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Isolation of Isosativan from Nigerian Red Propolis

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ABSTRACT

Propolis has been known for its medicinal use and as a source of diverse bioactive natural compounds. Phytochemical screening of propolis samples usually reveal the constituent compounds and the source of the sample. Chromatography is the simplest way of isolating natural compounds and thus the hexane and ethyl acetate extracts of propolis from Bonny in Rivers State, Nigeria were subjected to column chromatography and the isolated compounds were identified by LC-MS and NMR analysis. The compounds detected and identified were oleic acid, propyl stearate, *β*-amyrin, isosativan, calycosin, liquiritigenin, isoliquiritigenin, pinocembrin and medicarpin. The structure of Isosativan was further confirmed by HRMS and 2D NMR spectroscopy. This is the first report of Isosativan from a sample of Nigerian and African propolis.

Keywords: Isoflavan, *β*-amyrin, Propyl stearate, Oleic acid, Rivers State.

Introduction

Propolis produced by bees are well-known natural sources of a diverse and potentially bioactive compounds and their bioactivity studies coupled with their medicinal uses have been well reported.[1-2] Propolis from different parts and regions of the World have shown diversity in constituents and bioactivities.[3-5] Although Nigerian propolis samples have been investigated for their phytochemical constituents,[6-8] the diversity observed in these studies has necessitated a further investigation of the Nigerian red propolis from Bonny in Rivers State. This is to isolate more bioactive compounds and assay them against some disease pathogens. Nigerian red propolis is unique and attracting attention especially for the fact that its crude extracts and isolated compounds have been shown to be active against trypanosomiasis,[7,8] the crude extract being more active than the isolated compounds. Thus a further screening to isolate the more active compounds and a full identification of its constituents will enhance any drug discovery or development from it. A recent study of the Nigerian red propolis identified several flavonoids and isoflavonoids such as calycosin, liquiritigenin, pinocembrin, medicarpin, 6-prenylnaringenin, 8-prenylnaringenin, propolin D**,** macarangin and a novel dihydrobenzofuran, reverinol.[8] The extracts were also observed to possess significant anti-trypanosomal activity *in vitro*[7-8] and *in vivo*.[9] From previous studies, the exact constituents or compounds responsible for the activity observed have not been isolated or characterised. We hereby report on the further phytochemical screening and dereplication studies on the red propolis from Bonny which has yielded other compounds in addition to some of the previously reported ones. The isolation and characterization of these additional compounds from the Nigerian red propolis is hereby described.

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Materials and Methods

General

Nigerian red propolis sample was obtained from Bonny, Rivers State, Nigeria in April 2014. Solvents used were commercially obtained and re-distilled before use. NMR data were acquired on a JEOL (JNM LA400) 400 MHz spectrophotometer in $CDCl_3$ and TMS as internal standard. TLC were performed using pre-coated TLC grade silica gel on Aluminum sheets (Pre-coated Silica gel PF_{254}, Merck, Germany).

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Extraction and Isolation of Constituents

Dried and ground propolis (150 g) was placed in a clean container and extracted (72 h) successively with hexane, ethyl acetate and methanol (600 mL each) via maceration. The extracts were filtered and the solvents were evaporated. The dried hexane and ethyl acetate extracts were subjected to column chromatography over silica gel (230-400 mesh ASTM). The hexane extract column was eluted with an ethyl acetate in hexane gradient, with increasing amounts of ethyl acetate in hexane, collecting 20 mL fractions. The fractions were examined by TLC and those with similar TLC profiles after spraying with vanillin – sulfuric acid reagent and heating, were combined. The combined fractions were allowed to dry in a fume hood and thereafter examined by NMR. From these fractions, compounds **2** and **3** were obtained as yellowish oils and compound **4** (25 mg) as a white solid. The ethyl acetate extract column was similarly eluted with ethyl acetate in hexane gradient and then with methanol in ethyl acetate. Similar fractions on TLC were combined and allowed to dry in a fume hood and fraction 62 yielded compound **1** (30 mg) as a white crystalline solid.

