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ISOLATION AND CHARACTERIZATION OF CHEMICAL CONSTITUENTS FROM *Chrysophyllum albidum* G. DON-HOLL. STEM-BARK EXTRACTS AND THEIR ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES

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Abstract

Background: The plant, *Chrysophyllum albidum* is indigenous to Nigeria and its stem-bark has found relevance in folkloric medicine for infections and oxidative stress linked diseases medicaments. The study targets to isolate the chemical constituents accountable for the antioxidant and antibacterial actions of the plant stem-bark to substantiate some of its ethnomedicinal uses.
Materials and Methods: Stem-bark extract of *Chrysophyllum albidum* was obtained from 80 % ethanol was partitioned in sequence with ethyl acetate (EtOAc) and n-butanol. The solvent fractions and isolated compounds were verified for antioxidant chattels utilizing 2-2-diphenyl-1-picrylhydrazyl. Antibacterial actions were also assessed by agency of agar-diffusion and broth micro dilution methods. EtOAc fraction was on many occasions chromatographed on silica and Sephadex LH-20 column to afford four compounds and their chemical structures were proven with the employment of NMR (1D and 2D) and MS.
Results: Chromatographic fractionation of EtOAc fraction with the premier antioxidant and antimicrobial activities afforded stigmasterol (**1**), epicatechin (**2**), epigallocatechin (**3**) and procyanidin B5 (**4**). Procyanidin B5 isolated for the first time from Chrysophyllum genus proven the supreme antioxidant activity with IC_{50} values of 8.8 µM and 11.20 µM in DPPH and nitric oxide assays respectively and equally established the ultmost inhibitory activity against *Escherichia coli* (MIC 156.25 µg/mL), *Staphylococcus aureus* (MIC 156.25 µg/mL), *Pseudomonas aeruginosa* (MIC 625 µg/mL) and *Bacillus subtilis* (MIC 156.25 µg/mL).
Conclusion: The antibacterial and antioxidant activities of epicatechin, epigallocatechin and procyanidin B5 isolated from *Chrysophyllum albidum* stem-bark substantiate the folkloric uses.

Keywords: *Chrysophyllum albidum*, free-radical scavenging, antioxidant, antibacterial, isolation, characterization, procyanidin B5

Introduction

Chrysophyllum albidum G. Don-Holl. (Sapotaceae) is a tree with comestible fruits (Iwu, 1993). Diverse parts of the plant: fruits, leaves, root-bark and stem-bark are depleted for therapeutic purposes (Adewusi, 1997, Adewoye et al., 2010). The decoction of the leaves is employed in diarrhoea and stomach ache while the leaves are exhausted as emollient and for medication of skin eruptions (Idowu et. al., 2006, Adewoye et al., 2010). In the management of vaginal and dermatological infections, the cotyledons from the seeds are expended while pregnant women consume the fruit pulp to thwart nausea in Western Nigeria (Iwu, 1993; Onabanjo et al., 1979). The stem-bark is engaged in African trypanosomiasis, yellow fever and malaria alleviation (Postma et al., 1996, Iyamah et al., 2014). The methanol extract of the seed and root had been reported to spectacle antihistamine and anti-inflammatory activities (Onabanjo et al., 1979), while the exocarp fruit extract of *C. albidum* substantiated free radical scavenging activity (Orijajogun et. al., 2013). Studies carried out on the ethanol extract of the plant root by Onyeka et. al., 2012a, Onyeka et. al., 2012b displayed antioxidant and contraceptive activities.

The leaves extract had also been reported to spectacle antioxidant and antimicrobial activities (Adebayo et al., 2011, Ajetunmobi and Towolawi 2014) while the methanolic extract of the stem-bark upheld anti-plasmodial activity (Adewoye et al., 2010).

Myricetin rhamnoside, β-amyrin acetate and gentisic acid were chemical constituents earlier found in the leaves and stems of C. albidum (Iwu, 1993, Adebayo et al., 2011).

