Chemistry and Medicinal Potentials of the Seed Essential Oil of Eucalyptus Toreliana F. Muell Grown in Nigeria

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Abstract

E. toreliana is a distinct aromatic plant with several medicinal applications to cure many ailments. Chemistry and medicinal potentials of the seed essential oil of Eucalyptus toreliana F. Muell grown in Nigeria were examined in this study. The phytochemical composition of the seed essential oil was evaluated using multidimensional GCxGC-MS, MS and FT-IR. The seed oil was also investigated for its total phenolic content, antioxidant and acute toxicity, anti-inflammatory and antinociceptive potentials. Analyses of the seed essential oil extract resulted in the identification of 70 compounds representing 98.53%

Index terms—eucalyptus toreliana, seed essential oil, phytochemicals, phytotherapeutic.

1 Introduction

Eucalyptus toreliana (Myrtaceae) is a tall evergreen and a dense shade plant with an irregular crown, a very hard tree with smooth, tight and grey-green bark with persistent scaly, sub-fibrous base and tessellated. The leaves have a simple, leathery, variable but usually ovate, wavy margin, green above or with a pink tint, generally pubescent when young and with a wider leaf than other Eucalyptus. It processes attractive flowers with large creamy white clusters and numerous stamens, the creamy fruit is large and ovoid shape and with valves well below rim of the fruit.

Globally, there is a rapid increase in screening of plants that can lead to the discovery and development of novel therapeutics. Plants from different continents have shown considerable pharmacological activities such as antioxidant, antimicrobial, antiinflammatory, antiviral, anti-allergic and vasodilatory properties (Rustaiyan et al., 2011; Newman and Cragg, 2007).

The leaf essential oil of E. toreliana has been used in the treatment of lung diseases and was shown to have anti-tubercular properties (Alain et al., 2012).

The extracts of the leaf and stem of the plant were reported to have antibacterial and gastroprotective properties, it inhibits the growth of Helicobacter pylori (Adeniyi et al., 2006). The leaves extracts of the plant is applied over wounds and ulcers, also used to treat gastrointestinal disorders, they decrease gastric acid production and used for the treatment of gastric ulcers, cough associated with most pulmonary diseases and medically importance for the treatment of infections caused by the non-tuberculous mycobacteria . The plant is also used locally in the treatment bacterial infections of the urinary tracts, respiratory tracts, inflammation of the mucous membranes and sore throat (Farah et al., 2002).

Moreover, Eucalyptus essential oils has long history of safe use in food preservation, pharmaceuticals, phytherapies, pesticides and have attracted extra attentions for more intensive studies (Tepe et al., 2004). It was also reported that Eucalyptus leaf essential oil had a direct effect on the coxsakievirus B3 and ethno-pharmacologically been used to treat respiratory tract disorders such as pharyngitis, bronchitis, and sinusitis (Elaissi et al., 2012).

To best our knowledge, no literature on the chemistry, phenolic content, antioxidant, antiinflammatory and antinociceptive potentials of the seed essential oil of E. toreliana have been reported so far.
The present research was therefore undertaken for the first time with the main objective to isolate and characterize the seed essential oils of *E. toreliana* cultivated in Nigeria for their detailed chemical constituents, pharmacological properties.

2 II.

3 Materials and Methods

4 a) Plant Material

Seed of *E. toreliana* were collected from Ogbomoso, Nigeria. The plant was authenticated at the Forest Research Institute of Nigeria (FRIN), Ibadan, Nigeria.

b) Extraction of the Essential Oil

Seed of *E. toreliana* was air-dried in a well-ventilated place till when the moisture content reduced to a minimum suitable for grinding; the plant material was pulverized and used immediately. The crushed plant material (100 g) was subjected to hydrodistillation for 2.5 hours using a Clevenger-type apparatus (European Pharmacopoeia, 2004). The oil collected was stored in vial at low temperature.

