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THE BIOACTIVE POTENTIALS OF *OLEA EUROPAEA* SUBSPECIES AFRICANA A FOLKLORIC MEDICINAL PLANT AMONG KHOSA TRIBE IN THE EASTERN CAPE PROVINCE, SOUTH AFRICA

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Keywords:

Olea europaea, Solvent extracts, Essential oil, Antioxidant, Antibacterial

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ABSTRACT: The dramatic increase in the burden of chronic non-communicable diseases (NCDs) in emerging economies has become a huge challenge and as such led to increased reliance on folkloric medicine for NCDs management. Consequently, wild *Olea europaea* leaves were assessed for medicinal potentials, as they are commonly used as a traditional therapy for these diseases in Eastern Cape, South Africa. The antioxidant and antibacterial properties of the *O. europaea* extracts obtained using different solvents by maceration method were tested by spectrophotometric, disc diffusion and agar dilution methods respectively, while the essential oil obtained in a Clevenger modified apparatus was analyzed by GC-MS. The *O. europaea* extracts showed moderate to strong inhibitory potential in scavenging the three different tested radicals in a concentration dependant-manner. The ethyl acetate leaf extract (MEA) and ethanol extract (ME) were active against the growth of the six tested bacteria in particular *Klebsiella pneumoniae* and *Serratia marcescens*. Gas chromatography - mass spectroscopy studies on the essential oil revealed 61 compounds, accounting for 93.031% of the total oil. Nonanal, phytol and 2-isopropyl-5-methyl-9-methylene-bicycle [4.4.0] dec-1-ene were the main chemical compounds identified. The results suggest that the essential oil and solvent extracts of *O. europaea* contains bioactive compounds with antibacterial and antioxidant properties and which may give justification for its indigenous usage.

INTRODUCTION: The continuous rise of diverse chronic human pathologies caused by oxidative stress and microbial resistance to currently available antibiotics has become a huge challenge. Oxidative stress is considerably involved in the onset, progression and complications of diabetes as there are corroborative evidence available which supports that oxidative stress aggravates the adverse effects of diabetes [1, 2].

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Studies have shown that diabetics with reduced antioxidant status are at an increased risk for diabetic complications [3]. Consequently, more researches have emerged in the quest for novel antimicrobial agents and antioxidants from other origins such as plants, which have different mechanisms of action other than that of the currently used drugs, with which to combat pathogens as well as protect the human body against free radical induced damage diseases [4, 5, 6, 7].

Unlike conventional drugs, medicinal plants usually contain different phytochemicals which work synergistically to generate a combined effect that would exceed the total effect of the individual components.

The combined effect of these compounds leads to the increased activity of the main medicinal components by accelerating and delaying its assimilation in the body [8]. An individual plant may, for instance, possess alkaloids which improves the mood, phenols that act as an antioxidant, antibacterial and antifungal, tannins that can act out as natural antibiotics, diuretic substances that increase waste products and toxins elimination [8, 9]. Notwithstanding the numerous benefits of higher plants, their potential as sources of new drugs is still largely unexplored as only a small proportion of the reported 500,000 plant species have been investigated for pharmacological activities [10, 11].

The plant *Olea europaea* subspecies africana belongs to the family Oleaceae commonly referred to as "African wild olive", meanwhile in South Africa, it is locally called "motholoari" (Sotho and Tswana people) and "umnquma" (Xhosa, Zulu and Ndebele people) [12]. It is native to tropical and warm temperate regions of the world [13] and naturally distributed in South Africa [14, 15]. A decoction of the leaves is used in traditional medicine to treat diabetes and hypertension [16], as well as malaria and fevers [17, 18]. Jiang and Takamura [19] demonstrated that olive fruits possess high antioxidant activity. The ethanolic leaf extracts were also reported by Lafka *et al*., [20] to possess a high activity against DPPH radicals. Other extracts such as the aqueous olive leaves extract [21] and leaves infusion [22] have also been investigated. Oleuropein is considered to be the main active compound responsible for the biological activities of *O. europaea* leaves, although some of the other phytochemical compounds have been observed to contribute to the biological activities [23, 24].

O. europaea is commonly used in the folklore medicine in Cala community, Eastern Cape especially in the management of chronic non-communicable diseases such as diabetes. Although different studies have been carried out on *O. europaea*, the report on *O. europaea* from Cala community, Eastern Cape remains obscure. Hence, there is limited data available on the possibility of intraspecific chemodiversity for this species. This study therefore was aimed at investigating the medicinal potential of the *Olea europaea* subspecies africana growing in this community and validating its usage in traditional therapy by evaluating the antimicrobial and antioxidant activities of the leaves extracts as well as identifying the major compounds from the essential oil using gas chromatography mass spectrometry (GC-MS) analysis.

MATERIAL AND METHODS:

Collection and Identification of the Plant: The leaves of *O. europaea* were collected in May, 2016 from a mountain in Cala community, situated at geographical coordinates of 31.5230° S, 27.6980° E in the northern region of the Eastern Cape Province [25]. The plant was initially identified by its vernacular name "umnquma" and later authenticated in Selmar Schonland herbarium by a plant taxonomist in Botany Department, Rhodes University, Grahamstown. Then the voucher specimen was deposited in the Giffen Herbarium, University of Fort Hare under the accession ADE 2016/2.

Preparation of Plant Materials: The collected plant materials (leaves) were washed thoroughly with sterile water to remove dust and air dried at room temperature in the laboratory. The air-dried leaves were then pulverized using Polymix (PX-MFC 90 D model) till finely powdered and stored in an airtight container for further use. The extracts were then obtained using different solvents by maceration method [26]. Briefly the pulverized leaves (75 g) were prepared by soaking in 500 mL of organic solvents (n-hexane, ethanol and ethyl acetate) in 1000 mL glass bottles. The glass bottles were covered tightly to prevent spillage and then shaken at 100 rpm for 72 h at room temperature.

The extracts were filtered using a Buchner funnel and Whatman No.1 filter paper and then concentrated using vacuum rotary evaporator (BÜCHI Rotavapor R-200/205, Model R205V800). Stock solutions of crude n-hexane, ethanol and ethyl extracts were prepared by diluting each of the dried extracts with the solvents used for the extraction process to give the required concentrations needed for this study. Prior to the reconstituting of the extracts with the extracting solvents, the extracts were stored in pre-weighed screw capped bottles and the yield of extracts was weighed and then screw capped bottles were kept in refrigerator. Essential oil from the fresh leaves of *O. europaea* was extracted for 3 hours using a

hydro-distiller (Clevenger's-type apparatus) in a 5-L round bottom flask fitted in a condenser [27]. The extracted oil was treated with anhydrous sodium sulphate to remove the remaining water before collecting in tinted vials and storing in 4 °C.

