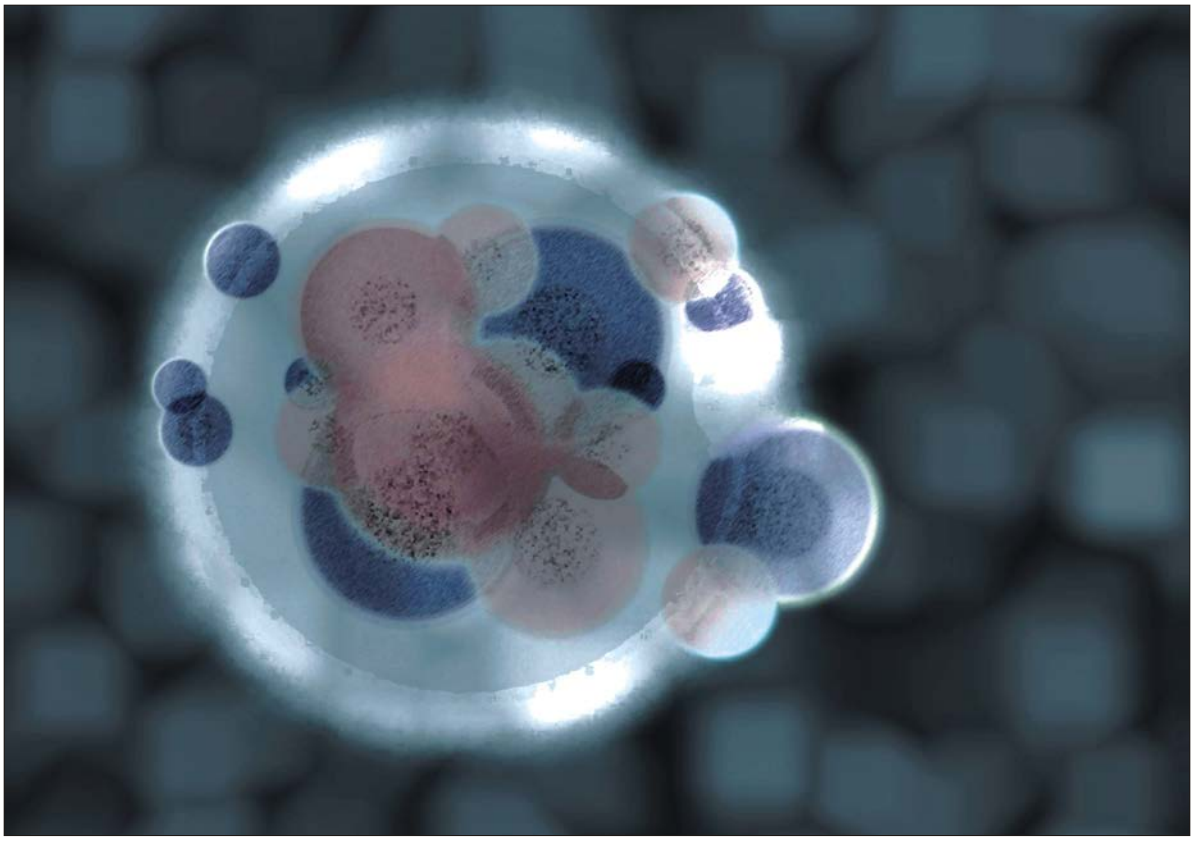

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Research Article
Therapeutic Evaluation of Plants Used Against Respiratory Infections and Related Symptoms in Limpopo Province, South Africa

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Abstract

Background and Objective: Respiratory infections (RIs) and related symptoms (RSs) are commonly treated by traditional healers (THs) using herbal remedies in the traditional primary health care sectors. The aim of this study was therefore, to evaluate the therapeutic value of most used plant species namely *Clerodendrum ternatum*, *Cryptocarya transvaalensis*, *Enicostema axillare* and *Lasiosiphon caffer* by Bapedi THs for these conditions. **Materials and Methods:** Qualitative phytochemical constitutes of the crude materials from the above-stated species were determined using the standard methods. The antioxidant activities of acetone, dichloromethane, hexane, methanol and water crude extracts were evaluated using qualitative and quantitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays. Antibacterial activities of these extracts was assessed using microdilution (minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) and bioautography assays). **Results:** The phytochemical screening of *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* crude extracts revealed the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, phlobatannin, saponins, steroids, tannins and terpenes. All plants including those that did not display any antioxidant activities using qualitative DPPH assay showed a certain level of scavenging activities when quantified, with the mentioned extracts from *E. axillare* (water and hexane), *L. caffer* (water and acetone) and *C. ternatum* (acetone) showing excellent activities almost comparable to a standard antioxidant drug (ascorbic acid). Plant extracts from all used solvents were able to completely exterminate *Mycobacterium smegmatis*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with MBC values between 0.019 and 2.5 mg mL^{-1} depending on the plant. Some of plant extracts were able to impede the growth of these bacteria with MIC values ranged between 0.63-2.5 mg mL^{-1}. **Conclusion:** Findings of the present study provide support for the use of *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* as medication for RIs and RSs by Bapedi THs. Studies focusing on isolation, identification and characterization of the pure compounds responsible for antibacterial activities of plants are in progress.

Key words: Antibacterial, antioxidant, Bapedi, medicinal plants, phytochemical analysis, respiratory infections, traditional healers

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Respiratory infections (RIs) and related symptoms (RSs) are major causes of mortality and morbidity in both developed and developing countries of the world, especially in the countries wherein there is a limited or lack of modern health care facilities[1]. The most common bacterial agents causing these ailments include *Acinetobacter baumanii*, *Bacillus cereus*, *Bordetella pertussis*, *Escherichia coli*, *Haemophilus influenza*, *Klebsiella pneumonia*, *Moraxella catarrhalis*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Pneumocystis jiroveci*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Streptococcus pyogenes*[2-5]. Clinically available drugs prescribed by western health care providers to RIs and RSs patients was primarily manufactured to cure or reduce both the spread and well-being threats caused by these organisms, amongst the others.

In developing continents, traditional medicine and traditional healers (THs) has been an important source of treating common infectious ailments including RIs and RSs[6]. This is true for Africa, where over 80% of the population rely on THs and their herbal medicines to treat prevailing ailments[7]. In South Africa, about 60-80% of the entire population utilise health services of an estimated 200,000 indigenous THs who prescribe herbal medicines made from over 30,000 native species[8]. Evidence such as this, substantiates the role of THs and herbal medicine in the South African primary health care sectors.

However, lack of scientific substantiation confirming the possible bioactivity properties and toxicity effects of a great number of folkloric herbal remedies commonly prescribed by THs to patients, is one of the setbacks in the development of African traditional healing practices and integration of THs in the local as well as national formal health care segments[9].

Clerodendrum ternatum Schinz, *Cryptocarya transva alensis* Burtt Davy, *Enicostema axillare* (Lam.) A. Raynal subsp. *axillare* and *Lasiosiphon caffer* Meisn are extensively employed by Bapedi THs practicing in the Limpopo Province of South Africa as medicine for RIs such as asthma, pneumonia, rhinitis, sinusitis, sore throat and TB as well as RSs (Table 1). However, as mentioned in Table 1, the therapeutic application/s of the aforesaid plant species in the treatment of other human ailments is a common practice amongst THs/lay people of other cultures. Sadly, despite the applications of most of these species as complementary medicine for diverse diseases, little is known about their pharmacological properties, particularly against RIs and RSs. To the best of our knowledge with the exception of *E. axillare*, there is presently no scientific study assessing the possible bio-activity properties (i.e., antioxidant, antibacterial and phytochemical constitutes, amongst the others) of the extracts from *C. ternatum*, *C. transvaalensis* and *L. caffer* (Table 2). As far as we know, all the scientific assessments were done on *E. axillare* occurring or harvested in India. There is therefore, a gap in the scientific literature validating extracts from this species against other prominent pathogens responsible for RIs and RSs in most African countries. The aim of the present study was to assess therapeutic application/s of the extracts from the above-mentioned plants used by Bapedi THs by analyzing the chemical profile and phytochemical constituents of the crude extracts, antioxidant activities of the extracts and assess the extracts for antibacterial activities.

