SHORT COMMUNICATION

CHEMICAL CONSTITUENTS OF EREMOMASTAX SPECIOSA (HOCHST.) CUFOd LEAVES AND ITS CYTOTOXIC POTENTIAL ON NIH-3T3 CELLS

Mboso Ofonime Eve1,1*, Tamfu Ngenge Alfred2*, Iwara Iwara Akrip1, Eyong Eyong Ubana1, and Iqbal Mohammad Choudhary3
1Department of Biochemistry, University of Calabar, Calabar, Nigeria
2School of Chemical Engineering and Mineral Industries, University of Ngaoundere, Ngaoundere, Cameroon
3International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

(Received January 17, 2019; Revised November 22, 2020; Accepted January 4, 2021)

ABSTRACT. This study aimed at assessing the cytotoxicity of Eremomastax speciosa crude extract on NIH-3T3 fibroblast cell lines and reporting the chemical constituents in the extract. The MTT assay on NIH-3T3 cells showed a significantly lower (p < 0.05) inhibition from E. speciosa (IC50 > 30 µg/mL) compared to cyclohexamide (IC50 > 0.8 µg/mL). This result validates the non-toxicity observed with the use of E. speciosa on normal cells at low to moderate doses. Four compounds were isolated and identified from their EIMS as well as 1D and 2D NMR spectroscopic data namely hydroxyandrographolide (1), stigmastanol glucoside (2), (Z)-4-coumaric acid 4-O-β-D-apiofuranosyl(1’’→2’’)-O-β-D-glucopyranoside (3) and 5-methoxy-4,4′-di-O-methyl-secolaricresinol-9′-monooacetate (4). These compounds are isolated from this species for the first time. Thirteen volatile constituents were detected in the extract using gas chromatography mass spectrometry (GC-MS). Besides 6,10,14-trimethyl-2-pentadecanone (12.63%), mostly fatty acid esters were detected in high amounts notably ethyl hexadecanoate (16.00%), ethyl-9,12,15-octadecatrienoate (11.51%) and 9,12-octadecadienoic acid ethyl ester (8.05%). This study revealed many unsaturated fatty acid esters in E. speciosa and is noteworthy that α-3 and α-6 fatty acid esters were predominant, hence an added nutritional value to this plant.

KEY WORDS: Eremomastax speciosa, Secondary metabolites, NIH-3T3 cytotoxicity, NMR, GC-MS

INTRODUCTION

Eremomastax speciosa (Hochst.) Cufo is a species of plants belonging to the Acanthaceae family [1, 2]. E. speciosa is widely distributed in West Africa where it is used traditionally, especially in Nigeria and Cameroon for various medicinal purposes. It is commonly used in pediatric care for conditions such as diarrhea, jaundice and fever [3]. Women of reproductive age in the Southern Regions of Nigeria and Cameroon use these leaves to enhance fertility and arrest postpartum bleeding [4, 5]. Reports have also shown an improved effect on male sexual behavior [6]. It is also known as the blood tonic plant due to its erythropoietic properties [7]. E. speciosa is known locally as ‘Ikpo Ikong’, ‘Mbimdmbid Ukebe’ amongst the Efiks and Ibibio tribes in Southern Nigeria. Other local names involve ‘Oyun’ in Yoruba, ‘Esinyin’ in Hausa and ‘Nkwukwo’ in Ibos [8]. In Cameroon, it is known as “Ntimir” in the Nso tribe, ‘Pang ndjenit’ (meaning ‘red on one side’) amongst the Bamileke, ‘purple leaf’ in pidgin English [8, 9], ‘mbezamou’ in Bafanji and ‘bgah’ in Mbam tribe. E. speciosa has well documented biological activities such as anti-ulcerative properties on the lining of the stomach [9, 10]. Its leaves have been reported to have anti-anemic properties and antimicrobial activity against Candida albicans, Escherichia coli and Staphylococcus aureus [11] as well as strong oxidative scavenging properties against hydrogen peroxide and superoxide anion [12]. Though it is

*Corresponding author. E-mail: evembose2@gmail.com; macntamfu@yahoo.co.uk
This work is licensed under the Creative Commons Attribution 4.0 International License
evident that these medicinal attributes are because of active biomolecules they contain [5], few studies exist on the chemical characterisation and on the bioactive molecules from this plant species. It is necessary to provide scientific basis for the therapeutic property of medicinal plants to avoid intoxication and poisoning and to improve safety and optimization of formulations [13]. It is therefore necessary to investigate bioactive molecules with potential pharmacological properties contained in medicinal plants. In this work, methanol extract of leaves of E. speciosa was obtained and characterized by GC-MS. Column chromatography was used to isolate major bioactive compounds from this extract.

