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Phytochemistry and Therapeutic Potentials of the Seed Essential Oil of *Eucalyptus maculata* Hook from Nigeria

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Abstract

The aim of this research was to study the phytochemicals, therapeutic potentials of the seed essential oil of *E. maculata* Hook grown in Nigeria. Composition of the seed essential oil was investigated by GC, GC-MS, MS and FT-IR. Analyses of the volatile oil resulted in the identification of fifty-eight phytocompounds representing 98.95% of the oil. Cyclofenchene (7.0%), *α*-pinene (8.0%), 1R-*α*-pinene (6.0%), 1S-*α*-pinene (7.0%), DL-pinene (6.0%) and *β-trans*-Ocimene (8.0%) were detected as the principal components accounted for 42% of the seed essential oil. None of these main compounds has ever been detected in the leaves extracts of *E. maculata* that had been investigated before except *α*-Pinene. The total phenolics content of the seed oil of *E. maculata* was estimated as 195.84 ± 0.002 µg/mg GAE. The results of free radical scavenging capacity using DPPH and FRAP methods showed that the oil possessed strong antioxidant activity with IC_{50} 8.0 and 10.0 $\mu g.ml^{-1}$ respectively. The acute toxicity test showed that the seed oil extract is safe to be used *in vivo*. The seed oil at 1000 $\mu g.kg^{-1}$ (*p.o.*) gave 87.50% significant inhibition of paw edema. In the antinociceptive assay the oil inhibited the licking time by 86.46% in first phase (neurogenic pain) and 60.10% in the second phase (inflammatory pain). These results showed that the seed oil of *E. maculata* could be an active source of substances with antioxidant, anti-inflammatory and analgesic activities. The pharmacological potentials of the investigated seed oil could be explained by their antioxidant properties, due to their high phenolic and terpenoids contents.

Keywords: *Eucalyptus maculata*; Seed essential oil; Antioxidant; Analgesic; Edema

Introduction

Eucalyptus maculata Hook commonly called spotted gum; a member of *Myrtaceae* family is an attractive with significantly larger vascular tissue was the better adaptation to a variety of environment types, fast growing and tall plant, usually about 35-45 metres in height and average width of 1.5 metres at breast height over bark. The immature leaves are glossy green and elliptic to ovate, while the adult leaves are lanceolate. The flowers are small, white and clustered which developed to ovoid or slightly urceolate fruits, which are disc depressed; valves enclosed, nectar from the flowers, even the seed was sometimes eaten. Trunks are relatively long and usually clean and straight without branches for more than half their height. Bark is smooth to ground level and greenish cream when fresh. It is shed in small irregular patches, leaving dimples that age to cream, grey, pink or coppery brown, giving trunks their characteristic spotted appearance. The wood is slightly greasy and gum veins are common. The plant is the adaptations to water limited environments like drought and salinity [1].

E. maculata is an odouriferous medicinal plant locally used for the treatment of asthma and chronic bronchitis [2]. The leaf extracts of this plant have antimicrobial properties [3]. The leaf essential oil of the plant is usually incorporated into soaps and disinfectants due to its antimicrobial and insecticidal potential of the oil. The oil is a natural source for the production of hydroxycitronellal, citronellylnitrile and menthol due to the high percentage of citronellal in the oil. The plant is considered a good source of natural antioxidants [4]. The resinous exudate from the trunk of the plant is also taken internally to cure bladder infections [5].

To best our knowledge, there is no literature on the phytochemicals, phenolic content, antioxidant, toxicity, anti-inflammatory and antinociceptive potentials of the seed essential oil of *E. maculata* so far. Therefore, the present research is the first report on the phytochemical and therapeutic potentials of the seed essential oil of this plant.

Materials and Methods

Plant material

Seed of *E. maculata* were collected from Kaduna, Nigeria. The plant was authenticated at the Forest Research Institute of Nigeria (FRIN), Kaduna, Nigeria. The seeds were air-dried in a well ventilation place until the moisture content reduced to a minimum suitable for grinding.