MATERIALS AND METHODS

Selection of four plant species: The choice of 4 species namely *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* implicated by Bapedi THs for RIs and RSs as candidates for further laboratory screening was based on their highest ethnobotanical indexes; (i) Fidelity Level, (ii) Use value and (iii) Preference ranking, compared to other species.

Plant collection and extracts preparation: Healthy anatomical part/s from *C. ternatum* (entire plant), *C. transvaalensis* (bark), *E. axillare* (entire plant) and *L. caffer* (root) as traditionally used by Bapedi THs to treat RIs and RSs (Table 1) were collected in the wilderness of Limpopo Province during May, 2018 and subsequently air dried at room temperature. Dried materials were deliberately utilised, firstly because they were used by THs and secondly due to the fact that there are fewer problems associated with their extraction compared to fresh plant materials[35]. Desiccated plant parts was cut into smaller pieces and then pulverized to a fine powder using an electric grinder at the Limpopo Agro-Food Technology Station (LATS) (University of Limpopo) and stored in an air-tight container which was kept at room temperature in the dark until extraction procedure.

Extraction procedures: Small scale extraction was done using several solvents, from non-polar to polar. The 10 g of pounded part/s of the 4 selected plant species for laboratory study were extracted thrice in 200 mL of various solvents of distinct

Table 1: Traditional use of *Clerodendrum ternatum*, *Cryptocarya transvaalensis*, *Enicostema axillare* and *Lasiosiphon caffer* by Bapedi healers to treat respiratory infections and related symptoms

Botanical family	Species	Habit	Plant parts and state of use	Methods of herbal preparation and administration	Aliment/s treated	Frequency of citation (sum of Bapedi THs interviewed = 240)	Other uses reported in literature
Gentianaceae	*Enicostema axillare* (Lam.) A. Raynal subsp. Axillare	Herb	Whole plant (dry)	Boiled for 5-14 min, extract is taken orally, thrice a day	Asthma	140	Abdominal ulcers, dyspepsia, rheumatism and stomach ache[10], arthritis[11], diabetes mellitus and swelling[12], blood purifier, hernia and itches[13], liver tonic[14] and skin disease[15]
				Or	Sinusitis	132	
				Pounded and taken orally with warm water, thrice a day	Sore throat	156	
					Tuberculosis	201	
Vitaceae	*Cyphostemma woodii* (Gilg and M. Brandt) Desc.	Dried powered root		Taken orally with warm water, thrice a day	Tuberculosis	1	
				Pounded and mixed with dried powdered bark of	Pneumonia	1	
Anacardiaceae	*Sclerocarya birrea* (A. Rich.) Hochst. subsp. caffra (Sond.)	Root	Whole plant	Taken orally with warm water, thrice a day			
Asteraceae	*Callilepislaureola* DC.						
Asteraceae	*Helichrysum caespititium* (DC.) Harv.						
Hyacinthaceae	*Drimia sanguinea* (Schinz) Jessop	Fresh bulb Dried twigs		Boiled for 3 min, stream is inhaled (nasally) under blanket, thrice a day	Pneumonia	1	
Asteraceae	*Kleinia longiflora* DC						
Hyacinthaceae	*Drimiaelata* Jacq.	Fresh bulb		Boiled for 5 min, extract is taken orally, thrice a day	Pneumonia	1	
				Boiled for 5-9 min, extract is taken orally, thrice a day	Pneumonia	125	
				Boiled for 5 min, steam is inhaled (nasally) under blanket, thrice a day	Rhinitis	105	
Lamiaceae	*Clerodendrum ternatum* Schinz	Herb	Whole plant (dry)	Boiled for 5-7 min, extract is taken orally, thrice a day	Chronic cough	358	Constipation[16], HIV/AIDS[17], epileptic fits and sore eyes[18], Malaria[19] and eczema[20], gastrointestinal diseases and sexually transmitted infections[21]
				or pounded and taken orally with warm water, thrice a day	Tuberculosis	202	
				Pounded and taken orally with warm water, thrice a day	Asthma	140	
					Pneumonia	128	
					Sore throat	156	
				Boiled for 5 min, steam inhaled (nasally) under blanket, thrice a day	Rhinitis	105	
					Sinusitis	132	
Lauraceae	*Cryptocarya transvaalensis* Burtt Davy	Tree	Bark (dry)	Boiled for 5-9 min, extract is taken orally, thrice a day	Asthma	140	Unspecified medicinal application for human ailments[22]
					Chest pain	202	
					Chronic cough	202	
					Fever	202	
					Pneumonia	128	
					Sore throat	156	
					Tuberculosis	202	
					Sinusitis	41	
				Pounded and poured in boiled water, steam is inhaled (nasally) under blanket, thrice a day	Sinusitis	91	
					Rhinitis	105	
					Lack of appetite	58	

Table 1: Continued

Botanical family	Species	Habit	Plant parts and state of use	Methods of herbal preparation and administration	Aliment/s treated	Frequency of citation (sum of Bapedi THs interviewed = 240)	Other uses reported in literature
Thymelaeacea	*Lasiosiphon caffer* Meisn.	Shrub	Root (dry)	Pounded and taken orally with warm water, thrice a day	Asthma	140	Unspecified medicinal application for human ailments[23]
					Chronic cough	358	
					Chest pain	202	
					Pneumonia	128	
					Sore throat	358	
					Tight chest	219	
					Tuberculosis	198	
Canellaceae	*Warburgia salutaris* (G. Bertol.) Chiov.	Dried powdered bark		Taken orally with warm water, thrice a day	Tuberculosis	4	
				Pounded and poured in boiled water, steam is inhaled (nasally) under blanket, thrice a day	Sinusitis	132	
					Rhinitis	105	

Table 2: Known therapeutic scientific assessments of *Clerodendrum ternatum*, *Cryptocarya transvaalensis*, *Enicostema axillare* and *Lasiosiphon caffer*

Species	Plant parts and state of use	Phytochemical constituent/s reported	Solvents	Activity/use assessed	Organisms and/used cells
Clerodendrum ternatum	-	-	-	-	-
Cryptocarya transvaalensis	-	-	-	-	-
Enicostema axillare	Whole plant (dry)	Alkaloids and tannins in methanol, hexane and chloroform	Chloroform, hexane and methanol	Anti-bacterial activity	+*Bacillus subtilis*[29]
			Aqueous, chloroform, hydro alcoholic, ethyl acetate and methanol	Anti-bacterial activity	+*Bacillus subtilis, Escherichia coli, Shigellasonnei*, +*Pseudomonas aeruginosa*[30]
				Anti-fungal activity	*Aspergillus niger* and *Candida albicans*[30]
		Alkaloids,	Petroleum ether[24]		
		Amino acid, saponins, steroids, phenolic and proteins	Ethanol and methanol	Anti-oxidant activity[14]	
			Aqueous, chloroform and methanol	Anti-arthritic activity[25]	
			Aqueous	Hepato-protective activity[26]	
			Ethyl acetate	Anti-edematogenic[27]	
			Methanol	Anti-inflammatory[28]	
			Methanol	Anti-tumor activity	Swiss albino mice[31]
			Methanol	Antibacterial activity	+*Bacillus subtilis* and+*Klebsiella pneumonia*[28]
			Chloroform, ethanol and hexane	Anti-bacterial activity	+*Pseudomonas aeruginosa*[32]
			Methanol	Cytotoxic activity	Hela cell line[33]
	Leaves (dry)		Ethanol	Anti-fertility activity	Male albino rat[34]
Lasiosiphon caffer	-	-	-	-	-

