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IRVINGIA GABONENSIS SEEDS EXTRACT FRACTIONATION, ITS ANTIOXIDANT ANALYSES AND EFFECTS ON RED BLOOD CELL MEMBRANE STABILITY

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Abstract

Isolating food constituents from *Irvingia gabonensis* seeds having anti-oxidant activity, showed the presence of tannins 1.37± 0.04 mg/g, saponins 0.09 ±0.08 mg/g, terpenoids 0.21±0.02 mg/g, flavonoids 0.18± 0.02 mg/g, phenols 0.12±0.01 mg/g, alkaloids 1.91±0.05 mg/g, sterols 0.14 ±0.01 mg/g, glycosides 0.22 ± 0.02 mg/g and anthraquinone 1.22± 0.01 mg/g. Methyl 2- [2-formyl-5-(hydroxymethyl)-1 H-pyrrol-1yl]-propanoate (1), kaempferol-3-o-β-D-6" (p-coumaroyl) glucopyranoside (2) and based on its structure, lupeol (3β-lup-20(29)-en-3-ol (3) were isolated after fractionation. Lupeol was most abundant and had better antioxidant activities than ascorbic acid at high concentrations. Kaempferol-3-o-β-D-6" (p-coumaroyl) glucopyranoside and Methyl 2- [2-formyl-5-(hydroxymethyl)-1 H-pyrrol-1yl]-propanoate also showed significant antioxidant activities. The erythrocyte osmotic fragility test showed the ability of the 3 compounds to protect membranes against lysis due to hypertonic environments. Therefore, *Irvingia gabonenis* seed has the potential for being considered a raw material for production of nutraceuticals or nutritional supplement.

Keywords: *Nuts, Kaempferol-3-o-β-D-6" (p-coumaroyl) glucopyranoside, Methyl 2- [2-formyl-5-(hydroxymethyl)-1-H-pyrrol-1yl]-propanoat,; Lupeol (3β-lup-20(29)-en-3-ol, antioxidants, osmotic fragility*

Introduction

Edible foods from the forest are a major source of food nutrients, providing energy for millions of peoples in the rain forest region of West Africa. In Nigeria, the economic growth attributable to the forest foods is estimated to range between 2 and 7 % per annum [1]. Seeds are a prominent feature in the peasants dietary especially in the developing countries and oilseeds are becoming valuable sources of nutrients for man, especially in countries where the diet is plant-based. *Irvingia gabonensis* is one of such seeds. *Irvingia gabonensis* (Irvingiaceae) fruit, also known as African mango or bush mango has fleshy mesocap and a nut. Use of the mesocarp in traditional medicine has been documented for the treatment of gastrointestinal or hepatic disorders, diarrhea, infections, and as a purgative [2, 3]. On the other hand, the nut has a seed of two cotyledons referred to as Dikanuts, Ogbono in Igbo, Apon in Yoruba and kuwing in Agoi, Nigeria. It is used as a soup and stew thickener, flavouring agent when roasted and as a spread on yam/plantain dishes when ground and fermented [4].

Recently, *Irvingia gabonensis* seeds have become available on the U.S. market as a dietary supplement and reports have shown they have high *in vitro* antioxidant capacity [5], significant effects on blood lipid decreases [6] and a lowering of plasma glucose [6] in experimental animal or human subject studies. Phytochemical investigations of the stem bark of *I. gabonensis* led to the isolation of antioxidant and hepatoprotective triterpenes and phenols [2] . Nevertheless, very limited studies on isolation of pure fractions of the seed constituents and characterization of its bioactive pure compounds have been reported. In 2011, Atawodi and co-workers published the first report on the phenolic compound profile of *I. gabonensis* seeds, in which methyl gallate, ellagic acid, and other ellagic acid derivatives were detected by LC-UV-MS in the methanol extract of the seeds [7].

This study was to isolate, characterize pure compounds of aqueous fractions of *irvingia gabonnensis* seeds and investigate their possible bioactive activities.

Methods

Plant Materials

Sun-dried seeds of *Irvingia garbonensis* were purchased from Marian Market, Calabar Municipality, Cross River state, Nigeria in June, 2017. A sample was deposited with code UCBCM/EL/119 in the herbarium of Department of Biochemistry, University of Calabar, Calabar, Nigeria. All solvents used for chromatographic separations were purchased from Zayo-Aldrich (Jos, Nigeria)

Extraction

The sun-dried seeds of *Irvingia gabonensis* (2 kg) were pulverized and extracted by cold maceration with 80 % ethanol: distilled water (3 × 2.5 L) at room temperature, for 72 h with intermittent shaking.

This was filtered and the filtrate was concentrated to dryness using a rotary evaporator (Büchi, Switzerland) at 40 °C. The obtained brown concentrate (350 g) was suspended in distilled water (1.5 L) and n-hexane (1.5 x 2.5 L) to yield 55.3 g (n-hexane extract) and 165 g of the aqueous extract. The aqueous extract was used for phytochemical analysis.

Phytochemical analysis

Qualitative analysis

The phytochemical composition of the aqueous extract was evaluated [8]. The UV spectra were compared and the phytochemical contents of the extract were determined.

Quantitative analysis

Total flavonoids were estimated using the method of Ordoñez [9].

Total phenol contents in the extracts were determined by the modified Folin-Ciocalteu method [10]. Total tannins were estimated according to the methods described by Van-Burden and Robinson [11]. Alkaloid, Anthraquinone and steroids contents **d**eterminations were by method described by Harborne [12]. Tannins were estimated according to the methods described by Van-Burden and Robinson [11]. Saponin determination was by the method by Gestetner et al. [13]. Determination of gylcosides was by method of El-Olemy et al [14] while total terpenoid content was by method of Ferguson [15].

Isolation of pure compounds

The final water-soluble extract was subjected to chromatography over a silica gel column, eluted with CHCl3/EtOH (50:1 to pure EtOH, stepwise).

Fractions Fii (ca. 1.8 g) was subjected to Sephadex LH-20 column chromatography, eluted by MeOH/H2O (50:50, 75:25, and pure MeOH) to remove pigments and afforded several subfractions. Subfraction Fiioi (ca. 100 mg) was purified further by HPLC on the same XBridge PrepC18 column by a gradient elution (A, CH3CN; B, H2O, 3–15% A over 70 min, 15–100 % A from 70 to 85 min; 8 mL/min), to give the purification of **Compound 1** (tR 49.5 min; 3.6 mg), a brownish gel-like solid [16].