Isolation of essential oil

The essential oil was extracted by subjecting the air-dried seed of *E. maculata* to hydrodistillation using a Clevenger-type apparatus. Fresh seed (100 g) were chopped and mixed with distilled water in a 5 litre round bottom flask. The hydrodistillation lasted for 3 hours and the oil collected was dried with sodium sulphate and stored at 4°C in a refrigerator for further use [6].

Instrumentation and analytical conditions

Gas Chromatographic (GC) analysis: The neat essential oil obtained from the seed of *E. maculata* was analysed using a GC-FTD

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with capillary column (30 m×0.25 mm, 0.25 µm film thickness). Oven temperature was kept at 60°C for 3 minutes initially, and then raised at the rate of 3°C/min to 250°C. Injector and detector temperatures were set at 250 and 290°C respectively. Ultra-high purity helium (flow rate: 1 ml/min) was used as carrier. Diluted samples 0.1 µl was auto-injected in the splitless mode. Peak area percent of each compound relative to the area percent of the entire spectrum (100%) were used for obtaining its quantitative data.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis: GC-MS analysis of the neat seed essential oil was carried out on a gas chromatograph with a capillary column (30 m×0.25 mm, 0.25 µm film thickness) equipped with a mass selective detector in the electron impact mode (Ionization energy: 70 eV). Helium was the carrier gas at a flow rate 0.99 ml/min. The GC was interfaced with mass detector operating in the EI^+ mode. Elutes were automatically passed into a mass spectrometer with a dictator voltage set at 1.5 kv and sampling rate of 0.2 sec. The mass spectrum was also equipped with a computer fed mass spectra data bank. The GC-MS was operated under the same conditions as described for GC above. The mass spectra were generally recorded over 40-700 amu that revealed the total ion current (TIC) chromatograms. Temperature program was used as the same as described above for GC analysis. The temperatures of the injector and ion source were maintained at 250 and 200°C, respectively.

Mass spectrophotometry analysis: The compounds were separated on 30 m×0.25 mm×0.25 µm column. Injection volume, 1 µl; transfer temperature, 280°C MS parameters were as follows: EI mode, with ionization voltage 70 eV, ion source temperature, 200°C. The MS fragmentation pattern was compared with those of pure compound, by matching the MS fragmentation patterns with NIST mass spectra libraries and with those given in literature.

Qualitative and quantitative analysis: Identification of the individual component in the seed oil was made by matching their recorded mass spectra with the NIST library provided by the instrument software, and by comparing their retention indices with literature. Relative area percentages of the individual components were obtained from GC analysis [7,8].

Fourier-Transform Infra-Red (FT-IR) analysis: The FT-IR analysis of the seed oil was conducted using FT-IR spectrophotometer, absorbance and functional groups determined with the help of correlation charts. The samples were examined neat by placing them in between potassium bromide cells. 0.25 µl of the samples was deposited in the middle of a KBr pellet and the IR spectrum was recorded at different times until total evaporation.

Total phenolic content and antioxidant capacity

Total phenolic content of the seed essential oil of *E. maculata* was measured by the Folin-Ciocalteu method [9]. A solution of the seed oil (0.2 ml) containing 1000 µg/ml of the oil in methanol was pipetted into a 50 ml volumetric flask, 46 ml distilled water and 1 ml Folin-Ciocalteu's phenol reagent were added, and the opaque flask was thoroughly shaken. 3 ml of (2% w/v) $Na_2CO_{3\ (aq.)}$ was added after 3 minutes and allowed to stand for 2 hours for incubation in dark with intermittent shaking. Absorbance values of the clear supernatants were measured at 760 nm against a blank (0.5 ml Folin-Ciocalteu's reagent + 1 ml Na_2CO_3) on a Spectrophotometer. The same procedure was repeated for the standard gallic acid solutions (0-1000 µg/0.1 ml); calculation of percentage total phenols content was based on Gallic Acid Equivalents (GAE). A standard curve obtained with the following equation:

Absorbance=0.0008×gallic acid (µg)+0.0068

In vitro antioxidant assays

***In vitro* DPPH free radical scavenging assay:** The 2,2´-diphenyl-1-picrylhydrazyl (DPPH) radical assay usually involves hydrogen atom transfer reactions and electron transfer mechanism, based on kinetic data. The free radical scavenging assay of seed oil against DPPH was determined spectrophotometrically. 1.0 ml of the seed *E. maculata* oils (10, 100 and 1000 $\mu g.ml^{-1}$) in methanol was added to 1.0 ml of a 0.004% w/v methanol solution of DPPH. The mixture was homogenized and the absorbance was monitored at 517 nm after 30 minutes of incubation, when the reaction reached a steady state. Ascorbic acid was used as reference compound. Assays were carried out in triplicate. The concentration which was responsible of half scavenging activity IC_{50} (concentration causing 50% inhibition) value of each extract was determined graphically and expressed as in µg/ml by using this formula [10].

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Where: A_{blank} was constituted by a same amount of methanol and DPPH solution without the oil and A_{sample} is the absorbance values of the test compounds.

***In vitro* Ferric Reducing Antioxidant Power (FRAP):** The antioxidant activity has been reported to be concomitant with the development of reducing power. The FRAP assay of the seed oil extract was carried out according by the method of [11]. 1.0 ml of different concentrations of the oil extract (10, 100 and 1000 $\mu g.ml^{-1}$) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6, 2.5 ml) and 2.5 ml of 1% aqueous potassium hexacyanoferrate $[K_3Fe(CN)_6]$ solution. After 20 minutes incubation at 50°C, 2.5 ml of trichloroacetic acid (10%) was added and the mixture was centrifuged at 1000 rpm for 10 minutes. Then, the supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared $FeCl_3$ solution (0.5 ml, 0.1%). Absorbances were read at 700 nm using an appropriate blank (containing all reagents except the test compound). Assays were carried out in triplicate. Ascorbic acid was used as a reference. The average values were plotted to obtain the half maximum effective concentration (EC_{50}) of Fe^{3+} reduction by linear regression.

Acute toxicity test

The acute toxicity of seed essential oil was determined according to [12]. Groups of rats received oral doses of the seed oil >1000 µg / kg. The groups were observed for 48 hours and mortality at end of this period was recorded for each group. The determination of LD_{50} served to define the doses used in experiments of pharmacological assays.

In vivo antiinflammatory assay

In vivo antiinflammatory potential was of the seed oil of *E. maculata* was tested in rat paw edema according to [13]. Healthy albino rats (200 ± 30 g) acclimatized to laboratory hygienic conditions were housed in clean cages under standard conditions of temperature (25 ± 2°C) and RH was % 55-60, 12 hours light/dark cycle were maintained in the quarantine and were fed with standard pellet diet and water *ad libitum*. The handling and uses of animals were in accordance to the institutional guidelines.

1% carrageenan (0.1 ml) was injected into the plantar surface of the rat hind paw 30 minutes after oral administration of the test compounds or vehicle. Indomethacin (25 $mg.kg^{-1}$) was used as reference drug. Paw volume was determined immediately after the

injection of the phlogistic agent and again 2 and 4 hours later by means of a digital vernier calliper. The antiinflammatory activity of the seed oil was expressed as the percentage of inhibition calculated from the difference between the responses of the treated and the control groups. The inhibition percentage of the inflammatory reaction which was calculated by the formula given in equation (1) below was determined for each rat by the comparison of each group with controls.

$$\% I = 1-(dt/dc)\times 100 \quad (1)$$

Where: I% = Percentage inhibition, 'd*t*' is the difference in paw volume in the drug-treated group and 'd*c*' is the difference in paw volume in control group.