-: Not found, +: Pathogens known to cause RIs and RSs

polarities (ranging from no-polar to more polar), namely; hexane, dichloromethane (DCM), acetone and methanol in 150 mL Erlenmeyer flask using IKA Magnetic Stirrers® (RH digital) overnight and plant residues were allowed to settle. With regards to the water extracts, it was done in 200 mL of distilled water in 250 mL round bottom flask using reflux apparatus for 30 min at 100°C and plant residues were allowed to settle. Generally, the extracts were filtered and solvents evaporated using a rotary evaporator (BUCHi Labotec rotavapor model R-205, Germany) and transferred into pre-weighed labelled glass beakers. Importantly, all the plant extracts were reconstituted with acetone to a final concentration of 10 mg mL^{-1}.

Phytochemical analysis: Chemical profile of the extracts from *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* materials were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates which included merck and silica gel 60 F254. The plant extracts were re-dissolved in acetone to give a final concentration of 10 mg mL^{-1}. Subsequently, 10 µL of the extracts was spotted onto a TLC plate. As described by Kotzé *et al.*[36], the TLC plates were developed in separation systems of varying polarities, namely, benzene/-ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic), chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic) and ethyl acetate/methanol/water (40:5:4:5): [EMW] (polar/neutral). The plates were then removed from the systems (tanks) and air dried under a fume-hood cabinet. Thereafter, the separated components were observed under visible and ultra-violet light at wavelengths of 254 and 365 nm. Separated compounds which were not visible under this light were detected using a method outlined by Wagner and Bladt[37]. For instance, TLC plates were sprayed with vanillin sulphuric acid reagent and carefully heated for about five minutes at 110°C for optimal colour development.

Phytochemical constitutes: The mentioned morphological parts of *C. ternatum*, *E. axillare* (whole plant), *C. transvaalensis* (bark) and *L. caffer* (root) as used by Bapedi THs were examined for the present of the following components and appropriate colour change were used for interpretation.

Alkaloids: The Dragendorff's reagent technique described by Harborne[38] was used. Milled parts (0.2 g) from the aforesaid species were extracted with 95% ethanol in a soxhlet extractor for 6 h. The ethanol extracts were evaporated to dryness using a vacuum evaporator at 45°C. Subsequently, the plant residues were dissolved in 5 mL of 1% hydrochloric acid and 5 drops of Dragendorff's reagent was added. The samples were observed for the formation of colour (orange to orange red precipitate) to draw inference.

Anthraquinones: The presence of these secondary metabolites in studied plant extracts was tested by weighing 0.5 g of ethanol extracts and boiled with 10 mL of 97% sulphuric acid and carefully filtered while hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer was pipetted into another test tube to which 1 mL of dilute ammonia was added. The resulting solution was observed for pink colour[39].

Cardiac glycosides: For cardiac glycosides test, the Keller-Killiani experiment was used, where 0.5 g of plant extracts was added to 5 mL of water. The mixture of 2 mL of glacial acetic acid containing one drop of 0.1% ferric chloride solution was added to the diluted extracts. This was underplayed with 1 mL of concentrated sulphuric acid. A brown ring (with a violet ring beneath) at the interface indicates the presence of a deoxysugar characteristic of cardenolides while in the acetic acid layer, a greenish ring may also form gradually throughout the layer[40].

Flavonoids: Diluted ammonia solution (5 mL) was added to a portion of the aqueous filtrate of each plant extracts, followed by the addition of concentrated sulphuric acid (1 mL). A yellow colouration that disappears on standing represented the presence of flavonoids[40].

Phlobatannin: Powdered plant part samples (0.2 g) of each investigated species used by Bapedi THs were dissolved in 10 mL of distilled water and filtered. The filtrate was boiled with 2% hydrochloric acid solution. The deposition of a red precipitate confirmed the presence of phlobatannins[40].

Saponins: Odebiyi and Sofowora[41]'s persistent frothing test for saponins was used. One gram of powdered materials from four selected plant species was mixed with 30 mL of tap water. The mixture was vigorously shaken and heated at 100°C. The sample was observed for the formation of a persistent froth to indicate the presence of saponins.

Steroids: This was tested by adding 2 mL of acetic anhydride to 0.5 g of each plant extract, followed by addition of 2 mL of sulphuric acid. Colour changes from violet to blue or green in some plants indicates the presence of steroids[40].

Tannins: The tannins were tested by gently boiling 0.5 g of powdered materials from the selected plant species, in 5 mL of distilled water in a test tube, then cooled and filtered. Three drops of 0.1% ferric chloride was added to 1 mL of solution in a test tube and observed for green coloured precipitate[42].

Terpenes: The Salkowski test was used to evaluate the presence of terpenes. Powdered plant part (0.5 g) of *C. ternatum*, *E. axillare*, *C. transvaalensis* and *L. caffer* were dissolved in 2 mL chloroform, followed by cautiously adding 3 mL of concentrated sulphuric acid, to form a layer. A reddish brown or greyish colouration of the interface was considered indicative of positive results[40].

Evaluation of the antioxidant activities: The antioxidant activities within the plant extracts were determined by using qualitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) on TLC and quantitative 2, 2-diphenyl-1-picrylhydrazyl assays.

Qualitative antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl: As described earlier under phytochemical analysis section TLC plates were used to separate crude extracts of different polarities. To detect any antioxidant activities presence in the separated plant extracts, the chromatograms were firstly air-dried and afterwards subjected to 0.2% (w/v) DPPH solution in methanol sprayed (until saturated) by using a spray gun[43]. Subsequently, yellow zones or spots (against purple background) appearing on the chromatograms indicated the presence of antioxidant.

Quantitative 2, 2-diphenyl-1-picrylhydrazyl assay: This assay was done on the studied medicinal plant extracts by adding 100 µL of distilled water into each well of 96-welled plate. These extracts were prepared at a concentration of 10 mg mL^{-1}. Subsequently, 100 µL of plant extracts were transferred into the first row of 96-welled plate and serially diluted 50%. Ascorbic acid (Vitamin C) at a concentration of 2 mg mL^{-1} was used as the antioxidant standard or positive control. Fifteen micro litres of 0.2% of 2, 2-diphenyl-1-picrylhydrazyl (sigma®) in methanol was added into each well of microtiter plate and allowed to react at room temperature in the dark for 30 min. The absorbance was measured at 540 nm using a microtiter plate reader (DTX 880 multimode detector; Beckman coulter, Inc.®) as previously used by Ayoola *et al.*[39]. Percentage antioxidant activities (AA %) values were calculated from the absorbance values using the equation:

$$AA\ (\%) = \frac{Absorbance_{control} - Absorbance_{experiment}}{Absorbance_{control}} \times 100$$

Antibacterial assay

Used microorganisms: Mycobacterium smegmatis ATCC 1441 (a potential *M. tuberculosis* surrogate), *Klebsiella pneumoniae*, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were chosen to test the antimicrobial activities of the 4 studied plants extracts. These are microorganisms that are known to cause RIs and RSs or responsible for the majority of their cases. The test organisms were sub cultured on nutrient broth and incubated at 37°C for 24 h and subsequently preserved at 4°C in the refrigerator as stock strains prior to screening testing.

