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Full Length Research Article

Isolation and Characterization of Stigmasterol and Bis-(5, 7-diacetyl-catechin-4'-α-rhamnopyranoside) from the Stem bark of *Neocarya macrophylla* (Sabine) Prance (Chrysobalanaceae)

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ABSTRACT: *Neocarya macrophylla* belongs to the Chrysobalanaceae family and is extensively used in folk medicine as an antibacterial, antivenin, antiasthmatic, anticancer, analgesic and anti-inflammatory agent. This study was aimed at isolation and characterization of compounds from the stem bark of *Neocarya macrophylla*. Pulverized plant material was exhaustively extracted with methanol using maceration method and concentrated *in-vacuo* with the aid of rotary evaporator at 40°C to afford a reddish brown crude methanol extract (ME). The methanol extract was successively partitioned into hexane, dichloromethane, ethylacetate, n-butanol and aqueous fractions. Stigmasterol was isolated from the hexane fraction and a catechin glycoside, Bis-(5,7-diacetyl-catechin-4'-α- rhamnopyranoside) was isolated from the ethylacetate soluble fraction using a combination of silica gel column, gel filtration (sephadex LH-20) and preparative thin layer chromatography. The structures of the compounds were established on the basis of chemical tests, spectroscopic analysis and by comparison with reference spectral data.
Key words: *Neocarya macrophylla,* phytochemical, stigmasterol, Bis-(5, 7-diacetyl-catechin-4'-α-rhamnopyranoside).

INTRODUCTION

Chrysobalanaceae is composed of 17 genera and about 525 species are found in tropical and subtropical regions (Yakandawala *et al*., 2010). *Neocarya macrophylla* (formerly *Parinari macrophylla*) known in Hausa language as Gawasa or Farar rura is widely distributed along coastal savannahs from Senegal to Liberia, woody savannahs of Southern Mali, Niger and Northern Nigeria (Burkill, 1985; Arbonnier, 2004). It is a shrub 6-10m high with short branches twisted with an open crown having densely pubescent and russet-brown stems. In Nigerian traditional medicine, the fruit is eaten fresh to treat diarrhoea and the leaves may be chewed or applied topically for the relief of pain (Tidjani *et al*., 2010). The kernels inside the seeds are usually roasted and enjoyed like cashews or almonds (NRC, 2008). Other traditional uses of the plant include; treatment of asthma, skin infections, cancer, pulmonary troubles, ear and eye infections (Warra *et al*., 2013). The stem bark is extensively used in ethno-medicine in Northern Nigeria to treat numerous diseases such as pain, inflammation and snakebites (Personal Communication).

Literature review of other members of the family revealed the occurrence of flavonoids and triterpenes in *Licania* genus (Castilho *et al*., 2005); Isocarthamidin and 4-hydroxybenzoic acid were isolated from the powdered stem of *P. anamense* (Werawattanachai and Kaewamatawong, 2010); diterpenes with molecular ent-kaurene derivative of 15-oxozoapatlin were isolated from *P. curatellifolia* and shown to possess cytotoxic activity (Lee *et al*., 1996; Garo *et al.,* 1997); six diterpenes kaurane derivatives were also isolated from *P. campestris* (Braca *et al*., 2005). Diterpenes found in *P. capensis* have antifungal activity (Garo *et al*., 1997) and antimalarial activity, but have high toxicity (Uys *et al*., 2002).

Neocarya macrophylla seeds are of high food value with about 40-60% oil and 21-25% Protein contents (Burkill, 1985; Amza *et al.,* 2010). The defatted seed meal contains 61% protein. In addition, the seeds are a good source of certain amino acids such as lysine, valine and phenylalanine (Amza *et al.,* 2010), which is important for balancing the deficiency of these essential amino acids in cereal-based diets. Research conducted on gingerbread plum fruit revealed its high nutritional values (Cook *et al.,* 2000; Audu *et al.,* 2005). The leaves have antihelminthic activities (Barnabas, *et al.,* 2010).

The decoction of the bark and leaves are used as mouth wash, internal troubles and for inflamed eye

(Fredrick, 1961). The fruits have antimicrobial activity against *Escherichia coli, Salmonella typhi, Candida albicans and Pseudomonas aeruginosa* (Audu *et al.,* 2005).