The seed cotyledons are characterized by the copious incidence of triacylglycerol, glycolipids and phospholipids and had also been certified to be opulent in oleic acid and linoleic acid (Essien et al., 1995). Nwadinigwe in 1988 also discovered a polymer, 1, 4- polyisoprene from the stem latex of C. albidum. Erstwhile phytochemical findings of the seed cotyledons in our laboratory described the isolation and characterization of three alkaloids: skatole (3-methylindole); tetrahydro-2-methylharman (1, 2, 3, 4-tetrahydro-1, 2 -dimethyl-β-carboline) and eleagnine (1, 2, 3, 4-tetrahydro-1-methyl-β-carboline). These alkaloids were found to retain antifungal and antibacterial activities safe skatole (Idowu et al. 2003). In our tireless quest for antioxidants and antimicrobial agents from natural sources (Adesina et al., 2000; Idowu et al., 2003; Adeloye et al., 2005; Soyinka et al., 2009; Idowu et al., 2010), we have surveyed the stem-bark of *C. albidum* with the aspiration of isolating and characterising the constituents responsible for the antioxidant and antibacterial principles from it. Hence, isolation of stigmasterol, epicatechin, epigallocatechin and procyanidin B5, all of which were foremostly isolated from the stem-bark of *C. albidum* is hereby reported in this study.

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Materials and methods

Instruments

Classical column chromatography (ccc) was executed using the Accelerated Gradient Chromatography (AGC), an orthodox medium pressure liquid chromatography which was adapted in 1995 by Dunstan. The AGC equipment was from Baeckstrom Separo AB, Lidingo, Sweden and used at Chemistry Department, University of Botswana, Gaborone, Bostwana and Pharmaceutical Chemistry Department, Obafemi Awolowo University, Ile-Ife, Nigeria. The ^{1}H-NMR spectra were documented at 300 MHz and ^{13}C-NMR at 75 MHz correspondingly, on Bruker Avance DPX 300 spectrometer, the ESI were done on Finnigan LQC Deca while the EIMS was executed on Finnigan MAT SSQ 7000 Single Quadruple and Optical rotation was carried out on AUTOPOL$^{(R)}$ IV optical rotation meter using acetone and methanol at 20 °C while UV spectra were measured in methanol, on a Shimadzu UV-2101PC spectrometer. Absorbance for antioxidant experiment was measured on spectrophotometer (Pharmacia Biotech Novaspec II) at the Biochemistry Department, Obafemi Awolowo University, Ile-Ife, Nigeria.

Reagents and solvents

Kieselgel 60 (ASTM 230– 400 mesh size, Merck), precoated silica gel GF_{254}, (Merck, Germany), Sephadex LH-20 (Amersham Pharmacia Biotech), anisaldehyde, vanillin, sulfuric acid, quercetin, sodium dodecyl sulphate, sodium nitroprusside, thiobarbituric acid, gallic acid and ascorbic acid. Solvents used for extraction and chromatography included ethanol, methanol, hexane, dichloromethane, chloroform, ethyl acetate n-butanol and were redistilled prior to usage.

Plant collection

The stem-bark of *C. albidum* was collected at Ede road, Ile-Ife in February 2009. A voucher herbarium specimen (FPH/S/001) identified by Mr. Oladele A. T. and preserved at the Herbarium of Pharmacognosy Department, Obafemi Awolowo University, Ile-Ife Nigeria.

Plant Processing and Isolation

The milled stem-bark (1.2 kg) was extracted with 5 litres of 80% ethanol in water three consecutive times at room temperature for 72 h and the combined extract was concentrated to dryness in vacuo on a rotavapor to get 132 g for the ethanol extract. 120 g of this was liquefy in distilled water and partitioned with ethyl acetate (3 x 500 millilitres) and n-butanol (3 x 500 millilitres) successively. These solvent fractions were processed to aridness using rotary evaporator under reduced pressure yielding the ethyl acetate fraction (26 g) and n-butanol fraction (31 g) in turn. A 20 g quantity of the ethyl acetate fraction was separated on silica gel column using a gradient of petroleum ether (PE), $CHCl_3$ and MeOH yielding six fractions **F1-F6**. **F2** (300 mg) was further fractionated on silica gel column using a gradient of PE and $CHCl_3$ ensuing in four fractions coded **F2a-F2d**. Subsequently, **F2d** (90 mg) was loaded on preparative TLC for purification using PE-chloroform (6:4) solvent system, which afforded **1** (31 mg). Fraction **F4** (1.3 g) was dissolved in a small amount of $CHCl_3$-MeOH (70:30) solvent mixture and loaded on a Sephadex LH-20 column hitherto equilibrated with $CHCl_3$-MeOH (70:30) solvent mixture and elution was effected isocratically to obtain **2** (280 mg). **F5** (2.2 g) was dissolved afterward in a minutest amount of $CHCl_3$-MeOH (70:30) solvent mixture and loaded on a Sephadex LH-20 column earlier equilibrated with $CHCl_3$-MeOH (70:30) and elution was carried out in an isocratic manner to yield six fractions, **F5a-F5f**. Fraction **F5e** (360 mg) was chromatographed on silica gel using a gradient of n-hexane, ethyl acetate and methanol leading to compound **3** (145 mg). **F6** was separated on a Sephadex LH-20 column using $CHCl_3$-MeOH (70:30) and this gave five fractions **F6a-F6e**. Subsequently, a recurrent purification of **F6e** (840 mg) on silica gel column using a gradient of PE, $CHCl_3$ and MeOH afforded **4** (71 mg).