LC-MS analysis

LC-HRMS analysis was performed on an Accela 600 HPLC system with an ACE C-18 column (150 × 3 mm, 3 µm particle size) (HiChrom, Reading UK) coupled to an Exactive (Orbitrap) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). About 2 mg of the hexane and ethyl acetate extracts were dissolved in 1 mL of methanol and filtered. 10 µL of the filtrate was used for the analysis. The mobile phase used was water with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B at a flow rate of 0.3 mL/min. The gradient elution was programmed as follows: 0 – 15 min linear gradient from 30% to 50% of B, 15 – 25

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min at 50% of B, 25 – 40 min linear gradient from 50% to 80% of B, 40 – 50 min at 80% of B, 50 – 51 min increasing to 100% of B, 51 – 59 min at 100% of B (with the flow rate increased to 0.5 mL/min) and at 61 min the solvent system was returned to 30% of B and held until the 70th min. The samples were run in duplicate, the MS detection range was from m/z 100 – 1500 and scanning was performed under ESI polarity switching mode. The needle voltages were −4.0 kV (negative) and 4.5 kV (positive) while the sheath and auxiliary gases were set at 50 and 17 arbitrary units respectively. The data obtained were split into positive and negative ions and the 'negative' dataset was processed using MZMine 2.14, with the masses selected between m/z 100–1200. Data were processed using Xcalibur 2.2 mass spectrometry software from Thermo Fisher Scientific.

Results and Discussion

The ethyl acetate extract yielded Isosativan (2′-hydroxy-7,4′-dimethoxyisoflavan) (**1**). The negative mode LC-HRESI-MS spectrum of the compound (Supplementary material F, Table 2) gave a molecular ion $[M-H]^-$ at m/z 285.1134 (Calc 285.1127, $C_{17}H_{17}O_4$) representing a molecular formula $C_{17}H_{18}O_4$. The ^{1}H-NMR spectrum (Supplementary material A, Table 1) of the compound showed two sets of ABX coupled protons at δ_H 6.98 (d, *J* = 9.4 Hz, H-5), 6.48 (dd, *J* = 9.4, 2.6 Hz, H-6), 6.43 (d, *J* = 2.6 Hz, H-8) and at 7.01 (d, *J* = 8.7 Hz, H-6′), 6.46 (dd, *J* = 8.7, 2.5 Hz, H-5′) and 6.35 (d, *J* = 2.5 Hz, H-3′). It also showed five coupled aliphatic protons at δ_H 4.34 (ddd, *J* = 10.3, 3.5, 1.9 Hz, H-2a), 4.04 (t, *J* = 10.2 Hz, H-2b), 3.51 (m, H-3), 3.00 (ddd, *J* = 15.8, 10.4, 1.1 Hz, H-4a), 2.90 (ddd, *J* = 15.8, 5.4, 2.0 Hz, H-4b) typical of a flavan or an isoflavan moiety.[6, 10] There were also two methoxy group protons at 3.75 and 3.77 (each 3H, s) ppm. The ^{13}C spectrum (Supplementary material C-G, Table 1) showed a total of 17 signals composed of six aromatic CH, two quaternary aromatic carbons, four phenolic, two methylene carbons at 70.2 and 31.6, one methine at 31.6 and two methoxy carbons. Using correlations in its 2D NMR spectra (Supplementary material C-E) the compound was identified as 2′-hydroxy-7,4′-dimethoxyisoflavan (**1**) as follows: the COSY spectrum linked H-2, H-3 and H-4 as a -CH_2-CH-CH_2- spin system and long range correlations from H-3 to C-2′ and C-6′ indicated the phenyl ring was substituted at C-3 confirming the compound to be an isoflavan. Identical correlations from H-5 to C-7 and H-6 to C-4′ as well as long range (HMBC) correlations (Figure 1) from a methoxy group to these carbons each, showed the methoxy groups were substituted at C-7 and C-4′, therefore C-2′ must have an –OH group as there are only two methoxy substituents in the compound. Other correlations (Figure 1) enabled the full chemical shift assignments (Table 1) and confirmed by literature reports.[11-12] The hexane extract yielded *β*-amyrin (**2**)[13,14] oleic acid (**3**)[15] and propyl stearate (**4**).[16]

Other compounds detected in the ethyl acetate extract were calycosin, liquiritigenin, pinocembrin, medicarpin and isoliquiritigenin. These were observed under LC-MS and were identified based on their high-resolution mass spectra.

Conclusion

Further phytochemical screening of Nigerian red propolis has led to the isolation of Isosativan, *β*-amyrin, oleic acid and propyl stearate. These compounds are reported from the Nigerian red propolis for the first time.