Inspite of its widespread use, there is no report yet on the isolation and characterization of any compound from the stem bark of the plant. We herein report the isolation and characterization of two compounds, stigmasterol and a catechin glycoside from the stem bark of *N. macrophylla*.

MATERIALS AND METHODS

Collection and Identification of Plant materials

The plant sample of *Neocarya macrophylla* was collected in November 2012 at Jega, Kebbi State, Nigeria. It was identified by U.S Gallah at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria by comparing with herbarium reference voucher specimen (No. 3197). The stem bark was shade dried, pulverized, labelled and stored at room temperature for use.

Preparation of the extract

The powdered stem bark (3000g) was extracted with methanol using maceration method. The extract was evaporated *in-vacuo* using rotary evaporator at 40^{o}C to yield a reddish-brown residue (396g) subsequently referred to as the crude methanol extract (ME). Two hundred gram of ME was suspended in distilled water, filtered and partitioned successively with n-hexane, dichloromethane, ethylacetate and n-butanol to obtain hexane fraction (HF, 2.1g), dichloromethane fraction (DF, 2.8g), ethylacetate fraction (EF, 13.0g), n-butanol fraction (BF, 32.0g) and the residual aqueous fraction (AF, 22g), respectively. The water insoluble portion of the extract was successively washed with n-hexane to afford (HF, 5.20g). The HF and EF portions were subjected to further phytochemical investigations.

Chromatographic Separation

The hexane fraction HF (5.20g) of the water insoluble residue was gradiently eluted in a silica gel packed column starting with n-hexane (100%), hexane: ethylacetate (9:1) to hexane: ethylacetate (1:1). Eluates were collected in 30ml portion. A total of 90 fractions were collected and combined based on their TLC profile to give (13) major fractions coded H1- H13. Fraction H7 (0.1g) was further subjected to gel filtration chromatography using sephadex (LH-20) eluting with acetone to give a white crystalline powder (9mg) coded A, which crystallizes on addition of methanol. TLC analysis of A using two solvent systems, viz; hexane: ethylacetate (5:1) and (9:1) and sprayed with 10% sulphuric acid revealed single homogenous spot. The compound (A) was further subjected to chemical test and spectroscopic analysis (1D and 2D-NMR) to elucidate its structure.

The ethylacetate portion, EF (13.0g) was subjected to column chromatography on a silica gel (60-120mesh) with gradient elution using hexane: ethylacetate (1:1), ethylacetate (100%) and ethylacetate: methanol (8:2). Thirty mililitres(30ml) each of a total of 426 fractions were collected and combined based on their TLC profile to give 12 major fractions EA-EL. Fraction EJ consisting of one major spot with little impurities was subjected to preparative thin-layer chromatography (PTLC) using pre-coated glass plate 20×20cm (0.25mm thick) and dichloromethane: methanol (4:1) as solvent system. The region containing band of interest was marked and scrapped off. The sorbent was size reduced using mortar and pestle, transferred to a sintered glass funnel and extracted with the mixture of dichloromethane and methanol; the solution obtained was evaporated to afford a reddish brown compound (7mg) coded B. The compound gave a single homogenous spot on TLC with dichloromethane: methanol (4:1) and ethylacetate: chloroform: methanol: water (15:8:2:0.5) solvent systems. Compound (B) was subjected to chemical test and spectroscopic analysis to elucidate its chemical structure.

RESULTS AND DISCUSSION

Compound A

Repeated gel filtration of the hexane fraction resulted in the isolation of a white-crystalline solid coded A(9mg). The uncorrected melting point of compound A was 135 – 136^{O}C and it showed positive test with Liebermann-Buchard reagent for steroidal nucleus (Silva *et al*., 1998). The IR spectrum of compound A (in KBr) exibited simple absorptions suggesting the purity of the compound. It showed absorption band at 3450 cm^{-1} characteristic of O-H stretching. (Jain and Bari, 2010). The bands at 2950cm^{-1}- 2925cm^{-1} and 1650cm^{-1}are due to aliphatic stretching and C=C olefinic stretching, respectively (Pateh *et al*., 2009). The absorption frequency at 1045cm^{-1} signifies cycloalkane.

These absorption frequencies resemble those of stigmasterol (Pateh *et al*., 2009; Jain and Bari, 2009; Arjun *et al*., 2010; Anjoo *et al*., 2011; Khanam and

Sultana, 2012).The ^{1}H-NMR spectrum of A (400Hz, $CDCl_3$) exhibited three olefinic resonances at δ5.35, δ5.10 and δ5.03, a proton on an oxygenated carbon at δ3.52 and a cluster of resonances up field between δ2.27 – δ0.69 suggesting steroidal nucleus (Agrawal *et al*., 1985; Pretsch *et al*., 2000).

