250
Bacillus cereus ATCC 10702	0.625	0.630	2.500	0.625	1.250	0.625

FLBG= fresh leaves Botanical Garden, DSBG=dry stem Botanical Garden, DLM=dry leaves Maphumulo, DSM= dry stem Maphumulo, Amp = Ampicillin.

The strong inhibition activity of the essential oil of DSM against *E coli* KZN at a lower concentration of 0.157mg/ml is worth noting, more especially when compared to 1.250mg/ml inhibitory concentration of the standard antibiotics. It is apparent that the oil can be a good source of antibacterial drug especially in cases where there are drug resistances against these standard known antibiotics. The dry stem from Botanical garden (DSBG) had lowest MIC value of 0.62 mg/ml against *Bacillus pumilus* ATCC 14884, *Pseudomonas aeruginosa* ATCC 19582 and *Bacillus cereus ATCC 10702.*

3.5 Antioxidant assay

Antioxidant assay was carried out using dry plant material.

3.5.1 *DPPH radical scavenging activity*

Table 11: **Percentage DPPH scavenging activities of the essential oil of *T. riparia* from Maphumulo**

Plant part used	50µl/mg	100µl/mg	250µl/mg	500µl/mg	1250µl/mg	2500µl/mg
DSM	6.20±0.057	4.50±0.152	8.90±0.050	9.20±0.100	11.20±0.208	26.70±0.100
DLM	4.55±0.071	3.24±0.064	8.88±0.028	7.85±0.078	17.29±0.0070	20.45±0.212
BHT	0.275±0.021	0.835±0.035	4.57±0.028	11.45±0.028	57.88±0.077	61.30±0.424

DSM = dry stem Maphumulo, DLM = dry leaves Maphumulo
BHT = butylated hydroxytoluene, Values represented as mean ± SD (n = 3)

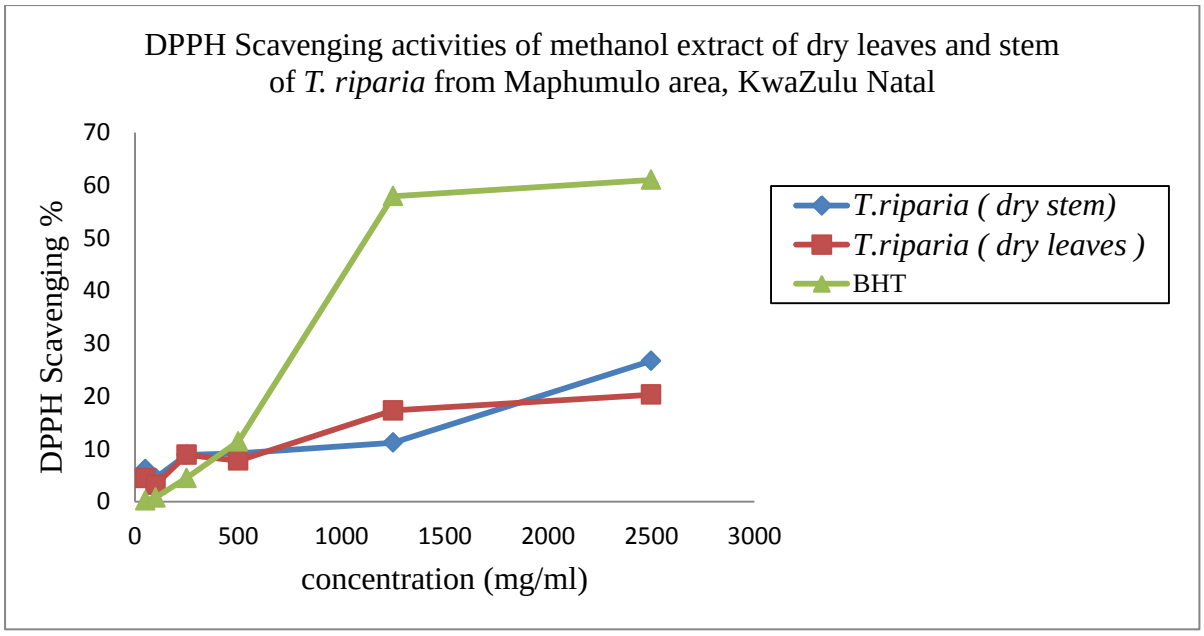

Figure 8. DPPH Scavenging activities of the methanol extract of the leaves and stem of *T. riparia* from Maphumulo area, KwaZulu Natal