Table 1: ^{1}H and ^{13}C-NMR Data ($CDCl_3$) for Isosativan (**1**)

Position	^{1}H (multiplicity, *J* (Hz)	^{13}C (multiplicity)
1	-	-
2a	4.34 (ddd, 10.3, 3.5, 1.9)	70.2 (CH_2)
2b	4.04 (t, 10.2)	
3	3.51 (m)	31.8 (CH)
4a	3.00 (ddd, 15.8, 10.4, 1.1)	30.4 (CH_2)
4b	2.90 (ddd, 15.8, 5.4, 2.0)	
5	6.98 (d, 9.4)	130.3 (CH)
6	6.48 (dd, 9.4, 2.6)	107.4 (CH)
7	-	159.1 (C)
8	6.43 (d, 2.6)	101.5 (CH)
9	-	155.1 (C)
10	-	114.9 (C)
1′	-	120.0 (C)
2′	-	154.4 (C)
3′	6.35 (d, *J* = 2.5)	102.2 (CH)
4′	-	159.4 (C)
5′	6.46 (dd, 8.7, 2.5)	106.1(CH)
6′	7.01 (d, 8.7)	128.3 (CH)
4′-OCH_3	3.75(s)	55.4 (CH_3)
8- OCH_3	3.77(s)	55.4 (CH_3)

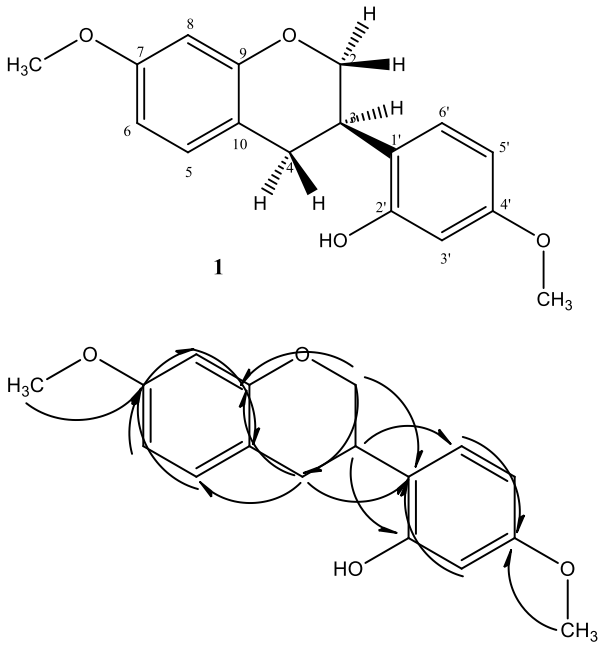

Figure 1: Selected HMBC correlations for compound 1

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Table 2: Retention time and mass ions detected on LC-MS of the ethyl acetate extract

S/No	Retention time (minutes)	Exact mass $[M-H]^-$	Molecular formula	Compound
1	7.7	283.0613	$C_{16}H_{12}O_5$	Calycosin
2	10.1	255.0664	$C_{15}H_{12}O_4$	Liquritigenin
3	13.2	255.0664	$C_{15}H_{12}O_4$	Pinocembrin
4	15.3	269.0820	$C_{16}H_{14}O_4$	Medicarpin
5	18.9	255.0665	$C_{15}H_{12}O_4$	Isoliquiritegenin
6	25.0	285.1134	$C_{17}H_{18}O_4$	Isosativan

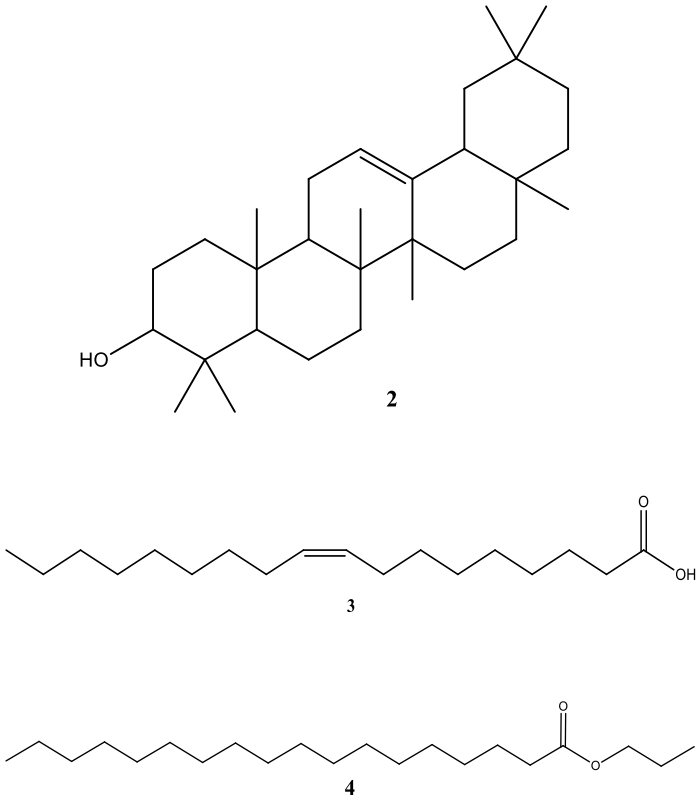

Figure 2: Compounds isolated from hexane and ethyl acetate extracts

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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