The ^{13}C-NMR (125MHz) and the DEPT experiments showed the structure of the compound consisting of an unsaturated aliphatic molecule with 29 carbons, comprising 6 methyl (CH_3) groups, 9 methylene (CH_2), 11 methine (CH) and 3 quaternary (C) carbons further indicating the steroidal nature of the compound (Agrawal *et al*., 1985; Pateh *et al*., 2009). The downfield resonances at *δ* 140.76(C-5), *δ* 121.71(C-6), *δ* 138.31(C-22) and *δ* 128.28(C-23) indicate unsaturation; the signals at 21.08 and 12.05 correspond to angular carbon atom C-19 and C-18 respectively (Habib *et al*., 2007; Pateh *et al*., 2009). The resonance at *δ*71.81(C-3) is due to C-3 β-hydroxyl group further suggesting the compound as stigmasterol (Figure 1) rather than a β–sitosterol (Pretsch *et* al., 2000; Li *et al*., 2006; Pateh *et al*., 2009; Jain and Bari, 2010).

The ^{1}H-^{1}H COSY established the correlations between protons that are situated in the same environment; major correlations observed include: δ_H 5.35 (H-6) and δ_H 2.27 (H-4); δ_H5.10 (H-22) and δ_H 2.02 (H-20); δ_H 3.52 (H-3) and δ_H 1.85 (H-1); δ_H 3.52 (H-3) and δ_H 2.27 (H-4); δ_H and 5.03 (H-22) and δ_H 5.10 (H-23).

The HMBC spectrum allowed establishing the long range connectivity between the various units of the molecule. Some major correlations observed between protons and carbon include; proton at H-4(2.27) correlated with C2, C3, and C6; H6(5.35) showed correlation with C5 and C7; and H22(5.10) showed correlation with C20 and C24. The results of the 1D and 2D NMR experiments, as summarized on (Table 1) establish the structure of A as stigmasterol. Comparison with a reference NMR data (Pateh *et al*., 2009, Table 1) showed a good match and further confirmed the proposed structure.