Qualitative antibacterial activities assay by bioautography: Thin layer chromatography plates were cut (10×10 cm) and loaded with 20 µL of each extract plant extract (10 mg mL^{-1}) dissolved in acetone in line with the method of Begue and Kline[44]. The plates were prepared, developed in the different solvent systems as stipulated earlier and were allowed to dried at room temperature under a stream of air for 2-3 days, in order to remove residual solvent, which might inhibit bacterial growth. The 4 bacterial species used in microplate serial dilution assay were used the test organism. The developed plates were sprayed with the concentrated suspension containing actively growing bacteria until they were entirely wet and thereafter incubated at 37°C for 24 h in 100% relative humidity in the dark. After incubation, the plates were sprayed with a 2 mg mL^{-1} solution of INT (Sigma®) and incubated for a further 2-3 h. The bioautograms were subsequently observed for bacterial growth. The white areas (clear zones) indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the test bacteria.

Quantitative antibacterial activities assay by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The microplate serial dilution method of Eloff[35] was employed to determine the minimum inhibitory concentration (values of the plant extracts against *M. smegmatis*, *K. pneumoniae*, *S. aureus* and *P. aeruginosa*). Residues of plant extracts were dissolved to a concentration of 10 mg mL^{-1} using the extracting solvent acetone. Consequently, the dissolved plant extracts were serially diluted (50%) with water in 96 well microtiter plates. Bacterial cultures were sub-cultured and thereafter transferred into fresh nutrient broth and 100 µL of the cultures were

transferred into each well and acetone was included blanks. The microtiter plate (cultures) was incubated for 24 h at 37°C. After incubation, 20 µL of 2 mg mL^{-1} p-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each microplate wells as an indicator of growth. The covered microtiter plates were incubated for 30 min at 35°C and 100% relative humidity and all determinations were carried out in triplicate. Microorganism growth was indicated by a purple-red colour resulting from the reduction of INT into formazan. Clear wells indicated the presence of compound in the extracts that inhibited the growth of the microorganisms tested. The MIC value of each plant extracts was determined as the lowest concentration that led to an inhibition of bacterial growth after 24 h of incubation at 37°C. The total activities of the plant extract in mL g^{-1} was calculated using a standard formula developed by Eloff[35]. For instance, the MIC values were divided with the quantity extracted from 10 g of plant material. The resultant value indicates the volume to which the extract can be diluted and still inhibits the growth of the bacterial isolate.

The MBC were determined by further incubating for another 24 h, the same 96 well microtiter plates used for MIC. The lowest concentration with no visible growth of *M. smegmatis*, *K. pneumoniae*, *S. aureus* and *P. aeruginosa* was defined as the MBC, indicating 99.9% reduction in colony forming units' number in the initial inoculum[45].

RESULTS AND DISCUSSION

Quantity of plant materials extracted: Table 3 depicts the percentage mass extracted from 10 g of *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* materials extracted in 5 different solvents namely acetone, dichloromethane, hexane, methanol and water. None of these solvents yielded the highest mass from these plant materials. In this regard, a wide range of quantity among extracts was observed depending on the extraction solvent and plant material used. Such disparity might be due to the variations in both particle sizes of grounded material and their phytochemical constituents, coupled with the ability of used solvents to solubilise compounds with similar polarity[46]. Generally, different mass yields obtained using various solvents in our study indicate that the investigated plant species possess diverse chemical compounds.

An analysis of our findings showed that water and methanol respectively was the best extracting solvents, followed by acetone and dichloromethane. Most of the hexane materials from the investigated species gave much lower extraction yields. In generally polar solvents (acetone, methanol and water) extracted more materials than non-polar, thus suggesting that the studied plant species mainly contain polar compounds. The strongest extractability of water and methanol as observed in this study might indicate that both these solvents have great ability to extract higher concentration of compounds from the investigated species compared to other solvents. Therefore, these solvents can be considered the best as far as the biochemical extraction of *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* is concerned. We perceive the highest extraction yields of water in this study, as an interesting finding since it is a standard method of extract preparation by Bapedi THs. Therefore, it can be said that water as used by questioned THs have great potential to recoup numerous phytochemicals including those that are effective against RIs and RSs.

To the best of our knowledge, with the exclusion of *E. axillare*, there is presently no study reporting on either the application of acetone, dichloromethane, hexane, methanol and water as extracting solvents for the rest of the species included in this study, hence there is no literature to compare and contrast our findings with. The present study in this regard provides a baseline data. Analogously to the outcomes of our investigation, methanol (45.8%) and water (43.6%) extracts of *E. axillare* also yielded more materials in an Indian study[47]. A comparable finding was reported by Gite *et al.*[26] in the same country. Findings from our research therefore, further buttress the significant of both water and methanol as the most efficient solvents for extraction of phytochemical constituents from *E. axillare* materials.

Table 3: Percentage mass extracted (g) from powdered material species using different solvents

	Quantity extracted (mg) and used solvent					
Plant species	Acetone	Dichloromethane	Hexane	Methanol	Water	Total yield
Clerodendrum ternatum	0.20	0.20	0.10	2.3	2.0	4.80
Cryptocarya transvaalensis	0.30	0.50	0.20	1.7	1.9	4.60
Enicostema axillare	1.50	0.50	0.50	3.1	2.1	7.70
Lasiosiphon caffer	1.40	0.10	0.10	1.9	8.7	12.20
Average	0.85	0.33	0.23	9.0	3.8	14.21

