

Full Length Research Paper

Phytochemical and biological investigation of chloroform and ethylacetate fractions of *Euphorbia heterophylla* leaf (Euphorbiaceae)

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Euphorbia heterophylla Linn (Euphorbiaceae) is a medicinal plant used by traditional herbal practitioners in Nigeria and some parts of West Africa for the treatment of constipation, bacterial and inflammatory disease conditions (arthritis and rheumatism). Powdered plant material was subjected to phytochemical screening using standard experimental procedures. The crude powdered sample of *Euphorbia heterophylla* leaves was extracted with n-hexane, chloroform, ethylacetate and methanol. The precipitates from the fractions were subjected to chromatographic and re-crystallization procedures. The structures of isolated compounds were characterized and elucidated with chemical and spectroscopic techniques such as IR, NMR and MS experiments. The *in vitro* biological activity of the isolated and characterized compounds were evaluated by superoxide scavenging assay using xanthine – xanthine oxidase system to generate superoxides. The result of the study showed that the crude plant material contained some secondary metabolites such as saponins, flavonoids and tannins. The phytochemical investigation led to the isolation of four known compounds stigmasterol, - stigmasterol glucoside, benzoic acid and 4 – hydroxyl benzoic acid. The four compounds exhibited good activity against the xanthine oxidase enzymes while the 4-hydroxybenzoic acid showed a marked activity. The isolation of the compounds from the leaves of *E. heterophylla*, which inhibited the xanthine oxidase enzyme, has justified the claims for which the plant is known and used.

Key words: *Euphorbia heterophylla*, euphorbiaceae, xanthine oxidase activity, superoxide scavenging, steriods.

INTRODUCTION

The use of herbal medicine for the treatment of diseases and infections is as old as mankind. The world Health Organization supports the use of traditional medicine provided they are proven to be efficacious and safe (WHO, 1995). In the developing countries, vast number of people lives in extreme poverty and some are suffering and dying for want of safe water and medicine, they have no alternative for primary health care (WHO, 1995). Therefore, the need to use medicinal plants as alternatives to orthodox medicines in the provision of

primary health care cannot be over-emphasized. More so, herbal medicines have received much attention as sources of lead compounds since they are considered as time tested and relatively safe for both human use and environment friendly (Fazly-Bazzaz et al., 2005). They are also cheap, easily available and affordable. There is therefore the need to look inwards to search for herbal medicinal plants with the aim of validating the ethno-medicinal use and subsequently the isolation and characterization of compounds which will be added to the potential lists of drugs.

Euphorbia plants are widespread in nature ranging from herbs and shrubs to trees in tropical and temperate regions all over the world (Bremer, 1994). The family Euphorbiaceae comprises of 280 genera and 730 spe-

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cies with the largest genus *Euphorbia* having about 1600 species. Generally, they have a characteristic milky latex (Mitich, 1992), sticky sap, co-carcinogenic, severe skin irritation and toxic to livestock and humans (Berry MI, 1984).

Euphorbia heterophylla leaf is used in traditional medical practices as laxative, anti gonorrhoeal, migraine and wart cures. The plant lattices have been used as fish poison, insecticide and ordeal poisons (Rodriguez et al., 1976, Falodun et al, 2003).

The leaves of *E. heterophylla* have been reported to contain quercetin (Falodun and Agbakwuru, 2004). Diterpenoids have also been reported in the root of *E. heterophylla* (Rowan and Onwukaeme, 2001).

The skin irritant, tumor-promoting and anti-tumor/anti-cancer and recently anti-HIV activities of *Euphorbia* species have also been reported in *E. heterophylla* leaf Linn (Williams et al., 1995).

The aim of this study was to isolate and characterize the chemical constituents of *E. heteropylla* leaf. The potential of these compounds as superoxide scavengers via enzymatic xanthine oxidase system was evaluated.

MATERIALS AND METHODS

General experimental procedures

Melting points were determined using a Fisher-Johns melting point apparatus and uncorrected. Optical rotations were measured on a Jasco-DIP-360 digital polarimeter. The UV and IR spectra were recorded on Hitachi -UV-3200 and Jasco -320-A spectrophotometer, respectively. NMR spectra were run on Bruker Avance-500 spectrometers. EIMS, ESI and HR spectra were recorded on a JMS-HX-110 spectrometer.

