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Isolation and Structural Elucidation of 20 Hydroxyecdystone from *Vitex doniana* Sweet Stem bark (Black plum) Mustapha

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Abstract

Vitex doniana Sweet, a plant commonly known black plum, in English, Prunier noir in French, dinya in Hausa, ucha koro in Igbo, oori-nla in yoruba and ngarmi in Kanuri is a medium-sized deciduous tree, 8-18 m high, with a heavy rounded crown and a clear bole up to 5 m. V. *doniana* is from *Verbenaceae* family and abundantly occurring in savannah regions. It can be found throughout tropical Africa**.** The ethanolic extract of Vitex *doniana* stem bark (11.9 g) was subjected to a silica gel accelerated column chromatography and eluents fractions (150 ml aliquots) obtained were collected and monitored with thin layer chromatography (TLC). Fractions with similar R_f values from same solvents system were poled together. Phytochemical test of all the fractions were perform. Complete elution yielded 48 fractions (150 ml/fraction) which were pooled to 24 fractions and finally to eight (8) eight fractions and coded. Fraction Vd_{8-a} (56 mg) has gave a single spot a white crystal compound coded V_1 on checking with TLC and observed under Ultraviolet lamp. The R_f values was calculated to be 0.433 and melting point was found to be 241-243°C uncorrected. The infrared spectrum of compound V_1 shows prominent peaks that corresponds to OHstr (3365 cm^{-1}) and C=0 (1652 cm^{-1}). The ^1H NMR (400 MHZ) spectrum of compound V_1 in DMSO-*d*6 displayed five singlet signals. It further showed a broad singlet at *δ* 5.58 integrated for 1 H is due to an olefinic H-atom adjacent to the carbonyl carbon atom. Three signals at δ 3.10` (d, *J* = 9.0 Hz, H-22), 3.59 (m, 1H, 2H-a) and 3.72 (m, 1H, 3H-e) each integrating for one proton is due to an oxymethine protons indicating that three oxymethine H-atoms were present in the compound. The ^{13}C-NMR spectrum showed the presence of 27 Carbon atoms, suggesting that may be steroid skeleton and the DEPT-135 spectra showed the presence of five CH_3, eight CH_2, and seven CH groups, and seven quaternary C-atoms. The Molecular formula was established as $C_{27}H_{44}O_7$ by HRES-MS positive ion mode *m/z* 481.3179. Based on the spectral analysis, the compound V_1 is thus concluded to have ecdysteriod skeleton and conclusively conforms with 2β, 3β 14α, 20R, 22R, 25- hexahydroxy-5 β cholest-7-ene-6- one, commonly known as 20-hydroxyecdysone. This is the first time this compound was isolated from *Vitex doniana* sweet.

Keywords: Vitex; Phytochemical; Purification; Isolation; Chromatography; Spectroscopy

Introduction

Nearly all cultures of the world, both ancient and recent have heavily depended on plants as a therapeutic agent used in various forms. Plants play a major role in the treatment of diseases and still remain the foremost alternative for a large majority of people [1]. The knowledge of plants if wisely utilized could bring out promising herbal leads. The World Health Organization (WHO) has reported that about 80% of the world population relies on traditional medicine to cure ailments [2,3]. Plants are used directly as therapeutic agents, as well as starting material for the synthesis of drugs or as models for pharmacologically active compounds [4]. The study of natural products has had a number of rewards. It has led to the discovery of a variety of useful drugs for the treatment of diverse diseases and contributed to the development of spectroscopic methods of structure elucidation and synthetic methodologies that is now the basis of analytical organic chemistry. *Vitex doniana* is a medium-sized deciduous tree, 8-18m high, with a heavy rounded crown. The V. *doniana* bark is rough, pale brown or greyish-white, rather smooth with narrow vertical fissures. *Vitex doniana* Sweet commonly known as black plum, in English, Prunier noir in French, "dinya" in Hausa, "ucha koro "in Igbo, " oori-nla" in yoruba and "ngarmi" in Kanuri. *Vitex doniana* can be found in deciduous forest, coastal woodland, riverine and lowland extending as high as upland grassland, secondary and dry forests. It can be found throughout tropical Africa [1,4].

Experimentation

Sample collection and identification

The stem bark of *Vitex doniana* leaves were collected in Kawuri village of Konduga Local Government Area of Borno state of Nigeria. The plant specimen was identified and authenticated by a Plant Taxonomist, Prof. S.S. Sanusi of the Department of Biological Sciences, Faculty of Science, University of Maiduguri. The herbarium specimen with a voucher number 555 C was deposited at the Post Graduate Research Laboratory, Department of Chemistry. The stem bark of the *Vitex doniana* was cleaned and air-dried in the laboratory. Two kilogram (2 kg) of the stem bark of *Vitex doniana* was pulverised into a coarse powder using mortar and pestle.

