Taxonomy and Pharmacognostic studies of *Tarchonanthus camphoratus* species complex (Tarchonanthea, Asteraceae)

by

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Co-supervisor: Prof F.C. Clarke

DECLARATION

I, **Abimbola Aro,** hereby declare that the work on which this dissertation is based, is original (except where acknowledgements indicate otherwise).

This dissertation is submitted for the degree Master of Science in Medicine at the University of Limpopo. Neither the whole work nor any part of it has been submitted before for any degree or examination at this or any other university.

Signed…… **Date…….**

Student Number:……

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SUMMARY

Taxonomy and Pharmacognostic studies of *Tarchonanthus camphoratus* species complex (Tarchonanthea, Asteraceae)

The genus *Tarchonanthus* belongs to the family *Asteraceae*, the subfamily *Cichorioideae* and the tribe *Tarchonantheae*. *Tarchonanthus* is one of the few Astareceae genera that includes a tree. Furthermore, it is dioecious, with male and female flowers produced on different plants. The specific name is usually chosen to indicate some striking characteristics of the plant. The name *camphoratus* refers to the strong smell of camphor given off when the leaves are crushed. Various parts of *Tarchonanthus* species are used medicinally. Infusions and tinctures of the leaves and twigs are used for stomach trouble, abdominal pain, headache, toothache, asthma, bronchitis and inflammation.

The main aim of this study was to improve the overall knowledge available on *Tarchonanthus camphoratus* and *T. parvicapitulatus* by resolving the uncertainties surrounding the taxonomy and also to check for the toxicity and the therapeutic effect of this plant.

A micromorphological and solubility studies proved useful in distinguishing between *T. camphoratus* and *T. parvicapitulatus* since the macromorphological characteristics of the plant did not prove useful due to overlapping keys used to distinguish the two species. A solubility studies also proved useful in differentiating between male, female or sterile plants of *T. camphoratus* and *T. parvicapitulatus.*

The results that were obtained from the TLC plates showed that there are differences in the chemical composition of the acetone extracts of *T. camphoratus* and *T. parvicapitulatus*. Moreso, this study showed that the acetone extract of *T. parvicapitulatus* has more antioxidant properties than *T. camphoratus*.

The therapeutic study also revealed that the plant extract from the leaves of *T. camphoratus* and *T. parvicapitulatus* showed no prophylactic protection against smooth muscle contraction of guinea-pig treachea but the plant extract of *T. parvicapitulatus* could relax pre-existing smooth muscle contraction of guinea-pig trachea.

Superoxide production was significantly inhibited by both *T. parvicapitulatus* and *T. camphoratus* plant extract, but the rate of superoxide inhibition of *T. parvicapitulatus* extract were more prominent than it was for *T. camphoratus* extract. The inhibitory effects of the water extract of *T. camphoratus* and *T. parvicapitulatus* on the isolated human neutrophils could be due to the activity of the intermediate polar compounds which include flavonoids. This explains why these plant extracts are used by traditional healers for the relieve of ailments such as abdominal pain, headache, toothache *T. parvicapitulatus* are toxic but the plant extracts of *T. camphoratus* did not show significant toxicity to isolated human neutrophils.

This study has helped to improve the overall knowledge available on *Tarchonanthus camphoratus* and *Tarchonanthus parvicapitulatus* by using different pharmacognostic tools to resolve the uncertainties surrounding the taxonomy and to determine the toxicity and the therapeutic effects of this plant.

Keywords

Tarchonantheae, Asteraceae, *Tarchonanthus camphoratus*, *Tarchonanthus parvicapitulatus*, micromorphology, macromorphology, solubility, taxonomy, thin layer chromatography, therapeutic, prophylactic, ATP, antioxidant, neutrophils, superoxide production, toxicity.

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BEA	Benzene:Ethanol:Ammonium hydroxide
$CaCl_2$	Calcium chloride
CEF	Chloroform:Ethylacetate:Formic Acid
CH_3COOH	Ethanol
CO_2	Carbondioxide
EMW	Ethylacetate:Methanol:Water
g	Gram
HCL	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Hydrochloric Acid
H_2O	Water
$HOCl^-$	Hypochlorous acid
TLC	Thin layer chromatography
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
O_2^-	Superoxide anion
OH^-	Hydroxyl radical
$NAHCO_3$	Sodium hydrocarbonate
PMA	Phorbol myristate acetate
µl	Microlitre
ng	Nanogram

ml	Millilitre
M	Mole
N_2	Nitrogen gas
rpm	Revolutions per minute
NaCl	Sodium chloride
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
NaH_2PO_4	Sodium hydrophosphate

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CHAPTER ONE

1.1 INTRODUCTION

"Throughout the ages, humans have relied on nature for their basic needs such as the production of food-stuffs, shelter, clothing, means of transportation, fertilizers, flavors, fragrances, and not the least medicine" (Ameenah Gurub-Fakim, 2006). Plants having been the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies and today, almost 80% of the "world's population relies on plants as a major part of their primary health care because medicinal plants constitute an effective source of traditional and modern medicines" (Ameenah Gurub-Fakim, 2006). Also, plant products play a vital role in the health care systems of the remaining 20% of the population, mainly residing in developed countries (Cragg and Newman, 2001).

Plants have always been used for treating various disease conditions "either in the form of traditional preparations or as pure active principles" (Farnsworth, 1994). A large number of modern drugs have been isolated from natural sources in which many base their use on traditional medicine (Cragg and Newman, 2001). The importance of medicinal plants and traditional health systems in solving the health care problems of the world is gaining increasing attention (Alluri *et al*., 2005). Farnsworth and Soejarto, (1991) state that historically, "all medicinal preparations were derived from plants, whether in the simple form of raw plant materials or in the

refined form of crude extracts or mixture". The vast majority of traditional remedies contain substances of plant origin but only about 25% of all known plant species have ever been investigated for their medicinal potential. An estimated 122 drugs from 94 plant species have already been discovered through ethnobotanical leads and these plant-based systems play an important role in health care (Fabricant and Farnsworth, 2001).

Medicinal plants still form the principal component of traditional medicine. This means that in the order of 3,300 million people use medicinal plants on a regular basis (Eloff, 1998). People who use traditional remedies may not understand the scientific rationale behind these medicines, but they know, from personal experience that some medicinal plants can be highly effective if used in therapeutic doses (Van Wyk *et al*., 1997). Medicinal plants typically contain mixtures of different compounds that may act individually, addictively or in synergy to improve health (Ameenah Gurib-Fakim, 2006). A single plant may, for example, contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swelling and pain, phenolic compounds that can act as antioxidants and venotonics, anti-bacterial and anti-fungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give a sense of well-being.

In developing countries, the use of traditional medicine is gaining increasing attention and this is probably due to the increase in population and the patriotic desire of the

people to revive and maintain their traditional culture (Ricardo *et al*., 2004). Developing countries are often subject to shortage of funds, medical facilities and modern medicine, which make them more dependent on their natural resources (Mammem and Cloete, 1996; Shale *et al*., 1999). In many cases developing countries cannot simply afford to spend millions of dollars on imported medicines. Several African and Asian nations are now encouraging the use of traditional medicines as an integral component of their public health care programs. Indigenous medicines are relatively inexpensive, locally available and are readily accepted by the local population (Ameenah Gurib-Fakim, 2006).

Africa has a rich biological and cultural diversity but unfortunately, this system of medicine is poorly recorded and remains so to date. Yet the documentation of medicinal uses of African plants is becoming increasingly urgent because of the rapid loss of the natural habitat of some of these plants because of anthropogenic activities. The African continent has one of the highest rates of deforestation in the world. Herbal medicines are an important part of the culture and tradition of the African people. The healer usually diagnoses and treats the psychological basis of an illness before prescribing medicines to treat the symptoms. 60% of the South Africa's population usually consults one of an estimated 200,000 traditional healers, in preference to, or in addition to Western medical doctors, especially in the rural areas because medicinal plants are an important aspect of the daily lives of many people and an important part of the South African cultural heritage (Van Wyk *et al*., 1997).

Natural products are becoming important in our modern-day society as man is moving away from synthetic products, which can be detrimental to the environment and human health (Louw *et al*., 2002). Although natural products, particularly secondary metabolites, formed the basis of medicines and the presence of these compounds in the biochemistry of the plant is very often difficult to determine. It has been suggested that these compounds may have been synthesized by the plant as part of the defense system of the plant; for example, plants are known to produce phytoalexins as a response to attack by bacteria and fungi (Ameenah Gurib-Fakim, 2006). The "Zulu, Xhosa and Sotho people of South Africa use around 147 plant families for medicinal purposes" (Hutchings *et al*.,1996). The most prominent of these, listing over 50 plant species each, are the Fabaceae, Asteraceae, Euphorbiaceae, Rubiaceae and Orchidaceae families. The wide spread global use of the majority of these families highlight the traditional focus on herbal plants and trees for healing purposes. One-third of the most frequently used indigenous plants in South Africa are tree species (Louw *et al*, 2002; Van Wyk, *et al*., 1997).

1.2 LITERATURE REVIEW

Plants can be used as taxonomic markers for the discovery of new compounds. All plants possess hundreds of characters of a morphological, histological, embryological, serological, chemical and genetic nature which are potentially available for building up a classification of the plant kingdom (Trease and Evans, 2002). Plants and their derivatives contribute to more than 50% of all medicine used worldwide so in this way it can be said that traditional healers and their medicine has played a major role in the development of western medicine.

Because medicinal plants have chemical compounds that are useful to treat many diseases, they are widely recognized as "chemical factories" capable of synthesizing large numbers of highly complex and new chemical substances (Farnsworth, 1984). Plant extracts are used particularly because they may possess notably biological activity including antioxidants, antibacterial and antifungal properties. These plant extracts may serve as templates for synthesis of pharmaceuticals. It is also cheaper and less time is spent in the development of an extract as compared to the isolation or synthesis of pure compounds. It is important and economical to come up with biologically active extracts as there may be fewer undesirable side effects.

The traditional healer (sangomas, inyanga, Rastafarians, bossiedokters, amaQuira) usually prescribes herbs and various other natural remedies to cure the diseases of their people. Currently there is a worldwide surge of interest in the screening systems for the evaluation of plant extracts for their healing and poisoning activity. There is a

growing interest in natural and traditional medicines as a source of new commercial products in South Africa. Watt and Breyer-Brandwijk (1962) reported on toxic symptoms caused by plants when ingested by animals but very little is known about the toxicity of herbal remedies in man. The concern about reports of toxicity or poisoning in patients taking traditional medicines must therefore be taken seriously.

Acute toxicity due to the use of traditional medicines is common in South Africa, "but due to insufficient data, estimates of mortality vary widely from 8,000 to 20,000 per annum" (Thomson, 2000). By way of indication, 43% of poisoning cases recorded in a forensic database in Johannesburg from 1991 to 1995, were caused by traditional plant medicines (Stewart and Steenkamp, 2000). Most cases of poisoning are not properly documented and so mortality rate from traditional medicinal plants may be higher than what is currently recorded (Thomson, 2000 and Popat *et al*., 2001). Several plants used in South African 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 (Fennel *et al*., 2004)

1.3 AIM AND OBJECTIVES

The main aim of this study is to improve the overall knowledge available on *Tarchonanthus camphoratus* L. complex and *Tarchonanthus pavicapitulatus* by resolving the uncertainties around the taxonomy and also to determine the toxicity and the therapeutic effects of these plants.

This study has the following objectives:

I. To study the micromorphology of *Tarchonanthus camphoratus* and *Tarchonanthus pavicapitulatus.*

II. To compare the solubility of powdered plant material in different solvents.

III. To study the TLC profile of recognized taxa within the *Tarchonanthus camphoratus* and *Tarchonanthus pavicapitulatus.*

IV. To investigate the effects of plant extracts from each taxon on the guinea pig trachea.

V. To assess the toxicological properties of *Tarchonanthus camphoratus* and *Tarchonanthus pavicapitulatus* on isolated human neutrophils.

1.4 RESEARCH HYPOTHESIS

I. Is there a difference in the micromorphology of *Tarchonanthus camphorates* and *Tarchonanthus pavicapitulatus*?

II. Is there a difference in the solubility of powdered plant material in the different solvents?

III. Is there a difference in the TLC profile of recognized taxa within the *Tarchonanthus camphorates* and *Tarchonanthus pavicapitulatus*?

IV. Is there a difference in the plant extracts of *Tarchonanthus camphoratus* and *Tarchonanthus pavicapitulatus* on the guinea pig trachea?

V. Is there a difference in the toxicological properties of *Tarchonanthus camphoratus* and *Tarchonanthus pavicapitulatus* on isolated human neutrophils?

CHAPTER TWO

2 TAXONOMY OF THE GENUS *TARCHONANTHUS*

2.1 INTRODUCTION

The potential economic uses of plants may not be immediately evident, but it is necessary to know the plants that are related to one another in order to predict their properties. Taxonomy is the broad field concerned with the study of the diversity of plants and their identification, naming, classification and evolution (Radford *et al*., 1974). Before the widespread acceptance of the principle of evolution and biologists, being convinced of the fixity of species and lacking much of the information available today, confined themselves to more or less artificial methods of classification, their systems being frequently based on one or a few characters instead of upon the organism as a whole (Trease and Evans, 2002).

The genus *Tarchonanthus* belongs to the family *Asteraceae*, the subfamily *Cichorioideae* and the tribe *Tarchonantheae*. *Tarchonanthus* is one of the few Asteraceae genera that include a tree. Furthermore, it is dioecious, with male and female flowers produced on different plants. The name *Tarchonanthus* is derived from a Greek word meaning funeral flower. The specific name is usually chosen to indicate some striking characteristics of the plant for example; the name *camphoratus* refers to the strong smell of camphor given off when the leaves are crushed.

A number of different names have been published for various *Tarchonanthus* species but they were all put into synonomy under *T. camphoratus* by Paiva (1972) and

subsequently by Pope (1992) and Beentje (1999). Many taxonomists maintained that there were different taxa under *T. camphoratus* (Acocks, 1988). Until recently the genus *Tarchonanthus* consisted of two species, viz. *T. camphoratus* and *T. trilobus.* Herman (2002) in his revision of *Tarchonanthus camphoratus* complex recognized five species: *T. camphoratus*, *T. obovatus*, *T. littoralis, and T. minor and T. parvicapitulatus*. He resurrected *T. minor* and *T. obovatus* and described two new species; *T. littoralis* and *T. parvicapitulatus*. He based his decisions mainly on the following characters: Type of synflorescences, flowering times, leaf shape, margin and distribution.

According to the description Herman (2002), *Tarchonanthus camphoratus* is a "multi-stemmed, rounded dioecious shrub or small tree bearing a male and female flower on different trees". It can grow up to 6m high with a moderate to strong odor of camphor. It is widely distributed in the northern part of Southern Africa; Namibia, Botswana, the Northern Province, North-West, Gauteng, Free State and it also occurs in tropical and North Africa. This species is the most common representative of the genus in the northern parts of Southern Africa. It can be distinguished by the narrowly elliptic to slightly oblanceolate, entire leaves with a grey-green or khaki-green color. The cypselas are enveloped by yellowish cottony hairs. It also occurs in a variety of habitats and soil types for example, savanna, bushveld, woodland, grassland, on flat rocky hills, mountain slopes and hillsides, riverbanks on sandy, loamy, gravelly, calcrete, quartzite and dolomite soils. It is not browsed by live stock except as a last resort in times of drought. It flowers between March and August with a peak from March to July (autumn to winter).

Tarchonanthus parvicapitulatus is one of the first newly described species by Herman (2002). It is a dioecious shrub or small, multi-stemmed tree, up to 8m high with a moderate to strong camphor odor. The leaves are alternate, oblanceolate or rarely obovate and it is widely distributed in the Northern Province, North-West, Gauteng, Mpumalanga, Swaziland, KwaZulu-Natal and Eastern Cape. It grows in forests, valleys and bushveld on mountain slopes, hills and riverbanks. This species is recognized by the oblanceolate, obtuse-mucronate leaves mostly with denticulate margins towards the apex. It flowers between March and October with a peak from April to June (autumn to winter).

Tarchonanthus littoralis is the second newly described species that was described by Herman (2002). *T. littoralis* is a large, dense, bushy shrub or small, shapely tree which is 1 - 8m tall. The trunks are often crooked, and the trees often multi-stemmed. The bark is vertically fissured and cracked, flaking off in narrow strips. Leaves are strongly aromatic and leathery but the colors 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 has often been confused with *Brachylaena* species. *Tarchonanthus littoralis* is a dioecious plant having its male and female flowers on different trees with peak flowering time during mid to late summer (December- March). It occurs from the Cape Pennisula in the Western Cape along the coast, on hillsides, dunes and river banks, through the Eastern Cape to KwaZulu-Natal. Because of its habitat it is also called coastal camphor bush. This plant is

important in binding sand on coastal dunes. It is also valuable source of food for wild animals and domestic stock.

Tarchonanthus minor is the first species resurrected by Herman (2002). This species is also a dioecious shrub with alternate, aromatic and small narrowly elliptic leaves. It flowers between August and December with a peak from September to December (spring to early summer). *Tarchonanthus minor* occurs in the Free State, Lesotho, Northern, Western and Eastern Cape on hillslopes, mountainsides, rocky ridges and hills. This species is distinguished from other *Tarchonanthus* species by the small, narrowly elliptic, entire leaves.

Tarchonanthus obovatus is the second species resurrected by Herman (2002). It is mostly a multi-stemmed dioecious tree up to 2m high with aromatic, alternate and obovate leaves. It flowers from March to May and it is restricted to the Northern Cape where it occurs on hillsides, rocky outcrops or flat surface often on an ironstone or limestone base in sandy soils. *Tarchonanthus obovatus* occurs in the same region with *Tarchonanthus camphoratus* but it is distinguished by its obovate leaf shape. The leaf is also enveloped by white, cottony hairs contrary to *Tarchonanthus camphoratus*, which is enveloped by yellowish hairs. *Tarchonanthus obovatus* is readily browsed by both game and domestic stock all year round.

Figure 1: ***Tarchonanthus camphoratus*** (Photograph was taken on the 17th of August, 2006 at Kaalplaas farm, Onderstepoort, Pretoria).

Figure 2: ***Tarchonanthus parvicapitulatus*** (Photograph was taken on the 17th of August, 2006 at Kaalplaas farm, Onderstepoort, Pretoria).

2.2 AIM

The present study aims to employ other sources of taxonomic evidence such as micro morphology and chemistry to ascertain whether *T. camphoratus* is a variable species. Chemical characters have proved useful in solving different kinds of taxonomic problems (Stuessy 1990). This study also aims to devise ways of identifying a crude drug from *Tarchonanthus* taxa using organoleptic and chemical characters.

Plant material were collected from different localities in Gauteng and the North West province and the plants where authenticated by Prof. P.P.J. Herman of the South African National Botanical Institute, Pretoria. Herbarium specimens were kept in the herbarium of the Department of Pharmacology and Therapeutics, Medunsa campus, University of Limpopo.

2.3 MATERIAL

Scanning Electron Microscope, plant press, GPS, mounting board, vehicle, Olympus vanox – S camera, slides, light microscope, mortar and pestle, electric grinder, bottles, test tubes, heat source, filter paper, sulphuric acid, hydrochloric acid, acetic acid, benzene, glass beakers, distilled water, 70% ethanol.

