Differentiation of *Tarchonanthus camphoratus* L. and *Tarchonanthus parvicapitulatus* P.P.J. Herman (Asteraceae) using electron microscopy, and comparison of their biological activities

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Abstract

Ethnopharmacological relevance: Tarchonanthus camphoratus L. complex has numerous medicinal uses amongst the sub-Saharan African populace, including treatment for bronchospasm. This study focused on providing scientific rationale for the traditional use of the extracts of *T. camphoratus* and *T. parvicapitulatus*. *T. camphoratus* L. complex has been published under diverse names by various taxonomists. *Tarchonanthus parvicapitulatus* was one of the newly described taxa, leaving *Tarchonanthus camphoratus* L. sens. strict. as a homogenous taxon. However, some of the morphological characters used tend to overlap, making it difficult to identify the different taxa.

Aims: The aim of this study was to evaluate the bronchodilatory, antioxidant and toxicological properties of the leaves of *T. camphoratus* L. and *T. parvicapitulatus*. This study also aimed to use scanning electron microscopy (SEM) to assess the differences between *T. camphoratus* L. and *T. parvicapitulatus*.

Materials and methods: Thin layer chromatography (TLC) with vanillin as visualizing agent was used to qualitatively compare the phytoconstituents of the plant acetone extracts. The free radical scavenging antioxidant qualitative assay was done by spraying TLC plates with DPPH free radical. The bronchodilatory effects of the aqueous extracts were assessed using pre-contracted guinea pig trachea. The effects of the extracts of *T. camphoratus* L. and *T. parvicapitulatus* on superoxide and ATP production was also investigated on isolated human neutrophils. A micromorphology study was done using scanning electron microscopy to study the leaves.

Results: Different compounds were visualized on the TLC plates with more than 40 compounds of intermediate polarity. The TLC plates sprayed with DPPH revealed the presence of 20 and 23 antioxidant compounds for *T. camphoratus* and *T. parvicapitulatus* respectively. Upon pre-contraction of the tracheal smooth muscles, the aqueous extracts of *T. parvicapitulatus* significantly relaxed the trachea while the relaxation observed for *T. camphoratus* was not significant. All the tested concentrations had a dose dependent inhibitory effect on superoxide production. The crude extract of *T. parvicapitulatus* at the highest concentration (10 mg/ml) significantly decreased ATP production while a non-significant increase in ATP production was observed for *T. camphoratus* at the highest concentration (10 mg/ml) when compared with the control. The micromorphology study was useful in revealing the presence of trichomes on the upper leaf surface of the studied taxa.

Conclusions: The results obtained from this study showed that the studied plant extracts had bronchodilatory effects on contracted guinea pig trachea and could also inhibit the production of free radicals including superoxide anions. To the best of our knowledge, this is the first report on the bronchodilatory activity of *T. camphoratus* and *T. parvicapitulatus*. The micromorphological studies were useful in distinguishing between the two species, confirming that *T. camphoratus* L. and *T. parvicapitulatus* are different taxa. This study provides evidence to support the traditional use of *T. camphoratus* and *T. parvicapitulatus* in managing bronchospasm.

Keywords: *Tarchonanthus camphoratus*, *Tarchonanthus parvicapitulatus*, electron microscopy, taxonomy, ATP production, asthma, bronchodilatory, superoxide production.

List of abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; BEA, benzene:ethanol:ammonia; CEF, chloroform:ethyl acetate:formic acid; COPD, chronic obstructive pulmonary disease; DPPH, diphenyl-1-picrylhydrazyl; EMW, ethyl acetate:methanol:water; HBSS, Hanks Balanced Salt Solution; PMA, phorbol myristate acetate; R_f, retention factor; SEM, scanning electron microscope; TCm, *Tarchonanthus camphoratus* male; TCf, *Tarchonanthus camphoratus* female; TPm, *Tarchonanthus parvicapitulatus* male; TPf, *Tarchonanthus parvicapitulatus* female; TLC, thin layer chromatography; UV, ultraviolet

Introduction

*Tarchonanthus camphoratu*s L. complex, commonly called camphor bush, has significant medicinal importance amongst the sub-Saharan African populace. Decoctions or extracts from *Tarchonanthus camphoratus* L. complex are traditionally employed in the treatment of abdominal pain, headache, toothache, chest ailments, asthma, cough, cold and flu, bronchitis and inflammation (Watt and Breyer–Brandwijk, 1962; Palmer and Pitman 1972; Hutchings and van Staden, 1994). The Tswana and Venda people of South Africa use the woolly seeds to stuff pillows to relieve headache and manage insomnia (Roberts and Roberts, 2017). The genus *Tarchonanthus* belongs to the family Asteraceae, subfamily Cichorioideae and tribe Tarchonantheae (Keeley and Jansen, 1991). Furthermore, they are dioecious, with male and female flowers produced on different plants (Herman, 2002). A specific name is usually chosen to indicate some striking characteristics of the plant. For example, the specific name *camphoratus* refers to the strong smell of camphor given off when the leaves are crushed and *parvicapitulatus* means 'small capitula'.

Asthma, a bronchospasm-associated disorder is one of the many respiratory diseases that affects approximately 300 million people globally, with about 250 000 deaths annually (WHO, 2007). Asthma is associated with upregulation in the levels of mast cells, eosinophils, lymphocytes, cytokines and inflammatory products leading to airway constriction (Taur and Patil, 2011). The release of mediators (histamines, prostaglandins and leukotrienes) and activation of various inflammatory cellular reactions often leads to contraction of smooth muscle of the airway and bronchoconstriction, resulting in a pronounced asthma episode (Del Donno et al., 2000; Holgate and Polosa, 2008). The current drugs (albuterol, levalbuterol and dexamethasone) used in the treatment or prevention of asthmatic attacks have adverse effects which include tachycardia, arrhythmias, hypotension, throat irritation and restlessness. Complications often associated with anti-asthmatic drugs are of concern, hence the need to discover efficacious and safe alternative drugs (Slader et al., 2006).

Synthetic drugs or medicinal plants used for the treatment of asthma should have pharmacological properties such as immunomodulation, anti-histamine, anti-inflammatory and smooth-muscle relaxant in order to alleviate the symptoms associated with asthma (Greenberger, 2003). Anti-asthma drugs such as theophylline, atropine, scopolamine, khellin (a naturally occurring chromone) and ephedrine isolated from plants such as cocoa beans

and tea, leaves of *Datura* spp., *Amni visnaga* and leaves of *Ephedra sinica* respectively are on the List of Essential Medicines (Hirsch, 1922; Hansel and Barnes, 2002; Barger and Dale, 1910). This is because they possess bronchodilatory properties and hence are employed in the treatment or prevention of asthma attacks. Antioxidant properties of natural products have been investigated for their potential to alleviate bronchoconstriction by preventing a cascade of pro-inflammatory events, thereby mitigating the excessive effects of obnoxious free radicals such as reactive oxygen and nitrogen species (Henricks and Nijkamp, 2001; Oghale and Idu, 2016).