Antioxidant activity assessment

Antioxidant activity has been determined by different methods, which relate to the mode of action of antioxidants. Essentially three methods have been employed in this study and these are the measurement of their radical scavenging ability, inhibition of lipid peroxidation and inhibition of nitric oxide radical when in the presence of these antioxidants.

Free radical scavenging by the use of DPPH

TLC autography assay was effected as follows. A minutest amount of the sample was reconstituted in a suitable organic solvent. The solution was spotted on silica gel sheet, allowed to dry and developed using apposite solvent system. The spots on dried plate was derivatized with 0.2% methanol solution of stable radical DPPH•. The derivatizing agent was employed to authenticate the presence of antioxidant spots (Burits and Bucar, 2000). Activity is indicated by yellow to whitish spot on a purple background. Quantitative free radical scavenging assays were implemented as follows: the radical scavenging ability of the isolated compounds, was fixed using the stable radical DPPH• in 1995 as explained by Brand-Williams et al 1995. The experiments on the samples were run three times and the percentage free radical inhibition was determined as expressed in the equation below.

$$\text{DPPH \% scavenging capacity} = \left(\frac{A_0 - A_S}{A_0}\right) \times 100$$

or

$$\left(1 - \frac{A_S}{A_0}\right) \times 100$$

A_0 indicates absorbance of the negative control (1.0 mL of DPPH solution + 1.0 mL of MeOHl)
A_s represents the absorbance of the positive control (1.0 mL of DPPH solution + 1.0 mL of sample solution)

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The 50% inhibition concentration (IC_{50}) was got from a linear regression graph of concentration of each of the test samples (µM) alongside the average percentage of the antioxidant activity (% inhibition) gotten from three replicate assays.

Inhibition of Lipid peroxidation

The lipid peroxidation inhibition potential of the isolated compounds was determined using a improved thiobarbituric acid reactive species (TBARS) assay of Ohkowa et al., (1979) as explained by Nabasree & Bratati, (2004). The percentage lipid peroxidation inhibitory activity was calculated as expressed above with DPPH radical scavenging.

Inhibition of Nitric oxide radical

The nitric oxide radical inhibiting activity of the extracts was carried out according to the method of Green et al., (1982) as depicted by Marcocci et al., (1994). Percentage inhibition nitric oxide radical formation was calculated as shown above with DPPH radical scavenging.

Determination of Antibacterial Activity
Agar-diffusion method

The antibacterial activities of the test samples (crude/fractions/pure compounds) realized from the stem-bark of C. albidum and the standard agent (Streptomycin) were determined using the agar diffusion method (Agyare et al., 2013).

Broth cultures (18 hr) of the test organisms; Staphylococcus aureus (NCTC 6571), Escherichia coli (ATCC 25922), Bacillus subtilis (NCTC 8263) and Pseudomonas aeruginosa (ATCC 10145) were used to seed molten nutrient agar and sanctioned to set. Thereafter, wells (8 mm diameter) were cut out using a sterile cork borer. The test solutions, 20 mg/mL (100 µL), Streptomycin, 1 mg/mL (100 µL) and the solvent, 50% methanol (100 µL), were introduced into each of the wells and allowed to diffuse for 1 hr before incubation at 37°C for 24 hr. The diameter of the zones of inhibition were measured to the nearest mm.

Determination of minimum inhibitory concentrations (MICs) using the broth microdilution method:

The MICs were determined utilizing the broth microdilution method as described by Amsterdam (1996). Mueller-Hinton broth (100 µL) was dispensed into the wells of a 96-well microtitre plate. 100 µL each of the test samples (20 mg/mL) and the standard agents, ciprofloxacin and streptomycin (1 mg/mL) were added to the first well in each row with adequate mixing. The test sample in each row was serially diluted two-fold to achieve concentration ranges of 10 – 0.0390625 mg/mL and 500 – 0.9765625 µg/mL for the standard agents. 5 µL of an overnight broth culture ($2x10^4$ cfu/mL) of the test bacteria was added to each well and the plate was incubated at 37°C for 24 hr. Each well was observed for the presence or absence of growth by addition of 20 µL of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) and incubation at 37°C for 30 min. The MIC is the lowermost concentration that inhibits the alteration in colour from yellow to blue-indicating prevention of growth. The above experiments were repeated three times.