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Extraction of stem bark of *V. doniana* and phytochemical analysis

The weighed powdered air-dried sample (2 kg) was macerated (cold extreaction) with 95% ethanol for five days filtered and evaporated in vacuo at 40°C using a rotary evaporator. The extract concentrate was labeled and the percentage yield were calculated in $^{w}/_{w}$. The ethanolic extract was subjected to qualitative chemical screening for identification of the primary and secondary metabolites such as flavonoids, alkaloids, sterols, triterpenes, saponins, anthracenosides, tannins, polyuronides, emodol, as described by Safowora and Evans [5-7].

Isolation of compound from ethanolic extract of *V. doniana* stem bark

The ethanol extract (11.9 g) was fractionated on a silica gel column chromatography using solvents such as n-hexane, ethyl acetate and methanol. Three hundred grams (300 g) silica gel 60-120 mesh was packed manually into a column (75 cm × 3 cm). The silica gel was first swell in n-hexane and filled into the column to a bedding height of 60 cm and then allowed to settle and packed. The ethanolic extract was then dissolved in 7 ml of n-hexane, mixed with silica gel subsequently applied on the top of the column. The ethanolic extract was gradiently and successively eluted with n-hexane, ethyl acetate and methanol 100% at a flow rate of 1ml/min. Each eluents fraction (250 ml aliquots) was collected and monitored with thin layer chromatography by making slurry of silica gel 60G merck, Germany on 20 cm × 20 cm and 7 cm × 5 cm precoated silica gel TLC plates. Fractions with similar R_f values from same solvents system were poled together. Phytochemical test of all the fractions were performed using standard procedure. The melting points of the isolated compounds were determined using Gallenkamp capillary melting point apparatus and the results were uncorrected.

Characterization of the isolated compound coded V_1

Compound V_1 was subjected to structural elucidation using infra-red spectroscopy, Nuclear Magnetic resonance (both ^{1}H and ^{13}C) and Mass spectroscopy. The Infra-red (IR) spectra of the isolated compound was recorded in a thin film in Nujol using Genesis series Fourier transform-infrared spectrophotometer (FT-IR), HNMR (one and two dimension spectra) was recorded using 400 MHz (Varian Instruments) DRX-500 spectrometer. ^{13}C NMR was recorded using variant instruments 100 MHz. High- resolution TOF- MS were measured on an Aglient series with and ESI (HRESI-MS).

Results and Discussion

Isolation of compound from *V. doniana* stem bark

Complete elution yielded 48 fractions (150 ml/fraction) which were pooled to 24 fractions base on the R_f values. It was further recombined and 12 fractions were obtained on the basis on R_f values and coded Vd_1 to Vd_{12} fractions. Vd_8 was further eluted with ethylacetate and methanol and gave fourteen 14 sub fractions $Vd_{8\text{-}a,}$ to $Vd_{8\text{-}m}$. Fraction $Vd_{8\text{-}a}$ (56 mg) has gave a white crystal compound coded V_1. It was further checked on TLC solvent system (Hexane, ethylacetate and methanol 7:3:1) and was found to give a single spot. The R_f values was calculated to be 0.433. The melting point was determined to be 241-243°C uncorrected.

Phytochemical analysis of fractions fractions Vd_1 to Vd_{12}

The result of the preliminary phytochemical analysis is shown here below in Tables 1-4. The result indicates that only fraction Vd_1 shows positive test of flavonoid. Carbohydrates is present in Vd_2 $Vd_{5,}$ and Vd_{10}.

Class of Chemical Components	Vd_1	Vd_2	Vd_3	Vd_4	Vd_5	Vd_6	Vd_7	Vd_8	Vd_9	Vd_{10}	Vd_{11}
Test of Alkaloids	-	-	-	-	-	-	-	-	-	-	-
Test for Flavonoid	+	-	-	-	-	-	-	-	-	-	-
Test for carbohydrates	-	-	+	-	-	+	-	-	-	+	+
Test for tannins	-	-	-	-	+	-	-	-	+	-	-
Test for free Anthraquinones	-	-	-	-	-	-	-	-	-	-	-
Test for Steroidal nucleus	-	+	-	-	-	-	-	+	-	-	-
Test for Terpenoids	-	+	-	-	+	-	-	-	-	-	-
Test for soluble starch	-	-	-	-	+	-	-	-	+	-	-
Test for saponins	-	-	-	-	-	-	-	-	-	-	+

Key: - Absent, + Present

Table 1: Phytochemical analysis of ethanolic extract fractions Vd_1 to Vd_{12}.

Functional group	Wave number cm^{-1}
CH_{3Vasy}	2962
Ohstr	3363
$C=C_{bend,str}$	1422
C=O	1652
$C\text{-}H_{bend}$	879.57

Table 2: Analysis of IR spectra of compound V_1.