Acocks (1988) reported that many taxonomists were convinced that there were different taxa under *T. camphoratus* but that these have been published under diverse names by various taxonomists (Paiva, 1972; Pope, 1992; Beentje; 1999). The genus *Tarchonanthus* previously consisted of two species, namely *T. camphoratus* L. and *T. trilobus* DC. until Herman in 2002 revised the *T. camphoratus* L. complex. Using macromorphological characters such as type of synflorescences, flowering times, leaf shape, leaf margin and habitat led to the resurrection of two species: *T. minor* Less. and *T. obovatus* DC. while *T. parvicapitulatus* P.P.J.Herman and *T. littoralis* P.P.J.Herman were two newly described species, leaving *T. camphoratus* sens. strict as a smaller, more homogenous taxon. However, some of the characters used in the key overlapped, making it difficult to identify the different taxa. *Tarchonanthus camphoratus* L. is a multi-stemmed, rounded dioecious shrub or small tree which is the most common species and a representative of the genus in the northern parts of southern Africa (Smith 1966). *Tarchonanthus parvicapitulatus* is one of the newly described species having a moderate to strong camphor odour in common with other *Tarchonanthus* species (Herman, 2002).

The antimicrobial and larvicidal activities of the leaves and stems of members of the *T. camphoratus* L. complex have been reported (Van Vuuren and Viljoen, 2009; Nanyonga et al., 2012; Nanyonga et al., 2014). Phytochemicals such as tannins, saponins, reducing sugars and terpenes have been reported to be present in the leaves of *T. camphoratus* L. complex (Van Wyk et al., 1997; Watunga et al., 2014). Within the limits of our literature search there are no scientific reports on studies validating or supporting the use of *Tarchonanthus camphoratus* L. complex in the treatment of bronchospasm. Therefore, this study was designed to evaluate the bronchodilatory, antioxidant and toxicological properties of the leaves of *Tarchonanthus camphoratus* L. and *Tarchonanthus parvicapitulatus*. In addition to the above aim, this study aimed to use scanning electron microscopy (SEM) to assess the morphological characteristics in order to determine the differences between *Tarchonanthus camphoratus* L. and *Tarchonanthus parvicapitulatus*.

Materials and Methods

Plant Collection

Different plant specimens were collected from Kokoriba game reserve, North West province, South Africa and were identified as *Tarchonanthus camphoratus* L. while specimens collected from Ga-Rankuwa and Onderstepoort in Pretoria, Gauteng were identified as *Tarchonanthus parvicapitulatus* P.P.J. Herman by Mr P.P.J. Herman from the South African National Biodiversity Institute, Pretoria. Herbarium specimens (Table 1) were deposited in the

herbarium of the Department of Pharmacology and Toxicology, Sefako Makgatho University of Health Sciences.

Table 1: Localities of plant specimens and voucher specimen numbers

Specimen No	Species	Sex	Localities Collected	Habitat
AA01	*Tarchonanthus camphoratus*	Male	North West Province	Lowland
AA02	*Tarchonanthus camphoratus*	Female	North West Province	Lowland
AA03	*Tarchonanthus camphoratus*	Female	North West Province	Lowland
AA14	*Tarchonanthus parvicapitulatus*	Male	Gauteng Province	Lowland
AA15	*Tarchonanthus parvicapitulatus*	Female	Gauteng Province	Mountain slope
AA16	*Tarchonanthus parvicapitulatus*	Female	North West Province	Mountain slope

Plant extraction

The plant material was air dried at room temperature and was milled into fine powder using a mortar and pestle. The powdered plant material was stored in a clean container to avoid contamination. The acetone crude extract was prepared from the powdered plant material by using acetone in a ratio of 1:10 (w:v), i.e. 1 g of powdered plant material was dissolved in 10 ml acetone (Eloff, 2000). The mixture was shaken overnight and filtered through Whatman No. 1 filter paper in a Buchner funnel. The process was repeated until complete extraction was achieved. The filtrate was placed in a pre-weighed glass beaker and dried completely under a stream of cold air. The water extract was prepared by adding 1 g of the crude plant material in 100 ml water. The solution was boiled for 5 min and left to stand for 24 h after which it was centrifuged at 450 x g for 15 min and the supernatant was collected and stored at 4^{o}C. This method was employed to replicate traditional preparation.

Phytochemistry of leaf extracts using thin-layer chromatography

The acetone extracts prepared above were dissolved in acetone to obtain 10 mg/ml stock solutions. The extracts were analyzed by thin layer chromatography (TLC) on silica-backed plates (Merck F254 10 × 20 cm) in three different solvent systems namely ethyl acetate:methanol:water (EMW) (10:1,35:1 v/v/v), benzene:ethanol:ammonia (BEA) (18:2:0,2 v/v/v), and chloroform:ethyl acetate:formic acid (CEF) (10:8:2 v/v/v). BEA was used for non-polar/basic compounds, EMW for polar and neutral compounds while CEF was used for intermediate polar and acidic compounds (Kotzé and Eloff, 2002) Briefly, aliquots of 10 µl of the extracts were loaded with a micropipette 1 cm from the bottom of a labelled TLC plate and the solvents were allowed to run up to 2 cm from the edge of the plate. The developed plates were air dried in the fume cupboard and thereafter visualized under UV light (254 and 360 nm Camac Universal UV lamp). For further detection of chemical compounds, the plates were sprayed with acidic vanillin spray reagent (Sigma-Aldrich, Germany) (0.1 g vanillin

powder was dissolved in 28 ml methanol with 1 ml sulfuric acid carefully added). The plates were heated to 100°C for 5 min for optimal colour development (Homans and Fuchs, 1970).

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging antioxidant qualitative assay

The acetone crude extracts were prepared as described above, as were the chromatograms, using the same solvent systems namely EMW, BEA and CEF. For further detection of chemical compounds, the developed TLC plates were sprayed with 0.2% DPPH in methanol. The plates were heated to 100°C for 5 min for optimal colour development. Chromatograms were examined for colour change over 30 min. Antioxidant compounds in the extracts changed the purple colour of DPPH to yellow. The DPPH radical is reduced from a stable free radical, which is purple, to diphenyl picrylhydrazine, which is yellow. Pairing off of the odd electron in a free radical scavenger results in reduced absorption and decolourization of DPPH solution that leads to colour changes from deep violet to light yellow (Blois, 1958).