Table 12: **Percentages for the DPPH method scavenging activities of the essential oil of *T. riparia* from Botanical garden**

Plant part used	50µl/mg	100µl/mg	250µl/mg	500µl/mg	1250µl/mg	2500µl/mg
DSBG	2.37±0.042	3.51±0.028	4.33±0.084	3.86±0.049	8.86±0.120	10.67±0.205
DLBG	1.23±0.042	2.43±0.339	7.65±0.035	8.62±0.042	10.29±0.106	15.78±0.028
BHT	0.535±0.035	0.46±0.042	5.65±0.021	12.67±0.035	33.59±0.403	40.78±0.028

DSBG = dry stem Botanical Garden, DLBG = dry leaves Botanical Garden
BHT = Butylated hydroxytoluene, Values represented as mean ± SD (n = 3)

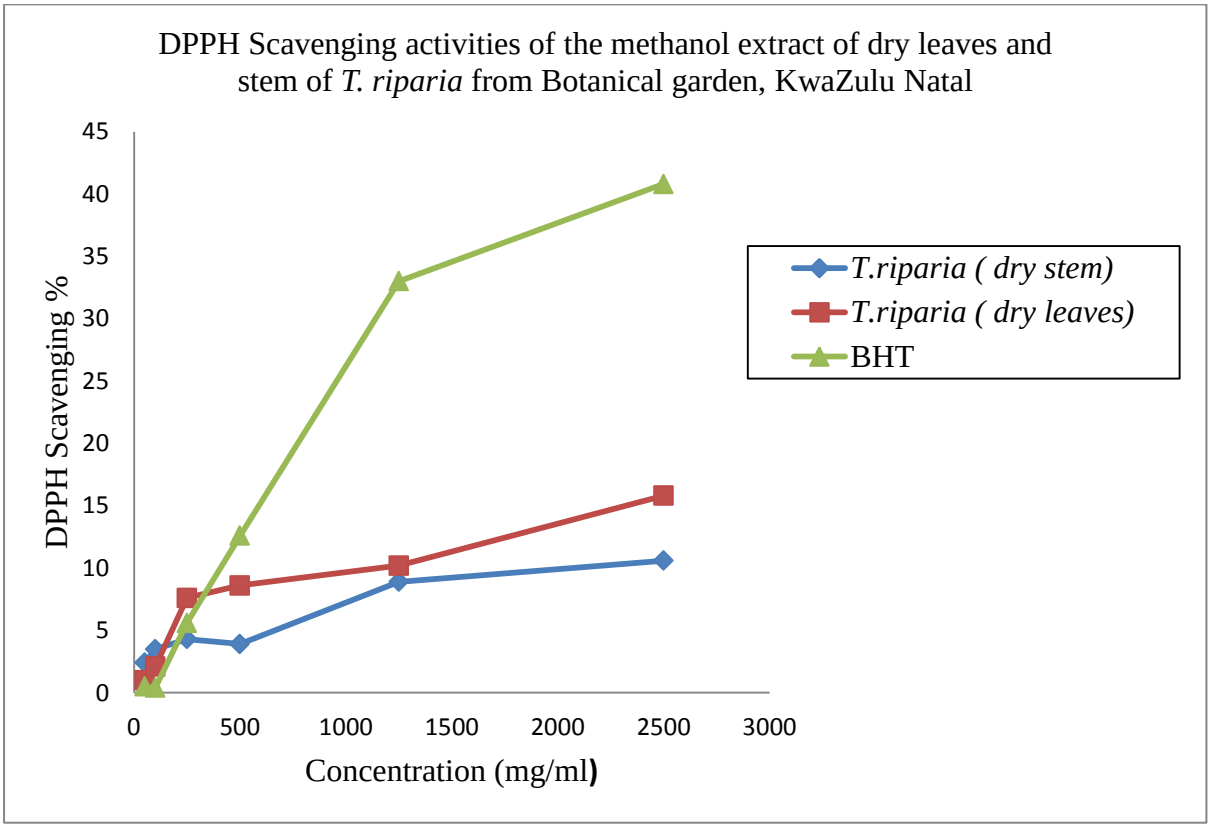

Figure 9. DPPH Scavenging activities of the methanol extract of the leaves and stem of *T. riparia* from Botanical garden, UZ

