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PHYTOSTEROLS FROM THE METHANOL ROOT EXTRACT OF *RAUWOLFIA VOMITORIA*, AFZEL (APOCYNACEAE)

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ABSTRACT

Phytosterols are recognized to promote positive biological activities such as hypocholesterolemic and anti-inflammatory activities amongst other functionalities still under investigation. They are found exclusively in the non-saponifiable fraction of plant oils. Preliminary investigation revealed the presence of phytosterols in hexane fraction of the crude methanol root extract of *Rauwolfia vomitoria*. The plant is used in traditional medicine for the treatment of snake bites, diarrhea, and skin related problems and as an anti-psychotic amongst other indications. Column chromatography by gradient elution method and preparative thin layer chromatography were used in the isolation of phytosterols from the plant. Further purification afforded a white crystalline needle-like compound coded HR5, which was completely soluble in dichloromethane with a melting point of 143- 145 ^{o}C. In comparison with literature, HR5 was identified as a mixture of stigmasterol and β- sitosterol (phytosterols) using IR, ^{1}H NMR and ^{13}C NMR spectroscopic techniques.

Keywords: Phytosterols, *Rauwolfia vomitoria,* Stigmasterol, β- sitosterol

INTRODUCTION

Phytosterols can be described as one of the groups of nutraceuticals exclusively from plant source and when incorporated into food, make it more nutritional [1]. The presence of these phytosterols in plants has increased their value and importance, thereby, their use as medicinal plants. *Rauwolfia vomitoria*, commonly known as swizzle stick in English, is an ever green perennial plant, a shrub to a medium sized tree of 0.5-20 meters in height [2]. The plant is used in traditional medicine in the treatment of a variety of illness. However, the parts commonly used for herbal remedies are the roots, leaves and stem bark with decoction and infusion as the mode

of preparation [3]. Powdered root in local gin is taken to treat hypertension, diarrhea, venereal disease, snake bite and as sedatives to calm people with epilepsy and psychotic [4]. Macerated powdered root and pulp fruit is used for skin problems like rash, pimples, chicken pox, and head lice [5].

Extensive phytochemical screening reports the isolation of more than 50 indole alkaloids which is the biomarker for *Rauwolfia* species [6]. Flavonoids like quercetin and rutin glycoside have been isolated from other species of the plant [7-8].

Despite several isolations and wide range medicinal potentials evaluated, there is a lacuna of data on the isolation and characterization of phytosterols from the root of the plant. Researches have shown the beneficial effect of phytosterols on human health based on their positive biological activities like hypo-cholesterolaemic action, antioxidant effect, immune modulatory effect and anti-inflammatory effect amongst others [9]. Hence, their use in the formulation of functional food is on the increase. It is therefore imperative, to screen medicinal plants for more phytosterols which could be responsible for observed biological activities when used in traditional medicine.

The aim and objectives of this paper, therefore, is to carry out preliminary screening, isolate and characterize some phytosterols from the hexane fraction of the crude methanol root extract of the plant.

MATERIALS AND METHODS

Sample Collection, Authentication and Preparation

The fresh plant materials comprising of only the root were collected from Ojo Local Government Area of Lagos State, Nigeria, in February, 2019. The plant sample was identified and authenticated by Mr. Namadi Sanusi of the Herbarium Section of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria. For herbarium reference, a voucher number of V/N 0610 was assigned. Dirt and earth materials from the plant root were removed; shade-dried for three weeks and pulverized using mortar and pestle to a suitable size prior extraction.

Sample Extraction and Partitioning

The pulverized root (1, 200 g) was extracted with absolute methanol using cold maceration technique for 8 days with occasional shaking. The extract was filtered and the residual solvent in

the crude extract was concentrated *in-vacuo* using rotary evaporator under reduced pressure to yield a reddish-brown crude extract. The crude extract (150 g), was further subjected to solvent partitioning using hexane, chloroform and butanol to obtain hexane fraction (HF), chloroform fraction (CF) and butanol fraction (BF) of the methanol root extract.

Phytochemical Screening

The crude methanol extract of the plant and the various fractions were screened for the presence of secondary metabolites, namely, alkaloids, carbohydrates, glycosides, saponins, flavonoids, tannins, triterpenes and steroids, using standard procedures [10] as shown in Table 1.