Gas Chromatography-Mass Spectrometry (GC–MS) Analysis: Chemical analysis of *O. europaea* essential oil was carried out as previously described by [28], using GC system (7890B), mass selective detector (Agilent 5977A) Chemettrix (pty) Ltd; Agilent Technologies, Deutschland (Germany) and a Zebron-5MS column (ZB-5MS 30 m × 0.25 mm × 0.25 um) (5%-phenylmethylpolysiloxane). The following conditions were used for the column and temperature: GC grade helium was at a flow rate of 2 mL/min and while splitless 1 ml injections were used. The injection port, ion source and oven temperatures were set at 280 °C, 280 °C and 70 °C respectively. The ramp settings were; 15°C/min to 120 °C, then 10 °C/min to 180 °C, then 20 °C/ min to 270 °C and held for 3 min. The mass spectra data were recorded using the Mass Selective Detector (MSD).

The various components present in the essential oil were identified by comparing the spectrum obtained through the GC-MS analysis with the spectrum of the known components stored in the National Institute Standard and Technology (NIST) database (NIST/EPA/NIH mass spectral library 2014). The quantity of compounds was estimated by integrating the peak areas of spectrograms.

Antioxidant Assay: The antioxidant potential of *O. europaea* solvent extracts were analyzed using three different antioxidant assays.

I) 2, 2-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid) Diammonium Salt (ABTS) Assay: The method of decolorization reaction as previously described by Re *et al*., [29] was adopted. Briefly, potassium persulfate solution (2.45 mM) was used to oxidize ABTS solution (7.0 mM) in equal amount forming pre-formed ABTS monocation (ABT). The mixture was then left for 12 h in a dark cupboard at ambient temperature for complete reaction. Thereafter, 1 mL of the solution was mixed with 60 mL methanol to attain 0.705 ± 0.001 at 734 nm, which is the absorbance required in performing the assay.

Then 100 µl of methanol was added to the wells except the second and third rows respectively. Thereafter, 200 µl of the *O. europaea* extracts and standard compound (SC) prepared in methanol were then added into the third row in triplicate. Starting from the first column in the third row, a twofold serial dilution was done by mixing the well contents and transferring 100 µl into the second well of the same column and this procedure was repeated until the 7th well of the same column with the remaining 100 µl from the 7th well discarded. The two fold dilution method was used to prepare the 0.03125 - 0.5 mg/mL concentrations of the standard (Vitamin C) and *O. europaea* extracts in the wells. Then 100 µl of the already prepared ABTS solution were added into all the wells. The mixture was allowed to react for seven minutes thereafter the absorbance was recorded at 734 nm spectrophotometrically. The percentage inhibition of ABTS by the extracts and Vitamin C were calculated using the following equation:

Inhibition (%) = [(Abs control – Abs sample) / (Abs control)] × 100

Where Abs control is the absorbance of the ABTS radical + methanol (control sample) and Abs sample is the absorbance of ABTS radical + extracts or standard (various samples).

A dose-response curve was plotted and used to generate the regression equation which was used to determine the IC_{50} values for each of the extracts tested and the standard compound (SC).

II) Lipid Peroxidation Assay: A modified thiobarbituric acid-reactive species (TBARS) protocol previously described by Badmus *et al.,* [30] was adopted with minor modifications (methanol was used for serial dilution of oils in place of distilled water), to measure the lipid peroxide formed using egg-yolk as lipid-rich media [31]. Ten percent egg yolk homogenate (0.5 mL) was mixed with the *O. europaea* extracts at varying concentrations of 0.03125 - 0.50 mg/mL and brought up to 1.0 mL. To induce the lipid peroxidation, 0.05 mL of $FeSO_4$ (0.07 M) was mixed with the solution above and incubated for 30 min. Then, 1.5 mL of 10% acetic acid (pH 3.50) and 1.5 mL of 0.80% 2-thiobarbituric acid in (1.1% sodium dodecyl sulphate and 20% trichloroacetic

acid) was added, vortexed and allowed to complete reaction in water bath at 65 °C for one hour. On cooling, n-butanol (0.5 mL) was mixed with individual tubes and subjected to centrifugation for ten minutes at three thousand revolutions per minute. Finally, at 532 nm organic layer absorbance of each tube was then recorded. Methanol was employed as the blank. The percentage inhibition of lipid peroxide was obtained using the equation stated for ABTS assay. The results were in triplicate and average values computed.

III) 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay: The effect of *O. europaea* extracts on DPPH radical was estimated using the method of Odeyemi [32]. The DPPH solution (2.7 × 10-6 M) was prepared at a 0.1 mM concentration in methanol and protected from light after preparation. Then 100 µl of methanol was added to the wells except the second and third rows respectively. Thereafter, 150 µl of methanol was added into the third row. 50 µl of *O. europaea* extracts prepared in methanol was then added into the third row in triplicates. Starting from the first column in the third row, a twofold serial dilution was done by mixing the well contents and transferring 100 µl into the second well of the same column and this procedure was repeated until the 7th well of the same column with the remaining 100 µl from the 7th well discarded.

The two fold dilution method was used to prepare the 0.3125 - 0.5 mg/mL concentrations of the standard and *O. europaea* extracts in the wells. Then 100 µl of the already prepared DPPH radical was added into all the wells. Afterwards, the reaction mixture was vortexed, and left for half an hour at ambient temperature in the dark. The ability of *O. europaea* extracts to scavenge DPPH radical was calculated using the same equation as stated for ABTS assay.

Antibacterial Activity:

Test Organisms: The antibacterial activity was done using the American Test Culture Collection (ATCC) and clinical isolates obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, South Africa. The microorganisms used were *Klebsiella pneumoniae* (ATCC 4352), *Serratia marcescens* (ATCC 9986), *Pseudomonas aeruginosa* (ATCC 19582), *Enterococcus faecalis* (ATCC 29212), *Shigella flexneri* and *Proteus vulgaris*.

Preparation of Bacterial Suspensions: A suspension of the bacterial strains was freshly prepared by inoculating fresh stock culture from each strain into separate broth tubes, each containing 7 mL of Muller Hinton Broth. The inoculated tubes were incubated at 37 °C for 24 h. After 24 h of incubation, the bacterial suspensions (inoculums) were diluted serially to an optical density of McFarland 0.5 using isotonic sodium chloride solution. Dilutions matching with 0.5 McFarland scale standards were then selected for screening of antibacterial activities. Ciprofloxacin 100 µg/mL was used as reference drug [33].

Disc Diffusion Method: The antimicrobial activity of *O. europaea* extracts against bacterial strains was tested using disc diffusion method [34]. Sterile disks with 6 mm in diameter of Whatman No. 1 filter paper were used. Sterile plates of Mueller-Hinton agar was seeded with 150 µl cell culture suspension matching 0.5 McFarland of target microorganisms. 0.4 g of each sample was dissolved in 1 mL of the respective solvents used for extraction. The disc was soaked with 0.01 mL of the extracts and the solvent was allowed to dry. The disc was then placed on the Mueller Hinton agar plate previously inoculated with 0.5 McFarland suspensions and kept at 4 °C for 48 hours to allow the extracts to diffuse into the media. The plates were incubated at 37 °C for 24 h and the inhibition zones in mm were checked. The tests were carried out in triplicates and the data were presented in average. The positive antibacterial activity of *O. europaea* extracts were established by the presence of measurable zones of inhibition.