In the present study, free radical scavenging activities of four oil extracts of the medicinal plants were evaluated. Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS (Veeru *et al*., 2009). Antioxidants, due to their free radicals scavenging activity, are useful for the management of those diseases. DPPH stable free radical method is a sensitive way to determine the antioxidant activity of plant extracts (Koleva *et al*., 2002, and Suresh *et al.*, 2008). Figure 8 and figure 9 show the amount of each essential oil extract required for 50% inhibition of DPPH activity, whilst Table 11 and Table 12 show the percentage scavenging activities represented as mean ± SD (n = 3) of the essential oil extracts and the standard antioxidant.

When the percentage scavenging activity is compared for Table 11 and that of Table 12, the free radical scavenging action**s of the oil extract in methanol were increasing in this order dry stem collected from Botanical garden (DSBG) < dry leaves collected from Botanical garden (DLBG) < dry leaves collected from Maphumulo area (DLM) < dry stem collected from Maphumulo area (DSM). The standard antioxidant (BHT) showed the highest activity against all oil extracts. The oil extract which showed the strongest DPPH radical scavenging activity is dry stem from Maphumulo area (DSM) whilst others showed moderate antioxidant properties.** The therapeutic potential of natural medicinal plants as an antioxidant in reducing such free radical induced tissue injury, suggests that many plants have antioxidant activities that can be therapeutically useful (Kanatt *et al*., 2007). **One can conclude that this oil extracts, especially DSM can be used for antioxidant activity.**

3.5.2 *Reducing power*

Table 13: The Reducing power method of the essential oil of *T. riparia* from Botanical garden and Maphumulo

Conc. (µg /ml)	*Plant part used*					
	DLM	**DSBG**	**DSM**	**DLBG**	**BHT**	**ASC.ACID**
50	0.0249 ± 0.0006	0.0249± 0.0009	0.0249± 0.00036	0.0249± 0.00098	0.0214± 0.0026	0.413± 0.002
100	0.0249± 0.0007	0.0223± 0.0005	0.0226± 0.0011	0.0226± 0.0030	0.2143± 0.0015	0.4563± 0.0037
250	0.0316± 0.0003	0.0316± 0.0002	0.0316± 0.0002	0.0316± 0.0002	0.3283± 0.0011	0.486± 0.002
500	0.056± 0.001	0.032± 0.001	0.0327± 0.0015	0.0323± 0.0015	0.598± 0.001	0.678± 0.001
1250	0.2033 ± 0.0057	0.1200± 0.0100	0.0489± 0.0002	0.0489± 0.00015	0.754± 0.001	0.8453± 0.0015
2500	0.263± 0.001	0.19± 0.0100	0.1933± 0.0152	0.098± 0.0010	1.1076± 0.0005	1.369± 0.001

DLM = dry leaves Maphumulo, DSM = dry stem Maphumulo, DSBG = dry stem Botanical garden, DLBG = dry leaves Botanical garden, Asc.acid = Ascorbic acid and BHT = butylated hydroxytoluene, Values represented as mean ± SD (n = 3)