c) Instrumentation and Analytical Conditions

i. Multi-Dimensional GCxGC-MS Analysis

Analysis of the seed essential oils of *E. toreliana* was performed using multi-dimensional gas chromatograph coupled with Gas Chromatography-Mass Spectrometer (Shimadzu, Japan) equipped with double capillary columns (25.0 m × 0.25 μm i.d., 0.25 μm df) that have different characteristics (non-polar and polar). High purity helium was used as the carrier gas at a constant flow rate of 0.99 ml/min. A total of 1 μl sample was injected (split ratio 100:1) into GCxGCMS using AOC20i auto injector for analysis. The initial temperature was set at 60 °C, heated at a rate of 3 °C/min to 280 °C and held isothermally for 6 minutes. Ion source temperature for these analyses was set at 200 °C, while the interface temperature was set at 250 °C, solvent cut time was 3.0 minutes and the mass spectrometer was set to operate in electron ionization mode with an ionizing energy of 70 eV as acquisition mass range from 40-700 amu at 0.50 scan/s.

ii. Mass Spectra Data Analysis

MS parameters were as follows: EI mode, with ionization voltage 70 eV, ion source temperature, 180 °C. The mass spectra were generally recorded over 40-700 amu that revealed the total ion current (TIC) chromatograms. 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.

of n-alkanes internal standard analyzed under the same operating conditions and calibrated based on the Automatic Adjustment of Compound Retention Time (AART) function of the GC-MS. Relative concentrations of the essential oil components were calculated based on GC peak area.

5 Fourier-Transform Infra-Red (FT-IR) Analysis

The IR spectra in KBr pellets were recorded using a spectrophotometer. 0.25 % of the seed essential oil was deposited in the middle of a KBr pellet and the IR spectrum was recorded at different times. The FT-IR conditions were: 4 cm⁻¹ spectral resolution, 20 kHz scan speed, 128 scan co-additions and scanning range 400-400 cm⁻¹.

Total Phenolic Content (TP) and Antioxidant Capacity equation:

Absorbance = 0.0008 × gallic acid (μg) + 0.0008 × Tannic acid (μg) + 0.0068

Calculation of percentage total phenols content was based on Gallic Acid Equivalents (GAE). The antioxidant activity of the seed oil extract was measured using the stable radical 2,2´-diphenyl-1-picrylhydrazyl (DPPH). 1.0 ml of the seed *E. toreliana* essential oils (10, 100 and 1000 μg/ml) in methanol was added to 1.0 ml of a 0.004% w/v methanol solution of DPPH. The mixture was shaken vigorously 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 inhibition percentage (%) of radical scavenging activity was calculated by using following formula (Oladade et al., 2012).

\[
\text{Inhibition Percentage} = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100
\]

Where A blank is the absorbance value of the control reaction (containing all reagents except the test compound) and A sample is the absorbance values of the test compounds.

Total phenolic content of the seed oil of *E. toreliana* was analysed by the Folin-Ciocalteu method (Wua and Ng, 2008). A solution of the seed oil (0.2 ml) containing 1000 μg/ml of methanol pipetted into a 50 ml volumetric flask, 46 ml distilled water and 1ml Folin-Ciocalteu’s phenol reagent were added, and the opaque flask was thoroughly shaken. After 3 minutes, 3 ml of (2% w/v) Na₂CO₃ solution was added and the mixture was 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 Na₂CO₃) on UV-Visible spectrophotometer. The same procedure was repeated for all the standard gallic acid solutions (0-1000 μg/0.1 ml) and a standard curve obtained with the following ii. In vitro Ferrous Reducing Power Assay (FRAP)

The reducing power of the seed oil was determined by the method of (Saeed et al., 2012). An aliquot of the sample (1.0 ml) at various concentrations (10, 100 and 1000 μg/ml) were mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 ml) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50 °C for 20 minutes.