Table 1: Localities of plant specimen

Specimen No	**Species**	**Sex**	**Localities Collected**	**Habitat**
1	*Tarchonanthus camphoratus*	Male	North West Province	Lowland
2	*Tarchonanthus camphoratus*	Female	North West Province	Lowland
3	*Tarchonanthus camphoratus*	Female	North West Province	Lowland
4	*Tarchonanthus camphoratus*	Female	North West Province	Lowland
11	*Tarchonanthus parvicapitulatus*	Female	Gauteng Province	Lowland
12	*Tarchonanthus parvicapitulatus*	Male	Gauteng Province	Lowland
13	*Tarchonanthus parvicapitulatus*	No inflorescence	Gauteng Province	Lowland

Specimen No	Species	Sex	Localities Collected	Habitat
14	*Tarchonanthus parvicapitulatus*	Male	Gauteng Province	Lowland
15	*Tarchonanthus parvicapitulatus*	Female	North West Province	Mountain slope
16	*Tarchonanthus parvicapitulatus*	Female	North West Province	Mountain slope
17	*Tarchonanthus parvicapitulatus*	No inflorescence	North West Province	Mountain slope

2.4 METHODOLOGY

This study was based on live material from different localities in Gauteng and North West province. At least three specimens were selected to represent each taxon.

2.4.1 FIELD WORK:

Field work was conducted between June and August 2006 in the North West (Kokoriba Game Reserve and Ga rankuwa) and Gauteng (Onderstepoort Nature Reserve) provinces of South Africa. Both flowering and sterile specimens were collected. From each locality specimens collected were grouped based on leaf morphology and inflorescence type. These were then taken to the South African National Botanical Institute, Herbarium Pretoria to confirm their identification.

2.4.2 MICROMORPHOLOGY:

Dried leaf, peduncle and involucral bract samples were examined under a Scanning Electron Microscope (SEM) using a standard SEM procedure. The photographs of the inflorescences and leaves were scanned by me while all the SEM pictures were taken by Dr Turner from the Electron Microscope Unit of University of Limpopo, Medunsa campus.

2.4.3 SOLUBILITY AND COLOUR RETENTION STUDIES:

Powdered leaf material of 0.5 g of a representative from each group was mixed in 10ml Sulphuric acid (H_2SO_4), Hydrochloric acid (HCl), ethanol (CH_3COOH) and water (H_2O) to test solubility and retention of original colour in these solvents under cold and hot conditions (Evers and Smith, 1955). Powdered plant material (0.5g) and 10ml of different solvents were heated using a burner to heat the mixture for hot condition but the mixtures were not heated for cold condition and the color changes were observed and recorded for both conditions. The mixtures were heated for 5 minutes under hot condition while the mixtures were allowed to stay for 5 minutes at room temperatures under cold condition.

2.5 RESULTS

2.5.1 FIELD WORK:

2.5.1.1 KOKORIBA GAME RESERVE:

Ten specimens were collected, one male, seven females and two sterile specimens. The female specimens were divided into two groups.

GROUP I consisted of specimens with more open inflorescence, with peduncles up to 25mm long and cypsela covered with white cottony hairs (Figure 3). The leaves in this group are greyish green (Figure 4).

GROUP II consisted of specimens with relatively shorter peduncles, up to 10mm long with cypsela covered with yellow cottony hairs (Figure 5). The leaves in this group are khaki green (Figure 6). The sterile specimens seem to belong to this group.

All groups were identified as *Tarchonanthus camphoratus* by Prof. P.P.J. Herman from the Pretoria Botanical Garden.

2.5.1.2 GARANKUWA:

Three specimens were collected; one male, one female and one sterile. All specimens looked similar except for the size of the leaves. The sterile specimen has large leaves up to 70mm long (Figure 7) whilst the female and male specimens have relatively smaller leaves up to 30 mm long (Figure 8). Leaf apices also vary from obtuse (sterile specimen) to acute (male and female specimens). All specimens were identified as *Tarchonanthus parvicapitulatus* by Prof. P.P.J. Herman from the Pretoria Botanical Garden.

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2.5.1.3 ONDERSTEPOORT:

Four specimens were collected, two females, one male and one sterile. Although all specimens were identified as *Tarchonanthus parvicapitulatus*, the leaves on male and female specimens are elliptic and the margins are not prominently serrated as in the specimens collected from Garankuwa (Figure 9, pg 23). The leaves of the sterile specimens resemble those of the sterile specimens collected at Garankuwa (Figure 10, pg 24).

2.5.2 MICROMORPHOLOGY

In both *Tarchonanthus camphoratus* and *T. parvicapitulatus* two types of trichomes, glandular and non-glandular were observed. In the upper leaf surface, glands are mostly distributed along the ridges.

Tarchonanthus camphoratus:

SEM studies confirm macromorphological groupings. In all GROUP I specimens, the upper leaf surfaces are densely hairy with glands not prominent (Figure 11, pg 25). In GROUP II specimens the upper leaf surface is sparsely hairy with prominent glands (Figure 12, pg 25). The lower leaf surface is also non glandular (Figure 13, pg 26). The involuncral bract (Figure 14, pg 26) and peduncles (Figure 15, pg 27) of male plants are non glandular.

***Tarchonanthus parvicapitulatus*:**

In all specimens the upper leaf surface is subglabrous to glabrous (Figure 16, pg 27). The lower leaf surface is glandular (Figure 17, pg 28). This also confirms macromorphological groupings. According to Figure 18, the involuncral bract and peduncles (Figure 19, pg 29) of the male plants are glandular.

Figure 3: Inflorescence of Group 1 (Female specimen from Kokoriba Game Reserve, North – West Province).

Figure 4: Leaves of group 1 (Female specimen from Kokoriba Game Reserve, North – West Province).

Figure 5: Inflorescence of Group 2 (Female specimen from Kokoriba Game Reserve, North – West Province).

Figure 6: Leaves of group 2 (Female specimen from Kokoriba Game Reserve, North – West Province).

Figure 7: Leaves of Sterile specimen (Sterile specimen from Ga-Rankuwa, Pretoria).

Figure 8: Leaves of *T. parvicapitulatus* (Male specimen from Ga-Rankuwa, Pretoria).

Figure 9: Leaves of *T. parvicapitulatus* (Female specimen from Kaalplaas farm, Onderstepoort, Pretoria).

Figure 10: Leaves of *T. parvicapitulatus* (Sterile specimen from Kaalplaas farm, Onderstepoort, Pretoria).

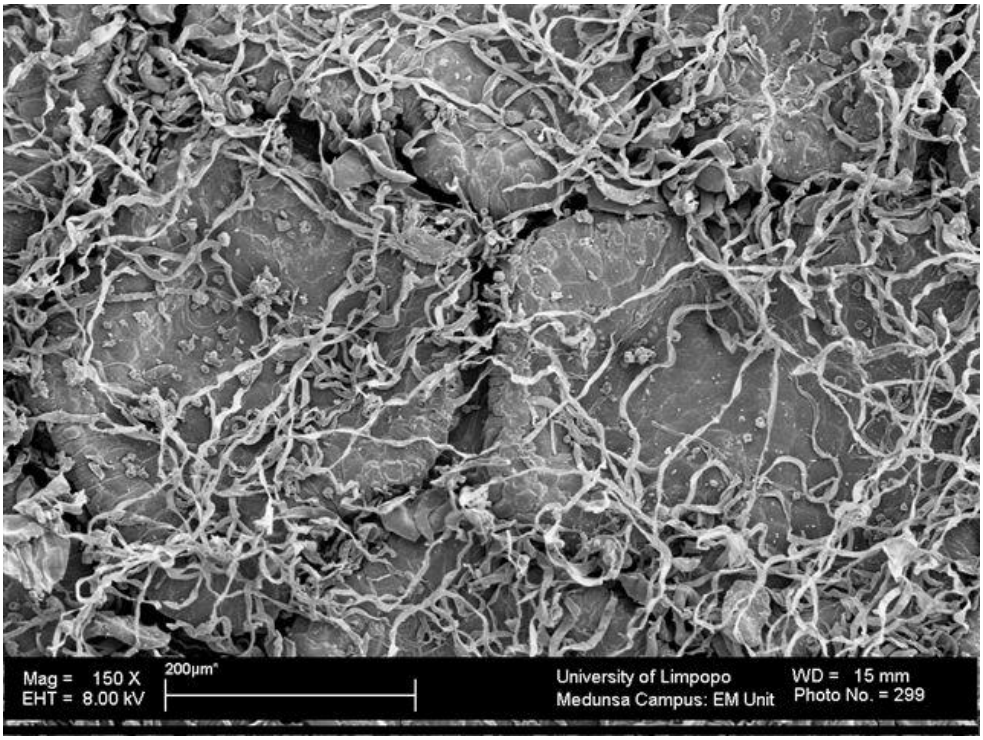


Figure 11: Upper leaf surface of Group 1 (Female *T. camphoratus*).

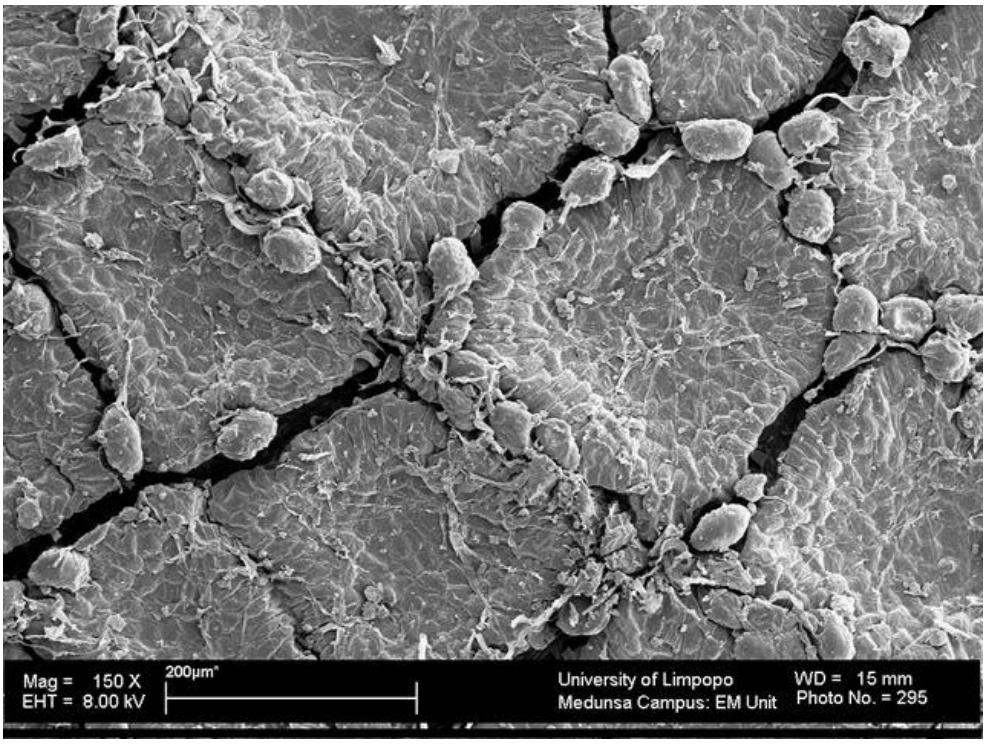


Figure 12: Upper leaf surface of Group 2 (Female *T. camphoratus*).

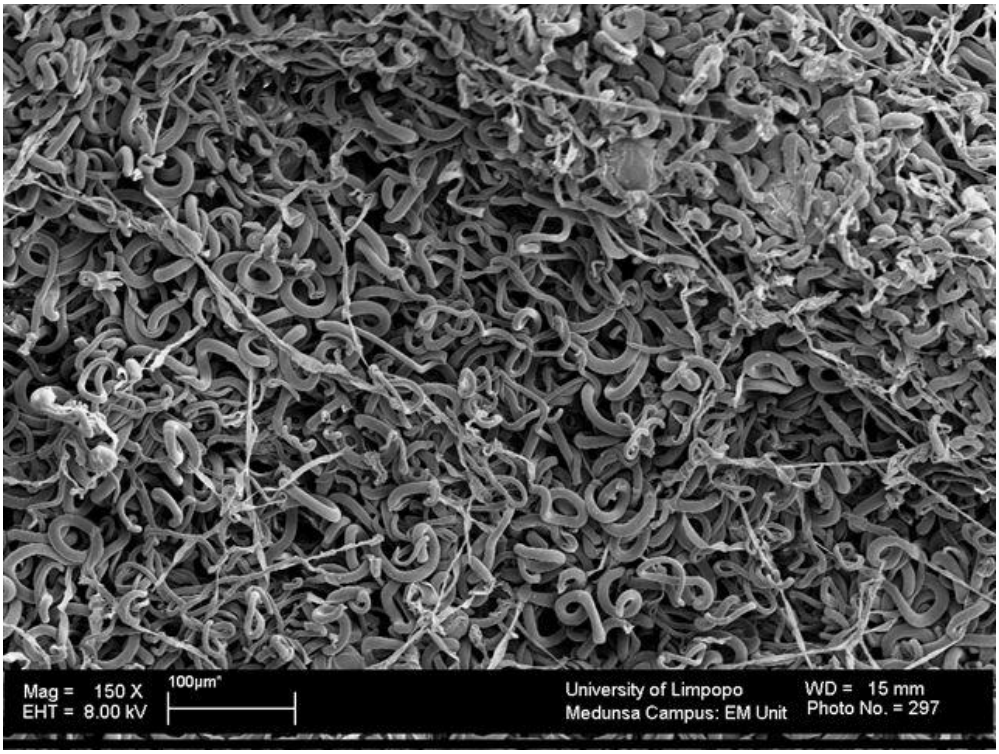


Figure 13: Lower leaf surface (Female *T. camphoratus*).

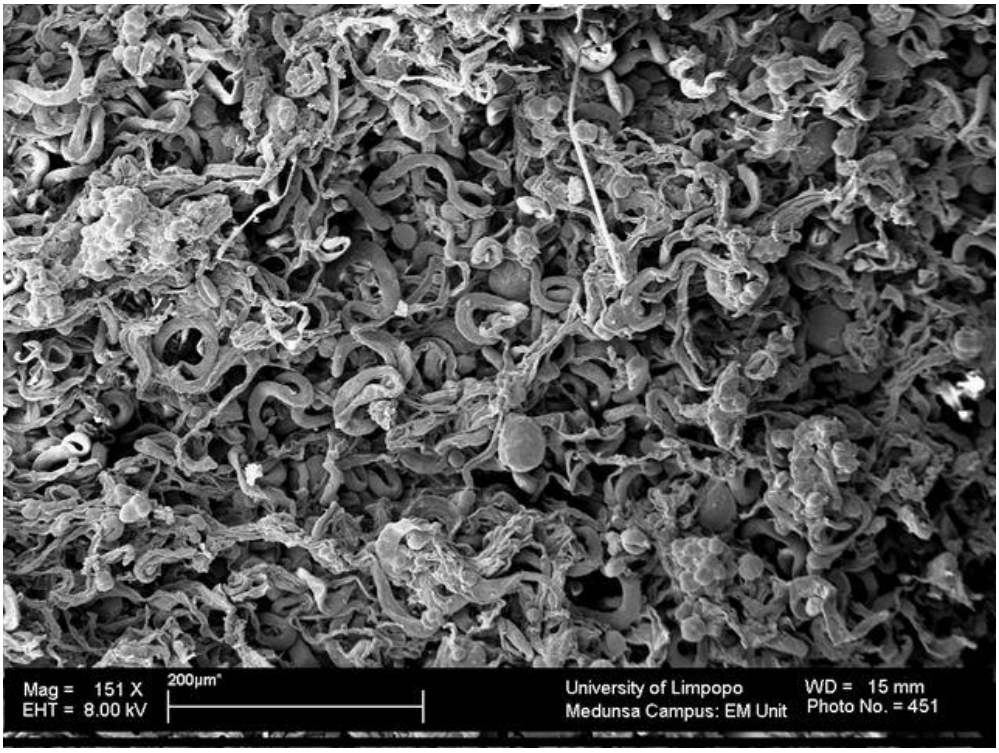


Figure 14: Glandular trichomes on the outer involucral bracts (Male *T. camphoratus*).

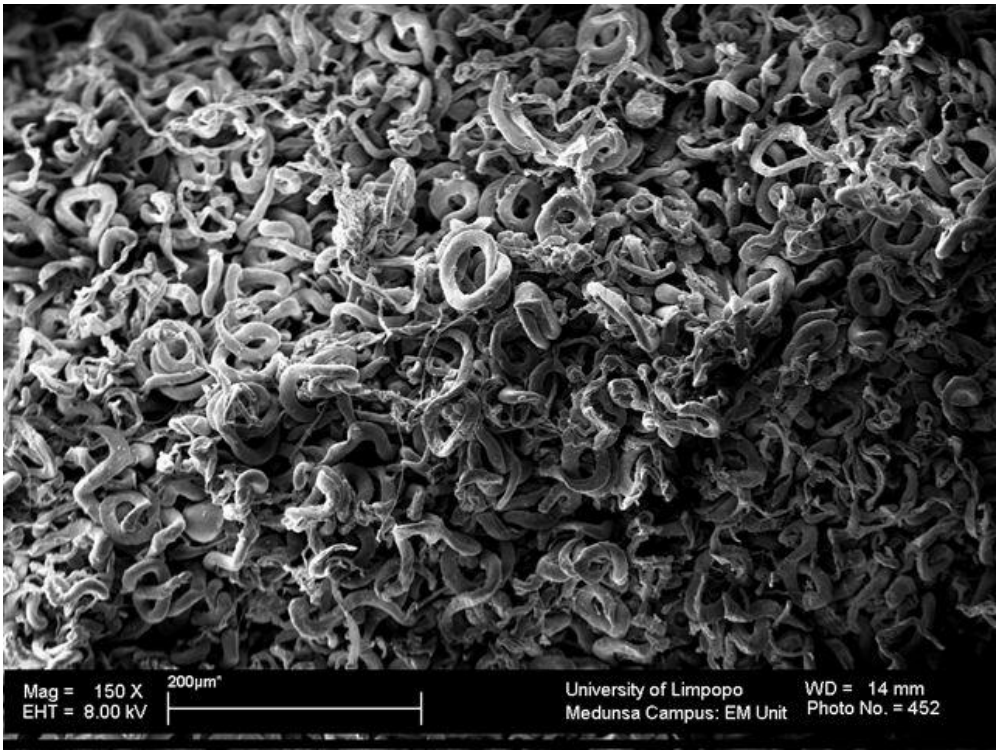


Figure 15: Peduncle (Male *T. camphoratus*).

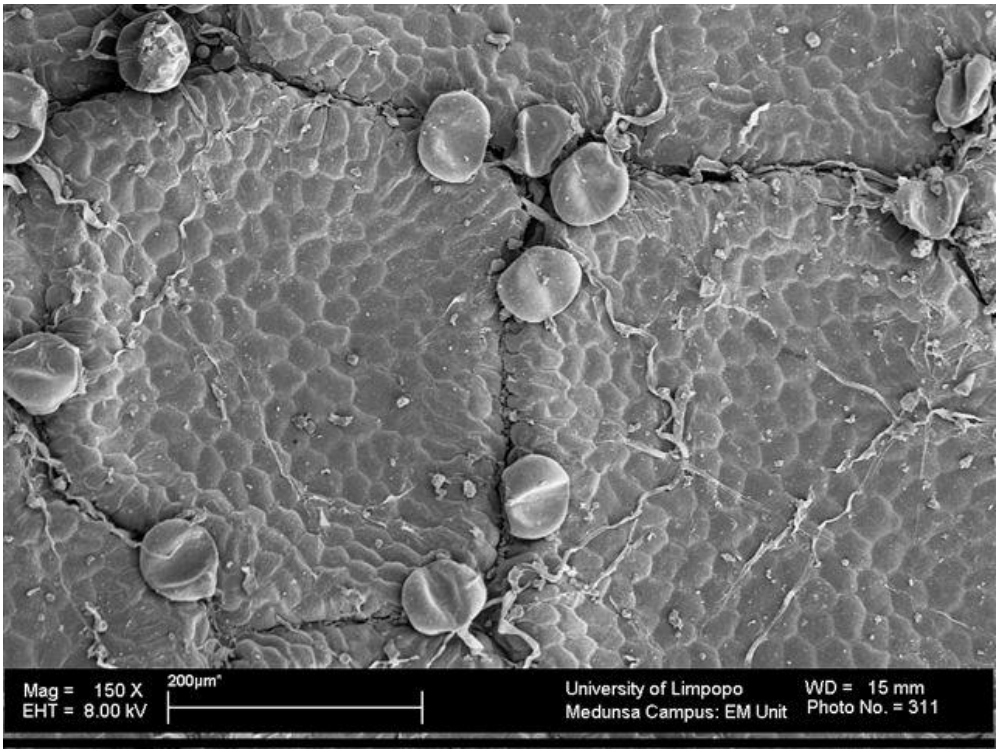


Figure 16: Upper leaf surface of *T. parvicapitulatus*.

