In vitro antimycobacterial and adjuvant properties of two traditional South African teas, *Aspalathus linearis* (Burm.f.) R. Dahlgren and *Lippia scaberrima* Sond

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Highlights

- *In vitro* studies conducted on two South African medicinal teas indicated potential candidates for further investigation as adjuvant for TB patients.
- The *in vitro* studies done include antimycobacterial, antioxidant, hepatoprotective and anti-inflammatory activities.
- Hepatoprotection was observed for both *A. linearis* and *L. scaberrima* with a percentage protection above 30% at 100 µg/mL.

ABSTRACT

Many plant extracts have been studied for their ability to treat tuberculosis and its associated symptoms. Both *Lippia scaberrima* Sond. and *Aspalathus linearis* (Burm.f.) R. Dahlgren, are popular forms of health teas in South Africa. This study focused on the ability of the ethanolic plant extracts of *L. scaberrima* and *A. linearis*, as well as the essential oil of *L. scaberrima* to act as adjuvants in host-directed therapy against tuberculosis. The ethanolic extract of *L. scaberrima* was found to have a minimum inhibitory concentration (MIC) of 125 µg/mL against *Mycobacterium tuberculosis* H37Rv, whereas the antiproliferative activity on HepG2 hepatocyte cells ranged between 109.20 ± 8.05 µg/mL and >400 µg/mL for all the samples tested. Due to the high antimycobacterial properties of the ethanolic extract from *L. scaberrima*, the sample was further tested for its cyclooxygenase (COX) II inhibitory potential and found to have an IC_{50}

value of 36.39±1.62 µg/mL. The essential oil of *L. scaberrima* and the ethanolic extract of green *A. linearis* exhibited good hepatoprotective activity, with up to 34% and 40% protection against acetaminophen-induced toxicity, respectively. Additionally, the study investigated the composition of the essential oil of *L. scaberrima* through gas-chromatography mass spectrometry. Limonene was found to be the main component for the essential oil. The essential oil from *L. scaberrima* showed promising results with noteworthy hepatoprotective activity as well as moderate antimycobacterial activity. These herbal teas showed potential as an adjunct host-directed therapy in tuberculosis patients through the demonstration of its biological activities and should be considered for further investigation.

Keywords: adjuvant, AHDT, antimycobacterial, inflammation, tuberculosis

1. Introduction

Adjunct host-directed therapy (AHDT) has been proposed many times for the treatment of tuberculosis, either through accessing the host immune system to eliminate the pathogenic bacteria efficiently or through limiting the subsequent damages as a result of infection (Tobin, 2015). Adjunct host-directed therapies have the potential of enhancing an existing immune response against tuberculosis or have the ability to create new ones (Tobin, 2015). Host-directed therapy has only been laid out theoretically and only in the instance of tuberculosis has it been utilized. This therapeutic strategy has been proposed due to the high incidence of treatment failure. This failure is due to the side-effects experienced during treatment for tuberculosis infection. By incorporating host-directed therapy the possibility exists in enhancing treatment outcome through assisting with limitation of side-effects experienced during treatment.

Inflammation has been one of the major causes of morbidity in severe cases of tuberculosis infection. Glucocorticoids are now included as part of the standard of care regimen to aid in these severe cases (Tobin, 2015). Nonsteroidal anti-inflammatory drugs such as aspirin are one of the host-directed therapies in pre-clinical trials for the limitation of prostaglandin synthesis through inhibition of cyclooxygenase (COX) I and COX-II. Through the inhibition of these pathways, the inflammatory response can be limited during tuberculosis infection and as such has been tested in a zebrafish model for hyperinflammatory conditions during infection with mycobacteria (Tobin et al., 2012). One clinical trial involving aspirin indicated a beneficial effect

as adjunct therapy on the survival rate (Misra et al., 2010; Schoeman et al., 2011). Another study conducted in a murine model of active TB by Vilaplana et al., (2013) showed the anti-inflammatory properties of Ibuprofen by decreasing the amount and size of lung lesions found within *M. tuberculosis* infected C3HeB/FeJ mice as well as lowering the bacillary loads found in the mice lungs. Other host-directed therapies for tuberculosis currently in phase 1, 2 and 3, involve the enhancement of host immunity and proper elimination and containment of the bacteria itself (Tiberi et al., 2018). Various other pathways of small molecules that act as host-directed therapeutics have been proposed. These include therapeutics that have the ability to target processes including; cell-mediated immunity, autophagy, granuloma formation and the vitamin D pathway (Kolloli and Subbian, 2017).