EXPERIMENTAL

General experimental procedures. Column chromatography was carried out on silica gel (70-230 mesh, Merck). Thin-layer chromatography (TLC) was performed on silica gel precoated plates (Merck, PF254, 20x20, 0.25 mm). TLC plates were revealed using ceric(IV) sulfate and H2SO4 10% spray reagent. 1H NMR spectra were recorded in deuterated solvents such as CD3OD and DMSO with TMS as internal standard at 400 MHz on Bruker Avance-400. The 13C NMR spectrum was recorded at 100 MHz using CD3OD and DMSO. EI-MS and was obtained with a JEOL JMS-600H mass spectrometer. Optical densities for the bioassay of cytotoxicity screening were measured on a Thermo Scientific Multiskan FC, Vantaa, Finland spectrophotometer. GC-MS was done on a Hewlett-Packard 5890 (Bunker Lake Blvd, Ramsey, MN) gas chromatograph coupled to JEOL MS-600H mass spectrometer.

Plant material. The plant material was collected from Akai Effa in Calabar municipality, River State, Nigeria, during the month of June-August 2016, authenticated and compared with an existing herbarium voucher specimen in the herbarium unit of the Department of Botany, Faculty of Sciences in the University of Calabar, Nigeria with voucher specimen: Herb/Bot/ucc/363.

Extraction and isolation. The leaves of E. speciosa were dried in a shed and grounded to powder. 1 kg of plant powder was then extracted by maceration with methanol (1:10, v/w) for 48 h at the room temperature. The supernatant in each extraction was filtered out and the solvent removed using a rotary evaporator to yield crude extract. This process was repeated three times. From this process, a crude MeOH extract of E. speciosa (20.5 g) was obtained.

A portion (10 g) of the MeOH extract of E. speciosa (extract obtained in larger amount) was subjected to column chromatography on silica gel using DCM-MeOH (0-100%) gradient with increasing polarity to give 35 fractions which were regrouped into 4 major fractions F1 (200 mg), F2 (350 g), F3 (4 g) and F4 (2.5 mg). Fraction F2 was rechromatographed on a silica gel using a DCM-MeOH (25-30%) gradient to afford compound 3 (13.5 mg) which was identified as (Z)-4-coumaric acid 4-O-β-D-apiofuranosyl-(1’’→2’’)-O-β-D-glucopyranoside [14]. Fraction F4 was purified on silica gel column using DCM-MeOH (50:50, v/v) isocratic gradient to give 15 subfractions. These obtained subfractions were crystallized on standing and were filtered out to give E-7 (65 mg) identified as a mixture of compound 1, hydroxyandrographolide [15] and compound 2, stigmasterol glucoside [16]. Purification of F3 using DCM-MeOH (40-60%) in a silica gel column yielded compound 4 (8 mg) identified as 5-methoxy-4,4′-di-O-methylsecolariciresinol-9′-monooacetate [17]. The structures of the isolated compounds were elucidated by the interpretation of their EIMS, 1D and 2D NMR data and by comparison with some data reported in literature.

Gas chromatography–mass spectrometry (GC-MS) analysis of volatile constituents. The profile of the extract was firstly performed using gas chromatography-flame ionization detection (GC-FID) on a Shimadzu GC-17 A (Shimadzu Corp., Kyoto, Japan) equipped with a SPB-5VR capillary column (30 m, 0.25 mmid). The carrier gas used was helium at a flow rate of 1
Identification of compounds. For the identification of compounds, electronic mass spectral search on NIST library and by comparison with EIMS (electronic impact mass spectrometry) data reported in literature. Area normalization method for each peak was used to quantify constituents and reported in terms of percentage composition.