Chemical Shifts multiplicities (ppm)	
Compound V_1 in DMSO-d6	20 Hydroxy ecdysterod
0.72 (3H, S H-18)	0.75 (3H, S H-18)
0.79 (3H, S, H-19)	0.79 (3H, S H-19)
1.01 (3H, S, H-21)	1.03 (3H, S H-21)
1.03 (3H, S, H-26)	1.04 (3H, S H-26)
1.18 (3H, S, H-27)	1.06 (3H, S H-27)
2.99 (1H, m, H-19)	2.99 (1H, m H-19)
3.1 (d, j=10.0 Hz, H-22)	3.1 (d, j=10.0 Hz H-22)
3.50 (m, 1H, 2H-a)	3.5 (m, 1H, 2H-a)
3.75 (m, 1H, 3H-e)	3.75 (m, 1H 3H-e)
5.6 (m, 1H, H-7)	5.6 (lH, m H-7)

Multiplicities of signals: s, singlet: d, doublet: m, multiplet.

Table 3: H^1 NMR Data for compound V_1 in DMSO-d_6 and compared with the Literature.

Tannins is found in fractions Vd_5 and Vd_9 while steroidal nucleus is present in $Vd_{2,}$ Vd_6 and Vd_8. Terpenes is found in fractions Vd_2 and Vd_5 while soluble starch and saponins can be found in fractions Vd_5 and Vd_8 as well as fractions Vd_1 and Vd_{12}. Alkaloids and anthraquinones were absent in all the fractions.

Discussion on the characterization of compound V_1

The Infra–red (IR) spectrum of compound V_1 shows prominent peaks at 3363 cm^{-1}, 2963 cm^{-1}, 1652 cm^{-1} and 1421 cm^{-1}. The peaks corresponds to OHstr (3365 cm^{-1}), $CH3_{Vas,}$ (2962 cm^{-1}), C=0 (1652 cm^{-1}) and $C=C_{bend,stre}$ (1421). This spectrum suggests that among the functional moiety in compound V_1 are the carbonyl and hydroxyl group. The UV spectra of compounds were consistent with the presence of an α, β- unsaturated ketone chromophore group.

The ^{1}H NMR (400 MHZ) spectrum of compound V_1 in DMSO-*d*6 displayed five singlet signals at δ 0.72 (3H, s, H-18), 0.79 (3H, s, H-19), 1.03 (3H, s, H-21), 1.04 (3H, s, H-26), 1.06 (3H, s, H-27) each integrating for three protons indicating the five methyl functional groups are

Carbon	Chemical Shift Multiplicities (δ)	
	Compound V_1	20 hydroxy ecdysteroid
C-1	36.6t	36.8t
C-2	66.8d	66.9d
C-3	66.7d	66.7d
C-4	31.7t	31.7t
C-5	50.5d	50.4d
C-6	202.8	202.9
C-7	120.5d	120.7d
C-8	165.3s	165.4s
C-9	33.2d	33.4d
C-10	37.7s	37.8s
C-11	20.3s	20.5s
C-12	31.5t	31.1t
C-13	46.9s	47.0s
C-14	83.0s	83.1s
C-15	30.0t	30.0t
C-16	20.3t	20.5t
C-17	50.1t	50.3t
C-18	17.2q	17.3q
C-19	23.9q	24.0q
C-20	75.7s	75.8s
C-21	21.0q	21.1q
C-22	69.0d	70.3d
C-23	26.1t	26.2t
C-24	41.5t	41.5t
C-25	68.8s	68.9s
C-26	29.1q	29.2q

q=CH_3; t=CH_2; d=CH; s=C

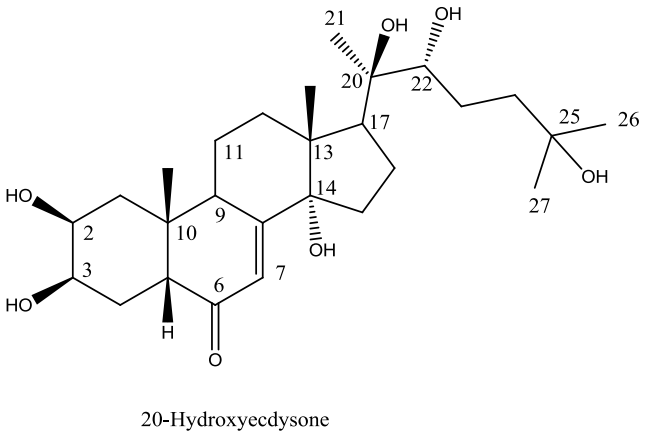
Table 4: ^{13}C NMR Data of compound V_1.

present in the compound. It further showed a multiplet at δ 5.58 integrated for 1 H is due to an olefinic H-atom adjacent to the carbonyl carbon atom. Three signals at δ 3.10` (d, *J*=9.0 Hz, H-22), 3.59 (m, 1H, 2H-a) and 3.72 (m, 1H, 3H-e) each integrating for one proton is due to an oxymethine protons indicating that three oxymethine H-atoms are present in the compound. These all signals are characteristic to the ecdysteroid skeletons.

