Antimicrobial activities of *Lippia javanica* from Eastern Cape Province of South Africa

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Research Article

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Abstract

Background and Aims

Lippia javanica is an aromatic plants with several biological active compounds including the essential oils. The antimicrobial activity of the essential oil extract from the fresh and dried leaves of *Lippia javanica* was validated.

Methods

The essential oil was extracted from fresh and dried leaves and the composition were identified using GC-MS. The brine shrimp lethality test was used to assess the toxicity of the oils. Antibacterial and antifungal activity of the plant extracts were determined on bacteria and fungi strains. One-way analysis of variance (ANOVA) was used to compare the data among the plant fractions at 0.05% alpha level.

Results

The essential oils obtained from the fresh and dried leaves of *Lippia javanica* have similar chemical composition with most documented reports on other *Lippia* species. Nineteen active compounds were found to be present in all test oils of *Lippia javanica*. No significant variations between the aromatic profiles of the fresh and dried leaves, but the extracted oil yielded more in the dried leaves than the fresh leaves (2.7%: 0.7%). The brine shrimp assay of the essential oils extracted from both fresh and dried leaves of *Lippia javanica* showed LC_{50} values of 35.7 and 48.7 µg/mL. The oils exhibited a concentration dependent toxicity against brine shrimp. *Lippia javanica* shows more antibacterial and antifungal potency than the control drug.

Conclusion

Based on this study, it can be inferred that the essential oil obtained from the leaves of *Lippia javanica* are less toxic and have high antimicrobial potency.

Introduction

Lippia javanica (Burm.f.) Spreng *javanica* is an erect woody perennial herb or shrub plant species which belongs to the family of Verbenaceae and the genus *Lippia* with more than 200 varied species and several taxa (Asowata-Ayodele et.al. 2016; Maroyi 2017). *Lippia javanica* can be found to occur naturally in sub- Saharan Africa where it is native to coutries like Nigeria, Botswana, Angola, Democratic Republic of Congo, Central African Republic, Ethiopia and Kenya. It was also recorded to be abundant in Southern Africa such as Malawi, Mozambique, South Africa and in tropical Indian subcontinent (Kumar and Dash 2012; Narayana and Narasimharao 2015). Locally, *Lippia javanica* is known as Izinziniba in South Africa, Kesse in Ethiopia, in Nigeria and some other tribes in Africa. *Lippia javanica* is generally known as the fever tea tree or lemon bush due to its strong aromatic and scented leaves which give off a lemon-like fragrance odour when pressed (VanWyk and N. Gericke 2000). *Lippia javanica* can grow up to 1–2 m

high (Fig. 1) and is characterized by brownish stems which is usually erect, spreading with short stiff tubercle-based whitish hairs and small glands, and branched inflorescences nearly in all axils. The leaves of *Lippia javanica* are opposite and in whorls of 3, blades are lanceolate to oblong shape and densely pubescent, rounded and cuneate at the base. The margins are usually crenate-serrate or closely serrulate except near the leaf base (Fernandes 2005; Sandasi et al. 2013; Endris et al. 2015). Flowers of *Lippia javanica* are usually whitish in color and can be yellowish-white to greenish. The fruit is usually a conical or oblong spikes, purple or dull-reddish, dark brownish on drying (Verdcourt 1992; Fernandes 2005)

Lippia javanica has been to proven to have several biological active compounds such as volatile and nonvolatile secondary metabolites including alkaloids, amino acids, flavonoids, triterpenes and iridoids, essential oils and several minerals nutrients (Shikanga et al. 2010;. Madzimure et al. 2011; Katerere et al. 2012; Narzary and Basumatary 2015). Observations from previous studies indicated that the leaves, twigs and flowers of *L. javanica contains* wide variety of nutrients, such as carbohydrates, proteins, fats, and vitamins. Some known minerals including cadmium, chromium, cobalt, copper, manganese, selenium, and zinc (Mahlangeni et al. 2013) which plays certain roles in metabolism have been reported to be present at appreciable amounts in the leaves of *Lippia javanica*. Previous investigation showed that *Lippia javanica* has medicinal value, pharmacological and therapeutic activities. It has been shown that the plants have pesticidal effects (Nyahangare et al. 2015), hepatoprotective effects (Kumar and Pandey 2013), antimalarial (Govere et al. 2000), anticancer (Fouche et al. 2008) antiviral, antioxidant (Shikanga et al. 2010), antiplasmodial (Prozesky et al. 2001) and cytostatic activities (Pascual et al. 2001). Other economic importance which has been reprted on *Lippia javanica* were that the leaves can be use as is used as remedy for a variety of wounds, Pain, Injuries, and skin infections (Coopoosamy and Naidoo 22012), leaves, whole parts of the plants are used as ethnoveterinary medicine (Hutching, 2007; Nyahangare et al. 2012), and also the fresh or air dried leaves are used to treat fever, malaria, and as an insect repellent.