Aspalathus linearis, commonly known as Rooibos, can be found growing in the Western Cape region of the Cedarberg Mountains in South Africa. Currently, this endemic herbal tea is in high demand across the world. Many studies have included the health benefits provided by the intake of Rooibos tea, many of which involve its antispasmodic potential (Van Wyk et al., 1997). *Aspalathus linearis* is found commercially available in two forms namely fermented (Rooibos) or unfermented (green Rooibos). It is during a fermentation process that the polyphenols within the rooibos plant are oxidized and it then turns into its characteristic red colour.
Lippia scaberrima is an aromatic shrub that is also enjoyed as a herbal tea and goes by the name of 'Musukudu' in southern Africa (Shikanga et al., 2010). Other *Lippia* spp. has been investigated for its biological activities to a greater extent when compared to *L. scaberrima* . In addition, most studies on *L. scaberrima* have been done on the essential oil and its ability to act as an antibacterial and protect against fungal pathogens (Regnier et al., 2008; Shikanga et al., 2010). Extracts and the essential oil of *L. scaberrima* have not yet been investigated for their antimycobacterial, anti-inflammatory and hepatoprotective activities. Due to both of these species of indigenous plants that are already known as health teas, the possibility exists for an easier method of incorporation of an AHDT to assist in the treatment of tuberculosis.

A more detailed look into the potential biological properties from the extracts of *L. scaberrima* and *A. linearis* is needed to understand their potential as adjunct host-directed therapy for tuberculosis patients. The current study investigated the essential oil and of *L. scaberrima* and the ethanolic extract of *A. linearis* for their antimycobacterial, hepatoprotective and anti-

inflammatory properties. Furthermore, the antiproliferative activity was evaluated to facilitate in the selectivity.

2. Materials and methods

2.1. Bacterial strains, cell lines, chemicals and reagents

Mycobacterium tuberculosis H37Rv (ATCC 27294), in MGIT media, and *M. smegmatis* (MC^2155) were kindly donated by the South African Medical Research Council, Pretoria, and Department of Medical Microbiology, University of Pretoria, respectively. The HepG2 cell line (HB-8065) was obtained from the American Type Tissue Culture Collection (ATCC). Dimethyl-sulfoxide (DMSO), Middlebrook 7H9 broth, Middlebrook 7H11 agar base and Middlebrook OADC (Oleic Albumin Dextrose Catalase) growth supplement were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Cell culture materials and reagents such as Fetal Bovine Serum (FBS), DMEM media and antibiotics were supplied by Highveld Biological. The PANTA plus antibiotic mixture was purchased from BD Biosciences (San Jose, CA, USA). PrestoBlue was purchased from Thermo Fisher (Carlsbad, CA, USA). All reagents obtained were of analytical grade.

2.2. Plant material, collection and extraction

Lippia scaberrima was collected in autumn from the Kopela community situated nearby Delareyville, North West Province, South Africa. The plant was identified, and a voucher specimen deposited at the H. G. W. J Schweickerdt herbarium, University of Pretoria (PRU) (Table 1). Powdered green and fermented *A. linearis* leaf material were kindly donated by Rooibos Limited, Clanwilliam, Western Cape, South Africa. The collected aerial parts of *L. scaberrima* (stem, flowers, and leaves) were mechanically ground to a uniform size of 0.2 mm. The powdered plant material from both plants was extracted with absolute ethanol at a ratio of 1:10 (weight: volume) and macerated for 72 hours. The extracts were filtered, and the plant material was subsequently extracted with fresh ethanol at a ratio of 1:5 for 48 hours, followed by another filtration. The filtrates were combined and dried under reduced pressure using a rotary evaporator which resulted in a dark green extract for *L. scaberrima* and reddish extracts for the green and fermented *A. linearis*. The three extracts were stored in airtight containers at 4°C for the duration of the study.

