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Seed Essential Oil of *Eucalyptus citriodora*: Evaluation of the Secondary Metabolites, Radical Scavenging, Antioxidant, Anti-inflammatory, Analgesic, Antimicrobial Potential and Benefits

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ABSTRACT

Eucalyptus essential oil is an important aroma-therapeutic agent in the treatment of diverse diseases and ailments in human and animals. The aim of this study was to determine the composition and pharmacological properties of the seed essential oil of *E. citriodora* from Nigeria. The essential oil was extracted by hydro-distillation using Clevenger-type apparatus and analyzed using GC-MS. The total phenolic content, antioxidant, anti-inflammatory, analgesic and antimicrobial activities were measured by Folin-Ciocalteu's, 2,2′-diphenyl-1-picrylhydrazyl, reduction antioxidant, carrageenan, formalin and agar-well diffusion assays respectively. The GC-MS result showed the presence of 22 organic compounds making up 98.8% of the percentage composition of the essential oil. The main components of the oil were palmitic acid (29.00%), oleic acid (10.00%), E,E,E-α-springene (9.00%), 2-ethenyl-2,5-dimethyl-4-hexen-1-ol (8.00%), 2,4-dimethylheptane (6.00%), hexahydrofarnesyl acetone (5.00%), geranyl butanoate (4.00%), farnesol (4.00%), geranylgeraniol (4.00%) and trans-2-methyl-2-(4-methyl-3-pentenyl)-cyclopropanecarboxaldehyde (4.00%). The TPC value was 175.84 ± 0.00 µgmg-1 GAE. The DPPH IC_{50} and AAI values of the essential oil were 3.00 µgml-1 and 13.30, respectively. The essential oil was capable of scavenging free radicals in a range of 67.77%-71.95%, while the FRAP EC_{50} value was 2.00 µgml^{-1}. The essential oil exhibited high anti-inflammatory effect with value of 43.80% and analgesic potential by inhibition of both neurogenic (41.32%) and inflammatory pains (11.11%). The zones of inhibition were between 0.80-18.00 mm. The seed essential oil of *E. citriodora* consists of useful phyto-therapeutic compounds that may be of great use in the food and pharmaceutical industries.

Keywords: *Eucalyptus citriodora*; Seed essential oil; Phytochemicals; Pharmacological activity

INTRODUCTION

Essential oils recently received a higher interest by scientists because they have some useful therapeutic components that act synergistically for preservation and drug formulations [1-5]. Essential oils are used as flavouring and preservative substances in both food and pharmaceutical industries [4,6-8]. Odoriferous plants possess essential oils that are very important to the plants in diverse ways such as defence mechanism against pest attacks, and covering of cell membrane [7,9]. Essential oils are valuable natural products used by man and livestock for aromatherapy, phytotherapy, spices, addictive in feed stocks and nutrition [10,11]. Eucalyptus essential oils are used medicinally to treat many symptoms and ailments among human such as sore throat, cold, insomnia, cough, bronchitis, coughing, fever, flu, poor circulation, stress, depression, arthritis, pains, headaches, migraines, inflammation and dental problems [12-15]. Essential oils can have a single, synergistic or multiple targets of their activities. Essential oil as a complex mixture provides more effective activity than the activity of the single major component used alone. This means that minor constituents may be very important for the activity of essential oil and may have a synergistic effect with other component [2,16-18]. Treatment of chicken meat products with essential oils from medicinal plants improves the antioxidant property during refrigeration at very low temperature for a long period of time [19-20]. *Eucalyptus citriodora* Hook, commonly called lemon-scented gum is native to Australia. It is an evergreen tree 20-50 m in height. It is a tree that has different forms of leaves: the juvenile leaves and adult (phyllodes) leaves which are light green, glossy, and lanceolate. The foliage and flowers are of great interest, they are attractive to bees, and therefore they are used as beekeepers for the production of honey

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[21-22]. Seeds are shiny irregularly ellipsoid blackish in nature and 3-7 mm long dimension [23-25].

So far, there is scanty information on the seed of E. citriodora. Therefore, this study was aimed at screening the chemical composition and pharmacological properties of the essential oil obtained from the seeds of *E. citriodora* from Nigeria.

MATERIALS AND METHODS

Plant materials

The sample of the plant was collected from the Afforestation Research Station Kaduna, Nigeria and it was identified as *E. citriodora* Hook by Mr. Sylvester Boye of the same Institute.