Results

The exhaustive fractionation and decontamination of the ethyl acetate fraction from the stem-bark of *C. albidum* on varied chromatographic techniques led to the isolation of the two focal types of chemical constituents viz: a steroid and flavan-3-ol derivatives. The spectroscopic and physical data of the isolated compounds are as documented below while the structures are certified in Figure 1. Table 1 flaunts the antioxidant activities of the isolated compounds while Tables 2 and 3 depict the antibacterial activities of various fractions and the isolated compounds respectively. Table 4 shows the minimum inhibitory concentrations (MICs) of various fractions and two isolated compounds against selected typed bacterial strains using the broth microdilution method.

Table 1: The *in vitro* antioxidant activities of compounds 2, 3 and 4 isolated from *Chrysophyllum albidum* in different antiradical test systems

Tested compounds	DPPH radical scavenging	Lipid peroxidation Inhibition	Nitric oxide inhibition
	IC_{50} (µM)	IC_{50} (µM)	IC_{50} (µM)
1	ND	ND	ND
2	19.02±0.17	436.98±4.73	27.34±1.02
3	15.88±0.13	437.61±5.61	22.54±0.41
4	8.80±0.11	244.36±4.33	11.20±0.09
(Gallic acid)	12.82±0.09	ND	45.15±1.46
(Quercetin)	ND	201.29±3.58	ND

Values are expressed as mean ± SD of triplicate experiments.
IC_{50} values were calculated from regression equations of compound's concentrations against % inhibition of free radical formation/prevention of lipid peroxidation.
ND – not determined.

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Table 2: Antibacterial activity of the crude ethanol extract, ethyl acetate, n-butanolic, and the aqueous fractions obtained from the stem-back of *Chrysophyllum albidum*

Test Samples / Standard		Zones of Inhibition (mm)*			
	Conc. (mg/mL)	E. coli (ATCC 25922)	S. aureus (NCTC 6571)	Ps. aeruginosa (ATCC 10145)	B. subtilis (NCTC 8263)
Crude ethanolic extract	20	0	10	4.5	12
Ethyl acetate fraction	20	4	7.5	2	7.5
n-butanolic fraction	20	0	7	3	7
Aqueous Fraction	20	0	4.5	0	5.5
Streptomycin sulphate (Standard)	1	17.5	15	4	19
Solvent MeOH:H_2O (1:1)	0	0	0	0	0

MeOH: H_2O (1:1) - The zones of inhibition recorded are less the diameter of the cup, which is 8 mm.
* Represents the mean of three determinations.

Table 3: Antibacterial activity of the isolated compounds from *Chrysophyllum albidum* stem-bark.

Test Samples / Standard	Zones of Inhibition (mm)*				
	Conc. (mg/mL)	E. coli (ATCC 25922)	S. aureus (NCTC 6571)	Ps. aeruginosa (ATCC 10145)	B. subtilis (NCTC 8263)
1	20	0	0	0	0
2	20	5	0	0	5
3	20	0	0	0	0
4	20	0	4	0	9
Streptomycin sulphate (Standard)	1	17.5	18	3.5	18.5
Solvent MeOH:H_2O (1:1)		0	0	0	

MeOH: H_2O (1:1) - The zones of inhibition recorded are less the diameter of the cup, which is 8 mm.
* Represents the mean of three determinations.

Table 4: Minimum Inhibitory Concentrations (MICs) of various fractions and isolated compounds from *Chrysophyllum albidum* stem-bark.

Test Samples / Standard	Minimum Inhibitory Concentrations (MICs) in µg/mL against			
	E. coli **(ATCC 25922)**	***S. aureus*** **(NCTC 6571)**	***P. aeruginosa*** **(ATCC 10145)**	***B. subtilis*** **(NCTC 8263)**
Crude ethanolic extract	625	625	1250	625
Ethyl acetate fraction	156	312	625	156
n-butanolic fraction	312	312	625	312
Aqueous Fraction	1250	1250	1250	1250
Epicatechin **(2)**	625	312	1250	625
Procyanidin B5 **(4)**	156	156	625	156
Ciprofloxacin	3.91	< 0.977	< 0.977	< 0.977
Streptomycin	7.81	< 195	500	15.6