Table 1
Identified compounds with the greatest contribution within the essential oil of *L. scaberrima*, determined by GC-MS analysis (>1%)

RT[a]	Name of the compounds	Synonym of compounds	Molecular Formula	Peak area (%)[b]	Nature of the compounds	Match Score (%)
11.93	(1R)-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene	Pinene	$C_{10}H_{16}$	1.74	Terpene	97.6
12.71	Camphene	Camphene	$C_{10}H_{16}$	6.53	Bicyclic monoterpenoids	97.6
14.25	Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-	Sabinene	$C_{10}H_{16}$	2.16	Terpene	98.4
17.31	p-Cymene	Cymene	$C_{10}H_{14}$	5.12	Hydrocarbon-related to monoterpene	97.2
17.64	D-Limonene	Limonene	$C_{10}H_{16}$	35.84	Terpene	92.7
24.22	(+)-2-Bornanone	Camphor	$C_{10}H_{16}O$	12.72	Terpene	95.0
25.50	endo-Borneol	Borneol	$C_{10}H_{18}O$	3.57	Terpene	98.2
27.01	L-. alpha -Terpineol	Terpineol	$C_{10}H_{18}O$	2.60	Monoterpene alcohol	97.6
28.67	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	Carveol	$C_{10}H_{16}O$	1.28	Monoterpene alcohol	97.1
30.06	(-)-Carvone	Carvone	$C_{10}H_{14}O$	14.30	Terpene	96.8
31.51	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethenyl)-, (S)-	Isopiperitenon	$C_{10}H_{14}O$	3.77	Menthane monoterpenoids	91.7

[a]RT: retention time, [b]Constituents contributing >1% adds up to a total of 90%.

2.3. Essential oil isolation by hydro-steam distillation and yield calculation

Lippia spp. are known for their essential oil content. The aerial parts of *L. scaberrima* were subjected to essential oil isolation using hydro-distillation. Air-dried plant material (stem, flowers, and leaves) (0.415 kg) were subjected to a Clevenger apparatus for hydro-steam distillation for one and a half hour. The isolated essential oil was collected and stored at –20 ⁰ C until further use. The yield was determined by the amount of essential oil obtained from 0.415 kg of plant material and was calculated by the following equation:

$$Yield = \frac{VEO \times 100}{M}$$

Where VEO is the volume of the extracted oil (in mL) and M, the initial biomass of the plant material (in g)

2.4. Gas chromatography-mass spectrometry (GC-MS) of the essential oil of Lippia scaberrima

For the probable identification of compounds within the essential oil, gas chromatography-mass spectroscopy was carried out using an Agilent 7000 C Triple/ Quadrupole GC-MS (Agilent, Santa Clara, United States) with the analysis carried out in scan mode. Mass spectroscopy scan conditions were as follows; an ion source temperature of 200° C, interface temperature of 280° C, and ionization energy of 70 eV. The separation of the compounds was achieved using an Agilent J&W DB-5ms Ultra Inert fused silica column 5% phenyl-poly-dimethyl-siloxane (DB-5ms) 30m x 0.25 mm i.d. with 0.25 µm film thickness. The GC oven temperature and programme were as follows; from 40º C (5 min hold), raised at 2.5ºC/ min to a final temperature of 300º C with a 20º C/ min hold. The injection mode was a split-splitless injection at a split ratio of 1:20 and an injector temperature of 250º C. The essential oil was diluted with GC-MS grade hexane in a 1: 200 µL ratio and 1 µL injected. Data acquisition was performed with MassHunter software with mass scan ranges of 30 – 600 u and a scan speed of 380 ms. The compounds were identified with an Agilent MassHunter analysis of the unknowns by use of the mass spectra with data collected from a NIST 14 Mass spectral library. The relative percentage of the compounds present in the essential oil was computed from a peak area of the GC-MS analysis.

2.5. Bacterial strain and growth conditions

Both *Mycobacterium tuberculosis* and *M. smegmatis* were cultured in Middlebrook 7H9 media supplemented with glycerol, OADC and PANTA (1%) and left to incubate for three weeks and 48 hours, respectively, at 37° C. Both bacteria were adjusted to a 0.5 McFarland standard of (1.5 x 10^8 colony-forming units/mL) [CFUs/mL]). The bacteria were further diluted a 50-fold to obtain the final test inoculum (1.5 x 10^6).

