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RESEARCH ARTICLE

A novel sesquiterpene acid and an alkaloid from leaves of the Eastern Nigeria mistletoe, *Loranthus micranthus* with potent immunostimulatory activity on C57BL6 mice splenocytes and CD69 molecule

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Abstract

Context: The Eastern Nigeria mistletoe, *Loranthus micranthus* Linn. (Loranthaceae), is used in the treatment of several diseases including immune-modifying diseases and thus there is a need to identify the immunoactive constituents.

Objective: This research isolated and characterized the immunoactive constituents in the Eastern Nigeria mistletoe.

Materials and methods: Bioassay-guided fractionation was employed in the isolation and purification of the constituents. The characterized compounds were screened for immunostimulatory activities on isolated C57BL/6 mice splenocytes and early activation marker, CD69 at concentrations of 10, 25, and 100 µg/mL using flow cytometry techniques and compared to lipopolysaccharide (LPS; 10 µg/mL) and concanavalin A (ConA; 2 µg/mL) as standards.

Results: Two compounds, a novel sesquiterpene, 2, 3-dimethoxy-benzo [a, b] cyclopentenyl-3′,3′,5′-trimethyl pyran-4-carboxylic acid (**1**), and a known alkaloid, lupinine (**2**) were isolated and characterized. The compounds (25 µg/mL) showed statistically significantly ($p < 0.05$) stimulatory activity on the splenocytes with values of 56.34 ± 0.26% and 69.84 ± 0.19%, respectively, compared to 7.58 ± 0.42% recorded for the unstimulated control. Similarly, the CD69 expression assay showed immunostimulation with statistically significant values ($p < 0.05$) of 2.31 ± 0.07% and 2.71 ± 0.03%, respectively, compared to 1.69 ± 0.05% recorded for the nonstimulated control.

Discussion: These data suggest that the isolated compounds possess immunomodifying abilities. In addition, the activation of the CD69 molecule is possibly one of its mechanisms of action.

Conclusion: These compounds may be responsible in part, for the immunostimulatory activities already established for the Eastern Nigeria mistletoes.

Keywords: Sesquiterpenoid, immunomodulation, C57BL6 splenocytes, natural product, cell proliferation, chromatography, medicinal plants

Introduction

Medicinal plants, which have been described as natural chemical factories, remain the main sources of bioactive molecules used to sustain health, and indeed huge researches are dedicated to lead finding from nature. Although, in 1828 when the German chemist,

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Wöhler accidentally synthesized urea (Ricardo, 2006), emphasis on lead finding shifted to synthesis, and unfortunately, phytomedicine was largely forgotten by Western science. In the 1980s, however, there was a resurgence of interest in the use of natural substances, generally known today as bioactive compounds. This development, within the past three decades, has witnessed a large output of research data on natural products, especially those with immune-modifying abilities. One of the starting points in the field of immunomodulation has been the search for agents that could be used against diseases and infections especially for the treatment of residual cancer (Yamamoto, 1996). Plant-derived substances have been repeatedly reputed for its immunomodulatory potentials (Wagner et al., 1999). Modulation of the immune system, therefore, offers unlimited strategies for therapy and management of diseases. The present study describes the isolation, characterization, and assessment of the immunomodulatory potentials of a sesquiterpene acid and an alkaloid from the chloroform fraction (CF) of the Eastern Nigeria mistletoe, *Loranthus micranthus* Linn. (Loranthaceae) epiphyting *Kola acuminata*, Schott & Endl. (Malvaceae).

Materials and methods

Instruments

Gallenkamp melting point apparatus (England; used uncorrected), high resolution electron impact mass spectrophotometry (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 Inorganic 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. The UV/visible spectra were obtained in a UV2102PC spectrophotometer with integrated data station (UNICO, USA) at the Faculty of Pharmaceutical Sciences, University of Nigeria, and Nsukka. Others were electronic analytical balance (Metler Toledo, 0.001 max, England), glass columns (4 × 150 cm; 2.7 × 70 cm), silica gel G_{60}, and precoated G_{254} plates.

Solvents and reagents

Analar grade methanol, *n*-hexane, ethyl acetate, acetone, chloroform (Sigma Aldrich, Germany) were used. Distilled water, normal saline (DANA Ltd), dimethylsulfoxide (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), sodium acetate powder, C57BL/6 mice (Janvier, France), aluminium chloride, boric acid (Sigma-Aldrich), LPS, and ConA (all 99% pure; Sigma-Aldrich) were used as reference drugs. All other reagents were of analytical grade or freshly prepared when needed.