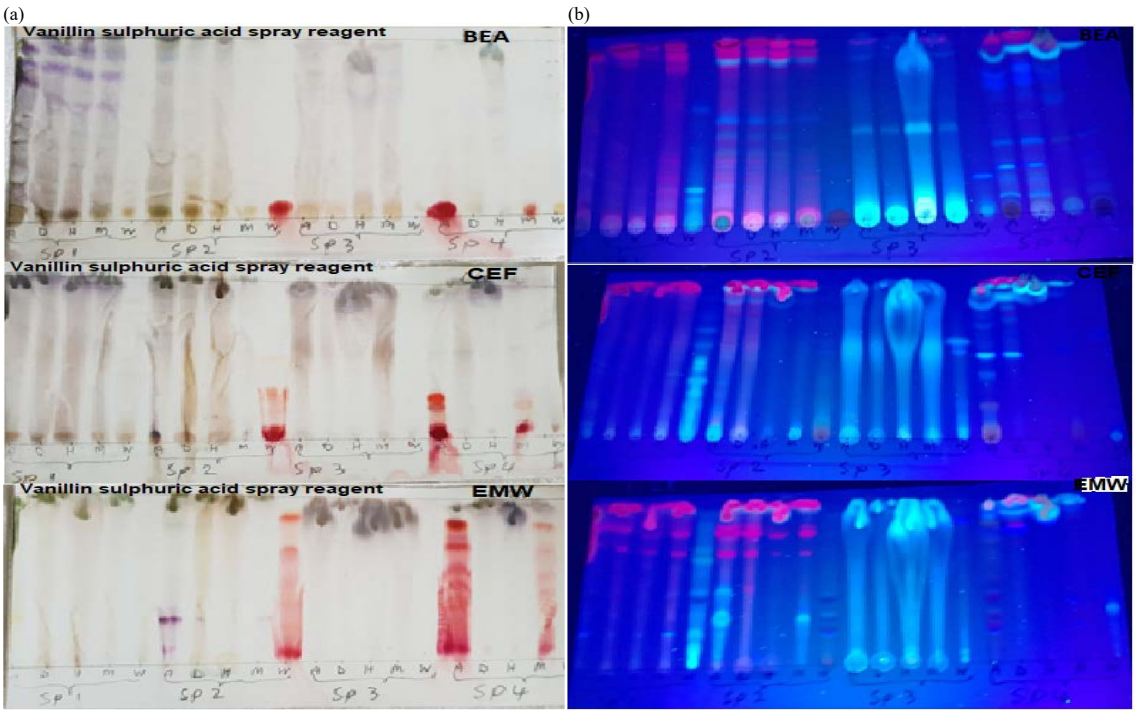

Fig. 1(a-b): Thin layer chromatographic profiles, (a) Sprayed with vanillin-sulphuric acid reagent and heated in the oven to show compounds and (b) Prior spraying with this reagent and heating of investigated plants

Species 1: *E. axillare*, Species 2: *C. ternatum*, Species 3: *C. transvaalensis*, Species 4: *L. caffer*, crude extracts A: Acetone, D: Dichloromethane, H: Hexane, M: Methanol, W: Water separated with 4 solvent systems namely: BEA, CEF and EMW and sprayed with vanillin-sulphuric acid reagent, to show compounds

Thin layer chromatography finger print profile of the plants: The separation of compounds from crude extracts of 4 studied medicinal plant species was determined using the thin-layer chromatography (TLC) and accordingly, TLC revealed complex mixture of compounds which exhibited different coloured reactions with and without the vanillin spray reagent (Fig. 1).

Generally, BEA (a non-polar), followed by EMW (polar/neutral) and CEF (intermediate polarity/acidic) respectively, proved to be a better solvent system for extraction of wide range of bioactive principles from all/most plants. Therefore, future studies further investigating the pharmacological activities of specific secondary metabolites from the 4 studied medicinal plants should utilize BEA and EMW TLC elution systems for column chromatography. However, the ability of the specific solvent used for each species to better isolate some compounds should not be overlooked by these studies. It worth mentioning that all water extracts from the studied plants displayed the presence of bioactive components, suggesting that the crude herbal remedies prescribed by Bapedi THs might be of help in the treatment and management of RIs and RSs.

Preliminary phytochemical analysis of crude extracts: Phytochemical constituents of 4 studied plant species exhibited the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, phlobatannin, saponins, steroids, tannins and terpenes. With the exclusion of flavonoids, saponins, steroids, tannins and terpenes, which were found in all four plants, the rest of these bioactive compounds was present in certain species. Generally, the referred secondary metabolites were not detected in all 4 investigated species and their concentration (based on colour intensity) within some of these plants were either in low, moderate or high concentrations (Table 4). The latter might be attributed to variation in genetic makeup and chemical composition of plant specimen used, ascribed to the dissimilar geographical areas (i.e., altitude, climate and rainfall) were they were collected.

With the exception of *E. axillare*, to date there is no work done on the rest of the species namely *C. ternatum*, *C. transvaalensis* and *L. caffer* as far as phytochemical constituents are concerned. The presence of alkaloids, flavonoids, glycosides, saponins, steroids and tannins within *E. axillare* extracts was previously reported in India[14,29]. Therefore, our findings further buttress this.

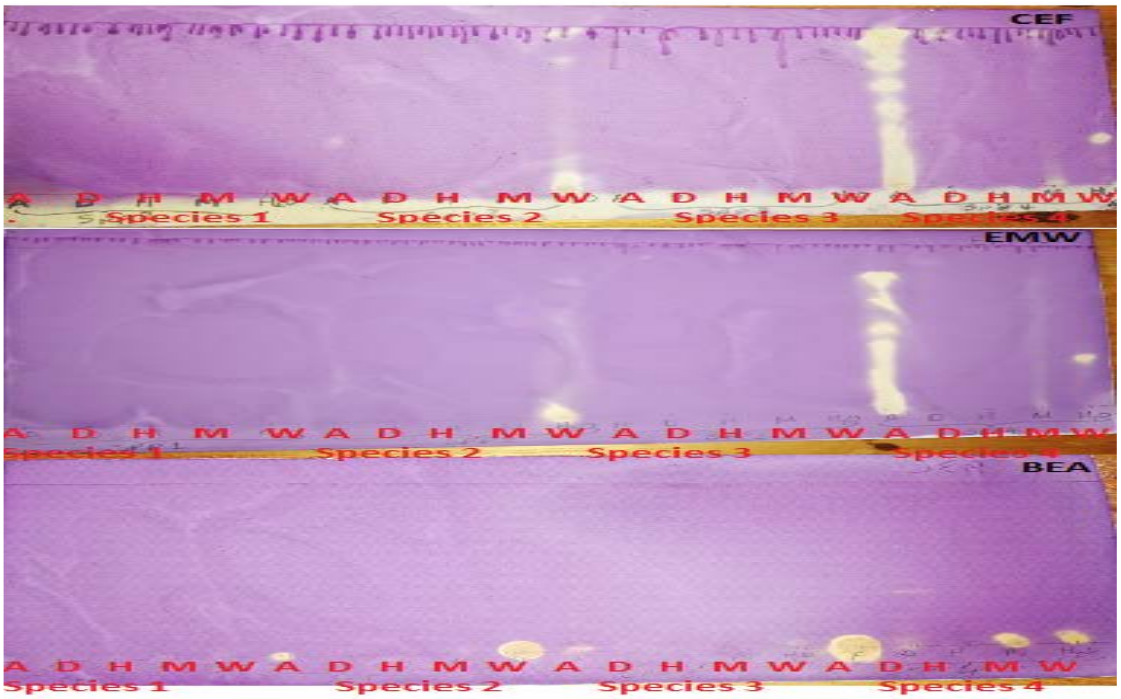

Fig. 2: Chromatograms of investigated species

Species 1: *E. axillare*, Species 2: *C. ternatum*, Species 3: *C. transvaalensis*, Species 4: *L. caffer* crude extracts (A, D, H, M and W) developed in four solvents systems (BEA, CEF and EMW) from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, with yellow zones indicating antioxidant activities exhibited by compounds

Table 4: Preliminary phytochemical analysis of screened therapeutic plant species

Species	A	AN	CA	F	PH	SA	ST	TA	TE
C. ternatum	-	-	+++	++++	-	++++	++++	++++	++++
C. transvaalensis	++	-	-	++++	-	++++	+++	++++	++++
E. axillare	+	-	+++	++++	+++	++++	+++	++++	++++
L. caffer	-	+++	+++	++++	++++	++	+++	++++	++++

+: Presence of phytochemicals, ++: Low concentration, +++: Moderate concentration, ++++: High concentration, A: Alkaloids, AN: Anthraquinones, CA: Cardiac glycosides, F: Flavonoids, PH: Phlobatannin, SA: Saponins, ST: Steroids, TA: Tannins, TE: Terpenes