The polar fraction (aqueous-ethanol extract) was further fractionated using preparative TLC method. The slurry for the preparative TLC was prepared by uniformly dissolving silica gel 60 GF_{254} (Merck, Darmstadt, Germany) in distilled water. This was poured on 20 x 20 cm glass plates using a spreader and allowed to air-dry. The plates were activated in the oven at 110 °C before streaking. The solvent system was chloroform: ethyl acetate: methanol (10:4:1) and chloroform: methanol (7:1). The fraction bands were identified under UV lamp and later sprayed at the edge with concentrated H_2SO_4-vanillin reagent. The isolated bands (R_f = 0.438) were scrapped off the plate, eluted in absolute methanol, filtered and concentrated to dryness [7]. This was done on about 20 prepared plates to obtain a substantial quantity. This fraction was purified further by HPLC on the XBridge PrepC18 column, by a gradient elution (A, CH3CN; B, H2O, 3–15% A over 70 min, 15–100% A from 70 to 85 min; 8 mL/min) to give **Compound 2,** a bark brown powder

Fraction of the ethanol extract was subjected to another series of pilot thin layer chromatographic (TLC) separations using different proportions (0:10 to 10:0) of various solvents of petroleum ether, carbon tetrachloride, toluene, diethyl ether, dichloromethane, *n*-butanol, chloroform, ethyl acetate, acetone and methanol as the mobile phase. Upon observing the results of TLC, 15 g dried chloroform fraction of the petroleum ether extract was subjected to column chromatography and loaded on a glass column (60 cm) packed with silica gel G (40 g, 60–120#, Spectrochem Pvt. Ltd.) as the stationary phase. Gradient elution was performed using toluene:methanol (10:0, 9.5:0.5, 9:1 up to 0:10) as the mobile phase. A total of 200 aliquots were collected in test tubes. Upon evaporation of the mobile phase from the test tubes, pure, white crystals of a compound were obtained in test tubes of toluene: methanol (9:1) fraction. A single spot resolved at Rf 0.65 using the mobile phase toluene: methanol (9:1). This gave **Compound 3,** (white lumps) [18].

Characterization of compounds

Optical rotations were measured using a PerkinElmer 343 automatic polarimeter (PerkinElmer, Waltham, MA, USA). UV spectra were collected on a Hitachi U-2910 spectrophotometer (Hitachi, Tokyo, Japan). IR spectra were obtained with a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA). NMR spectroscopic data were recorded at room temperature on a Bruker Avance DRX-400 MHz spectrometer (Bruker, Billerica, MA, USA) using standard Bruker pulse sequences. High-resolution electrospray ionization mass spectra (HRESIMS) were obtained on a Micromass Q-Tof II (Micromass, Wythenshawe, UK) mass spectrometer operated in the positive-ion mode, with sodium iodide being used for mass calibration. Column chromatography was performed with Sephadex LH-20 (Supelco, Bellefonte, PA, USA) and 65 × 250 or 230 × 400 mesh silica gel (Sorbent Technologies, Atlanta, GA, USA). Analytical thin-layer chromatography (TLC) was conducted on precoated 250 µm thickness Partisil Si gel 60F254 glass plates. A 150 mm × 19 mm i.d., 5 µm, XBridge PrepC18 column with a 10 mm × 19 mm i.d. guard column of the same material (Waters, Milford, MA, USA) was used for semipreparative HPLC, along with a Hitachi system composed of an L-2130 prep pump, an L-2200 autosampler, and an L-2450 diode array detector (Hitachi, Tokyo, Japan) as described by Li et al [16].

Sample Preparation and HPLC-PDA, LC-IT-MS, and 1H NMR Fingerprinting Analysis.

Each pure compound (3 g) was extracted with 10 mL of methanol by centrifuged at room temperature for 30 min. The methanol extracts were dried in vacuo (40 °C), followed by another centrifuge extraction with 10 ml of chloroform at room temperature for 30 min, to obtain a chloroform-soluble extract of each sample, which

was then dried in vacuo at 40 °C and stored at –20 °C before analysis. All analyses were completed within 24 h of extraction.

The HPLC-PDA analysis was performed using a 150 mm × 4.6 mm i.d., 5 µm, XBridge C18 analytical column (Waters), along with a Hitachi system composed of an L-2130 pump and an L-2450 diode array detector (Hitachi). The mobile phase consisted of 0.05% trifluoroacetic acid in water (A) and acetonitrile (B) using a gradient program of 3–20 % B from 0 to 60 min and 20–100 % B from 60 to 70 min. The mobile phase flow rate and the injection volume were 1 ml/ min and 10 µl, respectively. The dried chloroform-soluble extract of each sample was dissolved in 1 ml of HPLC grade methanol, and filtered through a Fisher Scientific 13 mm syringe filter (0.2 µm) prior to injection. After comparison of the chromatograms of the chloroform-soluble extract solution recorded at wavelengths within 200–550 nm, it was found that 254 nm could best represent the profile of the analytes.

The LC-IT-MS analysis employed the same separation conditions and Xbridge C18 analytical column as used for the HPLC-PDA analysis mentioned above, on a Waters Alliance 2690 separation module. The injection volume was 10 µl. The mobile phase flow rate was maintained at 1 ml/min and was split approximately 50:1 postcolumn using a microsplitter valve (Upchurch Scientific, Oak Harbor, WA, USA) for the introduction to the ESI source. The electrospray ionization ion trap mass spectrometry (ESI-IT-MS) was performed on a Bruker dual funnel amaZon ETD ion trap mass spectrometer (Bremen, Germany) equipped with an orthogonal electrospray source operated in positive-ion mode. Sodium iodide was used for mass calibration for a calibration range of m/z 100–1000. Optimal ESI conditions were as follows: capillary voltage, 4500 V; source temperature, 250 °C; ESI drying gas (nitrogen), 4.0 l/min; and nebulizer, 10 psi. The ion trap was set to UltraScan mode with a target mass of m/z 500 pass ions from m/z 100 to 1000.

The 1H NMR spectroscopic fingerprinting was conducted using the previously described methods for compounds 1 - 3. The samples were dissolved in 600 µL of $CDCl_3$, and spectra were measured at 300 K using a Bruker Avance-III HD400 spectrometer [16].

***In vitro* antioxidant analysis**

Evaluation of antioxidant capacity using the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) spectrophotometric assay

The method of Mensor *et al.* was adopted [19]. The percentage antioxidant activity was calculated as follows:

% antioxidant activity [AA] = 100 – ([absorbance of sample-absorbance of blank] * 100)

Absorbance of control

1 ml of methanol plus 2 ml of the extract was used as blank while 1 ml of 0.5 mM DPPH solution plus 2 ml of methanol was used as control. Ascorbic acid was used as reference standard.

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant potential of sample was determined using a ferric reducing ability of plasma (FRAP) assay of Benzie and Strain [20] as a measure of "antioxidant power". The results were corrected for dilution and expressed in µmol Fe^{11}/L. Vitamin C was measured within 1 h after preparation. The sample to be analyzed was first adequately diluted to fit within the linearity range. All determinations were performed in triplicate.

FRAP value of sample (µM) = changes in absorbance from 0-4 min × FRAP value of std (1000mM)

changes in absorbance of standard 0 min - 4 min

Hydrogen peroxide scavenging assay

An aliquot of 50 mM H_2O_2 and various concentrations (0–2 mg/ml) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90 µl of the H_2O_2-sample solution was mixed with 10 µl HPLC-grade methanol and 0.9 ml FOX reagent was added (prepared in advance by mixing 9 volumes of 4.4 mM BHT in HPLC-grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was

then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm. All tests were carried out 3 times and sodium pyruvate served as reference compound.