Plant material

The fresh leaves of *E. heterophylla* were obtained from the Ugbowo campus of the University of Benin, in March, 2007, identified and authenticated by the Mr. A. Sunny of Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Phytochemical screening

The crude plant powdered sample was subjected to phytochemical, screening, testing for the presence of Alkaloids, Tannins, Flavonoids and Saponins using standard experimental procedure (Sofowora et al., 1982; Harborne, 1973; Trease and Evans, 1989).

Molisch's test: powdered sample (0.1 g) was weighed into a beaker and 20 ml of distilled water was added. The beaker was heated in a water bath for over 5 min. 2 mls of the filtrate in a test tube and 2 drops of alcoholic solution of - naphthol added, concentrated sulphuric acid added down the side into the test tube. The reaction was observed for colour change.

Saponins: to 2 mls of the filtrate was added 10mls of distilled water, shaken vigorously for 2 min and observed for frothing.

Tannins: powdered *E. heterophylla* leaves (0.2 g) was weighed into a conical flask and mixed with 50 mls of water, boiled in a water bath for 5 min. The mixture was filtered hot using a filter paper and the filtrate collected in a beaker. 2 mls of the filtrate was mixed with 10mls of distilled water and then a drop of iron (III) chloride was added.

Flavonoids: dilute ammonia (5 ml) solution was added to a portion of the aqueous filtrate, followed by the addition of concentrated sulphuric acid. 1ml concentrated sulphuric acid was added to 2 mls of KOH solution and allowed to mix. Then, into the acid base mixture, a small quantity of aqueous filtrate of the sample was added and observed for colour change.

Alkaloids: Extraction of 5 g of the powdered sample was carried out by boiling in 50 ml of distilled water in a water bath for 30 minutes and filtered. The filtrate was tested with alkaloidal reagents (Dragendorff's, Wagner's and Mayer's reagents) and results compared with blanks.

Extraction and Isolation

The powdered leaves (600 g) were extracted first with n-hexane, chloroform, ethyl acetate and methanol at room temperature. The extracts were evaporated to dryness using a rotary evaporator at reduced pressure, to give 13.06, 16.22, 5.64 and 10.12 g of hexane, chloroform, ethylacetate and methanol fractions respectively.

Stigmasterol 1: Colourless crystalline solid from chloroform fraction, 25mg, MP 170-171°C. $[\alpha]_D^{25}$ 51.6° (CHCl₃, c 0.28); IR (CHCl₃) ν_{max} cm⁻¹: 3432 (OH), 1648 (C=C)

¹H-NMR (CDCl₃, 400 MHz), : 5.33 (1H, m H- 6), 5.15 (1H, dd, J = 15.3, 8.0 Hz, H-22), 5.02 (1H, dd, J = 15.3, 8.0 Hz, H-23), 3.28 (1H, m, H-3), 0.90 (3H, d, J = 6.5 Hz, Me-21), 0.83 (3H, d, J = 6.6 Hz, Me-26), 0.84 (3H, t, J = 7.0 Hz, Me- 29), 0.81 (3H, d, J = 6.5 Hz, Me-27), 0.80 (3H, s, Me-19), 0.65 (3H, s, Me-18).

¹³C- NMR (CDCl₃, 400 MHz), : 140.9 (C-5), 138.4 (C-22), 129.4 (C-23), 121.7 (C-6), 71.9 (C-3), 57.0 (C-14), 56.0 (C-17), 51.3 (C-24), 50.3 (C-9), 42.5 (C-13), 42.2 (C-4), 40.5 (C-20), 39.7 (C-12), 37.5 (C-1), 36.6 (C-10), 32.2 (C-8), 32.0 (C-25), 31.9 (C-7), 31.8 (C-2), 28.9 (C-16), 25.4 (C-28), 24.4 (C-15), 21.2 (C-27), 21.1 (C-21), 21.0 (C-11), 19.4 (C-19), 19.0 (C-26), 12.4 (C-18), 12.0 (C-29).

EIMS *m/z* (rel. int. %): [M]⁺ 412 (8), 396 (12), 394 (20), 379 (27), 369 (35), 351 (71), 327 (60), 301 (18), 300 (67), 273 (35), 270 (24). HREIMS *m/z*: 412.3920 (calcd. for C₂₉H₄₈O, 412.3926).