However, prior reports on the chemistry and pharmacology of the essential oil of *L. javanica* justify its ethnopharmacological uses in variety of cultures (Morayi 2017). Essential oil from *Lippia species* has been extensively studied but limited information is available on *Lippia javanica*. Fewer reports are also available on the variation in its chemical components, biological activities of oil extracted from both its fresh and dried leaves of *Lippia javanica*. Deep study on the antimicrobial action of the essential oil of *L. javanica* has not been validated. Although, *Lippia javanica* contains potentially active compounds, the antimicrobial activities of this plant particularly the species collected from Eastern Cape Province of South Africa must be proven, hence this study.

Materials and Methods

Extraction of the Essential oils by steam distillation

One hundred and sixty grams (160 g) of each plant materials were oven dried to constant weights at 40^{O}C. The fresh and dried leaves of *Lippia javanica* were separately subjected to hydro-distillation for 3 h

using a Clavenger unit as described by the British Pharmacopoeia (1980).

Chemical analysis of the essential oils

GC-MS analysis of the oil was carried out using a Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column. Initial temperature was 70%, maximum temperature 325°C, equilibration time 3 min, ramp 4°C/min, final temperature 240°C; inlet: split less, initial temperature 220°C, pressure 8.27 psi, purge flow 30ml/min, purge time 0.02 min, gas type helium; column: capillary, 30 m × 0.25 mm i.d., film thickness 0.25 µm, initial flow 0.7 ml/min, average velocity 32 cm/s; MS: EI method at 70eV.

Identification of components

The individual constituents of the oil were identified by matching their mass spectra and retention indices with those of Wiley 275 library (Wiley, New York) (Joulain and Konig 1998). The yield of each component was calculated per gram of the plant material while the composition was calculated from the summation of the peak areas of the total oil composition.

Brine shrimp lethality test

The brine shrimp lethality test was used to assess the toxicity of the oils and was conducted as described by Okoh and Afolayan (2011) using brine shrimp eggs obtained from Ocean Star International, Inc. Company USA.

The Shrimp eggs were hatched in sea water over 48 hours at room temperature in a glass vials. The naupili (newly hatched shrimps) were attracted to one side of the vials with a light source. Solutions of the oils were made in dimethyl sulfoxide (DMSO), at varying concentrations (100, 80, 60, 40, 20 µg/ml) and incubated in triplicate vials with the brine shrimp larvae. Twenty brine shrimp larvae were placed in a mixture of sea water and amphotericin as a control. After 48hours, the vials were examined against a lighted background and the average number of larvae that survived in each vial was counted.

Antibacterial activity

The reference strains used in this study were chosen based on their pathological effects on human and deterioration of food products: two gram positive (*Staphlococcus aureus* and *Listeria monocytogenes*) and two gram negative (*Salmonella typhimurium* and *Escherichia coli*) bacteria were obtained from the Department of Microbiology, University of Fort Hare.

The agar well diffusion based method of Deans and Ritchie (1987) modified by Oyedeji et al. (2009) was used to determine the susceptibility of bacteria to the essential oil. A 100 µL of 18 hours bacterial cultures were used to spread a bacterial lawn on nutrient agar. The cultures were adjusted to approximately 10^5 CFU/mL using McFarland standard. Twenty five microliters (25 µL) of various concentrations of plant extracts were added to each well (diameter of 4 mm) bored on nutrient agar plates under aseptic condition. The plates were left for 30 minutes at room temperature for the diffusion of the extracts and incubated at 37 ℃ for 18 hours. The diameters of zones of inhibition (mm) were

measured after 18 hours using a ruler. Each concentration of the extract was repeated three times. The minimum inhibitory concentration (MIC) of *Lippia javanica* extracts against the test bacteria was determined by agar dilution method as described by the National Committee for Clinical Laboratory Standards (2004). The MICs were determined as the lowest concentrations of the extract resulting to complete inhibition of visible growth of the test organisms.