ABTS radical cation scavenging activity

Re et al. [21] methodology with slight modification was followed for ABTS (2, 2 azobis, 3-ethylbenzothiozoline-6- sulphonic acid) radical cation scavenging activity. Percent inhibition was calculated by following formula:

ABTSscavenging effect (%) = [(control absorbance - sample absorbance) x 100

control absorbance]

Anti-lipid peroxidation assay

This assay was performed as illustrated by Dorman et al [22]. The percent anti lipid peroxidation was determined by

formula (1 - S/C) × 100

Where

C = Absorbance of control and S = Absorbance of test sample

Osmotic Fragility Test

Blood was collected from pig in to 5 heparinized tubes. each test tube contained each of compound 1, 2, 3 at 5mg/ml. For each blood sample, test tubes were numbered from 1 to 17. Five ml of buffered NaCl ranging from 0.1 to 0.85% was placed in tubes 1 through 16. Five ml of distilled water was placed in tube 17.

0.1 ml of blood was added to each tube and mixed gently and then incubated at 37 °C for 1 h. The tubes were centrifuged at 2000 rpm for 10 min to sediment any intact red cells. The haemoglobin content of the supernatant was measured at 540 nm using a spectrophotometer and the 0.85%-saline tube as a blank and the distilled water tube as the 100%-haemolysis as standard. The highest value of optical density which corresponded to an incubation concentration of 0.1 % NaCl was taken as 100 % haemolysis.

Percentage lysis was calculated using the formula:

Abs of test x 100 = % haemolysis

Abs of standard

% haemolysis was plotted against % NaCl concentrations. The mean corpuscular fragility (MCF), which is the concentration of NaCl producing 50% lysis was extrapolated. Analysis was done using the student t-test was applied at 5% confidence level [36].

Statistical analysis

All data were expressed as Mean ± S.E.M. or % mean. Data were analyzed using one way analysis of variance (ANOVA) at 5% level of significance. Statistical analysis was performed using SPSS statistical package. The IC_{50} values were calculated by the formula Y = 100*A1/(X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t tests. $P < 0.05$ was considered significant.

Results

Phytochemical analysis

The qualitative phytochemical analysis revealed the presence of tannins, saponins, terpenoids, flavonoids, phenols alkaloids, sterols, glycosides and anthraquinones. The quantitative phytochemical analysis showed tannins (1.37± 0.04 mg/g), saponins (0.09 ±0.08 mg/g), terpenoids (0.21±0.02 mg/g), flavonoids (0.18± 0.02 mg/g), phenols (0.12±0.01 mg/g), alkaloids (1.91±0.05 mg/g), sterols (0.14 ±0.01 mg/g), glycosides (0.22 ± 0.02 mg/g) and anthraquinone (1.22± 0.01 mg/g)

Pure compounds

Three pure compounds were isolated from the aqueous-ethanol crude extract of *Irvingia gabonensis* seed extract.

Methyl 2-[2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl]- propanoate, **Compound 1**, was isolated as a transparent brown resin: [α]20 D +28.0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 291 (4.09) nm; IR (film) νmax 3424, 3120, 2946, 2850, 2803, 2727, 1749, 1655 cm−1; 1H NMR (400 MHz, CD3OD) δ 9.31 (1H, s, CHO), 7.07 (1H, d, J = 4.0 Hz, H-3), 6.28 (1H, d, J = 4.0 Hz, H-4), 5.43 (1H, q, J = 7.0 Hz, H′), 4.63 (2H, ABq, Δν = 8.1 Hz, J = 13.9 Hz, H-6), 3.67 (3H, s, OCH3), 1.67 (3H, d, J = 7.0 Hz, CH3); 13C NMR (100 MHz, CD3OD) δ 180.5 (CHO), 172.5 (COO), 144.6 (C-5), 133.4 (C-2), 127.4 (C-3), 111.4 (C-4), 56.6 (C-6), 55.8 (C-1′), 52.8

(OCH3), 18.0 (CH3); HRESIMS m/z 234.0733 [M + Na]+ (calcd for C10H13NO4Na, 234.0742) as shown by Li et al [16].

Kaempferol-3-o-β-D-6" (p-coumaroyl) glucopyranoside, Compound 2, was a dark brown powder: $[\alpha]^{20}_{D}$= -62.5° (C. 0.5 MeOH); IR ν_{max} 3262 (OH), 1654 (C=O), 1606 (C=C) cm^{-1}; $C_{30}H_{26}O_{13}$; ^{1}H-NMR (CD_3OD) δ: 3.21 (2H, m, C3", 4"), 3.37 (1H,m,C2"), 3.38 (1H, m, C5"), 4.19 (2H, m, C6"), 5.13 (1H, m, C1"), 5.99 (1H, d, CΥ), 6.02 (1H, d, C6), 6. 18(1H. d, C8), 6.71 (4H, t, C3', 5', 3"', 5"'), 7.20 (2H, d, C2"', 6"'), 7.31 (1H, Cβ), 7.86 (2H, d, C2', 6'); ^{13}C-NMR: 63.06 (C6"), 70.42 (C4"), 74.44 (C3"), 74.50 (C2"), 76.71 (C5"), 93.57 (C8), 98.70 (C6), 102.72 (C1"), 104.28 (C10), 113.44 (CB), 114.74 (C3, 5'), 115.48 (C3"',5"'), 121.40 (C1'), 125.78 (C1"'), 129.89 (C2"', 6"'), 130.93 (C2',6'), 133.93 (C3), 145.26 (CΥ), 157.07 (C9), 158.01 (C2), 159.87 (C4"'), 160.21 (C4'), 161.63 (C5), 164.65 (C7), 167.53 (Cα), 178.09 (C4)

The ^{13}C-NMR spectrum showed 2 intense signals at δ 130.9 and at δ 114-7. These were assigned to H2'/H6' and H3'/H5' respectively. The signals at δ 121.4 and δ 160.2 are typical of an unsubstituted β-ring of a kaemferol-like structure. The signal at 63.6 (CH_2) shows that the P-coumaroyl linkage is at C-6 of a glucose unit.

In the HREI-MS, the molecular ion peak was seen at 594 a.m.u. After distortionless enhancement by polarization transfer (DEPT), the molecular formula of the compound was determined to be C_{30} $H_{26}O_{13}$.

Using HREI-MS and NMR data, it is confirmed as kaempferol-3-o-β-D-6" (p-coumaroyl) glucopyranoside.

While Lupeol (3β-lup-20(29)-en-3-ol), Compound 3, a white crystal was identified via spectral analysis: ultraviolet (UV), infrared (IR; KBr), gas chromatography-mass spectroscopy (GC-MS) and 1H-nuclear magnetic resonance (1H-NMR) (CDCl3). Upon the analysis of melting point and spectral data, the compound was suspected to be a sterol and triterpenoid. This was confirmed when the compound gave Salkowski test and Liebermann–Burchard test positive. The structure of the compound was elucidated on the basis of the spectra [18].

On TLC, the compound resolved at Rf 0.65 using the mobile phase toluene: methanol (9:1). Upon the analysis of melting point and spectral data, the compound was suspected to be a sterol and triterpenoid. This was confirmed when the compound gave Salkowski test and Liebermann–Burchard test positive [18].