Stigmasterol 3- O- -glucoside (2) crystallized as colourless needles crystals from the ethyl acetate fraction. The molecular formula was established as C₃₅H₅₈O₆ by HR-FAB-MS which showed molecular ion peak at *m/z* 574.4231 (calcd. for C₃₅H₅₈O₆, 574.4233)

28 mg, MP 289 - 290°C. $[\alpha]_D^{25}$ - 51.4° (CH₃OH c 0.22); IR (KBr) ν_{max} cm⁻¹: 3458 (OH), 1646 (C=C). EIMS *m/z* (rel. int. %): [M-Glu]⁺ 412 (72), 397 (15), 394 (22), 379 (28), 369 (35), 351 (71), 300 (67), 327 (55), 301 (67), 273 (21), 271 (26).

HREIMS *m/z*: 575.4231 [M+H]⁺ (calcd.for C₃₅H₅₉O₆, 575.4233).

¹H-NMR (CDCl₃, 400 MHz), : 5.23 (1H, br d J = 5.4 Hz, H-6), 5.14 (1H, dd, J = 15.2, 8.0 Hz, H-22), 5.02 (1H, dd, J = 15.3, 8.0 Hz, H-23), 4.78 (1H, d, J = 7.5 Hz, H-1), 3.83 (1H, m, H-3), 3.84 - 4.44 (m, Glc-H'), 1.01 (3H, s, Me-19), 0.90 (3H, d, J = 6.2 Hz, Me-21), 0.83 (3H, d, J = 6.6 Hz, Me-26), 0.82 (3H,t, J = 7.0 Hz, Me- 29), 0.80 (3H, d, J = 6.5 Hz, Me-27), 0.66 (3H, s, Me-18).

¹³C-NMR (CD₃OD ,400 MHz), : 141.5 (C-5), 138.9 (C-22), 129.1 (C-23), 121.1 (C-6), 102.8 (C-1), 79.8 (C-3), 76.7 (C-5), 74.2 (C-2), 70.6 (C-4), 62.2 (C-6), 57.0 (C-14), 56.1 (C-17), 52.1 (C-24), 50.8 (C-9), 43.9 (C-4), 43.1 (C-13), 40.5 (C-20), 39.9 (C-12), 37.8

Table 1. Phytochemical analyses of *Euphorbia heterophylla* leaf.

Secondary metabolites	leaf
Alkaloids	-
Saponins	+
Tannins	+
Flavonoids	+
Carbohydrate	+++

(C-1), 36.9 (C-10), 32.9 (C-25), 32.8 (C-2), 31.9 (C-7), 31.7 (C-8), 28.9 (C-16), 25.6 (C-28), 24.5 (C-15), 21.9 (C-21), (C-24), 21.7 (C-27), 21.5 (C-11), 19.5 (C-19), 19.1 (C-26), 12.6 (C-18), 12.1 (C-29).

Benzoic acid 3: Crystalline solid from chloroform fraction. Mp 122 – 123°C, UV (CD₃OD) λ_{\max} log : 228 (3.62), 272 (3.77), 300 (4.01) nm. IR (KBr) λ_{\max} cm⁻¹: 3260 - 2610 (COOH), 1705 (C=O). HREIMS *m/z*: 122.0367 (calcd. for C₇H₅O₂, 122.0360).

¹H-NMR (CDCl₃, 400 MHz): 11.92 (1H, s, O-H), 8.12 (2H, t, J = 8.5 Hz, H- 2, 6), 7.60 (1H, t, J = 8.5 Hz, H-4), 7.46 (2H, t, J = 8.5 Hz, H- 3, 5).

¹³C-NMR (CDCl₃, 400 MHz): 181.0 (C-7), 134.5 (C- 4), 131.1 (C-1), 130.7 (C-2,6), and 129.7 (C-3,5).

4-Hydroxybenzoic acid 4: White crystalline solid from EtOAc fraction. Mp 213 – 214°C, HREIMS *m/z*: 122.0367 (calcd. for C₇H₅O₃, 122.0367). UV (CD₃OD) λ_{\max} log : 223 (3.89), 310 (3.96) nm. IR (KBr) λ_{\max} cm⁻¹: 3330 (OH of carboxylic acid), 1705 (C=O).

¹H- NMR (CDCl₃, 400 MHz): 11.92 (1H, s, O-H), 8.12 (2H, t, J = 8.5 Hz, H- 2, 6), 7.60 (1H, t, J = 8.5 Hz, H-4), 7.46 (2H, t J = 8.5 Hz, H- 3, 5).

¹³C-NMR (CDCl₃, 400 MHz): 181.0 (C-7), 134.5 (C- 4), 131.1 (C-1), 130.7 (C-2,6), and 129.7 (C-3, 5).