Generally, the presence of phytochemicals within the investigated 4 crude plant extracts were mainly in high concentrations and none of the species had all the nine investigated secondary metabolites. *Enicostema axillare* and *L. caffer* were endured with more of these metabolites (n = 8) while *C. ternatum* and *C. transvaalensis* had six. Traces of several diverse biologically active phytochemicals reported in Table 3 within these species, justifies their use as treatment of various RIs and Rss. These phytochemicals serve as natural antibiotics, which help the body to fight infections and microbial invasion[48]. Alkaloids, terpenes and steroids are reported to have anti-inflammatory and anti-bacterial activities[49-51], thus are helpful in the treatment of asthma and other respiratory infections (i.e., pneumonia and sore throat) which are caused by bacterial infections. Similarly, glycosides have been found to decrease inflammatory symptoms in different animal models of acute and chronic inflammation[52]. Tannin is known to hasten the healing of inflamed mucous membranes[53]. Amutha and Doss[54] found that saponin fraction possess significant activities against *K. pneumonia* and *S. aureus*. Flavonoid supplementation decreased upper respiratory tract infections incidence by 33% (95% CI: 31-36%) compared with control in healthy adults, with no apparent adverse effects[55]. Therefore, it can be said that the bioactive principles present in 4 investigated therapeutic plants prescribe by Bapedi THs might be of benefit for respiratory sicknesses patients.

Antioxidant activities of plant extracts: Qualitative antioxidant compounds activities of the extracts from studied plants were examined using the DPPH assay on TLC plates. The presence of these compounds with scavenging activities is indicated with yellowish bands on a purple background on TLC plates (Fig. 2). The presence of antioxidant compounds

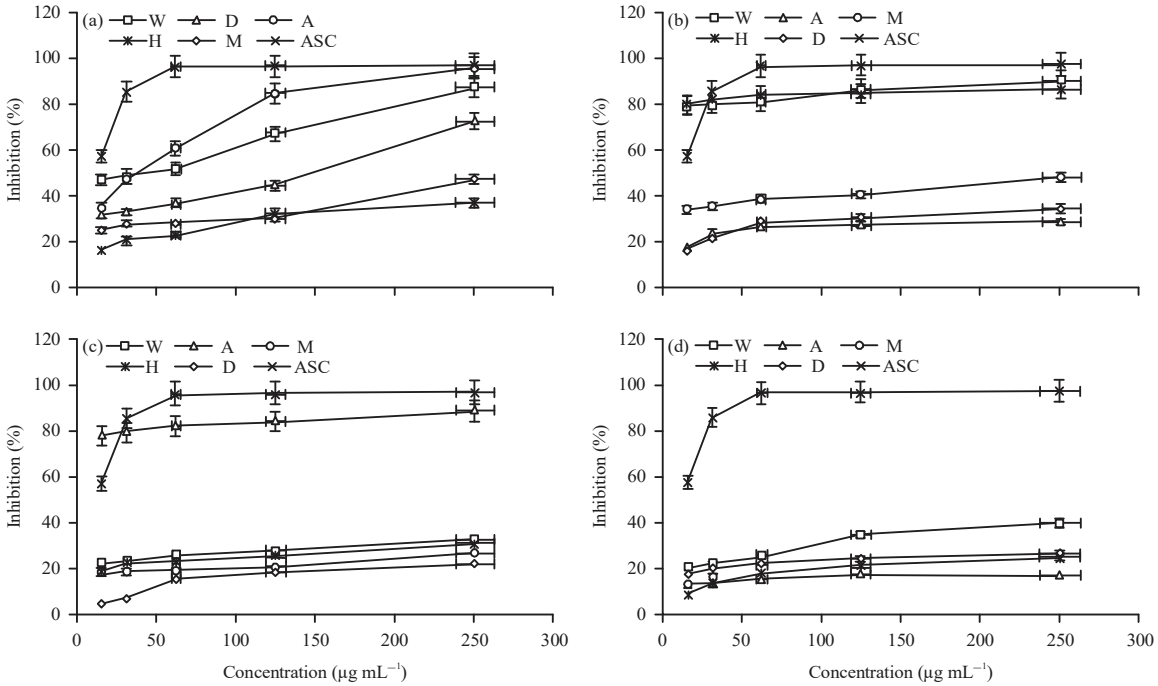

Fig. 3(a-d): Reducing power potential of 4 studied plant extracts, (a) *L. caffer*, (b) *E. axillare*, (c) *C. ternatum* and (d) *C. transvaalensis*

A: Acetone, D: Dichloromethane, H: Hexane, M: Methanol, W: Water, ASC: Ascorbic acid with absorbance increasing with increasing concentration

activities of the studied plant extracts per solvent used on 3 separation systems (BEA, CEF and EMW) was also determined. Extracts from certain species alienated by these systems had antioxidant compounds. On the CEF chromatogram, *C. ternatum* water extract, acetone, dichloromethane, methanol and water extracts from *L. caffer* showed traces of these compounds. Same species extracted with these solvents but using EMW separation systems also showed some antioxidants activities. The mobile phase system; BEA exhibited antioxidant activities of compounds from *C. ternatum* (acetone and water), *C. transvaalensis* (acetone) and *L. caffer* (acetone, methanol and water) extracted with the mentioned solvents. Therefore, it can be said that *C. ternatum* and *L. caffer* are good candidates to isolate antioxidant compounds from. No yellow bands were observed when the plates were resolved on the BEA, CEF and EMW using all extracts from *E. axillare* and *C. transvaalensis* on CEF and EMW. Overall, with the exception of *E. axillare* which were previously reported by Krishnaveni and Mohandass[14] as having antioxidant activities, the antioxidant capacity of the rest of the species investigated are mentioned for the first time in the present study.

Antioxidant activities (expressed as percentage) of *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* was quantified using quantitative 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activities assay, by comparing it to the activities of standard antioxidant drug, namely ascorbic acid (Fig. 3). Surprisingly, all plants including those that did not displayed any antioxidant activity using qualitative DPPH assay on TLC (Fig. 2) showed a certain level of scavenging activities when quantified via quantitative DPPH radical scavenging activities assay. Indeed, as reported by various researchers[56,57], one assay cannot reveal the presence of antioxidant compound in all plant extracts because the sensitivity of some methods depends on various factors, such as media pH, the presence of lipophilic and/or hydrophilic compounds and amongst the others. Therefore, antioxidant capacity of plant extracts should be evaluated by more than one method.

The mentioned extracts from *E. axillare* (water and hexane), *L. caffer* (water and acetone) and *C. ternatum* (acetone) showed excellent antioxidant activities almost comparable to ascorbic acid. Therefore, enquiries in to the possibilities of developing natural antioxidant drugs from these species should be a subject for future study. All *C. transvaalensis* extracts were less effective as opposed to ascorbic acid. Generally, the present of certain traces of anti-oxidants activities in all studied plants is very fundamental from the therapeutic stand-point pertinent to RIs and RSs. This is because the crucial role of this activities in preventing or

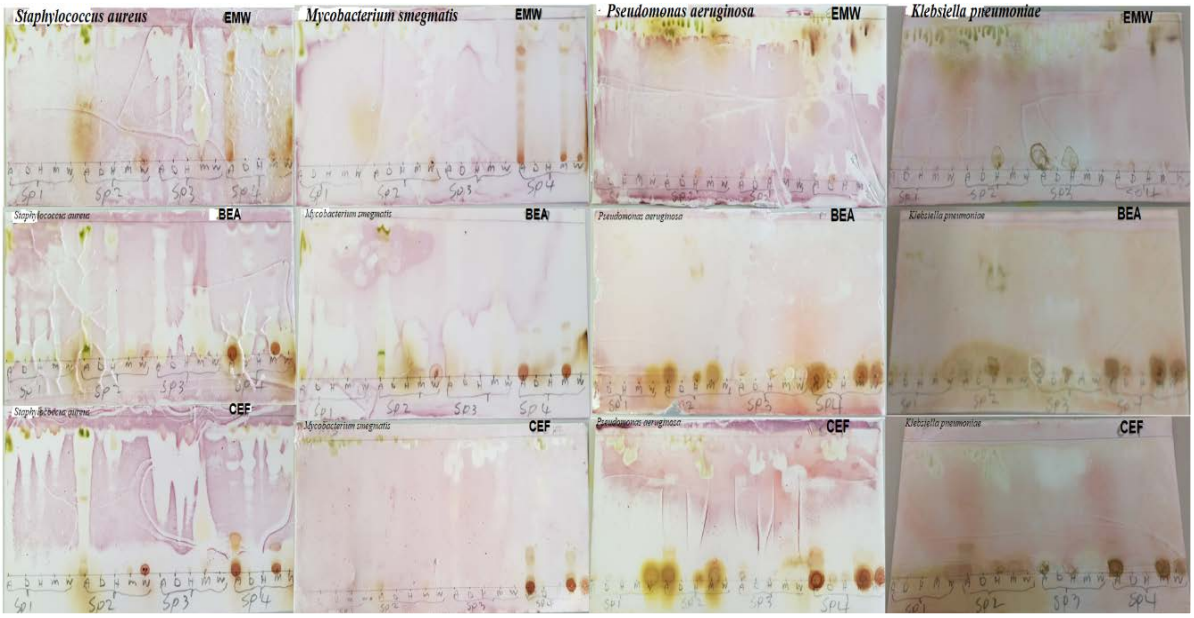

Fig. 4: Chromatograms of crude extracts of investigated species

Species 1: *E. axillare*, Species 2: *C. ternatum*, Species 3: *C. transvaalensis*, Species 4: *L. caffer*, crude extracts A: Acetone, D: Dichloromethane, H: Hexane, M: Methanol, W: Water developed in three solvents systems (BEA, CEF and EMW) sprayed with *S. aureus*, *M. smegmatis*, *P. aeruginosa* and *K. pneumonia*, respectively (starting from right)

slowing oxidative damage linked to asthma, infant and adult respiratory distress syndromes and chronic obstructive pulmonary disease were scientifically demonstrated[58].

Antibacterial activities of plant species

Qualitative antibacterial activities assay by bioautography: Different extracts from *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* were examined for antibacterial activities using the bioautography method. The appearances of a clear zones/white spots against the purple-red background on the bioautograms are areas of inhibition of the growth of used bacteria by certain compounds within the plant extracts. The bioautography results displayed a broad range of antibacterial activity compounds present in these extracts (Fig. 4). It worth mentioning that the position of most of these compounds (retention factor values of compounds was calculated but not presented) tallies with those observed during phytochemical profiling of the investigated plant species (Fig. 1). Thus, corroborating the assertion by the Bapedi THs that they prefer *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* due to their efficacy in the treatment and management of RIs and RSs.

There was a differential activity of various extracts against used bacterial species across the 3 mobile phases (BEA, EMW and CEF). This perhaps can be explained by the dissimilar sensitivity of used micro-organism towards the potential anti-bacterial phytochemicals present in the crude extracts on TLC mobile phases (Fig. 1). However, all bioautogram of *S. aureus* had more clear zone inhibition that corresponded with those noted in Fig. 1, thus indicating high level of antibacterial activities compared to mobile phases saturated with other micro-organisms. Analysis of the efficacy of the studied plant species extracts per three mobile phases used in this study showed a clear zones of inhibition by most extracts, that can be compared with bands displayed in Fig. 1. It should be stated that antibacterial activities observed within the plant extracts (at the top of the TLC plates eluted in some mobile phases especially EMW, might be due to synergy of various plant bioactive molecules which did not separate well, hence there are traces of chlorophyll. Importantly, a number of clear zones of inhibition (particularly those that correspond with bands in Fig. 1 exhibited by numerous studied therapeutic extracts against *M. smegmatis*, *K. pneumoniae*, *S. aureus* and *P. aeruginosa* across the different TLC elution systems (Fig. 4), show that the investigated plant species proffer beneficial effects towards alleviation or treatment of bacterial RIs and RSs.

Quantitative antibacterial activities assay by minimum inhibitory concentration: The bioautography results of the different extracts from *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* against tested bacteria strains was complemented by measuring the MIC values (Table 5).

Table 5: Minimal inhibitory concentration (MIC) values and total activity values of four studied plant against four bacterial test organisms

			Species name and MIC values (mg mL^{-1})					
Test organism	Concentration	Plant extracts	*E. axillare*	*C. ternatum*	*C. transvaalensis*	*L. caffer*	Average	Control (µg mL^{-1})
Klebsiella pneumoniae	2.50	A	0.63	1.25	>2.5	1.25	1.41	Ampicillin (0.13)
	1.25	DCM	0.63	1.25	>2.5	1.25	1.41	
	0.625	H	1.25	1.25	>2.5	0.63	1.41	
	0.313	M	0.63	2.5	>2.5	1.25	1.72	
	0.156	W	0.63	>2.5	>2.5	0.63	1.57	
Staphylococcus aureus	2.50	A	2.5	1.25	>2.5	0.63	1.72	Ampicillin (0.08)
	1.25	DCM	>2.5	1.25	>2.5	>2.5	2.19	
	0.625	H	>2.5	>2.5	>2.5	>2.5	10	
	0.313	M	>2.5	>2.5	>2.5	2.5	10	
	0.156	W	>2.5	>2.5	>2.5	>2.5	10	
Pseudomonas aeruginosa	2.50	A	>2.5	>2.5	>2.5	0.63	18.9	Ampicillin (0.13)
	1.25	DCM	>2.5	>2.5	>2.5	>2.5	10	
	0.625	H	>2.5	>2.5	>2.5	>2.5	10	
	0.313	M	>2.5	>2.5	>2.5	>2.5	10	
	0.156	W	2.5	>2.5	>2.5	>2.5	10	
Mycobacterium smegmatis	2.50	A	2.5	1.25	>2.5	0.63	1.72	Rifampicin (0.13)
	1.25	DCM	1.25	>2.5	>2.5	2.5	2.19	
	0.625	H	2.5	>2.5	>2.5	1.25	2.19	
	0.313	M	>2.5	>2.5	>2.5	1.25	2.19	
	0.156	W	1.25	>2.5	>2.5	>2.5	2.19	
Total activities (mL g^{-1})								
Klebsiella pneumoniae	2.50	A	238	016	012	112	378	
	1.25	DCM	079	016	02	008	105	
	0.625	H	04	008	008	015	35	
	0.313	M	492	092	068	152	804	
	0.156	W	333	08	076	696	1113	
Staphylococcus aureus	2.50	A	06	016	012	222	256	
	1.25	DCM	02	016	02	004	24	
	0.625	H	02	004	008	004	18	
	0.313	M	124	092	068	076	360	
	0.156	W	084	08	076	348	516	
Pseudomonas aeruginosa	2.50	A	06	008	012	222	248	
	1.25	DCM	02	008	02	004	16	
	0.625	H	02	004	008	004	18	
	0.313	M	124	092	068	076	360	
	0.156	W	084	08	076	348	516	
Mycobacterium smegmatis	2.50	A	06	016	012	222	256	
	1.25	DCM	04	008	02	004	18	
	0.625	H	02	004	008	004	18	
	0.313	M	124	092	068	152	436	
	0.156	W	168	08	0.76	696	948	

