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Immunomodulatory activity of a lupane triterpenoid ester isolated from the eastern Nigeria mistletoe, *Loranthus micranthus* (Linn)

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ABSTRACT

Objective: To provide further evidence for the ethnomedicinal use of the Eastern Nigeria mistletoe, *Loranthus micranthus (L. micranthus)*, as an immunostimulant. **Methods:** Solvent fractions from the crude extract of the mistletoe plant was obtained and screened by the cell mediated delayed type hypersensitivity reaction (DTHR) model in mice. Then the immunomodulatory potentials of a major lupane triterpenoid ester isolated from an active hexane fraction of the Eastern Nigeria mistletoe was investigated. Three lupeol-based triterpenoid esters: 7 β 15 α -dihydroxyl-lup-20(29)-ene-3 β -palmitate (Ⅰ), 7 β , 15 α -dihydroxyl-lup-20(29)-ene-3 β -stearate (Ⅱ) and 7 β , 15 α -dihydroxyl-lup-20(29)-ene-3 β -decadecanoate (Ⅲ) were isolated from the plant leaves epiphyting on a local kola nut tree and were characterized. Compound 1 was subjected to cell proliferation studies using C57Bl/6 splenocytes at three dose levels (5, 25 and 100 μg/mL) in presence of controls. Furthermore, the effect of this compound on IL-8 receptor expression was evaluated at three doses (1, 5 and 10 μg/mL) using the real time polymerase chain reaction assay. **Results:** This triterpenoid ester produced some enhancement of the splenocytes at the tested doses but at doses higher than 5 μg/mL caused inhibition of the IL-8 receptor expression. **Conclusions:** The present findings support the ethnomedicinal use of the Eastern Nigeria Mistletoe in the management of diseases affecting the immune system. The triterpenoid(s) have some immunomodulatory abilities on splenocytes and IL-8 receptors and may partly account for the overall immunomodulatory activity of this plant.

1. Introduction

The complex human immune system could be described aptly as an orchestra for the maintenance of homeostasis by its intricate ability to modulate myriads of immune responses. This action of the immune system precipitates strong defense against pathogens that frequently assaults the human health integrity[1]. Modulation of the immune system involves either stimulation on one part, or suppression at the other extreme[2]. The stimulation of defense mechanistic pathways has been recognized as a possible means of inhibiting disease progression in humans without, *per se*, eliciting harmful effects[3,4]. It is now recognized that immunomodulatory therapy could provide an alternative to conventional chemotherapy of a variety of disease conditions[5]. Essentially, immunomodulatory agents of plant origin are claimed to induce paraimmunity, the non-specific immunomodulation of granulocytes, macrophages, natural killer cells and complement functions[6,7]. They do so by maintaining body homeostasis[8] and through adaptogenic activities[9,10]. Furthermore, the emergence of acquired immunodeficiency syndrome (AIDS), in the 1980s and the concern about bioterrorism have increased the emphasis on the role of immune system in defending individuals against diseases[11]. Mistletoes are semi-parasitic ever-green plants which depend on their host tree for minerals and water only but photosynthesize their carbohydrate by means of its green leaves[12]. They grow on a variety of evergreen and deciduous trees. Over 700 species

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of the mistletoe plants are known world-wide[13]. Scientific studies, especially of the European mistletoe have shown that composition or activities of mistletoe are dependent on the host tree, species and harvesting period[14-18]. The macromolecular and micromolecular components of different species of mistletoes sourced from various host trees may also vary. The Eastern Nigeria mistletoe, *Loranthus micranthus (L. micranthus)* has been employed traditionally in management of various ailments, notably diabetes, high blood pressure and conditions affecting human immune system for many years. Aqueous decoctions or alcoholic extracts of *L. micranthus* have diverse folkloric claims of effectiveness for treatment of epilepsy, diabetes, hypertension, cardiovascular diseases, menopausal syndrome, infertility, rheumatism, agglutination and in conditions generally requiring modulation of the immune system. It is equally used locally as an antimicrobial and antispasmodic agent. The morphology, distribution and some biological activities of mistletoes in Nigeria, and in particular, Eastern Nigeria has been well-documented[19]. The present study involves the bio-assay guided isolation and characterization of the immunomodulatory constituents of the Eastern Nigeria mistletoe from the *n*-hexane fraction of the crude extract obtained from the host tree, *Kola acuminate (K. acuminata)* using cell proliferation studies by flow cytometry technique and real time polymerase chain reaction assay. To the best of our knowledge, this is the first attempt to isolate and characterize chemical constituents from Eastern Nigeria mistletoe, *L. micranthus*.

2. Materials and methods

2.1. Plant material

L. micranthus Linn. (Loranthaceae) leaves parasitic on the host tree *K. acuminate* were collected in April, 2007 from different locations in Nsukka LGA, Enugu State. The leaves were identified and certified by Mr. A. O. 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 BDC-532-07 for reference purposes.