Superoxide scavenging assay

The method of Ferda (2003) was used in the determination of superoxide scavenging activity of the isolated and characterized Figures 1 - 4.

Superoxide scavenging was assayed in phosphate buffer (0.1 M, pH 7.5). Xanthine oxidase (0.003 unit /well) and test samples in DMSO were mixed in 96 – well microliter plate and pre-incubated for 10 min at room temperature, WST-1 (15 M) was added. The reaction was initiated by adding 0.1mM of xanthine and uric acid formation was measured spectrophotometrically at 295 nm and the reduction of WST-1 was read at 450 nm by using molecular devices, spectramax 340. The percentage inhibitory activity by the samples was determined against DMSO blank and calculated using the formula:

% Inhibition = 100 - {(OD test compound/ OD control) x 100}

IC₅₀ of samples was determined by using EZ-FIT windows-based software.

RESULTS AND DISCUSSION

The results of the phytochemical screening (Table 1) revealed the presence of saponins and flavonoids. The present study on the chloroform and ethyl acetate soluble parts of *E. heterophylla* leaves collected from Nigeria, resulted in the isolation and characterization of Figures 1 - 4 for the first time from the mentioned source. These

compounds were characterized on the basis of spectroscopic analysis and comparison with reported data in literature. The chloroform soluble fraction gave Figure 1, isolated as colourless crystals. It gave positive Liebermann- Burchard and Salkowski tests for steroid. The molecular formula was established as C₂₉H₄₈O by high resolution mass spectrometry which indicated M⁺ peak at *m/z* 412.3920 (calcd. for C₂₉H₄₈O, 412.3926). The IR spectrum showed conspicuously the nature of hydroxyl group as absorption of 3432 cm⁻¹. The fragmentation pattern was in line with that of sterols. The ¹H -NMR showed peaks for two tertiary methyl groups 5.33 (1H) and 5.15 (2H) for three olefinic protons and another multiplet at 3.28 for the carbinyl proton (Rubinstein et al., 1976). The data thus established the structure of 1 as stigmasterol. The nature of oxygen was shown to be hydroxyl group as depicted by IR spectrum (3442 cm⁻¹). The mass spectrum showed characteristic fragmentation pattern of ⁵22 sterol (Bernard, 1977). The ¹³C NMR (Broad band and DEPT) displayed 29 carbon signals corresponding to stigmasterol moiety.

Compound 2 was isolated as amorphous solid and re - crystallized as colourless crystals from methanol. Its molecular formula was determined as C₃₅H₅₈O₆ by HR-MS which displayed molecular ion peak at *m/z* 574.4231 (calcd. for C₃₅H₅₈O₆ 574.4233). The mass spectrum also showed characteristic fragmentation pattern similar to compound 1.

The ¹H NMR of 2 was similar to 1 except additional resonances at 5.23 (1H, d, J= 5.4Hz) and glucose signals between 3.84 – 4.44 corresponding to the sugar molecule. The correlation between carbon 3 of aglycone and the H-1' of glucose revealed the point of attachment of glucose. The ¹³C-NMR displayed 35 carbon signals, 29 carbons for the stigmasterol moiety while the remaining 6 for the sugar molecule. The orientation of the glucose was confirmed to be a linkage due to the coupling con-stant of the anomeric proton (J = 7.5 Hz).The structure of 2 was then determined as Stigmasterol 3-O- -D-glucoside.

The structure of 3 was determined by combined interpretation of ¹H NMR, ¹³C NMR and other spectroscopic techniques. It was isolated as needle like crystalline solid from the chloroform fraction of *E. heterophylla* leaf extract. Its UV absorption at 272 and 300 nm was characteristic of an aromatic carboxylic acid group. The ¹H NMR showed aromatic protons signals at 8.12 (2H, t, J = 8.5Hz), 7.60 (1H, t, J = 8.5 Hz), 7.46 (2H, t J = 8.5 Hz) for positions (H-2, 6) and (H-4), (H-3, 5) respectively. A chelated OH was also displayed at 11.92 in the proton NMR spectrum. ¹³C NMR displayed 5 carbon signals resonating at 180.0, 134.5, 131.1, 130.7 and 129.7 for C=O, (C-4), (C-1), C-2, 6 and (C-3, 5) respectively. The structure was established as benzoic acid.