2.6. Antimycobacterial activity

The minimum inhibitory concentration (MIC) values of all the samples were determined, including 1:1 combinations of *L. scaberrima* and *A. linearis*. according to the method of Lall et al., (2013). All samples were dissolved in 20% DMSO, in sterile Middlebrook 7H9 media. Sterile, distilled water (200 µL) was added to the outer wells of the plate to compensate for

evaporation during the incubation period for *M. tuberculosis*. Serial, two-fold dilutions of each sample were made in sterile Middlebrook 7H9 media to yield a final test concentration range of 31.25 to 1000 µg/mL. The diluted inoculum (100 µL) was added to each well to yield a final assay volume of 200 µL. Isoniazid (INH), at a final concentration range of 0.03 to 2.5 µg/mL served as the positive drug control for *M. tuberculosis,* whereas ciprofloxacin, at a concentration range of 0.078 to 10µg/mL served as the positive drug control for *M. smegmatis.* Untreated bacterial control, as well as a solvent control (DMSO 5%), were included in triplicate. The plates were sealed with parafilm and incubated at 37° C for seven days and 24 hours for *M. tuberculosis* and *M. smegmatis*, respectively. Prestoblue (20 µL) was added to each well, and the plates were incubated for a further 2 to 24 hours. The MIC value was defined as the lowest concentration where no colour change from blue to pink could be observed.

2.7. Antiproliferative activity

Cell viability was determined by the method of Berrington and Lall (2012) of all the samples including the 1:1 combinations of *L. scaberrima* and *A. linearis*. The liver hepatocellular carcinoma (HepG2) cells (100 µL) were counted and seeded in 96 well plates with a cell density of 10 000 cells/well and left to incubate overnight at 37° C and 5% CO_2 to allow for attachment.

The samples were prepared to a stock solution of 2000 µg/mL. Serial dilutions of the extracts were made with final test concentrations ranging from 1.53 to 400 µg/mL. Plates were incubated for 72 hours at 37° C and 5% CO_2. An untreated cell, solvent control (DMSO 2%) and actinomycin D (positive control) with a final test concentration range of 0.002 to 0.5 µg/mL were included. After incubation, PrestoBlue (20 µL) was added to each well and incubated for 4 hours. The absorbance was read at 490 nm with a reference wavelength of 690 nm using a BIO-TEK Power-Wave XS multi-well reader. The assay was performed in triplicate in three independent assays, and the mean 50% inhibitory concentration (IC_{50}) values calculated.

2.8. COX-II inhibitory activity

Due to the high antimycobacterial activity that was found for *Lippia scaberrima,* it was further investigated for its COX-II inhibitory activity according to the method by Reininger and Bauer (2006). Briefly, in a 96-well plate, TRIS buffer at pH 8.0, COX-II (0.5 units/reaction), porcine

hematin (5 µM), L-epinephrine (18 mM) and NA_2EDTA (50 µM) was added. Thereafter, the plant extract at a stock solution of 10 mg/mL was serially diluted and added, with final test concentrations ranging from 2.5 to 160 µg/mL. DMSO (1.6 %) as a solvent control was also added. Ibuprofen (10 µM) was used as the positive control. After 5 min of incubation at room temperature, the reaction was initiated by the addition of arachidonic acid (10 µM) to a final assay volume of 200 µL and incubated for 20 min. To stop the reaction, 10 µL of formic acid (10%) was added to all the wells. For the measurement of the amount of prostaglandin E2 (PGE_2) formed, a PGE_2 ELISA kit was used, following the manufactures instructions. The absorbency values were measured using a BIO-TEK Power-Wave XS multi-well reader at a wavelength of 405 nm. The results are presented as the percentage PGE_2 synthesis inhibition when compared with the blank. The IC_{50} values were calculated by the use of Microsoft Excel 2010.

2.9. Hepatoprotective activity

The hepatoprotective ability of the samples including 1:1 combinations of *L. scaberrima* and *A. linearis* were evaluated against an acetaminophen-induced toxicity assay according to Lall et al., (2016) with modifications. Briefly, HepG2 cells were seeded at a density of 10 000 cells/well within a 96-well plate and incubated overnight at 37ºC in a humidified atmosphere of 5% CO_2 to allow for the attachment of the cells. All samples were prepared to a stock solution of 2000 µg/mL. The final test concentrations were 25, 100 and 400 µg/mL for all samples. Silymarin at 50 mM was included in the assay as the positive control. The samples and controls were added to the cells in triplicate. Cellular toxicity was induced by the addition of acetaminophen at 25 mM, to a final test volume of 200 µL and incubated for 3 hours at 37ºC in a humidified 5% CO_2 incubator. After incubation, PrestoBlue (20 µL) was added to each well and incubated for 4 hours. The absorbance was read at 490 nm with a reference wavelength set to 690 nm using a Bio-Tek Power-Wave XS multi-well reader. The percentage protection was calculated by using the acetaminophen-treated cells as the baseline.