Antifungal assay

Pathogens and media

The fungi used in this study were chosen primarily on the basis of their importance as pathogens of humans. Strains from the American Type Culture Collection (ATCC) were used: *Aspergillus fumigatus* ATCC 204305, *Aspergillus niger* ATCC 16888, *Microsporium canis* ATCC 36299, *Microsporium gypseum* ATCC 24102, *Trichophyton tonsurans* ATCC 28942, *Trichophyton rubrum* ATCC 28188, *Trichophyton mucoides* ATCC 201382, *Pennicullin aurantiogrieseum* ATCC 16025 and *Pennicullin chrysogenum* ATCC 1010. Both Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were prepared according to the manufacturer's instructions. The fungi were maintained at 4°C on SDA plates and the inoculum for the assays was prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to 0.5 McFarland standards and confirmed by spectrophotometric reading at 580 nm (Afolayan, 2003; Duarte et al. 2005). Cell suspensions were finally diluted to 10 4 CFU mL − 1 for the use in the assays.

Antifungal susceptibility assays

The agar diffusion and microdilution methods were used to determine the antifungal activities of the plant extracts against the fungi (Shad et al. 2008; Otang et al. 2012).

Agar well diffusion assay

The agar diffusion assay was carried out with slight modifications (Samie et al. 2010). Using the micropipette, 100 µL of 0.5 Mcfarland solution of each fungus culture in 0.85% sterile distilled water (SDW) was placed over the surface of an agar plate, and spread using a sterile inoculation loop. The same procedure was followed for the other fungi. Using a sterile cork borer, four holes (5 mm in diameter) were punched in each of the culture plates. In the first hole, 50 µL of a positive control drug was added (Nystatin); 50 µL of the corresponding extract solvent was added as a negative control in the second hole; and 50 µL of the plant extract was added in the third and last holes at concentrations of 25 and 50 mg/mL respectively. Each test was duplicated. The culture plates were then incubated at 37°C and the results were observed after 24 hours to 6 days depending on each fungal growth. The clear zone around each well was measured in mm, indicating the activity of the plant extracts against the fungal organisms.

Microdilution assay

The microdilution method was employed to determine the minimum inhibitory concentration (MIC) of the plant extracts using 96 well microtitre plates (Samie *et al.*, 2010). Initially, 120 µL of sterile distilled water

was added into each well of the first (A) and last (H) rows and also into all the wells of the last column (Dagne et al. 1996). Then, 120 µL of Sabouraud dextrose broth was added into each well of the second row (B) and 150 µL of Sabouraud dextrose broth was added into the remaining wells of the first column and 100 µL into the rest of the wells from the second column rightward. Fifty microliters of the plant extract were then added into the third well of the first column while 50 µL of the positive and negative control were separately added into the remaining wells of the first column. A two-fold serial dilution was done by mixing the contents in each well of the first column (starting from the third row) and transferring 100 µL into the second well of the same row and the procedure was repeated up to the 11th well of the same row and the last 100µL from the 11th well was discarded. Hence various concentrations of the plant extracts ranging from 0.005 mg/mL to 5 mg/mL were prepared in the wells, following the two-fold dilution method. Thereafter, 20 µL of 0.5 McFarland fungal suspensions was inoculated into the wells. The growth of the fungi was measured by determining the absorbance at 620 nm with a microtitre plate reader before and after incubation. The plates were incubated at 37°C at various durations (24 hours to 48 hours). The lowest concentration which inhibited the growth of the fungi was considered as the MIC of the extract.

Determination of the minimum fungicidal concentration (MFC).

The MFC was determined by inoculating the contents from the MIC plates onto Sabouraud dextrose agar plates, and the results were observed after incubation at 37°C at various durations depending on the fungi. The presence of the fungal colonies on the agar plates was an indication that the plant extracts only inhibited the growth of the fungi without killing them and the absence indicated that the plant extracts were able to kill the fungal organisms (Marimon et.al. 2007). The smallest concentration of the plant extracts that was able to kill the microorganisms was considered as the minimum fungicidal concentration (MFC).