Minimum Inhibitory Concentration (MIC): The MIC was performed on the ethyl acetate and ethanol extracts which on screening was found to be moderately active, using agar dilution method. Mueller-Hinton agar was firstly prepared according to manufacturer's instructions and sterilized by autoclaving. The sterilized media was then allowed to cool to about 50 °C, and 19 mL of the molten agar was added to sterile conical flasks, in addition to 1 mL of different concentrations of the *O.*

europaea extracts, standard and the control (DMSO) to make a final volume of 20 mL. The contents of the flasks were then thoroughly mixed and gently poured into properly labeled sterile petri dishes. The concentrations of the extracts used therefore ranged from 0.625 to 10 mg/mL. The plates were inoculated with 0.5 McFarland suspensions and then incubated at 37 °C for 24 h. The lowest concentration which was able to inhibit visible growth of the tested organisms was recorded as the MIC [35, 36].

Statistical Analysis: The IC_{50} value was calculated using linear regression analysis after generating the standard curve for the extracts and the standard compound. Analysis of variance was performed by one way ANOVA using MINITAB Release [17]. The significant levels were tested at $P < 0.05$ and Microsoft Office Excel was used to plot bar charts.

RESULTS: Effects of extracting solvent on the extracts yields from the medicinal plant materials. The leaves and stems of *O. europaea* ethanol extracts gave the highest yield 11.66 and 8.0 % respectively, while n-hexane extracts had the lowest yield of 1.89 and 1.04 % respectively. The yield of percentage is shown in **Fig. 1**.

Chemical Analysis of the Essential Oil of *O. europaea*: Sixty one compounds were identified representing 93.031 % of the total oil content.

Table 1 shows the chemical composition of *O. europaea* essential oil, including their retention time (RT), molecular formula, molecular weight (MW) and peak area in percentage. Based on the mass spectra peak areas (**Fig. 2**), the major compounds revealed were the nonanal (10.57%), phytol (6.40%), 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec- 1- ene (5.65%), 4- tert- Butyl-catechol, dimethyl ether (4.63%), octadecane (4.40 %), 2-Hexanal, (E)- (2.95%), 6, 10-dimethyl-5, 9-Undecadien-2-one (2.91%), 1, 2, 4a, 5, 6, 8a-hexahydro- 4, 7- dimethyl- 1- (1- methylethyl)-naphthalene (2.91%), 2- Decanal, (E)- (2.78%) (**Table 1**); and the minor compounds were 2, 6-Octadien-1-ol, 3, 7-dimethyl-, (Z)- (0.39%), heptadecane (0.39%), isopropyl palmitate (0.29 %) and silicic acid (0.20%).

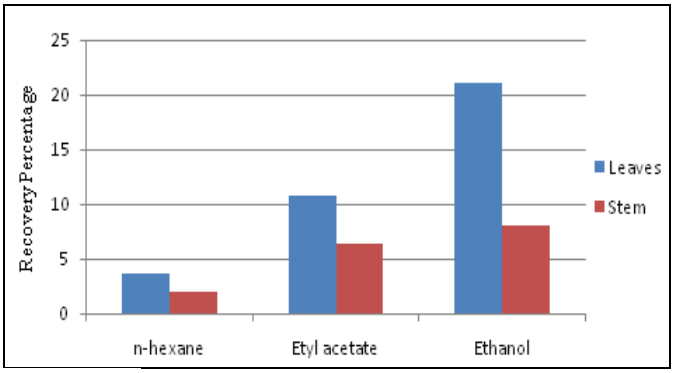

FIG. 1: EXTRACT YIELD PERCENTAGE OF DIFFERENT EXTRACTS OF *O. EUROPAEA*

TABLE 1: CHEMICAL COMPOSITION OF ESSENTIAL OIL IN LEAVES OF *OLEA EUROPAEA* SUB SP. AFRICANA

S. no.	Retention time (min)	Name of the Compounds	Molecular Formula	Molecular Weight	Composition of constituent
1	3.255	2-ethenyl-2-Butenal	C_6H_8O	96.127	1.56
2	3.292	(E)- 2-Hexenal	$C_6H_{10}O$	98.143	2.95
3	3.365	1-Hexanol	$C_6H_{14}O$	102.175	0.75
4	3.638	Heptanal	$C_7H_{14}O$	114.185	1.00
5	3.975	α-Pinene	$C_{10}H_{16}$	136.234	1.89
6	4.204	3-ethenylpyridine	C_7H_7N	105.137	2.21
7	4.412	(E,E)- 2,4-Heptadienal	$C_7H_{10}O$	110.154	1.58
8	4.450	Octanal	$C_8H_{16}O$	128.212	0.77
9	4.530	α-Phellandrene	$C_{10}H_{16}$	136.234	1.72
10	4.729	(+)-4-Carene	$C_{10}H_{16}$	136.234	0.79
11	4.908	(E)- 2-Octenal	$C_8H_{14}O$	126.196	0.50
12	4.972	1-Octanol	$C_8H_{18}O$	130.228	1.87
13	5.198	5-ethenyl-2-methylpyridine	C_8H_9N	119.164	0.88
14	5.271	Nonanal	$C_9H_{18}O$	142.239	10.57
15	5.674	2,5,5-trimethyl-1,6-Heptadiene	$C_{10}H_{18}$	138.249	0.731
16	5.720	(E)- 3,3 dimethylcyclo hexylidene-acetaldehyde	$C_{10}H_{16}O$	152.233	1.07
17	5.772	1-Methylpentyl cyclopropane	C_9H_{18}	126.239	0.80
18	6.059	Decanal	$C_{10}H_{20}O$	156.265	2.23

19	6.278	3-Isopropylidene-5-methyl-hex-4-en - 2-one	$C_{10}H_{16}O$	152.233	0.99
20	6.418	Geraniol	$C_{10}H_{18}O$	154.249	0.39
21	6.485	(E)- 2-Decenal	$C_{10}H_{18}O$	154.249	2.78
22	6.724	(E,E)-2, 4-Decadienal	$C_{10}H_{16}O$	152.233	0.76
23	6.809	4-tert-Butylcatechol	$C_{12}H_{18}O_2$	194.270	4.63
24	6.892	9-Oxabicyclo[4.2.1]non-7-en-3-one	$C_8H_{12}O$	124.180	1.80
25	6.995	2,6,10,10-tetramethyl-1-Oxaspiro[4.5]dec-6-ene	$C_{13}H_{22}O$	194.313	1.10
26	7.206	2-Undecenal	$C_{11}H_{20}O$	168.276	0.78
27	7.422	(E)-beta-farnesene	$C_{15}H_{24}$	204.351	0.60
28	7.631	(E)-α-damascone	$C_{13}H_{20}O$	192.297	0.50
29	7.751	Caryophyllene	$C_{15}H_{24}$	204.351	2.04
30	7.798	Geranylacetone	$C_{13}H_{22}O$	194.313	2.91
31	8.093	trans-.beta.-Ionone	$C_{13}H_{20}O$	192.297	2.39
32	8.150	21.xi-methyl-17-isocholest-16-en-3.beta.-ol	$C_{27}H_{46}O$	386.654	0.47
33	8.232	Selinene	$C_{15}H_{24}$	204.351	0.69
34	8.323	α-cadinene	$C_{15}H_{24}$	204.351	2.91
35	8.508	Nerolidol	$C_{15}H_{26}O$	222.366	1.82
36	8.631	Benzoic acid, nonadecyl ester	$C_{26}H_{44}O_2$	388.626	0.79
37	8.750	Spathulenol	$C_{15}H_{24}O$	220.351	0.80
38	8.809	Caryophyllene oxide	$C_{15}H_{24}O$	220.351	1.49
39	8.961	Alloisolongifolene	$C_{15}H_{24}$	204.351	0.52
40	9.089	2-isopropyl-5-methyl-9-methylene bicycle[4.4.0]dec-1-ene	$C_{15}H_{24}$	204.351	5.65
41	9.281	7-epi-cis-sesquisabinene hydrate	$C_{15}H_{26}O$	222.366	0.94
42	9.424	Farnesol	$C_{15}H_{26}O$	222.366	0.46
43	10.058	6,10,14-trimethyl2-Pentadecanone	$C_{18}H_{36}O$	268.478	1.66
44	10.235	1,2-Benzenedicarboxylic acid,bis (2-methylpropyl) ester	$C_{16}H_{22}O_4$	278.344	0.72
45	10.467	2,6,10,14,18-Pentamethyl-2,6,10,14, 18-eicosapentaene	$C_{25}H_{42}$	342.601	0.97
46	11.388	Phytol	$C_{20}H_{40}O$	296.531	6.40
47	14.659	Octadecane	$C_{18}H_{38}$	254.494	3.37