2.2. Solvents and reagents

The following chemicals were procured and used in the research: Analar grade methanol, n-hexane, ethylacetate, acetone, chloroform (Sigma Aldrich; Germany). Distilled water (Department of Pharmaceutical & Medicinal Chemistry, University of Nigeria), normal saline (DANA Ltd), dimethylsulphoxide (DMSO), Tween 20 or 80 solution (BDH, England), silica gel (70-230 and 60-120 mesh sizes), silica gel G60, precoated silica gel GF254, (Merck, Germany) were also used. All other reagents were of analytical grade or freshly prepared. Absence of water in analar grade solvents was confirmed by charge transfer complexation process using chloranilic acid as the π-electron acceptor. When necessary, such solvents were redistilled.

2.3. Equipment

The following items of equipment were used: Gallenkamp or Electrothermal melting point apparatus (England; used uncorrected), HREIMS and EIMS (mass spectrometers) linked to a MATT 8200 recorder, at the Institute of Anorganic Chemistry and Structure Chemistry, Heinrich-Heine-Universität, Düsseldorf, Germany and FT-IR spectrometer (Shimadzu, Japan) at the Department of Chemistry, Usmanu Dan Fodiyo University, Sokoto, GC-MS analyses were done with (GCMS-QP2010 Plus, Shimadzu, Japan) with a mass selective detector coupled to GC-2010 gas chromatograph at the National Research Institute of Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria.

^{1}HNMR, ^{13}CNMR, were recorded with BRUKER-500 MHz spectrophotometer in CD3OD 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. Correlations (COSY; HH, HMBC, HQSC *etc*) and variants (DEPT-135) experiments were also done in these institutions. 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. Other equipment used were soxhlet apparatus, rotary evaporator, separatory funnel, capillary tubes (0.5 mm id), electronic analytical balance (Metler Toledo, 0.001 max, England), Beam balance (Ohaus, England), venier calipers, glass columns for chromatography (4 cm × 150 cm; 2.7 cm × 70 cm), analytical and preparatory thin layer chromatographic tanks, 20 cm × 20 cm, 20 cm × 10 cm or 20 cm × 5 cm chromatographic plates, amber coloured bottles, cannulated or normal syringes (1 mL, 2 mL, 5 mL, 10 mL and 20 mL).

2.4. Preliminary extractions from mistletoe parasitic on K. acuminata

2.4.1. Preparation of crude methanol extract

Leaves of *L. micranthus* parasitic on *K. acuminata* were cleansed and dried under shade. They were then pulverized in mechanized laboratory grinder to produce fine powder. A total of 200 g of the powder was extracted in batches with 500 mL methanol using a soxhlet extractor. The solvent was removed in vacuo, using rotary evaporator at (45 ± 5) ℃ to dryness under dry air to give a dry extract which was weighed and its percentage yield was calculated. The dry extract was placed in a clean bottle container and stored in a refrigerator until use.

2.4.2. Experimental animals used

Groups of albino mice (20-33 g) or rats (120-165 g) of both sexes were procured from the animal house, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical

Sciences, and from the animal house, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The blood specimens used in the study were collected from sheep in the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The animals were kept in standard laboratory conditions and fed with pelletized rodent commercial diet (Vital Feed Nig., Ltd) and water *ad libitum* throughout the study. They were exposed to 12 h light-dark cycles. These investigations were conducted following laid down procedures by the relevant Ethical Committee on laboratory animal use and international rules were observed.

2.4.3. Preparation of antigen

Fresh blood was obtained from a male sheep in the animal farm of the Faculty of Veterinary Medicine, University of Nigeria. The sheep red blood cells (SRBCs) were washed three times in a large volume of pyrogen-free sterile normal saline by centrifugation at 3 000 × *g* for 10 min on each occasion. The washed SRBCs were adjusted to a concentration of 10^9 cells/mL with normal saline and used for immunization and challenge[20].

2.4.4. Solvent fractionation of extract of mistletoe from K. acuminata

Exactly 50 g of the dried aqueous methanol extract was re-dissolved in minimal quantity of methanol and adsorbed on 250 g of silica gel (Silica gel 60G); that is in a ratio of 1:5. This was dried and finely pulverized to provide a free flowing powder mass. This was poured into a stoppered 1 L-size flat bottom flask. Then equal volumes (500 mL) of each solvent were used in repeated succession with strong but uniform agitation until the extract so obtained with the solvent became convincingly clear. Each time, the extract was filtered using filter papers. The next solvent higher in polarity was introduced for further extraction after drying off the preceding solvent. Five solvents in increasing order of polarity namely; *n*-hexane, chloroform, ethylacetate, acetone and methanol were used in the fractionation. Subsequently, the fractions obtained were evaporated to a small pourable mass under reduced pressure (rotary evaporator) maintained at a temperature of (40 ± 5) ℃. The dried fractions obtained thereafter were weighed and the percentage yields calculated. The dry fractions were placed in clean plastic containers and stored in a refrigerator until use.