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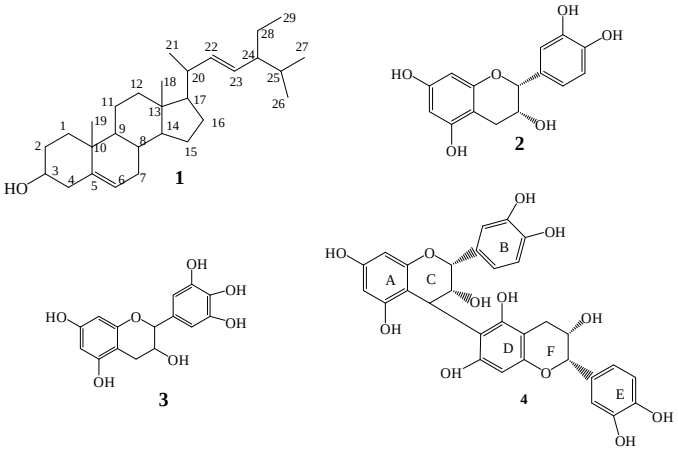

Figure 1: Structures of compounds 1-4 isolated from *Chrysophyllum albidum* stem-bark

Characterization of stigmasterol (1)
White crystal, ESI-MS m/z (rel. int. %): $[M + H]^+$ 413 (10), 395 (12), 369 (6), 327 (7) , IR V_{max} KBr cm^{-1}:3416.93, 2.956.78, 1634.83, 1458.19, 1375.93, 1043.12; UV λ_{max} MeOH nm 200.80, 209.20; 221.80; ^{1}H- and ^{13}C-NMR ($CDCl_3$):
^{1}Hnmr (300MHz, $CDCl_3$) δ ppm: 1.8 (2H, m), 3.60 (1H, m), 1.70 (1H, m, H-4a), 1.32 (1H, m, H-4b), 5.17 (1H, t, H-6), 1.29 (1H, H-17), 0.55 (3H, s, 18-CH_3), 0.81 (3H, s, 19-CH_3), 1.03 (3H, d, J= 6.64, 21-CH_3), 5.20 (1H, d, J=15.14, H-22), 5.06 (1H, dd, J=15.16, H=23), 0.86 (3H, 26-CH_3), 0.83 (3H, 27-CH_3), 0.79 (3H, 29-CH_3).
^{13}Cnmr (75 MHz, $CDCl_3$) δ ppm: 30.05 (C-1), 31.90 (C-2), 71.45 (C-3), 38.42 (C-4), 139.96 (C-5), 117.86 (C-6), 37.56 (C-7), 41.20 (C-8), 49.88 (C-9), 34.63 (C-10), 21.96 (C-11), 39.88 (C-12), 43.70 (C-13), 56.33 (C-14), 23.41 (C-15), 28.88 (C-16), 55.54 (C-17), 12.62 (C-18), 13.42 (C-19), 40.68 (C-20), 21.46 (C-21), 138.55 (C-22), 129.88 (C-23), 51.65 (C-24), 32.27 (C-25), 21.76 (C-26), 19.37 (C-27), 25.78 (C-28), 12.44 (C29).

Characterization of epicatechin (2)
Brown amorphous powder, $[\alpha]_D^{20}$ -70^0 (Me_2CO, c=1.00); ESI-MS $[M-H]^-$ 289.1, IR V_{max} KBr cm^{-1}: 3454.93, 2927.45.91, 1620.74, 1522.08, 1468.43, 1386.61, 1259.37, 1144.73; ^{1}H- and ^{13}C-NMR (CD3OD).
^{1}Hnmr (300MHz, CD_3OD) δ ppm: 4.73 (1H, bs, H-2), 4.10 (1H, bs, H-3), δ 2.69 (1H, dd, J= 16.83, 2.57 Hz, H-4a), 2.82 (1H, dd, J= 16.78, 4.35 Hz, H-4b), 5.88 (1H, d, J=2.26 Hz, H-6), 5.85 (1H, d, J= 2.24 Hz, H-8), 6.91 (1H, d, J= 1.46 Hz, H- 2′), 6.72 (1H, d, J= 8.14 Hz, H- 5′), 6.75 (1H, dd, J= 8.27, 1.72 Hz, H-6′)
^{13}Cnmr (75MHz, CD_3OD) δ ppm: 78.87 (C-2), 66.49 (C-3), 28.26 (C-4), 156.65 (C-5), 95.44 (C-6), 156.99 (C-7), 94.93 (C-8), 156.38 (C-9), 99.13 (C-10), 131.30 (C-1′), 114.34 (C-2′), 144.77 (C-3′), 144.93 (C-4′), 114.95 (C-5′), 118.45 (C-6′)