Melting point - 215–219 °C; UV (λmax) 235 nm and 270 nm in chloroform; IR peaks (cm^{-1}) 3347, 3068, 2945, 2869, 1640, 1456, 1380, 1189, 1106, 1038, 880, 690, 640, 599, 545; NMR peaks: δ 4.69 and δ 4.57 (each 1H, m, 29), δ 3.18 (1H, tdd, 3), δ 2.39 and δ 1.92 (each 1H, m, 19), δ 1.68 (1H, t, 15), δ 1.66 (3H, s, 30), δ 1.60 (1H, d, 2), δ 1.59 (1H, q, 2), δ 1.42 (1H, d, 16), δ 1.39 (1H, q, 6), δ 1.36 (1H, t, 18), δ 1.33 (1H, m, 21), δ 1.20 (1H, m, 22), δ 1.03 (1H, q, 12), δ 0.99 (3H, s, 23), δ 0.97 (3H, s, 27), and δ 0.83, δ 0.79 (3H, s, 25, 28, 24); GC-MS peaks (*m/z*) M+ peak: 426; Base peak: 95. Other fragments: 411, 383, 370, 358, 315, 247, 218, 207, 189, 175, 161, 147, 135, 121, 109, 95, 81, 69, 55, 41 as shown by Sanusi et al [18].

Antioxidant effects and Osmotic Fragility

The results of the andtioxidant activities (DPPH and FRAP) are shown in Figure 2 and 3 respectively, while the result on red blood cell membrane stability is shown in Table 2.

Discussion

Nine phytochemicals were quantified, the most abundant being alkaloids (1.91±0.05 mg/g), tannins (1.37± 0.04 mg/g), anthraquinone (1.22± 0.01 mg/g) while others in decreasing abundance included glycosides (0.22 ± 0.02 mg/g), terpenoids (0.21±0.02 mg/g), flavonoids (0.18± 0.02 mg/g), sterols (0.14 ±0.01 mg/g), phenols (0.12±0.01 mg/g) and saponins (0.09 ±0.08 mg/g).

Alkaloids have diverse and important physiological effects on humans. Tannins, on the other hand, have beneficial properties that are both therapeutic and pharmacological thereby confirming earlier research which classified *Irvingia gabonensis* seed as possessing physiological, therapeutic and pharmacological potentials. Furthermore, saponins have been associated with laxative properties [23, 24]. The quantification of glycosides, terpenoids, flavonoids, sterols and phenols in *I. gabonensis* indicates their abundance.

Their presence in remarkable quantities highlights this source as having future roles in the production of anti-tumour, anti-inflammatory and anti-oxidant drugs or supplements [25, 26, 27].

The results indicated that *Irvingia gabonnensis* seed contained significant amounts of flavonoids and phenolic compounds. Both classes of compounds have good antioxidant potential and their effects on human nutrition and health are considerable. The mechanism of action of flavonoids is through scavenging or chelation [28]. Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability [29]. Flavonoids and phenols have been known to possess free radical combating potential *in vitro* and *in vivo* as a result of their ability to mop up reactive oxygen species with the aid of their hydroxyl functional groups. This has made them important to researchers, nutraceutical and pharmaceutical companies.

In this study, Compound 3 showed an increased antioxidant activity across the concentrations when compared with standards (ascorbic acid) in the DPPH and FRAP analytical assessments (Figure 2 and 3). This is possible since the compound possess a lot of OH^- groups which are involved in free radical neutralization or chelation. Also, the IC_{50} of Compound 3 showed concentrations comparable to those of the standards (Table 1). That shows that Compound 3 could be a good antioxidant.

The antioxidant activity has been studied by the inhibition of ascorbic acid-induced lipid peroxidation and Catechin has been used as the standard for phenolic content, whereas in the present study, the antioxidant capacity of Compound 3, crude extract and other compounds isolated from *Irvingia gabonnensis* seed was measured by an improved ABTS radical cation decolorization assay and gallic acid was used as the standard. Therefore, it is clear that both the extract and isolated compounds from *Irvingia gabonnensis* seed aqueous have good antioxidant activities. $ABTS^{\cdot+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Addition of the isolated compounds to this pre-formed radical cation reduced it to ABTS in a concentration-dependent manner.

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects [30].

Hydrogen peroxide scavenging was assayed by the FOX reagent method [31]. Table 1 shows that the Compound 3 is a very good scavenger of H_2O_2 (IC_{50} = 98.12 ± 0.77 µg/ml) compared to standard sodium pyruvate (IC_{50} = 53.00 ± 0.00 µg/ml). This was also observed with the ABTS and anti-lipid assay methods.

From the results, it appeared that the H_2O_2 scavenging activity of the plant extract is comparable to that of the standard sodium pyruvate. At sites of inflammation, the oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase results in the production of another harmful ROS, hypochlorous acid [32]. HOCl has the ability to inactivate the antioxidant enzyme catalase through breakdown of the heme prosthetic group. Catalase inactivation can inhibited in the presence of these isolated compounds, signifying its HOCl scavenging activity. The results suggest that these isolated compounds are efficient scavenger comparable to the standard ascorbic acid.

Iron can stimulate lipid peroxidation by the Fenton reaction ($H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + OH^{\cdot}$) and can also accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction [33]. Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation [34]. According to the results, compounds isolated from *Irvingia gabonnensis* seed aqueous are not as good as the standard EDTA except for Compound 3; but the decrease in concentration-dependent color formation in the presence of the compounds isolated from *Irvingia gabonnensis* seed aqueous indicates that it has iron chelating activity.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as

prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging [35]. As shown in Figure 3, the reducing power of the extract and isolated compounds were compared with the standard BHT and found to be superior.

The osmotic fragility of red blood cells and generally, the body cells reflect their ability to take up water without lysis and also, withstand hypotonic environments [36]. Sujatha and Srivinas showed that the aqueous extracts inhibited lipid peroxidation (LPO) in human erythrocyte membrane [37]. Osmotic fragility is experimentally denoted by MCF values which was significantly high in Compounds 2 and 3. This implies that the red blood cells in these groups were able to retain more water volume in a hypotonic solution before 'stretching' of the membrane that progresses to lysis. Previous studies have shown that the cytoskeletal proteins of red blood cells from diabetic patients are heavily glycosylated and that spectrin is oxidatively damaged [38]; also several lipids (free cholesterol, sphingomyelin, and phosphatidylcholine) on the outer surface of the phospholipid bilayer is significantly decreased [39, 40]. The efficacy of treatment regimens on the integrity, cell shape, and health status of red blood cells should be tracked, as these cells' health status is crucial to the overall wellness of a disease infected individual.

Conclusion

On the basis of the results obtained in the present study, it is concluded that *Irvingia gabonnensis* seed contain large amounts of flavonoids and phenolic compounds that exhibited high antioxidant and free radical scavenging activities. They also chelates iron and have reducing power. This *in vitro* assay indicate that *Irvingia gabonnensis* seed or isolated compounds are significant source of natural antioxidant, which could be helpful in preventing the progress of various oxidative stresses. The *in vivo* antioxidant activity of this *Irvingia gabonnensis* seed or isolated compounds need to be assessed prior to clinical use.

