Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Assessment of the Polyphenolic Content, Free Radical Scavenging, Anti-inflammatory, and Antimicrobial Activities of Acetone and Aqueous Extracts of *Lippia javanica* (Burm.F.) Spreng

Abiola M. Asowata-Ayodele, Gloria A. Otunola, Anthony J. Afolayan

Department of Botany, Medicinal Plants and Economic Development (MPED) Research Centre, University of Fort Hare, Alice 5700, South Africa

Submitted: 16-07-2015 Revised: 03-09-2015 Published: 07-07-2016

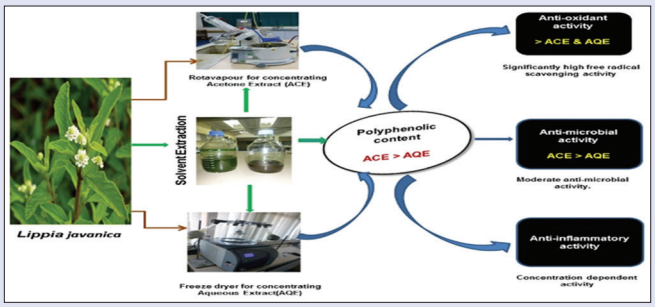
ABSTRACT

Background: *Lippia javanica* (Burm.F) Spreng is one of the spice plants commonly found in almost every part of South Africa. Apart from its culinary uses, it is also traditionally used as an insect repellant and infusion for fever, flu, kidney stone treatment, cough, common cold, and chest pain. **Materials and Methods:** The antioxidant activities of the aqueous and acetone extracts were determined by measuring their effects against 1,1-Diphenyl-2-picryl-hydrazyl, 2,2′azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), nitric oxide, phosphomolybdate, lipid peroxidation, hydrogen peroxide, and reducing power. The antimicrobial activities were evaluated against four bacterial (two Gram-positive, two Gram-negative) strains and 9 fungal pathogens using the agar well diffusion and microdilution methods. Anti-inflammatory activity was assessed by determining the inhibition against protein denaturation and membrane stabilizing effects. **Objective:** The polyphenolic content, free radical scavenging, anti-inflammatory, and antimicrobial activities of the aqueous and acetone extracts of the plant were evaluated. **Results:** A significantly high total phenolic content and free radical scavenging activities were observed in the acetone extracts of the plants. The study also revealed a concentration-dependent inhibition of protein denaturation and membrane stabilization effects by both the aqueous and acetone extracts at the concentrations studied. The ability of *L. javanica* extracts to inhibit protein denaturation and maintain membrane stability could be responsible for its folkloric use. The overall antimicrobial activity indicates that both extracts were active against the bacterial strains but the acetone extract exhibited the most potent antifungal activity higher than even the reference drugs. **Conclusion:** Overall, the acetone extract of *L. javanica* exhibited a more pronounced antioxidant, anti-inflammatory, and antimicrobial effects than the aqueous extract.

Key words: Anti-inflammatory properties, antimicrobial, antioxidant activities, *Lippia javanica*, membrane stability, protein denaturation

SUMMARY

- The polyphenolic content and biological activities of the spice plant, Lippia javanica from South Africa was evaluated
- Significantly high polyphenolic content and free radical scavenging activities were observed for both extracts
- Moderate antimicrobial action, concentration-dependent inhibition of protein denaturation and membrane haemolysis were also observed.



Abbreviations used: AA: ascorbic acid, ABTS: 2,2′azino-bis (3-ethylbenthiazoline-6-sulfonic acid), BHT: Butylated hydroxytoluene, DPPH: 1,1-Diphenyl-2-picryl-hydrazyl, NBT: 2,2′-di-pnitrophenyl-5,5′-diphenyl-(3,3′-dimethoxy-4,4′-diphenylene)- ditetrazolium chloride, PMS: Potassium metabisulfite, ROS: Reactive oxygen species, TBA: Thiobarbituric acid, TCA: Trichloroacetic acid.

Correspondence:

Dr. Gloria A. Otunola,
Medicinal Plants and Economic Development (MPED) Research Centre, Department of Botany, University of Fort Hare, Alice 5700, South Africa.
E-mail: gotunola@ufh.ac.za

DOI: 10.4103/0973-1296.185770

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INTRODUCTION

Biologically active compounds from natural sources have always been of great interest to scientists. It has been documented severally that some naturally occurring substances in plants possess antioxidant activity.[1-4] These naturally occurring substances include a wide variety of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, dietary glutathiones, vitamins, and endogenous metabolites.[5] A large number of phytochemicals belonging to several chemical classes have been reported to show biological activities such as antioxidants, antimicrobial, anti-inflammatory, antiviral, antitumor, antimalarial, antiurolithiatic, and analgesic.[6] It has been reported that plants owe their antimicrobial properties mostly to the presence of alkaloids, phenols, glycosides, steroids, essential oils, coumarins, and tannins.[4,6] Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown

Cite this article as: Asowata-Ayodele AM, Otunola GA, Afolayan AJ. Assessment of the polyphenolic content, free radical scavenging, anti-inflammatory, and antimicrobial activities of acetone and aqueous extracts of *Lippia javanica* (Burm.F.) spreng. Phcog Mag 2016;12:S353-62.

to have radical scavenging activity as part of their mechanism of action.[7]

Many pathological disorders have been associated with oxidative stress and inflammation. Reactive oxygen species (ROS) are involved in a diversity of important pathological processes such as inflammatory and neurodegenerative diseases, atherosclerosis, cancer, and reperfusion injury. Inflammation is the foundation of most chronic diseases; the presence of inflammation makes most disease perceptible to an individual. It can often occur for years before it exists at levels sufficient to be apparent or clinically significant. How long it has been smoldering really determines the degree of severity of a disease and often the prognosis assuming the inflammation can be controlled. Hence, one could also argue that without inflammation most disease would not even exist.[8]

The mechanism of inflammatory injury is attributed in part to the release of ROS from activated neutrophils and macrophages. This overproduction leads to tissue injury by damaging macromolecules and lipid peroxidation of membranes.[7] Free radicals are important mediators that provoke or sustain inflammatory processes, and consequently, their neutralization by antioxidants and radical scavengers can reduce inflammation.[9] Currently, researchers are searching for powerful and nontoxic natural antioxidants from edible plants not only to prevent autoxidation and lipid peroxidation but also to replace synthetic antioxidants.