Extraction of the essential oil

The fresh seeds were pulverized and the essential oil was obtained by hydro-distillation using all-glass Clevenger-type apparatus according to European pharmacopoeia [26]. The essential oil collected was then stored in vial in a refrigerator at 8°C to prevent evaporation.

Gc-ms analysis

The seed essential oil of *E. citriodora* was analysed using Shimadzu GC-MS-QP2010 Plus (Japan). The separations were carried out using a Restek Rtx-5MS fused silica capillary column (5%-Diphenyl-95%-dimethylpolysiloxane) of 30 mm × 0.25 mm internal diameter (di) and 0.25 mm in film thickness. The conditions for analysis were set as follows; column oven temperature was programmed from 60°C-280°C (temperature at 60°C was held for 1.0 min, raised to 180°C for 3 min and then finally to 280°C held for 2 min); injection mode, Split ratio 41.6; injection temperature, 250°C; flow control mode, linear velocity (36.2 cm/sec); purge flow 3.0 ml/min; pressure, 56.2 kPa; helium was the carrier gas with total flow rate 45.0 ml/min; column flow rate, 0.99 ml/min; ion source temperature, 200°C; interface temperature, 250°C; solvent cut time, 3.0 min; start time 3.5 min; end time, 24.0 min; start m/z, 50 and end m/z, 700. Detector was operated in EI ionization mode of 70 eV. Components were identified by matching their mass spectra data with those of the spectrometer data base using the NIST computer data bank, as well as by comparison of the fragmentation pattern with those reported in literature.

Total Phenolic Content (TPC) assay: The TPC of the essential oil was determined using the Folin-Ciocalteau method. 1 ml aliquot solution of the essential oil was mixed with 46 ml distilled water and 1 ml of Folin-Ciocalteau solution, then 3 ml of (2% w/v) Na_2CO_3 solution was added after 3 min, the mixture was thoroughly mixed, and incubated in dark at room temperature for 2 h; absorbance of the mixture measured at 760 nm using SM 7504 UV-vis spectrophotometer. Gallic acid was used as a reference; the index of TPC was expressed as µgmg-1 Gallic Acid Equivalent (GAE) [27].

In vitro antioxidant assays

***In vitro* 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) assay:** The free radical scavenging and antioxidant activities of the sample against the stable free radical 2,2′-diphenyl-1-picrylhydrazyl were measured. Briefly, different concentrations (1000-10 µgml^{-1}) of the oil in methanol were incubated with a methanol solution of DPPH (0.1 mM). After 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm using Uniscope SM 7504 UV-visible spectrophotometer. Ascorbic acid was used as reference compound. The assay was carried out in triplicate. Scavenging effect was calculated by the percentage (I%) of faded purple 2,2- Diphenyl-1-picrylhydrazyl solution colour into yellow by the tested sample against the control.

$$\%Radical\ scavenging = \frac{A_{blank} - A_{eo}}{A_{blank}}$$

Where: A blank is the absorbance of blank solution and Aeo is the absorbance of the sample. The dose-response curve was plotted and IC50 value for the sample and the standard were calculated.

Antioxidant Activity Index: The Antioxidant Activity Index (AAI) was calculated using Scherer and Godoy's criteria:

$$AAI = \frac{DPPH\ initial\ concentration}{IC_{50}}$$

Where: weak activity <0.5, moderate <1.0, strong <2.0 and very strong >2.0 [28].

***In vitro* reducing power assay:** Various concentrations of the sample (1000-10 µgml^{-1}) were dissolved in 1.0 ml of distilled water, followed by the addition of 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% w/v $K_3Fe(CN)_6$], and the resultant mixture was incubated at 50°C for 20 min. After addition of 2.5 ml of 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of deionised water and a freshly prepared 0.5 ml of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm using uniscope SM 7504 UV Spectrophotometer. A higher absorbance value indicates higher reducing power. Ascorbic acid was used as a standard. The assays were analysed in triplicate and IC_{50} of the oil value was determined [29].

In vivo anti-inflammatory and analgesic (anti-nociceptive) assays

Experimental animals: Abino rats (200g ± 30 g) used in this study were kept in controlled cycles (12/12 hours light/dark) with free access to food and water. The experiments were carried out in strict compliance with the principle of laboratory animal care [30].