Experimental Animals

Ethics approval for the study was obtained from the Animal and Ethics Committee of the University of Limpopo, Medunsa campus (MREC/M/188/2008:PG). Specially bred Dunkin Hartley guinea pigs were obtained from South Africa Vaccine (Johannesburg, South Africa). Male guinea pigs between 400-500 g were used for the study. Though gender was not a criterion 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 two weeks before commencing with the study. The guinea pigs were housed under standard conditions and supplied with food and water *ad libitum* in the Animal Unit of the Medunsa Campus, University of Limpopo.

Guinea pig tracheal preparation

The trachea of the guinea pig was removed after sacrificing by a blow on the neck, and placed in cold Krebs-Henseleit solution. No anaesthetic was used before killing the guinea pigs to avoid interference of the drugs with the plant extracts. All excess fat and tissue were removed carefully while the trachea was cut on the opposite side of the smooth muscle. After dividing the trachea into two or three parts, crosscuts were made between every two cartilage rings so that the trachea could be extended to form a chain. The extent of damage was minimized considerably by cutting the trachea transversely. Tissue was then suspended in a 10 ml organ bath (Schuler organ bath type 809, March-Hugstetten, Germany) containing Krebs–Henseliet solution of the following composition (mM): NaCl 120, NaH_2PO_4 1.2, $MgCl_2$ 1.2, KCl 4.7, $CaCl_2$ 2.5, $NAHCO_3$ 25 and glucose 11. The Krebs solution was maintained at 37°C and aerated with 95% O_2 and 5% CO_2. Tissues were suspended under isotonic resting tension of 1 g and equilibrated for at least 45 min. The Krebs-Henseleit solution was replaced every 15 min during equilibration (Kahler, 1994, Boskabady et al., 2004b).

Methacholine-induced contraction

The tracheal smooth muscle was primed with methacholine to induce maximal contraction. After priming the tracheal smooth muscle with methacholine, the muscle was maximally contracted with methacholine (10-5 M in organ bath). The first contraction was achieved, and subsequently a wash out period was ensured using Krebs-Henseleit solution before inducing a second contraction. Krebs-Henseleit solution was used for the washing step to allow the tracheal muscle to relax completely before the second contraction. The trachea was treated with 100 µl of the plant extracts for 30 min in ascending order after the second contraction. The dose response of the aqueous plant extract was assessed to determine whether the plant extract could actively relax the precontracted trachea smooth muscle. The experiment was repeated and a fixed dose of the extract (40 mg/ml, 20 mg/ml, 2 mg/ml, 0.2 mg/ml) was added respectively after the second contraction and incubated for 20 min (Tschirhart et al., 1987; Kahler, 1994). This was done to determine if the extract could prevent contraction of the smooth muscle.

Neutrophil isolation

A heparinized tube was used to draw 30 ml of blood from six healthy human volunteers (n=6) upon obtaining consent from all volunteers before participating in the study. The method described by Hansel et al. (1989) was used to isolate viable neutrophils (Hansel et al., 1989). The whole blood was diluted with RPMI-1640 medium (with $NAHCO_3$ and L-glutamine) (Sigma-Aldrich, South Africa) at 1:1 in 50 ml graduated plastic tubes. A 15 ml Percoll solution was prepared by mixing 9.5 ml Percoll stock solution with 1.5 ml Hanks Balanced Salt Solution (HBBS; pH 7.2) (Sigma-Aldrich, South Africa) (without calcium, magnesium or bicarbonate) and 4 ml distilled water (dH_2O) to obtain a density of 1.088 g/ml. Thirty ml of diluted blood was layered onto 15 ml Percoll (1.088 g/ml) in separate 50 ml graduated plastic tubes and centrifuged for 30 min at 450 x g. The mononuclear layer containing serum and Percoll were aspirated and discarded until 5 mm from the pellet which contained the granulocytes. The sides of the tubes were wiped clean with gauze to prevent contamination. In order to lyse the red cells, the pellet was resuspended in lysis buffer (8.3 g NH_4Cl and 1.0 g $KHCO_3$ together was dissolved in 800 ml of dH_2O) and left on wet ice for 5 min after which the tubes were centrifuged for 5 min at 1000 rpm. 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 min on wet ice. The pellet was suspended in 5 ml RPMI-1640 medium and transferred to 15 ml plastic test tubes. The Coulter system (Beckman, USA) was used to count the cells by adding 50 µl of cells/RPMI mixture in 10 ml isotone while the viability of the neutrophils was assessed using trypan blue exclusion.

Superoxide production from neutrophils

Superoxide anion (O_2-) generation by neutrophils (2.5×10^6 cells/ml) was measured using a reaction catalysed by the peroxidase enzyme luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) at a concentration of 10^{-4} M. The chemotactic factor phorbol myristate acetate (PMA, Sigma-Aldrich South Africa) at a final concentration of 0.2 ng/ml was added to the reaction to stimulate O_2- production. The cells were incubated with 100 µl of the different concentrations of the water extracts for 20 min at room temperature. The tested sample tubes contained 300 µl RPMI 1640 medium, 100 µl cells,100 µl plant extract, 200 µl luminol and 300 µl PMA while the control contained

400 µl RPMI 1640 medium, 100 µl cells, 200 µl luminol and 300 µl PMA with no extract. The bio-orbit 1251 Luminometer and 5211 dispensing system (OEN Enterprises, South Africa) were used to measure the amount of superoxide production in each sample tube. 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. Results were expressed as mean of maximum superoxide production (mV) ± S.D., n=6, 10^6 cells (Nadeem et al., 2003).

Bioluminescent ATP Assays

The Cytotoxicity and Cell Proliferation Kit (Labsystems) was used to measure the amount of adenosine triphosphate (ATP) production in the neutrophil cells. Manufacturer's instructions were followed in preparation of the reagents. Sample tubes consisted of 180 µl of cell suspension in RPMI incubated with 20 µl of different concentrations (0.1 mg/ml, 1 mg/ml, 10 mg/ml) of the plant extracts for 20 min at room temperature. The blank consisted of 200 µl of RPMI only while the control consisted of 180 µl of cell suspension in RPMI and 20 µl of distilled water. A volume of 100 µl of ATP reagent SL (containing D-luciferin, luciferase and stabilizers) 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 was normally obtained within a few seconds after which the light emission would start decaying. The second dispenser added 20 µl 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).

I_{smp} : Light emission corresponding to sample ATP

$I_{smp+std}$: Light emission corresponding to sample plus standard ATP

Morphology study

Dried leaf samples were examined using scanning electron microscope (SEM) procedures. Chemical fixation was done by soaking leaf specimens in 2.5% glutaraldehyde. The samples were then dehydrated in a graded series of ethanol (10%, 20%, 30%, 50% and 70%—once for 10 min at each step). Finally, specimens were immersed in 100% acetone twice for 30 min each and simple air-drying in a desiccator under vacuum. Prepared samples were mounted on a gold coated stub for examination under SEM (Pathan et al., 2008).