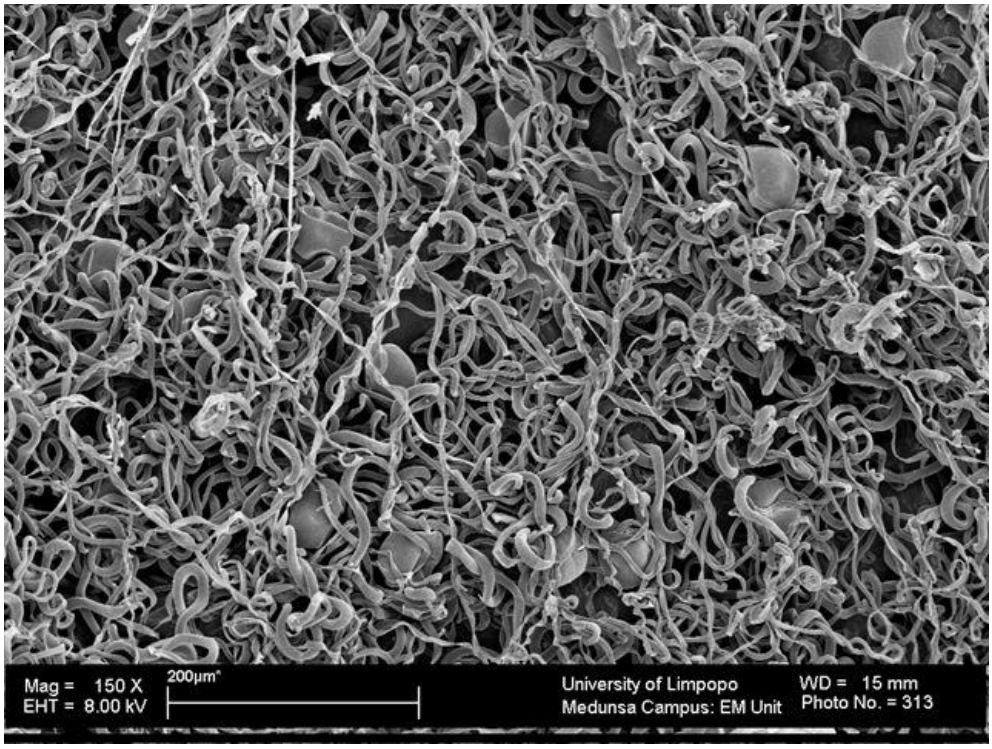


Figure 17: Lower leaf surface *T. parvicapitulatus*.

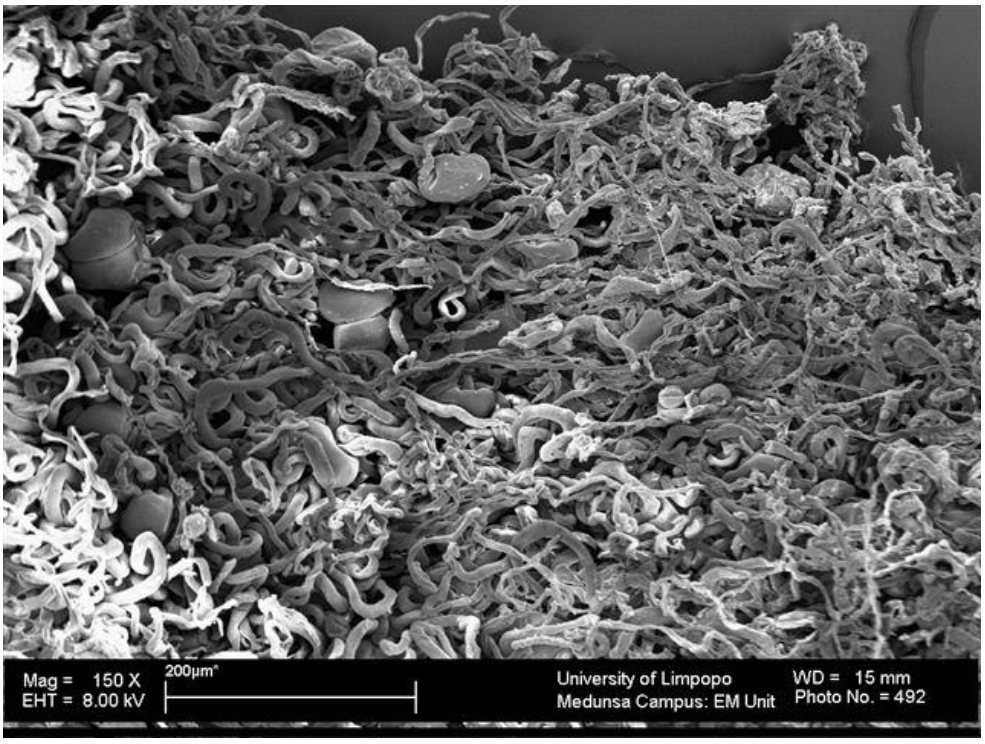


Figure 18: Glandular trichomes on the outer involucral bracts (Male *T. pavicapitulatus*).

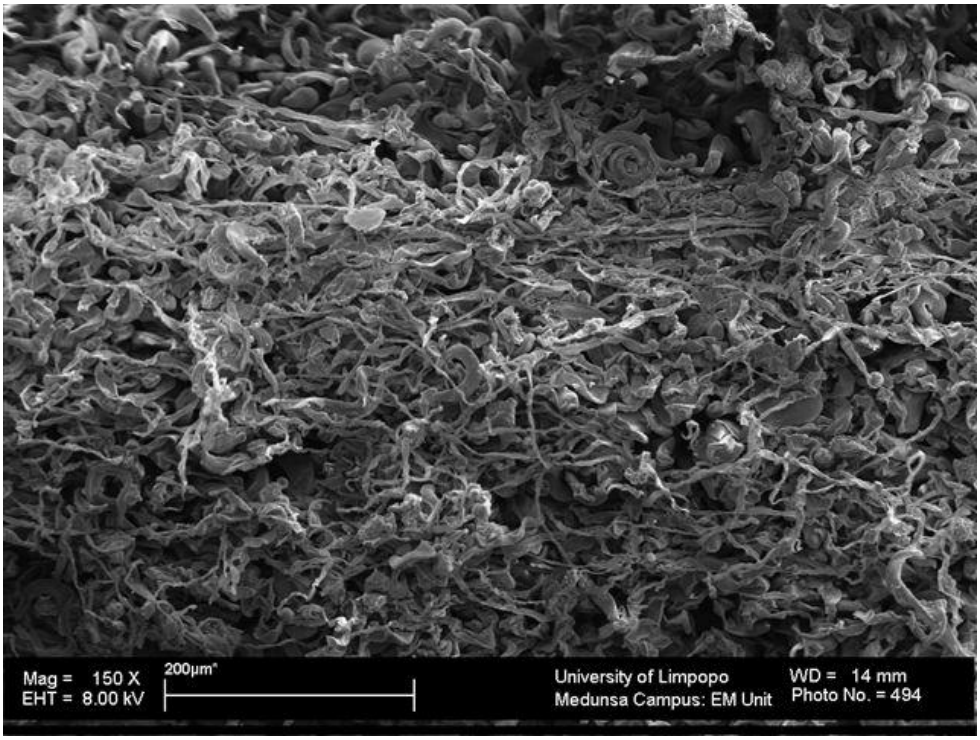


Figure 19: Peduncle (Male *T. pavicapitulatus*).

2.5.3 SOLUBILITY AND COLOUR RETENTION STUDIES:

***Tarchonanthus camphoratus*:**

H_2SO_4: The powdered plant material became warm on addition of H_2SO_4. All specimens were insoluble in both cold and hot conditions (Table 2, pg 32). The solvent turned brown in all specimens in the cold test (Table 3, pg 33).

HCl: All specimens were insoluble (Table 2, pg 32). In the female and sterile specimens the solvent turned light green while it turned pink in the male specimens (Table 3, pg 33).

CH_3COOH: All specimens were insoluble (Table 2, pg 32). The solvent turned olive green in all specimens (Table 3, pg 33).

H_2O: All specimens were insoluble (Table 2, pg 33). The solvent turned tea-brown (Table 3, pg 32).

Tarchonanthus parvicapitulatus:

H_2SO_4: The powdered plant material remained cold on addition of H_2SO_4 (Table 1, pg 31). All specimens were soluble in the hot condition. The solvent turned light brown in the cold test and black in the hot test (Table 2, pg 32).

HCl: All specimens were insoluble (Table 1, pg 31). In all the specimens including the male specimens the solvent turned light green (Table 2, pg 32).

CH_3COOH: All specimens were insoluble (Table 1, pg 32). The solvent turned olive green in all the specimens (Table 2, pg 32).

H_2O: All specimens were insoluble (Table 1, pg 31). The solvent turned tea-brown in all the specimens (Table 2, pg 32).

TABLE 2 Solubility analysis of powdered specimen of medicinal plants in various solvents by cold and hot method.

	COLD TEST				HOT TEST			
Specimen	CH_3COOH	H_2SO_4	HCl	H_2O	CH_3COOH	H_2SO_4	HCl	H_2O
1	*	*	*	*	*	*	*	*
2	*	*	*	*	*	*	*	*
3	*	*	*	*	*	*	*	*
4	*	*	*	*	*	*	*	*
9	*	*	*	*	*	*	*	*
11	*	*	*	*	*	**	*	*
12	*	*	*	*	*	**	*	*
13	*	*	*	*	*	**	*	*
14	*	*	*	*	*	**	*	*
15	*	*	*	*	*	**	*	*
16	*	*	*	*	*	**	*	*
17	*	*	*	*	*	**	*	*

* Powdered plant material insoluble

** Powdered plant material soluble

TABLE 2. Color analysis of powdered specimens of *T. camphoratus* and *T. parvicapitulatus* in various solvents using cold and hot methods.

	COLD TEST				HOT TEST			
Specimen	CH_3COOH	H_2SO_4	HCl	H_2O	CH_3COOH	H_2SO_4	HCl	H_2O
1	Yellowish Green	Light brown	Pink	Colorless	Dark green	Black	Rust	Light brown
2	Light green	Light brown	Light green	Colorless	Green	Black	Brown	Light brown
3	Green	Colorless	Colorless	Colorless	Green	Black	Yellowish green	Dark brown
4	Yellowish green	Colorless	Colorless	Colorless	Olive green	Black	Yellowish green	Brown
9	Light green	Colorless	Light green	Light green	Green	Brownish black	Yellowish green	Dark brown
11	Yellowish green	Light brown	Colorless	Colorless	Green	Colorless	Pale green	Brown
12	Yellowish green	Colorless	Colorless	Colorless	Green	Brownish black	Pale green	Brown
13	Yellowish green	Colorless	Colorless	Colorless	Light green	Black	Pale green	Brown
14	Yellowish green	Colorless	Colorless	Colorless	Light green	Black	Pale green	Brown
15	Green	Brown	Light green	Green	Pale green	Black	Green	Dark brown
16	Yellowish green	Brown	Yellowish green	Brown	Pale green	Black	Pale green	Dark brown
17	Yellowish green	Brown	Yellowish green	Light Brown	Pale green	Black	Pale green	Dark brown

2.6 DISCUSSION AND CONCLUSION

The specimens within each taxa vary even within the same locality. Differentiating between the two species proved difficult since the macromorphological characters tend to overlap between the species. For example, the main characters used to differentiate *T. camphoratus* from *T. parvicapitulatus* are serrated leaf margins and cypsela hair color, but serrated leaf margins were found in both *T. camphoratus* (Figure 4, pg 22) and *T. parvicapitulatus* (Figure 9, pg 25). *T. camphoratus* is supposed to have yellowish cypsela hairs while *T. parvicapitulatus* is supposed to have whitish hairs according to Herman (2002). Both cypsela hairs colors were found in *T. camphoratus* .In this study two distinct groups were identified within *Tarchonanthus camphoratus sensu stricto* based on the details of the inflorescence and ultrastructure of the upper leaf surface. The first group has a white inflourescence (Figure 3, pg 22) while the second group has a yellowish inflourescence (Figure 5, pg 23). Also, the upper leaf surface of group 1 is hairy (Figure 11, pg 26) while the second group is similar to *T. pavicapitulatus* rather than *T. camphoratus* due to the presence of grooves around the ridges (Figure 12, pg 26). This plant can be said to be a different species or a subspecies belonging to the *T. camphoratus* taxa. The macromorphological study did not prove useful in differentiating between the male and female plants of *T. camphoratus* and *T. parvicapitulatus*.

Micromorphological studies proved useful in distinguishing between the two species. The upper leaf surface is hairy in *T. camphoratus* (Figure 11, pg 26) while it is subglabrous to glabrous in *T. parvicapitulatus* (Figure 16, pg 28). The lower leaf surface is prominently glandular in *T. parvicapitulatus* (Figure 17, pg 29) but none was seen in *T. camphoratus* (Figure 13, pg 27). However, the outer involucral bracts of the two specimens show glandular trichomes but it was more prominent in. *T .pavicapitulatus* (Figure18, pg 29) than it is in *T. camphoratus* (Figure 14, pg 27). Therefore, micromorphological studies proved useful in distinguishing between the two species.

Chemical characters proved useful to some extent in differentiating between the two species. All specimens identified as *T. parvicapitulus* were soluble in H_2SO_4 (Table 2, pg 32) whereas those identified as *T. camphoratus* were insoluble in the same solvent. In addition, HCl turned pink in the presence of the male plant of *T. camphoratus* (Table 3, pg 33) whereas it remained colorless in the presence of the male plant of *T. parvicapitulatus*.

The micromorphological studies and the solubility tests proved useful in differentiating between *T. camphoratus* and *T. parvicapitulus* and also in differentiating between the male and female plants.

CHAPTER THREE

3. PHARMACOGNOSY

3.1 INTRODUCTION

According to Bruneton (1999), pharmacognosy is the study of staring material and substances intended for therapeutics, and the study of biological origin, in other words obtained from plants, animals or by fermentation by micro-organisms. In pharmacognosy, to study a plant is, among others, to define its identity, describe its morphology and anatomy, to know its origin and production methods, to appreciate their impact on the plant's quality, to determine its chemical composition and the factors that may affect it and to know the pharmacological activity of the active ingredients. Pharmacognosy during the 19^{th} century, was the "most important pharmaceutical discipline and the basis of all present-day pharmaceutical disciplines" (Ameenah Gurib-Fakim, 2006).

Van Wyk and Wink (2004) states that since we have a better understanding of how the human body functions today we are in a better position to fully appreciate the healing power of plants and their potential as multi-functional chemical entities for treating complicated health conditions. People who use traditional remedies may not understand the mechanism of action of their medicines, but they know from personal experience that some medicinal plants can be very effective if used at therapeutic doses. Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health

(Van Wyk and Wink, 2004). Medicinal plants are those plants, which are used directly or indirectly in the extraction of drugs for the treatment of ailments. But many of the plants which are used for treating various ailments have either not been properly investigated or the findings have not been correlated with phytochemical and pharmacological studies (Khan *et al*., 1979). A plant is said to be a biosynthetic laboratory, not only for a chemical compound but also a multitude of compounds. In most societies today, allopathic and traditional systems of medicine occur side by side in a complementary way while the allopathic medicine treats serious acute conditions, the traditional medicine is used for treating chronic illnesses, and it also reduces symptoms and improves the quality of life in a cost-effective way.

The medicinal properties of plants have been investigated throughout the world. This is due to their potential pharmacological activities, low toxicity (though not always), and economic viability. Because of the potential benefits of plants, their extracts may be developed and used as adjunctive therapy in the treatment of individuals for example, diabetes mellitus (Wolf & Weisbrode, 2003). Most medicinal plants grow wild and no systematic attempt has been made to carry out pharmacognostic investigation of these medicinal plants (Ahmad *et al*., 2003). Most of these plants have not been properly investigated or the findings have not been correlated with phytochemical and pharmacological studies.

3.1.1 CHEMICAL COMPOUNDS FOUND IN MEDICINAL PLANTS

Thousands of diverse chemical compounds are produced by higher plants (Hamburger and Hostettmann, 1991). Medicinal plants have active chemical compounds that may act directly or indirectly to prevent or treat diseases and maintain health (Hostettmann *et al.*, 1996). There are different classes of chemical compounds used as phytomedicine which are sold as extracts and powders in which the concentration of the active ingredients is standardized to ensure safety and efficacy (Van Wyk *et al*., 1997). Plant extracts may therefore have potential application in the pharmaceutical industry for the treatment of infectious diseases caused by bacteria, fungi, viruses and other microorganisms. Three large classes of secondary metabolites extracted from plants are phenolic derivatives, nitrogen-containing compounds (alkaloids) and terpenoids.

Phenolics are widespread in nature and are found in many natural compounds with aromatic rings and hydroxyl groups. This group of secondary metabolites includes anthcyanidins, anthocyanins, anthraquinones, coumarins, falconoids, naphthaquinones, simple phenolic compounds and tannins (Evans, 1989). Tannins are phenolic compounds that can combine with protein, starch, cellulose and the bacterial cell membrane. They are widely distributed in the plant kingdom and are common both in angiosperms and gymnosperms (Tyler *et al*., 1998). They are divided into two chemical classes namely, proanthocyanins (often called condensed tannins) and hydrolysable tannins. Hydrolysable tannins are compounds where one or more sugars (frequently glucose) are bonded to a phenolic acid molecule that could either be gallic

acid or ellagic acid (Tyler *et al.*, 1998).

Flavonoids are major metabolites of numerous plants and play a role as pigments in several fruits and vegetables. Flavonoids include chacones, flavones and flavonols. Flavonoids represent a significant source of antioxidant, anti-inflammatory, antibacterial, anti-allergic and antiviral properties (Olejniczak *et al*., 2002). The basic structure of these compounds contains phenolic rings connected with three carbon atoms. The derivatives of the flavonoids differ in their constituents, the number and position of hydroxyl and methoxyl groups and the number of sugars in the molecules. In addition, plants from different localities may contain varied amounts and quality of flavonoids (Suntornsuk, 2002).

Terpenoids can be divided into two groups according to their structures.Terpenoids are formed when five isoprene units are linked together. They occur as sesquiterpenoids (C_{15}), diterpenoids (C_{20}), triterpenoids (C_{30}) and tetraterpenoids (C_{40}). Triterpenoids are present in plants in the form of saponins, monoterpenoids and sesquiterpenoids and are common constituents of volatile oils (essential oils) (Van Wyk *et el*., 1997). Terpenoids may also have antibacterial and antifungal properties (Taylor *et al*., 1995). Many types of terpenoids have been isolated in various classified and recognized medicinal plants.

3.1.2 ACTIVE INGREDIENT IN *TARCHONANTUS* SPECIES

If a plant has any physiological effect on the body, it means that it has at least one bioactive chemical. Natural products and their derivatives represent more than 50% of all medication in clinical use in the world. Higher plants contribute no less than 25% to the total (Kinghorn *et al*., 1993). Well-known examples of plant-derived medicines include quinine, morphine, codeine, aspirin, atropine, reserpine and cocaine.

