Journal of
Bioresearch Communications

Ogechukwu et al., J Biomol Res Ther 2012, 1:1
DOI: 10.4172/2167-7956.1000102

Research Article Open Access

Three - (-) Catechin-O-Rhamnosides from the Eastern Nigeria Mistletoe with Potent Immunostimulatory and Antioxidant Activities

Omeje Edwin Ogechukwu[1]*, Osadebe Patience Ogoamaka[1], Akira Kawamura[2], Amal Hassan[3], Abdessamad Debbab[3], Esimone Charles Okechukwu[4], Nworu Chukwuemeka Sylvester[5], Nwodo Ngozi[1] and Proksch Peter[3]

[1]*Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. 410001, Enugu, Nigeria*
[2]*Department of Chemistry, Hunter College of CUNY, The City University of New York, USA*
[3]*Department of Pharmaceutical Biology, Heinrich-Hein University, Bilk, 40225 Düsseldorf, Germany*
[4]*Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, PMB 6025, Awka*
[5]*Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. 410001, Enugu, Nigeria*

Abstract

In an attempt to provide further convincing evidence for the variously reported immunomodulatory potentials of mistletoes, bioassay-guided fractionation of the eastern Nigeria mistletoe afforded three compounds: - (-) catechin-7-O-rhamnoside (1), - (-) catechin-3-O- rhamnoside (2) and a 4′-methoxy-7-O-rhamnoside (3). Their effects on C57BL6 mice splenocytes proliferation and expression of CD69 molecule were determined using flow cytometry techniques and compared to Lipopolysaccharide (LPS; 10 µg/ml) and Concanavalin A (ConA; 2 µg/ml) as standards. The antioxidant study was by the DPPH model with ascorbic acid as standard. The compounds (1-3) at 100 µg/ml showed statistically significant ($p<0.05$) stimulatory activity with values of 91.49 ± 0.22%, 95.17 ± 0.01% and 94.23 ± 0.07% respectively compared to 2.65 ± 0.33% for the unstimulated control. However, the CD69 expression assay showed only moderate stimulation. Their measured antioxidant potentials (effective concentration; EC_{50}) were high (≤ 55.42 ± 0.99 mg/ml) when compared to ascorbic acid (17.6 ± 1.78 mg/ml). Characterization of the compounds was achieved by chromatographic and spectroscopic methods.

Keywords: Immunomodulatory; Catechin; Rhamnoside; Mistletoe; Antioxidant; Eastern Nigeria; C57BL/6 splenocytes

Introduction

The unmatched availability and chemical diversity characterizing natural products provide unlimited opportunities for development of new drug leads [1]. Beside small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases [2]. Despite the huge resources on research involving the use of conventional targets and therapeutic approaches to fight diseases of man and animal, mortality and morbidity rates resulting from some of these diseases are still unacceptable. There are today, newer frontiers and paradigm shifts in therapy being investigated as better alternatives to conventional therapy. Modulation of the immune system as well as optimizing oxidative processes of the body with the aid of natural products represents a field of drug development-based research witnessing unprecedented upsurge in recent times [3]. In addition, the human immune system is intricately interwoven with oxidative processes in the body. High oxidative stress usually breaks down immune system, precipitates radicals as well as severe diseases and this must be prevented [4]. Even though, the body has developed a variety of ways to deal with damaging free radicals, antioxidants from dietary sources also play important role in their control, thus limiting cellular damage [5]. More recent studies have also emphasized the therapeutic importance of plant derived immunomodulants and antioxidants [6,7]. A newer approach to therapeutics is now the search for safe and potent immune modulating substances preferably with synergistic antioxidant activity. Interestingly, there has been growing interest in isolating and characterizing natural compounds with immunomodulatory and antioxidant activities [8]. It has also been established that most pharmacological activities are related to the immunostimulatory and antioxidant properties of plant secondary metabolites [9]. Mistletoes grow in different continents of the world and in Eastern Nigeria, a species, *Loranthus micranthus* have been used traditionally for the management of various ailments, notably diabetes, high blood pressure and conditions affecting human immune system for many years [10,11]. We recently reported the immunomodulatory potentials of mistletoes of Eastern Nigeria origin, *Loranthus micranthus*, parasitic on five different host trees with mistletoe from *Kola acuminata* exhibiting the highest activity [12]. A preceding study had established a potent up-regulatory immune response activity of *Loranthus micranthus* and indicated that these species of mistletoe is highly safe and obviously contains no active proteins or lectins [13]. Further preliminary studies indicated that steroids, triterpenoids, alkaloids and flavonoids were the possible immunostimulants in *Loranthus micranthus* [14]. Unpublished data (using the radical scavenging (DPPH)-based model) also suggested that all the mistletoes possessed moderate antioxidant activity, with that from *Citrus* sp. exhibiting highest activity. The present study was aimed at isolating and characterizing the immunomodulatory and antioxidant activities of catechin rhamnosides from the eastern Nigeria mistletoe.