The ^{13}C-NMR spectrum showed the presence of 27 Carbon atoms, suggesting that may be steroid skeleton. A signal at δ 202.77 (C-6) indicating that a carbonyl carbon atom is present in compound. Two signals at 165.32 (C-8) and 120.50 (C-7) due to an olefinic carbon atoms suggesting that double bond is present. Three signals at δ 83.03 (C-14), 75.74 (C-20), and 68.76 (C-25), is due to three quaternary oxygenated carbon atoms. Three signals at δ 76.23 (C-22), 66.82 (C-3), 66.63 (C-2) is suggesting the three oxymethine carbon atoms. Further, the ^{13}C-NMR spectrum showed signals characteristic of ecdysteroid skeleton.

The DEPT-135 experiment showed the presence of five CH_3, eight CH_2, and seven CH groups, and seven quaternary C-atoms.

The DEPT spectrum peaks for the five CH_3 at C-19 (24.1 ppm), C-21 (21.1 ppm), C-26 (29.2 ppm), C-27 (29.1 ppm) and C-18 (17.3 ppm). CH peaks are at C-2 (66.9 ppm), C-3 (66.7 ppm), C-5 (50.3 ppm), C-7 (120.7 ppm), C-9 (33.4 ppm), C-17 (50.3 ppm) and C-22 (76.3 ppm). The Spectrum further indicates that there are eight peaks at 36.8 ppm (C-1), 31.7 ppm (C-4), 20.6 ppm (C-19), 31.1 ppm (C-12), 30.5 ppm (C-15), 20.5 ppm (C-16), 26.5 ppm (C-23), and finally 41.5 ppm (C-24). The quaternary carbon atom peaks at 202 ppm (C-6) which is the carbonyl atom, 165.4 ppm (C-8), 37.8 ppm (C-10), 47.0 ppm (C-13), 83.1 ppm (C-H), 75.8 ppm (C-20) and 68.9 ppm (C-25).



20-Hydroxyecdysone

Figure 1: 2β 3β 14α, 20R, 22R, 25- hexahydroxy-cholest-7-ene-6- one (20 hydroxyecdystone).

The 2-D NMR clearly indicated the connectivities between the carbon atoms in the skeleton of the molecule and between the C-H bonds as well as the position of the unsaturation. The heteronuclear multiple bond quantum coherence (HMQC) spectra indicated that there is an interaction between the proton at 0.72 ppm (H-18) and that of C-18 at 17.3 ppm. There is connectivity for proton H-19 at 0.79 ppm and C-19 at 24.19 ppm. Connectivties at 1.06 ppm for H-27 and 29.2 ppm for C-27 ppm was also recorded. Proton H-22 at 3.16 ppm is connected to C-22 at 76.3 ppm while proton H-2 and H-3 at 3.50 ppm and 3.75 ppm is connected C-2 and C-3 at 66.9 ppm and 66.9 ppm.

The Molecular formula was established as $C_{27}H_{44}O_7$ by High resolution Electron spray ionization-Mass spectroscopy (HRESI-MS) positive ion mode *m/z* 481.3179 $[M+H]^+$ The signals in Mass spectrum 463, 445, and 427 peaks corresponding to losses of one, two, three, or four water molecules are characteristic for Ecdysterone skeleton reported in the literature. In the commonly used electron- impact (EI) mode, a mass spectrometer bombards molecules in the vapour phase with a high-energy electron beam and records the result of the electron impact as a spectrum of positive ions separated on the basis of mass/charge (m/z); most of the ions are singly charged.

Based on the spectral analysis (^{1}H NMR, ^{13}C NMR, DEPT, HMQC, IR, HRESI-MS) the compound V_1 is thus concluded to have ecdysteriod skeleton and conclusively conforms with 2β 3β 14α, 20R, 22R, 25-hexahydroxy-5β cholest-7-ene-6- one, or 2, 3,14, 20, 25 Hexa hydroxyl cholest-7-ene-6-one commonly known as 20-hydroxyecdysone as shown in Figure 1. The structure was earlier established by Zhu et al. and Bathori et al. in other plants [8,9]. However, this is the first time this structure or compound was established in *Vitex doniana* sweet. Over 250 ecdysteroid analogs have been identified so far in plants, and Dinan et al. [10] has indicated that there are over 1,000 possible structures which might occur in nature. 20-hydroxyecdysone, possessing the cholesterol nucleus, is present in *Ajuga turkestanica, Vitex glabrata), Tapinella panuoides* [10].

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