Table 1: Phytochemical constituents in methanol root extract and hexane fraction of *Rauwolfia vomitoria*

CONSTITUENTS	TEST	OBSERVATIONS	
		MRE	HF
Alkaloids	Dragendoff	+	+
Carbohydrate	Molisch	+	-
Cardiac glycoside	Keller-kiliani	+	-
Saponins	Frothing	+	-
Flavonoids	Sodium Hydroxide	+	-
	Shinoda	+	-
Tannins	Lead sub-acetate	+	-
Triterpenes	Liebermann-Burchard	+	+
Steriods	Salkowski	+	+

KEY: + Present; - Absent

Column Chromatography of Hexane Fraction

On the basis of the TLC profile and compound of interest, hexane fraction (8.89 g) was chromatographed over silica gel packed column of dimension 75 by 3.5 cm using gradient elution method; starting with hexane 100% followed by hexane:ethyl acetate (95:5). The polarity was gradually increased by 5 up to 100% ethyl acetate. Finally the column was washed with methanol. A total of 73 collections of 100 ml each were made. Column fractions were pooled together to give eleven (11) major fractions coded RV1-RV11 based on their TLC profile [11].

Purification of Compound HR5

Fraction RV5, showed two distinct spots on the TLC plate. When dissolved in hexane, it was found to be sparingly soluble but completely soluble in dichloromethane. On the basis of differences in solubility, fraction RV5 was first washed with hexane and thereafter dichloromethane severally (thrice), which afforded a white crystalline needle-like compound coded HR5 when concentrated (7 mg) and gave a single homogenous spot in different solvent system using 10% sulphuric acid as the spraying agent on the TLC plate. The solvent system and the R_f values are shown in Table 2.

Table 2: TLC profile of HR5

Solvent system	**No of spots**	**Colour of spot after heating**	**R_f Value**
DCM (100 %)	1	Deep red	0.39
DCM:EA (9:1)	1	Deep red	0.52
HX:EA (6:4)	1	Deep red	0.70

Test for steroids/triterpenes

Salkowki's test

A small portion of the extract was dissolved in 2 ml of chloroform, 3 drops of concentrated sulphuric acid (Salkowki's reagent) were added at the side of the test tube. A red-brown colouration at the interface indicates the presence of steroids [10].

Liebermann-burchard's test

To the portion of the extract, equal volume of acetic anhydride was added and mixed gently. Exactly 1 ml concentrated sulphuric acid was added down the test tube. This was observed for instant colour changes and over a period of one hour. Blue to blue-green colour in the upper layer and reddish, pink or purple colour at the junction of the two layers indicated the presence of triterpenes [10].

Spectroscopic characterization

Different spectroscopic methods were used to elucidate the structure of HR5. Among the spectroscopic techniques IR, ^{1}H-NMR, and ^{13}C-NMR were carried out. The IR spectrum was recorded on Happ-Genzel 4000-650 at Multiuser Research Laboratory, Department of Chemistry Ahmadu Bello University, Zaria. NMR spectra were recorded on 400 MHz-Bruker ((Bruker Bio

Spin, Rheinstetten, Germany) at Natural Product Laboratories, Institute of Pharmacy and Biomedical Sciences (SIPBS), University of Strathcyde Glasgow, United Kingdom. The chemical shifts (δ) were reported in parts per million (ppm) and the coupling constants (J values) were reported in hertz.

The IR absorption spectrum of HR5, showed stretching and bending vibration frequencies at 3399.3 cm^{-1}, 2933.4 cm^{-1}, 2862.6 cm^{-1}, 1640.0 cm^{-1} , 1461.1 cm^{-1}, 1379.1 cm^{-1} and 1051.1 cm^{-1}