After adding 10% Trichloroacetic acid (2.5 ml), the mixture was centrifuged at 1000 rpm for 10 minutes. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% FeCl₃ (0.5 ml) and the absorbance was measured at 700 nm against a blank (0.5 ml Folin-Ciocalteu’s reagent + 1 ml Na₂CO₃).
measured at 700 nm using an appropriate blank. 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.

iii. In vivo Anti-inflammatory Assay Healthy rats (200 ± 30 g) acclimatized to laboratory hygienic conditions were housed in polycarbonate 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. The in vivo toxicity of seed essential oil E. toreliana was also observed during and after the experiment (Santin et al., 2011).

In vivo antiinflammatory assay of the seed oil of E. toreliana was studied in rat paw edema. The rats of were divided into three groups of five animals each and the rats were fasted for 12 hours in order to avoid food interference with substance absorption, ensure uniform hydration and minimize variability in edematous response. 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 below was determined for each rat by the comparison of each group with controls (Sousa et al., 2010).

\[ I \% = 1 - \left( \frac{dt}{dc} \right) \times 100 \]

Where: I % = Percentage inhibition 'dt' is the difference in paw volume in the drugtreated group and 'dc' is the difference in paw volume in control group.

7 iv. In vivo Antinociceptive Assay

In vivo antinociceptive activity of the seed oil of E. toreliana was studied in rats according to (Ouedraogo et al., 2011). The rats were divided into three groups of five animals each and the rats were fasted for 12 hours in order to avoid food interference with substance absorption, ensure uniform hydration and minimize variability in response. The rats were treated respectively with 1000 µg/kg of E. toreliana seed essential oil or indomethacin.

Thirty minutes later, the pain was induced by injecting 0.05 ml of 2.5% v/v formalin (formaldehyde) in distilled water into the subplantar right hind paw of rat, immediately placed in a transparent plastic cage separately; 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 injection of formalin.

The percentage inhibition (I) was calculated accordingly.

8 III.

Results and Discussion a) Identification and Quantification of the Essential Oil v/w yield per 100g of dried seed sample and possessed a distinct sharp aromatic scent. The analyses of the seed essential oil were carried out using GCxGC-MS, MS and FT-IR systems. The percentage composition and retention index are given in Table 1. Seventy compounds were identified from the seed essential oil of E. toreliana amounting to 98.53% of the oil. The seed oil was dominated by ?-Pinene (16.0%), Copaene (10.0%), 1R-?-Pinene (8.0%), DLPine (8.0%), ?-trans-Ocimene (5.0%), ?-Bisabolol oxide B (5.0%), Oleamide (5.0%), 2fluoro-?-3,4-trihydroxy-N-isopropyl-Benzenethanamine (4.0%) and Globulol (4.0%). None of these principal compounds has ever been detected in the leaves extracts of E. toreliana that had been investigated before except ?-Pinene. Monoterpenes (43.40%) dominated the seed essential oil, because of the remarkable proportions of Pinene derivatives. The percentage composition of monoterpenoid was very low (3.50%), while the level of sesquiterpenoids was relatively high (20.70%), sesquiterpenoids constituted (10.50%), but few diterpenoids (2.30%) were also available in the seed oil of E. toreliana. We also identified some new phytocompounds which are not reported in the previous study on the leaf essential oil of this plant. Most of the principal components present in the seed oil were not available in the leaf oils and many components: (Chalchat et al., 2000).

From the mass spectrometry analysis, Compound 5, 7 and 8 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 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-?-pinene which is not feature in ?-pinene and 1R-?-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 The FT-IR spectra of the seed oil of E. toreliana revealed some prominent peaks especially in the regions around 3700-2933 cm-1, 1300-1447 cm-1 and 1440-1090 cm-1 the oil showed a peak. The band at 3500-3400 cm-1 was due OH stretching vibration, 3600 (sharp) was
due to unassociated OH, while 3400 cm\(^{-1}\) (broad) was due to associated (hydrogen bonded) OH; both bands
frequent 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 associated N-H; Peaks at 1750 and 1447 cm\(^{-1}\) were attributed
to \(^{\text{C}=\text{O}}\) stretch and \(^{-\text{C}=\text{C}}\)-stretch and can be used as an indicative for the presence of unsaturated bonds in
the oil. These functional groups detected by FT-IR are futures of the compounds found in the seed essential oil.