The health benefits of these known phytochemicals aside the medicinal and pharmacological potentials of *I. gabonensis*, positions it as a raw material for the production of nutraceutical. *Irvingia gabonensis* kaempferol-3-0-β-D-6" (p-coumaroyl) glucopyranoside, Methyl 2- [2-formyl-5-(hydroxymethyl)-1 H-pyrrol-1yl]-propanoate and Lupeol (3β-lup-20(29)-en-3-ol have been implicated in the seed's antioxidant effect. Isolation of Lupeol (3β-lup-20(29)-en-3-ol, a triterpenoid and sterol, Kaempferol-3-0-β-D-6" (p-coumaroyl) glucopyranoside a flavonoid and Methyl 2-[2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl]-propanoate a pyrrole alkaloid, enriches the body of knowledge on constituents of *I. gabonensis* seed.

Conflict of interests

The authors declare no competing financial interest. The authors solely funded the project.

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Table 1. Table 1: IC_{50} values for different antioxidant assays of *Irvingia gabonensis* ethanol seed extract, Compounds 1, 2 and 3

	IC_{50} (µg/ml)				
Activity	Crude extract	Compound 1	Compound 2	Compound 3	Standard
H_2O_2	303.33±1.44*	133.48±1.33	151.22±0.18*	98.12±0.77	53.00±0.00
ABTS	244.52±1.81*	101.41±11.34	217.00±1.61*	88.00±2.12	51.01±0.22
Anti-lipid	171.41±1.17*	97.18±1.21*	138.41±2.13*	69.31±1.57	34.12±0.13

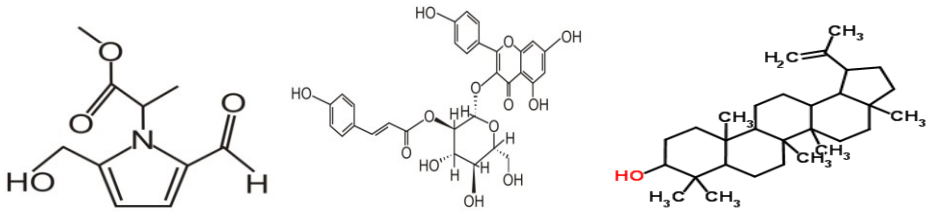
Values are expressed as mean±SD (n=3). * Means in rows significantly (P<0.05) different from the standard.

Table 2. Median Corpuscular Fragility (MCF) values and % stabilization Compounds 1, 2, 3

	Conc (mg/ml)	MCF (% [NaCl])
1	5	0.320±0.018*
2	5	0.401±0.016*
3	5	0.433±0.012*
Control (distilled water)	-	0.212±0.014

Values are mean±S.E.M.; * p<0.05 compared to distilled water (negative control)

Figure 1. Structural formulas of Compounds 1, 2 and 3.



Compound 1 **Compound 2** **Compound 3**

Figure 2. Comparison of DPPH antioxidant activities of the isolated pure compounds with the crude extract

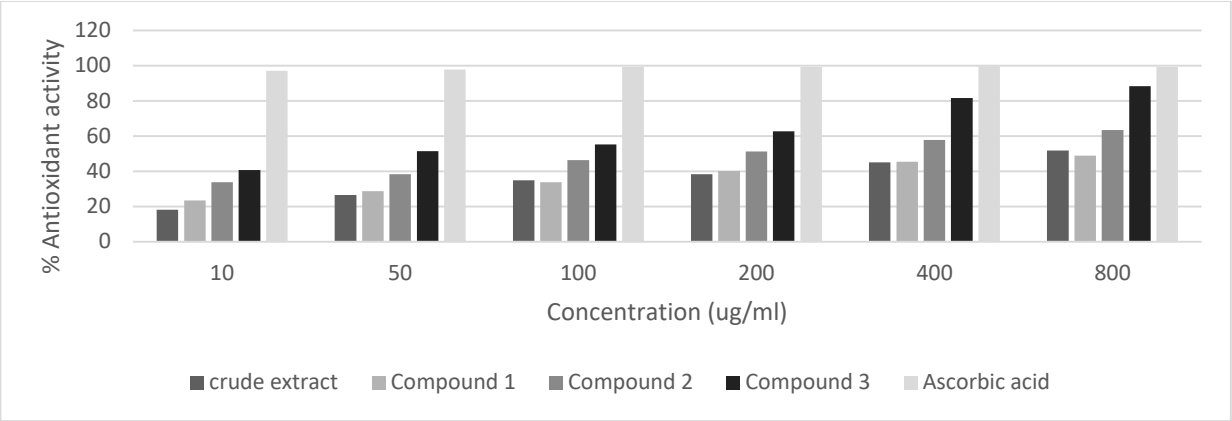


Figure 3. Comparison of FRAP values of the isolated pure compounds with the crude extract

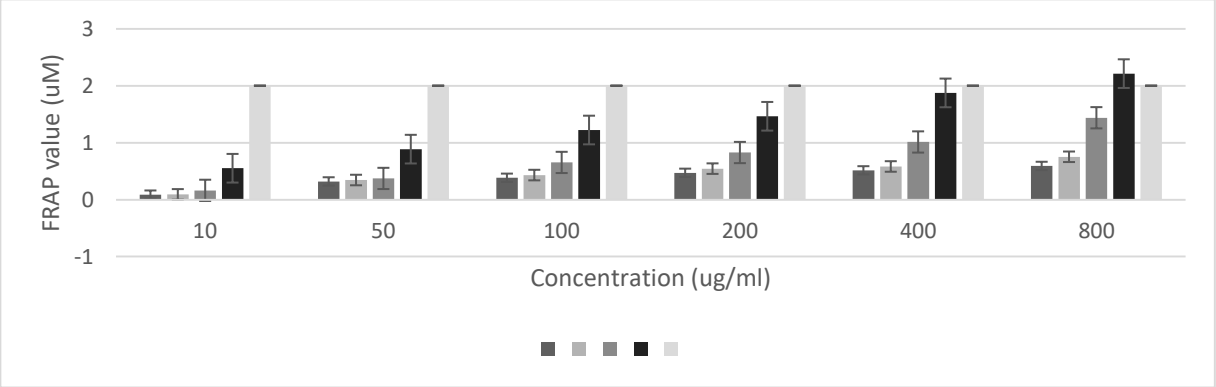


Figure 4. 1H NMR spectrum of compound 1 (CD3OD, 400 MHz).

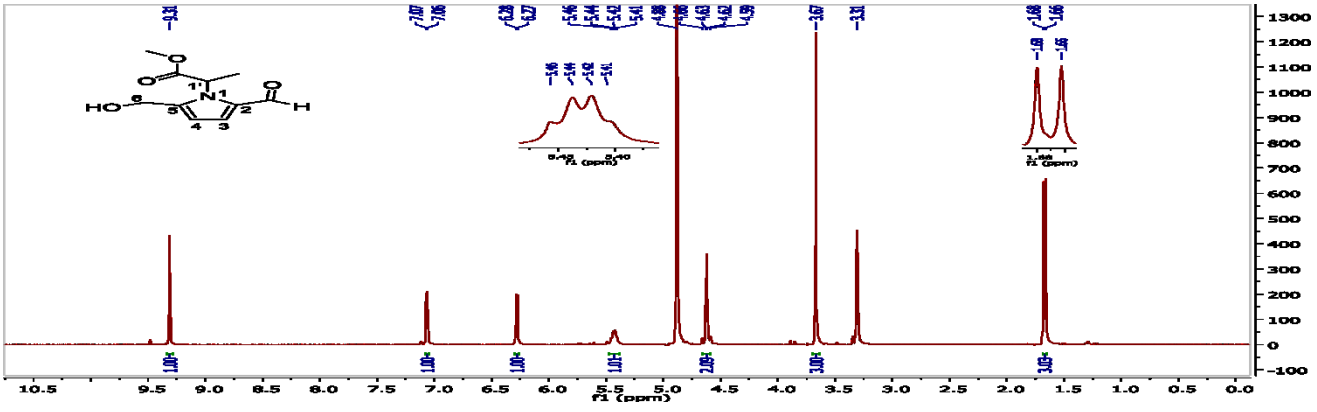


Figure 5. 1H-1H COSY spectrum of compound 1 (CD3OD, 400 MHz).