In vivo antinociceptive assay

In vivo antinociceptive activity of the seed oil of *E. maculata* was studied in rat according to [14]. The rats were divided into four groups of three animals each and fasted for 12 hours and deprived of water only during the experiment to ensure uniform hydration and minimize variability in edematous response. The rats were treated with 1000 $\mu g.kg^{-1}$ (*p.o.*) of essential oil and indomethacin. Thirty minutes later, the pain was induced by injecting 0.05 ml of 2.5% v/v formalin in distilled water into the sub-plantar right leg paw of the rats, the amount of time spent in licking the injected paw was monitored and was considered as an indicative of pain and frequency of the injected paw were recorded for 30 minutes. The number of lickings from 0-5 minutes (first phase) and 15-30 minutes (second phase) were counted after injectior of formalin. The percentage inhibition (I) was calculated accordingly.

Results and Discussion

Identification and quantification of the seed essential oil

Hydrodistillation of the seed of *E. maculata* gave a pale-yellow coloured essential oil with about 1.60% v/w yield per 100 g of air-dried seed sample and with a pleasant odour characteristic of the oil. GC, GC-MS, MS and FT-IR analyses of the seed essential oil enabled us to identify fifty-nine components (Table 1) amounted 98.95% of the total content. In the seed oil extract different groups of compounds were present. The main compounds in *E. maculata* seed essential oil were: Cyclofenchene (7.0%), *α*-pinene (8.0%), 1R-*α*-pinene (6.0%), 1S-*α*-pinene (7.0%), DL-pinene (6.0%) and *β-trans*-Ocimene (8.0). The major class of substances in the essential oil of *E. maculata* was monoterpene (48.50%), followed by oxygenated monoterpenoids (9.65%), sesquiterpenes (8.75%) and oxygenated sesquiterpenoids (4.60%).

The report from the literature showed that no part of this plant has been investigated before in Nigeria, some new phytocompounds were detected in the seed essential oil of this plant which were not present in the leaf essential oil of the same plant analysed from other part of the globe, these include compound: 1, 13, 14, 15, 16, 18, 28, 29, 31, 34, 35, 36, 37, 38, 39, 41, 45, 48, 49, 51, 52, 53, 54, 55 and 58.

According to [15], leaves essential oil of this plant grown in the western region of Cuba contained *α*-pinene (49.7%), *α*-eudesmol (18.0%) and *β*-eudesmol (11.3%) as major constituents. Moreover, [16] also reported their findings on the seasonal changes of leaf oil composition of the same plant from Khuzestan provinces in Iran where the major compositions were eucalyptol (in winter), citronellal (in spring) and citronellol (in summer). Elaissi et al. reported the constituent of *E. maculata* from Korbous arboreta Northeast Tunisia, whose leaf essential oil was characterized by two major components: eucalyptol and limonene [17].

From the mass spectrometry analysis of the principal compounds were discussed as following. Compound 1 is Cyclofenchene, a tricyclic monoterpene, with molecular formula of $C_{10}H_{16}$ (m/z 136), weak bond is broken to give a fragment at m/z 93 as the base peak, m/z 93 (M-43) is due to loss of $(H_3C)_2CH$ from the molecular ion m/z 136, m/z 121 (M-15) is due to loss of CH_3 group from m/z 136, other prominent peaks observed in Cyclofenchene occurred at 27, 39, 79, 105. Compound 5, 6 and 7 are pinene derivatives, they are bicyclic monoterpenes with molecular ion peak 136, the relatively low abundance of the molecular ion peak is consistent with the view that the molecular structure of the compound is rather strained or crowded; the base peak m/z 93 corresponds to the loss of 43 mass units and relatively abundance of the ion m/z 41 is about one quarter of the base peak. A point of distinction between the isomers arises from the abundance of the ion m/z 29 and 39 in 1S-*α*-pinene which is not feature in *α*-pinene and 1R-*α*-pinene. The failure to detect the isopropyl ion strengthens that the loss of 43 mass units is not an entity. Therefore, the groups elided may be obtained by the breaking of two tertiary bonds with the removal of or concomitant hydrogen migration. The occurrence of gem dimethyl group as a part of ring system is common feature of many monoterpenes. Compound 8 is a monocyclic monoterpene, with molecular formula of $C_{10}H_{16}$ (m/z 136) is *β-trans*-Ocimene, m/z 43 ($C_3H_7^+$) which is due to the detachment of isopropyl [$(H_3C)_2C$-] group attached to the quaternary carbon of the compound and weak bond is broken to give a fragment at m/z 93 as the base peak.