2.10. Statistical analysis

Statistical analysis was performed on the mean IC_{50}'s and percentages with GraphPad Prism (Version 7) using one-way analysis of variance (ANOVA). A Dunnett's multiple comparison test

was performed to identify significance compared to a control value. The data is expressed as the mean ± standard deviation, n= 3 or more.

3. Results and discussion

3.1. Essential oil isolation by hydro-steam distillation and yield calculation

The hydro-steam distillation of 415 g of dried plant material produced 2.5 mL of essential oil, which translates to a yield of 0.60% (v/w). The amount of essential oil isolated can be influenced by many factors such as humidity, temperature, the age of the plant parts collected and season (Bueno Da Costa et al., 2014; Sebei et al., 2015). Oil yields may also be lowered upon mechanical damage of the leaves after drying (Combrinck et al., 2006).

3.2. Gas chromatography-mass spectrometry of the essential oil of Lippia scaberrima

The identified compounds that had a contribution of more than 1% are represented in Table 1 for the essential oil. Limonene was found to be the major component within the essential oil (35.84%) which agrees with Regnier et al., (2008) and Combrinck et al., (2006). Compounds such as phenylethanoid glycosides which are known to be present within *Lippia* spp. were not identified as compounds after GC-MS analysis of the plant extract and the essential oil. This may be due to the fact that GC-MS analysis is mostly used for non-polar, volatile components whereas HPLC-MS analysis is used for more polar, non-volatile compounds such as the phenylethanoid glycosides such as verbascoside and isoverbascoside. The treatment of various ailments with *L. scaberrima* was shown to be due to the presence of verbascoside and isoverbascoside, that are present within the leaves, flowers and twigs (albeit in smaller quantities in the latter) (Olivier et al., 2010). It has been suggested that several chemotypes of *L. scaberrima* may exist due to inter- and intraspecies variation. These distinct chemotypes could present different compositions of compounds due to variations in their metabolic pathways (Viljoen et al., 2005; Maroyi, 2017).The chromatogram for the essential oil is found in the supplementary material together with the complete list of identified compounds (Figure 1A; Table 1A).

3.3. Antimycobacterial activity

All samples were tested against both *M. tuberculosis* and *M. smegmatis* including 1:1 combinations of *L. scaberrima* and *A. linearis*. The lowest MIC value was observed by the ethanolic extract of *L. scaberrima* which showed a MIC value of 125 µg/mL against *M. tuberculosis* (isoniazid (INH), MIC of 0.32 µg/mL (Table 2)). Due to the known antibacterial properties of *A. linearis*, 1: 1combinations of the ethanolic extracts of *L. scaberrima* and green and fermented *A. linearis* extracts were also tested. Both combinations exhibited a MIC value of 500 µg/mL against *M. tuberculosis*. The essential oil of *L. scaberrima* showed no activity at the highest tested concentration against *M. tuberculosis.* However, the essential oil showed the highest activity against *M. smegmatis* with a MIC value of 1000 µg/mL (ciprofloxacin MIC of 0.63 µg/mL). All the other samples showed no activity at the highest tested concentration against *M. smegmatis*.

Table 2
The antimycobacterial, antiproliferative and anti-inflammatory properties for *L. scaberrima*, *A. linearis* and combinations of *L. scaberrima* with both fermented and green *A. linearis.*

Sample	PRU number[a]	*M. smegmatis* MIC[b] (µg/mL)	*M. tuberculosis* MIC[b] (µg/mL)	Antiproliferative activity IC_{50}[c] (µg/mL)± SD	COX-II inhibition IC_{50}[d] (µg/mL)± SD
***L. scaberrima* (EtOH extract)**	119010	>1000	125	109.20±8.05	36.39±1.62
***L. scaberrima* (Essential oil)**	-[e]	1000	>1000	244.90±4.97	-
***A. linearis* (fermented)**	122176	>1000	1000	230.6±3.31	-
***A. linearis* (green)**	122176	1000	1000	>400	-
***L. scaberrima* and fermented *A. linearis* (1:1)**	-	>1000	500	163.30±2.53	-
***L. scaberrima* and green *A. linearis* (1:1)**	-	>1000	500	191.20±9.37	-
Ciprofloxacin[f]	-	0.63	-	-	-
Isoniazid[g]	-	-	0.31	-	-
Actinomycin-D[h]	-	-	-	8.56±8.24	-
Ibuprofen[i]	-	-	-	-	0.61±0.10

[a]Voucher specimen code for the H. G. W. J. Schweickerdt Herbarium, [b]Minimum inhibitory concentration, [c]Fifty percent inhibitory concentration of HepG2 cell viability, [d]Fifty percent inhibitory concentration of cyclooxyegnase II, [e]Not applicable or not available, [f]Positive control for *M. smegmatis* inhibitory assay, [g]Positive control for *M. tuberculosis* inhibitory assay, [h]Positive control for antiproliferative activity, [i]Positive control for COX-II inhibition assay.