2.4.5. Phytochemical test on the crude extract and solvent fractions

Phytochemical tests on the crude extract and all five solvent fractions were carried out. This was done as described by previous workers[21]. All reagents for the phytochemical tests were freshly prepared.

2.4.6. Immunomodulatory studies on solvent fractions

The cell-mediated delayed type hypersensitivity reaction (DTHR) model was used to screen all the solvent fractions (n-hexane (HF), chloroform (CF), ethylacetate (EF), acetone (AF) and methanol (MF), at two dose levels of 250 and 500 mg/kg body weight. Briefly, the method described by Chong and co-workers[22] in 1998 was used for the DTH studies. Briefly, 10 mL of blood was obtained from sheep and was washed three times with 20 mL of normal saline each for 10 min in a centrifuge at 2 500 rpm. After washing and packing the cells at the same speed, a 2.5 % v/v SRBC suspension (1.0×10^9 cells/mL) was then made in normal saline. Sixteen albino mice of either sex were selected at random and distributed into four groups (four mice per group). Starting with the methanol extract, the test animals in groups one to three received the extract at doses of 100, 200 and 400 mg/kg intraperitoneally (i.p.) respectively for eight consecutive days while the fourth group served as the negative control. The diameters of the mice hind footpads were measured using a venier calliper. On the last day of treatment, the mice were all sensitized with a subcutaneous injection of 0.1 mL of the freshly prepared SRBC suspension into the right hind footpad (RHFP). Five days post-sensitization, a challenging dose of 0.1 mL of the freshly prepared SRBC suspension was injected into the left hind footpad (LHFP). The induration was read 24 h later using a venier calliper. The above procedure was repeated using the fractions on separate groups of animals and fresh SRBC suspensions.

2.4.7. Isolation and purification of constituents from n-hexane fraction (HF)

Exactly 10.5 g of the hexane fraction was chromatographed on silica gel (70-230 mesh, 1.2 kg) packed in a glass column (4 cm × 150 cm) with the bed of 105 cm in height. The elution was performed with gradient mixtures of hexane, hexane: ethyl acetate, ethyl acetate. Aliquots of 25 mL were collected and monitored as previously described. Fourteen (14) fractions F1-F14 (1-5, 6-25, 26-37, 38-41, 42-65, 66-73, 74-93, 94-120, 121-136, 137-167, 168-175, 176-225, 226-260, 261-269). These fractions were screened again by DTHR assay. F12 (176-225), 0.9 g the most active fraction in terms of immunostimulation, was chromatographed on silica gel (70-230 mesh, 650 g) packed in a glass column (2.5 cm × 85 cm) with the bed of 65 cm in height using gradient elution technique as above. This repeated chromatographic process afforded compounds I (34 mg), II (12 mg) and III (8 mg) which were recrystallized in acetone-methanol mixture. Further purification of the compounds was achieved by a combination of analytical and prep-TLC. The phytochemical identities of the compounds were ascertained by standard procedures.

2.5. Cell proliferation studies with isolated compounds

2.5.1. Proliferation assay

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.) The splenocytes were thereafter incubated with CFSE cell tracer (3 μ mol/L) 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 after which 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 compound I (5, 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 mmol/L sodium azide (PBS/BSA/Azid), cell proliferation was measured by flow cytometry and FACS analysis using FACScaliburTM.

2.5.2. Quantification of CD69 expression

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 test extracts (5, 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 mmol/L 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 ℃. Expression of CD69 was measured by flow cytometry and FACS analysis on FACS FACScalibur™ and expressed as mean fluorescence intensity.