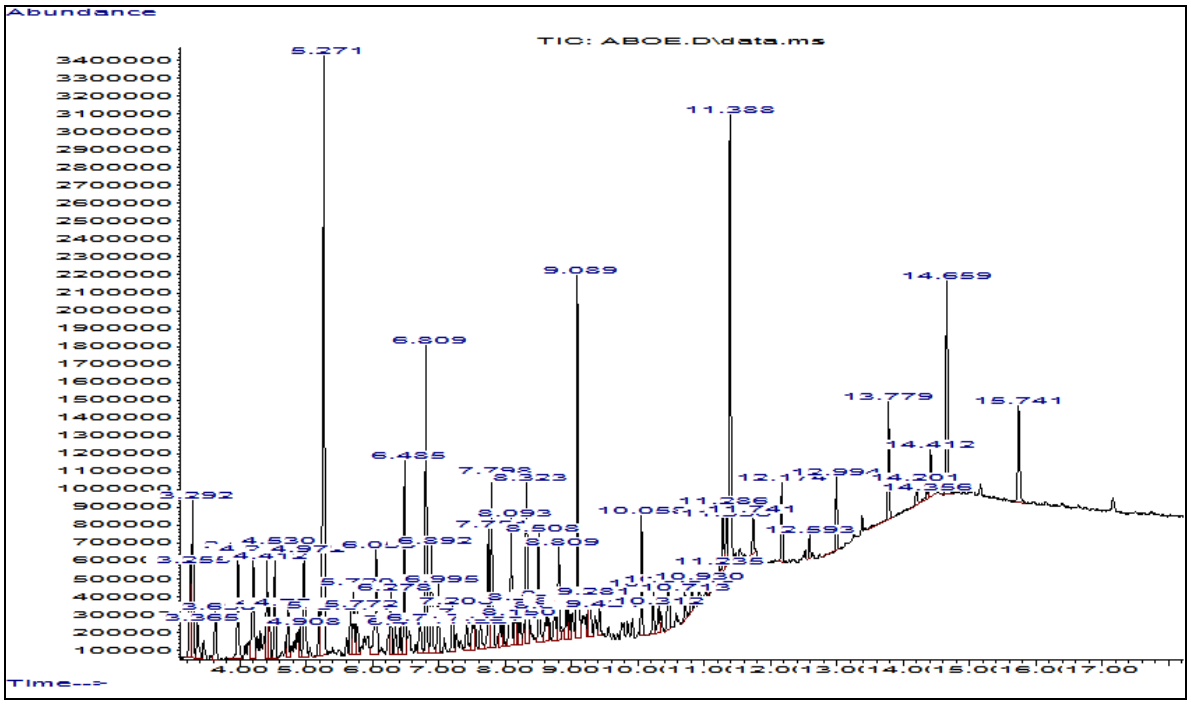

FIG. 2: TOTAL IONS CHROMATOGRAPH OF GC/MS SHOWING THE CHEMICAL ANALYSIS OF *O. EUROPAEA* ESSENTIAL OIL

***In vitro* Antioxidant Activity:** The extracts from *Olea europaea* stem and leaves exhibited ABTS and DPPH radical scavenging effects in a concentration-dependent manner. The extracts also significantly decreased lipid peroxidation in the lipid-rich egg yolk (**Fig. 3 - 10**).

ABTS: The quantitative determination of the radical scavenging activity of the ABTS free radical involved measuring the disappearance of the colored free radical, ABTS. **Fig. 3** represents the concentration response curve used for the indication of decreased ABTS concentration obtained as the sample concentration increased.

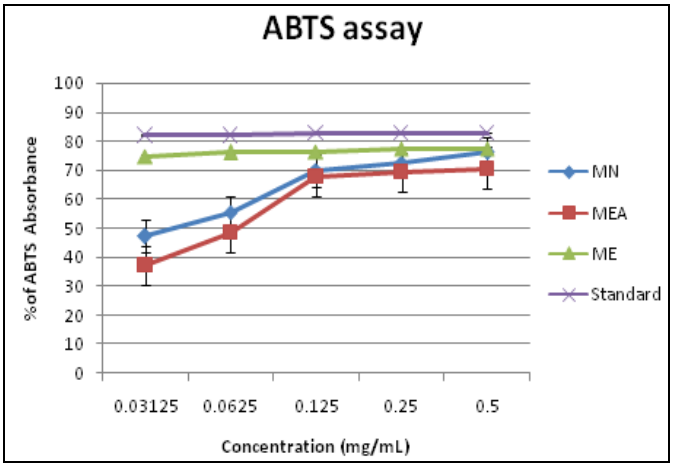

FIG. 3: DOSE RESPONSE CURVE FOR EACH OF THE EXTRACTS TESTED. This indicates the relationship between the concentration of the extracts (mg/mL) and the percentage of ABTS after incubation. MEA-Ethyl acetate extracts, MN-N-hexane extracts, ME-Ethanol extracts, Standard-Vitamin C.

In the ABTS scavenging activity, ethanol stem extract (MSE) significantly scavenged ABTS free radical (60.57 - 79.27%) more than n-hexane stem extract (MSN) (16 - 72%), while ethyl acetate (MSEA) had the least scavenging capacity (16.06 - 68.07%). Overall, the standard, Vitamin (C) was more effective than all extracts at all concentrations tested (82 - 83%). The linear regression equation generated from each extracts and standard compound were used to determine the IC_{50} value. The IC_{50} obtained were 9.18 mg/mL for MN, 4.58 mg/mL for MEA, 3.72 mg/mL, 3.45 mg/mL for MSN, 0.64 mg/mL for MSEA, 2.31 mg/mL for MSE and 1.3 x 10 - 5 mg/mL for Vitamin C.