Natural products that show MIC values of 1000 µg/mL and lower are considered as noteworthy activity according to (Gibbons, 2004). A study conducted by Shikanga et al., (2010), indicated the antibacterial potential of the methanolic extract of *L. scaberrima* against other gram-positive bacteria, showing MIC values of 1300 and 630 µg/mL against *S. aureus* and *E. faecalis,*

respectively. Many studies have indicated the antibacterial potential of both verbascoside and isoverbascoside. Although not shown in the GC-MS analysis, verbascoside is known to be a constituent found within the *Lippia* spp. indigenous to South Africa (Shikanga et al., 2010), which was present in the extract after co-TLC analysis with the pure compound of verbascoside as a standard (Fig 2A). *Lippia javanica* showed much higher activity against the same bacterial pathogens than *L. scaberrima*, but much of the activity is attributed to verbascoside and apigenin found within this species of *Lippia* (Shikanga et al., 2010, Sandoval-Montemayer et al., 2012). The activity found for the ethanolic extract of *L. scaberrima* seems to be due to the presence of the compounds, verbascoside and isoverbascoside being major constituents found within this plant and ethanolic extract of *L. scaberrima* (Olivier et al., 2010).

Andrade-Ochoa et al., (2015), hypothesized that the mechanism of action of the essential oils tested for their antimycobacterial activity may lie within the effects that they have on the structure and functioning of the mycobacterial cell wall and membrane, especially for the essential oils that are monoterpic in nature. The monoterpenoids have the ability to interact with the lipid membranes as an impurity within the ordered structure of the bilayer due to their lipophilic nature (Sikkema et al., 1995; Trombetta et al., 2005). Mycobacterial cell walls are known for their lipophilicity due to the presence of mycolic acids. Essential oils interacting with the cell walls of the bacteria have the ability to change the permeability factors and ultimately result in mycobacterial death (Andrade-Ochoa et al., 2015).

3.4. Antiproliferative assay

All samples were tested against liver hepatocellular carcinoma (HepG2) cells for their antiproliferative activity and found to have IC_{50} values ranging from between 109.20 ± 8.05 µg/mL and >400 µg/mL after 72 hours of incubation (Table 2). Plant extracts with an IC_{50} value of more than 100 µg/mL after 72 hours of incubation are considered as non-cytotoxic to the specific cell line tested (Geran et al., 1972). The green *A. linearis* extract, among the plant samples, showed the lowest toxicity against the HepG2 cells with an IC_{50} value greater than 400 µg/mL. *Lippia scaberrima* essential oil exhibited an IC_{50} value of 244. 90 ± 4.97 µg/mL. The combinations of the ethanolic extract of *L. scaberrima* with fermented and green *A. linearis* extracts showed higher IC_{50} values when compared to *L. scaberrima* on its own with IC_{50} values

of 163.30 ± 2.53 µg/mL, 191.20 ± 9.37 µg/mL and 109.20 ± 8.05 µg/mL, respectively. This may be due to the synergistic activity of the compounds found within *A. linearis* with those within the *L. scaberrima* ethanolic extract. No published data on the cytotoxic potential of *L. scaberrima* against any cell lines has been published with the exception of components isolated from the essential oil extract of *L. javanica*.