The FT-IR analysis of the seed essential oil *E. maculata* gave the following absorption signals, especially in the regions around 3500 cm^{-1}, 2945 cm^{-1} and 1650 cm^{-1}. Moreover, at frequency region of 1447-452 cm^{-1}, *E. maculata* showed more peaks. The band at 3600-3400 cm^{-1} was due OH stretching vibration, 3600 (sharp) was due to free OH, while 3400 cm^{-1} (broad) was due to associated OH; both bands frequency present alkanol spectra; bands at 3400-3200 are due to N-H stretching vibrations, 3400 (sharp) was due to free N-H, while 3200 cm^{-1} (broad) was due to hydrogen bonded N-H; 2945 cm^{-1} was due OH stretching vibration, strongly hydrogen bonded, a very broad band in this region superimposed on the C-H stretching frequencies is characteristic of fatty acids. Peak at 1651 cm^{-1} was attributed to C=O stretching vibration of amides or lactams. This band is lower in frequency by about 20 cm^{-1} by conjugation. The frequency of the band is raised about 35-70 cm^{-1} in lactam, this can be used as an indicative for the presence of esters in the *E. maculata* seed essential oil. 1447-1370 cm^{-1} were due to the presence of $-CH_3$ stretching vibration.

Phenolic content and antioxidant property

The result of absorbance value of the seed oils solution reacting with Folin-Ciocalteu phenol reagent and compared with the absorbance values of standard solutions of Gallic acid, total phenolics content of the seed oil of *E. maculata* was estimated as 195.84 ± 0.002 μg/mg of GAE. This might be due to the presence of low molecular mass phenolic compound such as 4-Isopropenyl-1-cyclohexen-1-ylmethyl benzoate in the seed essential oil. The Folin-Ciocalteu assay is based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic or phosphotungstic acid complexes to form blue complexes. Phenoloids constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators [18]. Phenoloids have inhibitory effects on mutagenesis and carcinogenesis in humans and may contribute directly to antioxidative action, when included in daily meal mostly from plant source [19].