The IC_{50} value determination indicates that the scavenging activity was significantly higher with Vitamin C, followed by MSEA, MSE,MSN, ME, and then MEA and MN ($p < 0.05$) (**Fig. 4** and **5**) (**Fig. 11**).

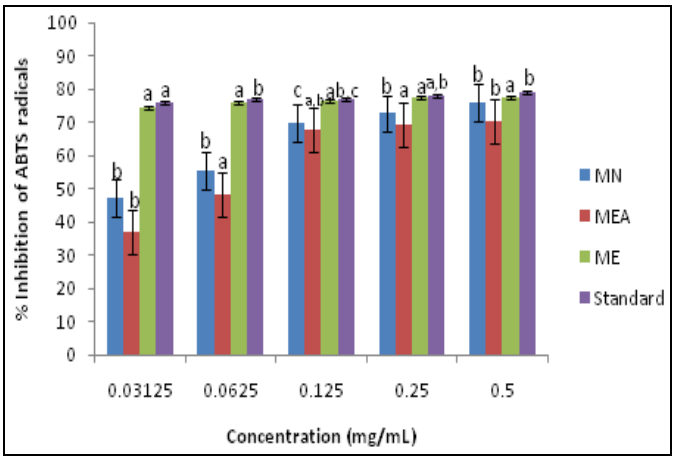

FIG. 4: ABTS RADICAL SCAVENGING ACTIVITY OF DIFFERENT *O. EUROPAEA* LEAF EXTRACTS AND THE STANDARD ANTIOXIDANT, VITAMIN C. Bar graphs with different letter superscript within the same concentration are significantly different ($p < 0.05$).

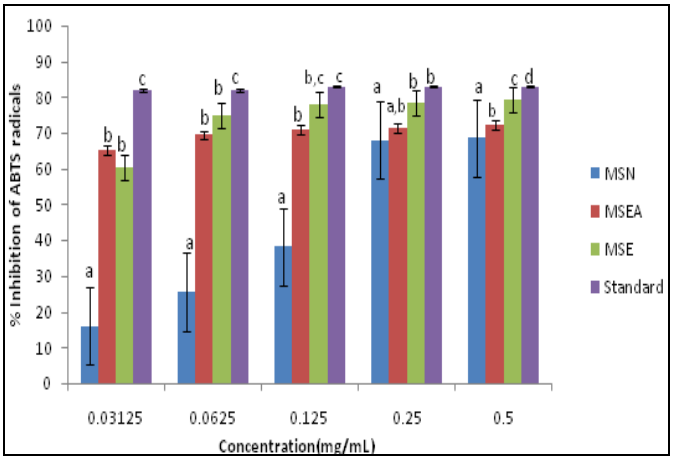

FIG. 5: ABTS RADICAL SCAVENGING ACTIVITY OF DIFFERENT *O. EUROPAEA* STEM EXTRACTS AND THE STANDARD ANTIOXIDANT, VITAMIN C. Bar graphs with different letter superscript within the same concentration are significantly different ($p < 0.05$).

Lipid Peroxidation Test: The antioxidant activity of the different extracts of the plant *O. europaea*, as measured by the ability to scavenge lipid peroxides, was compared with the standard Vitamin C. N-hexane extracts of the leaf and stem demonstrated overall higher activity than that of the ethyl acetate and ethanol extracts with percentage inhibitions ranging from 52.05 - 67.25%. At the 0.5 mg/mL, the scavenging activity of the ethanol and ethyl acetate reached 64.91% and 64.56% which was comparable to the standard Vitamin C with 64.91% at the same concentration for the leaf extracts (**Fig. 6**).

Although only ethyl acetate stem extract was close to that of the Vitamin C with 60.28% while the Vitamin C was 64% (**Fig. 7**). The results from the different concentrations were used to determine the concentration needed to attain 50% lipid peroxide scavenging effect (IC_{50}). The IC_{50} value evaluation indicated that for the leaf and stem extracts, the n-hexane extract had the highest inhibitory capacity (0.065 mg/mL and 0.17 mg/mL respectively) followed by ethyl acetate (1.27 mg/mL) for the leaf extract and ethanol (1.28 mg/mL) for the stem extract (**Table 2**).

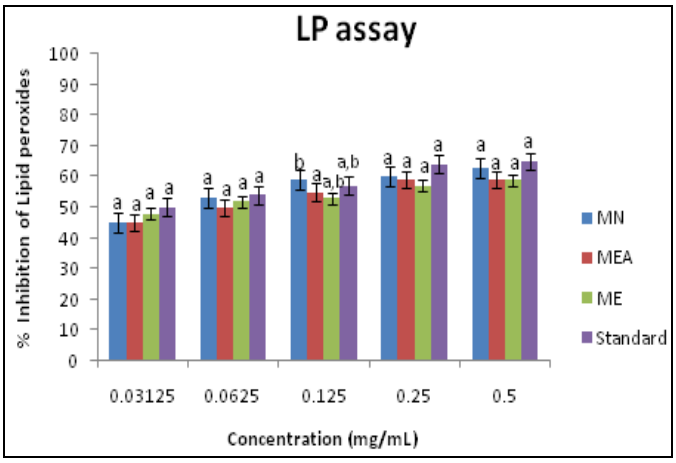

FIG. 6: LIPID PEROXIDE RADICAL SCAVENGING ACTIVITY OF *O. EUROPAEA* LEAVES EXTRACT AND THE STANDARD ANTIOXIDANT, VITAMIN C. Bar graphs with different letter superscript within the same concentration are significantly different ($p < 0.05$).

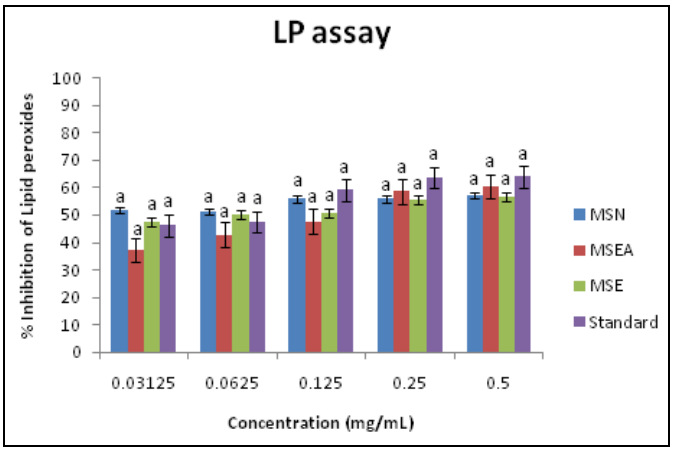

FIG. 7: LIPID PEROXIDE RADICAL SCAVENGING ACTIVITY OF DIFFERENT *O. EUROPAEA* STEM EXTRACTS AND THE STANDARD ANTIOXIDANT, VITAMIN C. Bar graphs with different letter superscript within the same concentration are significantly different ($p < 0.05$).

DPPH: The DPPH radical scavenging activity was represented as a concentration response curve which indicated that the sample concentration increased as the DPPH concentration decreases (**Fig. 8 - 10**).

The regression equation generated from the dose response curve enabled the calculation of the IC_{50} value, the concentration of the extracts that caused the inhibition of 50% of the initial concentration of the DPPH radicals (**Fig. 11**, **Table 2**).

