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Isolation of Steroid from Unsaponifiable Portion of *Trichilia emetica* Seed Oil

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ABSTRACT

Trichilia emetica (Vahl) seed oil has a wide folk medicinal usage in Africa. The aim of this study was to phytochemically investigate the unsaponifiable portion of *Trichilia emetica* seed oil. The portion was subjected to repeated column chromatography and preparative thin layer chromatography (PTLC) using hexane, chloroform, ethyl acetate and methanol. Final purification was achieved using an isocratic gradient elution of $CHCl_3$ and CH_3CN at a ratio of 3 to 1, in Reveleris Flash System fitted with C18 column. The structure of the isolated compound was elucidated on the basis of high resolution mass spectrometry as well as 1D and 2D NMR spectroscopic data analysis. Also, by direct comparison of the data obtained with those reported in the literature. It was then resolved that the compound isolated was stigmasterol, a steroid present in plant fats or oils. This compound has not been previously reported from this species.

1. Introduction

Trichilia emetica Vahl, is an evergreen tree belonging to the Family Meliaceae. The generic name '*Trichilia*' is derived from the Greek "tricho" referring to the 3-lobed fruits and "emetica" relating to the emetic properties of the tree [1]. This species is native to Africa and it grows widely throughout sub-Saharan African extending from Ghana to the Red Sea, throughout East and Central Africa to Zambia and KwaZulu-Natal in South Africa. It also grows naturally in Yemen and has been introduced as an ornamental tree in Cape Verde [2-4]. They are propagated by cuttings and regenerate naturally by root suckers, and seeds [5].

T. emetica seeds have a seed weight between 0.35-1.0 g. The seed is made up of 21–29% oily shell like husk called sarcotesta and 71–79% kernel. The kernel also contains 55-65% of a brownish fat that melts between 35-41 °C [6]. The seeds, when pressed, give two kinds of oil: 'mafura oil' from the seed coat (sarcotesta) and 'mafura butter', from the kernel. These oils may be separately extracted in traditional extraction or combined as a single product as in commercial extraction. The mafura oil is edible, but mafura butter is not suitable for consumption because of its bitter taste [1, 3, 7].

In Africa traditional medicine, the seed oil is rubbed into cuts made on a fractured limb in order to hasten healing, and it is also taken internally to treat rheumatism [4]. The seed oil is used in combination with *Cyathula natalensis* Sond to treat leprosy [8]. The oil is sometimes combined with coconut oil to provide an emollient and moisturising effect on the skin [9].

This paper describes the isolation and structure elucidation of stigmasterol from unsaponifiable fraction of *T. emetica* seed oil.

2. Experimental Methods

The melting point was determined by a Stuart instrument and is uncorrected. The NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using standard pulse sequences at ambient temperature. Trimethylsilane (TMS) was used as an internal standard, while chemical shifts are given in (ppm), and coupling constants are reported in Hertz. Low pressure chromatography was performed on Reveleris Flash System fitted with Grace Reveleris C18 column. Flash column chromatography was performed on Fluorochem silica gel 60 Å. Thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC) was conducted on precoated E. Merck TLC silica gel 60 F_{254} glass plates, and identification of the spots on the TLC plate was carried out by spraying with phosphomolybdic acid and heating the plate at about 100 °C for 5 minutes.

2.1 Collection of Plant Material

*T. emetic*a seeds were collected from Kumasi, Ghana, in February 2013 and authenticated by a botanist Mr. Martin A. Arkoh of Kwame Nkruma University of Science and Technology, Kumasi, Ghana. A voucher specimen TBG-2014-1 was deposited at the herbarium of Treborth Botanical Garden Bangor, UK.

2.2 Preparation of the Plant Material

2.2.1 Determination of Percentage Unsaponifiable Matter

The method by Cañabate-Díaz [10] was used with modification. *Trichilia emetica* oil (100 g) was dissolved in 100 mL of ethanolic potassium hydroxide (0.5 M) and refluxed for 2 hours at 70 °C, with constant stirring. After cooling, the mixture was diluted with 200 mL deionised water and partition with diethyl ether (400 mL × 5). The ether extract was first washed with (0.5 M) potassium hydroxide (400 mL × 2) and then deionised water (400 mL × 7). The ether extracts was dried over $MgSO_4$, filtered under gravity and the solvent removed under vacuum at 40 °C. The unsaponifiable matter is then calculated as percentage.