SN	Compounds	Percentage composition	Retention index
1	Cyclofenchene	7.0	729
2	3,4-Dimethyl-(Z,Z)-2,4-Hexadiene	2.0	750
3	3,5-Dimethylcyclohexene	4.0	838
4	*β*,*β*-dimethyl-1H-Imidazole-4-ethanamine	3.0	850
5	D-Sabinene	0.4	897
6	1R-*α*-pinene	6.0	937
7	1S-*α*-pinene	7.0	941
8	DL-pinene	6.0	943
9	*α*-pinene	8.0	948
10	*β*-pinene	1.0	970
11	*β*-*trans*-Ocimene	8.0	976
12	L-*β*-pinene	0.4	978
13	2,3,4-trimethylthiophene	0.5	993
14	Phenyltrimethylmethane	0.4	1007
15	2-(1,1-Dimethyl-2-pentenyl)-1,1-dimethylcyclopropane	2.0	1040
16	3,7,7-Trimethyl-1,3,5-cycloheptatriene	0.5	1010
17	*α*-Limonene	0.6	1018
18	1,5-Dimethyl-1-vinyl-4-hexenylbutyrate	0.1	1022
19	*o*-Cymene	0.5	1029
20	L-Limonene	0.5	1031
21	D-(*E*)-Limonene oxide	0.6	1030
22	2-Isopropylimidazole	3.0	1038
23	*m*-Cymene	0.5	1042
24	*p*-Cymene	0.6	1045
25	Limonene epoxide	0.8	1048
26	Eucalyptol	1.45	1059
27	L-Linalool	0.2	1083
28	1,5-Dimethyl-1,5-cyclooctadiene	0.5	1103
29	2-Ethyl-*p*-Xylene	0.6	1119
30	*β*-Terpineol	0.7	1158
31	3,4,4-trimethyl-5-oxo-(Z)-2-hexenoic acid	2.0	1200
32	*α*-Copaene	3.0	1221
33	*cis*-Geraniol	0.6	1228
34	Ethenyldimethylester Phosphoric acid	2.0	1233
35	2,3-Dimethylcyclohexanol	2.0	1240
36	4,4-Dimethyl-2-cyclopenten-1-one	3.0	1250
37	1,1,3-Trimethyl-1-silacyclo-3-pentene	0.5	1260
38	Phellandral	3.0	1280
39	2-Cyclopentylcyclopentanol	0.6	1289
40	Nopol	0.4	1290
41	2-Hydroxymethyl-5-(1-hydroxy-1-isopropyl)-2-cyclohexen-1-one	0.5	1300
42	*α*-Cubebene	1.0	1344
43	Aromadendrene	0.2	1386
44	L-*allo*-Aromadendrene	0.8	1452
45	(2*E*,6*E*)-2,6Dimethyl-2,6-octadiene-1,8-diol	0.7	1471
46	*α*-Selinene	0.7	1474
47	*α*-Bulnesene	1.0	1490
48	1R,3Z,9s-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-3-ene	0.2	1494
49	2-isopropyl-5-methyl-9-methylenebicyclo[4.4.0]dec-1-ene	0.6	1503
50	*trans*-*Z*-*α*-Bisabolene epoxide	1.0	1531
51	3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methylcyclohexene	3.0	1533
52	1-(1-methylene-2-propenyl)cyclopentanol	1.0	1560
53	2-isopropyl-5-methyl-9-methylenebicyclo[4.4.0]dec-1-ene	0.5	1563
54	N-Acetyl-3-propoxyamphetamine	0.8	1580
55	5-Chloromethyl-2-oxazolidinone	1.0	1600
56	*α*-Eudesmol	0.6	1650
57	*β*-Eudesmol	1.0	1652
58	4-Isopropenyl-1-cyclohexen-1-ylmethylbenzoate	0.4	1958
Percentage Total		**98.95**	

Table 1: Composition of the seed essential oil of *Eucalyptus* maculata Hook.

In vitro antioxidant potentials by dpph and frap methods

In this research, two most widely used assays, namely, DPPH and FRAP methods, were applied to evaluate the antioxidant potentials of the seed essential oil. DPPH measures the oil's free radical scavenging, whereas FRAP measures the ability of the essential oil to reduce metal ions. DPPH involves single electron transfer (SET) and hydrogen atom transfer reactions (HAT), while in FRAP there is a single electron transfer (SET) [20].

The seed essential oil was able to inhibit the formation of DPPH radicals in a concentration dependent manner. The percentage inhibitions of the essential oil at various concentrations (10, 100 and 1000 µg.ml^{-1}) were 59.46 ± 0.000, 62.82 ± 0.0006 and 82.84 ± 0.003% respectively; while the IC_{50} values was found to be 8.0 µg.ml^{-1} in comparison to ascorbic acid which gave 54.37 ± 0.00, 84.51 ± 0.001 and 95.50 ± 0.00 as the percentage inhibitions with IC_{50} value of 9.0 µg.ml^{-1}. The DPPH radical scavenging potential of the seed essential oil was at same range as observed for ascorbic acid (synthetic antioxidant) as shown in table 2.

The solution of radical was decolourized after reduction with the antioxidant (AH) or the radical (R•) in accordance with the following scheme [21]:

$DPPH^{\bullet}+AH \rightarrow DPPH^{\bullet}-H+A^{\bullet}$

$DPPH^{\bullet}+R^{\bullet} \rightarrow DPPH^{\bullet}-R$

The result of reducing power (FRAP) of the seed oil of *E. maculata* in comparison with ascorbic acid as a standard antioxidant is also shown in table 2. The reducing power of ascorbic acid used as standard in this study was ascorbic acid was EC_{50}: 20.00. The seed oil exhibited excellent reducing potential value at concentrations of 10, 100 and 1000 µg.ml^{-1} with effective dose value at (EC_{50}: 10.00 µg.ml^{-1}). Reducing power of *E. maculata* seed oil increases from 0.31 ± 0.005 at 10 µg/ml to 0.43 ± 0.002 at 100 µg/ml and finally to 0.60 ± 0.005 at 1000 µg.ml^{-1} in a concentration dependent manner. At tested concentrations the oil possessed the ability to reduce Fe^{3+}. It was observed that the seed oil of *E. maculata* showed high Fe^{3+} reducing power comparable to ascorbic acid activity. The reducing power of the seed oil increased with concentrations in a strongly linear manner. The reducing power assay measures the electron donating ability of antioxidants using the potassium ferricyanide reduction method. Antioxidants cause the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form and activity is measured as the increase in the absorbance at 700 nm.

The antioxidant power increased as the essential oil concentration increased, indicating some compounds in *E. maculata* are electron donors and could react with free radicals to convert them into more stable products and to terminate radical chain reactions.

Compared to reported extracts antioxidant of different *Eucalyptus* species, it was shown that the seed oil of this plant is more active, *E. maculata* gave lower IC_{50} values than other species studied before; *E. oleosa* with IC_{50}: >1000 [22], *E. Globules* (leaf) IC_{50} : 57.00 µg.ml^{-1} (DPPH); IC_{50}: 48.00 µg.ml^{-1} (FRAP) [23].

The free radical scavenging potentials could be attributed to the presence of some components that have antioxidant activities, possible antagonistic and synergistic effects of compounds and functional groups in the seed oil could also be taken into consideration [24]. These results showed that the seed oil of *E. maculata* potentially exert its radical scavenging effects at a much lower concentration. This observed effect is certainly associated with high phenolic content, terpenes, terpenoids and carbonyl compounds in the seed oil. The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated in situ (endogenous antioxidants), or externally supplied through natural products (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention. The results clearly showed that the seed oil of *E. maculata* possesses strong antioxidant activity and can be considered as good sources of natural antioxidants for therapeutic purposes such as reactive oxygen species ailments including chronic inflammatory joint disease such as rheumatoid arthritis.

Acute toxicity test

The acute toxicity test showed that the seed oil of *E. maculata* was not toxic to rat at the doses administered per oral route (*p.o.*). During this experiment, no apparent behavioural side effects were observed in the animals; they were very active. This shows that the seed oil was relatively non-toxic and safe.

In vivo antiinflammatory activity

The carrageenan assay was used because of its sensitivity in defecting orally active anti-inflammatory agents mainly in the acute phase of inflammation. The anti-inflammatory effects of the seed oil of *E. maculata* and standard antiinflammatory drug (indomethacin) on carrageenan induced edema in rats hind paws were presented in table 3. The antiinflammatory activity of oil was found to have effect in dose-time manner. There was a significant decrease in edema paw volume of rats in the test group. However, there was no reduction in inflammation found in case of control group. The results showed that the seed oil of *E. maculata* causes significant reduction in inflammation i.e. 87.50% (1000 µg.kg.ml^{-1} *p.o*), while the standard anti-inflammatory drug indomethacin gave 93.75% (25 mg.kg^{-1}). The lower the paw volume the better the activity, the inhibitory activity of the oil is very close to indomethacin. There was no reduction in inflammation found in case of rats treated with 10% DMSO.

Seed oil and reference compound	DPPH IC_{50} µgml^{-1}	FRAP EC_{50} µgml^{-1}
E. maculata	8.00	10.00
Ascorbic acid	9.00	20.00

Data are presented as triplicate of the mean ± S.E.M

Table 2: IC_{50} of the DPPH and FRAP assays of the seed essential oil of *E. maculate*.