***In vivo* carrageenan-induced anti-inflammatory assay:** 0.1 ml (1000 µgkg^{-1}) of the solution sample was administered orally 30 min. before carrageenan injection. 0.1 ml of freshly prepared 1% carrageenan was injected into the sub-plantar region of the right hind paw of the rat. 3 groups of 5 rats in each group were used. Indomethacin 1000 µgml^{-1} was used as reference drug. Control group received the vehicle only. Paw sizes were measured at time intervals of 0-4 hours using a digital vernier calliper. Increases in the linear diameter of the hind paws were taken as an indicator of paw oedema. Results were expressed as the increase in paw volume (mm) calculated after subtraction of basal paw volume prior to carrageenan irritant injection. The inhibition percentage (I%) of the oedema was determined for each rat by comparing each group with controls and calculated by the formula below:

$$I\% = \frac{H_o - H_t}{H_o} \times 100$$

Where: Ho was the average inflammation of the control group at a given time 0. Ht is the mean inflammation of the rats treated with drug at time (t) [31].

***In vivo* analgesic (antinociceptive formalin licking assay):** This test was based on the method described by Onifer et al. [32] with slight modification. Abino rats (n-5 per group) were treated respectively

with 1000 µgkg^{-1} each of the sample and indomethacin. 30 min later, the pain was induced by injecting 0.05 ml of 2.5% v/v formalin in distilled water into the sub-plantar right hind paw of rat, and was placed immediately in a transparent plastic cage separately. The time (sec) spent in licking the paw and the biting responses of the injected paw were taken as an indicator of pain response. The rats were observed for 30 min after the injection of formalin and the amount of time spent licking the injected hind paw was recorded. The first 5 min of post formalin injection is referred to as the early phase and the period between 15 and 30 min as the late phase. The test was performed at room temperature and strict actions were taken to exclude environmental disturbances that might interfere with the animals' response. The percentage inhibition (I%) of pain was calculated as the reduction in the number of licking compared to the control using the formula below:

$$I\% = \frac{J_o - J_t}{J_o} \quad 100$$

Where: Jo represents the vehicle treated control group value for each phase while Jt represents the treated group value for each phase.

***In vitro* antimicrobial assay:** This was done using agar-well diffusion method against multi-drug resistance Gram-positive organism (*Streptococcus agalactiae* and *Staphylococcus aureus*), and Gram-negative organisms (*Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). The bacteria isolates were first sub-cultured in Nutrient agar and incubated at 37°C for 24 hours. All the bacteria cultures were adjusted to 0.5 McFarland standards, 20 ml of sterilized nutrient agar medium was poured into each petri dish aseptically and plates were then swabbed with inocula of the test organisms, and kept for 15 minutes for adsorption. Using sterile cork borer of 6 mm diameter wells were bored into the seeded agar plates, and these were loaded with different concentrations (1000-10 µgml-1) of the sample in DMSO. The plates were allowed to stand in the refrigerator for 1 hr to allow proper diffusion of the sample into the medium and incubated at 37°C for 16-24 hours before visual assessment of the Zones Of Inhibition (ZI). Ofloxacin (OFL) and Cefuroxine (CRX) were used as reference drugs [33].

RESULTS AND DISCUSSION

Chemical composition of the seed essential oil

The GC-MS result of seed essential oil showed that the sample consist of 22 components, representing 98.80% of the total components identified (Table 1). The major phytochemical in the oil were: palmitic acid (29.00%), oleic acid (10.00%), E,E,E- α-springene (9.00%), 2-ethenyl-2,5-dimethyl-4-hexen-1-ol (8.00%), 2,4-dimethylheptane (6.00%), hexahydrofarnesyl acetone (5.00%), geranyl butanoate (4.00%), farnesol (4.00%), geranylgeraniol (4.00%) and trans-2-methyl-2-(4-methyl-3-pentenyl)-cyclopropanecarboxaldehyde (4.00%). Previous studies on the leaf essential oil of *E. citriodora* from Brazil showed that the major components found in *E. citriodora* essential oil were citronellal (29.31%), geraniol (27.63%), β-citronellol (14.88%) and δ-cadinene (6.32%) [34] while the leaves essential oil of *E. citriodora* from Eygpt mainly comprised of 3-hexen-1-ol (31.26%), cis-geraniol (19.66%), citronellol acetate (13.68%), 5-hepten-1-ol, 2,6-dimethyl (13.14%) and citronellal (9.36%) [35].