A: Acetone, DCM: Dichloromethane, H: Hexane, M: Methanol, W: Water, MIC values>2.5 were included as >2.5 for the calculation purposes

These values were considered as the lowest concentration of the extract/s that completely inhibits the bacteria growth. In this regards, the lower MIC values depict greater antibacterial effectiveness of the crude extract/s and the opposite of this is true for high values. Extracts with MIC values $\leq$0.1 mg mL^{-1} were considered to have significant activities; moderate activities were between 0.1 and 0.63 mg mL^{-1} and weak or poor activities with MICs[59] >0.63 mg mL^{-1}. Generally, the MIC values of plants in the present study ranged between 0.63-2.5 mg mL^{-1} after 24 h of incubation and there was no substantial difference in values observed between most plant extracts against tested strains, thus indicating that these extracts have the same inhibitory potential. With the exception of *L. caffer*, no other record, except our observations amongst the Bapedi, could be located regarding the MIC of the investigated plants. Pavithra *et al.*[29] found that hexane and methanol extracts of *E. axillare* harvested in India are ineffective against *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. Likewise, in the present study these extracts did show activities against *S. aureus* and *P. aeruginosa*, but were effective against *K. pneumoniae* with MIC values of 1.25 and 0.625 mg mL^{-1}, respectively. This disjuncture may be due to the absence of antibacterial constituents present in the crude extracts of *E. axillare* collected in Indian. This is because the number of *E. axillare* phytochemical constituents in our study were higher compared to Pavithra *et al.*[29] study.

Table 6: Minimum bactericidal concentration (MBC)

		Bacterial strains and plant extracts																			
		M. smegmatis					*K. pneumoniae*					*S. aureus*					*P. aeruginosa*				
Species	Concentration	A	D	H	M	W	A	D	H	M	W	A	D	H	M	W	A	D	H	M	W
E. axillare	2.50	+	+	+	+	+		+			+						+	+	+		
	1.25						+			+			+								
	0.625								+			+			+	+					
	0.313													+						+	+
	0.156																				
C. ternatum	2.50					+	+				+					+	+	+			
	1.25							+													
	0.625		+						+	+		+			+					+	
	0.313													+							
	0.156	+			+							+									
	0.078			+																	
	0.039																				
	0.019																				+
C. transvaalensis	2.50	+	+		+	+	+	+		+			+								
	1.25													+							
	0.625			+					+												
	0.313														+	+					
	0.156											+					+	+	+	+	+
L. caffer	2.50		+			+	+	+		+	+	+			+						
	1.25			+																	
	0.625	+			+				+				+	+							
	0.313															+					
	0.156																+	+	+	+	+

A: Acetone, D: Dichloromethane, H: Hexane, M: Methanol, W: Water

With the exception of all *C. transvaalensis* extracts which did not show any activities against four used bacteria species perhaps due to the insensitivity of this species to substances contained in the extracts, some extracts from the rest of the plants exhibited certain degrees of antibacterial activities against a particular bacteria strain/s tested (Table 5). None of the extract displayed noteworthy antibacterial activities. The moderate MIC value of 0.63 against the bacteria species were obtained with the stated extracts of *E. axillare* (acetone, dichloromethane, methanol and water against *K. pneumonia*) and *L. caffer* (hexane and water against *K. pneumonia* and acetone against *S. aureus*, *P. aeruginosa* and *M. smegmatis*). This finding confirms that *E. axillare* and *L. caffer* extracts contains secondary metabolites with potent antibacterial activities. However, it should be reported that the resistance of used bacteria in this study to some of the plant extracts using MIC assay may be due to insufficient amount of active ingredients. This speculation is based on the fact that same extracts portrayed antibacterial activities on bioautography assay.

The total activities values of four medicinal plant extracts investigated are also given in Table 5. Generally, these activities indicate the volume to which the bioactive compounds extracted from 10 g of plant material can be diluted and still inhibits growth of bacteria. The highest total activities were found in water extract of *L. caffer* (696 mL g^{-1}) against *M. smegmatis* and *K. pneumonia* compared with the other extracts, followed by same extract from this species (5125 mL g^{-1}) against *S. aureus* and *P. aeruginosa*. Other extracts which showed greater total activities includes *E. axillare* methanol (492 mL g^{-1}) and water (333 mL g^{-1}) against *K. pneumonia*. This finding came as no shock as both species had high percentage extract yield (Table 3). According to Eloff[60], total activities is dependent on the quantity of material extracted from the dried plant parts, meaning that the higher the total activities, the greater is the potential for application of the specific plant extract. Therefore, it is acceptable to state that all plant species extracts which yielded the moderate or lowest total activities against one or more bacteria is due to the lowest number of antibacterial compounds extracted. Overall, the plant extracts with higher total activities values against the tested bacterial strains should be selected for further fractionation.

Quantitative antibacterial activities assay by minimum bactericidal concentration: Minimum bactericidal concentration (MBC = lowest concentration that killed the bacteria) of all *C. ternatum*, *C. transvaalensis*, *E. axillare* and *L. caffer* extracts were determined at various concentrations tested over a 42 h period (Table 6), to

further assess the therapeutic value of the plants implicated by Bapedi THs in the treatment of RIs and RSs. Minimum bactericidal concentration of these extracts ranged from 0.019-2.5 mg mL^{-1}.

Unexpectedly, the MBC values were greater than the MIC values and all extracts which depicted poor or no activities on the MIC were bactericidal. Although not specifically for the investigated species, this finding was previously reported by other researchers[61-63]. Nevertheless, the observed disjunction between MBC and MIC values in the present study might be due to the strains high resistance, attributed to thick outer membrane in their cell wall acting as an obstacle for the plant extracts permeability over 24 h of exposure via MIC assay. Therefore, as these extracts perhaps fully came in contact with the test organisms over a longer duration (42 h period), they were able to completely eliminate organisms at various concentration. Generally, *C. ternatum* hexane and water extracts showed strongest bactericidal activities with MBC values of 0.078 and 0.019 mg mL^{-1} against *M. smegmatis* and *P. aeruginosa*, respectively. All extracts from *C. transvaalensis* and *L. caffer* were also more effective (0.156 mg mL^{-1}) in killing *P. aeruginosa*. This suggests that these plants, when prescribed orally by Bapedi THs as antibacterials for RIs and RSs, might inhibit bacterial growth and ultimately kill it.

CONCLUSION

Findings of the present study indicated that crude extracts from four medicinal plants commonly used by Bapedi THs to cure and manage RIs and RSs contain diverse secondary metabolites active against bacterial strains causing these diseases. These findings therefore, provide support for the use of these plants in traditional medicine for RIs and RSs and could potentially be used as alternative therapeutic agents. Studies focusing on isolation, identification and characterization of the pure compounds responsible for antibacterial activities of *C. ternatum*, *C. transvaalensis* and *L. caffer* extracts and cytotoxicity of these compounds to determine any potential toxicity are in progress.

SIGNIFICANCE STATEMENT

This study is the first to report on the phytochemical constituents and biological activities of *C. ternatum*, *C. transvaalensis* and *L. caffer*. Overall, the study showed that the extracts from all the investigated four species can be beneficial in the treatment of RIs and RSs. A better understanding of these species' phytochemical constitutes and biological activities will help the researchers to further investigate the possibilities of developing new herbal drugs that can be utilized against RIs and RSs.

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