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10 Compounds

Percentage Composition Retention Index Folin-Ciocalteu measured both total phenolics and antioxidant
strength base on the nature of its chemistry, to prevent inhibitory effects due to the oxidants competing with
Folin-Ciocalteu reagent and/or air oxidation after the sample is made alkaline, the Folin-Ciocalteu reagent is
added before the alkali (Singleton et al., 1999).

Overall, IC 50 values of the seed essential oils of the E. toreliana examined was more effective than oils of some
other related species: E. toreliana (leaf from Republic of Benin) IC 50 : 2.90 gl l\(^{-1}\) (Alain et al., 2012), E. oleosa
with IC 50 : 71000 (Marzoug et al., 2011), E. globulus(leaf) IC 50 : 57.00 µgml\(^{-1}\) (Noumi et al., 2011). These
findings in DPPH and FRAP assays are in agreement with Vardar-Unlu et al., 2003, who reported that the entire
essential oil showed greater antioxidant activity than individual components, indicating the possible synergistic
interaction of the essential oil constituents. These results showed that the seed oil of E. toreliana potentially exert
its radical scavenging effects at a much lower concentration. This observed effect is certainly associated with high
phenolic content and sesquiterpenoids components in the oil. The results clearly showed that the seed oil of E.
toreliana possesses strong antioxidant activity and can be considered as good sources of natural antioxidants for
medicinal purposes such as reactive oxygen species ailments including chronic inflammatory joint disease such as
rheumatoid arthritis. Studies have showed that the electron donating capacity, reflecting the reducing power of
bioactive compounds, is associated with antioxidant activity. 9.00 µgml\(^{-1}\) ). Reducing power of E. toreliana oil
increases from 0.641±0.008 at 10 µgml\(^{-1}\) to 1.016±0.02 at 100 µgml\(^{-1}\) and finally appreciated to 1.566±0.004
at 1000 µgml\(^{-1}\) 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. toreliana showed higher 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. Data are presented as triplicate of the mean ± S.E.M
ii. In vivo Anti-inflammatory Activity

The anti-inflammatory effects of the seed oil of E. toreliana on carrageenan induced oedema in rats hind paws
is presented in Table ???. The anti-inflammatory activity of oil was found to have effect in time manner.
There was a significant decrease in oedema 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. toreliana
causes significant reduction in inflammation i.e.
99.61% (1000 µgkg\(^{-1}\) p.o), while the standard anti-inflammatory drug indomethacin gave 93.75% (25 mgkg \(-1\)
). The seed oil of E. toreliana proves its antiinflammatory potential in in vivo study by controlling biphasic
inflammatory events induced by carrageenan. The carrageenan induced oedema shows to be a multimediated
phenomenon that liberates diversity of mediators which could be in two phases. Degree of inflammatory immune
responses is controlled by involvement of inflammatory cells into inflammatory lesions (Solanki and Jain, 2010).
The early phase (one hour) of the inflammation is due to the release of serotonin, histamine and related substances.
The later phase (over one hour) is mediated by prostaglandins, proteases and lysosome (Ayoola et al., 2009). The
seed oil extract promptly controlled both the phases of inflammation.

11 iii. In Vivo Antinociceptive Activity

The antinociceptive activity of the seed oil of E. toreliana measured on abino rat by using injection of formalin
solution is shown in Table 4. The extracts exhibited significant dose related reduction of hind paw licking caused
by formalin. Interestingly, the seed oil at the concentration of 1000 µgkg\(^{-1}\) exhibited high inhibitory effect 88.69
and 75.90% in early and late 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 in pain and inflammatory problems. 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 probably 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. These phases represented neurogenic and inflammatory pain responses, respectively (Chen
et al., 1995). During and after the in vivo 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. This is in
agreement with the report on the Eucalyptus leaf essential oil (Silva et al., 2003 Eucalyptol which is one of
the principal components of the seed oil and by far the most known naturally occurring oxide as it is the most common in essential oils as an oxygenated monoterpenoid has can readily penetrate tissue, one of the reasons for its efficacy in various decongestants and pain relief products and has anticitarrhale, mucolytic, antimicrobial, antiviral and as a stimulating expectorant in cases of chronic bronchitis IV.