Lippia javanica (Burm.F.) Spreng belongs to the family *Verbenaceae,* and the genus includes about 200 species.[10-13] It is a woody shrub with aromatic leaves that gives a lemon-like smell, which probably accounts for its common name lemon bush plant. The plant is widely distributed in the Eastern Cape Province of South Africa, and different bioactive compounds have been isolated from it. The plant is traditionally used as a culinary spice and medicinally to treat coughs, colds, fever, chest ailments, kidney stones, measles, rashes, and stomach problems.[12] The main objective of this study was to evaluate the phenolic content, antioxidant, anti-inflammatory, and antimicrobial activities of the acetone and aqueous extracts of the aerial parts of this plant to validate some of its traditional medicinal uses.

MATERIALS AND METHODS

Collection and preparation of the plant

The fresh leaves of *L. javanica* were collected in June 2014 from the University of Fort Hare research farm in Alice, Eastern Cape. It was authenticated by Tony Dold in Albany Herbarium, Rhodes University, and voucher specimen (ASO/01) was deposited at Giffen herbarium of the University of Fort Hare. The aerial parts of *L. javanica* were oven-dried at 40°C, milled into fine powder, packed into airtight plastic bottles, and stored at 4°C until needed. The acetone extract was obtained by extracting 100 g of the powdered plant in 70% acetone (560 mL) and shaking for 24 h in an orbital shaker. The extract was filtered using a Buchner funnel and a Whatman No. 1 filter paper. The filtrate was then concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heidolph, Germany). For the aqueous extract, 50 g of the powdered plant was extracted in 1000 mL of distilled water heated at 100°C for 10 min. It was allowed to cool, filtered, and then freeze-dried (Vir Tis bench top K, Vir Tis Co., Gardiner, NY, USA). The freeze-dried sample was reconstituted with distilled water to give the desired concentrations used in the study.

Chemicals and reagents

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), absolute ethanol, ascorbic acid (AA), Folin–Ciocalteu phenol reagent, sodium carbonate, aluminum chloride, vanillin, sodium acetate, phosphate buffer, potassium ferricyanide ($K_3Fe[CN]_6$), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), potassium metabisulfite, 2,2'-di-p-Nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenyl)- ditetrazolium chloride catechin, quercetin, 2,2'azino-bis (3-ethylbenthiazoline- 6-sulfonic acid) (ABTS), potassium persulfate, sodium nitroprusside, hydrogen peroxide (H_2O_2), sulfanilic acid, glacial acetic acid, butylated hydroxytoluene (BHT), and tannic acid were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Butanol-HCl reagents (butanol-HCl, 95:5 v/v) and ferric reagents were also purchased from Merck Chemical Supplies (Darmstadt, Germany). All chemicals used including the solvents were of analytical grade.

Determination of total phenols

The amount of phenol was determined spectrophotometrically using the modified method of Wolfe *et al.*,[14] with Folin–Ciocalteu reagent. About 1 mg/mL aliquot of the extract was mixed with 5 mL Folin–Ciocalteu reagent (previously diluted with water at a concentration of 1:10 v/v) and 4 mL (75 g/L) of sodium carbonate. The tubes were vortexed for 15 s and left to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using the AJI-C03 ultraviolet-visible spectrophotometer. Results were expressed as mg/g of tannic acid equivalent (TAE) using the calibration curve:

$Y = 2.0573x + 2.635$, $R^2 = 0.9985$

where x is the concentration and Y is the TAE.

Estimation of total flavonoids

Total flavonoid content was determined using the method of Ordonez *et al.*[15] A volume of 0.5 mL of 2% $AlCl_3$ ethanol solution was added to 0.5 mL of the sample solution and allowed to stand for 1 h at room temperature. The absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Plant extracts were evaluated at a concentration of 0.1 mg/mL. Total flavonoid content was calculated as mg/g of quercetin using the following equation based on the calibration curve:

$Y = 0.3705x + 1.1779$, $R^2 = 0.7601$

where x is the concentration and Y is the quercetin equivalent.

Determination of total flavonols

Total flavonol content was determined by adopting the procedure described by Kumaran and Karunakaran.[16] The mixture consisted of 2.0 mL of the sample, 2.0 mL of $AlCl_3$ which was prepared in ethanol, and 3.0 mL of (50 g/L) sodium acetate solution. The absorbance at 440 nm was measured after 2.5 h at 20°C. Total flavonol content was calculated as mg/g of quercetin equivalent from the calibration curve using the equation:

$Y = 0.3705x + 1.1779$, $R^2 = 0.7601$

where x is the concentration and Y is the quercetin equivalent.

Determination of total proanthocyanidin

The method of Sun *et al.*[17] was used for the determination of total proanthocyanidin. To 0.5 mL of 1 mg/mL of the extract, 3 mL of vanillin-methanol (4% v/v) was added; 1.5 mL of hydrochloric acid was also added and vortexed. The mixture was allowed to stand for 15 min at room temperature, and the absorbance was measured at 500 nm. Total proanthocyanidin content was evaluated at a concentration of 0.1 mg/mL and expressed as catechin equivalent (mg/g) using the calibration curve equation:

$Y = 0.6845x + 0.7147$, $R^2 = 0.7932$

where x is the concentration and Y is the catechin equivalent.

Antioxidant assays

The antioxidant activities of the aqueous and acetone extracts were determined by measuring their effect against DPPH, ABTS, reducing power, lipid peroxidation, H_2O_2, nitric oxide (NO), and phosphomolybdate.

Determination of ferric reducing power of the extracts

The reducing power of the plant extracts was evaluated according to the method described by Shiddhuraju *et al.*[18] The mixture containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 mL of the extracts and standards (0.025–0.5 mg/mL) prepared in distilled water. The resulting mixture was incubated for 20 min at 50°C, followed by the addition of 2.5 mL of TCA (10% w/v), which was then centrifuged at 3000 rpm for 10 min. A volume of 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1% w/v). The absorbance was then measured at 700 nm against the blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

1,1-Diphenyl-2-picryl-hydrazyl radical scavenging assay

The method of Liyana-Pathirana and Shahidi[19] was used for the determination of scavenging activity of DPPH free radical. DPPH (1 mL, 0.135 mM) prepared in methanol was mixed with 1.0 mL of aqueous extract ranging from 0.025 to 0.5 mg/mL. The mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured at 517 nm. The scavenging ability of the plant extract was calculated using the equation:

DPPH scavenging activity (%) = ([Abs control − Abs sample]/[Abs control]) × 100

where Abs control is the absorbance of DPPH + methanol and Abs sample is the absorbance of DPPH radical + sample (sample or standard).