4.76 (1H,
Characterization of epigallocatechin (3)
Brown amorphous powder, $[\alpha_D^{20}$ 110^0 (Me_2CO, c=1.00); EIMS m/z (rel. int. %) 306.07(22), 168 .04 (19), 139.04 (100), 126.03 (11); IR V_{max} KBr cm^{-1}: 3345, 2923.45.9, 1614.3, 1519.8, 1463.9, 1353.9, 1282.6, 1195.8; ^{1}H- and 13C-NMR (CD3OD): ^{1}Hnmr (600MHz, CD_3OD) δ ppm: 4.76 (1H, bs, H-2), 4.18 (1H, d, J=3.0 Hz, H-3), 2.86 (1H, dd, J= 4.8, 16.8, Hz, H-4a), 2.74 (1H, dd, J= 3.0, 16.8, Hz, H-4b), 5.96 (1H, d, J=2.4 Hz, H-6), 5.93 (1H, d, J= 2.4 Hz, H-8), 6.53 (2H, s, H- 2′ / H-6′). ^{13}Cnmr (150MHz, CD_3OD) □ ppm: 78.44 (C-2), 66.09 (C-3), 27.76 (C-4), 156.65 (C-5), 94.98 (C-6), 156.24 (C-7), 94.50 (C-8), 155.91 (C-9), 98.73 (C-10), 130.14 (C-1′), 105.59 (C-2′), 145.28 (C-3′), 132.20 (C-4′), 145.28 (C-5′), 105.59 (C-6′)

Characterization of procyanidin B5 (**4**)
Brown amorphous powder, $[\alpha_D^{20}$ $+15^0$ (Me_2CO, c=1.00); ESI-MS m/z (rel. int. %) $[M-H]^-$ 577.2 (39), 451.2 $[M+H-126]^+$ (3), 425.2 $[M+H-152]^+$ (12), 407.3 $[M+H-126-152-18]^+$ (7), 289.2 (11); IR V_{max} KBr cm^{-1}: UV λ_{max} Me_2CO nm 210.00, 323.50; 325.50; ^{1}H- and ^{13}C-NMR (CD_3OD).
^{1}Hnmr (300MHz, CD_3OD) δ ppm: 5.08 [1H, bs, H-2 (C)], 3.94 [1H, bs, H-3, (C)], 4.60 [1H, bs, H-4, (C)], 6.03 [1H, d, J=2.19, H-6 (A)], 6.09 [1H, d, J=2.18, H-8 (A)], 7.01 [1H, d, J= 1.63 Hz, H- 2′ (B)], 6.75 [1H, d, J= 6.91 Hz, H- 5′ (B)], 6.73 [1H, bs, H-6′ (B)], 4.83 [1H, bs, H-2 (F)], 4.29 [1H, bs, H-3, (F)], 2.84 [1H, m, H-4 (F)], 5.75 [1H, d, J=2.32, H-8 (D)], 6.93 [1H, d, J= 1.58 Hz, H- 2′ (E)], 6.75 [1H, d, J= 6.91 Hz, H- 5′ (E)], 6.76 [1H, d, J= 6.91, 1.11 Hz, H-6′ (E)].
^{13}Cnmr (75MHz, CD_3OD) δ ppm: 78.78 [C-2 (C)], 72.50 [C-3 (C)], 36.16 [C-4 (C)], 154.47 [C-5 (A)], 95.82 [C-6, (A)], 157.03 [C-7 (A)], 95.19 [C-8 (A)], 155.44 [C-9 (A)], 99.72 [C-10 (A)], 131.11 [C-1′ (B)], 114.31 [C-2′ (B)], 144.62 [C-3′ (B)], 144.62 [C-4′ (B)], 115.02 [C-5′ (B)], 118.48 [C-6′ (B)], 76.11 [C-2 (F)], 66.02 [C-3 (F)], 28.71 [C-4 (F)], 154.47 [C-5 (D)], 106.07 [C-6, (D)], 157.03 [C-7 (D)], 95.50 [C-8 (D)], 154.86 [C-9 (D)], 99.60 [C-10 (D)], 131.35 [C-1′ (E)], 114.31 [C-2′ (E)], 144.94 [C-3′ (E)], 144.87 [C-4′ (E)], 115.02 [C-5′ (E)], 118.25 [C-6′ (E)]

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Discussion

Recurrent chromatography of the ethyl acetate portion from the stem-bark of C. albidum on silica gel and Sephadex LH-20 produced compounds 1–4 (Figure 1).