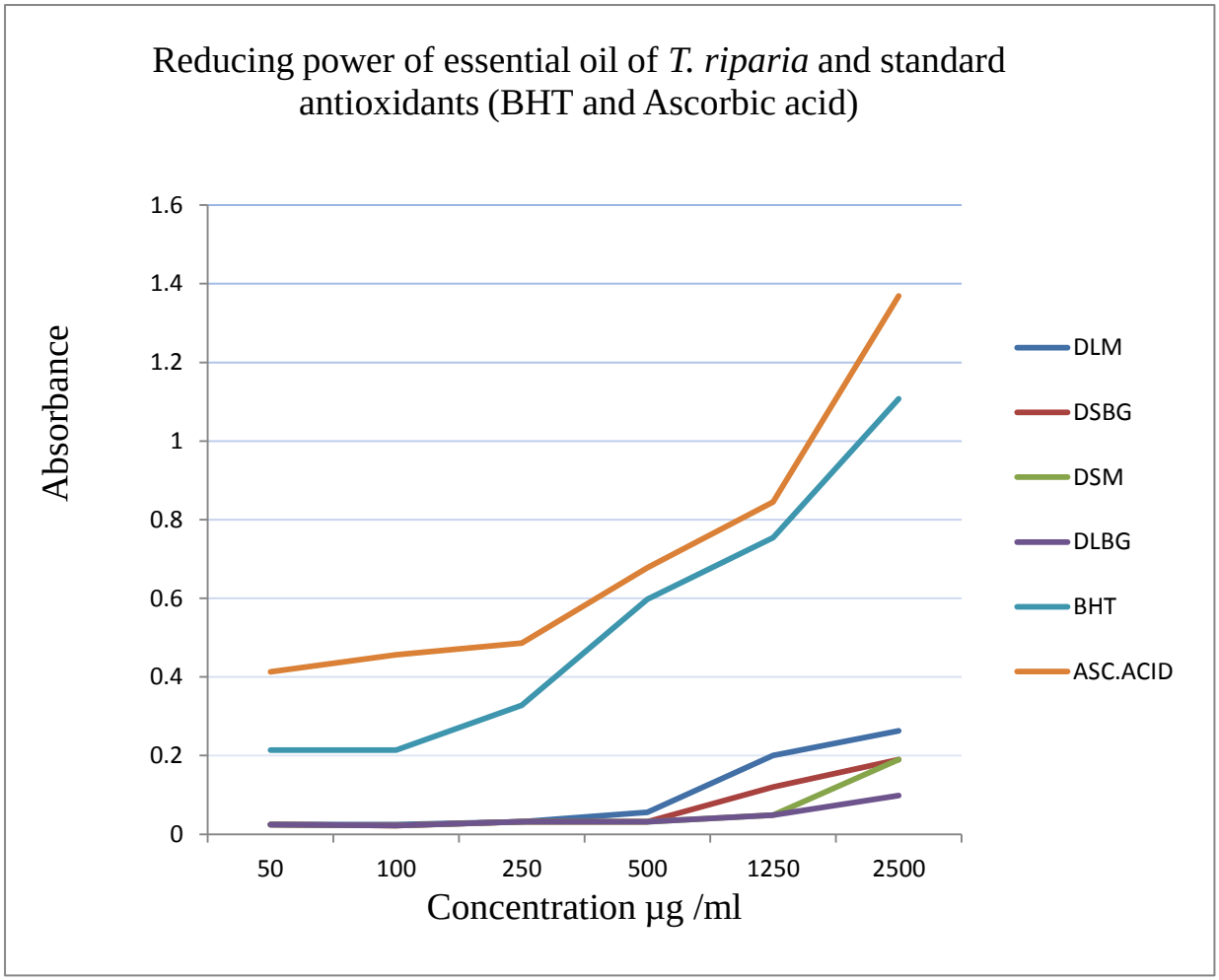

Figure 10: **Reducing power of essential oil of *T. riparia* and standards antioxidants (BHT and Ascorbic acid).**

DLM = dry leaves Maphumulo, DSM = dry stem Maphumulo, DSBG = dry stem Botanical garden, DLBG = dry leaves Botanical garden, Asc.acid = Ascorbic acid and BHT = butylated hydroxytoluene

The reducing power of a compound is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity. Figure 10 and Table 13 shows the reducing power of the oil extract and standard antioxidants (BHT and Ascorbic acid) as function of it concentration. From the graph represented in Figure 10 it is apparent that the standard antioxidants Ascorbic acid and BHT had the higher activity than the extracts.

For the oil extract the reducing power increased in the order DLM > DSBG > DSM > DLBG. The reducing power was concentration dependent. The oil extract collected from Maphumulo area, KwaZulu-Natal proves to be better source of antioxidant than the Botanical garden extracts although the difference is insignificant. This behavior could be attributed to the difference on local soil and environmental conditions where the plants were raised.

3.6 Anti-ulcer assay

The anti-ulcer activity studies could only be carried out with the dry leaves extract of the Maphumulo samples owing to the fact that there were little or no more leaves on the shrub from the University of Zululand Botanic garden. Also, in view of the distance of the Maphumulo village from the University, fresh plant extracts could not be tested, hence only the use dry leaves were used for the study.