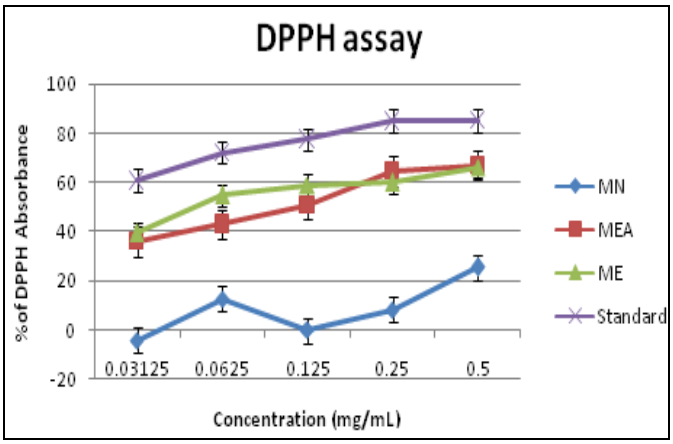

FIG. 8: DOSE RESPONSE CURVE FOR EACH OF THE *O. EUROPAEA* EXTRACTS TESTED. This indicates the relationship between the concentration of the extracts (mg/mL) and the percentage of DPPH after incubation. MEA-Ethyl acetate extracts, MN-N-hexane extracts, ME-Ethanol extracts, Standard-Vitamin C.

A linear correlation between the extract concentration and scavenging activity was observed when the free radical scavenging activity of each extract was compared. The extracts effectively reduced DPPH radical with ethanol and ethyl acetate leaf extracts having higher scavenging activity. However, none of the extracts had a higher activity than the standard, Vitamin C, used.

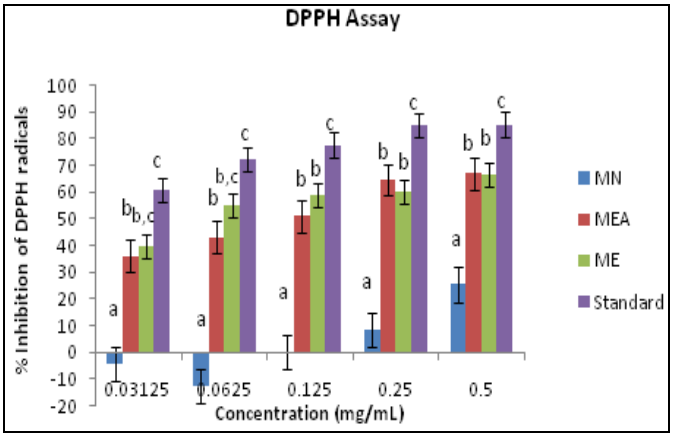

FIG. 9: COMPARISON OF THE DPPH RADICAL SCAVENGING EFFECT OF DIFFERENT *O. EUROPAEA* LEAVES EXTRACT WHERE THE INDICATED AMOUNT OF EXTRACTS WAS ADDED TO THE DPPH SOLUTION

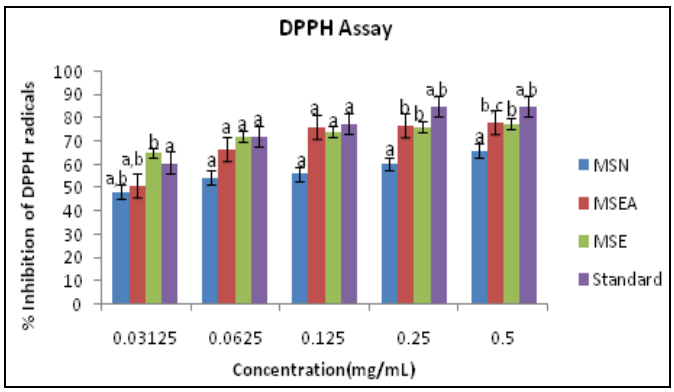

FIG. 10: COMPARISON OF DPPH RADICAL SCAVENGING EFFECT OF THE DIFFERENT *O. EUROPAEA* STEM EXTRACT, WHERE THE INDICATED AMOUNT OF EXTRACTS WAS ADDED TO THE DPPH SOLUTION

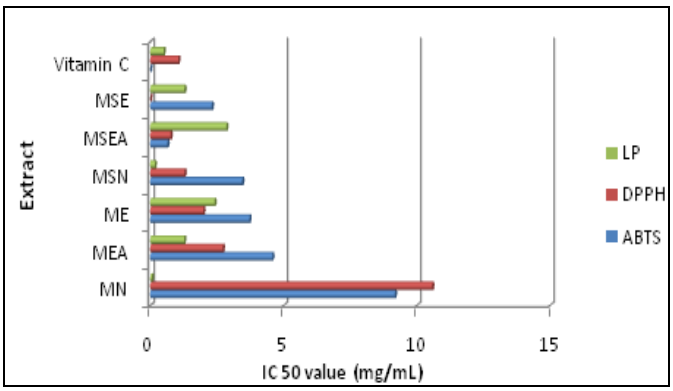

FIG. 11: COMPARISON OF THE ANTIOXIDANT ACTIVITY OF THE DIFFERENT *O. EUROPAEA* EXTRACTS WITH VITAMIN C. LP (Lipid Peroxidation), DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2, 2-azinobis-(3-ethylbenzothiazolin - 6-sulfonic acid) di-ammonium salt).

TABLE 2: ANTIOXIDANT CAPACITY OF THE DIFFERENT *O. EUROPAEA* EXTRACTS AND STANDARD (mg/ml)

	ME	MEA	MN	MSE	MSEA	MSN	SC
ABTS (IC_{50})	10.58	2.72	1.99	1.28	0.76	2.74×10^{-3}	1.05
LP (IC_{50})	0.065	1.27	2.41	0.17	2.85	1.28	0.5
DPPH (IC_{50})	9.18	4.58	3.72	3.45	0.64	2.31	1.3×10^{-5}

LP (Lipid Peroxidation), DPPH (2, 2-Diphenyl-1-picrylhydrazyl), ABTS (2, 2-azinobis-(3-ethylbenzothiazolin - 6-sulfonic acid) diammonium salt) determination in different solvent extracts of *O. europaea*

Antibacterial Activity of the Extracts: The *O. europaea* extracts exhibited inhibitory effects against some of the tested microorganism (**Table 3**). However, higher activity was observed in the ethanol and ethyl acetate leaf extracts. Antibiotic disks of ciprofloxacin were also tested against the microbes.