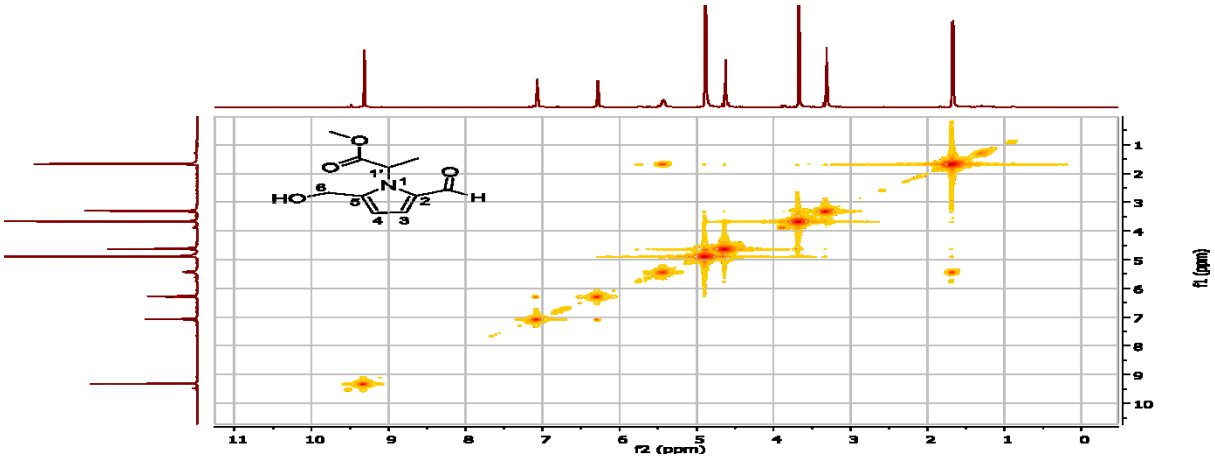


Figure 6. 13C DEPT 135 NMR spectrum of compound 1 (CD3OD, 400 MHz).

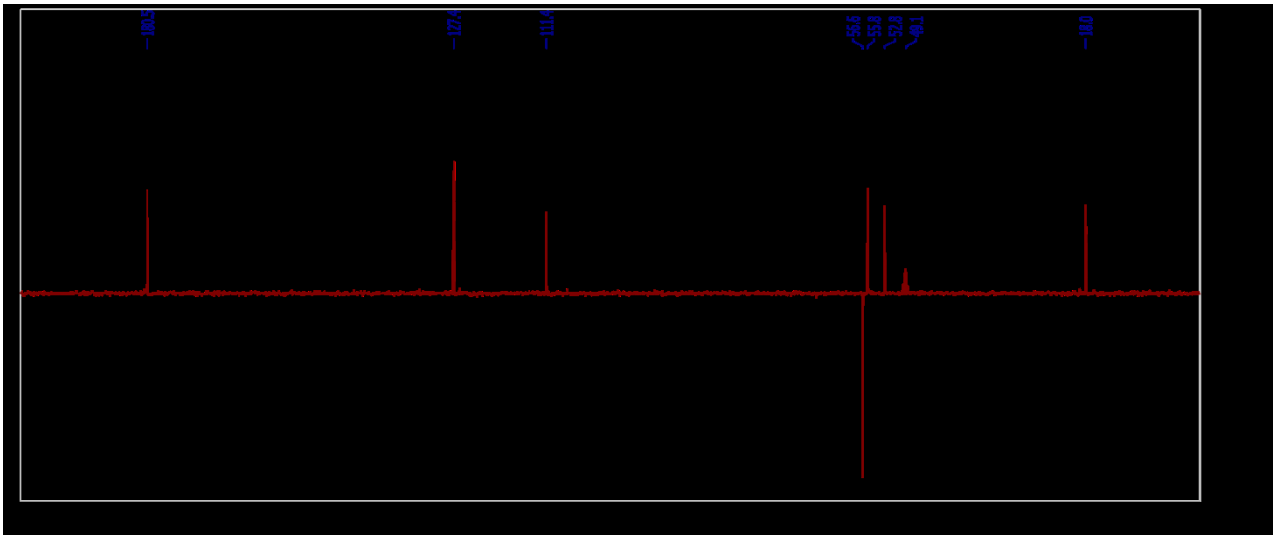


Figure 7. 13C NMR spectrum of compound 1 (CD3OD, 400 MHz)

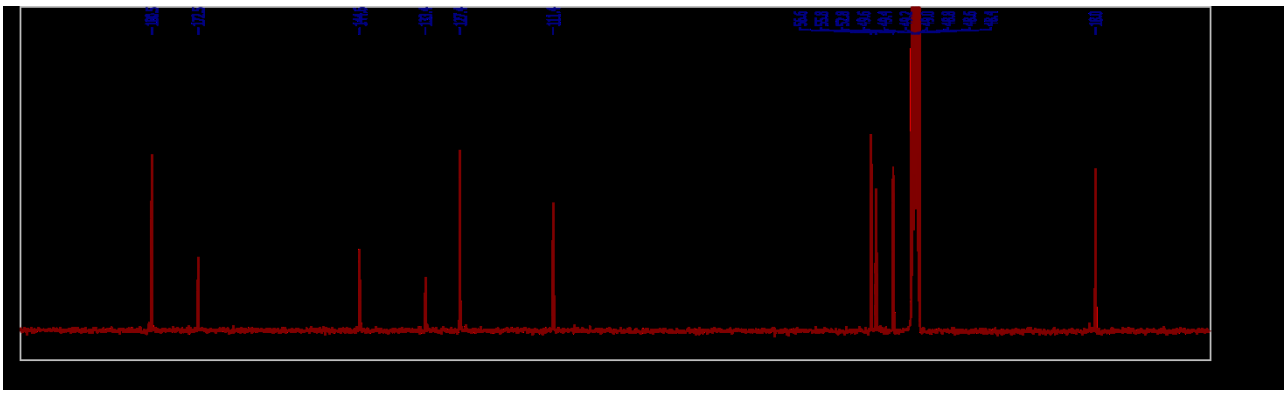


Figure 8. HMBC spectrum of compound 1 (CD3OD, 400 MHz).