Materials and Methods

Collection and identification of plant material

Loranthus micranthus Linn. (Loranthaceae) leaves parasitic on the

***Corresponding author:** Omeje Edwin Ogechukwu, Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. 410001, Enugu, Nigeria, Tel: +234-803-549-5441; E-mail: winnomeje@yahoo.com; edwin.omeje@unn.edu.ng

Received December 19, 2011; **Accepted** February 21, 2012; **Published** February 25, 2012

Citation: Ogechukwu OE, Ogoamaka OP, Kawamura A, Hassan A, Debbab A, et al. (2012) Three - (-) Catechin-O-Rhamnosides from the Eastern Nigeria Mistletoe with Potent Immunostimulatory and Antioxidant Activities. J Biomol Res Ther 1:102. doi:10.4172/2167-7956.1000102

host tree (*Kola acuminata*) were collected in April, 2007 from different locations in Nsukka LGA, Enugu State. The leaves were identified and certified by Mr. AO Ozioko, a taxonomist of the Bioresources Development and Conservation Programme (BDCP), Nsukka, Enugu State. Voucher specimens were kept at the BDCP Centre with the number BDCP-532-07 for reference purposes.

Instruments

Gallenkamp melting point apparatus (England; used uncorrected), HREIMS and EIMS (mass spectrometers) linked to a MATT 8200 recorder, ^{1}HNMR, ^{13}CNMR and correlation studies were recorded with BRUKER-500 MHz spectrophotometer in CD_3OD or $CDCl_3$ with or without internal standards at the Institute of Anorganic Chemistry and Structure Chemistry, Heinrich-Heine-Universität, Düsseldorf, Germany or the Department of Chemistry, City University of New York (CUNY), USA. FT-IR spectrometer (Shimadzu, Japan) at the Department of Chemistry, Usmanu Dan Fodiyo University, Sokoto. UV/visible spectra were obtained in a UV2102PC spectrophotometer with integrated data station (UNICO, USA) at the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. Others were electronic analytical balance (Metler Toledo, 0.001 max, England), glass columns (4x150 cm; 2.7x70 cm), silica gel G_{60} and precoated G_{254} plates.

Solvents and reagents

Analar grade methanol, n-hexane, ethylacetate, acetone, chloroform (Sigma Aldrich; Germany). Distilled water, normal saline (DANA Ltd), dimethylsulphoxide (DMSO), Tween 20 or 80 solution (BDH, England), silica gel (70-230 and 60-120 mesh sizes), silica gel G_{60}, precoated silica gel GF_{254}, (Merck, Germany), sodium acetate powder, Aluminium chloride, Boric acid (Sigma-Aldrich), LPS and Con A (all 99% pure; Sigma-Aldrich) were used as reference drugs. All other reagents were of analytical grade or freshly prepared when needed.

Extraction, fractionation and isolation of the constituents

The methods of extraction and fractionation recently reported by some authors were used to obtain the partially purified ethyl acetate fraction from the crude aqueous methanol extract of the Eastern Nigeria mistletoe [12,14].