$$\text{Percentage unsaponifiable matter (\%)} = K / K_0 \times 100 \qquad (1)$$

where, K = weight of unsaponifiable matter and
K_0 = weight of oil

2.3 Isolation

The separation of unsaponifiable matter (2.1 g) was carried out by column chromatography [11]. The column was run using a gradient mixture of hexane and chloroform (100:0 to 0:100), and chloroform and methanol (100:0 to 90:10). Fifty three (53) eluates were collected (50 mL

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each) and the eluted fractions were monitored by TLC. Fractions 35-39 obtained on elution with 2 % methanol in chloroform were combined to get 0.25 g residue, and was subjected to sub-column chromatography for further fractionation. The column was eluted with chloroform and ethyl acetate of increasing polarities to provide 26 fractions. Fraction 19 (41 mg) obtained on elution with 8 % EtOAc in $CHCl_3$ was purified by PTLC using ethyl acetate-hexane (7:3) as developing solvent to give an impure stigmasterol (25 mg). Final purification was achieved using Grace Reveleris® C18 column with ELSD (Evaporation Light Scattering Detection) and 2 selected UV-VIS wavelength detectors at 215 and 250 nm, using an isocratic elution of $CHCl_3$-CH_3CN (3:1) resulted in the isolation of stigmasterol (12 mg).

2.4 Test for Steroid

The method which was proposed by Harbone [12] was used.

2.4.1 Salkowski Reaction

2 mg of the compound was dissolved in 1 mL of chloroform and 3 drops of concentrated sulfuric acid was added. A reddish colour was seen in the upper chloroform layer.

2.4.2 Liebermann-Burchard Reaction

2 mg of the compound was dissolved in 1 mL of chloroform and 3 drops of concentrated sulfuric acid was added followed by 2 drops of acetic anhydride. The solution turned violet blue and finally green.

2.5 Characterisation of the Compound

The structure of the compound according to spectroscopic data was found to be as follows:

IR ($CHCl_3$) cm^{-1}: 3368, 2934, 2852, 1654, 1456, 1377, 1030, 760. ^1H NMR ($CDCl_3$, 400 MHz): 3.48 (1H, m, H-3), 5.37 (1H, t, J= 5.4 Hz, H-6), 0.72 (3H, s, H-18), 1.03 (3H, s, H-19), 0.92 (3H, m, H-21), 5.15 (1H, dd, J= 15.5, 8.6 Hz, H-22), 5.01(1H, dd, J= 15.5, 8.6 Hz, H-23), 0.85 (3H, d, J = 7.1 Hz, H-26), 0.81 (3H, d, J = 7.1 Hz, H-27), 0.83 (3H, d, J= 6.5 Hz, H-29), 1.52 (2H, m, H-2). ^{13}C NMR ($CDCl_3$, 100 MHz): 140.8 (C-5), 121.7 (C-6), 71.8 (C-3), 56.9 (C-14), 56.0 (C-17), 50.2 (C-9), 51.3 (C-24), 42.3 (C-4) 42.2 (C-13), 39.7 (C-12), 37.3 (C-1), 36.5 (C-10), 40.5 (C-20), 138.3 (C-22), 31.9 (C-8), 31.9 (C-7), 28.9 (C-25), 31.7 (C-2), 28.9 (C-25), 28.9 (C-16), 129.3 (C-23), 24.4 (C-15), 25.4 (C-28), 21.2 (C-11, C-21), 19.0 (C-27), 19.4 (C-19), 21.1 (C-26), 12.3 (C-29), 12.1 (C-18).

3. Results and Discussion

Stigmasterol (12 mg) was isolated as a white crystalline powder and recrystallized from methanol. It has a melting point of 164-165 °C and gave positive Salkowski and Lieberman-Burchard test for steroids. It has a molecular formula of $C_{29}H_{48}O$ which was established on the basis of ESI-HRMS at m/z 413.3780[M + H]$^+$(Calculated for 413.3784). The IR spectrum of this compound showed a band at 3368 (broad, O-H stretching), 2934 and 2852 (C-H stretching), 1654 (weak, C=C stretching), 1456 (C-H deformation), 1377 (methyl group symmetrical deformation), 1114, 1030 (C-O stretching) and 760 (C-H out of plane deformation) cm^{-1}. The DEPT spectrum [Fig. 1] showed twenty nine carbon signals, which consist of six methyl, nine methylenes, eleven methines and three quaternary carbons.