Statistical Analysis

The statistical paired t-test was used for statistical analysis (n = 6). The control was used to determine statistical differences and significance using two-way ANOVA. The value $p < 0.05$ was considered significant. The result of the effect of the plant extract on methacholine contractions of the trachea tissue were presented as histograms. The bars were expressed as the mean ± SEM, n = 6. All data were analyzed with Dunnett's tests using GraphPad

Prism version 5.01 (GraphPad Software Inc., San Diego, CA, United States).

Results and Discussion

Phytochemistry of leaf extracts

The varying polarities of the compounds present in the acetone leaf extracts of *T. camphoratus* and *T. parvicapitulatus* were visualized on the TLC plates sprayed with vanillin-sulphuric reagent. Different compounds were visualized on the TLC plates using the BEA and CEF mobile solvent systems with more than 40 compounds of intermediate polarity separated by CEF (Fig. 1a). The retention factor (R_f) values of the different bands were then calculated using the equation:

$$R_f = \frac{\text{Distance of spot from origin}}{\text{Distance of solvent front from origin}}$$

The R_f values of the separated compounds ranged from 0.1 to 0.8. There appeared to be common compounds with an R_f value of 0.7 present in both *T. camphoratus* and *T. parvicapitulatus* (Figure 1a). The TLC fingerprint revealed the presence of intermediate polar compounds as the extracts were poorly separated in the polar EMW solvent. There was a blue coloured compound in the *T. camphoratus* extract but this was absent in the *T. parvicapitulatus* extract. This result obtained from the TLC plates showed that there are differences in the chemical composition of the acetone extracts of *T. camphoratus* and *T. parvicapitulatus*. Terpenoids and flavonoids are known to be intermediately polar compounds that separate well in solvents of intermediate polarity such as acetone, methanol, ethanol, and ethyl acetate (Masoko, 2007).

Various methods have been employed for the determination of antioxidant potential of different biological samples. The use of the DPPH method has been widely applied for estimating antioxidant activity. Based on the chromatogram, both the leaf extracts of *T. camphoratus* and *T. parvicapitulatus* showed the presence of antioxidant compounds with R_f values ranging from 0.1 to 0.89. There was an antioxidant compound with an R_f value of 0.59 present in both species. The TLC plates revealed the presence of 20 and 23 antioxidant compounds for *T. camphoratus* and *T. parvicapitulatus* respectively (Fig 1b). Hence, the antioxidant compounds present in the leaf extracts of *T. parvicapitulatus* were more abundant than those of *T. camphoratus*. Due to the presence of odd electrons, DPPH is strongly absorbed at 517 nm but in the presence of free radical scavenging antioxidants, the odd electron of DPPH will become paired, thereby reducing the intensity of the absorption at 517 nm (Hirano et al., 2001). The change in colour of compounds to yellow on TLC plates sprayed with DPPH has been a widely used method for the identification of flavonoids (Gwatidzo et al., 2018; Mittal, 2013).

The qualitative DPPH antioxidant assay conducted therefore revealed that the acetone crude extracts of *T. camphoratus* and *T. parvicapitulatus* both possess free radical scavenging compounds as evidenced in the decolourization of DPPH solution from purple to yellow indicating the possible presence of flavonoids. Studies

conducted by Wetungu and colleagues reported the presence of tannins, phenolics and flavonoids in the acetone fractions of *T. camphoratus* L. (Wetungu et al., 2014). The phytochemical profiling proved useful in differentiating the species. Plant extracts with antioxidant properties have been proven to be able to regulate the levels of eosinophils and cytokines due to the presence of high amounts of secondary metabolites such as saponins, lignans, flavonoids and phenols (Ferlazzo et al., 2015; Terahara, 2015).

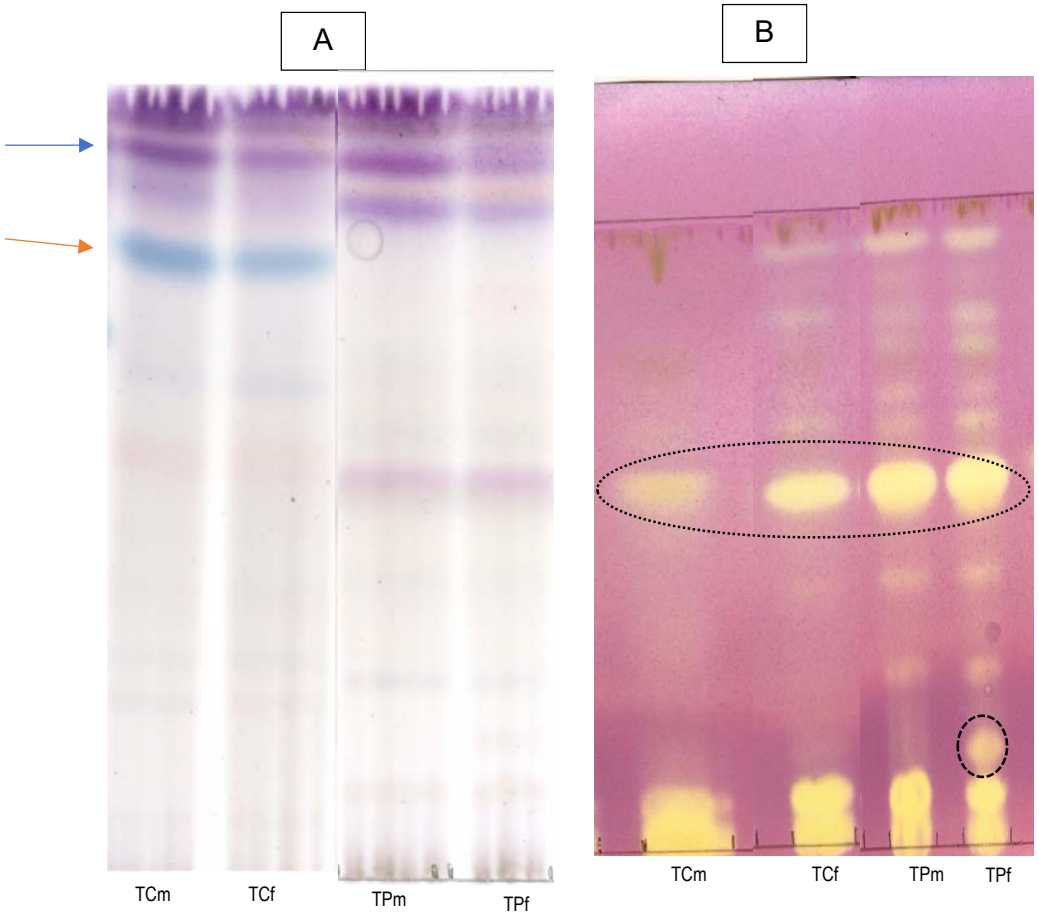

Figure 1: (A) Thin-layer chromatography profile of leaf extract of *T. camphoratus* and *T. parvicapitulatus* developed in chloroform/ethyl acetate/formic acid [CEF] sprayed with Vanillin (B) Thin-layer Chromatography plates sprayed with DPPH.