Isolation and purification of constituents from ethyl acetate fraction (EAF)

A portion of ethyl acetate fraction, 4.2 g was chromatographed on silica gel (70-230, 500 g) in a glass column (2.7 X 70 cm) with the bed height of 45 cm. The elution was performed with gradient mixtures of chloroform: methanol 100:0 (1950 mL), 95:5 (2200 mL), 90:10 (1250 mL), 85:15 (850 mL), 80:20 (1050 mL), 75:25 (830 mL), 70:30 (650 mL), 60:40 (700 mL); 50:50 (500 mL) and 0:100 (900 mL), at an approximate flow rate of 2.5 mL/min. Aliquots of 25 ml were collected and monitored by analytical thin layer chromatography, UV spectral (with shift reagents) and phytochemical analyses. Similar fractions were combined and were screened by DTHR and DPPH assays as models of cell-mediated immunomodulation and antioxidation respectively. Six fractions [F1 (1-16; 0.3 g), F2 (17-30; 0.12 g), F3 (31-45; 0.95 g), F4 (46-57; 0.61 g), F5 (58-102; 0.85 g), and F6 (103-131; 1.1 g)] were obtained. The most active fraction with respect to the activity guides, F3 (31-45; 0.95 g) was subjected to reverse phase HPLC and was shown to be a mixture of closely related compounds (Figure 1). Further column fractionation of F3 on ethyl acetate: methanol gradient system [100:0 (750 mL), 95:5 (900 mL), 85:15 (750 mL), 70:30 (600 mL), 0:100 (200 mL) and in the column dimensions above afforded a major fraction F3A (0.420 g) from the ethyl acetate: methanol, 70:30 ratio. The fraction F3A was then purified on preparatory plates (0.5 mm) in ethyl acetate: methanol (5:1) to afford phenolic compounds 1 (35 mg), 2 (115 mg) and 3 (11 mg), all dried in a desiccator charged with silica gel. Detection of these eluates by TLC (SiO_2, ethyl acetate/methanol 5/1 v/v; aqueous ammonia, Shinoda's reagent), Rf 1: 0.50 and 2: 0.65 and 3: 0.70. Further confirmation was achieved by the use of Shift's reagents in the UV spectrophotometry. Their purities were examined by both TLC precoated with 0.25 mm of silica gel GF_{254} and chromatographed in ethyl acetate: methanol 85/15. The phytochemical identities of the compounds were ascertained by standard procedures. All the isolated compounds tested positive to either aqueous ammonia or Shinoda's reagent.

Antioxidant studies on crude extract and solvent fractions

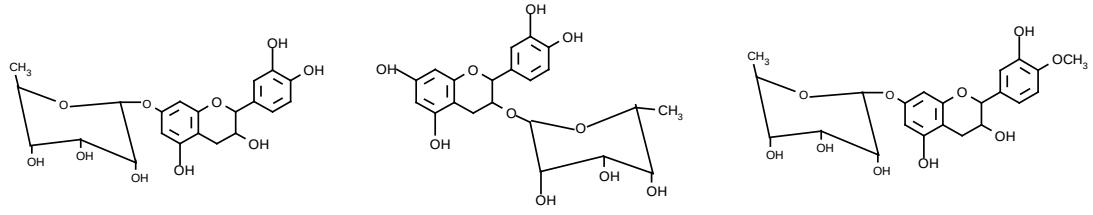
This was carried out using the DPPH radical-based assay procedure. Exactly 15 mg or 7.5 mg of DPPH was weighed on an analytical balance and dissolved in 10 ml of Analar methanol contained in a 250 ml volumetric flask. The volume was made up to mark with fresh methanol to produce a concentration of 0.4 or 0.2 mM solution respectively. This solution was kept in the dark to avoid degradation.

Preparation of standard antioxidant solution

Exactly 1.0 M (0.176 mg/ml) solution of ascorbic acid was prepared by dissolving 8.06 mg (accurately weighed on Mettler balance, Toledo) in 50 ml volumetric flask and making up to volume with fresh Analar methanol. This served as the standard antioxidant solution. It was diluted serially to obtain other concentrations for analyses.