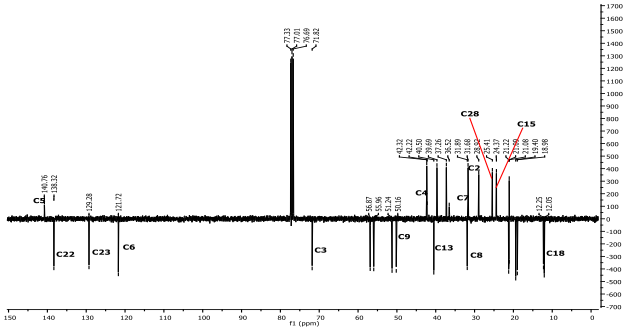

Fig. 1 DEPT spectrum of stigmasterol

The ^1H NMR spectrum [Fig. 2], has a proton signals at δ_H 3.48 (H-3) which is a carbinolic proton on ring A and it occurred as a multiplet. The spectrum also showed the presence of six methyl peaks at δ_H 0.72 (H-18), δ_H 1.03 (H-19), δ_H 0.92 (H-21), δ_H 0.85 (H-26), δ_H 0.81 (H-27) and δ_H 0.83 (H-29). The endocyclic double bond proton appeared as a doublet at δ_H 5.37 with a coupling constant of 5.4 Hz. The presence of a double bond between H-22 and H-23 occurred as a result of splitting of proton at H-22 by protons H-20 and H-23, while proton H-23 was splitted by H-24 and H-22 both resulting in a doublet of doublet at δ_H 5.01 and 5.15 with coupling constant of 8.6 and 15.49 Hz each.

In the ^{13}C NMR spectrum, the carbinolic proton and hydroxyl group in ring A are both attached to C-3 and the signal appeared at δ 71.8. An endocyclic carbon-carbon double bond signal in ring B appeared at δ 121.9 and 141.0 assigned to C-6 and C-5. Other olefinic carbon signals at C-22 and C-23 appeared at δ 121.9 and 141.0. The side chain occurring at C-17 was identified as 5- ethyl-6-methylhept-3-ene and was confirmed from the HMBC correlation of Me-21 (δ_H 0.92) with C-17 (δc 56.0) (Fig. 3).

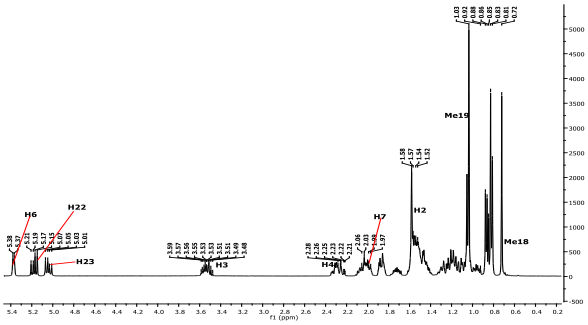

Fig. 2 ^1H NMR spectrum of stigmasterol

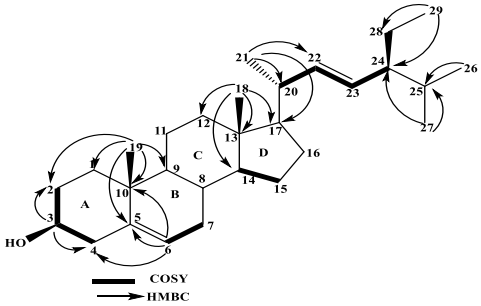

Fig. 3 HMBC and COSY correlation of stigmasterol

This compound has been reported from the leaves of an endemic plants *Glyptopetalum calocarpum* and it showed anti-leptospiral activity [13]. Similarly, it has been reported in the rhizomes of *Etlingera sphaerocephala* Var. *Grandiflora* [14] and also from the leaves of *Rubus suavissimus* S. Lee [15]. The NMR data are comparable with those reported in the literature.

4. Conclusion

The isolation and identification of stigmasterol from the unsaponifiable portion of *T. emetica* seed oil was the first ever to be reported from this plant. The work was carried out by utilizing several kinds of chromatographic separation techniques such as TLC, CC, PTLC and Flash system. And several spectroscopic analytical techniques were also used. Also direct comparison of the data obtained with those reported in the literature. It was concluded that the compound isolated was stigmasterol.

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