TCm: *Tarchonanthus camphoratus* male; TCf: *Tarchonanthus camphoratus* female; TPm: *Tarchonanthus parvicapitulatus* male; TPf: *Tarchonanthus parvicapitulatus* female

Blue (top) arrow and Black solid circle indicates presence of common and antioxidant compounds of the same Rf value

Orange (bottom) arrow indicates compound present in *T. camphoratus* species but absent in *T. parvicapitulatus*

Black dotted circle indicates that TPf has more antioxidant compounds than TPm, TCf and TCm

Guinea pig tracheal smooth muscle relaxation

The use of the guinea pig model for preclinical studies of asthma and chronic obstructive pulmonary disease (COPD) is increasingly popular due to the fact that the animal seldom bites and its allergen-induced bronchoconstriction and that of human bronchial asthma are similar (Canning and Chou, 2008; Hong and Chang, 2008). It is a widely accepted method to use the guinea pig tracheal chain preparation as an *in vitro* model to evaluate the potency of plant extracts or compounds having bronchodilatory effect (Ahuja et al, 2011). Acetylcholine can activate efferent cholinergic fibres by inflammatory mediators such as histamine which in turn causes bronchoconstriction. However, the action of methacholine is significantly more long-lasting than that of acetylcholine (Flenley, 1990).

In this study, the bronchodilatory effects of aqueous extracts of male and female plants of *T. camphoratus* and *T. parvicapitulatus* were evaluated on methacholine-induced contraction of the tracheal tissue of guinea pigs. The tracheal smooth muscle was primed with methacholine to induce maximal contraction. The results of the effect of the extracts on the methacholine pre-contracted trachea are presented in Figure 2. The extract of *T. camphoratus* showed a tendency to relax methacholine pre-contracted guinea pig trachea, however the relaxation was not significant ($p > 0.05$). The EC_{50} values obtained were TCM = 19.90 µg/mL, TCF = 10.27 µg/mL, TPM =3.15 µg/mL and TPF= 5.10 µg/mL. None of the plant extracts of *T.camphoratus* and *T. parvicapitulatus* significantly inhibited methacholine contraction of guinea pig tracheal smooth muscle (Figure 3). Upon pre-contraction of the tracheal smooth muscles, both the female and male specimen of *T. parvicapitulatus* significantly ($P < 0.05$) caused relaxation at the tested concentrations (40, 20, 2 mg/mL). This study suggests that extracts from these plants do not have prophylactic protection for smooth muscle contraction at the tested concentration *in vitro*. As observed with the DPPH antioxidant assay, the presence of more antioxidant compounds in *T. parvicapitulatus* could be aiding the potent relaxant effect recorded. Therefore, the plant extract of *T. parvicapitulatus* has the potential to relieve pre-existing smooth muscle contraction. However, there was no significant difference in the relaxant effects of both the male and female species of *T. camphoratus* on the pre-contracted trachea. The results obtained from this study are comparable to the potent relaxant effect of the water extract of *Bunium persicuam* Boiss. and *Adhatoda vasica* Nees on pre-contracted tracheal smooth muscles of guinea pigs (Boskabady and Talebi, 1999; Mahapatra and Pradhan, 2012b). The observed bronchodilatory effect of the aqueous extracts of *T. parvicapitulatus* might be associated with the good free radical scavenging ability of the extracts as shown in the DPPH assay. This can be compared to the study conducted by Oghale and Idu (2016) with the water extracts of *Anchomanes difformis* (Blume) Engl. possessing good smooth muscle relaxant effect as well as antioxidant activity (Oghale and Idu, 2016). Natural antioxidants in the form of flavonoids can impair Ca^{2+} release and the utilization mechanisms in smooth muscle, and can be used for the treatment of degenerative diseases (Ali et al., 2008; Ghayur and Gilani, 2006). Other possible mechanisms could be due to inhibitory effects on muscarinic receptors or potassium channel opening effect and calcium channels (Boskabady et al., 2004a; Miyahara et al., 1993).

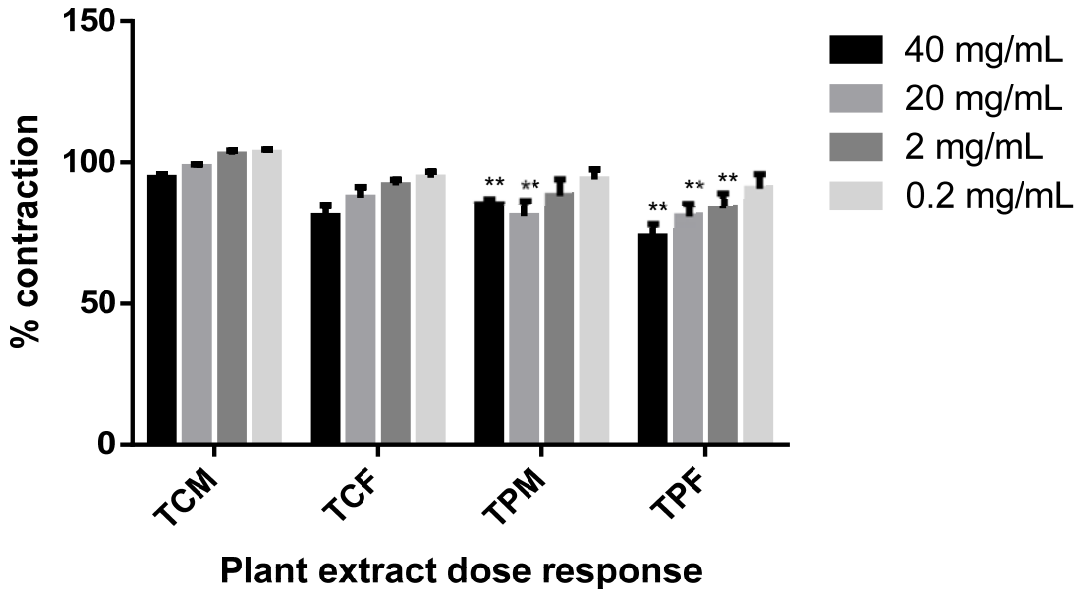

Figure 2: The effect of the aqueous extracts of *T. camphoratus* and *T. parvicapitulatus* on the methacholine pre-contracted guinea-pig trachea.

Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils.