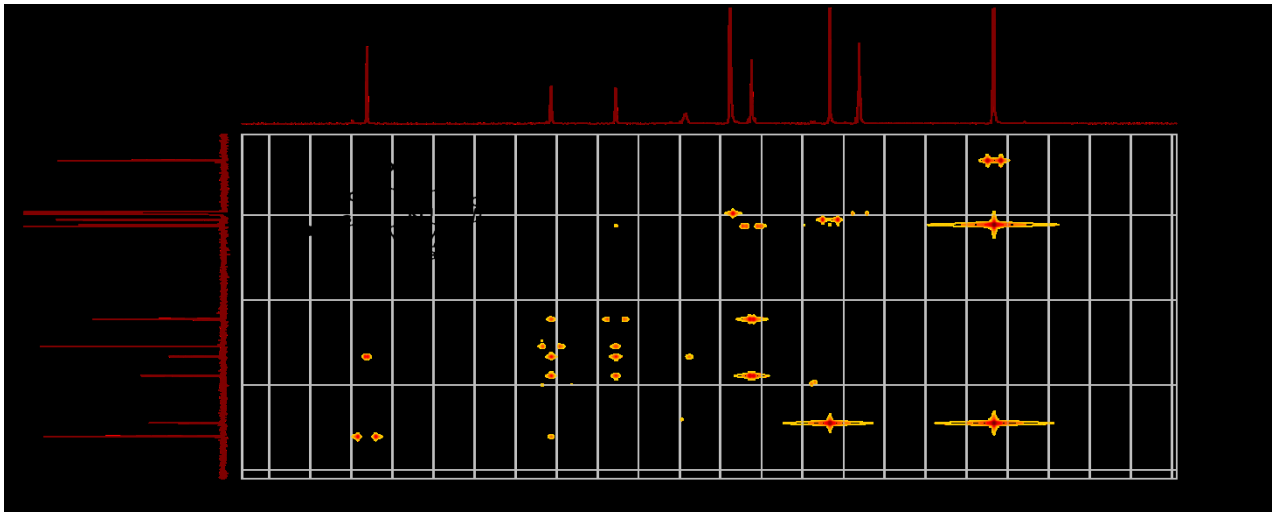


Figure 9. HRESIMS spectrum of compound 1

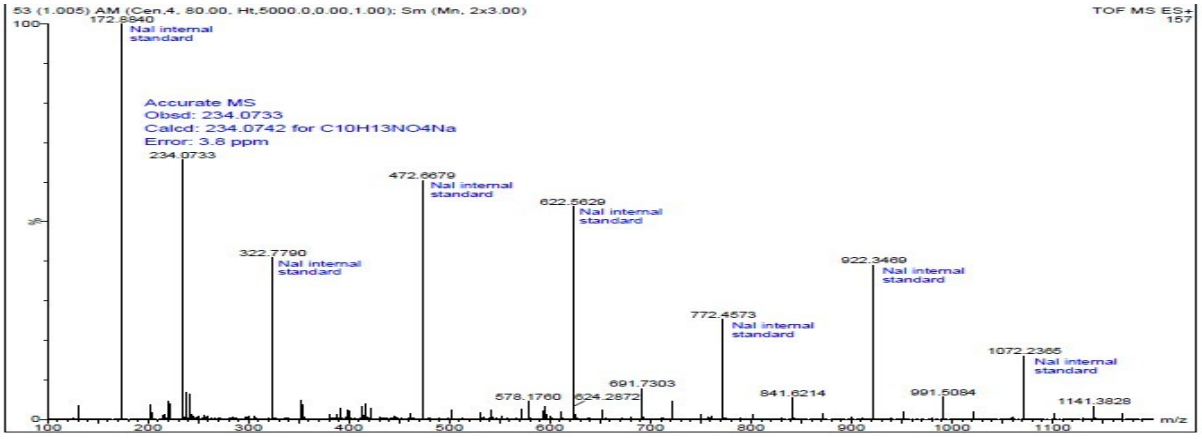


Figure 10. HSQC spectrum of compound 1 (CD3OD, 400 MHz)