2,2′azino-bis (3-ethylbenthiazoline-6-sulfonic acid) radical scavenging activity

The method described by Pandey *et al.*[20] was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate in equal amounts and allowed to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1 mL ABTS + solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm after 7 min using a spectrophotometer. The percentage inhibition (% inhibition) of ABTS + by the extract was calculated as follows:

% inhibition = ([Abs control − Abs sample]/[Abs control]) × 100.

Nitric oxide scavenging activity

The modified method described by Green *et al.*[21] was used to determine the NO radical scavenging activity of aqueous and acetone extracts. A volume of 2 mL of 10 mM of sodium nitroprusside prepared in 0.5 mM phosphate buffered saline (pH 7.4) was mixed with 0.5 mL of plant extracts, gallic acid, and BHT individually at 0.025–0.5 mg/mL. The mixture was incubated at 25°C for 150 min. Then, 0.5 mL of the incubated solution was mixed with 0.5 mL of Griess reagent (1.0 mL sulfanilic acid reagent [0.33% prepared in 20% glacial acetic acid] at room temperature for 5 min with 1 mL of naphthylene diamine dichloride [0.1% w/v]). The mixture was incubated at room temperature for 30 min followed by the measurement of the absorbance at 540 nm. The amount of NO radicals inhibited by the extract was calculated using the following equation:

NO radical scavenging activity (%) = ([Abs control − Abs sample]/[Abs control]) × 100

where Abs control is the absorbance of NO radicals + methanol and Abs sample is the absorbance of NO radical + extract or standard.

Hydrogen peroxide scavenging activity

H_2O_2 scavenging activity of the plant extract was estimated as described by Ruch *et al.*[22] Plant extract (4 mg/mL) prepared in distilled water at various concentrations was mixed with 0.6 mL of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated for 10 min. The absorbance of the solution was measured at 230 nm using the Biomate thermospectronoic, against a blank solution containing the plant extract without H_2O_2. The amount of H_2O_2 radical inhibited by the extract was calculated using the following equation:

H_2O_2 radical scavenging activity = ([Abs control − Abs sample])/(Abs control) ×100

where Abs control is the absorbance of H_2O_2 radicals + methanol and Abs sample is the absorbance of H_2O_2 radical + sample or extract or standard.

Estimation of lipid peroxidation

A modified TBA-reactive species assay described by Dasgupta and De[23] was used to measure the lipid peroxide formed, using liver homogenates as lipid-rich media. Liver homogenate (0.5 mL, 10% in distilled water, v/v) and 0.1 mL of the plant extracts were mixed in a test tube, and the volume was made up to 1 mL with distilled water. Finally, 0.05 mL $FeSO_4$ (0.07 M) was added to the mixture and incubated for 30 min to induce lipid peroxidation. Thereafter, 1.5 mL of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8% TBA (w/v) (prepared in 1.1% sodium dodecyl sulfate) and 0.05 mL 20% TCA were added, vortexed, and heated in a boiling water bath for 60 min. After cooling, 5.0 mL of n-butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. The control was made up of $FeSO_4$ + acetic acid + TBA + SDS + TCA + butanol − the extract.

Phosphomolybdate assay

The total antioxidant activity of the plant sample was determined using phosphomolybdenum method according to the protocol of Rabia *et al.*[24] The plant extract 0.1 mL was taken into test tubes and dissolved in 1 mL of reagent solution containing 0.6 M sulfuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate. Then, the test tubes were covered with silver foil and incubated in a water bath at 95°C for 95 min. The sample was allowed to cool at room temperature after which the absorbance of the mixture was measured at 765 nm against a blank. AA was used as standard. Higher absorbance indicates higher total antioxidant potential.

Anti-inflammatory assay

In vitro protein denaturation

The reaction mixture (0.5 mL, pH 6.3) consisted of 0.45 mL of bovine serum albumin (5% aqueous solution) and 0.05 mL of distilled water; pH was adjusted at 6.3 using 1 N HCl (83 mL of concentrated HCl was diluted in a volumetric flask to 1000 mL). Around 1000 µg of *L. javanica* aqueous and acetone extracts (mg/mL of respective organic solvents) was added to the reaction mixture and was incubated at 37°C for 30 min and then heated at 57°C for 5 min. After cooling the

samples, 2.5 mL of phosphate buffer was added. Turbidity was measured spectrophotometrically at 600 nm. For negative control, 0.05 mL distilled water and 0.45 mL of bovine serum albumin were used. The % inhibition of protein denaturation was calculated as follows.[25]

% inhibition of protein denaturation = ([Abs control – Abs sample]/[Abs control]) × 100

Ex vivo membrane stabilizing activity

Preparation of erythrocyte suspension

The red blood cell (RBC) was obtained with heparinized syringes from a rat through cardiac puncture. The blood was washed 3 times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 min at 3000 g.

Induced rat erythrocyte hemolysis

Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte hemolysis.[26] The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (0.25–2.0 mg/mL) or diclofenac (0.1 mg/mL). The control sample consisted of 0.5 mL of RBC mixed with hypotonic buffered saline solution alone. The mixtures were incubated for 10 min at room temperature (25°C) and centrifuged for 10 min at 3000 g (13,200 rpm). The absorbance of the supernatant was measured at 540 nm. Membrane stabilization of the extract was determined to be the % inhibition of hemolysis and calculated as follows:

% inhibition of hemolysis = 100 × (OD1 − OD2/OD1)

where OD1 = optical density of hypotonic buffered saline solution alone and OD2 = optical density of test sample in hypotonic solution.

Antimicrobial assay

Bacterial strains

The reference strains used in this study were chosen based on their pathological effects on human and deterioration of food products: Two Gram-positive (*Staphylococcus 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[27] modified by Oyedeji *et al.*[28] was used to determine the susceptibility of bacteria. A 100 µL of 18 h bacterial cultures was 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 was added to each well (diameter of 4 mm) bored on nutrient agar plates under aseptic condition. The plates were left for 30 min at room temperature for the diffusion of the extracts and incubated at 37°C for 18 h. The zones of inhibition were measured after 18 h using ruler. Each concentration of the extract was repeated 3 times. The minimum bactericidal concentration of *L. javanica* extracts against the test bacteria was determined by agar dilution method as described by the National Committee for Clinical Laboratory Standards.[29] The minimum inhibitory concentrations (MICs) were the lowest concentrations of the extract resulting in completing 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 opportunistic pathogens of humans. Strains from the American Type Culture Collection (ATCC) were used: *Aspergillus fumigatus* ATCC 204305, *Aspergillus niger* ATCC 16888, *Microsporum canis* ATCC 36299, *Microsporum gypseum* ATCC 24102, *Trichophyton tonsurans* ATCC 28942, *Trichophyton rubrum* ATCC 28188, *Trichophyton mucoides* ATCC 201382, *Penicillium aurantiogriseum* ATCC 16025, and *Penicillium 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.[30,31] 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 opportunistic fungi.[32,33]

Agar well diffusion assay

The agar diffusion assay was carried out with slight modifications.[34] 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; 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 h, up 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 MIC of the plant extracts using 96-well microtiter plate.[34] Initially, 120 µL of SDW was added into each well of the first (A) and last (H) rows and also into all the wells of the last column.[35] Then, 120 µL of SDB was added into each well of the second row (B) and 150 µL of SDB 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 was then added into the third well of the first column while 50 µL of the positive and negative control was separately added into the remaining wells of the first column. A 2-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 11^{th} well of the same row and the last 100 µL from the 11^{th} 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 2-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 microtiter plate reader before and after incubation. The plates were incubated at 37°C at various durations (24 h to 48 h). The lowest concentration which inhibited the growth of the fungi was considered as the MIC of the extract.

Determination of the minimum fungicidal concentration

The minimum fungicidal concentration (MFC) was determined by inoculating the contents from the MIC plates onto SDA 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 extract only inhibited the

growth of the fungi without killing them, and the absence indicated that the plant extract was able to kill the fungal organisms.[36] The smallest concentration of the plant extract that was able to kill the microorganisms was considered MFC.

Statistical analysis

Results were expressed as mean ± standard deviation of three determinations. One-way analysis of variance was used to determine the differences of means among the samples. A significant difference was considered at the level of $P < 0.05$.

RESULTS

Polyphenolic constituents

The flavonoid and flavonol contents of *L. javanica* acetone and aqueous extracts were expressed as quercetin equivalents while total phenol and proanthocyanidin were expressed as tannic acid and catechin equivalents, respectively. The acetone extract of *L. javanica* showed a higher content of phenol, flavonoid, and proanthocyanidin compared to the aqueous extract [Table 1]. This could be attributed to the better extracting power of acetone over water. According to Eloff,[37] acetone extracts are more bioactive components from plants. Further, the lower total polyphenolic content could be as a result of extraction by boiling which has been reported to reduce/destroy total phenolic contents in some foods.[38] The total phenolic content of acetone and aqueous extracts were 4.49 ± 0.411 mg/g and 3.73 ± 0.498 mg/g TAE, respectively.

Free radical scavenging activity

2,2'azino-bis (3-ethylbenthiazoline-6-sulfonic acid)

The % inhibition of ABTS by *L. javanica* extracts is shown in Figure 1. All the tested samples inhibited the ABTS radicals, but the acetone extract exhibited a higher inhibitory activity compared to the aqueous extract. The inhibition concentration (IC_{50}) values [Table 2] confirmed that the acetone extract exhibited a greater inhibition than the aqueous extract, as well as comparable activity with the standards. The ABTS radical scavenging ability of the samples can be ranked as rutin > acetone extract > BHT > aqueous extract.

Table 1: Polyphenolic constituents of *Lippia javanica*

Polyphenolic content (mg/g)	Aqueous	Acetone
Phenol	3.73±0.498[a]	4.49±0.411[a]
Flavonol	1.21±0.002[a]	1.53±0.060[b]
Flavonoid	1.30±0.002[a]	1.67±0.002[b]
Proanthocyanidin	0.73±0.002[a]	0.76±0.045[b]

Values on the same row with different superscripts are significantly different ($P \leq 0.05$)

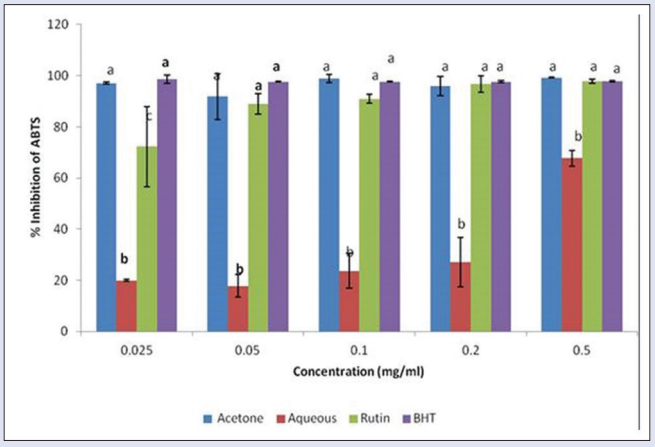

Figure 1: Inhibition of 2,2'azino-bis (3-ethylbenthiazoline-6-sulfonic acid) radicals by acetone and aqueous extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

1,1-Diphenyl-2-picryl-hydrazyl

Figure 2 shows the DPPH radical scavenging capacity of the extracts and standards. The extracts exhibited good DPPH scavenging capacity in the order rutin > aqueous extract > BHT > acetone extract. Although the antioxidant potential of fractions was found to be lower ($P < 0.05$) than those of rutin, the IC_{50} [Table 2] revealed that both the aqueous and acetone extracts have prominent antioxidant activities.

Nitric oxide

NO radical scavenging was in a concentration-dependent manner [Figure 3] and was in the order: Rutin > aqueous extract > BHT > acetone extract. The IC_{50} values obtained for the aqueous extract (0.205 mg/mL) and acetone extract (0.215 mg/mL) showed a comparable activity with the standards rutin (0.184 mg/mL) and BHT (0.222 mg/mL). The data demonstrated that both the aqueous and acetone extracts are potent scavengers of NO.

Lipid peroxidation

The liver homogenate from a rat was used for this assay. The extracts exhibited significant ($P < 0.05$) lipid peroxidation quenching activities in a concentration-dependent manner, reaching the peak at concentrations of 0.5 mg/mL [Figure 4]. The results from IC_{50} values [Table 2] showed that the aqueous extract showed a significantly higher scavenging capacity than the acetone extract though the standards BHT and Vitamin C exhibited the greatest inhibition. The inhibitory capacity is ranked in the order: BHT > Vitamin C > aqueous extract > acetone extract.

Phosphomolybdate

The extracts exhibited a concentration-dependent activity against phosphomolybdate radical in the order: Aqueous extract > gallic acid > Vitamin C > acetone extract [Figure 5]. The IC_{50} value [Table 2] for the aqueous extract was 0.007, while the acetone extract, Vitamin C, and gallic acid had values of 0.703, 0.113, and 0.184 mg/mL, respectively. This is an indication of the potent overall antioxidant capacity of the aqueous extract of *L. javanica*.

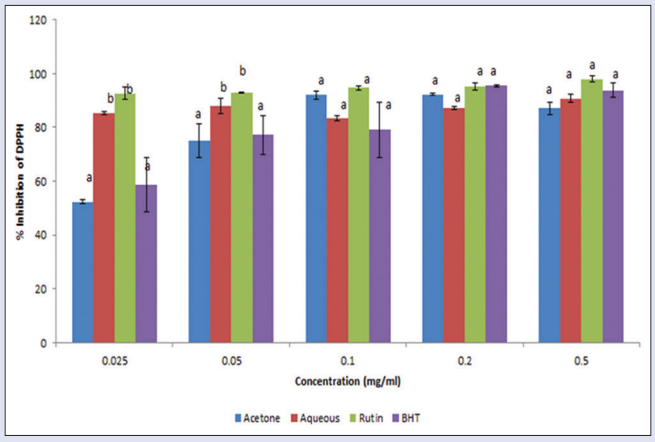

Figure 2: Inhibition of 1,1-Diphenyl-2-picryl-hydrazyl radicals by acetone and aqueous extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

Reducing power

The potentials of the plant extracts to reduce Fe^{3+} to Fe^{2+} by electron transfer are an indication of their antioxidant ability. The reducing power of the extracts in comparison with the standards (BHT, rutin, and Vitamin C) is presented in Figure 6. BHT and Vitamin C exhibited better-reducing power compared to the extracts while the aqueous extract exhibited a higher reducing power compared with the acetone extract.

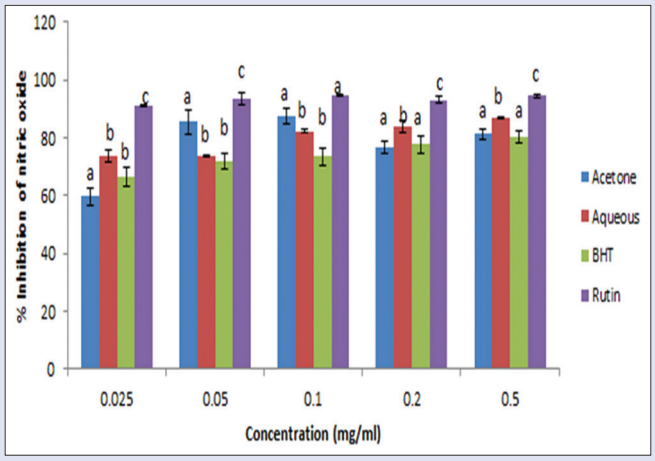

Figure 3: Inhibition of nitric oxide radicals by acetone and aqueous extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

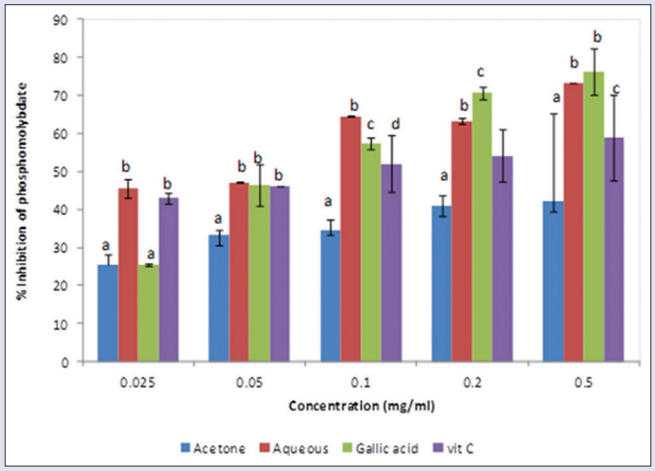

Figure 5: Percentage inhibition of phosphomolybdate radicals by acetone and aqueous extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

Hydrogen peroxide

The acetone extract exhibited a strong activity against H_2O_2 compared to the aqueous extract and standards [Figure 7]. The order of activity was aqueous extract > Vitamin C > BHT > acetone extract.

Anti-inflammatory activity

Results on anti-inflammatory activity of the plant extracts showed that both extracts were able to inhibit protein denaturation in a concentration-dependent manner [Figure 8]. Aqueous extract was very active at IC_{50} = 0.250 mg/mL followed by the acetone extract 0.300 mg/mL

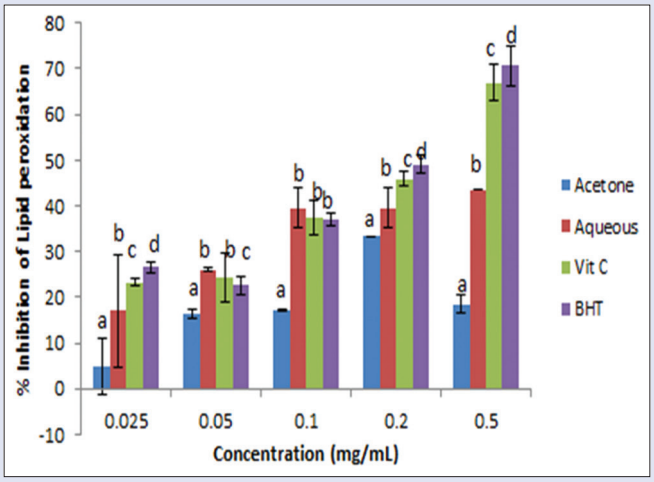

Figure 4: Percentage inhibition of lipid peroxidation by acetone and aqueous extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