Compounds **1** was identified as stigmasterol by contrast of the recorded spectral characteristics with those stated for the same compound in the collected works (Greca et al., 1990; Holland et al., 1978; Wright et al., 1978). Compound **2** was obtained as amorphous brown, and was distinguished as epicatechin by judgment of its spectral characteristics with literature (Porter et al, 1991 and Agrawal, 1989). Compound **3** gave a molecular ion peak at m/z at 306.07 in EIMS which is compatible with the molecular formula $C_{15}H_{14}O_7$. Other fragment ions were located at m/z 168.04, 139.04 and 126.03 correspondingly. The fragment ion [M-H - 126] at m/z 179.0 corresponded to the removal of a molecule, phloroglucinol from epigallocatechin. The benzofuran ring forming fragmentation (BFF) produced the fragment ion at 168.04 while the ion at m/z 139.4 resulted from a retro-Diels–Alder (RDA) fragmentation. ^{1}H- and ^{13}C-NMR experiments, uniquely HMQC and HMBC, inveterate proton and carbon connectivity of **3** and the DEPT spectrum asserted its carbon multiplicities, which are analogous to the physical and spectra data reported in the literature for epigallocatechin (Agrawal, 1989; Luo et al., 2002; Cândida da Silva et al., 2006). Compound **4** was determined as $C_{30}H_{26}O_{12}$ by ESI-MS. The ESI-MS (negative-ion mode) of **4** displayed a pseudomolecular ion peak $[M-H]^-$ at m/z 577 and was assumed to be a dimeric proanthocyanidin. The presence of the AB coupling system due to H-2 and H-3 at δ 3.94 and 4.60 of the first (upper) monomer unit, the meta-coupled doublets δ 6.03 and 6.09 (each, d, J=2.19 Hz and 2.18 Hz) in turn, one aromatic singlet at δ 5.98 (ring D), and the presence of two ABX systems in the aromatic region (δ 6.73 - 7.01) due to rings B and E long-established the B-type proanthocyanidin dimeric structure. The ^{13}C-NMR spectrum indicated the presence of two flavan-3-ol units from the signals at δ 72.53 and 66.02 attributable to C-3 in the heterocyclic rings C and F and two carbon signals at δ 76.11 and 78.78 due to the C-2 in the heterocyclic rings C and F respectively. The upper and lower units were determined to be epicatechin from the C-2 and C-3 carbon signals of each unit.

The ^{13}Cnmr and DEPT NMR spectra flashed 30 carbon signals consisting of CH_2 (1), non-aromatic CH (5), aromatic CH (9), and quaternary carbons (15). In the HMBC spectrum, particularly diagnostic was the correspondence from the proton signal at δ 4.60 attributed to H-4, C-ring of upper monomer which showed cross peaks with δ 106.07, 154.47, 157.03 (C-6, C-5, C-7) of A-ring of lower monomer correspondingly. The uniqueness of this procyanidin as epicatechin- (4β→6) epicatechin (Procyanidin B5) was confirmed by contrast of its physical and spectra data with those described in the literature, remarkably the C-2, C-3, C-4, C-6 and C-8 ^{13}Cnmr signals (Agrawal et al., 1989; Porter et al., 1982) which is isolated for the first time from genus Chrysophyllum.

Antioxidant activities

The antioxidant activities of compounds **1–4** were evaluated using the DPPH, inhibition of lipid peroxidase and nitric oxide radical scavenging assays as IC_{50} values. Qualitative determination on TLC disclosed that all the compounds demonstrated varying antioxidant activity as they briskly bleached the purple colour background of DPPH on TLC within minutes except for stigmasterol (**1**), which had no antioxidant activity. Quantitatively, the effects for the three antioxidant test systems for compounds **2, 3** and **4** are reported in Table 1. In all the three in vitro corresponding assays, the isolated compounds expressed their antioxidant effects in a concentration-dependent manner and the antioxidant activities in DPPH parallel those of lipid peroxidation inhibitory and nitric oxide radical scavenging assay. The utmost free radical scavenging activity was shown by procyanidin B5 (**4**) in DPPH and nitric oxide test systems with IC_{50} of 8.80 µM and 11.20 µM when compared with corresponding IC_{50} values of 12.82 µM and 45.15 µM for gallic acid that was used as standard. The free radical scavenging activity of the isolated compounds decreased in the following order: procyanidin B5 (**4**) with IC_{50} of 8.80 µM, 244.36 µM and 11.20 µM > epigallocatechin (**3**) with IC_{50} of 15.88 µM, 437.61 µM and 22.54 µM > epicatechin with IC_{50} of 19.02 µM, 236.98 µM and 27.34 µM in DPPH, lipid peroxidation, nitric oxide test systems respectively except in lipid peroxidation in which **2** is marginally higher than **3.**