Table 1: Chemical composition of the seed essential oil of *E. citriodora*.

Compound	Percentage composition
2,4-Dimethylheptane	6
A-pinene	4
P-cymene	0.4
1,8-Cineole	0.5
Phthalic acid, di(1-hexen-5-yl) ester	1
Palmitic acid	29
Oleic acid	10
Geranyl butanoate	4
Hexahydrofarnesyl acetone	5
2,4-Dibromopentane	0.4
2,4-Dimethyl-2,4-heptadiene	0.05
Levomenol	1
E,e,e- α-springene	9
2-Ethenyl-2,5-dimethyl-4-hexen-1-ol	8
5-Bromo-n-pentanol-cyclohexyl ether	2
Threo-2,3-dibromopentane	2
Farnesol	4
Lavandulol	1
1,1'-Bicyclooctyl	1
Geranylgeraniol	4
(E,e)-geranyl linalool	2
Trans-2-methyl-2-(4-methyl-3-pentenyl)-cyclopropanecarboxaldehyde	4
Percentage total	98.8

Total Phenolic Content (TPC)

The TPC of the essential oil was 175.84 ± 0.00 µgmg-1 GAE. The sample had a high value for phenolic compound(s) when compared with the previous study on the related species such as the leaf oil of E. globulus from Greece with 10.50 ± 0.30 mg^{-1} GAE [36]. Moreover, literatures showed that the TPC for the commercial Eucalyptus leaf extract from Japan was 11.90 mgg^{-1} GAE [37,38]. This study indicates that there is a correlation between the TPC, antioxidant and therapeutic activities of the essential oil. Polyphenols are very useful phytochemicals in food and pharmaceutical industries due to their beneficial effects on human health [39,40].

Antioxidant potential

DPPH free radical scavenging and antioxidant activities: The essential oil scavenges the radicals using DPPH in a concentration dependent manner with the percentage inhibitions as 71.95% ± 0.00% , 68.08% ± 0.00% and 67.77% ± 0.00% respectively; while the DPPH IC_{50} and AAI values of the essential oil were 3.00 µgml^{-1} and 13.30, respectively, in comparison to ascorbic acid which gave IC_{50} value of 9.00 µgml^{-1}. The antioxidant IC50 value of the seed essential oil was lower than the leaf essential oil of *E. globulus* from Pakistan (IC_{50} =15.27 ± 1.77) [41]. The lower the IC_{50} value, the higher the antioxidant potential. The result obtained in this study was found to be five times more active than that of leaf essential oil Likewise, *E. globulus* the essential oil investigated in this study was more active than the extracts of different parts (stems>10000, adult leaves 1536.30 ± 40.50, fruits 441.10 ± 12.70, immature flowers 1270.40 ± 33.40 of *E. oleosa* from south Tunisia with IC_{50}: 1,536.30 mg^{-1} [42]. The essential oil had a very strong AAI value

of 13.30, indicating that the presence of terpenoids played active roles in the antioxidant potential of the seed essential oil (Table 2).

Table 2: Antioxidant IC_{50} and AAI of the seed essential oil.

Essential Oil	DPPH IC μgml^{-1}	DPPH AAI	Reduction EC μgml^{-1}
E. Citriodora	3	13.3	2

Reduction antioxidant potential: Reduction antioxidant activity of the essential oil (EC50:2.00 μgml^{-1}) was 82% higher in reducing antioxidant potential than ascorbic acid (11.00 μgml^{-1}). The oil was more active than the leaf essential oil of *E. citriodora* from South-East Asia with reduction value of 95.80 µM at the concentration of 50.00 µl [43]. The presence of terpenoids in the oil contributed to its higher reduction antioxidant effect since these compounds are known to form chelate metal ions [44-46]. The reducing ability assay is based on the principle that substances, which have reduction potential, react with potassium ferricyanide ($K_3Fe^{3+}(CN)_6$) to form potassium ferrocyanide ($K_4Fe^{2+}(CN)_6$) (1), which then reacts with ferric chloride to form a ferric-ferrous complex (2) that has an absorption maximum at 700 nm [47].