TABLE 3: GROWTH INHIBITION EFFECT OF EXTRACTS OF *OLEA EUROPAEA*

Microorganism		Extract			
Gram-positive bacteria	**Culture collection and Ref. No.**	**Ethanol**	**N-hexane**	**Ethyl Acetate**	**Standard**
Serratia marcescens	(ATCC 9986)		-	+	+
Enterococcus faecalis	(ATCC 29212)	+	-	+	+
Proteus vulgaris	(lab isolate)	+	-	+	+
Gram-negative Bacteria					
Klebsiella pneumoniae	(ATCC 4352)	+	-	+	+
Pseudomonas aeruginosa	(ATCC 19582)	+	-	+	+
Shigella flexneri	(lab isolate)	+	-	+	+

Minimum Inhibitory Concentration (MIC): The concentrations between 0.0625 to 10 mg/mL of the ethanol and ethyl acetate *O. europaea* leaf extracts were evaluated in order to determine their minimum inhibitory concentration as presented in **Table 4**. It was observed initially in the disc diffusion assay, that the level of inhibitory activity was only exhibited by the ethanol and ethyl acetate leaf extracts while the n-hexane extract had no antimicrobial effect against the test organisms. The ethanol and ethyl acetate leaf extracts which exerted inhibitory activity against the test microorganism were then used for the MIC determination (**Table 4**). None of the test micro organisms showed insensitivity to both extracts in the MIC assay, as the ethanol extract and ethyl acetate leaf extracts were able to inhibit both the gram positive (*S. marcescens*, *E. faecalis*, *P. vulgaris*) and gram negative (*K. pneumoniae*, *P. aeruginosa*, *S. flexneri*). The extent of growth inhibition of the microorganisms tested was dependent on the concentration of plant extract used. Although both extracts exhibited antibacterial activity with MIC value of 10 mg/ml against the other test microorganisms, ethyl acetate leaf extract was observed to significantly inhibit the growth of *S. marcescens*.

The ethyl acetate leaf extract with a MIC value of 0.625 mg/mL had strong inhibitory activity and this was significantly similar with that of ciprofloxacin against *S. marcescens* (**Table 4**), while with ethanol leaf extract, the minimum inhibitory concentration value of 1.25 mg/mL was observed against both *K. pneumoniae* and *S. marcescens*.

TABLE 4: MINIMUM INHIBITION CONCENTRATIONS (mg/ml) OF *OLEA EUROPAEA* SUB SP. AFRICANA

Microorganism	Extracts (mg/mL)		Standard (mg/mL)
Gram-positive Bacteria	**Ethyl Acetate**	**Ethanol**	
Serratia marcescens	0.625	1.25	< 0.625
Enterococcus faecalis	10	10	< 0.625
Proteus vulgaris,	10	10	< 0.625
Gram-negative Bacteria			
Klebsiella pneumonia	10	1.25	< 0.625
Pseudomonas aeruginosa	10	10	< 0.625
Shigella flexneri	10	10	< 0.625

DISCUSSION:

Effect of Extraction Solvent on the Extract Yields: The results obtained shows that extract yield depends on solvents, time and temperature of extraction as well as the chemical nature of the sample (**Fig. 1**). This corroborates Shimada *et al*., [37] report that under the same time and temperature conditions, the solvent used and the chemical property of the sample are the two most important factors. The extraction yield and percentage yield of extract determined for the *O. europaea* stem and leaves are presented in **Fig. 1**.

The maximum amount of extract yield was obtained from the ethanol extract with 8% and 21.04% for the stem and leaves respectively. It was observed that the extraction yield increased significantly as the polarity of solvents employed increased [n-hexane (non-polar) to ethanol (polar)]. When the solvent n-hexane was used, an extract yield of 3.68% and 2% for leaf and stem respectively was recorded which was the least amount of recovered extract. Although the chemical components in the solvents extracts were not determined, the results suggest that more components may be present in the polar than non-polar solvent.

As the efficiency of extraction and biological potential of the plants are known to depend on the polarity of the extraction solvent, due to the occurrence of different compounds with varied chemical characteristics that may or may not be soluble in a particular solvent. Hence, extraction is the first critical step in drug discovery process from plants, considering this, this study employed solvents of different polarities.

Effect of Extraction Solvent on the Extract Yields: The result presented in **Table 3** shows that the extraction yield of *O. europaea* increased significantly as the polarity of solvents used in the extraction process changed from n-hexane (non-polar) to ethanol (polar). When ethanol was used, an extract yield of 15.78 g and 0.8 g for leaf and stem respectively was recorded which was the highest amount of recovered extract. The maximum amount of extract yield was recovered from the ethanol extract with a percentage yield of 8% and 21.04% for the stem and leaves respectively. This indicates that more polar compounds are present in the extracts and the different chemical components of the *O. europaea* may have resulted in the varied amounts of extraction yields.

GC - MS Analysis: Previous studies have shown that in the investigation of the components of plant origin, the GC-MS analysis performs a vital part, as plant materials are usually very complex making the GC-MS analysis suitable for plant analysis due to its high selectivity and sensitivity. Hence, the GC-MS analysis is considered to be the gold standard in scientific analysis [38]. GC–MS has been used in different studies which investigated phytochemical screening worldwide [39, 40]. The phytochemical analysis of *O. europaea* oil by GC-MS revealed the presence of aldehydes, monoterpene, sesquiterpenes, and alcohol as the major compound groups. Phytol, one of the most prevailing phyto-constituents is a significant therapeutic molecule identified with known activity. It is a precursor for Vitamins E and K, it has been reported to be an anticancer, anti-inflammatory and antimicrobial agent [41, 42].

It is able to reduce free radical production *in vitro* due to the activity of its hydroxyl group [43]. The potent antioxidant activity exhibited could be linked with the phytol content of the *O. europaea*. Although, the other components (major and minor) of *O. europaea* essential oil, such as caryophyllene, α-pinene, Selinene, α-farnesol, Nerolidol, Spatulenol, cis-β-Farnesene, α-phellandrene and their overall combined synergistic activity could also have enhanced the activity of the plant.

According to previous studies, it has been reported that the main active constituent of *O. europaea* leaves is oleuropein and it is responsible for activities such as antioxidant, antimicrobial, hypolipidemic and hypotensive [23, 24]. Amabeoku and Bamuamba [44] also reported on the possibility of oleuropein being present in the leaves of the South African species of *O. europaea* subsp. Africana. However, this active compound was not present in this sample. This finding partly conforms to the studies of Somova *et al*., [14] who reported that oleuropein occurs only in trace amounts in *O. europaea* leaves.

This absence may therefore be due to the observation of Gholivand *et al*., [45] who reported that the composition of essential oil vary from place to place, as it depends on variables such as soil biology, location, climatic conditions and habitat. Radulović *et al*., [46] also noted that these variables regulate the plant internal physiology leading to the various array of chemicals produced within the same plant species.

***In vitro* Antioxidant Activity:** Antioxidant therapies have been proposed to likely inhibit the onset of diabetes and in addition prevent the development of diabetes complications [47, 48]. Therefore, medicinal plants especially those with high content of antioxidants perform an integral part in the protection of the human body against harm by reactive oxygen species and in the amelioration of diseases associated with oxidative stress such as diabetes mellitus [5].

Plants contain a large number of antioxidants making it cumbersome to measure the antioxidant activity of each compound so therefore different methods have been developed to evaluate the antioxidant capacity of different plant materials [49].

In this study, three different free radical generating methods were used to assess the scavenging properties of the extracts of *O. europaea*. Different range of scavenging activities was exhibited by the extracts when tested against the different systems and this may be attributed to the various mechanisms of the radical antioxidant reactions in the different assays [50]. The extent of inhibition of these radicals by *O. europaea* extracts was dependent on the concentration used. From the antioxidant results presented in **Fig. 3 - 10**, the extracts effectively inhibited the ABTS, lipid peroxide and DPPH radicals when compared to Vitamin C which was used as standard. The antioxidant ability of the extracts was expressed as IC_{50}, the concentration necessary for the inhibition of radical formation by 50% (**Fig. 11**).