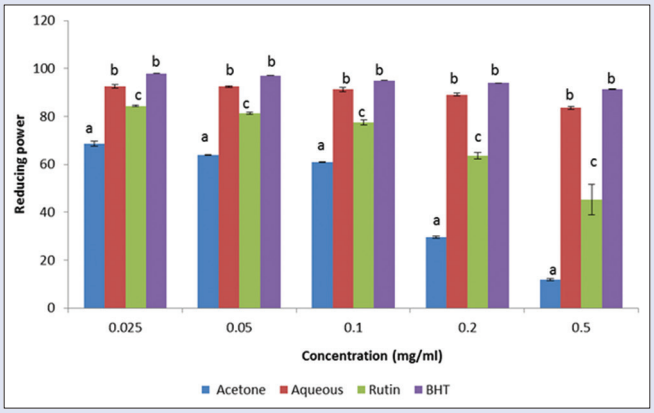

Figure 6: Ferric reducing power effect of acetone and aqueous extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

Table 2: Inhibitory concentrations at 50% of the antioxidant activities of *Lippia javanica* (mg/mL)

	ABTS	DPPH	Reducing power	Nitric oxide	Phosphomolybdate	Lipid peroxidation	Hydrogen peroxide
Acetone	0.177	0.198	0.185	<0.025	0.703*	>0.5*	>0.5*
Aqueous	0.354	0.195	0.200	<0.025	0.007	0.576	>0.5*
BHT	<0.025	<0.025	-	<0.025	-	0.290	>0.5*
Rutin	<0.025	<0.025	0.413	<0.025	-	-	-
Gallic acid	-	-	-	-	0.077	-	-
Vitamin C	-	-	0.220	-	0.09	0.265	>0.5*

*Standard not used or IC_{50} values higher than or lower than the range of concentration. ABTS: 2,2'azino-bis (3-ethylbenthiazoline-6-sulfonic acid); DPPH: 1,1-Diphenyl-2-picryl-hydrazyl; IC_{50}: Inhibitory concentrations at 50%; BHT: Butylated hydroxytoluene

and the anti-inflammatory drug diclofenac with IC_{50} of 0.6905 mg/mL [Figure 9]. The aqueous extract exhibited a higher membrane stability potential, compared with the acetone extract and the anti-inflammatory drug diclofenac [Figure 10]. The IC_{50} values [Figure 9] were 0.228, 0.314, and 0.314 mg/mL for the aqueous, acetone, and diclofenac, respectively.

Antimicrobial activities

The aqueous and acetone extracts of *L. javanica* exhibited antibacterial effect against *S. typhimurium*, *E. coli*, *L. Monocytogenes,* and *S. aureus.* The two extracts showed similar activities on the tested bacteria [Table 3]. In addition, both extracts were active against all the fungi with zones of inhibition varying from 5 to 35 mm; the reference drug, nystatin, was also active on all the tested pathogens, but the most active was the acetone extract [Table 4]. The most susceptible fungi based on the overall mean diameter of growth inhibition were *T. mucoides*, *T. rubrum, M. Gypseum,* and *T. tonsurans*. *A. niger*, *M. canis*, *P. aurantiogriseum,* and *A. fumigatus* were quite resistant to the extracts even at a highest concentration of 5 mg/mL [Table 5].

DISCUSSION

The data presented in this study demonstrate that both acetone and aqueous extracts of *L. javanica* possess excellent antioxidant and free

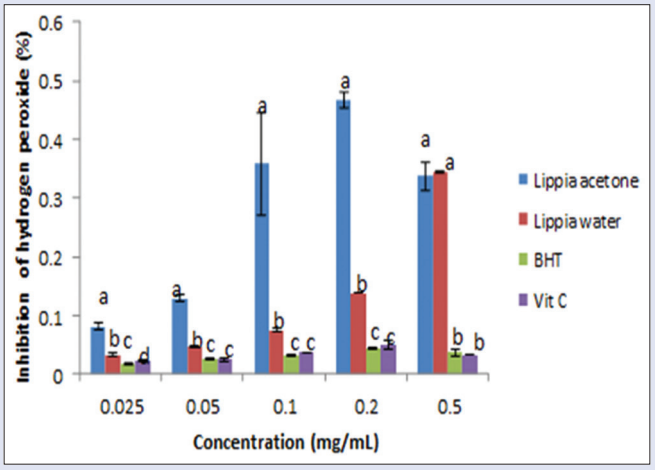

Figure 7: Effect of acetone and aqueous extracts of *Lippia javanica* on hydrogen peroxide radicals. Columns with different letters are significantly different ($P \leq 0.05$)

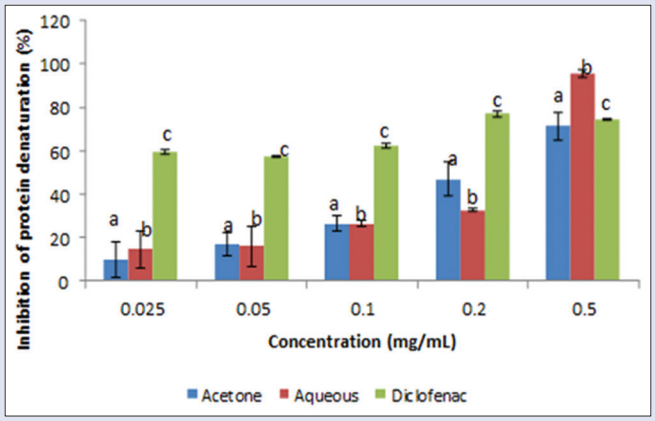

Figure 8: Inhibition of protein denaturation (%) by aqueous and acetone extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

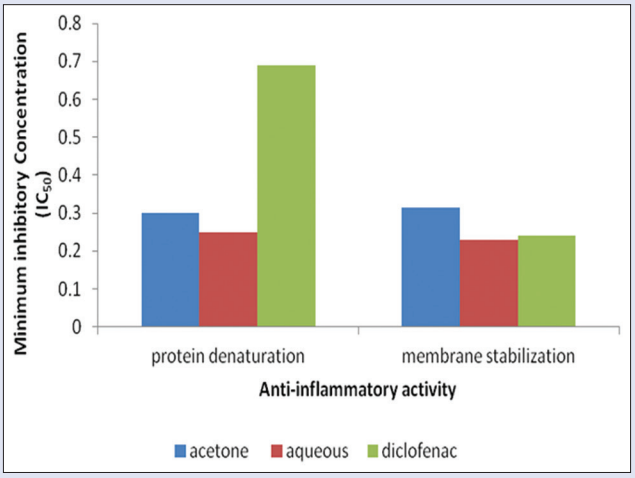

Figure 9: Minimum inhibitory concentration of anti-inflammatory activities of aqueous and acetone extracts of *Lippia javanica*

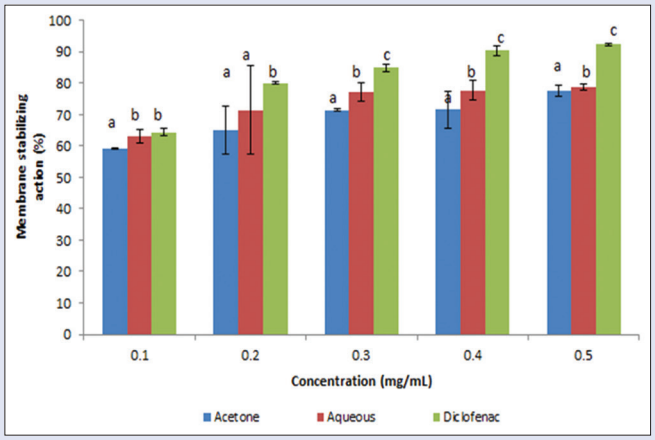

Figure 10: Membrane stabilizing action (%) of acetone and aqueous extracts of *Lippia javanica*. Columns with different letters are significantly different ($P \leq 0.05$)

Table 3: Zone of inhibition (mm) and antibacterial minimum inhibitory concentration of *Lippia javanica* extracts

Sample	*Salmonella typhimurium*		*Escherichia coli*		*Listeria monocytogenes*		*Staphylococcus aureus*	
	ZI (mm)	MIC	ZI (mm)	MIC	ZI (mm)	MIC	ZI (mm)	MIC
Acetone extract	25.0±1.5	0.625[a]	23.0±1.7	0.625[a]	14.3±1.5	1.25[b]	14.3±0.6	1.25[b]
Aqueous extract	24.0±1.0	0.625[a]	20.3±2.0	0.625[a]	14.4±1.5	0.625[a]	14.0±2.0	0.625[a]
Amoxiycillin (standard)	18.3±1.5	<1.25[a]	13.3±1.2	1.25[b]	16.0±0.0	1.25[b]	13.7±1.5	1.25[b]

Values are the mean±SD of the mean; Inhibition diameters with the same superscript letters in the same column are not significantly different from each other (P>0.05). ZI: Zone of inhibition; MIC: Minimum inhibitory concentration; Disc diameter: 4 mm; SD: Standard deviation

Table 4: Antifungal activity of *Lippia javanica* extracts

	Zone of inhibition (mm)								
	Microsporum gypseum	*Penicillium aurantiogriseum*	*Aspergillus niger*	*Trichophyton tonsurans*	*Aspergillus fumigatus*	*Penicillium chrysogenum*	*Trichophyton mucoides*	*Microsporum canis*	*Trichophyton rubrum*
Acetone	12.5±17.7[a]	-	-	12.5±17.7[a]	-	15±21.2[a]	10±14.14[a]	-	11±15.6[b]
Aqueous	-	-	-	12.5±17.7[a]	-	25±7.1[b]	10±0.7[c]	-	13±18.4[b]
Nystatin*	27.5±3.5[b]	25.5±0.71[a]	25±7.1[a]	27.5±10.6[a]	25±0.71[b]	22.5±3.54[c]	30±0.7[d]	-	-

*Concentration of positive control drug=0.03 mg/mL. -: Not active, [a,b,c,d]($P < 0.05$)

Table 5: Minimum fungicidal concentration of *Lippia javanica* extracts

	Aspergillus fumigatus	*Microsporum canis*	*Microsporum gypseum*	*Penicillium aurantiogriseum*	*Penicillium chrysogenum*	*Trichophyton mucoides*	*Trichophyton rubrum*	*Trichophyton tonsuran*	*Aspergillus niger*
Acetone	>5[a]	-	1.25[b]	-	1.25[b]	>5[a]	2.5[c]	2.5[c]	-
Aqueous	-	-	>5[a]	-	<0.005[b]	>5[a]	2.5[c]	2.5[c]	-
Control	>5[a]	2.5[b]	>5[a]	>5[a]	>5[a]	<0.005[c]	>5[a]	2.5[b]	>5[a]

Inhibitory concentrations with different superscript letters in the same row are significantly different from each other ($P<0.05$). -: Not tested

radical scavenging activities. The observed *in vitro* activities suggest that the plant extracts could exert protective effects also *in vivo* against oxidative and free radical injuries occurring in different pathological conditions. According to Saeed *et al.*,[39] substances that have low antioxidant activity *in vitro* will probably show little activity *in vivo*. It is therefore assumed that substances with high antioxidant activity *in vitro* will probably show the same type of activity *in vivo*.

The total phenol, flavonoids, flavonol, and proanthocyanidin contents of both extracts were determined in this study. Phenolic compounds aid in the preservation of food, fresh flavor, taste, color and help in prevention of oxidative deterioration. In particular, many phenolic compounds are attracting the attention of food and medical scientists because of their antioxidative, anti-inflammatory, antimutagenic, and anticancer properties as well as their capacity to modulate some key cellular enzyme functions.[40] Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of H_2O_2 into free radicals.[41] In addition, flavonoids and phenolic compounds are effective in preventing the formation of ROS and protecting low-density lipoprotein from iron- and copper-mediated free radical production.[42] Flavonoids are hydroxylated phenolics and are potent water soluble antioxidants which help in radical scavenging and the prevention of oxidative cell damage.[43] Proanthocyanidins are polyphenolic bioflavonoids which have a protective effect in eliminating hydroxyl radicals. The concentration of flavonoids and proanthocyanidin was higher in the acetone extracts compared with the aqueous extracts in this study which agree with several studies that acetone extracts had higher concentration of phenolic compound.[43,44] The presence of these polyphenols may be responsible for the therapeutic effects of *L. javanica*.

The scavenging activity of ABTS by the aqueous and acetone extracts was found to vary with concentration used and IC_{50} values; the acetone extracts exhibited better scavenging effect with ABTS compared to the aqueous extracts. The solubility of extracts in different testing systems and radical reactivity confirming the removal of odd electron are believed to be responsible for the higher scavenging activity of ABTS. The scavenging activity of ABTS radical by the plant extracts justifies the presence of compounds with free radical scavenging activity as well as the possibility of the extracts being used for treating radical-related pathological ailments.[43]

The electron donating ability of natural products can be measured by the decolorization of the DPPH radical. The degree of color change is proportional to the concentration and potency of the antioxidant. Both extracts and standards tested exhibited strong antioxidant effect against DPPH radicals. This implies that the high total polyphenol content could be responsible for the strong DPPH scavenging power since they can readily donate hydrogen atom, thereby quenching the radical.[45]

NO is an important chemical mediator generated by endothelial cells, macrophages, and neurons and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess NO to generate nitrite and peroxynitrite anions which act as free radicals. Although the standard (gallic acid) exhibited greater NO inhibition, both the aqueous and acetone extracts were also potent scavengers of NO and could be useful in the management of inflammatory reactions that are detrimental to human health.

Lipid peroxidation of cellular membranes by free radicals generates malondialdehyde which reacts with DNA to cause mutations.[46] Active components of *L. javanica* significantly inhibit the generation of lipid peroxides. This implies that the combination of phenols, flavonoids, and other bioactive compounds in the plant are actively involved in the antioxidant role of *L. javanica* extracts.

When converted to hydroxyl radical, H_2O_2 becomes toxic and may initiate lipid peroxidation and irreversible damage to DNA.[47] Both the aqueous and acetone extracts of *L. javanica* were able to quench these radicals. This is an indication of the presence of potent phytoconstituents which have the ability to scavenge hydroxyl radicals though the activities of such components may be shielded by the presence of other components in the extracts.

Phosphomolybdate assay is used to assess the overall antioxidant capacity of plant extracts. Extracts of *L. javanica* exhibit a high total antioxidant capacity against molybdenum radicals. This could be attributed to the presence of flavonoids in the extracts since many flavonoids and related phenols have been reported to contribute significantly to the phosphomolybdate scavenging activity of medicinal plants.[48]

Overall *L. javanica* exhibits high polyphenolic contents, outstanding reducing power, and good radical scavenging activity against DPPH, ABTS, NO, and inhibition of lipid peroxidation, H_2O_2, and phosphomolybdate. The free radical scavenging ability of *L. javanica* is dependent on the polyphenol content. Several studies have evaluated the relationships between antioxidant activity of plant products and their phenolic content. Some authors found a correlation between the phenolic content and antioxidant activity while others found no strong relationship.[49,50] In this study, a significant total phenolic content and free radical scavenging activities were found in both the acetone and aqueous extracts supporting the claim that a correlation exists between the total phenolic content and antioxidant activity.[7,50,51]

In addition, numerous monoterpenoids have been identified in the volatile extract of *L. javanica* including myrcene, caryophyllene, linalool, p-cymene, and ipsdienol.[41] *L. javanica* contains various organic acids and alcohols iridoid glycosides and toxic triterpenoids which have been detected in some *Lippia* species.[52-54] The presence of all these compounds in *L. javanica* could probably account for the medicinal uses of the plant as these metabolites are well known for their biological activities.[55] This finding seems to justify the folkloric use of infusing a combination of the aerial plant parts of *L. javanica* for therapeutic purposes. Previous studies have reported that the essential oils of *L. javanica* possess moderate antioxidant activity.[56,57]

Denaturation of protein is a well-documented cause of inflammation. Some anti-inflammatory drugs have been reported to show dose-dependent ability to prevent thermally-induced protein denaturation.[58,59] In the present investigation, the *in vitro* anti-inflammatory effects of aqueous and acetone extracts were evaluated against denaturation of bovine serum albumin and stability of rat erythrocyte membrane. Both extracts exhibited a concentration-dependent inhibition of protein denaturation and membrane stability within the concentration ranges studied and was compared with diclofenac, a standard anti-inflammatory drug. Protein denaturation and membrane lysis have been reported to be the cause of inflammation in conditions such as rheumatism and arthritis;[60,61] therefore, any substance that can prevent or inhibit protein denaturation and enhance membrane stability will be a good anti-inflammatory agent. The ability of *L. javanica* extracts to enhance these two as observed in this study could account for its reported folkloric use as an anti-inflammatory agent.[60,61] The precise mechanism of this anti-inflammatory action is yet to be elucidated and will be further investigated.

The results from the antimicrobial studies are quite encouraging as almost all the extracts exhibited moderate antimicrobial activity against most of the tested pathogens, which included Gram-negative and Gram-positive bacteria as well as fungi. Although some pathogens such as *M. canis*, *P. aurantiogriseum*, *A. niger,* and *A. fumigatus* showed resistance to both the aqueous and acetone extracts of *L. javanica.* This study agrees with the reports of Mangena and Muyima[62] that *Tagetes minuta*, *L. javanica,* and *Foeniculum vulgare* oils displayed remarkable antimicrobial activity against all the tested organisms.[62]

CONCLUSION

The polyphenolic content, free radical scavenging, anti-inflammatory, and antimicrobial activities of the aqueous and acetone extracts of *L. javanica* (Burm.F.) Spreng were evaluated. The data presented in this study demonstrate that both extracts possess excellent antioxidant, free radical scavenging, anti-inflammatory, and antimicrobial activities. There was correlation between the antioxidant activities and the total phenolic and flavonoids content. A concentration-dependent inhibition of protein denaturation and membrane stabilization of the aqueous and acetone extracts were also observed. Therefore, the ability of *L. javanica* extracts to inhibit protein denaturation and maintain stability as reported in this study could account for its folkloric use in the treatment of inflammatory diseases. The overall antimicrobial activity evaluated revealed that both extracts were active, but the acetone extract exhibited a higher antifungal activity compared to the reference drugs. Further studies on the characterization of various antioxidant compounds present and the mechanism of anti-inflammatory action are in progress.

Acknowledgments

This study was supported by the Govan Mbeki Research and Development Centre and Medicinal Plants and Economic Development Research Centre, both of the University of Fort Hare, South Africa.

Financial support and sponsorship

This study was supported by the Govan Mbeki Research and Development Centre (GMRDC) University of Fort Hare, South Africa; and Medical Research Council (MRC), South Africa.

Conflicts of interest

There are no conflicts of interest.

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Gloria A. Otunola

ABOUT AUTHOR

Gloria A Otunola, PhD, is a Researcher at the Medicinal Plants and Economic Development (MPED) Research Centre, Department of Botany, University of Fort Hare, South Africa. Her research interest is on the biochemical, pharmacological and metabolomic elucidation of medicinal plants and food fortification in the management of chronic and metabolic diseases.