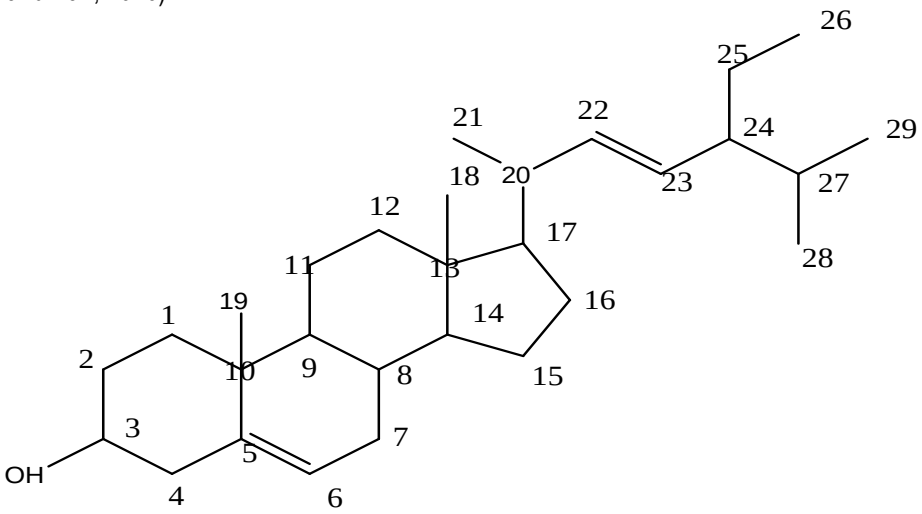

Figure 1: Stigmasterol: (Stigmast-5, 22-dien-3β-ol) $C_{29}H_{48}O$

Compound B

Compound B reacted positively for flavonoids (ferric chloride test) suggesting the presence of phenolic hydroxyl group. The presence of sugar moiety was discerned with Fehling's reagent. The ^{1}H-NMR and ^{13}C-NMR spectra of B exhibited chemical shift values typical of flavonoid (John *et al*., 2004; Wen-Lung *et al*., 2007; Hye *et al*., 2009; Jung *et al*., 2012). The presence of 1, 2, 4, 6- tetra-substituted benzene ring A was clearly discerned from the calculated ^{1}H-NMR J values via the protons at *δ*5.89 (1H, d, J = 2.1) and *δ*5.96 (1H, d, J = 2.1) representing H-6 and H-8, respectively. Similarly, an ABX system (1, 3, 4-trisubstituted benzene ring) was depicted by the protons at *δ*7.13 (1H, d, J = 8.28, H-5'), *δ*6.85 (1H, dd, J = 1.92, 8.40, H-6') and *δ*6.94 (1H, d, J = 1.92, H-2'). The proton chemical shift values at *δ*4.63 and *δ*3.97 corresponding to carbons at δ81.17 (C-2) and δ67.40 (C-3) represent protons on oxygenated carbons typical of a saturated ring C (Hye *et al*., 2009; Jung *et al*., 2012). Furthermore, the

chemical shift value of carbon C-4 (δ29.39) appeared unsubstituted and the presence of two hydrogens was confirmed through their chemical shift values as well as the J values δ2.88 (1H, dd, J= 5.5, 16.2 Hz) and δ2.55 (1H, dd, J=8.2, 16.2 Hz) characteristic of 3-flavan type flavonoids (Jennifer *et al*., 1999, Moharrama and Marzoukb, 2007). The higher chemical shift at H-2, H-3, H-4 and the corresponding J values exhibited by compound B suggest it to be a catechin rather than an epicatechin (Wen-Lung *et al*., 2007; Hye *et al*., 2009; Jung *et al*., 2012).

The ^{1}H-NMR and the ^{13}C-NMR spectra also indicated characteristic sugar absorptions around δ3.8 to 1.3 and δ73 to 69, respectively; the CH_3 absorption at δ16.6 suggests the sugar to be rhamnose (Surabhi and Bhadoria, 2005; Yaqing *et al.,* 2007; Jung *et al*., 2012). The 1D ^{13}C-NMR spectra indicated double chemical shift values at δ99.7 and 99.3, 94.9 and 94.1 signifying the structure is a dimer. The proton NMR showed the two meta-coupled protons at *δ*5.89 and *δ*5.96 integrated for one further suggesting B to be a dimer of catechin rhamnopyranoside. Comparison with several reported data, however, reveal the relatively low chemical shift value at C-3 indicating that the sugar was not attached at C-3 (John *et al.,* 2004; Surabhi and Bhadoria, 2005; Moharrama, and Marzoukb, 2007). The presence of an elevated (downfield) absorption at δ147.21 suggest the sugar is more likely to be attached to C-4' (Surabhi and Bhadoria, 2005; Jung *et al*., 2012). The ^{13}C-NMR chemical shift values at δ27.17 and δ22.68 presumably due to ester-methyl group absorption corresponding to ^{1}H-NMR around δ1.3 accounted for a total of 6 methyl groups, two of which are thought to attach to C-5 and C-7, and one methyl from the rhamnose part of each of the monomers. Based on the above, ^{1}HNMR, ^{13}CNMR and comparison with those in literature (Jung *et al*., 2012), a tentative structure of B, Bis-(5, 7-diacetyl-catechin-4'-α-rhamnopyranoside) has been proposed Figure 2.

CONCLUSION

Stigmasterol and Bis-(5, 7-diacetyl-catechin-4-α-rhamnopyranoside) were isolated from the stem-bark of *Neocarya macrophylla* and their chemical structures were elucidated. To the best of our knowledge, this is the first report of isolation of these compounds from the stem bark of *Neocarya macrophylla*.