Antioxidant studies

Stock solutions of isolated compounds under study were carefully made in Analar methanol. Serial dilutions (2 or10 fold) were achieved for each stock and labeled appropriately. The initial absorbance, (A_o) of the DPPH solution was taken at 517 nm using the JENWAY 680 spectrophotometer. Similarly, the absorbance of the standard ascorbic acid or pure compounds were taken [15]. Then the final absorbance (A_c) of



Catechin-7-O-rhamnoside (1) Catechin-3-O-rhamnoside (2) 4'-methoxy-catechin-7-O-rhamnoside (3)

Figure 1: Proposed structures of isolated catechin rhamnosides.

a mixture of 1 ml of DPPH solution and 1 ml of the respective solutions of test substances and standard antioxidant (stood for 5 min) were obtained at the same wavelength, taking into account the dilutions due to the mixing [16]. The percentage reduction of the DPPH activity by the standard and test solutions were estimated as "Quenching (Q)" using the formula,

$$Q = 100(A_o - A_c)/A_o \text{----------Equation 1}$$

The effective concentration required to reduce the activity of DPPH by 50% (EC_{50}) was estimated for all test extracts, fractions or compounds from the plot of percentage quenching (Q) versus concentration. The mean values compounds were compared to that of the standard antioxidant, ascorbic acid.

Proliferation assay

Proliferation assay using single cell suspension of spleen cells of C57BL/6 mice (Janvier, France) was done following a recently adapted method [17]. In summary, single cell suspension of spleen cells of C57BL/6 mice (Janvier, France) was prepared by dispersion, straining and suspending in R10 culture medium (RPMI 1640, supplemented with 10% FCS, 1% Penicillin/Strep., Gibco, Germany) The splenocytes were thereafter incubated with CFSE cell tracer (3μM) at a density of 8×10^7 cells/ml for 6 min at room temperature with gentle mixing. The labelling reaction was stopped by adding one volume of FCS (Gibco, Germany) and the cells were washed twice with PBS. Thereafter, cells were plated into 96-well plates at a density of 1×10^5 cells/well and incubated with different concentrations of compounds 1, 2 and 3 (10, 25 and 100 μg/mL), LPS (10 μg/mL), and ConA (2 μg/mL) for 72 h. After washing the cells twice with PBS containing 0.5 % BSA and 1 mM sodium azide (PBS/BSA/Azid), cell proliferation was measured by flow cytometry and FACS analysis using FACS calibur™.

Quantification of CD69 expression

The quantification of the CD69 expression was carried out following a recently adapted method [17]. Briefly, single cell suspension of spleen cells of C57BL/6 mice was prepared by dispersion straining and suspending in R10 culture medium (RPMI 1640, supplemented with 10% FCS, 1% Penicillin/Strep.) Thereafter, cells were plated into 96-well plates at a density of 1×10^5 cells/well and incubated with different concentrations of compounds above (10, 25 and 100 μg/mL), LPS (10 μg/mL), and ConA (2 μg/mL) for 24 h. After washing the cells twice with PBS containing 0.5% BSA and 1 mM sodium azide (PBS/BSA/Azid) and blocking FcR antibody; the expression of CD69 was determined by staining with FITCS-conjugated anti-CD69 antibody for 25 h at 4°C. Expression of CD69 was measured by flow cytometry and FACS analysis on FACS calibur™ and expressed as mean fluorescence intensity.

Statistical analyses

The results obtained (analysed by SPSS version 11), were recorded as the mean values with the standard error in mean (SEM) and statistical significance between treated and control groups were evaluated by the Students' t-test and one way analysis of variance (ANOVA; Fischer LSD post hoc test). Differences between means of treated and control groups were considered significant at $p<0.05$, 0.01 0r 0.001.