Lippia javanica is a well-known medicinal plant found within the same genus and is also indigenous to South Africa. Piperitenone, found to be a constituent within the extract and the essential oil has shown to be of low toxicity when tested against human adenocarcinoma (HCT-8) cells with an IC_{50} value of 265.6 ± 8.53 µg/mL. Similar results were found for limonene, the major constituent within the essential oil extract which had an IC_{50} value of 285 ± 49 µM (38.83 µg/mL) against human lung (CCD-19Lu) cells after 24 hours incubation (Rolseth et al., 2002). Individual compounds of limonene and carvone were tested against the HeLa and Vero cell line and found not be cytotoxic (Mesa-Arango et al., 2009). L-carvone showed considerable toxicity towards breast cancer cell lines and was explored for its apoptotic ability within these cell lines. The IC_{50} values against MCF-7, MDA MB 231 and MCF 10 A were 1.2 mM (180.26 µg/mL), 1.0 mM (150. 22 µg/mL) and 20 mM (3004, 4 µg/mL), respectively (Patel and and Thakkar, 2014). Cytotoxicity of fermented *A. linearis* and its major components were tested against immortalized keratinocytes (HaCat), fibroblast-like skin (CRL 7761) cells and basal carcinoma malignant (CRL 7762) cells. Both methanolic and aqueous extracts of fermented *A. linearis* were tested, and all showed IC_{50} values greater than 100 µg/mL on all cell lines (Macgwebeba et al., 2016).

3.5. COX-II inhibition

It is readily reported that compounds which are very good antioxidants exert effective anti-inflammatory properties, due to their phenolic content. An inflammatory response against invading bacteria (phagocytosis), such as *M. tuberculosis,* is mostly followed by an oxidative burst that results in the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Miguel, 2010; Naz et al., 2017). Most of the components found within the ethanolic and essential oil of *L. scaberrima* were found to be flavonoids, and they are known to have high antioxidative potential (Jung et al., 2003; Leyva-Lopez et al., 2016).

The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- Kβ) requires nuclear translocation which is ROS-dependent. After activation, NF- Kβ, initiates the activation of inducible nitric oxide synthase (iNOS) and cyclooxygenase II (COX-II). Arachidonic acid metabolism is one of the key processes during an inflammatory response (Miguel, 2010). During inflammation arachidonic acid is released from the cell membranes by phospholipase A_2 and is metabolized through COX or lipoxygenase (LOX) pathways within prostaglandins and leukotrienes. Due to the high antimycobacterial activity found for the ethanolic extract of *L. scaberrima* and the fact that natural products that show good antibacterial activities normally exhibited good anti-inflammatory activity due to the presence of phenolics (Naz et al., 2017), the COX-II inhibitory activity was studied. The inhibition was compared to the activity of ibuprofen (Table 2), a selective COX-II inhibitor. The IC_{50} value for the sample was found to be 36.39 ± 1.62 μg/mL, higher than the IC_{50} value found for ibuprofen at 0.61 ± 0.10 μg/mL. *Torreya nucifera* oils that mainly consist of limonene, δ-3-carene, and α-pinene, were found to be selective COX-II inhibitors with effective inhibition activity against PGE_2 production (Yoon et al., 2009). The compound; 1, 8-cineole which is a major component of the essential oil and the extract of *L. scaberrima* was reported as an inhibitor of leukotrienes and prostaglandin PGE_2 (Miguel, 2010). Further studies into the inhibitory potential of *L. scaberrima* against NF- Kβ are needed to determine if its high free radical scavenging potential has the ability to inhibit NF- Kβ and therefore, cause downstream inhibition of iNOS and as a result NO and COX-II.

3.6. Hepatoprotective activity

The hepatoprotective activity of the samples was calculated after acetaminophen was introduced to HepG2 liver cells to induce cellular toxicity. To assess the hepatoprotective ability of the samples, the viability of the toxic induced cells (acetaminophen, 0% protection threshold), was compared to the untreated cells (100% protection threshold)(Table 3). Significant protection was established when 20% or more protection against acetaminophen was shown. The ethanolic green *A. linearis* extract showed the highest level of hepatoprotection with 40.79±3.46%, 37.65±5.09%, and 32.02±2.23% for the concentrations 400 μg/mL, 100 μg/mL and 25 μg/mL tested, respectively.

Table 3
The hepatoprotective activity of the ethanolic extract of green *A. linearis* and the essential oil of *L. scaberrima* on acetaminophen-induced toxic HepG2 hepatocyte cells.

Sample	% Hepatoprotection		
	400 (µg/mL) ± SD	100 (µg/mL) ± SD	25 (µg/mL) ± SD
***A. linearis* (green)**	40.79±3.46*	37.65±5.09*	32.02±2.23*
***L. scaberrima* (Essential oil)**	17.79±4.63*	34.34±3.10*	33.20±3.91*
Acetaminophen[a]	0.00±1.00		
Silymarin[b] 50 mM	91.70±5.35*		
Untreated cells[c]	100±4.65*		

[a]Toxic inducer, used as the 0% protection value, [b]Positive control, [c]Untreated cells, used as the 100% protection value, Data is represented as mean±SD, n=3, ANOVA with Dunnett's multiple comparison test, *Statistically significantly different from acetaminophen-induced cells (p-value <0.05).

Many studies have revealed the hepatoprotective effects of the fermented extracts of *A. linearis*, although it was not prevalent in the current study. A study by Olawale et al., (2013) concluded that the hepatoprotective effects might be due to the inhibition of lipid peroxidation, thus stabilization of the membranes of hepatocytes. Many studies have involved different methods of studying hepatoprotection (*tert*-butyl hydroperoxide-induced oxidative hepatotoxicity), and this might give different and conflicting results, as seen in the previous study, whereby fermented *A. linearis* is known to be hepatoprotective and these results were not found when investigated in the current method of acetaminophen-induced toxicity.

The essential oil of *L. scaberrima* showed high levels of hepatoprotection with 17.79±4.63%, 34.34±3.10% and 33.20±3.91% for the concentrations 400 µg/mL, 100 µg/mL and 25 µg/mL, respectively. A significant increase in hepatoprotection can be observed as the concentration of the essential oil is lowered. This may in part be due to the many components present within the essential oil, including as limonene. No data on the hepatoprotective activity of limonene or for any species of *Lippia* could be found. Different approaches have been used to explain the mechanism of action of natural products and their ability to exert hepatoprotection against a variety of sources (CCl_4, APAP, H_20_2 etc.). The main mechanism of action is due to the antioxidative capacity, the ability to scavenge free radicals as well as the inhibition of CYP 2E1 enzyme (Jaeschke et al., 2010, Kumar et al., 2013). Kumar et al., (2013), indicated that an increase in the activity of various enzymes such as glutathione peroxidase (GPx), glutathione- S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) together with the reduction of lipid peroxide will manage the integrity of liver cells as well as decrease the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP).

Further research is warranted into the mechanism of action of the essential oil of *L. scaberrima* and its ability to protect the liver against induced toxicity.

4. Conclusions

Investigations into the constituents of the ethanolic and essential oil of *L. scaberrima* and, as confirmed by various publications and studies, are that they are rich in terpenoids. These terpenoids are lipophilic in nature and may interfere with the cell wall of bacteria and ultimately lead to the death of the bacteria, especially with mycobacterial spp. which possess mycolic acids. Mycolic acids are known to prevent the penetration of many compounds and therefore inhibit the activity of many of these compounds which need entry within these cells to exert their therapeutic action as seen by the lowered activity found in the current study. During the invasion of the host by bacterial pathogens such as *M. tuberculosis*, nitric oxide is produced in large quantities by the host's macrophages and neutrophils. The inflammatory process is facilitated by the initiation of several proinflammatory mediators such as reactive oxygen species (ROS) and nitric oxide (NO) (Kumar et al., 2013; Leyva-Lopez et al., 2016). These are all important mediators that need to be down-regulated to prevent an undesired inflammatory response, which leads to increased chances of a burst granuloma, risks of spreading the disease indirectly increasing the cases of morbidity. With regards to inflammation, reactive oxygen species (ROS) are a major part of the inflammation process, through the activation of transcription factors such as NF-Kβ, which has the ability to override proinflammatory gene expression (Grigore et al., 2013). Treatments that can inhibit ROS production may also restrict the development of inflammation (Leyva-Lopez et al., 2016). The chemical composition of plants or a compound can act as an anti-inflammatory agent by affecting the arachidonic acid pathway, cytokine production or through the modulation of pro-inflammatory gene expression. The ability of *L. scaberrima* and *A. linearis* are found to be effective, at least in an *in vitro* setting, as adjunct host direct therapy. The combination of the ethanolic extracts of *L. scaberrima* and green *A. linearis* can be considered as a key candidate for adjunct host-directed therapy in tuberculosis patients, as it exhibited both anti-inflammatory and has existing antioxidant potential. More in-depth information is, however, needed to show how these samples affect the host's ability to interact with invading bacterial pathogens such as *M. tuberculosis* with regards to which pathways are inhibited or induced. Tea is a beverage that is consumed across the world, and the introduction of

teas as additives may have the ability to aid in the treatment of various disorders and diseases. *Lippia scaberrima* is already well known as a health tea in the neighbouring countries of South Africa and the popular Rooibos tea is a productive export, endemic to the Cape areas of South Africa.

Conflict of interest

The authors declare that there is no conflict of interest.

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