2.5.3. Real time polymerase chain reaction (RT-PCR) test

The effect of 1 μg/mL, 5 μg/mL, and 10 μg/mL of compound I on interleukin-8 expression was assessed by real time polymerase chain reaction assay and compared with *Juzen-taito-to* (JTT; an oriental herbal formulation for immunostimulation) as positive standard. Briefly, human monocytic leukemia cells (THP-1) were plated onto each well of a 6-well plate at about 20 % confluence in 2 mL RMPI medium with 10 % foetal bovine serum (FBS) and incubated for 24 h to allow them to recover from passage. Cells were treated with compound I (1 mg/mL in DMSO; final concentrations 1, 5, 10 μg/mL) for 4 h. DMSO vehicle control was used as the negative control in this assay, whereas *Juzen-taito-to* was used as positive control. RNeasy mini kit (Qiagen) was used to purify the total RNA. RNA samples were quantified using UV absorbance at 260 nm. Samples with the 260 nm/280 nm ratio ~ 1.8 or higher were used for the subsequent study. The first strand cDNA samples were synthesized with Gibco BRL Superscript Choice System and oligo (dT) 12-18 primer. TaqMan® Gene Expression Assays (Applied biosystems) were carried out on Applied Biosystems 7500 Real-Time PCR system using pre-optimised assays for interleukin-8 (IL-8) and GAPDH (endogenous control). The ΔΔ CT method was employed to quantify differential gene regulation. The raw data were first normalised by the endogenous control (GAPDH). The relative quantification (RQ) values, *i.e.* the fold-change ratios, were obtained by comparing the normalized data against the DMSO control. Minimum triplicate experiments were carried out.

2.6. 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 group and also between solvents and extracts at $P<0.05$ or $P<0.01$ was considered significant.

3. Results

The phytochemical chemical profile of crude aqueous methanol extract of mistletoe is shown in Table 1. From the table, the Eastern Nigeria mistletoe is phytochemically, a richly constituted parasitic plant. In terms of quantities in gram weights of the recovered solvent fractions, the least polar of the solvents under consideration, n-hexane afforded the highest yield of 14.0 g or 28.0% yield while acetone gave the least yield of 2.2 g (4.4%). The n-hexane and chloroform fraction were both dark greenish and highly resinous while the ethylacetate fraction was moderate in resin content.

The result of the comparative phytochemical tests on all the different solvent fractions as well as the crude aqueous methanol extract is shown in Table 2. It is evident that the non-polar solvents extracted mainly the non-polar constituents while the moderately polar solvents, especially ethylacetate extracted the moderately polar constituents such as flavonoids and other polyphenolics. Acetone and methanol obviously showed presence of highly polar constituents.

Table 1
The result of the preliminary phytochemical analysis.

Constituent	Presence or absence
Carbohydrate	+++
Alkaloids	+++
Reducing sugar	++
Glycoside	+++
Saponin	++
Tannin	++
Flavonoids	++
Steroids	++
Resins	+++
Terpenoids	++
Carotenoids	++++
Fats and oil	−
Proteins	+
Acidic compound	+

+++ = present in very high concentration; ++ = present in moderately high concentration; + = present in small concentration; − = not present.

The result of the DTHR of the different solvent fractions is as shown in Figure 1. In summary, at 500 and 250 mg/kg

body weight dose levels, the percentage stimulation observed were as follows: chloroform fraction-311.11 and 122.22%, ethyl acetate fraction-193.38 and 95.56%, *n*-hexane-155.56 and 3.50%, acetone fraction-77.78 and 51.11% and methanol fraction-68.89 and 24.44% respectively. Levamisole, a known potent immunostimulant, afforded a stimulation of 68.89% at a dose of 2.5 mg/kg i.p. The order of potency is therefore, chloroform fraction>ethylacetate fraction>*n*-hexane fraction>acetone fraction>methanol fraction. All responses were dose-dependent. These values exhibit statistically ($P<0.05$) significant difference with that of the standard immunostimulant, Levamisole. Repeated fractionation and purification of the *n*-hexane fraction led to the isolation of a major triterpenoid ester alongside (compound I) two other minor closely related triterpenoidal esters (compounds II and III) all, of the lupane series, specifically lupeol. The general structure of the triterpenoids is shown in Figure 2. Compound I was obtained as a white amorphous compound; yield (34 mg), m.p = 107.0 ℃. A solution of Compound I in chloroform showed positive reaction to concentrated sulphuric acid (Salkowski test) indicating a terpenoid. UV λ_{max} ($CHCl_3$) nm (ε): 250 (11 100), 268 (1 890.4), 289 (1 160), IR (KBr) cm^{-1}: 3167 (OH), 2 872 (CH str), 1 728 (C=O), 1 645 (C=C), 1 384 (isopropylene side chain), HREIMS [M]+ m/z: 696.602 2 (calculated for $C_{46}H_{80}O_4$) with 7 double bond equivalent (DBE). [M+H]+ [$-H_2O$] m/z: 679.602 1 (loss of water), $[M+Na]^+$ m/z: 719.593 6, $[M^+HCOO]^-$ m/z: 741.604 5, $[M+Cl]^-$ m/z: 731.575 1, $[M^+CF_3COO]^-$ m/z: 809.592 0, (all calculated for $C_{46}H_{80}O_4$). EIMS (relative int.), M^+ (m/z): 696.