Results and Discussion

The proliferative effects of different concentrations (10, 25 and 100 μg/ml) of compounds I, II and III (Figure 1) on the C57BL/6 mice splenocytes are shown in Table 1. The compounds produced a dose-dependent and highly significant ($p<0.001$) stimulation of the target cells when compared to the response from the untreated group. At a 100 μg/ml concentration, compounds I, II and III produced stimulations of 91.49%, 95.17% and 94.23% respectively compared to 2.65% recorded for the untreated group. This implies an over 30 times potency of the compounds compared to the controls. Strikingly too, these compounds also performed better than the positive controls, LPS (10 μg/ml) and Con A (2 μg/ml) which exhibited stimulations of 16.09% and 20.57% respectively. The stimulation of C57BL/6 mice splenocytes suggests an up regulation of the immune function indicating an immunostimulatory potential of the compounds on these mice immune cells. It can be inferred from these data that the isolated catechin rhamnosides are potently immunostimulating on C57BL/6 mice splenocytes. It appears that the hydroxyl group at position 3 of the catechin nucleus is essential for activity as compounds II and III which had the position unsubstituted showed slightly higher activities than compound I with the position containing the glycone (rhamnoside). Methylation of the 4′-position did not produce any significant difference ($p<0.05$) in activity. These assumptions are however, subject to further detailed studies. Similarly, the stimulatory effects of the isolated compounds on the early activation marker, CD69 is shown in Table 2. The data revealed a moderate dose-dependent stimulation of this marker in manner higher than the unstimulated controls, however, lower than the positive control agents. CD69 (Cluster of Differentiation 69) is a human transmembrane C-Type lectin protein encoded by the CD69 gene. The activation of T lymphocytes, both *in vivo* and *in vitro*, induces expression of CD69. This molecule, which appears to be the earliest inducible cell surface glycoprotein acquired during lymphoid activation, is involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes, including natural killer (NK) cells, and platelets [18]. CD69 is also a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor [19]. The enhancement of expression of this early activation marker by molecules is therefore a direct measurement of the immunomodulatory potentials of such molecules. Although the stimulation produced by these compounds in their present molecular structure is moderate, detailed structure activity studies would likely produce analogues with higher potencies. Furthermore, the antioxidant potentials of the isolated catechin rhamnosides are depicted in Table 3. The antioxidant potentials, which were estimated by the DPPH free radical method, showed that the test-

Dose (μg/ml)	Compound I	Compound II	Compound III
10	82.24 ± 0.34*	86.29 ± 0.23*	85.69 ± 0.26*
25	87.51 ± 0.93**	89.42 ± 0.47**	89.95 ± 0.07**
100	91.49 ± 0.22***	95.17 ± 0.01***	94.23 ± 0.07***
LPS 2 μg/ml	16.09 ± 0.28	16.09 ± 0.28	16.09 ± 0.28
Con A 10 μg/ml	20.57 ± 0.25	20.57 ± 0.25	20.57 ± 0.25
Control (vehicle)	2.65 ± 0.33***	2.65 ± 0.33	2.65 ± 0.33

*, **, *** indicates values statistically significant at p<0.05, p<0.01 and p<0.001 respectively.

Table 1: Proliferation abilities of C57BL/6 mice splenocytes.

Dose (μg/ml)	Compound I	Compound II	Compound III
10	1.65 ± 0.03	1.90 ± 0.04**	1.76 ± 0.03**
25	1.80 ± 0.02*	1.95 ± 0.04	2.01 ± 0.01
100	1.97 ± 0.40**	2.10 ± 0.06**	1.99 ± 0.02**
LPS 2 μg/ml	5.42 ± 0.11	5.42 ± 0.11	5.42 ± 0.11
Con A 10 μg/ml	5.27 ± 0.08	5.27 ± 0.08	5.27 ± 0.08
Control (vehicle)	1.69 ± 0.05*	1.69 ± 0.05**	1.69 ± 0.05**

*, ** indicates values statistically significant at p<0.05 and p<0.01 respectively

Table 2: Stimulation of early activation marker (CD69) cells by the isolated compounds.

Compound	**EC50 value (mg/ml)	***Potency factor
Compound I	55.42 ± 0.99	3.14
Compound II	58.45 ± 1.07	3.32
Compound III	59.71 ± 1.63	3.39
Compound III	59.71 ± 1.63	3.39
Ascorbic acid (standard)	17.6 ± 1.78	1.00

EC_{50} is the effective concentration of a substance estimated to cause a 50 % reduction in the total free radical activity of DPPH. * Potency factors between 1.00 and 3.50 were considered suitable candidates or sources for possible antioxidant development.

Table 3: Antioxidant potentials of crude extract, solvent fractions and catechin rhamnosides.