Structural activity relationship study in printed literatures have confirmed that apart from 3–OH of ring-C and B-ring catechol groups in flavan–3-ol class of compounds, 4 → 8 and 4 → 6 linkages in their dimers, trimers and polymers had been corroborated to increase the stability of its radical and thereby endows a polymer with significant free radical scavenging properties (Morimoto et al., 1986, Castillo et al., 2000, Heim et al., 2002, Muselik et al., 2007) This clarifies why **4** demonstrated superior antioxidant property compared to **3**. Compound **3** in DPPH and nitric oxide assays likewise established stronger antioxidant activity compare to **2**. This observation is not astounding as 3′, 4′, 5′-triOH hydroxyl substitution pattern of ring–B (galloyl group) in a molecule has been reportedly certified to further boost free radical scavenging property of such molecule (Rice-Evans et al., 1996, Wang et al., 2007, Seyoum et al., 2006). This is a characteristic that is nonexistence in compound **2**.

Conversely, the reduced inhibitory effects of epigallocatechin (**3)** on lipid peroxidation which was somewhat different (unusually low and marginally lower than that of epicatechin), in contrast to what was observed in DPPH radical scavenging activity where the antiradical activity was significantly high almost comparable with the standard gallic acid used had been attributed to the presence of galloylation which had been reported in literatures to reduce the ability of a molecule to prevent peroxidation (Plumb et al., 1998, Muselik et al., 2007).

Antibacterial Activity

The consequence of the antibacterial activity tests of the raw extract, ethyl acetate, n-butyl ethanol and aqueous proportions at 20 mg/mL are as recapitulated in Table 2. Solvent partitioning of the crude extract revealed that the n-butanol and ethyl acetate fractions possessed almost equal antibacterial activity while the residues in the mother liquors flaunt little or no activity against test bacteria. Nonetheless, the ethyl acetate segment was active against all the test organisms with Gram-positive bacteria being more susceptible, while crude ethanolic extract, butan-1-ol and aqueous fractions had no activity against *E. coli*.

All the isolated compounds, stigmasterol (**1**), epicatechin (**2**), epigallocatechin (**3**) and procyanidin B5 (**4**) were equally subjected to antibacterial examination at 20 mg/mL as recorded in Table 3. However only two of them epicatechin (**2**) and

procyanidin B5 (**4**) demonstrated inhibitory activity. While epicatechin (**2**) was active against *E. coli* and *B. subtilis*, procyanidin B5 (**4**) was active against *B. subtilis* and *S. aureus* as revealed in Table 3. The lack of activity in epigallocatechin (**3**) is in line with published literature that galloyed molecules lack antibacterial activity except when alkylated with long chain aliphatic group such C8 (octanyl) and C10 (decanyl) (Stapleton et al., 2004, Tsuchiya et al., 1996).

The ethyl acetate fraction confirmed a broad spectrum of activities against the selected bacteria and the most promising with an MIC of 156 µg/mL against *E. coli* and *B. subtilis*. The MIC of the aqueous fraction was the same (1250 µg/mL) for all the test organisms while the MICs of all the fractions and isolated compounds against *P. aeruginosa* were the highest (1250 µg/mL). However, the procyanidin B5 isolated from the plant for the first time proven the highest inhibitory activity against E. coli (MIC 156.25 µg/mL), *S. aureus* (MIC 156.25 µg/mL), *P. aeruginosa* (MIC 625 µg/mL) and *B. subtilis* (MIC 156.25 µg/mL) when compared with crude and other fractions as shown in Table 4. Procyanidin B5 demonstrated activity which was 4 times better against the strains of bacteria tested when compared with the crude. Though, these activities were much lower compared to that observed for the standard, however the moderately good MIC values ranging from 156 to 625 µg/mL put on view by procyanidin B5 against the tested bacterial strains were noteworthy.

Conclusions

Three of the isolated compounds from *C. albidum* stem-bark, procyanidin B5 (**4**), epigallocatechin (**3**) and epicatechin (**2**) sustained moderate antibacterial and strong free radical scavenging activities, thus justifying some of its ethnomedicinal uses.

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Declaration of interest

The authors assert that no opposing interest exists. The authors alone are answerable for the content and writing of the paper.

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