ABTS Radical Scavenging Assay: The conventional ABTS spectrophotometric assay which is also referred to as the Trolox equivalent antioxidant capacity (TEAC) assay was used to quantify the antioxidant activity of the extract [51]. In this assay, the blue coloured ABTS radical converts to its colourless form when it reacts with antioxidants. **Fig. 4 - 5** shows the ABTS radical scavenging effect of different extracts of *O. europaea* in comparison with Vitamin C. The percentage inhibition values were noted to increase as the concentration of the extracts increased in the assay. A standard curve was plotted by using the different concentrations of extracts and Vitamin C against the percentage of inhibition obtained and this was further used to calculate the IC_{50} (mg/mL).

The IC_{50} value was used to quantify the antioxidant activity of the different extracts of *O. europaea* tested. The IC_{50} (mg/mL) of each extract was determined as 9.178 for n-hexane extract, 4.58 for ethyl acetate extract, 3.72 for ethanol extract of *O. europaea* and 0.00013 in case of Vitamin C respectively. The antioxidant activity of the extracts increased in the following order N-hexane<Ethyl Acetate <Ethanol <Vitamin C. The Vitamin C demonstrated a very strong antioxidant activity and this could be due to the fact that it was a pure compound; unlike the extracts which are crude in nature, consisting of various compounds with the active compound present in low quantities. It was observed that the ethanol leaf extracts had the highest antioxidant activity amongst the

extracts used, although none of the extracts could match the Vitamin C in its antioxidant activity.

Lipid Peroxidation Assay: Thiobarbituric acid (TBARS) is usually measured in order to monitor oxidative stress and lipid peroxidation both *in vivo* and *in vitro* [52]. The reduction of lipid peroxides was used to indicate the scavenging potential of the *O. europaea*. Lipid peroxidation assay was carried out *in vitro* by inducing egg-yolk homogenates using ferrous sulphate. The n-hexane extract of the plant had higher lipid peroxidation inhibition than ethanol extract of the plant. The scavenging activity of the lipid peroxide by the *O. europaea* leaf and stem extracts, at the various concentrations compared with Vitamin C in terms of inhibition percentage is presented in **Fig. 6** and **7**.

The potential of the *O. europaea* to scavenge lipid peroxides and inhibit the formation of ABTS reflects also in its ability to scavenge DPPH. The ABTS radical scavenging activity by the plant extract was noted to be appreciable; these results therefore imply that the *O. europaea* extract could be strong antioxidants and may be useful for the treatment of radical related pathological damage especially when used at higher concentration [53].

DPPH Radical Scavenging Assay: DPPH radical scavenging assay is a widely used parameter for evaluating the antioxidant potential of natural compounds in a relatively short period. DPPH is a stable light sensitive, nitrogen-centered free radical which produces violet color in methanol solution [54]. The scavenging potential of the antioxidant compounds causes it to be decolorized to yellow or colourless [55]. It is relative to the hydrogen donating ability and is indicated by the degree of discoloration [54].

The *O. europaea* extracts revealed their hydrogen donating ability when added to the DPPH radical, resulting in decreased absorbance and the production of a yellow colored diphenylpicryl hydrazine compound. **Fig. 9** and **10** represent the DPPH radical scavenging activity of *O. europaea* leaf and stem extracts, at the various concentrations compared with Vitamin C in terms of inhibition percentage, in which *O. europaea* leaf and stem ethanol and ethyl acetate extracts demonstrated stronger DPPH radicals scavenging activity (percentage inhibition values ranging from 67% to 36%) than the leaves of the n-hexane extracts which at 0.5 mg/mL (the highest concentration tested) had 25%.

Lower IC_{50} values indicate better DPPH radical scavenging activity and so for each extract the IC_{50} value was calculated from the curves plotted. For the stem and leaf, ethanol extract exhibited the lowest IC_{50} value (0.002 mg/mL and 1.99 mg/mL) while the n-hexane extract demonstrated the highest IC_{50} value (1.28 mg/mL and 10.58 mg/mL) respectively as shown in **Fig. 11**.

The result of DPPH scavenging activity assay in this study indicates that *O. europaea* was potently active suggesting that the plant extract contain antioxidant compounds that are have the ability to donate hydrogen to the DPPH free radical in order to remove the unpaired electron which is responsible for radical's reactivity. The antioxidant properties of various extracts of the plant have earlier been studied and it has been observed to have high antioxidant properties.

Antibacterial Assay: Extracts of *O. europaea* have earlier been studied and they have been shown to exhibit a wide range of pharmacological properties such as antibacterial, antifungal and antiviral [56, 57, 58]. From the antimicrobial results presented in **Table 4**, it was demonstrated that the ethanol and ethyl acetate leaf extracts of *O. europaea* exhibited activity against the growth of the pathogens tested in this study (**Table 4**). Both ethyl acetate and ethanol leaf extracts displayed strong antibacterial activity against *S. marcescens* and *K. pneumoniae*, thus indicating that the *O. europaea* is a good antibacterial source while confirming its traditional usage in the study area against infections associated with diabetes.

The inhibitory activity of *O. europaea* could be attributed to the presence of some of the essential oil compounds identified GC-MS analysis and ethanol and ethyl acetate extracts. Literature has shown that *O. europaea* leaf phenolics have strong antimicrobial activity than individual isolated phenolics when tested against microorganisms. The results of the findings of Lee and Lee [59] demonstrated that the combined phenolics showed evident activity against different microorganisms

than the isolated phenolic constituents. Hussain *et al*., [60] revealed that the ethanol and methanol crude extracts of leaves of cultivated and wild *O. europaea* strongly inhibited the growth of *K. pneumoniae*, *Proteus vulgaris*, *P. aeruginosa*, which confirmed the results of this current study.

The *O. europaea* leaf extracts have also been reported to strongly inhibit the growth of *Salmonella typhimurium*, *E. coli*, *S. aureus*, *Bacillus cereus*, *Listeria monocytogenes* including *P. aeruginosa* which was also tested in this study [61]. The ethyl acetate and ethanol leaf extracts were effective against the tested microorganisms due to the fact that the solvents ethyl acetate and ethanol permitted the extraction of the phenolic constituent contained in the *O. europaea*.

CONCLUSION: In view of the serious threats posed by NCDs, the salient pharmacological properties of medicinal plants imply a good alternative to failing antibiotics. *O. europaea* extracts showed significant activity indicating that it is a good source of antioxidant and antibacterial agents. The results of the present investigation also complement the ethnobotanical usage of the *O. europaea*, as the plants possess several chemical constituents revealed when elucidated by the GC–MS.

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AUTHORS' CONTRIBUTIONS: AAA and BCI designed the experiments, carried out the analysis, interpreted the results and wrote the manuscript, UUN assisted with writing and proof reading of manuscript LCO and AIO coordinated the research and manuscript preparation. All authors have read and approved the final manuscript.

CONFLICT OF INTERESTS: The authors declare that they have no conflicts of interests.

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