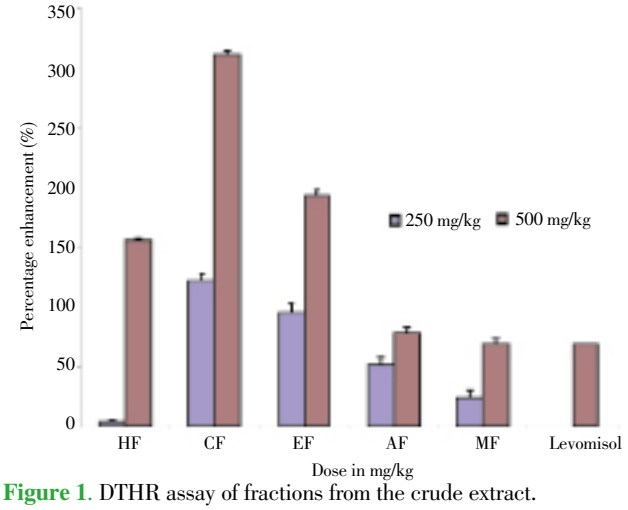

Figure 1. DTHR assay of fractions from the crude extract.

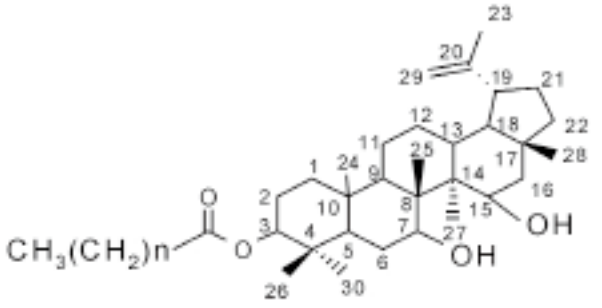

Figure 2. Structures of the triterpenoids.

^{1}H-NMR ($CDCl_3$, 600 or 500 MHz): δ 4.66(1H,d, H-29), δ 4.57 (1H, br,s, H-29), δ 4.45 (1H,dd, H-3), δ 4.14 (1H, dd, H-5), δ 3.79 (1H,dd, H-15) seven singlet methyls at δ (0.84, 1.67, 0.81, 0.83, 0.82, 1.06, 0.97, methyls of lupeol moiety) and a triplet-doublet methyl signal at δ 0.86 (terminal methyl of fatty acid chain), multiple flexible and overlapping methylenes of the fatty acid chain at δ ~1.22-1.27.

^{13}C-NMR ($CDCl_3$, 500 MHz) at δ 38.62 (C1), 23.96 (C2), 80.37 (C3), 37.77 (C4), 52.31 (C5), 28.25 (C6), 72.71 (C7), 48.05 (C8), 50.39 (C9), 37.42 (C10), 20.81(C11), 25.02 (C12), 37.63 (C13), 49.15 (C14), 68.25 (C15), 45.90 (C16), 42.78 (C17), 48.29 (C18), 47.77 (C19), 10.52 (C20), 30.33 (C21), 39.99 (C22), 16.65 (C23), 28.10 (C24), 15.82 (C25), 11.16 (C26), 8.60 (C27), 42.78 (C28), 109.96 (C29), 19.60 (C30); fatty acid chain: δ 173.91 (C1′-ester carbonyl), 35.05 (C2′), 25.38 (C3′), 29.82 (C4′), 29.69 (C5′), 29.59 (C6′), 29.49 (C7′), 29.39 (C8′), 29.92 (C9′), 29.90 (C10′), 29.89 (C11′), 29.87 (C12′), ? (C13′), 32.15 (C14′), 22.92 (C15′), 14.36 (C16′).

DEPT-135 signals observed for 8 methyls, 23 methylenes, 8 methines carbons. 7 quaternary carbons were not shown in the DEPT. 1H-COSY, HSQC, and HMBC studies confirmed all the fragments and connectivities. Positive NOE and NOESY confirmed spatial arrangements or configurations. Spectral data correlates well with literature data for dihydroxylated hydroxylated esterified lupane nucleus (lupeol) and was elucidated to be 7 β, 15 α-dihydroxy-lup-20(29)-en-3 β-O-palmitate.

Compound II was isolated as a white amorphous compound; yield (12 mg), m.p = 107.0 ℃. UV λ_{max} ($CHCl_3$) nm (α): 250 (11 100), 268 (1 890.4), 295 (1 160). IR (KBr) cm^{-1}: 3 167 (OH), 2 872 (CH str), 1 728 (C=O), 1 645 (C=C), 1 384 (isopropylene side chain). HREIMS $[M]^+$ m/z: 724. 637 4 (calculated for $C_{48}H_{84}O_4$) with 7 double bond equivalent (DBE). $[M+H]^+$ [$-H_2O$] m/z: 707.632 9 (loss of water), $[M^+NH4]^+$ m/z: 742.670 5, [M+HCOO]- m/z: 769.635 4, $[M^+Cl]^-$ m/z: 731.575 1, $[M^+CF_3COO]^-$ m/z: 837.621 3, (all calculated for $C_{48}H_{84}O_4$). EIMS (relative int.), M^+ (m/z): 724.

Compound III was observed as a white amorphous compound; yield (8 mg), m.p = 107.0 ℃. UV λ_{max} ($CHCl_3$) nm (ε): 250 (11 100), 268 (1 890.4), 295 (1 160). IR (KBr) cm-1: 3 167 (OH), 2 872 (CH str), 1 728 (C=O), 1 645 (C=C), 1 384 (isopropylene side chain). HREIMS [M]+ m/z: 752.667 1 (calculated for $C_{50}H_{88}O_4$). $[M^+H]^+$ [$-H_2O$] m/z: 735.663 6 (loss of water), $[M^+HCOO]^-$ m/z: 797.664 8, $[M^+Cl]^-$ m/z: 787.635 1, $[M^+CF_3COO]^-$ m/z: 865.652 1, (all calculated for $C_{50}H_{88}O_4$). EIMS (relative int.), M^+ (m/z): 752.

The ^{1}H-NMR and ^{13}C-NMR data of compounds II and III were found to be similar to those of Compound I except that two and four extra methylene groups were observed for them respectively. These extra methylene groups were revealed by 2-D correlation studies to be part of the fatty acid chain. Spectral data for compound II correlate well with literature data for hydroxylated esterified lupane nucleus and was elucidated to be 7 β, 15 α-dihydroxy-lup-20(29)-en-3 β-O-stearate while the spectral data of Compound III correlate well with literature data for hydroxylated esterified lupane nucleus and was elucidated to 7 β, 15 α-dihydroxy-lup-20(29)-en-3 β-O-decadecanoate. All the original spectra data are with the author for correspondence.

Table 3 and 4 show some potentiating abilities of compound

Table 2
Phytochemical analyses on crude and solvent fractions.

Constituent	Solvent fractions					
	Crude	*n*-Hexane	Chloroform	Ethylacetate	Acetone	Methanol
Carbohydrate	++++	-	-	++	++	+++
Alkaloids	++++	-	+	+	+	+++
Reducing sugar	+++	-	-	+	+	+
Glycoside	++++	-	+	+++	+++	+++
Saponin	++	-	-	-	+	++++
Tannin	+++	-	-	+++	+++	++++
Flavonoids	++	-	+	++++	+++	++
Steroids	+++	+	++++	+++	+	+
Resins	++++	++++	+	++	-	-
Terpenoids	+++	+	++++	+++	+	+
Proteins	+++	-	-	+++	++	++
Fats and oil	-	-	-	-	-	-
Acidic compound	+	-	-	-	+	+

Key: ++++ = present in very high concentration; +++ = present in high concentration; ++ = present in moderate concentration; += present in small concentration; - = not present.

Table 3
Proliferation potentials of Compound Ⅰ on C57B1/6 Spleenocytes.

Drug	Dose (μg/mL)	Percentage proliferation (%)
Compound Ⅰ	5	18.34 ± 1.56
Compound Ⅰ	25	20.08 ± 0.83
Compound Ⅰ	100	24.44 ± 2.58
LPS	10	34.61 ± 0.44
Con A	2	34.01 ± 0.32
Control	Vehicle	7.69 ± 0.41

Table 4
Proliferation potentials of Compound Ⅰ on CD69 cells.

Drug	Dose (μg/mL)	Stimulation	Percentage yield stimulation (%)
Compound Ⅰ	5	2.45 ± 0.07	33.14
Compound Ⅰ	25	2.71 ± 0.06	60.36
Compound Ⅰ	100	3.16 ± 0.06	86.98
LPS	10	5.42 ± 0.11	220.71
Con A	2	5.27 ± 0.08	211.83
Control	Vehicle	1.69 ± 0.05	-

1, in its present chemical structure, on mice splenocytes (C57B1/6) and the early activation marker (CD69) respectively. There was also observed, a poor action of the compound on IL-8 receptor expression using the real-time polymerase chain reaction assay. Compound I did not strongly potentiate these cells or mediator types at the tested doses. There were very significant difference in activities between the test compounds and the controls ($P<0.05$; $P<0.001$).

4. Discussion

The phytochemical profiles of extracts and solvent fractions of the Eastern Nigeria mistletoes as well as its acute toxicity have been reported severally[23]. The established data confirmed that the extracts and fractions of this species of mistletoes are optimally rich in phytoconstituents and exhibit high margin of safety in rodents (mice and rats). However, the quantities and qualities of these constituents are both dependent on season of harvest and also on the particular host tree from the mistletoes are harvested[24]. These findings, which make the Eastern Nigeria mistletoes better than its European counterpart, *Viscum album* in terms of safety, provide additional credence to its known multiple folkloric and ethno-medicinal uses. The extracts from the different host trees exhibited very high margin of safety (between 5 500 to 11 000 mg/kg). The apparently high LD_{50} implies that the extracts are very safe in mice when administered intraperitoneally[23]. These findings are in agreement with previous reports on the acute toxicities of these species of Eastern Nigeria mistletoe[16]. Furthermore, recent data suggest that both the crude extracts from leaves and berries of Eastern Nigeria mistletoes are moderately safe, exhibiting no sub-acute toxicity[25,26]. They reported that histopathogical examination of major organs after the toxicity studies revealed no damage to them by the extracts. Furthermore, it has been reported that mistletoe extracts do not produce adverse biochemical changes following a

maximal dose of 827 mg/kg body weight[27]. It is therefore reasonable to postulate at this time, that the Eastern Nigeria mistletoe might be safer than other continental mistletoes including the European version, *V. album* which has an established LD_{50} value of less than 1 000 mg/kg.

However, the chronic toxicity study should be carried out in order to validate its safety on long term use. Recent researches with crude extracts of mistletoes harvested from different host trees established their immunomodulatory properties in both cell- and humour-mediated immune responses[19,23,28-30]. Interestingly, we have been able to establish that heat (soxhlet extraction) does not destroy the immunomodulatory activities of the extracts obtained from mistletoe harvested from *K. acuminata*[31]. This suggests in strong terms that immunostimulating activity of the mistletoe extracts does not mainly depend on any possible heat labile constituents. These findings support the documented report on European and Korean mistletoes, which emphasized that their main immunomodulatory principles were glycoproteins and thionin toxins called lectins and viscotoxins respectively[32]. An elegant research also established the imunogenicity of mistletoe lectins following intranasal co-administration with herpes simplex virus glycoproteins D2[33]. The presence of these highly mitogenic (immunogenic) compounds in *V. album* may account for its low therapeutic index. Another group of authors assessed crude methanol extracts of mistletoe harvested from cashew and cocoa trees for antioxidant activities and found them active[34].

Antioxidant potentials of mistletoe extracts and fractions harvested from different host trees have been confirmed (Omeje and Osadebe, unpublished data). Mistletoe harvested from *K. acuminata* was therefore the overall most potent in terms of immunostimulation and antioxidation with enhanced macrophage activation and cell proliferative abilities. Further bioassay-guided solvent fractionation of the crude extract (using DTHR as model guide) showed potency in the order of chloroform fraction >> ethylacetate fraction > *n*-hexane fraction> methanol fraction > acetone fraction. At the dose levels of 500 and 250 mg/kg body weight, the percentage stimulation observed were as follows; chloroform fraction-311.11 and 122.22%, ethyl acetate fraction-193.38 and 95.56%, *n*-hexane-155.56 and 3.50%, acetone fraction-77.78 and 51.11% and methanol fraction-68.89 and 24.44% respectively. Levamisole, a known potent immunostimulant, afforded a stimulation of 68.89% at a dose of 2.5 mg/kg. The DTHR is mediated by interferon-gamma (IFN-γ) producing CD4+ (TH1) or CD8+ (TC1) T cells[35]. It usually takes 24 - 72 h to develop and involves activation of T cells, which results in the mobilization of monocytes and lymphocytes to areas requiring immune stimulation. DTHR is known to be initiated by reaction between antigen-specific T cells and the antigen, which results in the release of lymphokines that affect a variety of cell types, especially macrophages. Thus, DTHR is an enhancement of "lympho-proliferative" response. SRBC is a T-dependent antigen hence, the first dose sensitized the T-lymphocytes to become activated to synthesize and release physiologically active substances such as macrophages, monocytes, polymorphonuclear leukocytes and probably, other non-sensitized leucocytes into the area of antigen contact. The observed stimulation supports the use of mistletoe extracts as immune boosters.

The reduction in DTHR response at higher doses may be an indication that Eastern Nigeria mistletoe may as well possess anti inflammatory activity at such doses. The phytochemical composition of the three most active solvent fractions, revealed that steroids, terpenoids, alkaloids, flavonoids were the likely immunomodulatory constituents. There are documented evidences supporting the use of these constituents as immunomodulatory agents[36-40]. Bioassay-guided fractionation of the *n*-hexane fraction led to the isolation of a major triterpenoidal ester (compound I) and two minor ones (compounds II and III) of the same class. Compound I particularly could not enhance interleukin-8 (IL-8) expression using a real time PCR assay. IL-8, a T-helper cell type 2 (Th2) macrophage derived cytokine mediates the attraction of neutrophil (PMN) to target sites and thereby mediates anti-inflammation[11]. It is known that T-cells secrete a number of cytokines which mediate cellular immune functions at either ends[41]. This implies that compound I lacked pronounced immunostimulatory function on cytokine (IL-8) activation and other possible macrophage functions. It is therefore a possible anti-inflammatory agent. A recent elegant review provided a strong evidence for the relationship between inflammation and tumourigenesis pathways mediated by cytokines, chemokines and other immunogenic agents[42]. Similarly, compound I could not strongly potentiate the proliferation of mice splenocytes (C57B1/6) cells on the early activation marker, CD69 cells using flow cytometry techniques at the tested doses. These findings provide strong evidence that this major triterpenoidal ester may be a good anti-inflammatory candidate. These compounds alongside other numerous constituents of the Eastern Nigeria mistletoe therefore, may become good leads for the development of potent anti-inflammatory as well as anti-cancer agents in the near future. However, immunological reactions are intricately complex processes and it is obvious that this assumption will require further validation using multiple experimental models.

Compound I was recrystallized in acetone-methanol to yield white amorphous compound; yield (34 mg) and melting point 107.0 ℃. The spectroscopic were obviously typical of a triterpenoidal ester. Its ^{1}H-NMR spectrum exhibited signals due to seven singlet methyls and a triplet-doublet methyl at δ 0.86. The presence of a hydroxyl bearing methine at 4.14 ppm and a triplet-doublet methine at δ 2.32 are reminiscent of a lupeol-type triterpene[43,44]. Additionally, a terminal methyl around δ 0.86 and a strong methylene proton signals around δ 1.26 were indicative of a fatty acid which was supported by the appearance of the carbonyl ester in the ^{13}C-NMR spectrum. Furthermore, the signal at δ 4.45 (1H, dd, J=4.9, 12.6Hz) was ascribable to H-3 in the triterpene moiety by the ^{1}H-^{1}H COSY, HSQC and HMBC. ^{13}CNMR ($CDCl_3$, 500 MHz): δ 173.91(ester carbonyl), δ 150.52

(double bonded carbon), δ 109.96 (double bonded carbon), δ 80.37 (oxygenated carbon), δ 72.71 (oxygenated carbon), δ 68.25 (oxygenated carbon), δ 52.31 (methine carbon), δ 50.39 (methine carbon), δ 49.15 (quaternary carbon), δ 48.29 (methine carbon), δ 48.05 (quaternary carbon), δ 47.77 (methine carbon), δ 45.89 (methylene carbon), δ 42.78 (quaternary carbon), δ 39.99 (methylene carbon), δ 38.62 (methylene carbon), δ 37.77 (quaternary carbon), δ 37.63 (methine proton), δ 37.42 (quaternary proton) δ 35.05 (methylene carbon), δ 14.36 (terminal methyl of fatty acid chain).

Although the ^{13}C-NMR showed 44 carbons, the remaining carbons were revealed to have been in a highly flexible and overlapping methylene chain, a feature confirming the presence of traces of multiple fatty acid chain. The unequivocal structural build up was achieved with fragments formed from HSQC experiments. In summary, DEPT-135 signals of compound I were observed for 8 methyls, 23 methylenes, 8 methines carbons with 7 unobserved quaternary carbons. ^{1}H-COSY, HSQC, and HMBC studies confirmed all the fragments and connectivities. Positive NOE and NOESY confirmed spatial arrangements or configurations. Spectral data correlates well with literature data for hydroxylated esterified lupane nucleus (lupeol)[43-48] and was elucidated to be 7 β ,15 α -dihydroxy-lup-20(29)-en-3 β -O-palmitate.

Compounds II and III were similarly elucidated as 7 β ,15 α -dihydroxy-lup-20(29)-en-3 β -O-stearate and 7 β ,15 α -dihydroxy-lup-20(29)-decadecanoate respectively as was previously shown with compound I. The only difference in there spectral data was from the HREIMS which showed additional masses assignable to extra methylene groups in the fatty acid chain. The ^{13}C-NMR could not show these differences simply because of overwhelming overlap from the methylene groups. Although related compounds have been reported to occur in plant of other classes[48,49], it appears that this is the first report of their occurrence in mistletoe of Eastern Nigeria origin. Strikingly, the presence of two hydroxyl groups in the compound makes it a novel and unique lupeol based long-chain fatty acid ester isolated for the first time from the Eastern Nigeria mistletoe. However, compound I only has been reported present in Japanese mistletoe[45].

There is overwhelming evidence from the foregoing research that these triterpenoidal esters of the Eastern Nigeria mistletoes are good candidates of immunomodulation. However, it appears that fractionation and purification of the n-hexane extract produced single pure compounds that failed to strongly enhance certain cell types involved in the mediation of immunologic reactions and therefore will be good candidates of anti-inflammation and possible development as anti-neoplastic agents.

Conflict of interest statement

The authors declare that there is no conflict of interest of any kind.

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