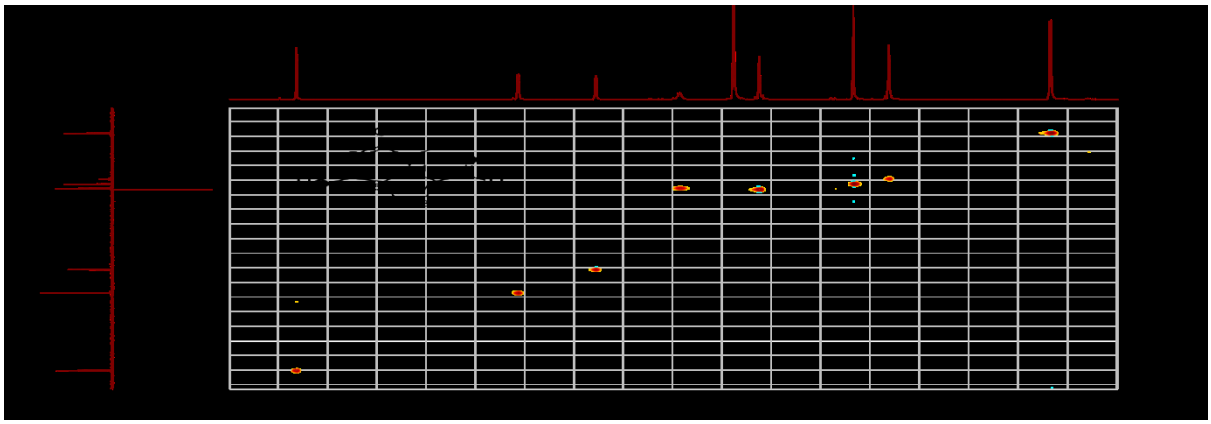


Figure 11. IR spectrum of compound 1

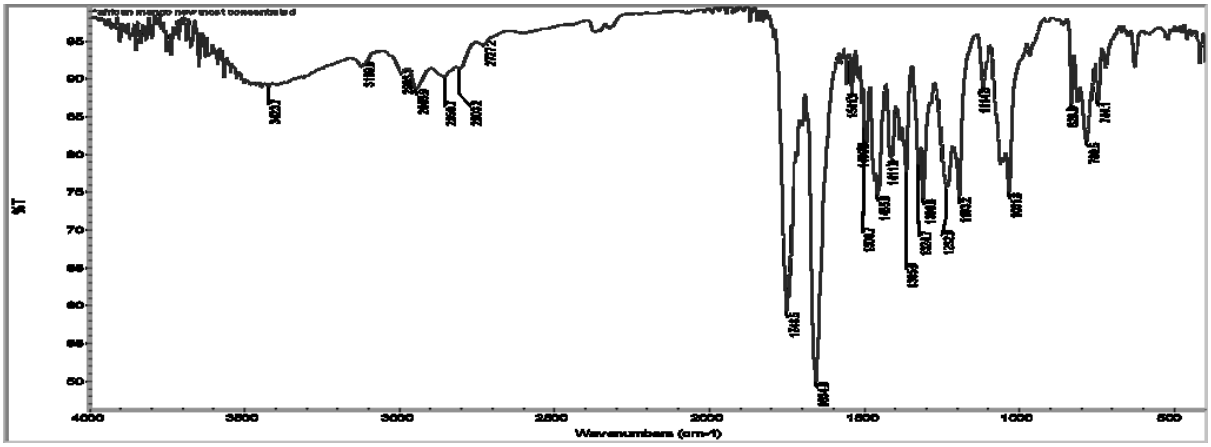


Figure 12. UV spectrum of compound 1

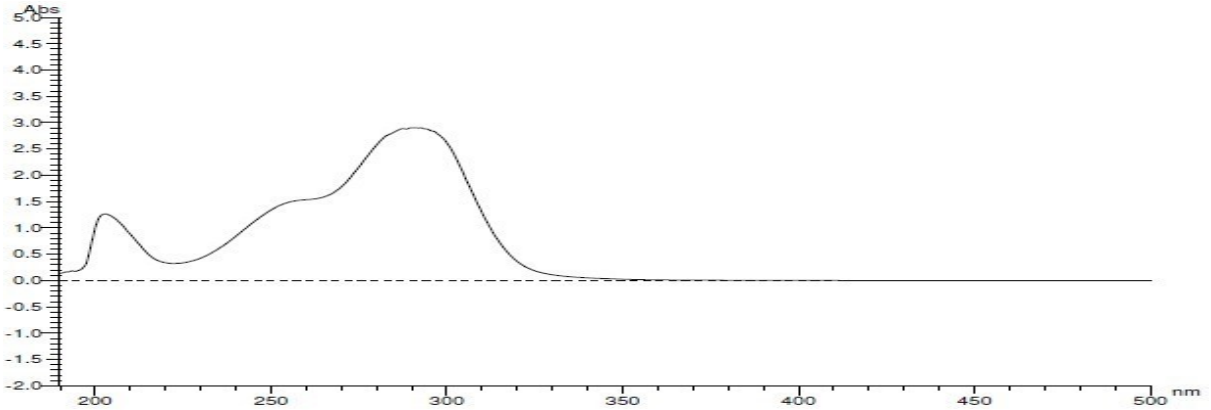


Figure 13. 1H-NMR SPECTRUM (CD3OD, 250MHz) of compound 2

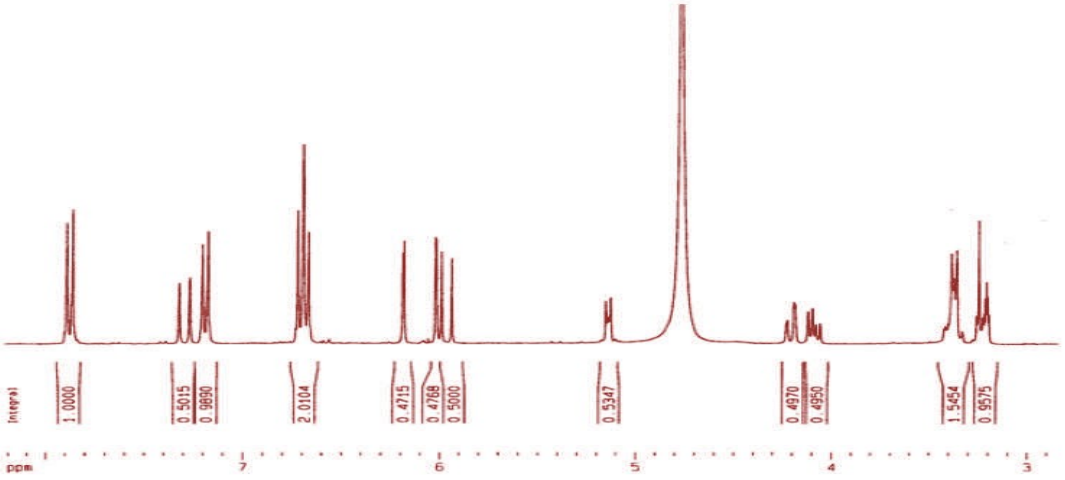


Figure 14. 2D–COSY SPECTRUM (CD3OD, 250MHz) of compound 2

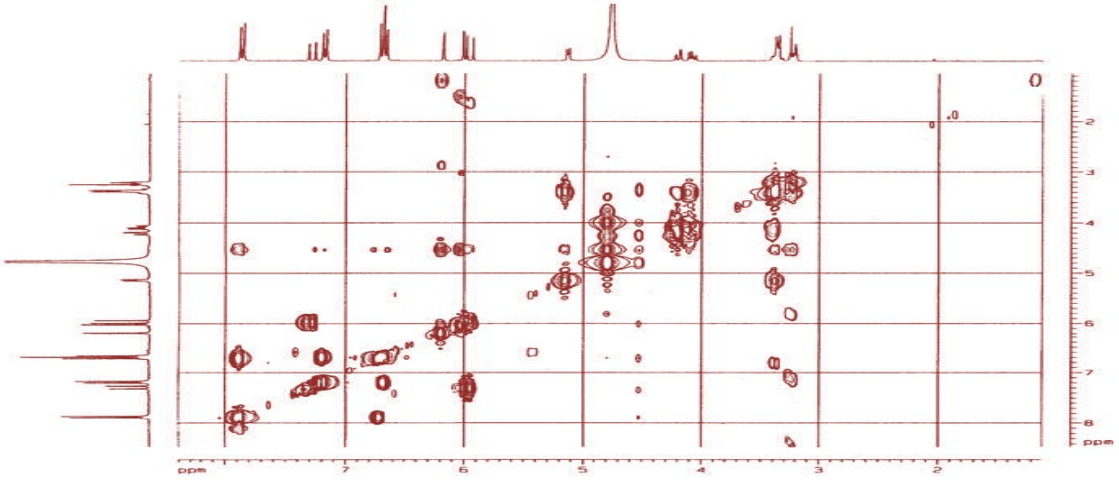


Figure 15. 13C–NMR SPECTRUM (CD3OD, 250MHz) of compound 2

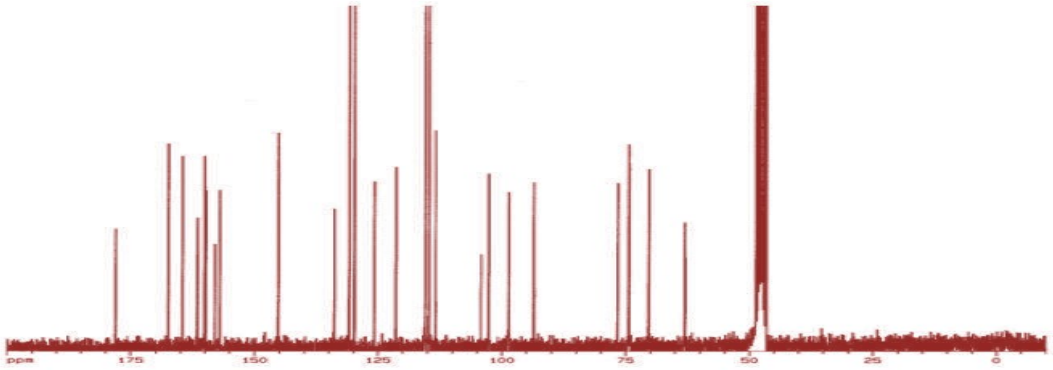


Figure 16. IR SPECTRUM of compound 2