Watt and Breyer-Brandwijk (1962) reported that compounds isolated from leaves of *Tarchonanthus camphoratus* L. complex in East Africa and Ethiopia include volatile oils and tarchonyl alcohol. The volatile oil is highly complex and variable, showing large differences between localities. The wood smells like camphor but despite the camphor-like smell, the plant contains only very small amounts of camphor (0,4%). The leaves are also reputed to contain camphor and are reported to have given negative antibiotic tests. Material from North Africa yielded α-fenchyl alcohol (29,1%), 1,8-cineole (eucalyptol: 16.5%) and α-terpineol (8,5%) as major constitutes, together with a large number of minor constituents. Van Wyk and Wink (2004) reported that the plant also contains a flavanone-pinocembrin. Various flavonones (luteolin, apigenin, nepetin and hispidulin) have been identified in Egyptian collections of this species, as well as the sesquiterpine lactone parthenolide and a quarternary alkaloid, tarchonanthine. It is possible that flavonoids and ingredients of the volatile oil are responsible for the reported analgesic, diaphoretic, decongestant and antispasmodic effects.

3.1.3 MEDICINAL USES OF *TARCHONANTUS* SPECIES

"Various parts of *Tarchonanthus* species are used medicinally" (Palmer and Pitman 1972). Infusions and tinctures of the leaves and twigs are used for stomach trouble, abdominal pain, headache, toothache, asthma, bronchitis and inflammation (Watt & Breyer–Brandwijk, 1962 and Hutchings & Van Staden, 1994). Leaves are also reported to have been smoked, chewed or applied as poultices for asthma, bronchitis, inflammation and plaque in the Western Cape. Dried leaves are reported to have slight narcotic effects when smoked (Watt & Breyer-Brandwijk, 1962).

3.1.4 OTHER USES OF *T. CAMPHORATUS*

Leaves are used by Zulu women to perfume their hair. The wood was used for turnery, boat-building, musical instruments, cabinet work, fence posts, shafts of spears and walking sticks (Smith, 1966; Palmer & Pitman, 1972 and Coates 1977). *T. camphoratus* is used as a fodder plant in dry areas (Smith 1966 and Palmer & Pitman 1972).

3.2 AIM

The aim of this study is to use chromatography study such as thin layer chromatography studies as a pharmacognostic tool to test for the presence of antioxidant compounds in *Tarchonanthus camphoratus* and *Tarchonanthus parvicapitulatus.*

3.3 THIN LAYER CHROMATOGRAPHY

Chromatographic techniques have been instrumental in the separation of natural products. Thin Layer Chromatography (TLC) is one of the fastest and most widely used chromatographic techniques and is also an extremely useful technique for monitoring reactions. Chromatographic separations are based on the dynamic equilibrium that is established between concentrations of a solute in two phases, the mobile and stationary phase. The stationary phase comprises thin plates that are commercially available whereas the mobile phase is a suitable solvent, which can be prepared and adjusted to separate the compounds of interest. The solutes or compounds migrate according to their differential interaction and affinity between the two phases.

TLC can be used for both qualitative and quantitative analysis of crude extracts for identification of compounds. In quantitative analysis, the size and color intensity of the zones on the TLC chromatogram are related to the amount of substance present, therefore the amount of substance applied to the plate must be accurately known. On the other hand, qualitative analysis is done by comparing the retention factor (R_f) value on the TLC against a reference value of a standard. The detection methods used in TLC are largely dependent on the nature of the substance present in the mixture. Despite the fact that some components of an extract may be colored and thus easy to visualize if the stationary phase is white, the vast majority will have little or no colour, hence other methods have to be used to visualize them. The most common

method used to examine the components of a mixture on a TLC plate is the use of a UV light and the use of spray reagents such as Vanillin to stain isolated compounds by heating it to 105^{o}C for 3-5 minutes.

3.4 ANTIOXIDANT ACTIVITY

Most polar plant extracts probably have antioxidant compounds present in them. Their role is at least partly to scavenge for free radicals. Free radicals are highly unstable and reactive molecules and are capable of damaging molecules such as DNA, proteins and carbohydrates (Surai, 2002). There is a worldwide trend towards the use of natural products present in, for example berry crops, teas, herbs, fruits and vegetables for prevention of oxidative damage to tissues that may lead to disease (Deiana *et al*., 1999 and Lee and Shibamoto, 2001). It has been reported that oxidation of lipoproteins contribute in all initial stages of atherosclerotic plaque formation, including activation of inflammation, endothelial damage and other events (Berliner and Heinecke, 1996). It would seem that oxidative damage predisposes to cardiovascular and other degenerative diseases, and hence leads to considerable interest in the isolation and role of antioxidants for the prevention of these ailments. The traditional health practitioners use a variety of plant materials for treatment of diseases not properly diagnosed; hence some of these diseases may be caused by oxidative damage.

Antioxidant activity is qualitatively determined by spraying TLC chromatograms with 2, 2-diphenyl-picryhydrazyl (DPPH). The radical and antioxidant activity is

displayed by the ability of extracts to reduce DPPH. The color of DPPH changes from red to clear. This indicates free radical scavenging capacity. DPPH is reduced from a stable free radical, which is purple in color to diphenylpicryl hydralazine, which is yellow in color. The DPPH method by Braca *et al*., (2002) was employed.

3.5 MATERIAL

Plant material, shaker, Buchner funnel, oven, electric grinder, TLC plate, UV light, Whatmann no 1 filter paper, vacuum drier, vials, Buchi rotarory evacuator, fume cupboard, distilled water, acetone, benzene, ethanol, ammonium hydroxide, ethyl acetate, methanol, chloroform, formic acid.

3.6 METHODOLOGY

3.6.1 Preparation of leaf extract

The plant material was air dried at room temperature and was milled into a fine powder using a mortar and pestle. The powdered plant material was stored in a clean container to avoid contamination. The powdered plant material was extracted using acetone in a ratio of 1:9 i.e. 10g of powdered plant material was dissolved in 90ml acetone. The mixture was shaken overnight using a shaker and filtered through Whatman no. 1 paper in a Buchner funnel. The extracting solvent was changed when the color of the mixture decreased substantially and the solvent was evaporated to concentrate the extract *in vacuo* at 40^{o}C using a Buchi Rotarory Evacuator. For complete drying, extracts were placed in a pre-weighed glass beaker and dried under a stream of cold air.

3.6.2 TLC Analysis

The extracts prepared above were dissolved in acetone to yield 10mg/ml stock solution. The extracts were analyzed by TLC on Merck TLC F_{254} 10 × 20cm in three solvent systems, namely: Ethyl acetate : Methanol : Water (E: M: W) (10:1, 35:1 v/v), Benzene : Ethanol : Ammonia (B: E: A) (18:2:0, 2), and Chloroform : Ethylacetate : Formic acid (CEF) (10: 8: 2). BEA was used for non-polar / basic compounds, EMW for polar and neutral compounds while CEF was used for intermediate polar and acidic compounds (Kotze and Eloff, 2002). Aliquots of 10µl of the extracts were loaded with a micropipette 1cm from the bottom of a labeled TLC plate and the solvents were allowed to run up to 2cm from the edge of the plate. The developed plates were air dried in the fume cupboard and thereafter visualized under a UV light (254 and 360nm Camac Universal UV lamp). For further detection of chemical compounds, the plates were sprayed with acidic vanillin spray reagent (Sigma-Aldrich, Germany) (0.1g Vanillin powder was dissolved in 28ml methanol with 1ml sulfuric acid carefully added). The plates were heated to 100^{o}C for 5 minutes for optimal color development. Vanillin sulphuric acid reagent was used for visualization of higher alcohols, phenols, steroids and essential oils.

3.6.3 Antioxidant Analysis

A solution of 10mg/ml was prepared from each extract. The TLC plates were developed in two solvent systems namely Ethyl acetate: Methanol: Water (E: M: W) (10:1, 35:1v/v) and Chloroform: Ethyl acetate: Formic acid (C: E: F). Aliquots of

10µl of the extracts were loaded with micropipette 1cm from the bottom of a labeled TLC plate and the solvents were allowed to run up to 2cm from the edge of the plate. The developed plates were air dried in the fume cupboard and thereafter visualized under a UV light (254 and 360nm Camac Universal UV lamp). For further detection of chemical compounds, the plates were sprayed with 0,2% DPPH in methanol. The plates were heated to 100^{o}C for 5 minutes for optimal color development. Chromatograms were examined for color change over 30 minutes. Antioxidant compounds in the extracts changed the purple color of DPPH to yellow. DPPH radical is reduced from a stable free radical, which is purple to diphenylpicrylhydrazine, which is yellow.

3.7 RESULTS

Leaf extracts were subjected to qualitative analysis using a thin layer chromatography (TLC) technique as described above. This was done to investigate the chemical composition of the extracts. There were many compounds on the chromatograms which were visible under the UV light and there were also differences in the composition of the extracts sprayed with Vanillin spray reagents.

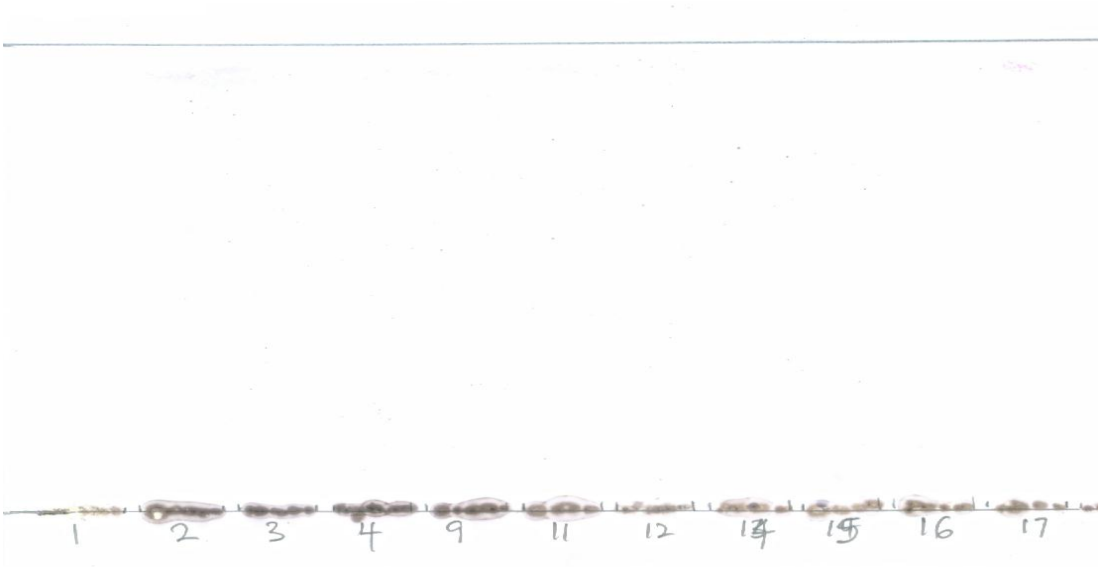


Figure 20

TLC profile of leaf extract of *T. camphoratus* and *T. parvicapitulatus* run in BEA solvent system sprayed with Vanillin reagent. Lanes left to right:

1	*T. camphoratus* (male)
2 – 4	*T. camphoratus* (female),
9	*T. camphoratus* (no inflourescence)
11	*T. parvicapitulatus* (female)
12 - 14	*T. parvicapitulatus* (male)
15-16	*T. parvicapitulatus* (female)
17	*T. parvicapitulatus* (no inflourescence)

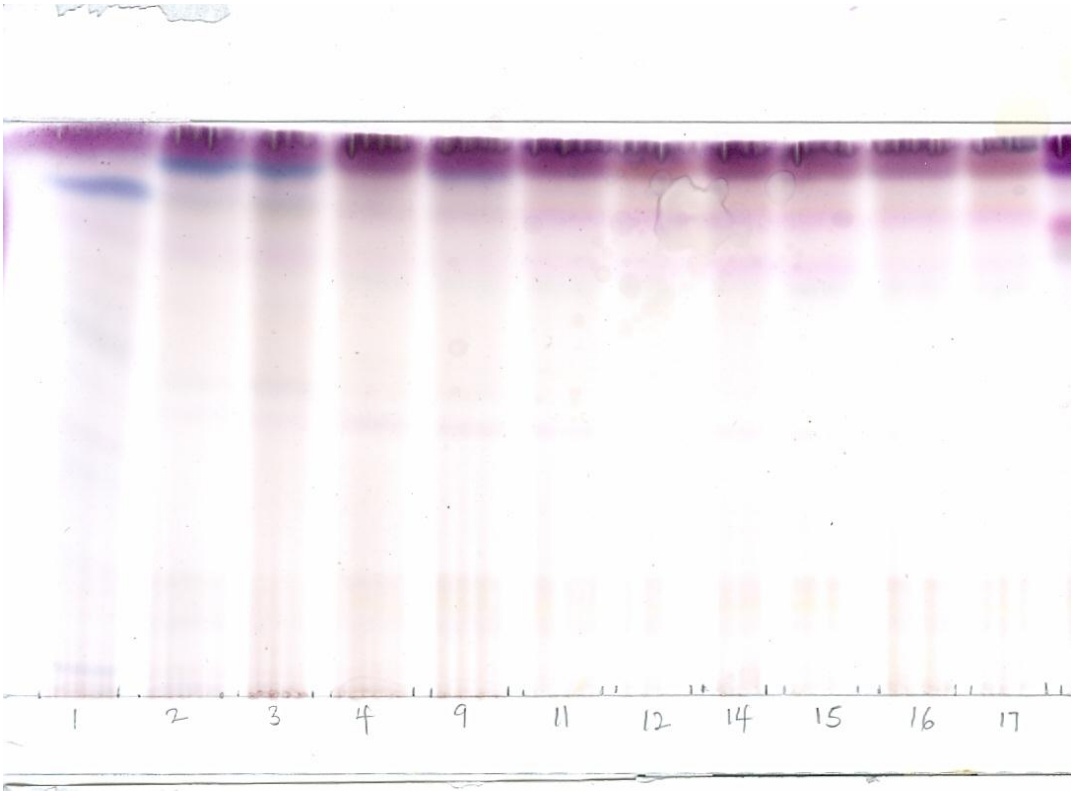


Figure 21

TLC profile of leaf extract of *T. camphoratus* and *T. parvicapitulatus* run in EMW solvent system sprayed with vanillin reagent. Lanes left to right:

1	*T. camphoratus* (male)
2 – 4	*T. camphoratus* (female),
9	*T. camphoratus* (no inflourescence)
11	*T. parvicapitulatus* (female)
12 - 14	*T. parvicapitulatus* (male)
15-16	*T. parvicapitulatus* (female)
17	*T. parvicapitulatus* (no inflourescence)

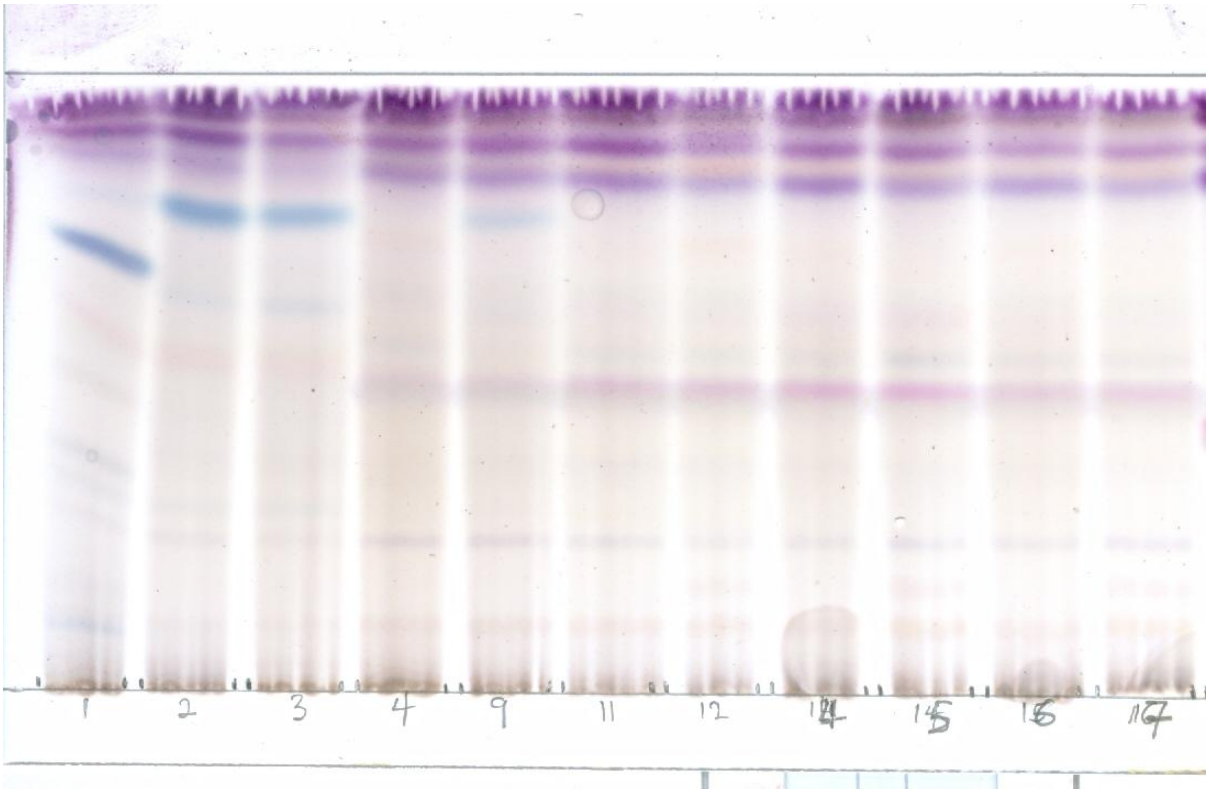


Figure 22

TLC profile of leaf extract of *T. camphoratus* and *T. parvicapitulatus* run in CEF system sprayed with vanillin reagent. Lanes left to right:

1	*T. camphoratus* (male)
2 – 4	*T. camphoratus* (female),
9	*T. camphoratus* (no inflourescence)
11	*T. parvicapitulatus* (female)
12 - 14	*T. parvicapitulatus* (male)
15-16	*T. parvicapitulatus* (female)
17	*T. parvicapitulatus* (no inflourescence)

3.7.1 Antioxidant activity

Polyphenolic compounds like flavonoids, proanthocyanidins and coumarins are typically antioxidant compounds. The TLC plates with plant extract were sprayed with 0,2% DPPH and this was done to determine the free radical scavenging ability of the compounds present in the different extracts. DPPH is a stable free radical and on interaction with plant extracts, antioxidants either transfer electrons or hydrogen atoms to DPPH thus neutralizing its free radical character (Naik *et al*., 2003). All of the extracts showed antioxidant activity as they changed the purple colour of diphenyl picrylhydralazine to the yellow diphenyl picrylhydrazyl.