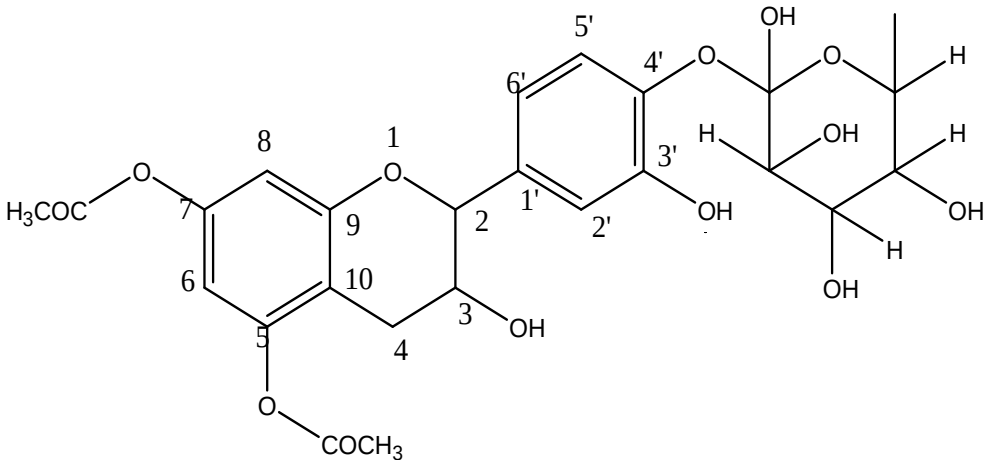

Figure 2: Bis-(5,7-diacetyl-catechin-4-α-rhamnopyranoside)

Table 1: ^{1}H &^{13}C-NMR data of A (Stigmasterol) compared with Pateh *et al*., (2009)

Position	^{13}C-NMR A	^{1}H-NMR A	^{13}C-NMR (Pateh *et al.,* 2009)	^{1}H-NMR
1	37.26	1.85	37.3	
2	31.67	1.46	31.6	
3	71.81	3.52 m	71.8	3.52 m
4	42.31	2.27	42.3	
5	140.76	–	140.8	
6	121.71	5.35 br s	121.7	5.35 br s
7	31.90	1.96	31.9	
8	31.90	1.48	31.9	
9	50.16	0.93	51.2	
10	36.51	-	36.5	
11	21.21	1.49	21.1	
12	39.68	1.16	39.7	
13	42.22	-	42.3	
14	56.87	1.05	56.9	
15	24.36	1.56	24.4	
16	28.92	1.70	28.4	
17	55.96	1.13	56.1	
18	12.05	0.69 s	11	0.69 s
19	21.08	1.03 s	21.2	1.01 s
20	40.49	2.02	40.5	
21	23.07	1.02	21.2	1.02(d, 7.5)
22	138.31	5.10	138.3	
23	129.28	5.03	129.3	
24	51.24	1.53	51.2	
25	29.15	1.65	31.9	
26	18.98	0.82	21.2	0.79 (d, 6.5)
27	19.40	0.78	19	0.84(d, 6.5)
28	25.40	1.15	25.5	
29	12.25	0.80	12.1	0.80(t, 7.5)

Measured in $CDCl_3$ (400Hz)

Table 2: ^{1}H and ^{13}C-NMR data of compound B compared with reported literature

Position	δ ^{13}C	δ ^{1}H (J in Hz)	δ ^{13}C (Jung *et al*., 2012)	δ ^{1}H (J in Hz)
1	-	-	-	-
2	81.17	4.63(d, 7.8)	83.1	4.62(d, 8.0)
3	67.40	3.97(m)	76.1	3.93(m)
4	29.29	2.88(dd, 5.5, 16.2) 2.55(dd, 8.2, 16.2)	28.1	2.88(dd, 5.5, 16.0) 2.64(dd, 8.5, 16.0)
5	-	-	157.1	-
6	99.74	5.89(d, 2.1)	96.5	5.94(d, 2.3)
7	-	-	157.0	-
8	94.9	5.96(d, 2.1)	95.6	5.86(d, 2.3)
9	-	-	158.1	-
10	-	-	100.8	-
1'	-	-	132.1	-
2'	114.68	6.94(d, 1.9)	115.2	6.84(d, 1.8)
3'	-	-	146.4	-
4'	147.21	-	146.5	-
5'	117.09	7.13(d, 8.3)	116.2	6.77(d, 8.0)
6'	118.45	6.85(dd, 1.9, 8.4)	120.0	6.72(dd, 1.8, 8.0)
Rhamnose				
1"	100.02	4.13(d, 1.4)	102.3	4.29(d, 1.4)
2"	70.56	3.95(d, 3.3)	72.1	3.51(dd, 1.8, 3.2)
3"	70.72	3.34(dd, 1.3, 2.7)	72.4	3.57(dd, 3.2, 9.6)
4"	72.50	3.82(dd, 6.2, 9.4)	74.1	3.31(m)
5"	69.32	4.00(m)	70.5	3.68(m)
6"	16.59	1.27(d, 6.4)	18.1	1.25(d, 6.2)

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