ed compounds were reasonably active compared to the standard drug, ascorbic acid. However, the standard, ascorbic acid was generally, 3 times more active than the respective compounds in their present structural forms. It is important to emphasize that the assessment model in this experiment is an *in vitro*-based type suggesting that a possible potentiation of activity when assayed by *in vivo* models. It is further envisaged that guided structural modifications of the compounds will afford more potent analogues. The very many documented biological activities of the eastern Nigeria mistletoe may be attributed largely to this established antioxidant property. In concrete terms, it is needful to assess this property with several other models depicting both *in vivo* and *in vitro* environment. Compound I was isolated and purified as yellow semi solid compound; yield (35 mg) and exhibited a positive reaction with $FeCl_3$, a yellow colour which deepens in alkaline solution. The UV $\lambda_{max\ (in\ methanol)}$ nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminium chloride $AlCl_3$ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH in A-ring of the flavonoid nucleus. The FT-IR $vmax^{KBr}$ showed values at 3971-3400 (OH), 2941 (CH_3), 1701 and 1614 (C=C), 1022-613 (aromatic finger print). These were assigned to position 6 and 8 in ring A of the flavonoid nucleus. The absence of a carbonyl functional group in both C-13 NMR and FT-IR suggested the absence of the usual ketonic group at position 4 of most flavonoids apart from catechins [20]. The HMBC spectra suggested that the sugar linkage was at the position 7 of the A-ring of the flavonoid nucleus. DEPT-135 studies of compound I also supported the proposed structure. The proposed structure for compound I was elucidated as catechin-7-O-α-rhamnoside based on available spectral data and comparison with other published data [21]. This was typical of 7-O-based catechin rhamnosides because they are known to have their rhamnoside methyl groups resonating above δ value of 1.00 [21]. In addition there were no further signals suggesting the presence of extra sugar moiety. Compound II was isolated and purified as a yellow semi solid compound; yield (115 mg). It showed a positive reaction with $FeCl_3$, producing a yellow colour which deepened in alkaline solution. The ^{1}H-COSY, HSQC and HMBC of compound II supported the fragments identified as sugar portion and the aromatic region. Connectivities were confirmed by the 2-D correlation. In addition, the DEPT-135 studies of compound II also supported the proposed structure. The proposed structure for compound II was elucidated as catechin-3-O-α-rhamnoside based on available spectral data and comparison with other published data [22]. The IH-NMR of compound II showed two meta coupled (J=1.2) protons resonating at δ 6.38 and 6.93. These were assigned to position 6 and 8 in ring A of the flavonoid nucleus. The absence of a carbonyl functional group in both C-13 NMR and FT-IR suggested the absence of the usual ketonic group at position 4 of most flavonoids apart from catechins [23]. Unequivocal evidence for this observation was further provided by the peaks resonating thus, at δ 2.48 (1H, dd, J=3.5, 16.3 Hz, δ 2.75 (1H, dd, J=4.6, 16.3) and representing the two protons at position 4 of the flavan nucleus. These observations describe B- and C-ring protons found in catechins [24]. It has been reported that methylation and or glycosylation of hydroxyl group(s) of ring B causes a downfield shift of ortho, meta and para protons [25,26]. The observation that all the B-ring protons resonated at normal ranges suggested also that there was no attachment in the OH's of ring B. The 1H-NMR spectrum also indicated occurrence of protons close to oxygenated carbons (δ 3.0-5.5 ppm) signifying the presence of sugar moiety [25]. Furthermore, the presence of signal resonating at δ 4.96 ppm is assignable to the anomeric carbon of a sugar moiety, likely rhamnose, further evidence of which is the presence of a rhamnose methyl signal at δ 0.86. The HMBC correlation of H1 of the sugar with H-3 of the catechin nucleus gave a clue that the position of the sugar was at position 3 in the flavonoid nucleus. As was mentioned above, the appearance of a methyl signal at δ 0.86, added credence to the argument that the attached sugar moiety was indeed a rhamnose. In addition, there were no other signals suggesting the presence of extra sugar moiety or further attachments. Furthermore, 3-O-rhamnosides based flavonoids have been reported to cause upfield shift of H-2′ and H-6′ signals of the flavonoid nucleus [25]. These observations suggested in strong terms, that the proposed structure of compound II was catechin-3-O-rhamnoside. Compound III was isolated as a yellow semi solid compound; yield (11 mg). It showed positive reaction with $FeCl_3$, a yellow colour which deepens in alkaline solution. UV $\lambda_{max\ (in\ methanol)}$ nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride $AlCl_3$ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH. With sodium acetate (NaOAc) powder, UV changes to 337, 372, and 382 (5-OH and 7-OH free). The FT-IR $vmax^{KBr}$ showed values at 3971-3400 (OH), 2941 (CH_3), 1701 and 1614 (C=C), 1022-613 (aromatic finger print). The DEPT-135 studies of compound III also supported the proposed carbon structure. The proposed structure for compound III was elucidated as 4′-methoxy-catechin-7-O-α-rhamnoside based on available spectral data [21]. In addition to the above arguments in favour of the catechin nucleus based rhamnosides, compound III showed a downfield shift of some B-ring protons indicating a methylation of the B-ring [26]. The signal at δ 3.82-3.90 was assignable to 4′-methoxy group [25,26]. For all these catechin-based flavonoids, the use of chemical shift reagents afforded further evidence that the 5-OH position of ring A was free for compound I, compound II and compound III. In compound I and compound III, the 7-OH of ring A was not free. The free 5-OH position was confirmed from the observation that addition of $AlCl_3$ (5 %) to the ethanol solution of the flavonoid glycosides caused a bathochromic shift in the UV absorption band [25]. In addition, a free 7-OH position is confirmed by the bathochromic shift caused by the introduction sodium acetate powder to an ethanolic solution of the flavonoid glycoside [25]. Although overwhelming evidence has been provided in this discuss supporting the proposed structures, further studies may be necessary to confirm an unequivocal proof of structure.