12 Conclusion

This research represents the first comprehensive study of the seed essential oil E. toreliana. The analysis of the seed essential oil from the plant indicates terpenes, terpenoids and phenoloids are the major constituents of this medicinal plant. Pharmacological activities of the oil may be due to the synergetic effects of these chemical constituents.

Therefore, the seed essential oil of E. toreliana can be used as natural therapeutic product that may serve as leads for the development of new pharmaceuticals that can handle many health problems.

Figure 1:

Figure 2:

Figure 3: 7 +) 7 +) 7 +)
species investigated in the leaves from Nigeria (30\%, 4.2\%) (Ogunwande et al., 2011), Ethiopia (44\%, 7\%) (Dagne et al., 2000), Congo-Brazzaville (78\%, 1\%) (Loumouamou et al., 2009), Brazil (40\%, 55\%; 24\%, 7\%), Morocco (14\%, 64\%) (Coffi et al., 2012), Republic of Benin (38, 18 and 14\%) (Alain et al., 2012; Sohounlhoue et al., 1996), Mali

Figure 5:
Z-5,17-Octadecadien-1-ol acetate 1.0 2082
Sulfurous acid, cyclohexylmethyltetradecylester 0.3 2100
Farnesy1-?-D-Mannofuranoside 0.2 2102
Geranylgeraniol 0.3 2201
Oleamide 5.0 2397
n-Heptacosane 0.2 2700
n-Octacosane 0.2 2800
n-Hentriacontane 0.2 3100
Percentage Total 98.53

Year 2013

involves single electron transfer (Prior et al., 2005). The reducing power of ascorbic acid used as standard in this study was EC 50 : 20.00. The seed oil exhibited the high of reducing power value at concentrations of 10, 61 00 and 1000 µg/ml with effective dose value at (EC 50 :

Figure 7:
Oil and Reference Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPPH IC 50</th>
<th>FRAP EC 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. toreliana</td>
<td>9.00</td>
<td>9.00</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>9.00</td>
<td>20.00</td>
</tr>
</tbody>
</table>

In vivo Anti-inflammatory Activity the Seed Essential Oil of E. toreliana Oil and Reference

<table>
<thead>
<tr>
<th>Compound</th>
<th>2 Hour % I</th>
<th>4 Hour % I</th>
<th>Mean Paw Diameter Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. toreliana</td>
<td>4.5±0.50</td>
<td>99.50</td>
<td>4.5±0.12 99.88 (mm)</td>
</tr>
<tr>
<td>Indomethacin (Standard)</td>
<td>4.7±0.21</td>
<td>87.50</td>
<td>4.6±0.35 99.65 4.65±0.29</td>
</tr>
<tr>
<td>(Control) 10% DMSO</td>
<td>5.5±0.07</td>
<td>-</td>
<td>5.5±0.00 - 5.50±0.31</td>
</tr>
</tbody>
</table>

Oil and Reference Compounds E. toreliana

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of Licking and Biting Percentage Inhibition Early Phase (0-5) min</th>
<th>Late Phase (5-30) min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin (Standard)</td>
<td>34.33±2.121</td>
<td>64.23 53.00±2.12</td>
</tr>
<tr>
<td>10% DMSO (Control)</td>
<td>96.00</td>
<td>- 117</td>
</tr>
</tbody>
</table>

Generally, phytocompounds found in the seed essential oil are very useful for various pharmacological purposes.

[Note: purposes]