TCM: *T. camphoratus* male; TCF: *T. camphoratus* female; TPM: *T. parvicapitulatus* male; TPF: *T. parvicapitulatus* female

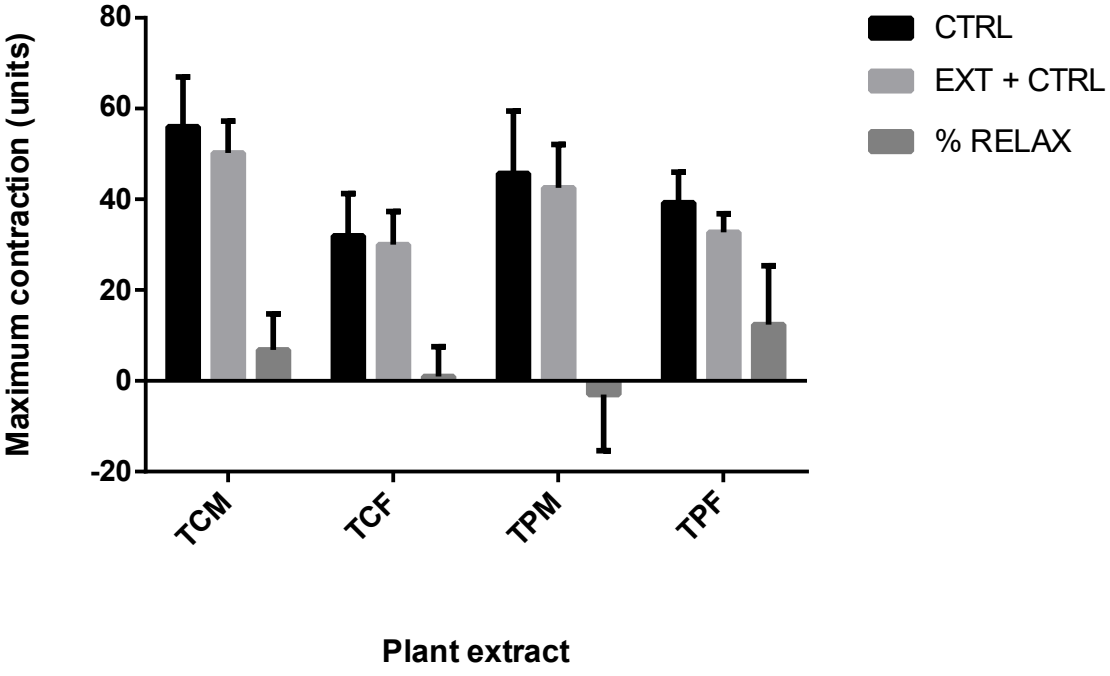

Figure 3: The effect of *T. camphoratus* and *T. parvicapitulatus* extracts on methacholine contraction of guinea pig tracheal smooth muscle.

Results were expressed as mean ± S.D., n=6, for 10^6 neutrophils.

TCM: *T. camphoratus* male; TCF: *T. camphoratus* female; TPM: *T. parvicapitulatus* male; TPF: *T. parvicapitulatus* female

The effect of superoxide and ATP production from neutrophils

The generation of superoxide anions can be initiated by activation of phagocytic cells such as macrophages, neutrophils, and eosinophils (Rahman, 1996). All the tested concentrations showed a dose dependent inhibitory effect on superoxide production. At the two highest concentrations (10 mg/ml and 1 mg/ml), superoxide production was significantly ($p < 0.001$) inhibited by the aqueous extracts of both *T. camphoratus* and *T. parvicapitulatus* (Fig. 4). The result showed that the control emitted the greatest percentage of light (mV) due to superoxide production followed by the lowest concentration of the plant extract (0.1 mg/ml). ATP bioluminescence has been used for determining levels of intracellular ATP production in different number of cell types including neutrophils (Wulf et al., 1984; Girotti et al., 1989, Crouch et al., 1993; Kahler, 1994). The effect of *T. camphoratus* and *T. parvicapitulatus* aqueous plant extracts on ATP accumulation on isolated human neutrophils was assessed. The data obtained revealed that the crude extract of *T. parvicapitulatus* (both male and female) at the highest concentration (10 mg/ml) significantly ($p < 0.001$) decreased ATP production when compared with the control (Fig. 5). A significant ($p < 0.05$) reduction in ATP production at 1 mg/ml and 0.1 mg/ml was observed for the crude extract of both male and female species of *T. parvicapitulatus*. The aqueous crude extract of *T. camphoratus* (female specimen) also decreased ATP production significantly at 10 mg/ml ($p < 0.001$) and 1 mg/ml ($p < 0.05$). However, the increased ATP production observed for the male species of *T. camphoratus* at the highest concentration (10 mg/ml) was not significant and was not dose-dependent.

Superoxide is rapidly reduced by the superoxide dismutase enzyme to generate membrane permeable hydrogen peroxide. Oxidative stress parameters such as the hydroxyl radical, superoxide anion or alkoxy radical can have deleterious effects on progression of asthma and has been reported by some researchers (Argüelles et al., 2004; Nadeem et al., 2014; Emin et al., 2015). Therefore, therapeutic strategies that can inhibit the production of ROS including superoxide anions can produce pharmacological effects useful as a therapy in the treatment of smooth muscle contraction or airway hyper-responsiveness. Interestingly, the aqueous extracts of both male and female species of *T. camphoratus* and *T. parvicapitulatus* inhibited the production of superoxide in a concentration-dependent manner. Significant inhibition was observed at the concentrations 1 mg/ml and 10 mg/ml. The result obtained from this study is close to the reported superoxide inhibitory effects of *Boophone disticha* on isolated human neutrophils (Botha et al., 2005). Inhibition of superoxide production in PMA stimulated neutrophils could be one of the possible mechanisms of action of both *T. camphoratus* and *T. parvicapitulatus*. The sexes of the species did not produce different biological effects. The results obtained from this study showed that the studied plant extracts could inhibit the production of free radicals such as superoxide anions.

In view of the above discussion, the aqueous plant extracts of *T. camphoratus* and *T. parvicapitulatus* can be said to inhibit the effect of oxygen free radicals via the receptor/G protein. This in turn leads to inactivation of the pathway that leads to superoxide production and therefore prevents 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). Therefore, the inhibitory effects of the aqueous extracts on the isolated human neutrophils could be due to the activity of the intermediate polar compounds which include flavonoids. This helps to explain why the extracts of these plants are used by traditional healers for the relief of stomach trouble, abdominal pain, headache, toothache, asthma, bronchitis, and inflammation.

Reduction of ATP production intracellularly could be due to toxicity to neutrophils as well as depletion of the oxygen substrate, thereby resulting in cellular injury (Crouch et al., 1993). Hence, measurement of ATP is fundamental to the study of living processes as well as being useful to evaluate possible cytotoxic effects on cells. The cytotoxic profile of the selected plant extracts was carried out by measuring ATP production on human neutrophils using a cytotoxicity and cell proliferation kit. A significant decrease in ATP production in human neutrophils was observed for the water extracts of both species of *T. parvicapitulatus* at all the tested concentrations when compared with the control. However, the male species of *T. camphoratus* did not significantly inhibit the production of ATP but rather enhanced its production at the tested concentrations, except for the lowest concentration (0.1 mg/mL). The ATP production results obtained from this study are comparable with the cytotoxic effect of the water extracts of *Boophone disticha* (Botha et al., 2005). Therefore, the observed therapeutic effects of *T. parvicapitulatus* could be due to cytotoxicity while that of the male species of *T. camphoratus* was not due to cytotoxicity. However, *in vivo* efficacy and toxicity of extracts or active compounds upon administration to animals or humans may differ substantially from their *in vitro* properties owing to pharmacokinetic and pharmacodynamic considerations (Aro et al., 2015).

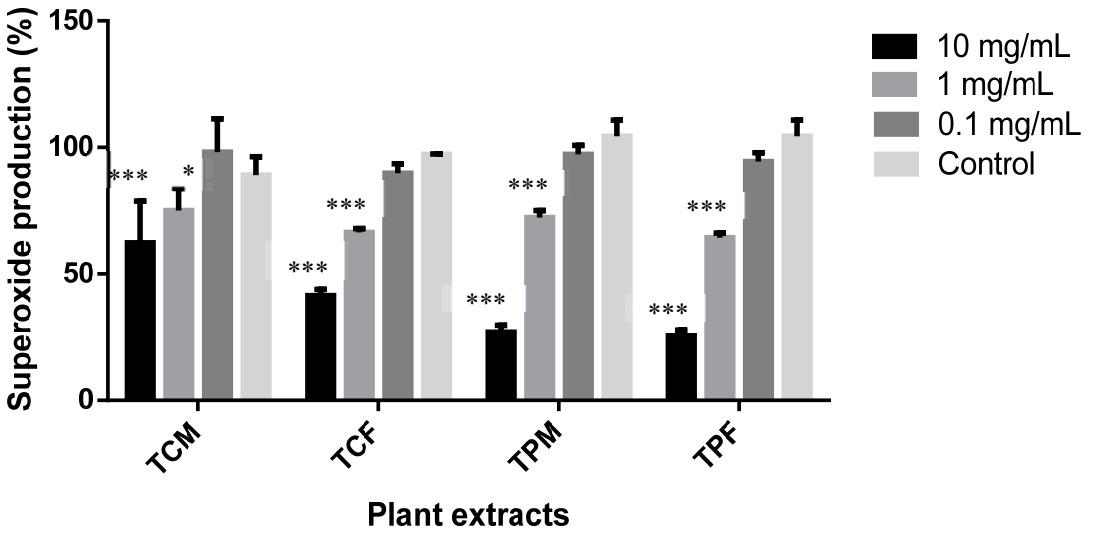

Figure 4: The effect of aqueous extract of *T. camphoratus* and *T. parvicapitulatus* plant extract on isolated human neutrophils superoxide production after PMA stimulation. Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils.

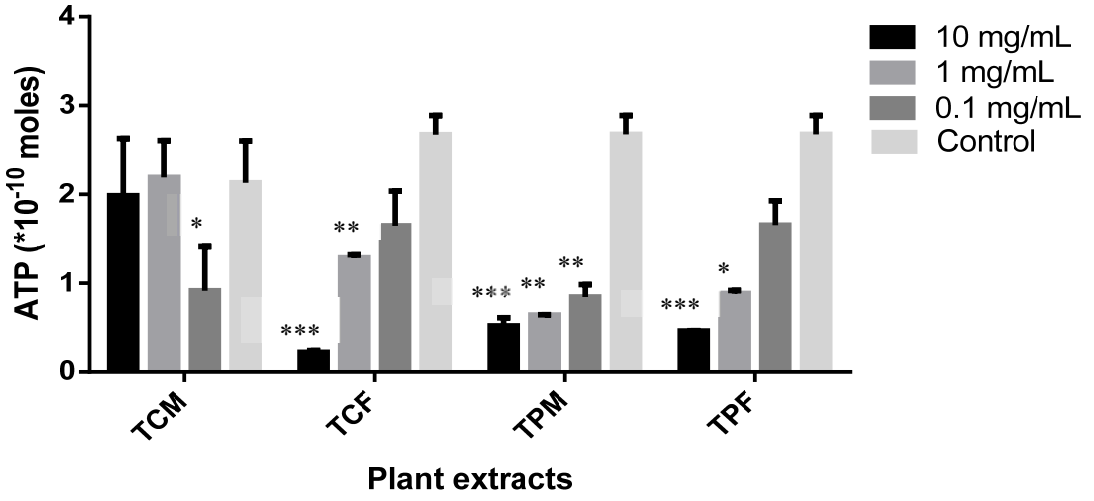

Figure 5: The effect of aqueous extract of *T. camphoratus* and *T. parvicapitulatus* on ATP production in human neutrophils. Result was expressed as mean ± S.D., n=6, for 10^6 neutrophils.

TCM: *Tarchonanthus camphoratus* male; TCF: *Tarchonanthus camphoratus* female;TPM: *Tarchonanthus parvicapitulatus* male; TPF: *Tarchonanthus parvicapitulatus* female

Morphology study

The micromorphological study using SEM revealed that both *T. camphoratus* and *T. parvicapitulatus* have two types of trichomes: glandular and non-glandular. On the upper leaf surface, glands were mostly distributed along the ridges for both species (Fig. 6). The upper leaf surface of *T. camphoratus* was sparsely hairy with prominent glands and grooves (Fig. 6a) while the the upper leaf surface of *T. parvicapitulatus* specimens was subglabrous to glabrous (Fig.6b). The glands on the lower leaf surface of *T. camphoratus* were not prominent, and absence of hair was observed (Fig 7a) while the glands on *T. parvicapitulatus* were prominent with presence of hair (Fig. 7b). Two new species (*T. parvicapitulatus* and *T. littoralis*) formerly grouped under the *T. camphoratus* L. complex were described using morphological characters such as flowering times, leaf shape, leaf margin and habitat (Herman, 2002). Differentiating between *T. camphoratus* and *T. parvicapitulatus* species proved difficult since the macromorphological characters tend to overlap between the species. For example, the main character used to differentiate *T. camphoratus* from *T. parvicapitulatus* is denticulate leaf margins. However, during the sample collection, denticulate leaf margins were found in both *T. camphoratus* and *T. parvicapitulatus* (Aro, 2010). The micromorphology study using SEM revealed that *T. camphoratus* and *T. parvicapitulatus* are separate species (Fig 6). The two species both have glandular trichomes around the ridges but *T. camphoratus* has grooves while these are absent in *T. parvicapitulatus*. The micromorphology study of *Tarchonanthus minor* revealed the presence of grooves around the ridges and absence of hair on the upper leaf surface (Herman, 2002). This observed feature might be peculiar to some taxa in the *T. camphoratus* L. complex. The lower leaf surface was prominently glandular in *T. parvicapitulatus* but this was not detected in *T. camphoratus*. Studies conducted on the pollen morphology of the *T. camphoratus* complex using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) also confirmed that there are different taxa grouped together (Zavada and Lowrey, 2010). The use of Scanning Electron Microscopy to study the micromorphology of eukaryotes including plants has proven useful as a tool to resolve many taxonomic uncertainties (Huri et al., 2016). Therefore, micromorphological studies were more useful in distinguishing between the two species than the macromorphological study.