Figure 4 Crystalline solid from ethylacetate fraction. It was similar to 3 except the OH group which gave a characteristic IR absorption at 3510 cm⁻¹. The chemical

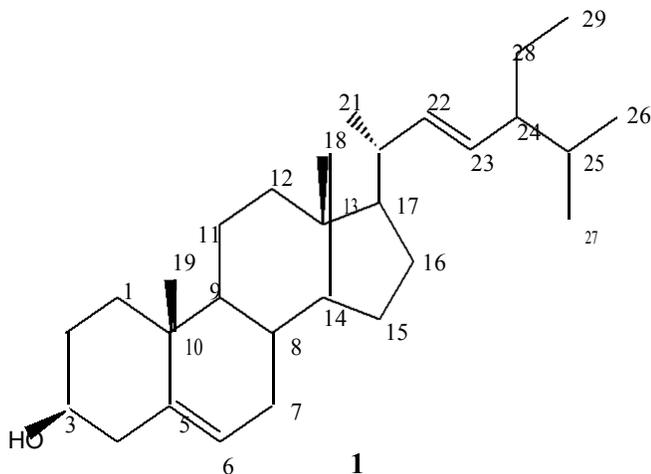


Figure 1. -stigmasterol glucoside

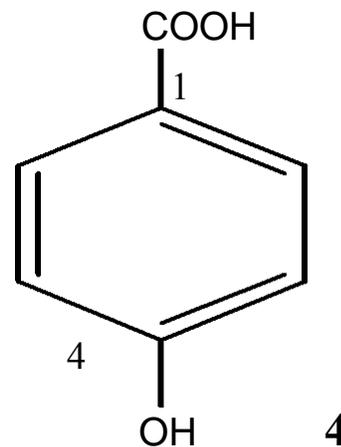


Figure 4. 4 – hydroxyl benzoic acid.

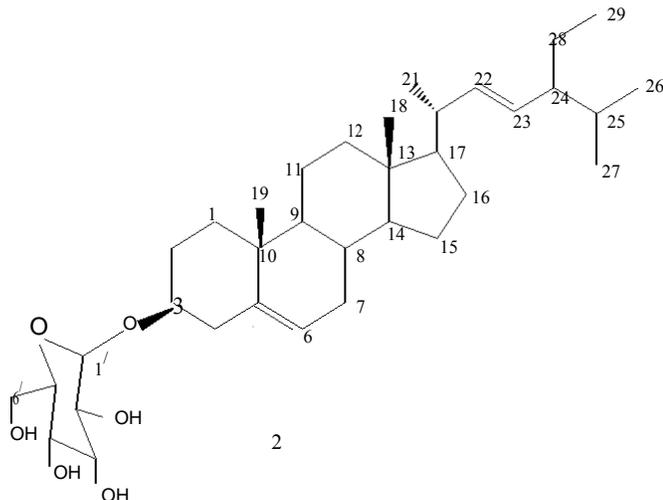


Figure 2. Stigmasterol

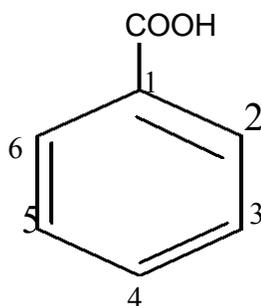


Figure 3. Benzoic acid

shift of H-4 was more downfield because of the contributory effect of the OH group. Its structure determined as 4- hydroxycarboxylic acid. The results of the superoxide scavenging activity is presented in Table 2.

Table 2. *In vitro* superoxide scavenging activity of compounds 1 – 4

Compound	IC ₅₀ ± SEM[m]
1	455.25 ± 5.23
2	585.30 ± 2.23
3	289.25 ± 2.56
4	115.64 ± 3.89
Allupurinol	10.23 ± 2.67

SEM, Standard Error of Mean.

Figure 4 showed the highest activity followed by 3 while 1 and 2 were moderately active when compared to the standard drug. Figure 3 and 4 are more active due probably to the presence of hydroxyl groups while, 4 had an extra phenolic OH group thus demonstrating high potency.

Conclusion

The research work through a systematic chemical and biological investigation has determined and identified for the first time the presence of four chemical constituents as carboxylic acids, 4-hydroxy carboxylic acid, and steroidal components such as stigmasterol and stigmasterol glucoside in the chloroform and ethylacetate fractions of *E. heterophylla* leaf respectively. The superoxide scavenging properties of the isolated compounds reveals that 1-4 were active, while 4 was the most active. Further investigation will aimed at determining mechanisms of action of the compounds and possible toxicity profile.

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