Cytotoxic assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxic effect of *E. speciosa* crude extract on NIH-3T3 mouse fibroblast cells (ATCC, Manassas, USA) based on the principle of detecting viable mitochondrial cells. The MTT is reduced to a colored product by the activity of NAD(P)H-dependent dehydrogenases and this indicates the level of energy metabolism in cells. Briefly cells were seeded in 96-well microplates with 1×10⁴ cells in 0.1 mL of DMEM medium supplemented with 10% FBS and routinely cultured in a humidified incubator (37 °C in 5% CO₂) for 24 h. After 24 h incubation, the old media was removed and various concentrations of *E. speciosa* (1–30 μg/mL), diluted in 200 μL of fresh media, were added. Then the medium was discarded and 200 μL of MTT solution (5 mg mL⁻¹ in cyclohexemidine) was added to every well and re-incubated for an additional 4 h. 100 μL of DMSO was added to dissolve the formazan crystals formed. The plate was then read on a microplate reader at 570 nm (Spectra Max plus, Molecular Devices, CA, USA) for the extent of MTT reduction to formazan within cells. The cytotoxicity was recorded as concentrations causing 50% growth inhibition (IC₅₀) on the NIH-3T3 cell lines. The results (% inhibition) were processed by using Soft-Max Pro software (Molecular Devices, CA, USA) [13]. MTT solution with DMSO (without cells and medium) as a blank control in microplate reading while the cyclohexemidine-treated cells served as a drug control [18, 19].

RESULTS AND DISCUSSION

*Isolated compounds.* The crude extract of *E. speciosa* was subjected to column chromatography and some pure secondary metabolites were obtained and their structures elucidated from their EIMS and NMR data and by comparison with some literature data. The compounds were identified as hydroxyandrogapholide [15] and stigmasterol glucoside [16], (Z)-4-coumaric Acid 4-O-β-D-apiofuranosyl(1''→2'')-O-β-D-glucopyranoside [14], 5-methoxy-4,4'-di-O-methyl-secolariciresinosinol-9'-monoacetate [17], whose structures are given in Figure 1.

**Hydroxyandrogapholide (I).** ¹H NMR (pyridine-d₅, 600 MHz): δH ppm 2.04 (H-1), 1.97 (H-2), 3.59 (H-3), 1.20 (H-5), 1.78 (H-6), 2.30 (H-7), 1.81 (H-9, d, J = 10.9 Hz), 1.22 (H-11), 4.62 (H-12, d, J = 6.3 Hz), 3.12 (H-13, d, J = 6.3 Hz), 5.11 (H-14), 4.41 (H-15, d, J = 10.6 Hz), 5.15/4.94 (H-17), 1.49 (H-18), 4.28 (H-19), 0.72 (H-20). ¹³C NMR (pyridine-d₅, 150 MHz): δC ppm 37.4 (C-1), 29.1 (C-2), 79.9 (C-3), 43.3 (C-4), 55.5 (C-5), 47.7 (C-6), 38.7 (C-7), 148.8 (C-8), 53.2 (C-9), 39.7 (C-10), 30.4 (C-11), 70.6 (C-12), 54.1 (C-13), 73.3 (C-14), 76.1 (C-15), 177.3 (C-16), 107.4 (C-17), 23.8 (C-18), 64.3 (C-19), 15.5 (C-20).

**Stigmasterol glucoside (2).** ¹H NMR (pyridine-d₅, 600 MHz): δH ppm 1.69 (H-1), 1.51 (H-2), 3.47 (H-3), 1.78; 1.56 (H-4), 5.33 (H-6), 1.92 (H-7), 1.48 (H-8), 0.93 (H-9), 1.22 (H-11), 1.93 (H-12), 1.11 (H-14), 1.48 (H-15), 1.70 (H-16), 1.07 (H-17), 0.65 (H-18), 0.96 (H-19), 1.98 (H-20).
Structures of compounds isolated from *E. speciosa.*

5-Methoxy-4,4′-di-O-methylsecolariciresinol-9′-monoacetate (4), EIMS M++ 462; 1H NMR (pyridine d6, 600 MHz): δH ppm 6.81 (1H, s, H-2, d, J = 1.7 Hz), 7.16 (1H, s, H-6, d, J = 1.7 Hz), 1.90 (2H, d, H-7), 1.09 (1H, m, H-8), 4.28/3.59 (2H, d, H-9), 7.19 (1H, s, H-2′), 7.86 (1H, s, H-5′), 7.56 (1H, s, H-6′), 1.94 (2H, d, H-7′), 1.09 (1H, m, H-8′), 4.64/4.41 (2H, dd, H-9′), 1.94 (3H, s, H-11′), 4.03 (3 MeO), 4.03 (4 MeO), 4.03 (5 MeO), 4.05 (3′ MeO), 4.05 (4′ MeO).

13C NMR (pyridine d6, 150 MHz): δC ppm 136.6 (C-1), 106.0 (C-2), 153.6 (C-3), 153.6 (C-4), 153.8 (C-5), 106.0 (C-6), 35.8 (C-7), 42.4 (C-8), 62.6 (C-9), 132.6 (C-1′), 111.9 (C-2′), 147.3 (C-3′), 148.8 (C-4′), 111.0 (C-5′), 121.0 (C-6′), 34.9 (C-7′), 39.6 (C-8′), 64.6 (C-9′), 172.1 (C-10′), 21.1 (C-11′), 60.8 (3 MeO), 60.8 (4 MeO), 60.8 (5 MeO), 55.8 (3′ MeO), 56.1 (4′ MeO).

The compounds 1, 3 and 4 are reported from this species for the first time and this study is one of the prime isolation studies done on this specie despite its long-standing applications in traditional medicine.

The compound 2 has been detected by GC-MS in the aqueous extract of *E. speciosa* but was not isolated [20]. Most chemical studies on this plant only report qualitative phytochemical screening using conventional reagents for qualitative test of classes of organic compounds. These qualitative tests may be misleading considering the colourful nature of the extracts that result from the aerial parts of this plant. It is therefore necessary to report these compounds isolated individually and characterized. This can justify some observed bioactivities as well as give orientations on expected bioactivities of this plant. These compounds either act in synergy or individually to confer the observed biological and therapeutic effects of *E. speciosa*. These compounds which are non-volatile occur in the plants alongside volatile compounds which can suitably be characterized using GC-MS.

**GC-MS profile.** The GC-MS profile of the crude extract of *E. speciosa* enabled the characterisation of 13 volatile constituents reported on Table 1.

Table 1. Volatile constituents of *E. speciosa* leave extracts detected by GC-MS with their percentage compositions.

<table>
<thead>
<tr>
<th>RT</th>
<th>Compound name</th>
<th>% Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.18</td>
<td>2,4-bis(1,1-dimethylethyl)-phenol</td>
<td>0.21</td>
</tr>
<tr>
<td>28.06</td>
<td>6,10,14-Trimethyl-1,2-pentadecanone</td>
<td>12.63</td>
</tr>
<tr>
<td>30.86</td>
<td>Methyl hexadecanoate</td>
<td>4.21</td>
</tr>
<tr>
<td>39.99</td>
<td>Ethyl hexadecanoate</td>
<td>16.00</td>
</tr>
<tr>
<td>40.44</td>
<td>(Z,Z)-9,12-Octadecadienoic acid methyl ester</td>
<td>1.83</td>
</tr>
<tr>
<td>43.39</td>
<td>9,12-Octadecadienoic acid ethyl ester</td>
<td>8.05</td>
</tr>
<tr>
<td>43.64</td>
<td>Ethyl-9,12,15-octadecatrienoate</td>
<td>11.51</td>
</tr>
<tr>
<td>44.55</td>
<td>Ethyl octadecanoate</td>
<td>2.62</td>
</tr>
<tr>
<td>55.95</td>
<td>2,2-Dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-oxirane</td>
<td>1.09</td>
</tr>
<tr>
<td>63.69</td>
<td>3,7,11,15-Tetramethyl-2-hexadecen-1-ol</td>
<td>6.67</td>
</tr>
<tr>
<td>67.95</td>
<td>i-Propyl-9,12-octadecadienoate</td>
<td>5.87</td>
</tr>
<tr>
<td>68.31</td>
<td>(E,E,Z)-1,3,12-Nonadecatriene-5,14-diol</td>
<td>3.71</td>
</tr>
</tbody>
</table>

GC-MS was used to characterize the volatile constituents in the extract of *E. speciosa*. Mostly fatty acid esters were abundant. Ethyl hexadecanoate was the most abundant constituent identified in the extract with a percentage composition of 16.00%. Other major fatty acid esters were ethyl-9,12,15-octadecatrienoate (11.51%), 9,12-octadecadienoic acid ethyl ester (8.05%), i-propyl 9,12-octadecadienoate (5.87%), (Z,Z,Z)-9,12,15-octadecatrienoic acid methyl ester (4.27%), methyl hexadecanoate (4.21%), ethyl octadecanoate (2.62%) and (Z,Z)-9,12-octadecadienoic acid methyl ester (1.83%). Most of these detected fatty acids esters are ω-3 and

o-6 fatty acids which can confer additional nutritive value to this plant. Previous studies reported 3,7,11,15-tetramethyl-2-hexadecen-1-ol, (E,E,Z)-1,3,12-nonadecatriene-5,14-diol, in an aqueous extract of *E. speciosa* [20] and generally corroborate with high amount of fatty acids reported herein. Other non-ester compounds included a fatty ketone 6,10,14-trimethyly-2-pentadecanone (12.64%) as one of the major constituents, two fatty alcohols 3,7,11,15-tetramethyl-2-hexadecen-1-ol (6.67%), (E,E,Z)-1,3,12-nonadecatriene-5,14-diol (3.37%), a fatty epoxide 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-oxirane (1.09%) and a phenol derivative, 2,4-bis(1,1-dimethylethyl)-phenol (0.21%).

**Cytotoxicity of crude extract on NIH-3T3 fibroblasts.** The cytotoxicity of the methanol extract of *E. speciosa* was evaluated on NIH-3T3 mouse fibroblast cells at concentrations of 1-30 µg/mL and cycloheximide was used as positive control. At the highest tested concentration of 30 µg/mL, there was no appreciable activity of the crude extract compared to the positive control and the IC₅₀ (concentration causing 50% growth inhibition on the NIH-3T3 fibroblasts) is therefore greater than 30 µg/mL as shown on Table 2.

Table 2. Cytotoxicity of *E. speciosa* leave extract on NIH-3T3 fibroblasts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/mL)</th>
<th>% inhibition</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol fraction of <em>E. speciosa</em></td>
<td>30</td>
<td>5</td>
<td>&gt;30±0.0</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>30</td>
<td>71</td>
<td>0.8±0.0</td>
</tr>
</tbody>
</table>

Data are presented in mean±SD all samples were compared to cycloheximide (p < 0.05).

The results from this screening of potential cytotoxicity of methanol fraction of *E. speciosa* on NIH-3T3 cell lines validates the non-toxicity associated with the use of *E. speciosa* leaves on living cells. This plant does not show any toxicological signs such as changes in appearance of internal organs or mortality even at a dose of 2 g/kg though minor changes were reported in hematological and biochemical parameters at the dose of 800 and 1600 mg/kg mostly in female animals [21]. The low toxicity of this plant is a favourable factor for its use in traditional medicine and no harmful chemical compound has been found as well.

**CONCLUSION**

*E. speciosa* is a highly valued medicinal plant widely used in Africa and other tropical areas in the world. However, chemical studies on this plant remains scanty and there is a great necessity to reports its chemical constituents so as to guarantee its safety for use in traditional medicine. One of such important chemical study is the isolation of the bioactive compounds found in this plant and their characterisation. In this study, four secondary metabolites were characterised namely, hydroxyandrographolide (1), stigmasterol glucoside (2), (Z)-4-coumaric Acid 4-O-β-D-apiofuranosyl(1′→2′)-O-β-D-glucopyranoside (3), 5-methoxy-4,4′-di-O-methylsecolariresinol-9′-monoacetate (4). These compounds are reportedly isolated from this species for the first time. The volatile constituents were characterized by GC-MS and found to be mostly fatty acid esters. Cytotoxic potential of the methanol fraction shows a non-toxic effect of this plant at low doses thereby validating the non-toxicity associated with the use of this plant on normal cells. This study and mostly the isolated compounds can pave a way for future chemical and biological investigations on this plant.

**ACKNOWLEDGEMENT**

This work was supported by The World Academy of Science (TWAS) through the TWAS-ICCBS Postdoctoral Fellowship (Grant No. 3240293183) offered to Dr. Mboso Ofonime Eve.
REFERENCES