Statistical analysis

The experimental data was expressed as mean ± standard deviation (SD) of the three replicates. Statistical analysis was done by using MINITAB program (version 12 for Windows) (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used to compare the data among the plant fractions with the controls. $p < 0.05$ was considered statistically significant.

Results and Discussion

Composition of essential oil extracted from both fresh and dried leaves of Lippia javanica

The results obtained on the composition of essential oil extracted from both fresh and dried leaves of *Lippia javanica* indicated that the dried leaves extracted yielded more oil yield than the fresh leaves (2.7%: 0.7%). This corroborate the previous study of Asekun et.al (2007) who indicated that the essential oil yield of *L. javanica* leaves using the hydrodistillation method was around 2.94%, and the drying

method before hydrodistillation was found to affect the oil yield and chemical constituents of the oil while SFME method yielded 19.75 and 13.46%, from fresh and dried leaves respectively. This results contradicts the report of Adeogun et.al., 2018 an in line with the assertion raised by Asekun et al (2007) that dried plants yielded more oils than the fresh plants.

GC/MS analysis had also identified several chemical compounds present in the essential oil, but 19 were documented in this work, chemotypes Verbanone and Butane were found in relative amounts, up to 26%. The ANOVA analyses revealed no significant variations between the aromatic profiles of the essential oils obtained from individual specimens growing in the two localities. The essential oils were dominated by sesquiterpene hydrocarbon, considerable amounts of monoterpene and oxygenated hydrocarbons, and ketones. The chemical profiles of the essential oils such as cymene, Verbanone, Nonyne, Myrcene, Isophorone, Copaene, Caryophyllene, Germacrene D, Thujene which was more profound in the oil extract from the the dried leaves of *L. javanica* were found to closely resemble that reported for *L alba*, which originating from South America (Linde et.al., 2010), another *Lippia* species. However, the genus Lippia is well known for chemotypical variability in the essential oil composition (Viljoen et al., 2005) and a more comprehensive study involving specimens from localities, differing in climate and geography, should shed more light on this aspect.

Table 1
Composition of essential oil extracted from both fresh and dried leaves of *Lippia javanica* (%)

Components	Oil extracted from fresh leaves samples	Oil extracted from dried leaves samples
Verbanone	5.37	21.46
Nonyne	-	1.40
Myrcene	2.86	-
Isophorone	-	2.70
Copaene	-	0.41
Caryophyllene	-	3.28
Germacrene D	-	3.74
Thujene	-	9.31
Methyl-2-butenoic acid, tridec-2-ynyl-ester	6.18	1.04
Methyl m –tolyl carbinol	-	6.31
Bicyclo –oct-1-ene, 7-exo-ethenyl	-	1.08
Phellandrene	-	3.09
o-cymene	-	1.76
Diazatricyclo dec-9-ene	1.64	4.55
Linalool	-	3.60
Butane	-	26.4
Butylphenol	2.60	8.26
Total (%)	0.7%	90.13
Unknown (%)		9.87
Yield of oil (%)		2.7%

Antimicrobial activity of the leaves of Lippia javanica

The essential oils extracted from dried leaves of *Lippia javanica* had more Zone of Inhibition (ZI) against the four tested human pathogenic bacterial and it gave varying activities on all twith mean zones of inhibition (ZI) ranging from 14.3 mm to 32 mm (Table 2). This implied that the antimicrobial studies shown that the oil of *L.javanica* shown more potency than the control drug used. According to Manenzhe et al. (2004), essential oil of *L.javanica* shown activity against *S. aureus* and *E. coli* with ZI of 18 mm and 16 mm at 10 mg/mL respectively. The same trend was observed in this study for *S. aureus* (14.3 mm).