$$K_3Fe^{3+}(CN)_{6(aq)} + Natural\ Phenolic\ Antioxidant[red]_{(aq)} + K_4Fe^{2+}(CN)_{6(aq)} + Natural\ Phenolic\ Antioxidant[OX]_{(aq)} \quad (1)$$

$$K_4Fe^{2+}(CN)_{6(aq)} + Fecl_{3(aq)} \rightarrow KFe^{3+}Fe^{2+}(CN)_{6(aq)} + 3KCl_{(aq)} \quad (2)$$

Anti-inflammatory potential

The essential oil investigated has a moderate anti-inflammatory property of 43.80% at 1000 µg (Table 3). The oil has a comparable activity with 2% isolates of *E. globulus* from India which caused inhibition of inflammation between 47.58%-49.18% [48]. This study has shown that the oil possessed a moderate and dose dependent anti-inflammatory effect on paw oedema induced by carrageenan due to the presence of terpenoids in the essential oil.

Table 3: Result of the Anti-inflammatory Activities.

Essential Oil	%I (2 Hrs)	%I (4 Hrs)	Mean %I
E. citrodora	25	62.6	43.8
Indomethacin	87.5	99.7	93.7

Analgesic potential

The essential oil showed a moderate analgesic property by inhibition of both neurogenic (41.32%) and inflammatory pain (11.11%) induced by injection of formalin (Table 4). This indicates the presence of analgesic phytochemical (s) in the essential oil. The seed oil investigated in this study had a lower analgesic activity at the concentration used compared to the leaf essential oil of *E. citriodora* from Brazil at concentration of 0.10 $mgkg^{-1}$ which caused inhibition of neurogenic pain by 57% [49]. This result showed that the oil was able to block both phases of the formalin response although, the effect was more pronounced in the first phase (neurogenic). The analgesic activity of the essential oil was in a correlation with its antioxidant activity.

Table 4: Result of the Analgesic Activities.

Essential Oil	Time of Licking and Biting Inhibition in Percentage			
	Early Phase	% Inhibition	Late Phase	% Inhibition
	(0-5) min.		(5-30) min.	
E. Citriodora	56.33 ± 0.00	41.32	130.00 ± 15.95	11.11
Indomethacin	34.33 ± 2.12	64.23	53.00 ± 2.12	54.7

Note: Mean value ± S.D. of triplicate.

Antibacterial potentials

The antibacterial activities of the oil were determined against five bacteria, including Gram-positive and Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. agalactiae*, and *S. aureus*) were shown in Table 5. The sample showed different potentials against the bacteria. The highest inhibitory effect of the oil was observed against *E. coli* (18 mm), *P. aeruginosa* (15.00 mm), *S. aureus* (15.00 mm), *S. agalactiae* (10.00 mm) and *K. pneumoniae* (13.00 mm). The bacteria were found to be sensitive to Ofloxacin (OFL) and some are resistant to Cefuroxine (CRX) conventional antibiotics (Table 5). The antibacterial properties of the oil were higher than that of essential oils of other Eucalyptus species such as leaf essential oil of *E. globulus* from Australia which showed no or very low ZI with on *S. aureus*, *B. subtilis*, *L. monocytogenes*, *E. coli* and *S. typhi* ranging between 2.20 mm-10.10 mm, but resistant to *S. typhi* [41]. The high antibacterial activities of the essential oil was most likely due to the presence of terpenoids which have antimicrobial properties, particularly, 1,8-cineole [50].

Table 5: Zones of Inhibition (mm) of the Antibacterial Activity.

	Seed Essential Oil			OFL	CRX
Conc. ($Mgml^{-1}$)/ organisms	1000	100	10	25 µg	30 µg
E. Coli (-)	18	15	10	14	14
K. Pneumoniae (-)	13	13	12	17	-
P. Aeruginosa (-)	15	15	15	18	17
S. Agalactiae (+)	10	8	8	20	-
S. Aureus (+)	15	15	15	25	18

Note: Resistant (-), not sensitive (<8 mm), sensitive (9-14 mm), very sensitive (15-19 mm) and ultrasensitive (>20 mm), (+) high antibacterial activity.

CONCLUSION

This study showed that the seed essential oil of *E. citriodora* could be considered as natural antioxidant and antibiotic agent. The synergistic activity of the phytochemicals in the oil would be of benefit in health and industrial applications. The terpenoids in the oil could lower the risk of serious health disorders. It can also be a good flavouring and preservative agents in the food and pharmaceutical industries for the production of drugs that can be used to treatment of pathogenic and reactive oxygen species related diseases.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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