Various research studies have established that glandular trichomes found in plants are responsible for the biosynthesis of essential oils and this has proven equally useful for taxonomic clarification of plant species (Venkatachalam et al., 1984; Liu et al. 2018; Yu et al., 2018). Compounds isolated from leaves of *T. camphoratus* L. complex in East Africa and Southern Africa include various components of essential oils (Van Wyk et al., 1997; Van Vuuren and Viljoen, 2009; Nanyonga et al., 2013. These authors attributed the various biological activities to the presence of essential oils such as 1,8-cineole (5.4 %), linalool (4.5 %) α-copaene (3.8 %), hexadecanoic acid (3.5 %), neoalloocimene (3.5 %), (-)-globulol (3.3 %) and δ-cadinene (3.1 %). Hence, more studies are needed to confirm the essential oil profile of the different taxa belonging to the *Tarchonanthus* genus.

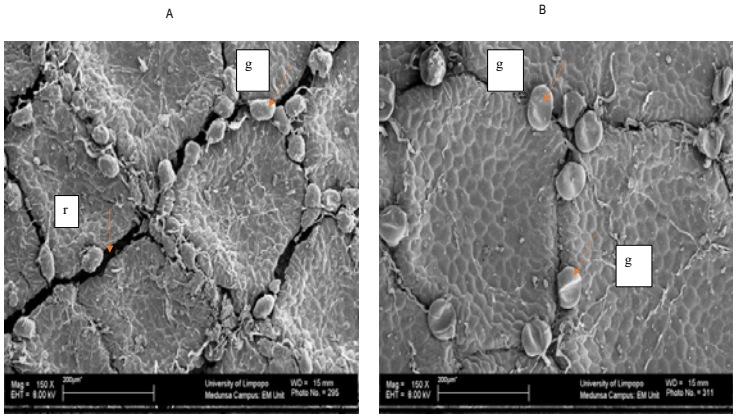

Figure 6: SEM micrographs of the upper leaf surface of (A) *T. camphoratus* and (B) *T. parvicapitulatus*. (r: ridges; g: glandular trichome)

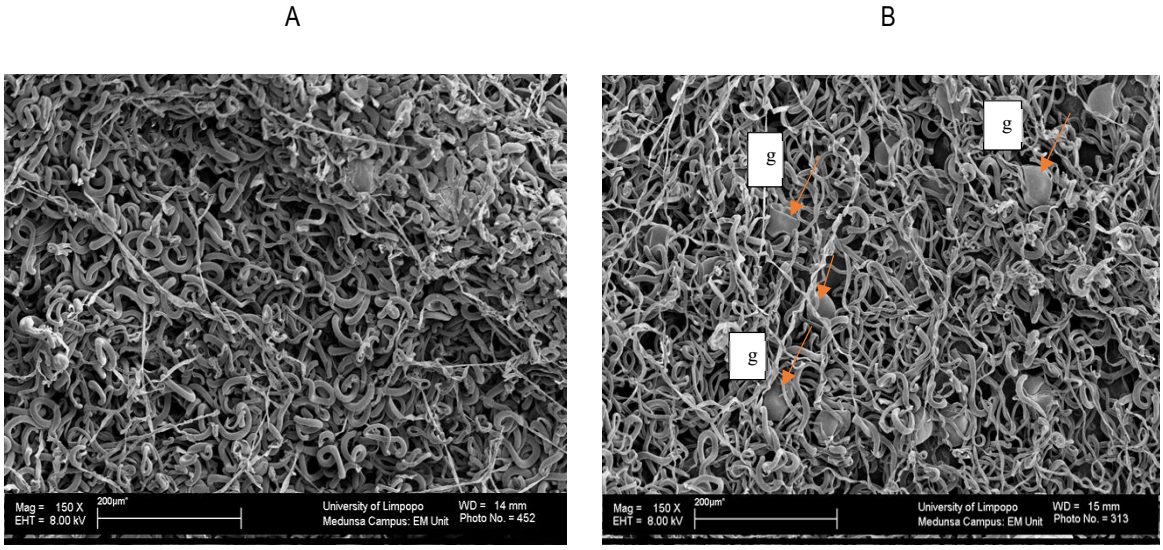

Figure 7: SEM micrographs of the lower leaf surface of (A) *T. camphoratus*; (B) *T. parvicapitulatus*. (g: glandular trichome)

Conclusion

This study provides evidence to support the traditional use of *T. camphoratus* and *T. parvicapitulatus* in managing respiratory disorders associated with bronchospasm such as asthma and bronchitis. Also, the micromorphological and phytochemical data proved useful in distinguishing between *T. camphoratus* and *T. parvicapitulatus*. The most

potent relaxant effect was seen for the aqueous extract of *T. parvicapitulatus*. Both the superoxide inhibitory, antioxidant, as well as smooth muscle relaxant effects of the extracts of *T. camphoratus* and *T. parvicapitulatus* potentially contribute to a synergistic effect to combat asthma and bronchitis. This study provides motivation for further studies on *T. camphoratus* and *T. parvicapitulatus* as therapy for the treatment of respiratory ailments associated with bronchospasm. To the best of our knowledge, this is the first report on the bronchodilatory activity of *T. camphoratus* and *T. parvicapitulatus*. More studies are needed to determine the exact mechanism(s) of action in an *in vivo* model. The bioactive compounds responsible for the observed pharmacological effects of the leaf extract should be studied and corroborated with the therapeutic effects. This study has helped to improve the overall knowledge available on *Tarchonanthus camphoratus* L. complex by using different pharmacognostic tools to determine the potential therapeutic effects of these plants.

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Author contributions

AOA was involved in plant collection. AOA and ILE carried out the experiments and drafted the first manuscript. IMF analysed the data. CPK, PNK and LJM designed and supervised the experiments. All authors read and approved the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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