However, the ZI for *E.coli* was high (32 mm) at 50 mg/mL.,this may be due to the active ingredients that were more profound in the oil extracted from dried leaves. This leaves can be physically compared with *Ocimum grattissicum*, a Nigeria plant commonly called scent leaves. The drier the leaves the better aroma it releases. Likewise the same trend were observed in the Minimum Fungicidal Concentration experiment conducted. According to Viljeon et al. (2005), L. *javanica* essential oil is mainly used traditionally to treat respiratory disorders such as coughs, colds and bronchitis. The moderate antimicrobial activity recorded in this study justifies its use in African traditional medicine for the treatment of colds and flus associated with microbial infections.

Table 2
Minimum fungicidal concentration of oil extracts from *Lippia javanica* from dried samples(mg/mL)

	Af	Mc	Mg	Pa	Pc	Tm	Tr	Tt	An
L. javanica oil	2.5^{a}	2.5^{a}	2.5^{a}	2.5^{a}	2.5^{a}	1.25^{b}	2.5^{a}	2.5^{a}	NT
Control	$> 5^{a}$	2.5^{b}	$> 5^{a}$	$> 5^{a}$	$> 5^{a}$	$< 0.005^{c}$	$> 5^{a}$	2.5^{b}	$> 5^{a}$

Inhibition diameters with the different superscript letters in the same row are significantly different from each other ($P > 0.05$). (NT = not tested, Af *= Aspergillus fumigatus*,Mc *= Microsporum canis*, Mg *= Microsporum gypseum*, Pa *= Penicillium aurantiogriseum*, Pc *= Penicillium chrysogenum*, Tm *= Tricophyton mucoides*, Tr- *T.rubrum*, Tt- *Tricophyton tonsuran*, An *= Aspergillus niger*).

Table 3
Minimum fungicidal concentration of oil extracts from *Lippia javanica* from dried samples(mg/mL)

	Af	Mc	Mg	Pa	Pc	Tm	Tr	Tt	An
L. javanica oil	2.5^{a}	2.5^{a}	2.5^{a}	2.5^{a}	2.5^{a}	1.25^{b}	2.5^{a}	2.5^{a}	NT
Control	$> 5^{a}$	2.5^{b}	$> 5^{a}$	$> 5^{a}$	$> 5^{a}$	$< 0.005^{c}$	$> 5^{a}$	2.5^{b}	$> 5^{a}$

Inhibition diameters with the different superscript letters in the same row are significantly different from each other ($P > 0.05$). (NT = not tested, Af *= Aspergillus fumigatus*,Mc *= Microsporum canis*, Mg *= Microsporum gypseum*, Pa *= Penicillium aurantiogriseum*, Pc *= Penicillium chrysogenum*, Tm *= Tricophyton mucoides*, Tr- *T.rubrum*, Tt- *Tricophyton tonsuran*, An *= Aspergillus niger*).

Brine shrimp assay of the essential oils extracted from both fresh and dried leaves of Lippia javanica

The brine shrimp assay of the essential oils extracted from both fresh and dried leaves of *Lippia javanica* showed LC_{50} values of 35.7 and 48.7 µg/mL respectively (Fig. 2). The oils exhibited a concentration dependent toxicity against brine shrimp, the least concentration of 20 µg/mL used for the cytotoxicity test for both the essential oil extracts and control (Amphotericin) was toxic on the brine shrimps except

for sea water which was used as the negative control. These result supported reports that the essential oils from *Lippia javanica* contains triterpenoids and iridoid glycosides (Olivier et al. 2010). As *Lippia javanica* is also administered in the form of tinctures and teas, it is most probably that the non- volatile compounds could be acting in a synergistic /additive manor to produce enhanced medicinal properties (Viljeon et al. 2005). This may be due to the presence of some monoterpenes in the oil, which are medicinally active to brine shrimp larvae (Okoh and Afolayan 2011). The low LC_{50} values of the essential oils extracted from fresh leaves of *Lippia javanica* probably means that the fresh leaves of *L. javanica* may be less toxic.

Conclusion

The overall antimicrobial activity indicates that the oil of this plant exhibited a higher antifungal and antibacterial activity compared to the reference drugs. This study also showed that the oil from fresh leaves of *Lippia javanica* may be less toxic. Therefore, it may be safer to use fresh leaves of *Lippia javanica* for medicinal and pharmaceutical purposes to reduce side effects. This implies that essential oil from this plant can be a potential source of raw materials for drug development and pesticides.

Declarations

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Conflict of interest: Authors declare no conflicts of interest

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