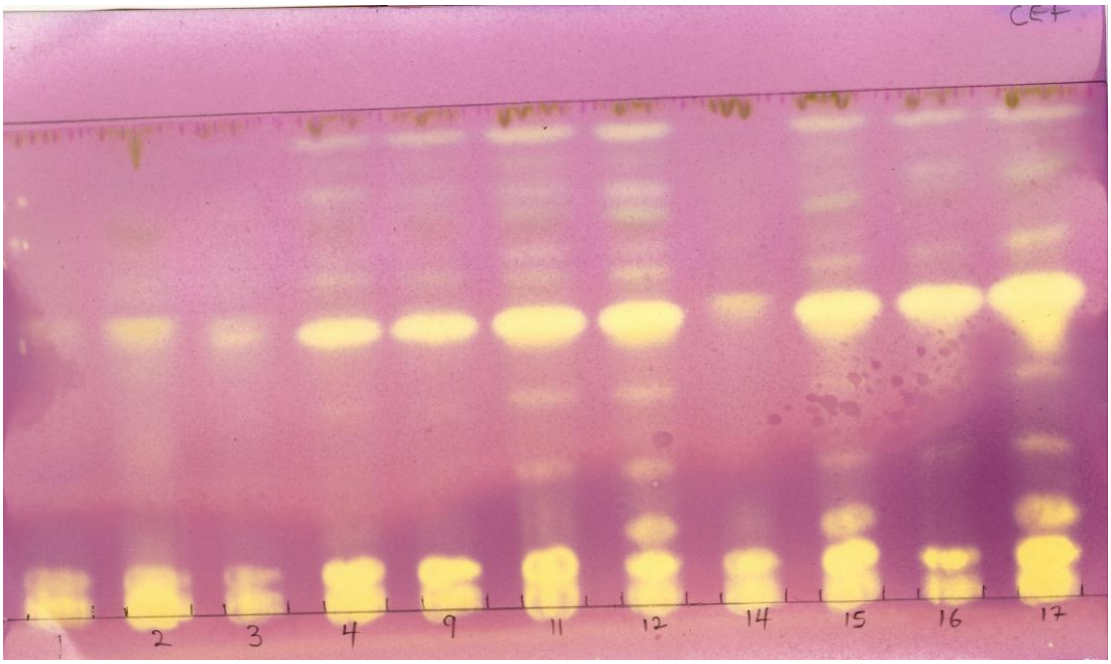


Figure 23

TLC profile of leaf extract of *T. camphoratus* and *T. parvicapitulatus* run in CEF solvent system and sprayed with 0, 2% DPPH in methanol. Lanes right to left:

1	*T. camphoratus* (male)
2 – 4	*T. camphoratus* (female)
9	*T. camphoratus* (no inflourescence)
11	*T. parvicapitulatus* (female)
12 - 14	*T. parvicapitulatus* (male)
15-16	*T. parvicapitulatus* (female)
17	*T. parvicapitulatus* (no inflourescence)

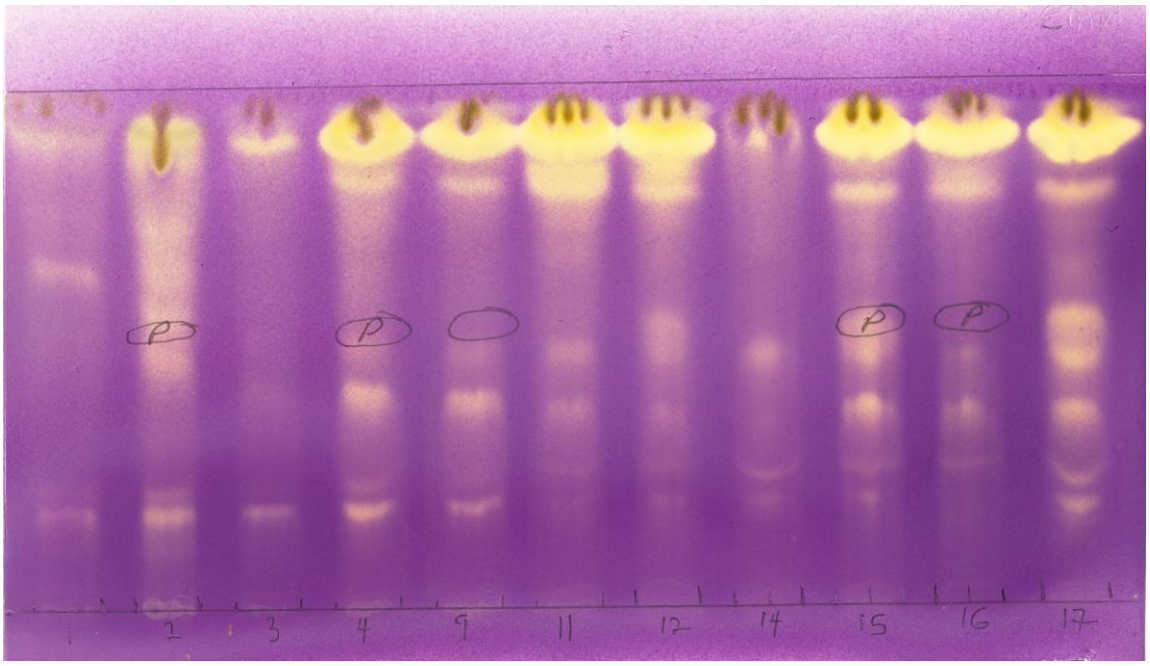


Figure 24

TLC profile of leaf extract of *T. camphoratus* and *T. parvicapitulatus* run in EMW solvent system and sprayed with 0,2% DPPH in methanol. Lanes left to right:

1	*T. camphoratus* (male)
2 – 4	*T. camphoratus* (female)
9	*T. camphoratus* (no inflourescence)
11	*T. parvicapitulatus* (female)
12 - 14	*T. parvicapitulatus* (male)
15-16	*T. parvicapitulatus* (female)
17	*T. parvicapitulatus* (no inflourescence)

3.8 DISCUSSION AND CONCLUSION

Phytochemical screening revealed the presence of varied chemical components in the different extracts of the plants. This is notable from the different color changes depicted by individual compounds due to their reaction with the spray reagent used (Vanillin/sulphuric acid). For example, terpenes showed red or blue colouration on the chromatograms when sprayed with Vanillin/sulphuric (Gibbons and Gray, 1998). Similarities exist between chemical compositions of the components of extracts separated using the same solvent system (Figure 20, pg 48) except for plant no 4 (*T.camphoratus* female) which has a different profile compared to other *T. camphoratus* profiles.

According to the chromatograms seen on the TLC plates, extract in BEA (Figure 20, pg 48) were not visible meaning that the non-polar compounds are absent in the plant extract or could be below detection level while the chromatograms of extract separated by EMW (Figure 21, pg 49) showed the range of polar compounds present in the plant extract. Chromatograms of extract separated by CEF (Figure 22, pg 50) showed that intermediate polar compounds are present in the plant extract. Intermediate polar compounds include flavonoids, coumarins, alkaloids, aglycones and glycosides and they are typically antioxidant compounds.

The TLC DPPH method of qualitative antioxidant detection showed that the acetone extracts of *T. camphoratus* and *T. parvicapitulatus* displayed antioxidant compounds due to their DPPH free radical scavenging activity. Antioxidant compounds were

seen as yellow bands against a purple background. The use of DPPH provides an easy and rapid way to evaluate antioxidants (Mensor *et al*., 2001). The extracts that showed antioxidant activity separated very well when applied and eluted on TLC in CEF (Figure 23, pg 52) but poorly on EMW (Figure 24, pg 53). Because the plant extracts separated well in an intermediate polar compound indicates that flavonoids are present in the plant extracts of *T. camphoratus* and *T. parvicapitulatus*.

It can be concluded from this study that the antioxidant activity found in the plant extracts of *T. camphoratus* and *T. parvicapitulatus* could be ascribed to the presence of flavonoids. Research supports that the antioxidant properties of flavonoids inhibit inflammation by decreasing the release of inflammatory mediators and by stabilizing cell membranes (Berkoff, 1998).

CHAPTER FOUR

4. THE EFFECT OF *T. CAMPHORATUS* AND *T. PARVICAPITULATUS* PLANT EXTRACTS ON CONTRACTION OF GUINEA PIG TRACHEA (*IN VITRO* STUDY).

4.1 INTRODUCTION

Bioassay is a very important stage in assessing the pharmacological actions of plant extracts and validating their ethnomedical uses. In the initial stages, *in vitro* testing has priority over *in vivo* studies involving laboratory animal models but this decision is usually based on scientific, economic and ethical grounds. *In vivo* studies may be preferable at later stages of the research project but still depends on the amount and the nature of evidence or bioactivity already collected by means of *in vitro* studies and the quest for additional information under life conditions. Before a bioactive component that has therapeutic application can be registered as medicines, it has to "undergo extensive clinical and toxicological screening programs" (Ameenah, 2006).

A suitable model had to be found to investigate the *in vitro* relaxation of the smooth muscle in the trachea. The use of guinea-pig as a research model has become increasingly popular (Hong and Chang, 2008). For the past 90 years, guinea-pig has been the most commonly used small animal species in preclinical studies related to asthma and chronic obstructive pulmonary disease (COPD) (Kahler, 1994; Canning and Chou, 2008). Good reasons for using the guinea-pig as model of asthma range from pragmatic benefits of economy to the ease of handling it.This animal seldom bites and its allergen-induced bronchoconstriction and that of human bronchial

asthma are common. Such features include the responsiveness of the airways to histamine (Pretolani and Vargaftig, 1993), allergen challenge and the characteristic eosinophilic nature of allergic bronchial inflammation (Campos and Church, 1992) and this is because the anatomy and physiology of guinea-pig lung closely resembles that of humans airway (Ressmeyer *et al*., 2006). These advantages out weigh the disadvantages to using guinea-pig which includes; a prominent axon reflex in the airways which is unlikely to be present in human, limited prospects for transgenic animals and limited number of guinea-pig strains for comparative studies (Canning and Chou, 2008).

Guinea-pigs possess a sensitive respiratory smooth muscle, which contracts intensively and rapidly in response to *in vivo* or *in vitro* exposure to antigens and other antagonists. Animals that have relatively insensitive airway smooth muscle, as reported for mice, might not be the first choice to study antigen-induced bronchoconstriction (Pretolani and Vargaftig, 1993). Lung hypersensitivity can be monitored easily *in vivo* or *in vitro* by following smooth muscle contraction and the formation or release of mediators from whole lungs or isolated tissues in the presence of the antigen. Models have been developed particularly using the guinea-pig, to investigate the mechanisms of the late phase reaction of asthma and of the accompanying bronchial hyperreactivity (Goldie *et al.*, 1986; Munakata *et al*., 1989).

4.2 AIM

The present study aims to investigate the therapeutic properties of *T. camphoratus* and *T. parvicapitulatus* in respiratory complaints such as bronchitis and asthma, to determine whether the plant extracts of these plants can be used prophylactically or

can be used as a bronchodilator in a pre existing asthma condition.

4.3 Source of guinea-pig for experiments

A proposal was submitted to the Animal and Ethics Committee of the University of Limpopo and it was approved (AEC No 01/07). Specially bred Dunkin Hartley guinea-pigs were obtained from South Africa Vaccine. Male guinea-pigs between 400-500g were used for the study. Though gender was not a criteria in the protocol, the reason for using one gender was due to caging problems as the mixing of genders could lead to reproduction for which the cages were not suitable. The animals were allowed to acclimatize to the environment for weeks before commencing with the studying.

4.4 Housing of the animals

The guinea-pigs were kept in standard plastic housing trays on a layer of vermiculite spread over a layer of cat-litter, serviced with plastic water bottles with stainless steel cannulas and food hoppers in the Animal Unit of the Medunsa Campus, University of Limpopo. The animals were kept in a separate room with ceilings and closed door to minimize dust.

The food was prepared by Epol (PTY) LTD and contained the following nutrients:

-Protein 160g/kg
-Moisture 120g/kg
-Fat 25g/kg
-Fiber 120g/kg
-Calcium 18g/kg
-Phosphorus 7g/kg

1g of Ascorbic acid (Univer) per liter was added to their drinking water daily, as guinea-pigs cannot synthesize ascorbic acid.

4.5 METHODOLOGY

4.5.1 Preparation of Plant Extract

Water is used in rural villages to extract plant materials for medicinal purposes because it mostly extracts sugars, amino acids and glycosides (Houghton, 2002). The dried leaves were grinded with pestle and mortar, 1g of the crude plant material was dissolved in 100ml of distilled water. The solution was boiled for 5 minutes and left to stand for 24 hours after which it was centrifuged at 2000rpm for 15 minutes and the supernatant collected and stored at 4^0C.

4.5.2 Laboratory set-up

A 10ml organ bath with a double wall through which water, at a constant temperature of 37^0C was circulated, was used. The temperature of the circulating water was controlled by a Laude Immersion Thermostat and water bath. The organ bath contained freshly prepared Krebs-Hensleit solution which was aerated with 95% O_2 and 5% CO_2 through a metal tube. One end of the tracheal chain was connected to the end of the metal tube and the other end to a UFI transducer, which was connected to a Beckman recorder; model R511A, to record the reaction of the tracheal smooth muscle to the specific stimulants. The reaction was displayed on paper.

4.5.2.1. Krebs-Henseleit Solution

The Krebs-Henseleit solution consisted of:

6.6g NaCl (BDH, Analar)

0.4g KCl (Saarchem, Univar)

0.3g $CaCl_2$ (BDH Analar)

0.1g $MgCl_2$ (Holpro Analytics)

0.1g NaH_2PO_4 (BDH, Analar)

2.0g Glucose (PAL Chemicals LTD)

All chemicals were dissolved in 1 liter of distilled water. The pH was adjusted with $NaHCO_3$ to between pH 7.3-7.4.

4.5.2.2 *In vitro* Guinea-Pig tracheal preparation

The trachea of the guinea pig was removed and placed in cold Krebs-Henseleit solution. All excessive fat and tissue were removed carefully. The trachea was cut on the opposite side of the smooth muscle. After dividing the trachea into two or three parts, cross cut were made between every two cartilage rings so that the trachea could be extended to form a chain. Care was taken not to damage the endothelial cells. The extent of damage was minimized considerably by cutting the trachea transversely. The tracheal segments were placed in 10ml organ bath. All preparations were suspended with an initial optimal resting tension of 1g and equilibrated for at least 45 minutes. The Krebs-Henseleit solution was replaced every 15 minutes during equilibration. The Krebs solution was maintained at 37^oC aerated with 95% O_2 and 5% CO_2. No anesthetic was used before killing the guinea pigs in order to avoid interference of the drugs with the plant extracts.

4.5.2.3 Methacholine-induced contraction

After priming the tracheal smooth muscle with methacholine, the muscle was maximally contracted with methacholine (10^{-5}M in organ bath). The first and second contraction was done with washing in between to allow the tracheal muscle to relax completely before the second contraction. The washing was done with Krebs-Henseleit solution. The trachea was exposed to the plant extracts (100µl) for 30 minute after the second contraction. The dose response of the plant extract was done to determine whether the plant extract can actively relax the precontracted trachea smooth muscle. The experiment was repeated and a fixed dose of the extract (10,10^{-1}, 10^{-2}, 10^{-3}) was added respectively after the first contraction and incubated for 20 minutes each before the second contraction was done, to determine whether the extract can prevent contraction of the smooth muscle (prophylactic action).

4.5.3 Statistical Analysis

The statistical paired t-test was used for statistical analysis (n = 6). The control was used to determine statistical differences and significance was taken as $p \leq 0, 05$. The result of the effect of the plant extract on Methacholine contractions of tracheas were presented as histograms. The bars were expressed as the mean $\pm$ SEM, n = 6.

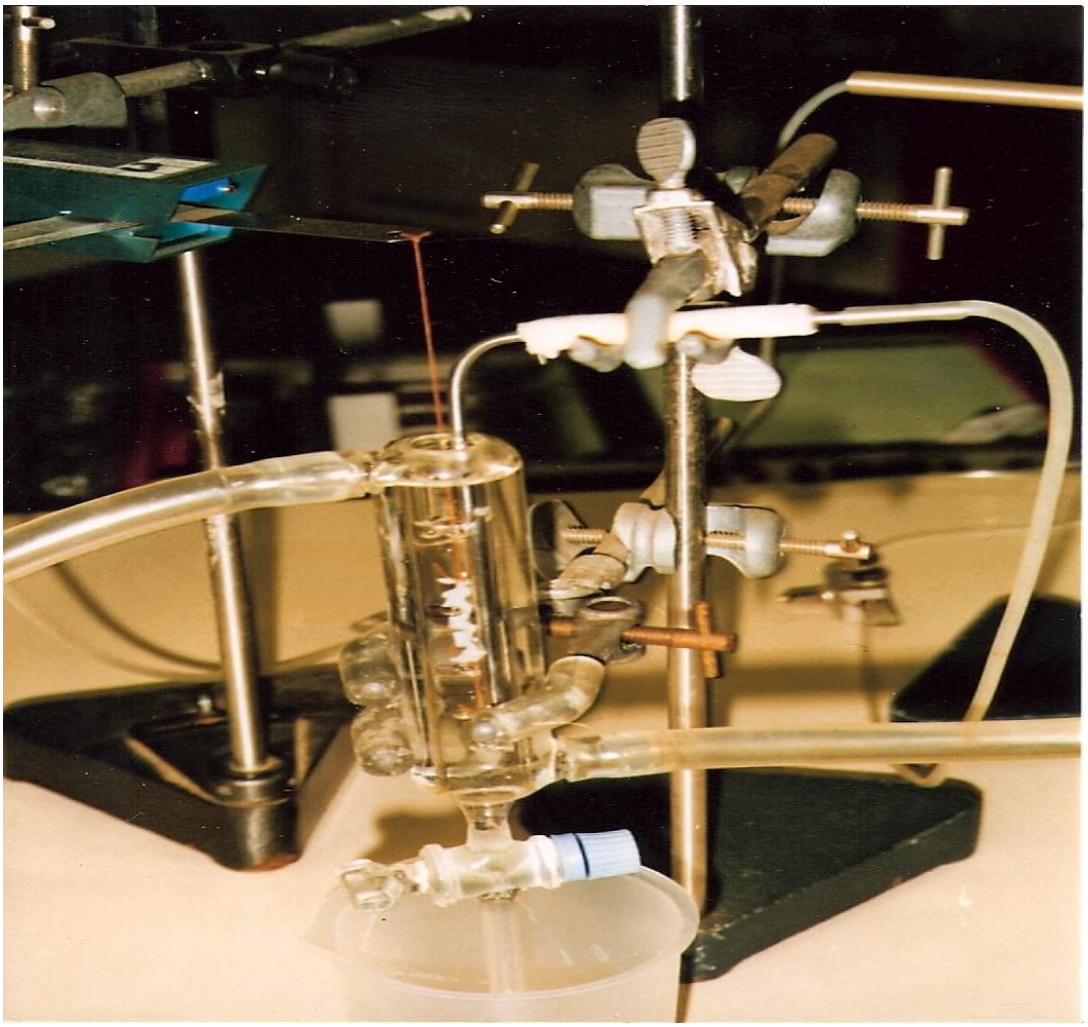


Figure 25: Tracheal setup **(**Photo by Prof Kahler-Venter of the Department of Pharmacology and Therapeutics *In-vitro* lab, used with her permission).

4.6 RESULT

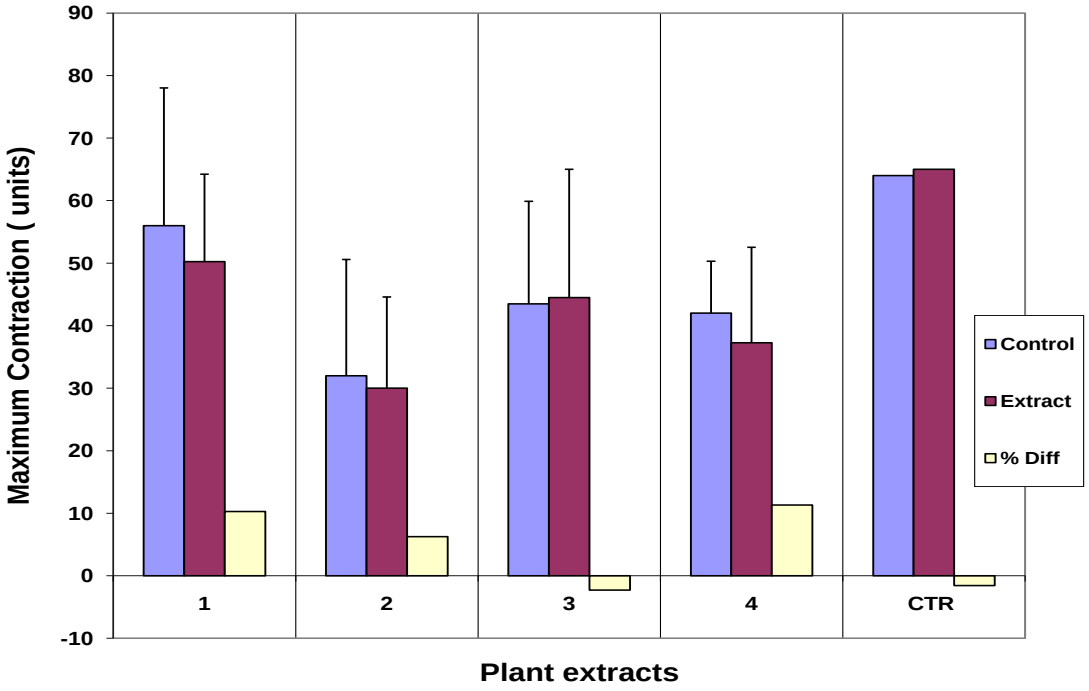


Figure 26: The effect of *T. camphoratus* extracts on methacholine contraction of guinea pig tracheal smooth muscle.