Table 14: Effect of *T. riparia* extract on gastric content, pH, total and free acidity for the ulceration induced in rats

Treatment	Dose (mg/kg)	Gastric content (ml)	pH of gastric content	Total acidity (meq./l)
Negative control	D/W[#]	17.05	7.24	6214.73
Propanolol	D/W[#]	11.58	7.32	4264.91
*T. riparia * (Dry M)*	100	11.7	4.65	4229.65
*T. riparia * (Dry M)*	200	15.68	4.07	4210.36
*T. riparia * (Dry M)*	400	9.03	4.01	3291.44

[#] Distilled water, Values in the results are expressed as mean of five readings (n=5)
**Dry M = Dry Maphumulo*

Table 15: Effect of *T. riparia* oil extract on gastric content in indomethacin induced ulceration in rats

Treatment	Dose (mg/kg)	Ulcer score	% of Incidence	Ulcer index	% Inhibition
Negative control	D/W[#]	0.5	75	0.38	-
Propanolol	D/W[#]	0	0	0	100
*T. riparia *(Dry M)*	100	0.25	50	0.13	65
*T. riparia *(Dry M)*	200	0.25	50	0.13	65
*T. riparia *(Dry M)*	400	0.25	50	0.13	65

[#] Distilled water, Values in the results are expressed as mean (n = 5)
**Dry M = Dry Maphumulo, [%] Incidence = % of animals with ulcer*

In most cases the etiology of ulcers is unknown, it is generally accepted that they are the results of an imbalance between aggressive factors and the maintenance of mucosa integrity through endogenous defensive mechanism (Akah *et al.,* 1998). To recover the balance, different therapeutic agents including plant extracts may be used (Gurbaz, 2003). *T. riparia* is one of the herbal drugs used traditionally to treat ulcer. In the present study the plant extrac.t of *T. riparia* showed significant (65%) anti-ulcer activity in indomethacin induced gastric ulceration in rats. The anti-ulcer activity of *T. riparia* is evident from its significant reduction in acidity, total acidity, number of ulcer and ulcer index shown in (Table 14 and Table 15) . The phytochemical screening analysis of *T. riparia* extract showed the presence of flavonoids. Flavonoids are among the cytoprotective materials for which anti-ulcerogenic efficacy has been extensively confirmed. The anti-ulcer activity of *T. riparia* may therefore be attribute to its flavonoids content and subsequently, to its antioxidant activity.

3.7 Conclusion and Recommendation

Analysis of all essential oils investigated in this study reveal the predominance of two major compounds farneol and caryophyllene which have strong biology activities. Farnesol dominancy in the *T. riparia* oil samples was in the following order; DSM(27.4%) > DSBG(12.3%) > FLM(6.3%) > FLBG(2.5%). Farnesol has been reported to have flavouring property and anti-bacterial properties (Hemmer *et al.*, 2000). Caryophyllene oxide is reported to display anti-inflammatory, anti-carcinogenic and skin enhancing properties (Opdycke and Letizia, 1983). In view of the anti-ulcer assay carried out on this oil extracts, the traditional uses of *T. riparia* therefore correlates with the major compounds found in this plant.

Furthermore, the presence of saponins at high concentration in the crude extract and tannins does support the enthobotanical claims on the plant. These two metabolites are known to be useful in the treatment of ulcerated tissue. The anti-ulcer assay showed 65% inhibition ulceration in the gastric content of the rats. This confirms that *T. riparia* can be used traditionally for management of ulcers. Flavonoids are reported to have anti-inflammatory (Just *et al.*, 1998) and antiviral properties, this compound was also present in large amount in the quantitative study carried out on this plant. The presence of this group of compounds further justify that South African *T. riparia* can be used traditionally for such related diseases.

The challenge now is to try and isolate some bioactive volatile compounds using their preparative GC and also carry out *in vivo* investigations so as to ascertain the potency of these compounds with the crude extract for possible drug formulation. Comparing the chemical composition and biology potential of the South African *T. riparia* with those

from other countries is eminent as this will establish the economic value of this plant species.

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25 March 2010

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **JK Khuzwayo** (Student No **20033252**), registered for a MSc Degree in the Department of Chemistry at the University of Zululand, in accordance with appropriate rules submitted a research project proposal to the Ethics Committee of the Faculty of Science and Agriculture at the University of Zululand. The research project will investigate: **Essential oil composition and selected biological assay of *Tetradenia riparia***. Based on the research protocol stipulated, this committee could find no reason from an ethical standpoint to reject the proposed research. There is however a stipulation that administration of any medicinal substances such as anesthetics should be supervised by a suitably qualified person, in this case Dr G. Fourie.

Yours sincerely

Mr L Vivier
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand