P Masoko et al /J. Pharm. Sci. & Res. Vol. 11(8), 2019, 3020-3029

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Phytochemical and biological investigations of *Olea europaea* subspecies *africana* leaves

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Abstract

The leaves of *Olea europaea* subspecies *africana* have been used in South Africa to treat various diseases traditionally. The study was aimed at screening the leaves for antioxidant, antibacterial, anti-inflammatory activities and cytotoxic effects. Phytoconstituent analysis and quantification were performed. Quantitative and qualitative antioxidant activities were determined. Antibacterial activity was also determined. Leaves were tested for anti-inflammatory potential and cell viability. Leaves contain tannins, terpenoids, steroids and flavonoids. Spectrophotometric analysis indicated high amounts of total phenolic (99.67 ± 2.52 mg of GAE/g) and tannin content (114.33 ± 9.02 mg of GAE/g). All crude extracts exhibited antioxidant activities with an EC_{50} value of 1.05 ± 0.0071 mg/mL. Crude extracts had antibacterial activity against test organisms. *O. europaea* extracts demonstrated anti-inflammatory potential. *Olea africana* contain compounds with antioxidant, antibacterial and anti-inflammatory activities. The leaves were non-cytotoxic to the normal cells. Further pharmacological studies of these plants are essential and significant.

Keywords: Antibacterial activity, antioxidant activity, anti-inflammatory activity, cytotoxicity

INTRODUCTION

From time memorable to humans, plants have been a source of medicinal agents used to treat and prevent various ailments [1]. Their healing ability is attributed to the different entities and bioactive molecules produced through the process of metabolism [2]. Secondary metabolites derived from plants are mostly phenols or oxygen-substituted derivatives. Structural variations and chemical composition of these compounds result in differences in their therapeutic relevance [2]. Phytochemicals have many health benefits on humans; these include protecting our bodies against the build-up of free radicals and fighting against infections caused by bacteria, fungi, and viruses. They also possess activities like lowering cholesterol, antithrombotic and anti-inflammatory responses [3].

Various parts of the plant contain different bioactive compounds, these include saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds found as secondary metabolites [4]. Oxidative stress, through the production of reactive oxygen species (ROS), has been proposed as the root cause underlying the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance and type 2 diabetes mellitus [5]. Oxidative stress has also been implicated as one of the causes of hypertension, as it amplifies blood pressure elevation in the presence of other pro-hypertensive factors [6]. Obesity as one the leading lifestyle diseases has been linked with macrophage accumulation in white adipose tissue leading to the development of adipose tissue inflammation in obesity patients [7].

All these lifestyle diseases and bacterial infections have been reported to be traditionally treated with the leaves of *Olea europaea* subspecies *africana* [8]. The genus *Olea* was derived from the Greek word “elaia” and the Latin word “oleum” but it is known by nearly 80 different names with the common one being the olive tree [9]. *Olea europaea* subspecies *africana* is an evergreen tree, previously known as *Olea europaea* subspecies *africana* (Mill) P.S. Green [10]. It is commonly known as the wild olive and its vernacular names include mohlware (Sotho), olienhout (Afrikaans) and umquma [11]. This plant has been stated to be “the most important plant” from 120 plants being used in traditional medicine [12]. It has a number of traditional uses medically that are summarised in table 1. The bark, leaves, roots and fruits are used in different forms, alone and/or sometimes in combination [13].

Although the leaves of this plant have been used traditionally for the longest of time, there is still little information about the antioxidant, antibacterial, and anti-inflammatory activities of the leaves in literature and their cytotoxic effects thereof. Hence the study was aimed at filling in those gaps in relation to their traditional use in southern Africa.

Table 1: Ethnobotanical uses of *Olea europaea* subspecies *africana* in southern Africa and the rest of Africa

Traditional uses in southern Africa
Dried and powdered leaf is applied on fresh wounds as a styptic.
Powered leaf stops nosebleeds by using it as a snuff.
Leaves are used as a treatment for malaria, urinary tract infections, backaches and kidney problems. Leaf infusions are used as lotion for the treatment of eye infections or to relieve sore throats. Bark, which is dried, pound and powdered, is applied for eye illnesses. Boiled bark is administered for itchy rashes. Decoction of stem bark is used to treat helminthiasis, asthma, rheumatism and lumbago in Samburu district, Kenya. Bark, root and leaf infusions are taken to relieve colic, leaf infusion taken to treat sore throats and diphtheria. Fruit infusion treats bloody stool and diarrhoea.
Decoction of the fruits and leaves is used in treating blood pressure in the Transkei region.

METHODS

Plant collection

The leaves of *Olea europaea* subspecies *africana* were collected from the University of Limpopo (Turfloop campus), South Africa in Summer, 2016. Plant identity was confirmed by Dr Brownyn Egan (Larry Leach Herbarium (UNIN)) and the herbarium voucher number was UNIN 11938. The leaves were dried at room temperature. The dried leave material were ground to fine powder using an electric grinder and stored in an air-tight container in a dark place until they are used.

Extraction procedure

The plant materials (1 g) were extracted with 10 mL of different solvents of varying polarity (n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol and water) and the supernatants of each plant material were filtered using a Whatman no.1 filter paper into pre-weighed vials and the filtrates were dried under a stream of air. The quantity of plant material extracted was determined. The extracts were reconstituted in acetone to a concentration of 10 mg/mL for the subsequent bioassays.

Phytochemical analysis of extracts using standard chemical tests

The following phytochemical analysis were performed; anthraquinones [14], saponins [15], tannins [16], alkaloids [17], terpenoids(Salkowski test), flavonoids, cardiac glycosides(Keller- Killiani test), Steroids and phlobatannin [18] .

Thin layer chromatography (TLC) analysis

The chemical constituents of the plant extracts were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F254). The reconstituted extracts (10 µL) were spotted onto TLC plates which were developed in solvent systems of varying polarity, i.e. ethyl acetate: methanol: water (10:5.4:4), [EMW] (polar/neutral); chloroform: ethyl acetate: formic acid (10:8:2), [CEF] (intermediate polarity/acidic) and benzene: ethanol: ammonium hydroxide (18:2:0.2) [BEA] (non-polar/basic) [19]. The plates were removed from the tanks, air dried under a fume hood cabinet and observed under ultraviolet (UV) light (254 and 365 nm). To detect non-fluorescing compounds, vanillin sulphuric acid reagent (0.1 g vanillin (Sigma ®): 28 methanol: 1 mL sulphuric acid) was sprayed on the chromatograms followed by heating at 110 °C for optimal colour development.

Quantification of major phytochemicals

Total phenolic content

The concentration of phenolic content in 70% aqueous acetone extracts of the selected plants was determined using spectrophotometric method described by Singleton *et al*. [20], with modifications. The determination of the total phenol content employed the Folin-Ciocalteau method, where 0.1 mL of extract and 0.9 mL of distilled water were mixed in a 25 mL volumetric flask. To this mixture 0.1 mL of Folin-Ciocalteau phenol reagent was added and the mixture shaken well. One millilitre of 7 % Sodium carbonate (Na_2CO_3) solution was added to the mixture After 5 minutes. The volume was made up to 2.5 mL with distilled water. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared as described above. The mixtures were incubated for 90 minutes at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV)/visible spectrophotometer. Total phenol content was expressed as mg of GAE/g of extract calculated from the equation, $y = 3.650x - 0.0811, R^2 = 0.996$, where y is the absorbance at 550 nm and x is the amount of gallic acid equivalence (mg/mL) [21].

Total tannin content

The tannin content was determined using Folin-Ciocalteau method. About 0.1 mL of the 70% aqueous acetone extracts of the selected plants was added to a 25 mL volumetric flask with 5 mL of distilled water. To this mixture 0.2 mL of 2 M Folin-Ciocalteau phenol reagent and 1 mL of 35 % Na_2CO_3 solution was added and this was made up to 10 mL with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared in the same manner as described above. Absorbance for test samples and standard solutions were measured against the blank at 725 nm with a UV/Visible spectrophotometer. The tannin content was expressed as mg of GAE /g of extract calculated from the equation, $y = 1.3263x + 0.0227, R^2 = 0.09975$, where y is the absorbance at 725 nm and x is the amount of gallic acid equivalence (mg/mL) [21].

Total flavonoid content

Total flavonoid content was determined by the aluminium chloride colorimetric assay. One millilitre of 70% aqueous acetone extracts of the selected plants was mixed with 4 mL of distilled water in a 25 mL volumetric flask. To the flask, 0.30 mL of 5 % sodium nitrite was added. About 0.3 mL of 10 % aluminium chloride was added to the mixture after 5 minutes, this was mixed. After 5 minutes, 2 mL of 1 M Sodium hydroxide was added and this was made up to 10 mL with distilled water. A set of reference standard solutions of quercetin (0.0625, 0.125, 0.25, 0.5 and 1 mg/mL) were prepared in the same manner as described above. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with a UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract calculated from the equation, $y = 3.448x + 0.0028, R^2 = 0.999$, where y is the absorbance at 510 nm and x is the amount of quercetin equivalence (mg/mL) [21].

Antioxidant activity assay

Qualitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC

The plant extracts were separated using TLC as described in thin layer chromatography (TLC) for phytochemical

analysis. The chromatograms were air dried and sprayed with 0.2 % (w/v) 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma®) to detect the presence of antioxidant compounds on TLC plates. The presence of antioxidant activity is indicated by yellow spots against a purple background on TLC plates sprayed with 0.2 % DPPH in methanol [22].

Quantitative antioxidant activity assay

Quantitative 2, 2-diphenyl-1-picrylhydrazyl DPPH radical scavenging activity assay

The free radical scavenging activity of the 70% aqueous acetone extracts of the selected selected plants was measured using the method described by Brand-Williams *et al*. [23] with modifications. The stock solution of 0.2% DPPH in methanol was prepared and this was kept in a refrigerator until use. A working solution was made by diluting the stock solution with methanol to make an absorbance of 0.98 (±0.02) at 517 nm. One millilitre of the DPPH working solution was mixed with 1 mL of the plant extract (1 mg/mL), or the standard solution in a test tube. The absorbance was measured at 517 nm. The free radical scavenging activity was represented as EC_{50} .

Reducing power method

The antioxidant capacity was evaluated using the reducing power assay described by Oyaizu [24] with modifications. A set of concentrations ranging from 0.0625 mg/mL to 1 mg/mL of the 70% aqueous acetone extracts of the selected plants was made. Two millilitres of each of the prepared concentration was added into a test tube, to this 2 mL of sodium phosphate buffer (1 M, pH 6.6) and 2 mL of potassium ferricyanide (1% cw/v in distilled water) were added and mixed well. This mixture was incubated in a water bath at 50 ^{0}C for 20 minutes. Following incubation 2.5 mL of trichloroacetic acid (10% w/v in distilled water) was added and the mixture was centrifuged at 650 rpm for 10 minutes. About 3 mL of the supernatant was and added into a test tube. To this 10 mL of distilled water and 1 mL of ferric chloride (0.1% w/v in distilled water) solution was added and mixed well. The absorbance of the solutions was measured at 700 nm against a blank prepared as described above but replacing the plant extract with an equal volume of a solvent.

Antibacterial activity assay

Microorganisms used in this study

The bacterial species were supplied by the Department of Biochemistry, Microbiology and Biotechnology section at the University of Limpopo (Turfloop campus). Two Gram positive bacteria (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) and two Gram negative bacteria (*Escherichia coli* ATCC 28922 and *Pseudomonas aeruginosa* ATCC 27853) were used as test microorganisms. The bacterial species were maintained on nutrient agar at 4 °C. The cells were inoculated and incubated at 37 °C in nutrient broth for 12 hours prior to screening tests.

Quantitative antibacterial activity assay by microbroth dilution method

Antibacterial activity was evaluated by the determination of the minimum inhibitory concentration (MIC) of each extract on *E. faecalis*, *E. coli*, *P. aeruginosa*, and *S. aureus* using the micro-dilution method developed by Eloff [25]. The extracts were re-dissolved in acetone to a final concentration of 10 mg/mL. Two-fold serial dilutions of the extracts (2.5 mg/mL to 0.02 mg/mL) were prepared in 96-well microtitre plates. The effects of the extracts were tested against each pathogen at a density of following exposure, and microtitre plates were incubated at 37 ^{o} C overnight. Densities of bacterial cultures for use in the screening procedures were as follows: *S. aureus*, 2.6×10^{12} CFU/mL; *E. faecalis*, 1.5×10^{10} CFU/mL; *P. aeruginosa*, 5.2×10^{13} CFU/mL; *E. coli*, 3.0×10^{11} CFU/mL. There after 40 µL of 0.2 mg/mL iodonitrotetrazolium chloride (INT) (Sigma-Aldrich) was added to each well and the plates were re-incubated for a further 30 min at 37 ^{o} C for *S. aureus* and *P. aeruginosa*, 1.5 hours for *E. coli* and 24 hours for *E. faecalis*. The formation of a red-pink colour signified microbial growth. All samples were assayed in triplicates.

Qualitative antibacterial activity assay by bioautography

The Bioautography procedure was done according to Begue and Kline [26]. The plates were developed as indicated above and allowed the solvents to evaporate for five days prior to spraying with test bacteria. Following spraying with test bacteria, the plates were placed in humidy and incubated at 37 ^{o} C for 24 hours. Following incubation, the plates were sprayed with 2 mg/mL ρ-iodonitrotetrazolium violet (INT) (sigma) dissolved in distilled water and further incubated for 2-3 hours. The bioautograms were observed for bacterial growth, and the clear zones against the red-pink background indicated growth inhibition by the compounds with antibacterial activity on TLC plates.

Anti-inflammatory activity assay using DCFHD-A assay

Anti-inflammatory assay was carried out according to the method described by Sekhar et al [27]. DCFHD-A assay uses stimulants such as lipopolysaccharide (LPS) to induce oxidative stress and dihydrodichlorofluorescein diacetate (H_2DCF-DA) to detect the presence of reactive oxygen species in excess. In the presence of reactive oxygen species H_2DCF-DA is oxidized to fluorescent 2, 7-dichlorofluorescein (DCF). Two hundred microliters of cells (Raw 264.7 macrophages) in RPMI-1640 was seeded in a 96-well plate. The cells were incubated at 37 °C, 5% CO_2 overnight for attachment. The medium was removed and the cells were washed with PBS. Cells were exposed to 100 µL of acetone extracts (8 mg/mL, 0.64 mg/mL, and 0.32 mg/mL) of *D. rotundifolia* and *S. pinnata* and 20 µL of LPS for 24 hours. Following incubation, the medium was aspirated a new medium without FBS was added and the cells were stained with 100 µL of 20 µM of H_2DCF-DA and incubated for 30 minutes in the dark. The fluorescence was measured at 480 nm. Curcumin (50 µM)

and untreated cells were used as positive and negative controls respectively.

Cell viability assay

The toxic effects of the selected plants on African green monkey kidney (Vero) cells was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [28]. The cells were maintained in a minimal essential medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). The cells suspension (5 x 10^4 cells/mL) was seeded in a sterile 96-well microtitre plate and incubated for 24 hours at 37 ºC in 5% CO_2 for the cells to attach. The MEM was aspirated and the cells were washed with 150 µL phosphate buffered saline (PBS, Whitehead Scientific). The cells were treated with different concentrations of the extracts (1-0.025 mg/mL) prepared in MEM. Doxorubicin was used as a positive control. The microtitre plates were incubated for 48 hours with the extracts in the same conditions as described earlier. Untreated cells were included negative control. After treatment, the treatment medium was aspired and replaced with 30 µL of MTT (5 mg/mL) in PBS (Sigma) and the plates were incubated further for 4 hours at 37 ºC. The medium was removed and replaced with 50 µL of DMSO to dissolve the MTT formazan crystals. The absorbance was measured in a microplate reader (BioTek Synergy) at 570 nm. Cytotoxicity was expressed as the concentration of test sample resulting in a 50% reduction of absorbance compared to untreated cells (LC_{50} values). All the analysis was made in quadruplicate. The selectivity index (SI) was expressed as LC_{50}/ MIC value

Statistical analysis

Each experiment was performed in triplicates and the results were expressed as mean values. Linear regression analysis was used to calculate LC_{50} values. Microsoft Excel® was used to enter and capture data. Various graphs and tables were extracted from this data. Data was then exported to SPSS for further analysis. The MIC for each microorganism was analyzed using one-way analysis of variance (ANOVA). P value < 0.05 was considered as significant. SPSS 25.0 was employed for statistical analysis.

RESULTS

Extraction procedure

Water extracted most of the plant material (158.2 mg), followed by methanol (109.1 mg) (Fig. 1). The majority of traditional healers use water to extract active compounds from these plants, because water is not harmful to domestic animals and humans and is generally the only extractant available.

Phytochemical analysis of extracts using standard chemical tests

The standard chemical test results indicated the presence of flavonoids, tannins, steroids and terpenoids in the leaf extracts (Table 2).

Table 2: Quantification of total phenolic, tannins and flavonoid content in the leaves of *Olea europaea* subspecies *africana*.

Phytochemicals	Quantity
Phenols (mg of GAE/g)	99.67 ± 2.52
Tannins (mg of GAE/g)	114.33 ± 9.02
Flavonoids (mg of QE/g)	23.97 ± 1.15

Results were represented as ± standard deviation; GAE: Gallic acid equivalence; QE: quercetin equivalence.

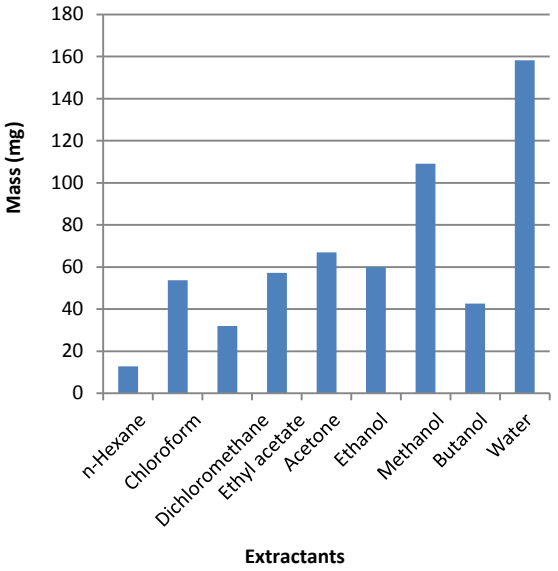

Fig. 1: The mass extracted from 1 g of plant dried leaves using different solvents.

Thin layer chromatography (TLC) analysis

The presence of the phytochemicals was further strengthened by TLC analysis, to demonstrate various constituents in the leaf extracts (Fig. 2). After spraying the chromatograms with vanillin-sulphuric acid reagent, it was revealed that BEA separated the phytochemicals better than EMW. However, more bands were also observed under UV light on the TLC plate developed in EMW. The results suggested that the plant contained more of both non-polar and polar compounds.

Quantification of major phytochemicals

The major phytochemicals present in the extracts were quantified to determine their concentration in the crude extracts (Table 3). Tannin content was the highest.

Table 3: The EC_{50} of the aqueous acetone extract and L-ascorbic acid. L-ascorbic acid was used as a standard control.

Sample	DPPH scavenging potential EC_{50}
Olea europaea subspecies *Africana*	1.05 ± 0.0071
L-Ascorbic acid (Standard)	0.10 ± 0.0014

Data is expressed as mean ± standard deviation of three independent experiments.

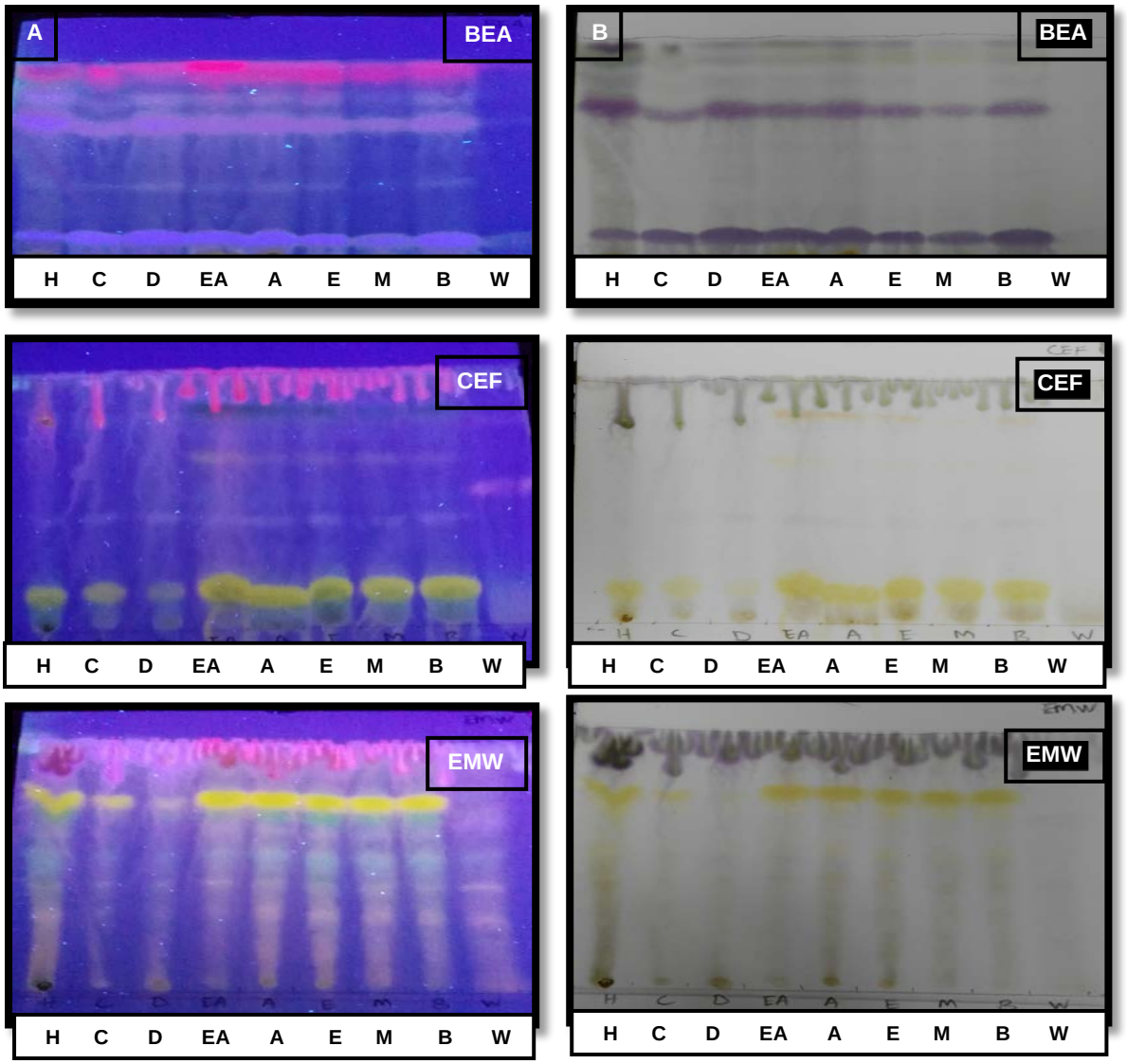

Fig. 2: TLC plates visualised under UV light at 365 nm (A) and sprayed with vanillin-sulphuric acid reagent (B). The compounds separated on the TLC plates were extracted with n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E), methanol (M), butanol (B) and water (W)

Qualitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC

Antioxidant compounds failed to separate in the BEA solvent system and only separated in CEF and EMW. These demonstrated that the active compounds were polar (Fig. 3).

Quantitative 2, 2-diphenyl-1-picrylhydrazyl DPPH radical scavenging activity assay

Prior quantification analyses, the powdered leaves were extracted with 70% acetone: 30% water. The observed antioxidant activity was quantified spectrophotometrically through the DPPH free radical scavenging activity assay. The aqueous acetone extract had an EC_{50} higher than L-ascorbic acid (control) (Table 4).

Table 4. The MIC and selectivity indices of the acetone extract against the four tested bacteria.

Microorganisms	MIC values (µg/mL)	SI values
E. coli	230 ± 0.01	1.23
P. aeruginosa	160 ± 0.00	1.77
E. faecalis	130 ± 0.04	2.77
S. aureus	160 ± 0.00	1.77

Sample	LC_{50}
Acetone extract	282.4
Doxorubicin	2.29

Table 5: LC_{50} values of the acetone extract and the doxorubicin in µg/mL.

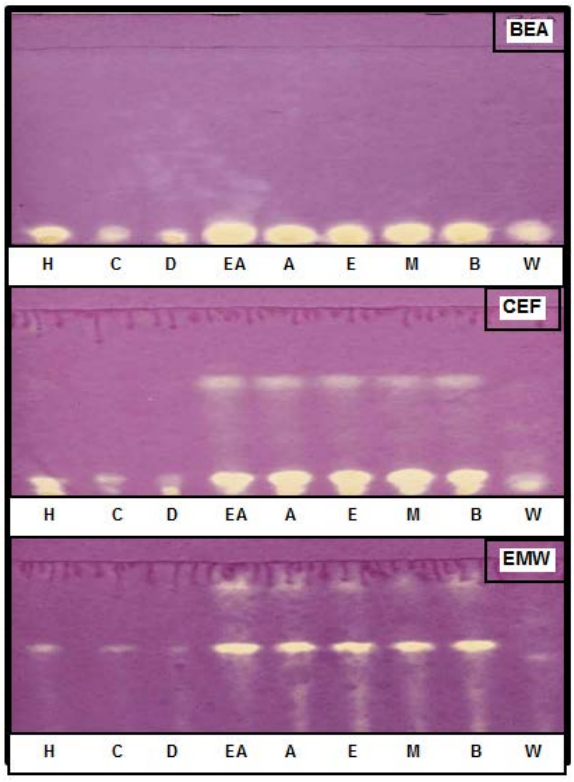

Fig. 3: Chromatograms of *Olea europaea* subspecies *africana* developed in BEA, CEF and EMW solvent systems and sprayed with 0.2% DPPH. The yellow spots indicate the extracts containing compounds with antioxidant activity. (n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E), methanol (M), butanol (B) and water (W)

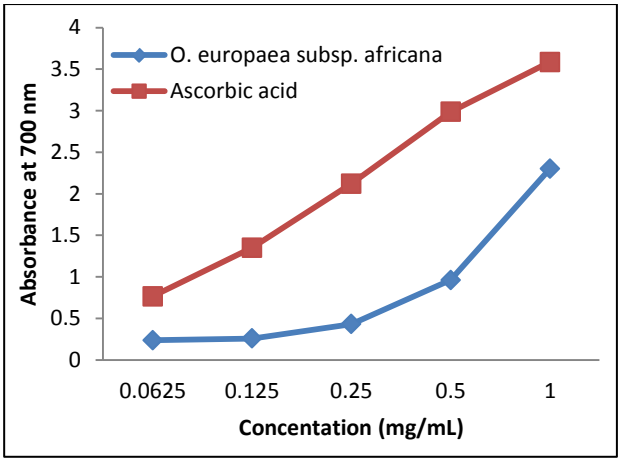

Fig. 4. The reducing power of the aqueous acetone extract at an absorbance of 700 nm with respect to increasing concentrations. L-ascorbic acid was used as a reference standard.

Reducing power method

At the lowest concentration of 0.0625 mg/mL, the reducing power was low, with an absorbance of 0.236 nm when compared to the reducing power of the standard (L-ascorbic acid) which had absorption of 0.765 nm at a similar concentration (Fig. 4).

Quantitative antibacterial activity assay by microbroth dilution method

MIC values of the tested bacteria ranged from 160 to 230 µg/mL (Table 5). Ampicillin was used as a positive control as it is regarded as the commonly used broad-spectrum antibiotic in most laboratory environments.

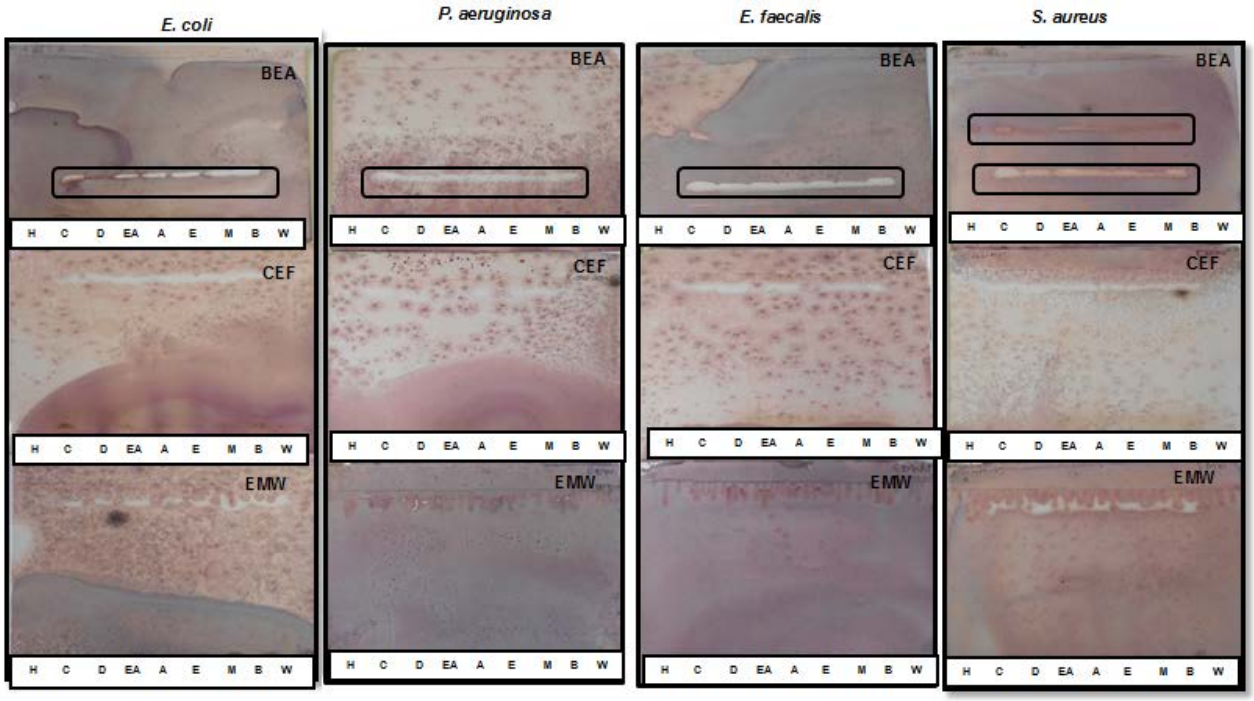

Fig. 5: Bioautograms of *Olea europaea* subspecies *africana* leaf extracts separated in BEA, CEF and EMW solvent systems and sprayed with *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. Clear spots against a purple background indicate antibacterial activity

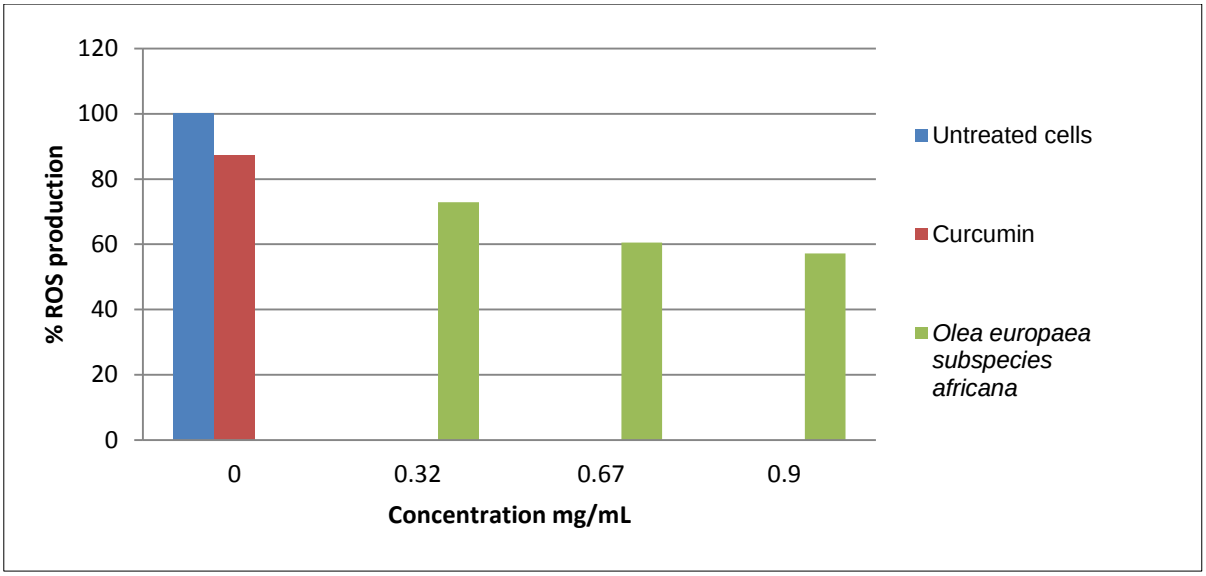

Fig. 6: Anti-inflammatory activity of acetone crude extract determined by measuring the production of reactive oxygen species (ROS) in percentages in lipopolysaccharide-stimulated RAW 264.7 cells. Curcumin was used as a positive control.

Qualitative antibacterial activity assay by bioautography

The appearance of clear zones indicated that at that particular band, INT reduction did not take place because the bacterial growth was inhibited. BEA effectively separated most compounds with antibacterial activities, followed by CEF and EMW which showed the least zones of inhibition (Fig. 5).

Anti-inflammatory activity assay using DCFHD-A assay

The investigation of the anti-inflammatory activity of the leaves of *Olea europaea subspecies africana* was done by evaluating the inhibition of ROS production in LPS-stimulated RAW-264.7 cells. Treatment of the RAW 264.7 cells with LPS only, resulted in generation of intracellular ROS and the ROS produced was assumed to be 100%. This was used as a negative control to determine how much ROS was produced in the treated cells. At the lowest concentration of 0.32 mg/ml (Fig. 6)

Cell viability assay

The leaves were also evaluated for cytotoxic effects by determining the LC_{50} of the acetone extract against the Vero kidney cells and calculating the selectivity index of the extract against the tested bacteria (*E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*). The acetone extract was found to have an LC_{50} of 282.492 µg/mL as shown in table 6. At this level, extracts are considered safe, considering that the value is greater than 100 µg/ml, which is considered safe for plant extracts[29].

DISCUSSION

Olea europaea subspecies *africana* was selected in this study based on its use by traditional healers in southern Africa and around the world for combating bacterial, free radical and inflammation related diseases [13]. Solvents of increasing polarities were used to extract bioactive compounds of varying polarities. The phytochemical groups of interest ranged from non-polar terpenoids extracted with n-hexane to polar saponins extracted with methanol and water. Acetone was used to reconstitute the plant extracts. The use of acetone to reconstitute has been reported by Eloff [30] to be non-toxic towards bacteria and fungi [31] and it is able to dissolve both hydrophilic and hydrophobic components in an extract.

In this study water extract was not considered for further the study, because water extract only polar compounds, it partially solubilises phytochemicals with antioxidant activity, its extracts have been found to possess less potent antimicrobial activities and it does not evaporate quickly from the extracts [32, 33, 34]. Methanol was quantitatively the best extractant, extracting a greater quantity (109.1 mg) of plant material than any of the other solvents used. This was also observed by Paulsamy and Jeeshna [35], they concluded that this may be due to methanol's high polarity which can extract a variety of plant constituents. The low extraction yield observed in n-hexane could be attributed its zero index of polarity and was only able to extract lipophilic compounds [36].

Preliminary phytochemical analysis is important in determining the chemical constituents present in plant materials. It is also useful in locating the source of pharmacologically active chemical compounds. These phytochemicals (Table 1) serve as defence mechanisms in plants against microorganisms, insects and other herbivores [37]. They were also reported to be responsible for scavenging free radicals in plants [38].

Tannin content was the highest, with a value of 114.33 ± 9.02 mg of GAE/g, followed by total phenolics (99.67 ± 2.52 mg of GAE/g) and flavonoids (23.97 ± 1.15 mg of QE/g) (Table 2). It was reported that the concentration of phenolic content is directly proportional to its antioxidant activity and that phenolic compounds have antimicrobial activities [39,40]. Since phenolic phytochemicals include flavonoids and tannins, then in this study it was evident that the tannin content dominated as phenolic compounds, whilst flavonoids were only constituted in low quantities. These findings suggested that, *Olea europaea* subspecies

africana could have strong antioxidant and antimicrobial activities.

Best separation of the extracts was seen on the TLC plates developed EMW, wherein all extracts separated into different compounds. On chromatogram developed in CEF, n-hexane, chloroform, dichloromethane, and water extracts failed to separate (Fig. 3). Chloroform, dichloromethane and water extracts had low antioxidant activities, indicated by the low yellow intensity. Most antioxidants have been reported to be polar[19].

Advantage of using this DPPH free radical scavenging activity assay is that it allows quantification of both lipophilic and hydrophobic compounds and not restricted by the nature of the antioxidants as compared to other available methods [41], hence it was selected to be used in this study. The activity was expressed as EC_{50}, which is a parameter that is typically employed to express the antioxidant capacity as well as to compare the activity of different compounds [42]. EC_{50} was defined as the concentration of the antioxidants required to scavenge 50% of DPPH, and the smaller the value, the higher the antioxidant activity of the plant extract [43]. The aqueous acetone extract had an EC_{50} of 1.05 ± 0.0071 mg/mL while L-ascorbic acid had a value of 0.10 ± 0.0014 mg/mL (Table 3); the results indicated that *Olea europaea* subspecies *africana* was a potent source of antioxidant compounds.

An increase in the absorbance of the reaction mixture indicated that the sample had greater reducing power [44], the reducing power of the extract increased with an increase in the amount of extract (Fig. 4). At the lowest concentration of 0.0625 mg/mL, the reducing power was low, with an absorbance of 0.236 nm when compared to the reducing power of the standard (L-ascorbic acid) which had absorption of 0.765 nm at a similar concentration. However, the reducing power of the leaves increased gradually at concentration 0.125 mg/ml, followed by a two-fold increase from concentration 0.5 mg/mL to 1 mg/mL. At the highest concentration, *Olea europaea* subspecies *africana* leaves showed a reducing power of about 2/3 of that of L-ascorbic acid, indicating good reducing power ability.

The results obtained in the DPPH and the reducing power assay done spectrophotometrically, are supported by the high amount of total phenolic content (99. 67 ± 2. 52 mg of GAE/g) that the plant contained as represented in table 3. It has been reported that plants demonstrating high amounts of phenolic contents, have high antioxidant capacity. This is because phenolic compounds are the main antioxidant components and their total contents are directly proportional to their antioxidant activity [45]. It has also been reported that there is a direct correlation between antioxidant activity and the reducing power of plants [46]. The high phenolic content was in correlation with the reducing power of the leaves as well, since this assay was based on the ability of an antioxidant to donate an electron, which is a very important mechanism of phenolic antioxidant action [47,48].

The inhibitory characteristics of the leaf extracts agree with the results obtained by Pereira and colleagues [49], where the extracts of *Olea europaea* subspecies *europaea*, a close relative of *Olea europaea* subspecies *africana*, were evaluated for their antibacterial activities against the Gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis*, and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumonia*). The results indicated good antibacterial activities.

Water extracts did not show any antibacterial activity against all tested bacteria. This observation was also done by Korukluoglu *et al*.[50], where the aqueous extract of the olive leaves showed no antibacterial effect against *Salmonella enteritidis*, *Klebsiella pneumonia*, *Bacillus cereus*, *Escherichia coli*, *Streptococcus thermophiles*, *Enterococcus faecalis* and *Lactobacillus bulgaricus*. The effectiveness of *Olea europaea* subspecies *africana* against *S. aureus* also validates the plant's uses to treat wound infection, throat infections, and serious inflammations.

In this study, the MIC values of 0.10 mg/mL or less were considered good while values up to 0.32 mg/mL were considered reasonable and the MIC values above 0.64 mg/mL were considered as having poor activity as described by Adamu *et al*. [51]. *E. faecalis* was the most susceptible bacterium (0.31 mg/mL), followed by *E. coli* (0.35 mg/mL) (Table 4). These microorganisms belong to the Gram-positive and Gram-negative groups, respectively. The results obtained indicated that the mechanism of bacterial inhibition might not be cell membrane targeted; hence, the extracts were effective against both Gram-positive and Gram-negative bacteria. It was reported that plant based-antimicrobials have different target sites in bacterial cells such as the disintegration of cytoplasmic membrane and coagulation of the cell content, unlike the conventional antibiotics, which act either by inhibiting cell wall, protein wall, DNA, RNA synthesis and other mechanisms [52, 53]. *P. aeruginosa* and *S. aureus* were the least sensitive microorganisms with the overall average MIC values of 0.90 and 0.67 mg/mL respectively.

The extracts that had the lowest average MIC values were dichloromethane and ethyl acetate; they both had MIC values of 0.16 mg/mL against *E. faecalis*. This demonstrated the effectiveness of the extracts. The extracts also exhibited stronger activity and a much broad-spectrum with an overall average MIC values of 0.30 and 0.32 mg/mL, respectively. The MIC and total antibacterial activity values are useful pharmacological tools in determining the activity of extracts in mg/mL (potency) and the (efficacy) in mL/g, which is useful for the selection of plant species [54]. Methanol extract, which had the highest total activity (1068 mL/g) against *E. faecalis*, could be diluted to 1068 mL/g and it would still inhibit the growth of *E. faecalis*. n-Hexane extract displayed the lowest total activity with the values of 25 mL/g against both *P. aeruginosa* and *S. aureus*. The high total activity of methanol extract may be due to the presence of tannins, flavonoids and terpenoids and the fact that it extracted much plant material (Results not shown).

Screening the leaf extracts qualitatively by bioautography (Fig. 5A B, C and D), revealed that the extracts possessed compounds with good antibacterial activities. It was therefore concluded that the antimicrobial compounds were non-polar in nature. All leaf extracts exhibited inhibitory characteristics towards the tested bacteria, except n-hexane and water extracts. The bioautograms sprayed with *E. coli* and *P. aeruginosa* showed similar profiles of clear zones. However, n-hexane extract indicated a bit of antibacterial activities against *E. faecalis and S. aureus* as represented in Fig. 5A and B, respectively.

In both the direct bioautography assay and the serial broth microdilution assay, n-hexane and water extracts showed less to no clear zones on bioautography and high average MIC values of 1.07 and 1.74 mg/mL, respectively. These gave an indication that for isolation of active compounds, these extracts should not be considered and only consider ethyl acetate or acetone extracts, which showed good antibacterial activity.

The investigation of the anti-inflammatory activity of the leaves of *Olea europaea subspecies africana* was done by evaluating the inhibition of ROS production in LPS-stimulated RAW-264.7 cells. ROS production in treated cells was not significantly different from cells treated with LPS only. However, at concentration 0.67 mg/mL, ROS production was inhibited to 60% and at the highest concentration (0.90 mg/mL), the percentage of ROS production was inhibited to 58 percent (Fig. 6). It was noted that as the concentration of the extract was increased, the ROS production decreased.

This study is the first to report on the anti-inflammatory activities of the leaves of *Olea europaea* subspecies *africana* in *in vitro* studies using LPS-stimulated RAW 264.7 murine macrophage cells. The therapeutic potential of these leaves has been credited to its capacity to neutralise ROS produced under *in vitro* conditions when RAW 264.7 cells were stimulated with bacterial LPS. The long term use of this plant to treat anti-inflammatory related ailments (eye injuries, enlarged tonsils, rheumatisms and arthritis) indicated the plant's potential to have anti-inflammatory properties.

Performing the *in vitro* cytotoxicity analysis is important; this is done to define the basal cytotoxicity of the extract such as the intrinsic ability of an extract to cause cell death as a result of damage to several cellular functions. Doxorubicin which was used as a positive control and had an LC_{50} of 2.29 µg/mL, this value is much lower than that of the extract. It indicated that the leaf extract was less toxic to Vero cells than the positive control, doxorubicin. This validated the safeness of the extract and showed that it is much more safer to use on mammal cells.

The selectivity indices (SI) determined, were represented in table 4, along with the MIC values of the acetone extract against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. The best SI value was seen against *E. faecalis* (2.77) and the lowest was demonstrated against *E. coli* (1.23). A high selectivity index is an indication of a large safety margin, while a low SI value is an indication of cytotoxicity. The SI values were low but not cytotoxic to animal cells since their SI values were greater than 1. According to Makhafola *et al*.[55], the *in vitro* toxicity does not equate to *in vivo* toxicity because of a difference in physiological microenvironment in live animal and tissue culture. Moreover, other factors relating to chemical kinetics which may include absorption, biotransformation, distribution and excretion, which influence the exposure at the level of target cells *in vivo*, cannot be adequately simulated *in vitro*. The toxic components in the crude extracts may be eliminated by manipulation of the extract and more suitable antibacterial extracts may be yielded [56].

CONCLUSION

The major phytochemicals were present in the extract and they exhibited antioxidant, antibacterial and anti-inflammatory activities. Good antibacterial activities explains the traditional uses of this plant. Anti-inflammatory drugs of high potency and of natural origin may be developed from the plant extracts. The anti-inflammatory properties documented in this study, validate the use of the leaves. The cytotoxicity analysis proved that the leaves were non-cytotoxic to the normal cells. Further pharmacological studies of these plants are essential and significant.

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