From left to right:

1 *T. camphoratus* (male) {Lowland }

2 – 4 *T. camphoratus* (female) {Lowland}

CTR Control

None of the plant extract of *T. camphoratus* showed a significant inhibition on methacholine contraction of guinea pig tracheal smooth muscle.

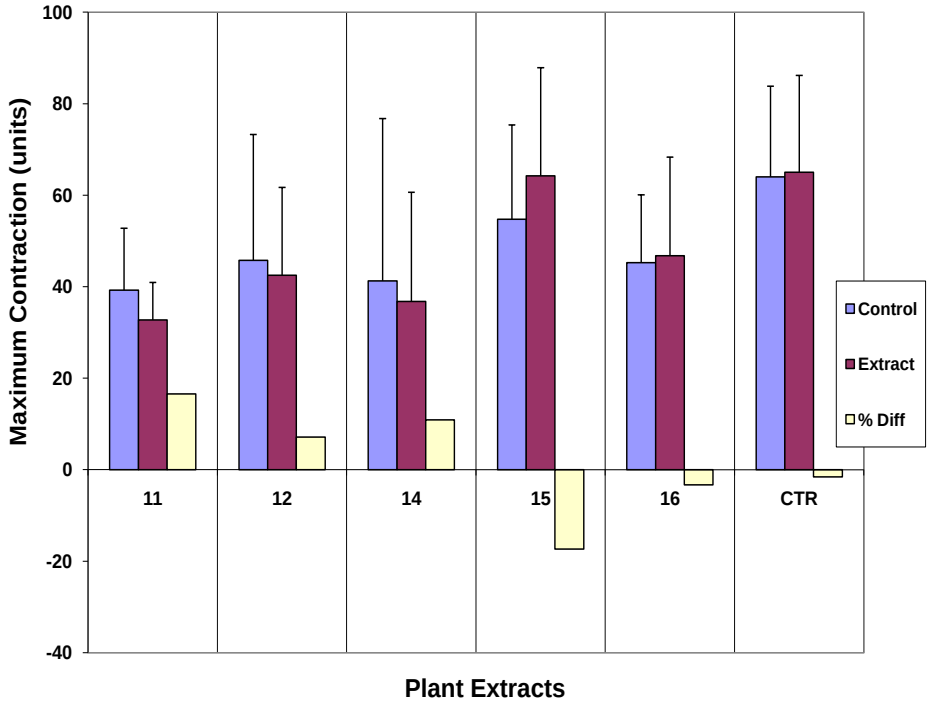


Figure 27: The effect of *T. parvicapitulatus* extracts on methacholine contraction of guinea pig tracheal smooth muscle.

From left to right:

11 *T. parvicapitulatus* (female) {lowland}

12 - 14 *T. parvicapitulatus* (male) {lowland}

15-16 *T. parvicapitulatus* (female) {mountain slope}

CTR Control

None of the plant extracts of *T. parvicapitulatus* showed a significant inhibition on methacholine contraction of guinea pig tracheal smooth muscle.

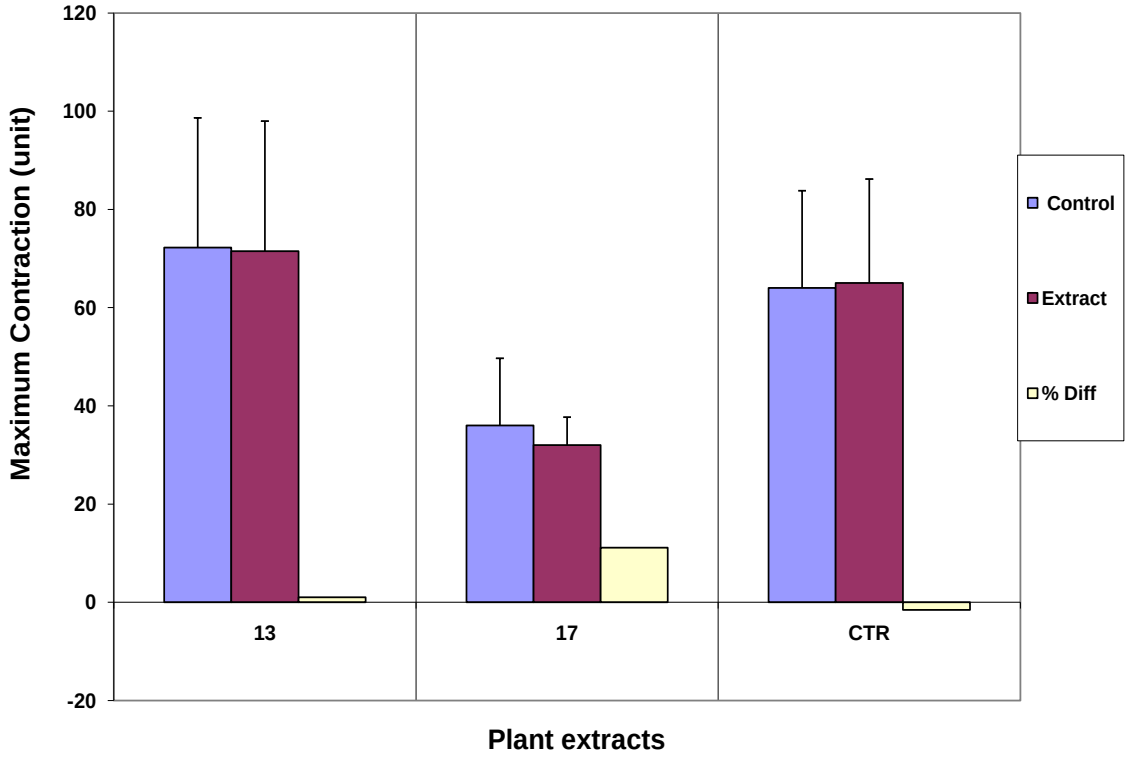


Figure 28: The effect of *T. parvicapitulatus* (sterile specimens) extracts on methacholine contraction of guinea pig tracheal smooth muscle.

13 *T. parvicapitulatus* (Sterile) {lowland}

17 *T. parvicapitulatus* (Sterile) {mountain slope}

CTR Control

None of the plant extract of *T. parvicapitulatus* showed a significant inhibition on methacholine contraction of guinea pig tracheal smooth muscle.

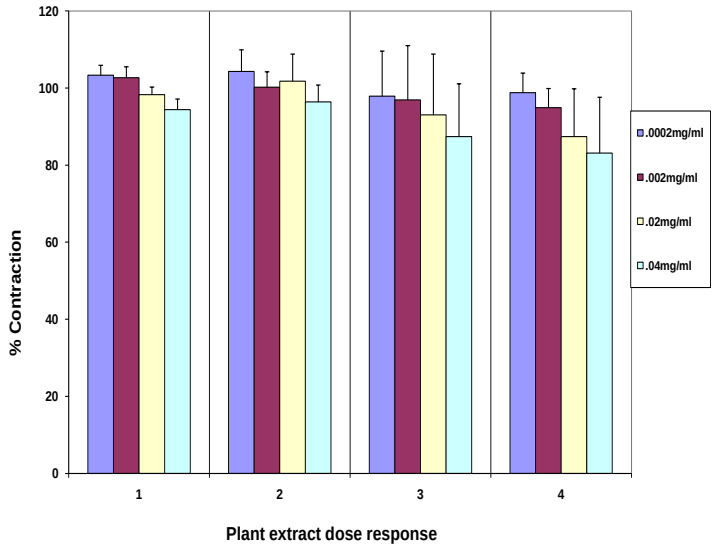


Figure 29: The effect of *T. camphoratus* extracts on the methacholine pre contracted guinea pig tracheal chain (Control 100%)

From left to right:

1 *T. camphoratus* (male) {lowland}

2 – 4 *T. camphoratus* (female) {lowland}

CTR Control

None of the plant extracts of *T. camphoratus* could significantly relax methacholine pre contracted guinea pig tracheal smooth muscle.

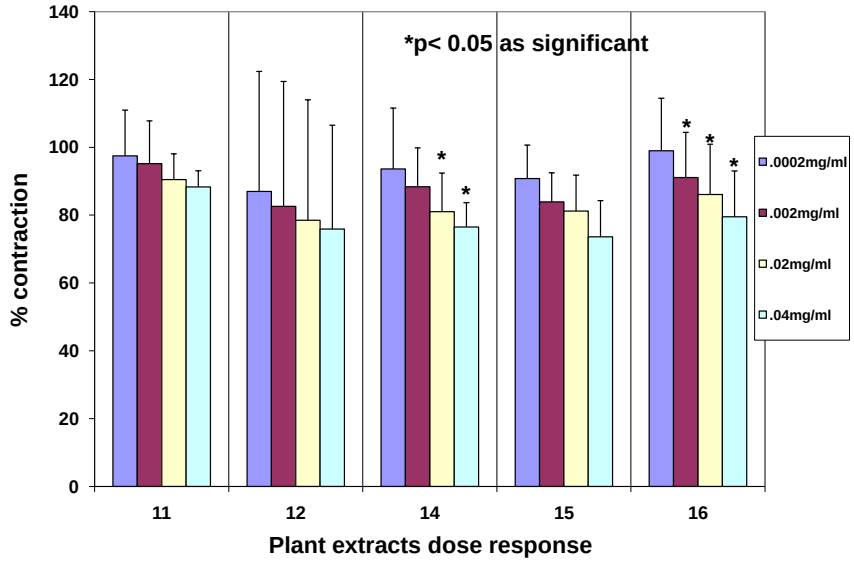


Figure 30: The effect of *T. parvicapitulatus* extracts on the methacholine pre contracted guinea pig tracheal chain. (*p< 0.05 as significant). (Control 100%).

11 *T. parvicapitulatus* (female) {lowland}

12 - 14 *T. parvicapitulatus* (male) {lowland}

15-16 *T. parvicapitulatus* (female) {mountain slope}

CTR Control

Plant extracts of specimens 14 and 16 significantly relaxed the methacholine pre contracted guinea pig trachea while the other plant extracts 11, 12 and 15 showed a tendency to relax the methacholine pre contracted guinea pig trachea but the relaxation was non significant.

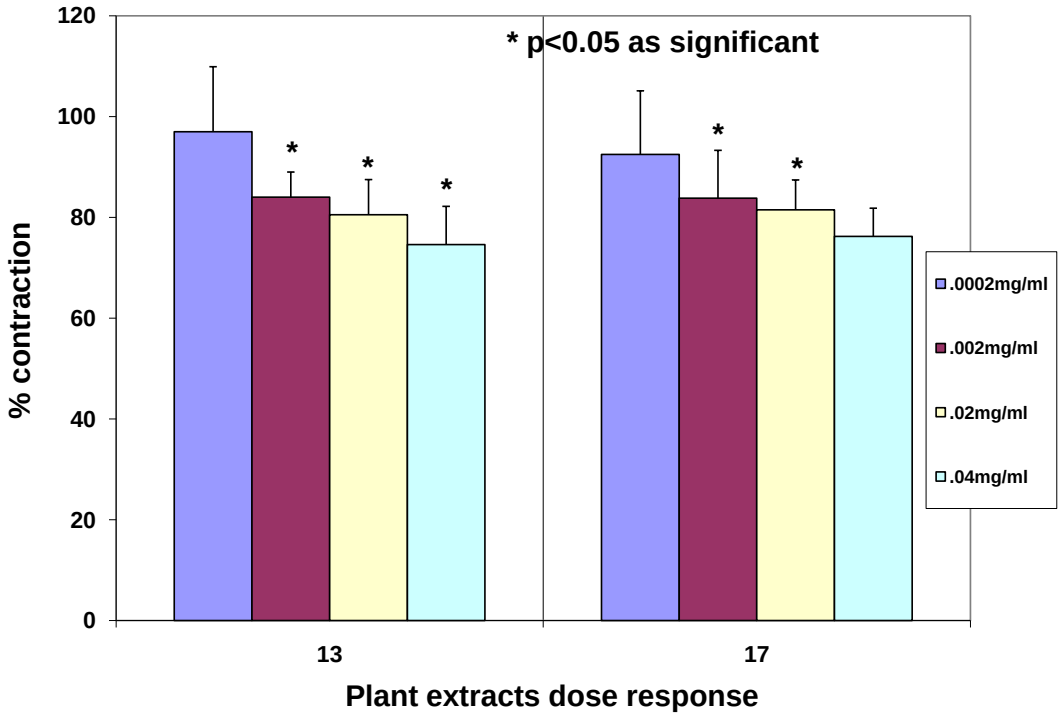


Figure 31: The effect of *T. parvicapitulatus* (sterile specimens) extracts on the methacholine pre contracted guinea pig tracheal chain. (*p< 0.05 as significant).

13 *T. parvicapitulatus* (Sterile) {lowland}

17 *T. parvicapitulatus* (Sterile) {mountain slope}

Both specimen 13 and 17 which are sterile plants significantly relaxed the methacholine pre contracted guinea pig trachea.

4.7 DISCUSSION AND CONCLUSION

The result revealed that none of the plant extracts of *T. camphoratus* and *T. parvicapitulatus* could significantly inhibit Methacholine contraction of the smooth muscle in Guinea-pig trachea while some of the extracts significantly relaxed methacholine pre-contracted trachea. Plant extract of *T. camphoratus* (Figure 29, pg 66) showed a tendency to relax methacholine pre contracted guinea pig trachea but the relaxation were non-significant while some of the plant extracts of *T. parvicapitulatus* (Figure 30, pg 67) showed a significant relaxation on the methacholine pre-contracted guinea pig trachea.

This study suggests that extracts from these plants do not have prophylactic protection for smooth muscle contraction. However plant extracts of *T. parvicapitulatus* are useful in relieving pre-existing smooth muscle contraction. This study supports the use of *T. parvicapitulatus* plant extracts as a bronchodilator but further studies need to be done to ascertain the use of these plant extracts for relieving asthma attack.

CHAPTER FIVE

5. THE EFFECT OF *T. CAMPHORATUS* AND *T. PARVICAPITULATUS* PLANT EXTRACTS ON SUPEROXIDE GENERATION AND ATP ACCUMULATION IN HUMAN NEUTROPHILS *IN VITRO.*

5.1. INTRODUCTION

Although plants used as medicine are widely assumed to be safe, many are potentially toxic. Some plants are very toxic not only to humans but also to livestock. Many plant species synthesize toxic chemicals, apparently as a primary defense against the hordes of bacterial, fungi, insect and other predators. Base on this, it is essential to evaluate their possible mutagenic and genotoxic effects. Although one expects a medicinal plant to be as little toxic as possible, experience shows that poisonous plants are the most important source of medicines in Africa (Neuwinger, 1996). Of nearly 4000 ethnomedicinal plant species in South Africa traditional healthcare (Arnold *et al*., 2000), relatively few are considered likely to give rise to serious toxicity. Poisoning from traditional medicines is usually a consequence of misadministration, incorrect preparation or inappropriate administration and dosage, and also frequently due to self-administration (Stewart and Steenkamp, 2000; Popat *et al*., 2001) rather than innate risks of using traditional healthcare. It is very important to know the toxic substance in plant material before using it as an herbal remedy.

Fennel *et al*., (2004) and Lewis, (2003) reported that significant risks are associated with the inappropriate use of medicines in all healthcare systems thus, death from

plant poisoning is infrequent in general and rare among medicinal plant users, if doses are not exceeded and the material is labeled correctly. In the South African traditional healthcare, these risks are of greater consequence due to the form in which plant medicines are sold.

Quite a number of traditional healers do not have formal training neither do they have a sufficient amount of knowledge, skill nor experience to practice successfully. Medicinal plants used regularly in "traditional medicine are assumed to be safe due to their long-term use" and are also considered to have no side effects because they are natural product (Elgorashi *et al*., 2002). This concept is largely circumstantial and the toxicology of plant extracts, especially those that are frequently used over long periods should be determined (Zampini *et al*., 2008). Some synthetic drugs have undesirable side effects and some are still abused by individuals and could be replaced by natural product (Chan, 2003). Medicinal plants used in traditional medicine should therefore be studied for safety and efficacy (Farnsworth, 1994) because little or nothing is known about the toxicity of most medicinal plant.

5.2 AIM

The aim of this study is to assess the superoxide production of *Tarchonanthus camphoratus* and *Tarchonanthus parvicapitulatus* plant extracts on isolated human neutrophils.

5.3 CHEMILUMINESCENCE

5.3.1 INTRODUCTION

Bioluminescence is light emitted from livings organism as a result of their biochemical (enzymatic) activities. Bioluminescence is primarily a marine phenomenon where it is the predominant source of light in the deep ocean. It is thus the term applied to chemiluminescent reactions that occur naturally in light-emitting organisms such as fireflies, fungi, jellyfish etc.

Luminescence analysis is used as a replacement for techniques such as colorimetry and spectrophotometry. The advantages of luminescence over other methods based on light absorption are stability, higher sensitivity, the use of non-toxic reagents, a wider measurement range, and the requirement highly specific and smaller specimen samples. Luminol-dependent chemiluminescence has been recognized as a useful tool for evaluating the phagocytic activity of granulocytes and lymphocytes (Eloff *et al*., 2004).

Adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (phosphate) NAD (P) H, and hydrogen peroxide (H_2O_2) are substances that are measured directly by light measurement. It is also possible to measure other rate-limiting metabolites, enzymes and substances that can be linked to the final light producing reaction by the use of specific coupling reactions. Luminescence analysis is based primarily on three light-producing systems namely, luminal chemiluminescence for the measurement of H_2O_2, firefly bioluminescence for the measurement of ATP and bacterial bioluminescence for the measurement of NAD(P)H (Allen *et al*., 1972). Luminol is

converted to an excited aminophthalate ion in the presence of an oxidizing compound and this reaction emits blue light that can be measured by the chemiluminometer. The chemical basis of the chemiluminescence reaction is not known in detail but superoxide anion and the myeloperoxidase product hypochlorite (HOCl) are necessary for generating luminal amplified chemiluminiscence (Wiik *et al*., 1996).

5.3.2 LEUCOCYTES

Cells in the blood consist of three major functional classes namely: erythrocytes, leucocytes and thrombocytes. Leucocytes (white blood cells) play an important role in the defense and immune systems of the human body, by means of phagocytosis and antibody production. These cells contain a nucleus and are 8-20µm in diameter. The engulfment and digestion of microorganisms is assigned to two major cell types namely the polymorphonuclear neutrophil and the macrophage. The polymophonuclear neutrophil is the dominant white cell in the blood stream and they form an important component in the antimicrobial defense system.

There are five types of leucocytes present in circulation. They are divided into two main groups based on their nuclei shape and cytoplasmic granules.