Seed Oil	2 Hour	% I (2 Hr)	4 Hour	% I (4 Hr)	Mean Paw (mm)	Mean % I
E. maculata	4.50 ± 0.67	87.50	4.10 ± 0.69	62.50	4.30 ± 0.53	87.50
Indomethacin	4.70 ± 0.21	87.50	4.60 ± 0.35	99.65	4.65 ± 0.29	93.70

Data are presented as triplicate of the mean with standard deviation
Key note: I%=% Inhibition; 70-100: very strong activity; 40-69: moderate activity; ≤ 39: weak activity

Table 3: *In vivo* anti-inflammatory activity the seed essential oil of *E. maculate*.

Seed oil and reference compound	Time of licking and biting percentage inhibition			
	Early phase (0-5) min.	Percentage inhibition	Late phase (5-30) min.	Percentage inhibition
E. maculata	13.00 ± 9.19	86.46	46.70 ± 5.92	60.10
Indomethacin	34.33 ± 2.12	64.23	53.00 ± 2.12	54.70
10% DMSO	96.00	-	117	-

Data are presented as triplicate of the mean ± S.E.M

Table 4: *In vivo* analgesic activity the seeds essential oil of *E. maculate*.

Inflammation is a complex physiopathological response to different stimuli. Formation of edema induced by carrageenan is commonly correlated with the early exudative stage of inflammation, one of the important processes of inflammatory pathology. In the early phase (one hour) of carrageenan injection, there is sudden elevation of paw volume as consequence of histamine, serotonin and related substances liberation from mastocyte cells. In the later phase (over one hour) the inflammation increases gradually and is elevated. This second phase is mediated by prostaglandins, cyclooxygenase, proteases and lysosome products. Continuity between the two phases is provided by kinins [25,26]. The seed oil extract promptly controlled both the phases of inflammation.

In vivo antinociceptive activity

Chemically induced visceral pain and paw nociception are very useful models for the study of nociception and the assessment of analgesic drugs. The antinociceptive activity of the seed oil of *E. maculata* measured on abino rat by using injection of formalin solution as shown in table 4. Interestingly, the seed oil at the concentration of 1000 $\mu g.kg^{-1}$ exhibited high inhibitory effect 86.46 and 60.10% in early (neurogenic) and late (inflammatory) phases respectively, while the standard anti-inflammatory drug (indomethacin) gave 64.23 and 54.70% in first and second phase respectively. The results showed that the seed oil is more active than the synthetic drug (indomethacin) commonly used as analgesic and anti-inflammatory drug. Drugs that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs inhibit the late phase. The early phase is a direct result of stimulation of nociceptors in the paw which reflects centrally mediated pain while the late phase is due to inflammation with a release of serotonin, histamine, bradykinin and prostaglandins. The seed oil exhibited significant dose related reduction of paw licking caused by formalin and was able to block both phases of the formalin response. The formalin test represents a more valid model for clinical pain. The formalin test is a very useful method, not only for assessing antinociceptive drugs, but also helping in the elucidation of the action mechanism [27,28].

Conclusion

The present study demonstrated for the first time the phytochemicals and therapeutic potentials of the seed essential oil of *E. maculata* grown in Nigeria. On the basis of this result the seed essential oil *E. maculata*, may be utilized as a source for the isolation of natural pinenes, cyclofenchene and other terpenoids. Pharmacological activities of the oil may be due to the synergetic effect of these chemical constituents. Terpenoids and esters are antiinflammatory, analgesic, antiseptic, expectorant and stimulating compounds. Some are antiviral and some help break down gallstones [29]. β-pinene, eucalyptol, *p*-menth-1-en-ol and ocimene, have strong antimicrobial activities and eucalyptol also used as therapeutic agent in cardiovascular effects [30]. The excellent antiinflammatory and remarkable antinociceptive potentials of the seed essential oil of *E. maculata* may provide supports in the treatment of pathologies such as painful and inflammatory disorder in which free radical oxidation plays a fundamental role. Moreover, the seed oil gives no sign of toxicity, which indicates that the oil is therapeutic safety for the pharmacologically active doses. The information on essential oil profile can be used for the possible exploitation of this species for various research and pharmaceutical purposes. This shows that the seed essential oil of *E. maculata* would exert several beneficial effects by virtue of its phytochemicals and pharmacological potentials and could be harnessed in drug formulation.

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