The ^{1}H NMR ($CDCl_3$, 400MH_Z) spectrum of HR5 varied between δ 0.71-5.38ppm. It revealed signals at δ 0.71 (3H, s, H-18), 0.77 (3H, t, H-29), 0.95 (1H, q, H-9), 0.82 (3H, d, H-27), 0.83 (3H, d, H-21), 0.86 (3H, d, H-26), 1.86 (1H, m, H-8), 5.18 (1H, dd, H-22), 5.05 (1H, dd, H-23), 1.08 (2H, t, H-1), 1.03 (1H, q, H-14), 1.04 (3H, s, H-19), 1.13 (1H, q, H-17), 1.47 (2H, t, H-7), 1.53 (2H, q, H-2), 1.56 (2H, q, H-11), 1.73 (2H, q, H-16), 2.02 (2H, t, H-12), 2.31 (2H, t, H-4), 3.55 (1H, m, H-3), and 5.38 (1H, Br s, H-6) ppm

The ^{13}C NMR ($CDCl_3$, 400 MH_Z) spectrum of HR5 revealed the presence of twenty nine carbon signals. It revealed signals at δ 37.27 (C-1), 31.69 (C-2), 71.82 (C-3), 40.49 (C-4), 140.76(C-5), 121.72 (C-6), 31.89 (C-7), 31.92 (C8), 50.15 (C-9), 36.52 (C-10), 21.09, 21.22 (C-11), 39.79 (C-12), 42.33 (C-13), 56.88 (C-14), 24.38 (C-15), 28.92 (C-16), 56.67 (C-17), 11.87 (C-18), 19.41 (C-19), 40.49, 36.16 (C-20), 19.05 (C-21), 138.31, 33.97 (C-22), 129.29, 26.10 (C-23), 51.25 (C-24), 31.89 (C-25), 21.22, 19.83 (C-26), 18.99 (C-27), 25.41, 23.09 (C-28), and 12.25 (C-29) ppm.

RESULTS AND DISCUSSION

Preliminary phytochemical screening revealed the presence of secondary metabolites: alkaloids, carbohydrate, cardiac glycoside, saponins, flavonoids, tannins, triterpenes, and steroids, in the methanol root extract of *Rauwolfia vomitoria* which is in accordance to those reported in literature [12-13]. However, the hexane fraction revealed the presence of alkaloids, triterpenes and steroids as presented in Table 1. The result shows that the presence of secondary metabolites in different extracts is dependent on the polarity of the solvents used for the extraction and also partitioning [14]. The column chromatographic separation of the hexane fraction on silica gel yielded HR5 (7 mg) which was completely soluble in dichloromethane but sparingly soluble in hexane. HR5 is a white crystalline needle-like compound with a melting point of 143-145 ^{o}C and

tested positive to Liebermann-Burchard test with a green colouration at the top layer of the test tube suggesting that HR5 contains a steroidal nucleus. This is similar to those reported in literatures [15-16].

IR spectrum in KBr showed absorption bands at 3399.3 cm^{-1} which is characteristic of O-H stretching. Absorption at 2933.4 cm^{-1} and 2862.6 cm^{-1} is due to aliphatic C-H stretching. At 1461.1 cm^{-1} is a bending frequency for cyclic $(CH_2)n$ and 1379.1cm^{-1} for $–CH_2(CH_3)_2$. The absorption frequency at 1051.1 cm^{-1} signifies cycloalkane. These absorption frequencies resemble those reported for stigmasterol [17].

^1H NMR (CDCl3, 400MHz) spectrum of HR5 revealed the presence of six tertiary methyl protons at; δ_H0.71 (H-18), 1.04 (H-19), 0.83 (H-21), 0.86 (H-26), 0.82 (H-27) and 0.77 (H-29), integrated for 3H each. Carbinoyl proton showed a multiplet at δ 3.52 and a broad singlet for H-6 at δ 5.38. This is typical for H-3 and H-6 for a steroidal nucleus [18]. Two olefinic protons were observed at characteristic downfield signals of δ 5.05 (1H, dd, J= 4.0 Hz) and δ 5.18 (1H, dd, J= 8.0 Hz) which were identical with the chemical shift of H-23 and H-22 of stigmasterol respectively in literatures [19-20]. Angular methyl protons at δ0.68 (s), 0.71 (s) and 1.04 (s) corresponds to C-18 and C-19 proton respectively. The proton also displayed three proton doublet at δ 0.83, 0.86 and 0.82, assignable to H-21, H-26, H-27 respectively and a three proton triplet at δ 0.77 (3H, t) which could be assignable to the primary methyl group at H-29.

The ^{13}C NMR spectrum revealed the presence of 29 carbon signals. Signal at δ 71.82 was assigned to oxymethine carbon. This is characteristic to spirostene [21]. Signals at δ 140.76 and 121.72 were assigned to C-5 and C-6 respectively. Also two angular methyl carbons, C-19 and C-18, were assigned δ 19.41 and 11.87. Two olefinic carbons appeared at δ 138.31 and δ 129.29 which were identical with the chemical shift of C-22 and C-23 respectively for stigmasterol. However, signals at δ 33.97 and δ 26.10 were also observed and assigned to C-22 and C-23 double bond which is typical of β- sitosterol. The assignment of signals to their respective carbons is in good agreement to those reported in literatures [22].

Identification and confirmation of HR5, as a mixture of stigmasterol and β- sitosterol with the following structures in Figures 1 and 2, was accomplished using IR, ^1H NMR and ^{13}C NMR spectra data. HR5 often present as mixture, having the same R_f value using different solvent system which makes them difficult to be isolated in the pure state. The only difference

between them is the presence of C-22/C-23 double bond in stigmasterol and C-22/C-23 single bond in β- sitosterol.

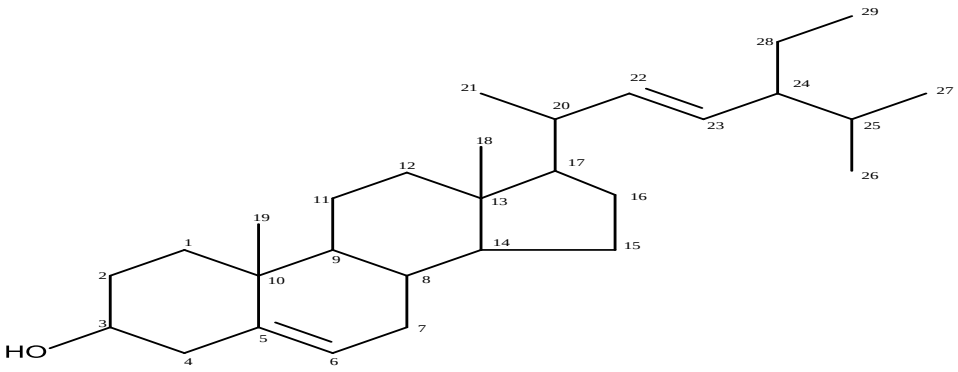

Figure 1: Stigmasterol

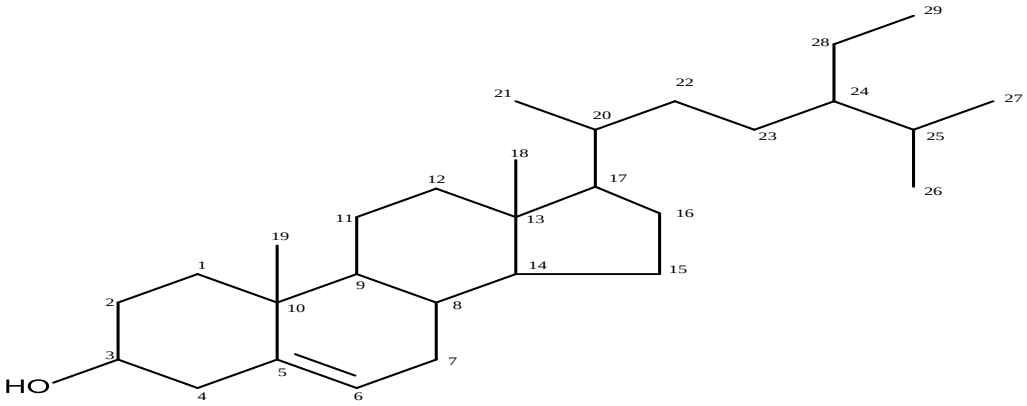

Figure 2: β- Sitosterol

CONCLUSION

Stigmasterol and β-sitosterol are well known phytosterols with anti-inflammatory and cholesterol lowering properties. They were isolated from the hexane fraction of the methanol root extract of *Rauwolfia vomitoria* and their chemical structures were elucidated using spectroscopic techniques: IR, ^{1}H NMR and ^{13}C NMR.

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