Table 4: The leucocytes

GRANULOCYTES	**MONONUCLEAR CELL**
Neutrophils	Lymphocytes
Eosinophils	Monocytes
Basophils	

Neutrophil is a type of white blood cell that is abundant in human blood stream. It is specifically granulocytes which forms an essential part of the immune system (www.medterms.com). However, during the beginning (acute) phase of inflammation, particularly as a result of bacterial infection, neutrophils leave the blood vessels and migrate towards the site of inflammation, by following chemical signals in a process called chemotaxis. Neutrophils granulocytes are the predominant cells in pus, accounting for its whitish/yellowish appearance. The stated normal range for human blood counts for neutrophils is 2,5-7,5 × 10^9/L but people of African and Middle Eastern descent may have lower counts which are still regarded as normal.

5.3.3 SUPEROXIDE PRODUCTION

When microorganisms enter the body, there are two defensive operations that come into play. These are the destructive effect of soluble chemical factors such as bactericidal enzymes and the mechanism of phagocytosis. The word phagocytosis is derived from a Greek word “phagein” meaning to eat and “cytosis” meaning the cell, is the process by which cells engulf particular material. Neutrophils play a major role during the process of phargocytosis. They are capable of ingesting microorganisms or particles. Phagocytotic event resulting in the formation of a “phagosome into which reactive oxygen species and hydrolytic enzymes are secreted”. The generation of reactive oxygen species which has been termed the “respiratory burst” involves the consumption of oxygen although this process has nothing to do with respiration or energy production.

The respiratory burst involves the activation of the enzyme NADPH oxidase, which produces large amount of superoxide, a reactive oxygen species. Superoxide dismutates, spontaneously or through catalysis via enzymes known as superoxide dismutates (Cu/ZnSOD and MnSOD), to hydrogen peroxide, which is then converted to hypochlorous acid (HOCl, also known as chlorine bleach) by the green heme enzyme myeloperoxidase. It is thought that the bactericidal properties of HOCl are enough to kill bacteria phagocytosed by a neutrophil, but this has not been proven conclusively.

HOCl may also be able to react with other oxygen metabolites generated by the phagocyte to produce new reactive intermediates. The ability of O_2^- to react with HOCl will result in the formation of the hydroxyl radical (Weiss and LoBuglio, 1982).

$$O_2^- + HOCl \longrightarrow OH^- + Cl^- + O_2$$

The formation of nitric oxide within macrophages generates a powerful antimicrobial system. The mechanism of action may be through degradation of the Fe-S prosthetic groups of certain electron transport enzymes and conceivably also by production of hydroxyl radicals (Roitt, 1994).

A flash light is associated with the release of oxidizing species for example, superoxide anion, hydrogen peroxide and hydroxyl radicals. Luminescence is an

energy product of the microbe-humoral-phagocyte reaction. Chemiluminescence is very weak and cannot be easily used in analytical systems as a means of measuring the phagocytic process. However, the production of light can be amplified by adding a chemiluminegenic probe such as Luminal to phagocytosing cells in biological fluids. The addition of compounds such as Luminol (Allen *et al*., 1972) or lucigenin (Weiss and LoBuglio, 1982) can enhance this light emission 10^3-10^4 times.

Hydrogen peroxide is an oxidant capable of injuring or altering cell function. It can destroy normal or malignant cells and can alter erythrocyte, neutrophil, lymphocyte or platelet function. Fluorescence and luminol-dependent chemiluminescence assays for H_2O_2 are more sensitive and they are suitable for the determination of the total H_2O_2 production (Wymann *et al*., 1987). The oxygen free radicals that are produced by phagocytes because of stimuli such as bacteria or phorbol-myristate-acetate (PMA) can cause cell death, cellular damage (inflammation) and it can destroy pathogens.

5.4 METHODOLOGY

5.4.1 Source of granulocytes (neutrophils)

Neutrophils are much more numerous than the long-lived monocyte phagocytes. The first phagocyte a pathogen is likely to encounter is a neutrophil. It is important that the cells must be viable, especially if the cells are to be stimulated to produce superoxides in the chemiluminescence assay. Neutrophils were isolated immediately after the blood was drawn from a healthy volunteer to increase the life span of the cells and to ensure that cells are highly viable.

Thirty milliliters of blood was drawn from six healthy volunteers (n=6) into heparinized tubes. Consent was obtained from all volunteers before participating in the study.

5.4.2 Isolation of Neutrophils

The method described by Hansel was used for this study. The whole blood was diluted with RPMI-1640 medium (with $NAHCO_3$ and L-glutamine) (Sigma-Aldrich, South Africa) at 1:1 in 50ml graduated plastic tubes. Thirty milliliters of diluted blood was layered onto 15ml percoll (1,088g/ml) in separate 50ml graduated plastic tubes. The tubes were centrifuged for 30 minutes at 2000rpm. The mononuclear layer which is the serum and the percoll were aspirated and discarded until 5mm from the pellet which contains the granulocytes. The sides of the tubes were wiped clean with gauze to prevent contamination. The pellet was then resuspended in lyses buffer with 1ml pipette. The tubes were filled with lyses buffer and left on wet ice for 5 minutes

after which the tubes were centrifuged for 5 minutes at 1000rpm. The supernatant was discarded and the dry pellet was left in the tube. This was repeated twice but the cells were only incubated for 2 minutes on wet ice. At this point most of the red cells were lysed. The pellet was suspended in 5ml RPMI-1640 medium and transferred to 15ml plastic test tubes. The Coulter system was used to count the cells by adding 50µl of cells/RPMI mixture in 10ml isotone. The mixture was stirred gently and counted 4 times and the average was divided by 200 which is the dilution factor. The cells were kept on wet ice during the experiment to prolong their lifespan.

5.4.3 Percoll Gradient Elution

Percoll is a density gradient centrifugation medium that was developed by Pertoft and Laurent, (1977). It is a colloidal suspension of silica particles (15-30nm in diameter), coated with polyvinylpyrrolidone (PVP), with 1-2% unbound polymer in solution. The function of the PVP coating is to stabilize the silica particles.

Percoll can be described as a medium that consists of two distinct compartments; an aqueous phase and a solid phase. Percoll has the advantage of being more physiological in terms of tonicity and pH. Percoll is non-toxic and possesses qualities such as low viscosity, low molarity and low buffering capacity, but high density. The low viscosity of Percoll permits cell separation at relatively low-g forces in a short period of time and it can also be autoclaved.

Vincent and Nadeau (1984) designed a formula for diluting Percoll with salt solutions to produce a solution of predefined osmolarity. This formula considers the volume of the silica particles within the percoll and it can be used to calculate the volume of a hypertonic salt solution required to add to a given volume of percoll to form an isotonic solution. The isotonic Percoll can be diluted with an isotonic salt solution to produce a percoll suspension of desired density (Venaille *et al*., 1994).

Osmolarity is a critical variable in isopycnic centrifugation which separates cells based on the differences in specific gravities. Strokes law states that the velocity of a cell in a centrifugal field is proportional to the difference between the specific gravity of the cell and that of the medium. Cells with specific gravity that is lower than that of the medium will float to the surface, and those with a specific gravity higher than that of the medium will be sedimented. After adjustment of the pH and osmolarity of the medium to physiological conditions, a spontaneous gradient can be formed that allows for the isopycnic separation of cell organelles and cells in moderate centrifugal fields (Ide *et al*., 1994). Density gradient centrifugation forms the basis for the isolation of blood cells. The pH and tonicity of the surrounding density gradient medium does however, have an influence on the density of granulocytes (Ide *et al*., 1994).

Percoll (Sigma-Aldrich, South Africa) was purchased as 100% Percoll (1,13g/ml). A 15ml Percoll solution was prepared by mixing 9,5ml Percoll stock solution with 1,5ml Hanks Balanced Salt Solution (HBBS; pH 7, 2) (Sigma-Aldrich, South Africa)

(without calcium, magnesium or bicarbonate) and 4ml dH_2O. It was diluted to 1,088g/ml. This solution of Percoll is of correct osmolarity and viscosity as can be calculated using the formula designed by Vincent and Nadeau (1984).

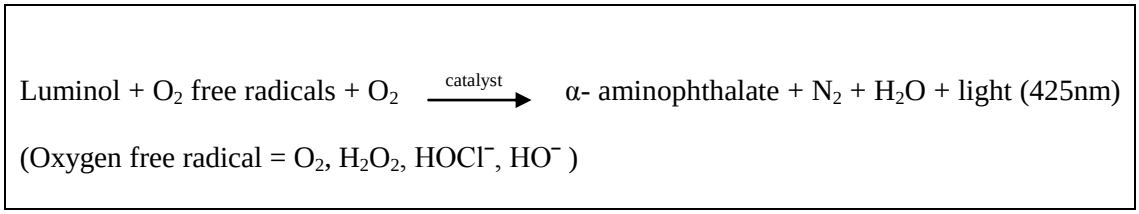
5.4.4 Erythrocyte lyses

The method used in this study was the specific lysis of erythrocytes by ammonium chloride (NH_4Cl) in the presence of potassium bicarbonate ($KHCO_3$) at pH 7, 4. The NH_3 and CO_2 penetrate into the cells and the resulting osmotic swelling is accelerated by chloride-bicarbonate exchange. The plasma membrane of leucocytes, in contrast to that of erythrocytes, does not contain carbonic anhydrase, and therefore the lyses do not affect leukocytes as long as the temperature is kept at 4^oC to minimize diffusion as the treatment of cells with NH_4Cl at room temperature leads to damage of granulocytes. The lyses buffer was prepared by adding 8.3g NH_4Cl and 1,0g $KHCO_3$ together and dissolving the two salts in 800ml of distilled H_2O. The mixture was stirred and then made up to 1000ml.

5.4.5 Chemiluminigenic probes

The two probes available for use are Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) and Lucigenin (10, 10-dimethyl-9, 9-biacridinium dinitrate DBA). Luminol is a synthetic compound, which emits light when oxidized by either peroxidases or oxygen radicals. The reaction is catalyzed by peroxidase enzymes or by appropriate inorganic oxidative catalysts. Molecular oxygen cannot be used to

achieve oxidation as in luciferase-catalysed bioluminescent reactions. The reaction can be written as:



The measurement of the light emitted during the reaction can be used to detect peroxidase and oxygen free radicals or systems producing these substances. The probe used in this study was Luminol which interacts with the oxidizing species to produce larger and more measurable amounts of light at a peak wavelength of 452nm. Luminal was weighed and 0,00199g was dissolved in 1ml of dimethylsulfoxide (DMSO). It was vigorously stirred for 5 minutes and 9ml RPMI-1640 medium (with $NAHCO_3$, without L-glutamine and phenol red) was added to make a stock solution of 10^{-3}M. From this stock solution, a working solution of 10^{-4} was made.

5.4.6 Chemotactic factor Phorbol Myristate Acetate (PMA)

Chemiluminescence can be produced by the soluble initiator of the metabolic burst, PMA (Phorbol-myristate-acetate). PMA is a co-carcinogen extracted from croton oil. It is known to cause specific degranulation with full activation of a granulocyte redox metabolism, but little azurophilic degranulation and no phagolysosome formation. PMA is thus a potent stimulator of the oxidative metabolism of neutrophils and the NADPH-oxidase system (Heyworth and Badway, 1990). PMA (Sigma-Aldrich, South Africa) was dissolved in 1ml of DMSO to prepare the stock solution. Two hundred microliter of the stock solution was dissolved in 800µl DMSO to make a

working solution. From the working solution, 10µl was taken and added to 10ml of RPMI-1640 medium (with $NAHCO_3$, without L-glutamine and phenol red).

5.4.7 Superoxide production detecting system

The bio-orbit 1251 luminometer and 5211 dispensing system were used (OEN Enterprises, South Africa). The 1251 luminometer is a bench top automatically operated luminescence photometer. The luminometer was run through a computer and the data automatically stored after each reading for further calculations. The 5221 dispensing system consists of glass syringe dispensers specially designed for the precise delivery of small volumes of liquid and a separate dispenser controller.

5.4.8 Methods

The cells were incubated with 100 µl of the different plant extracts for 20 minutes at room temperature.

The 1000µl in the cuvette was made up of:

- 300 µl RPMI 1640 medium
- 100 µl cells
- 100 µl plant extraction
- 200 µl Luminol (10^{-4}M)
- 300 µl PMA (0,2 ng/ml)

The control was made up of:

- 400 µl of RPMI-1640 medium

- 100 µl of cells
- 300 µl PMA (0,2ng/ml)
- 200 µl Luminol (10^{-4}M)
- No plant extract

5.4.9. STATISTICAL CALCULATIONS

The paired t-test was used for statistical analysis (n=6). Control was 100% and was used to determine the percentage inhibition. However, a second control was determined by adding the solvent (100 µl distilled H_2O) to the cells, and this control is indicated on the graphs. This control was used to determine statistical differences. Significant was taken at $p < 0, 05$.

5.5 RESULT

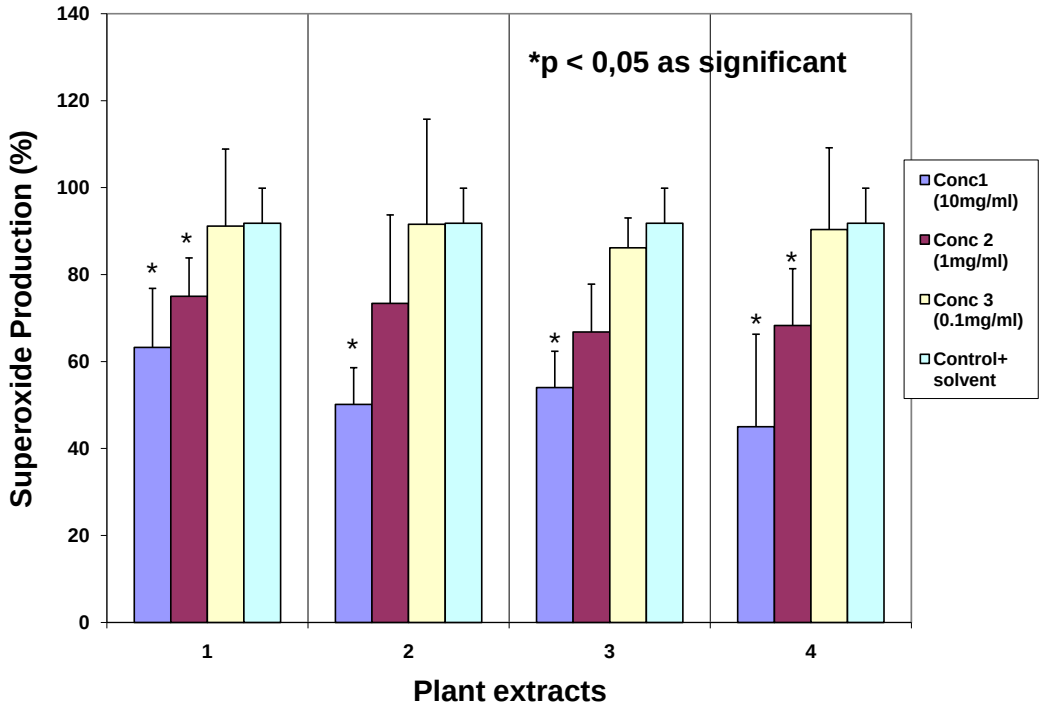


FIGURE 32: The effect of *T. camphoratus* plant extract on isolated human neutrophils superoxide production after PMA stimulation.

1 *T.camphoratus* (male) {lowland}

2 – 4 *T.camphoratus* (female) {lowland}

Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils. The paired T-test was used to determine statistical significance. (*p< 0.05 as significant). Control =100%

According to the results obtained from the above graph, the two strongest concentrations (Concentration 1 and 2) of Specimen 1 and Specimen 4 showed a significant superoxide inhibition while for specimen 2 and 3 only the strongest concentration significantly suppressed superoxide production. The result showed as expected that the control emitting the greatest percentage of light (mV) due to superoxide production was followed by the lowest concentration of the plant extract.

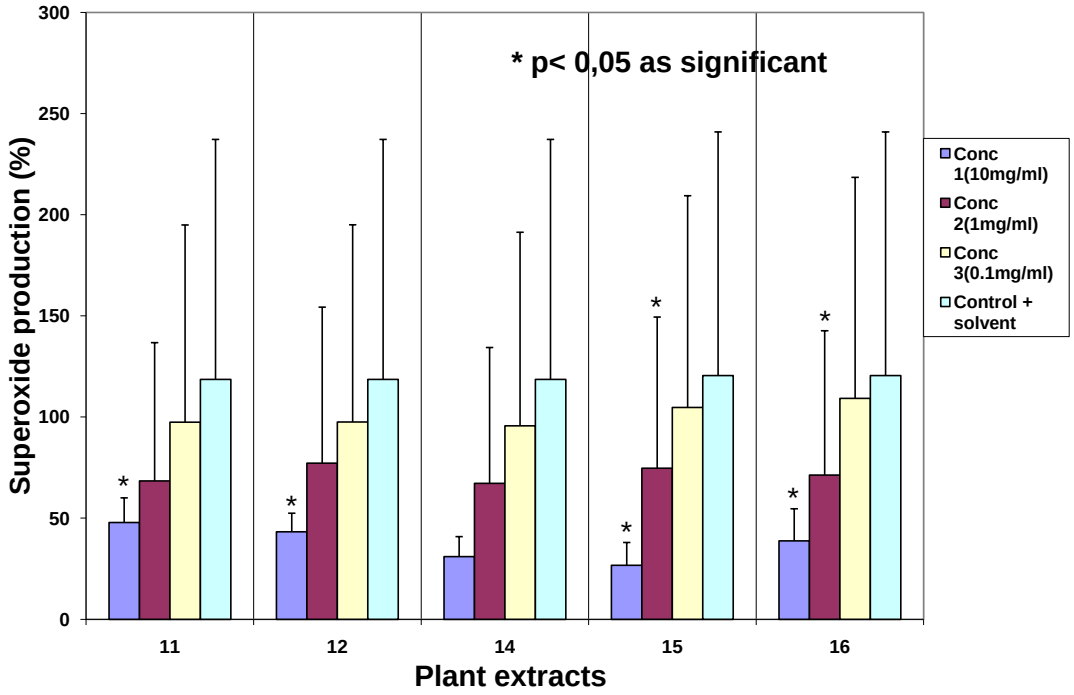


FIGURE 33: The effect of T. parvicapitulatus plant extract on isolated Human Neutrophils Superoxide production after PMA stimulation.

11 *T. parvicapitulatus* (female) {lowland}

12 - 14 *T. parvicapitulatus* (male) {lowland}

15-16 *T. parvicapitulatus* (female) {mountain slope}

Result was expressed as mean ± S.D., n = 6, for 10^6 neutrophils. (*p< 0.05 as significant). Control = 100%

Superoxide production was significantly inhibited by the strongest concentration (Concentration 1=10mg/ml) of the male plant except for specimen 14 which was not significant due to high standard deviation. There was a significant superoxide inhibition by the two strongest concentrations (Concentration. 1 and 2) of the female plants. There was non-significant superoxide suppression with the lowest concentration.