Spectral data of isolated compounds

Compound 1 was isolated as a yellow semi solid compound; yield (35mg). It showed positive reaction with $FeCl_3$, yellow colour deepens in alkaline solution an indication of a phenolic and or a flavonoidal compound.

CH_3OH

UV λ_{max} nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride $AlCl_3$ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH in A-ring of the flavonoid nucleus. IR $vmax^{KBr}$ 3971-3400 (OH), 2941 (CH_3), 1701 and 1614 (C=C), 1022-613 (aromatic finger print).

^{1}HNMR (DMSO, 500 MHz): δ 2.45 (1H, dd, H4), δ 2.75 (1H, dd, H4), δ 1.25 (1H, d, H-6 of a 7-O-rhamnoside), δ 6.60 (1H, dd, H-6), δ 6.80 (1H, dd, J=1.2, H-8), δ 3.84 (H-4 of rhamnoside) δ 5.25 (1H, dd, H1 on anomeric carbon of sugar) δ 3.92 (1H, dd-H2 of sugar), δ (3.70, 1H, dd, H3 of sugar), δ (6.70, 1H, d-H2′), δ 7.10 (1H, d, H5′), δ 7.25 (1H, H6′), δ 8.1 (1H, d, H3′-OH), δ 8.75 (1H, d,H4′-OH), δ 9.70 (1H, d, 5-OH). ^{13}CNMR (DMSO, 500 MHz): δ 60.58 (C2), δ 60.32 (C3), δ 42.54 (C4), δ 130 (C1′), δ 115.35 (C5′), δ 55.97 (C3′′), δ 56.40 (C4′′), δ 40.35 (C2′′), δ 40.36 (C5′′), δ 39.86 (C6′′).

The proposed structure for compound I was elucidated as catechin-7-O-α-rhamnoside based on available spectral data and comparison with other published data [21]. DEPT-135 studies of compound 1 also supported the proposed structure. Compound 2 was isolated as a yellow semi solid compound; yield (115 mg). A positive reaction with $FeCl_3$, yellow colour which deepens in alkaline solution was observed and indicated phenolic and or a flavonoidal compound.

CH_3OH

UV λ_{max} nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminum chloride $AlCl_3$ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH. With sodium acetate (NaOAc) powder, UV changes to 337, 372 and 382 (5-OH and 7-OH free).

IR *vmax^{KBr}* 3971-3400 (OH), 2941 (CH_3), 1701 and 1614 (C=C), 1022-613 (aromatic finger print). ^{1}HNMR (DMSO, 500 MHz): δ 2.48 (1H, dd, J=3.5, 16.3 Hz, H4), δ 2.75 (1H, dd, J=4.6, 16.3 Hz, H4), δ 6.38 (1H, d J=1.2, H6), δ 6.93(1H, dd, J=1.2-H8), δ 4.96 (anomeric proton), δ 4.50(1H, H1′′), δ 0.86 (3H, dd-methyl of rhamnose), 3-4.0 (1H, m, H5′′), δ 4.05 (1H, dd, H2) δ 3.61 (1H, dd, H3), δ (1H, dd, H3), δ 6.63 (1H, dd, H5′), δ 6.65 (1H, dd, H6′), δ 6.93 (1H, d, H2′). ^{13}CNMR (DMSO, 500 MHz): δ 77.14 (C2), δ 63.42 (C3), δ 34.57 (C4), δ 155.98 (C5), δ 108.20 (C6), δ 153.11 (C7), δ 142.52 (C9), δ 129.70 (C1′), δ 114.98 (C2′), δ (106.61 (C3′), δ 142.35 (C4′), δ115.38 (C6′), δ 110.60 (C6′), δ 39.73 (C7′) δ 73.28 (C1′′), δ 102.97 (C2′′), 56.25 (C3′′), δ 59.90 (C4′′), δ 69.89 (C5′′), 39.27 (C6′′).

^{1}H-COSY, HSQC and HMBC of compound 2 supported the fragments identified as sugar portion and the aromatic region. The proposed structure for Compound 2 was elucidated as catechin-3-O-α-rhamnoside based on available spectral data and comparison with other published data [21]. DEPT-135 studies of compound 2 also supported the proposed structure. Compound 3 also a yellow semi solid compound; yield (11 mg) was observed and showed a positive reaction with $FeCl_3$, yellow colour deepens in alkaline solution.

CH_3OH

UV λ_{max} nm (ε): 262 (15400), 338 (16250), 358, 391. With aluminium chloride $AlCl_3$ and HCl, UV changes to 320, 358 and 378 indicating a free 5-OH. With sodium acetate (NaOAc) powder, UV changes to 337, 372, and 382 (5-OH and 7-OH free). IR *vmax^{KBr}* 3971-3400 (OH), 2941 (CH_3), 1701 and 1614 (C=C), 1022-613 (aromatic finger print) ^{1}HNMR (DMSO, 500 MHz): δ 2.35 (1H, dd, J=3.5, 16.3 Hz, H4), δ 2.75 (1H, dd, J=4.6, 16.3 Hz, H4), δ 6.60 (1H, d J=1.2, H6), δ 6.80(1H, dd, J=1.2-H8), δ 4.96 (anomeric proton), δ 4.50(1H, H1′′), δ 0.86 (3H, dd-methyl of rhamnose), δ 3.84 (H-4 of rhamnoside) δ 5.75 (1H, dd, H1 on anomeric carbon of sugar) δ 3.92 (1H, dd-H2 of sugar), δ (3.70, 1H, dd, H3 of sugar), δ 3-4.0 (1H, m, H5′′), δ 4.05 (1H, dd, H2), δ 3.61 (1H, dd, H3), δ (1H, dd, H3), δ 7.10 (1H, dd, H5′), δ 7.30 (1H, dd, H6′), δ 6.70 (1H, d, H2′), δ 6.90 (1H, dd-H1′), δ 7.50 (1H, m, H1′), δ 3.82-3.90 (3H,s, 4′ methoxyl; the OH of the 4′ position is the most acidic), δ 9.2 (1H, d, H3′-OH), δ 9.70, (1H, d,H4′-OH). ^{13}CNMR (DMSO, 500 MHz): δ 130.30 (C1′), δ 115.36 (C2′), δ (106.61 (C3′), δ 142.35 (C4′), δ115.38 (C6′), δ 110.60 (C6′), δ 39.73 (C7′), δ 39.27 (C6′′). The proposed structure for compound 3 was elucidated as 4'-methoxy-catechin-7-O-α-rhamnoside based on available spectral data. DEPT-135 studies of compound 3 also supported the proposed structure.

Conclusion

The present study has demonstrated the *in vitro* antioxidant and immunostimulatory properties of three catechin rhamnosides obtained from the eastern Nigeria mistletoe. It has provided some logical evidence supporting the traditional uses of the eastern Nigeria mistletoe as an immunomodulants and antioxidant. Furthermore, this study suggests that, antioxidation; in addition to immunomodulation may be one of the mechanisms of action of mistletoe as an established antidiabetic agent.

Acknowledgements

The authors wish to thank Professor Dr. Peter Procksh, his sponsors and colleagues in Düsseldorf for their efforts and resources that led to the structural assignments of these compounds. Dr. CS Nworu is highly appreciated for performing the *in vitro* screening and FACS analysis on the compounds.

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