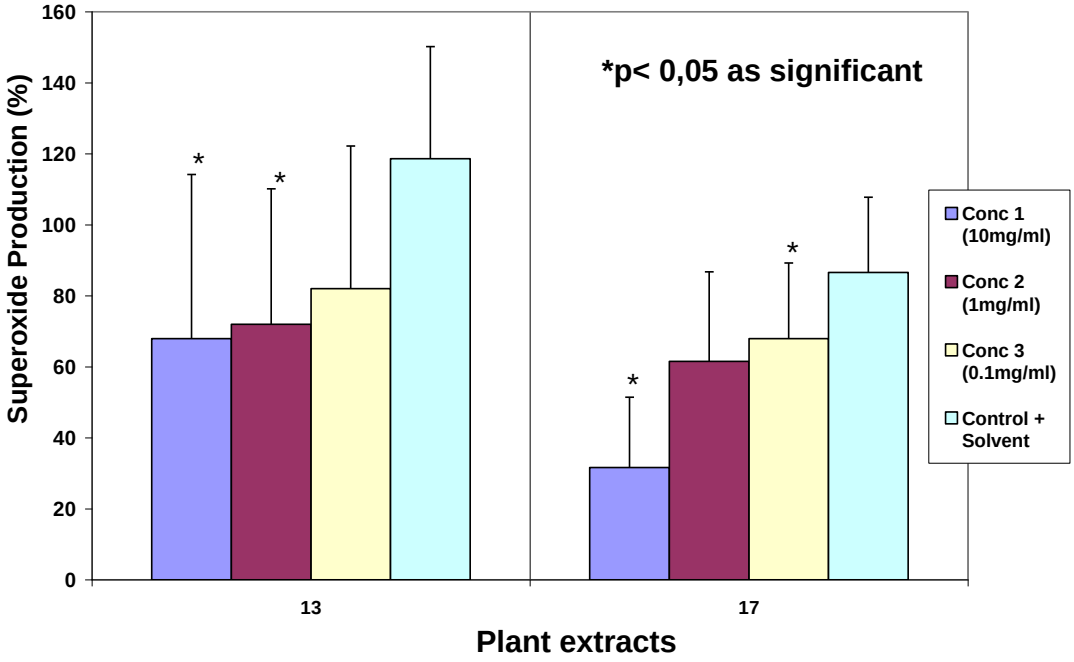


FIGURE 34: The effect of T. parvicapitulatus plant extract on isolated Human Neutrophils Superoxide production after PMA stimulation.

13 *T. parvicapitulatus* (Sterile) {lowland}

17 *T. parvicapitulatus* (Sterile) {mountain slope}

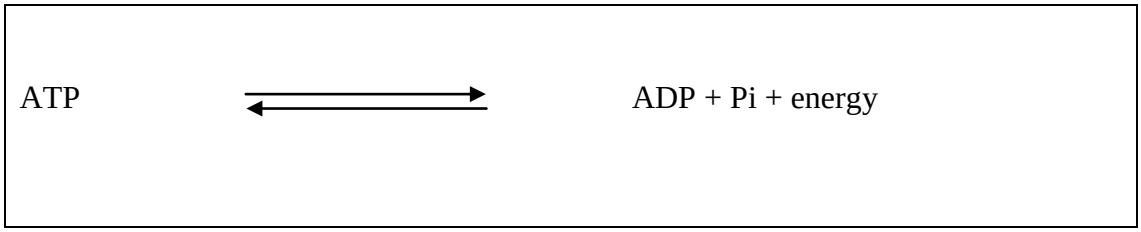
Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils. The paired T-test was used to determine statistical significance. (* p< 0.05 as significant). Control =100%

According to the result shown above, the sterile specimens showed superoxide inhibition which was statistically significant. Specimen 13 showed significant inhibition at concentration 1 and 2 while specimen 17 showed significant inhibition at concentration 1 and 3.

5.6 ATP DETERMINATION

5.6.1 Introduction

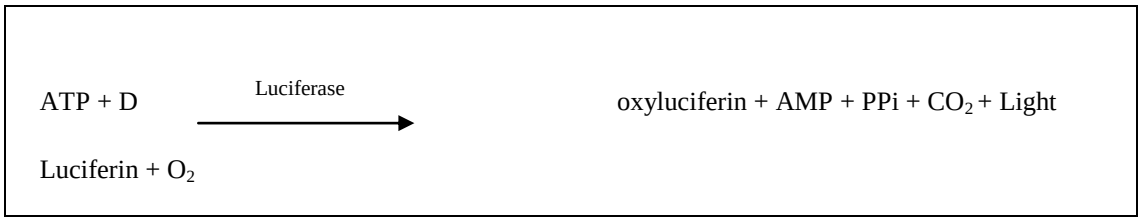
Adenosine triphosphate (ATP) is a carrier of energy in cells, and it is used for the transfer of energy from energy-yielding to energy-requiring molecular events. ATP is present in all metabolically active cells, and it supplies the energy needed for transport, as well as the energy needed for mechanical work for example muscle contraction. ATP is a high-energy molecule because its phosphate groups are easily removed. Its release of energy occurs by its hydrolysis to yield adenosine diphosphate (ADP) and inorganic phosphate (Pi). The reaction can be written as follows:



Living organism uses exergonic (energy-releasing) reactions to provide the energy requirements for life processes. As the predominant supplier of metabolic energy, ATP furnishes the chemical energy to drive endergonic (energy-requiring) reactions, provide heat, perform mechanical work and produce light (example fireflies) (Campbell, 1995).

ATP in solution can be measured using a bioluminescent assay method. This measurement of ATP is based on firefly bioluminescence, which is the most studied of all luminescent reactions. The bioluminescent measurement of ATP utilizes an

enzyme called luciferase, which catalyses the formation of light from ATP and D-luciferin. The assay has been optimized to give light at all ATP levels up to 10^{-6}mol/L. This enzyme reaction leading to light emission is:



The emitted light is linearly related to the ATP concentration and is measured using a luminometer. The stable light makes it possible to manually add ATP reagent and ATP standard using a microplate luminometer with automatic reagent dispensers. The luciferase enzyme, catalyses the activation of the substrate D-luciferin to oxyluciferin with the aid of ATP and molecular oxygen. The transition of excited oxyluciferin to its ground state results in light emission. Bioluminescence is the most efficient light-producing system because the enzymes specifically direct the reaction through a light-emitting pathway.

The sensitivity of ATP measurement is very high in assay systems where optimum reaction conditions exist. Amount as low as 10^{-15} moles of ATP can be measured when using a highly sensitive luminometer. ATP monitoring can be useful in drug discovery, toxicity testing, hormone effects, tumour chemosensitivity testing and for cell proliferation, coupled kinetic determinations of enzymes and metabolites derived from biological material.

5.7 METHODOLOGY

5.7.1 Source of granulocytes (neutrophils)

It is important that the cells must be viable, especially if the cells are to be stimulated to produce superoxides in chemiluminiscence studies. The cells were isolated immediately after blood was drawn to increase the life span of the cells and to ensure highly viable cells. Thirty milliliter of blood was obtained from four healthy volunteers (n=6) into heparinized tubes. Consent was obtained from all volunteers before participating in this study. The same procedure for isolation of neutrophils (5.4.2) for superoxide production was followed.

5.7.2 Cytotoxicity and cell Proliferation Kit (Labsystems)

This kit provides rapid analysis of cytotoxicity of proliferation in cell studies. The assay of cellular ATP replaces methods such as titrated thymidine incorporation and tetrazolium salt reduction. If a stable light emission is not obtained, the ATPases are not completely inhibited. The optimal working temperature for all reagents is 25^0C. In this procedure, a known amount of ATP is added in the assay of each individual sample. This strongly increases the reliability of the assay and makes it possible to express ATP results in moles rather than rlu or other non-chemical units.

Kits contents:

a) ATP Reagent SL; 5 vials of lyophilized reagent containing D-luciferin, luciferase and stabilizers. The lyophilized reagent was reconstituted by adding the entire content of the brown Lysing Diluent vial (12ml) to the reagent vial

and contamination was avoided by using a clean pair of tweezers to remove the rubber stopper from the ATP reagent vial. After reconstituting the reagent it was protected from sunlight by pouring it back into the brown Lysing Diluent vial.

b) Lysing Diluent 12ml; 5 vials containing Tris (hydroxymethyl) aminomethane, EDTA buffer adjusted to pH 7.75 with acetic acid, lysing agent and an ATPase inhibitor.

c) ATP Standard 5ml: 5 vials containing 10^{-5} moles/L of ATP.

The kit was stored at +4 ^{0}C but the reagents were allowed to attain room temperature before the assay was done.

5.7.3 ATP determination equipment:

The Bio-orbit 1251 luminometer and 5211 dispensing system were used for this procedure.

5.8 AIM

The aim of this study is to assess the effect of *Tarchonanthus camphoratus* and *Tarchonanthus parvicapitulatus* plant extracts on ATP accumulation on isolated human neutrophils.

5.9 Method

The cells were incubated with 20µl of different concentrations of the plant extract for 20 minutes at room temperature.

In the cuvette the 200µl was made up of:

- 180µl cells /RPMI
- 20µl of plant extract

The blanks consist of 200µl of RPMI while the controls consist of 180µl of cell/RPMI and 20µl of distilled water.

100µl of ATP reagent SL was dispensed by the dispenser of the luminometer into the cuvette and after 10 seconds the light emission was measured by the equipment and it was recorded as ATP $_{smp}$. Maximum light emission is normally obtained within a few seconds after which the light emission would start decaying. The 2nd dispenser added 20µl of ATP standard reagent and after 10 seconds the light emission was measured and recorded as ATP, $I_{smp + std}$. The equation for calculating the amount of ATP (moles) in the sample is as follow: $ATP_{smp} = 10^{-10} \times I_{smp} / (I_{smp+std} - I_{smp})$. The factor 10^{-10} is the amount (moles) of ATP standard in the well (10 µL of 1×10^{-5} mol/L).

5.10 RESULT

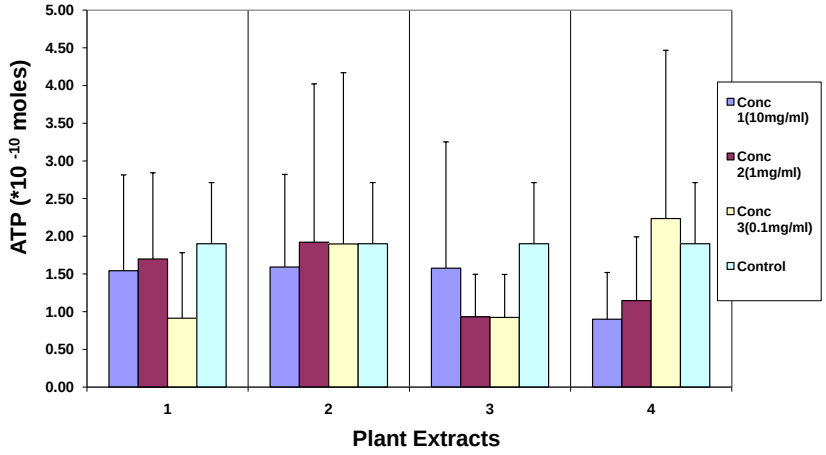


FIGURE 35: The effect of *T. camphoratus* plant extract on ATP extraction from human neutrophils.

1 *T.camphoratus* (male) {lowland}

2 – 4 *T.camphoratus* (female) {lowland}

Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils. The paired T-test was used to determine statistical significance (* $p < 0.05$ taken as significant).

A slight but non-significant decrease in ATP production in human neutrophils were found after incubating with specimen 1 and 3 plant extracts but the lowest concentration of specimen 4 showed increased ATP production. Specimen 2 showed no decrease in ATP production compared to the control except for the strongest concentration which showed a slight non significant decrease in ATP production.

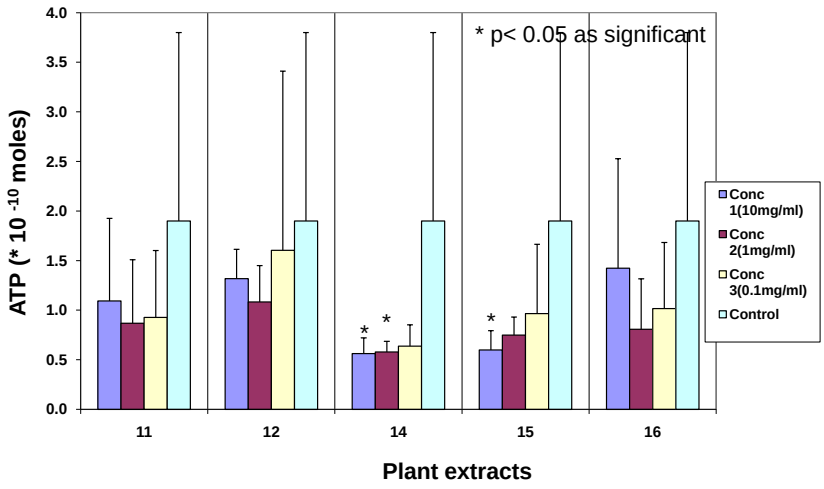


FIGURE 36: The effect of *T. parvicapitulatus* plant extracts on ATP extraction from human neutrophils.

11 *T. parvicapitulatus* (female) {lowland}

12 - 14 *T. parvicapitulatus* (male) {lowland}

15-16 *T. parvicapitulatus* (female) {mountain slope}

Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils. The paired T-test was used to determine statistical significance (* p< 0.05 taken as significant).

T. parvicapitulatus plant extract showed a non-significant decrease in ATP production in human neutrophils due to high standard deviation but specimen 14 and 15 showed significant decrease in ATP production.

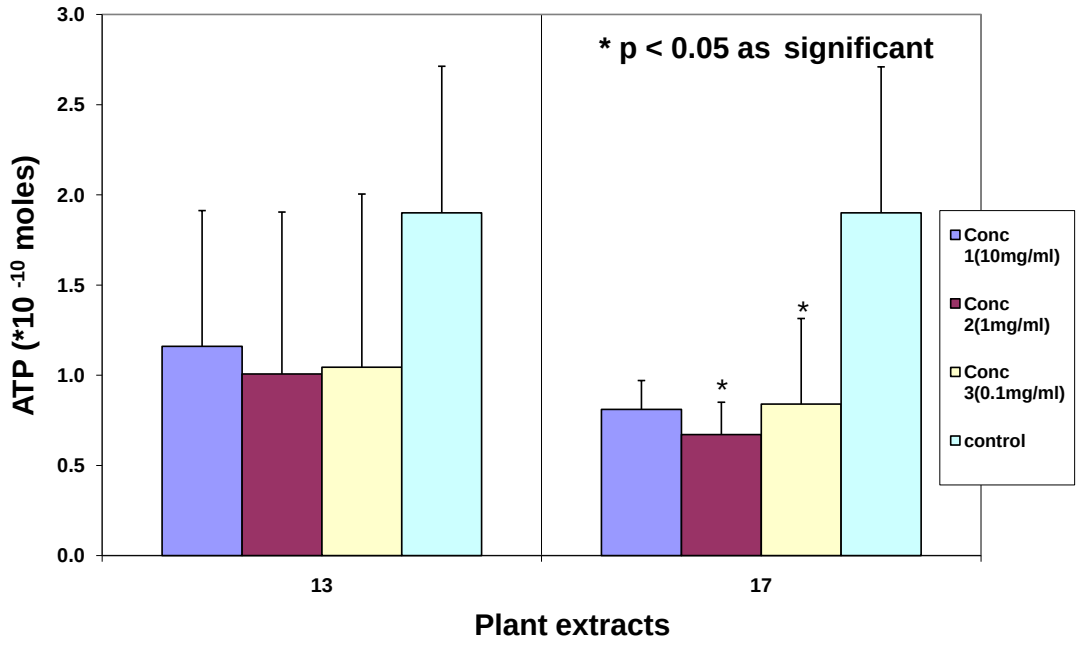


FIGURE 37: The effect of *T. parvicapitulatus* plant extract on ATP extraction from human neutrophils

13 *T. parvicapitulatus* (Sterile) {lowland}

17 *T. parvicapitulatus* (Sterile) {mountain slope}

Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils. The paired T-test was used to determine statistical significance (* $p < 0.05$ as significant).

According to the result of the graph, the two sterile specimens showed decreased ATP production but only specimen 17 showed a significant decrease in ATP production.

5.11 DISCUSSION AND CONCLUSION

Cells were stimulated with phorbol myristate acetate (PMA) as cell injury or stimulants of the inflammatory process would cause the release of superoxides (Botha *et al*., 2005). Superoxide production was significantly inhibited by both *T. parvicapitulatus* and *T. camphoratus*, but the rate of inhibition of *T. parvicapitulatus* (Figure 33, pg 85) was more prominent than it was in *T. camphoratus* (Figure 32, pg 84). The strongest concentration (10mg/ml) of *T. camphoratus* and *T. parvicapitulatus* significantly suppressed superoxide production. The lowest concentration (0.01mg/ml) of *T. camphoratus* (Figure 32, pg 84) when compared to the control did not suppress superoxide production. The lowest concentration of *T. parvicapitulatus* (Figure 33, pg 85) plant extract did however, suppress superoxide production.

A decrease in intracellular ATP reflects possible cell injury due to toxicity to neutrophils or when oxygen substrate depletion occurred (Crouch *et al*., 1993). This assay reflects the intracellular ATP concentration of isolated human neutrophils after incubation with different *T. camphoratus* and *T. parvicapitulatus* plant extracts. ATP production was decreased by both plant extracts but only the *T. parvicapitulatus* plant extracts (Figure 35, pg 92) showed significant reduction of ATP production indicating its toxicity to human neutrophils.

It can be concluded from this study, that the plants extracts of *T. camphoratus* and *T. parvicapitulatus* have significant antioxidant properties which explains traditional

healers use it in the treatment of headache, toothache, asthma, bronchitis and inflammation. *T. parvicapitulatus* however showed stronger antioxidant properties than *T.camphoratus*, this could be due to higher toxicity levels seen in *T. parvicapitulatus.* Also, *T. parvicapitulatus* can be said to be more toxic to human neutrophils than *T. camphoratus*.

CHAPTER SIX

GENERAL CONCLUSION

The micromorphological study proved useful in distinguishing between *T. camphoratus* and *T. parvicapitulatus* since the macromorphological characteristics did not prove useful, due to overlapping keys used to distinguish the two species. Solubility study also proved useful in differentiating between *T. camphoratus* and *T. parvicapitulatus* and also their sexes. Based on the macromorphological and micromorphological study, two distinct groups were identified within *T. camphoratus sensu stricto*. The second group looks more like *T. parvicapitulatus* rather than *T. camphoratus*. The TLC chromatogram also supports the macromorhological and micromorphological study. It can be concluded that this group may be a subspecies belonging to the *T.camphoratus* taxon or it could have been misidentified as *T. camphoratus*.

Studies done by other researchers however, suggest that flavonoids are responsible for the antioxidant properties of these plant extracts. The results that were obtained from the TLC plates showed that there are differences in the chemical composition of the acetone extracts of *T. camphoratus* and *T. parvicapitulatus*. This study also showed that the acetone extract of *T. parvicapitulatus* has more antioxidant properties than *T. camphoratus*.

The therapeutic study revealed that the plant extract of *T.camphoratus* and *T. parvicapitulatus* showed no prophylactic protection for smooth muscle contraction

but the plant extract of *T. parvicapitulatus* was useful in relieving pre-existing smooth muscle contraction.

The water plant extracts of *T. camphoratus* and *T. parvicapitulatus* inhibit the effect of oxygen free radicals via the receptor/G protein which led to inactivation of the pathway that lead to superoxide production and therefore prevented the formation of free radicals. Many flavonoids exhibit remarkably high radical-scavenging activity indicating that they could reduce the potential neoplastic and inflammatory effects attributed to free radical formation (Sawa *et al*., 1999). The inhibitory effects of the water extract on the isolated human neutrophils could be due to the activity of the intermediate polar compounds which include flavonoids. This explains why the extract of these plant is used by traditional healers to relieve for stomach trouble, abdominal pain, headache, toothache, asthma, bronchitis and inflammation.

The measurement of the ATP did show that the plant extract of *T. parvicapitulatus* is toxic but the plant extract of *T. camphoratus* did not show significant ATP reduction. Further research should be done to ascertain the active chemical components present in *T. parvicapitulatus*. This study has helped to improve the overall knowledge available on *Tarchonanthus camphoratus* L. complex by using different pharmacognostic tools to resolve the uncertainties surrounding the taxonomy and to determine the toxicity and the therapeutic effects of this plant.

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