Plant material

Loranthus micranthus leaves, parasitic on the 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 A. O. Ozioko, a taxonomist of the Bioresources Development and Conservation Programme (BDCP), Nsukka, Enugu State. Voucher specimens were kept at the BDCP Center with the number BDCP-532-07 for reference purposes.

Extraction, fractionation, and isolation of the constituents

The method of sequential solvent extraction and fractionation recently reported was used to obtain the partially purified CF from the crude aqueous methanol extract of the Eastern Nigeria mistletoe (Osadebe & Omeje, 2009a, b; Omeje et al., 2011). Briefly, leaves of *Loranthus micranthus* parasitic on *Kola acuminata* were cleansed and dried under shade. They were then pulverized in mechanized laboratory grinder to produce fine powder. A total of 1 kg of the powder was extracted in batches with a total of 6500 mL of 98% aqueous methanol using a Soxhlet extractor. The extract was recovered *in vacuo*, using rotary evaporator at 45 ± 5°C and dried under forced dry air. Exactly 50 g of the extract (210.5 g; 21.05% yield) was adsorbed on silica Gel-60 grade, dried, pulverized, and washed repeatedly with analar grades of *n*-hexane, chloroform, acetone, ethyl acetate, methanol, and water in that polarity order to afford the respective solvent fractions. The washings continued until all the extractable constituents were evidently retrieved by a particular solvent before introducing the next solvent.

Phytochemical evaluation of the partially purified CF

This was carried out according to the procedure described by Harborne (1984). All reagents for the phytochemical tests were freshly prepared.

Isolation and purification of the constituents from CF

Exactly 6.0 g of CF was chromatographed on silica gel (60–120, 1 kg) packed into a glass column (4 × 150 cm) with a bed of 95 cm in height. The elution was performed with isocratic mixture of chloroform: methanol (19:1). Aliquots of 25 mL were collected and monitored as previously described (Omeje et al., 2011). This afforded three fractions, F1 (1–6; 150 mL), F2 (7–19; 325 mL), and F3 (20–60; 525 mL). The column was further eluted isocratically with chloroform: methanol (9:1), (8:1), and (7:1) to yield F4 (61–70; 250 mL), F5 (71–103; 825 mL), and F6 (104–124; 525 mL), respectively. The F1 (1.8 g; 30.0% yield) was chromatographed on silica gel (70–230, 500 g) in a glass column (2.7 × 70 cm) with the bed height of 43 cm. Gradient elution of chloroform, chloroform: methanol (49:1; 39:1; 29:1; 19:1; 9:1) and methanol (100%) were used and aliquots of 25 mL were collected and fractions monitored as previously described. Three major fractions E1 (1–11; 275 mL), E2 (12–36; 375 mL), and E3 (37–63; 675 mL) were obtained. The E2 was strongly steroidal and lipophilic.

It was subjected to gas chromatography mass spectrometry (GC/MS) analysis. The E3 was the carotenoid fraction and was confirmed by UV analysis to be predominantly β-carotenoid and epiphasic in nature. An alkaloidal compound, lupinine was isolated from E2 (12–36; 375 mL) as white brittle crystals following a phase separation and after drying off the solvent. The crude lupinine was purified by simple alkaloid precipitation technique (in organic phase) and purity probed by melting point determination (yield was ca.150 mg; 10.05%). A fresh portion of the CF (11 g) was chromatographed on silica gel (70–230, 1.2 kg) packed into a glass column (4 × 150 cm) with a 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. Eleven fractions (F1–F11) were evidently pooled as follows: 1–13, 14–23, 24–32, 33–36, 37–75, 76–86, 87–126, 127–187, 188–202, 203–224, and 225–236. From F4 (100 mL), F9 (375 mL), and F10 (550 mL) eluted with *n*-hexane: ethyl acetate (8:2, 1:9, 0:10), respectively, compounds CF6 (loranthoic acid; 43 mg; 4.34%), CF3 (26 mg), and CF4 (15 mg) were isolated. All compounds were recrystallized in either acetone or ethanol. Purity was probed by melting point determination. Characterization of CF3 and CF4 is ongoing.

Proliferation assay

The proliferation potentials of the two compounds on spleen cells of C57BL/6 mice (Janvier, France) were determined following a recently reported method (Omeje et al., 2011). Briefly, 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 (3 μM) 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 lupinine and loranthoic acid (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

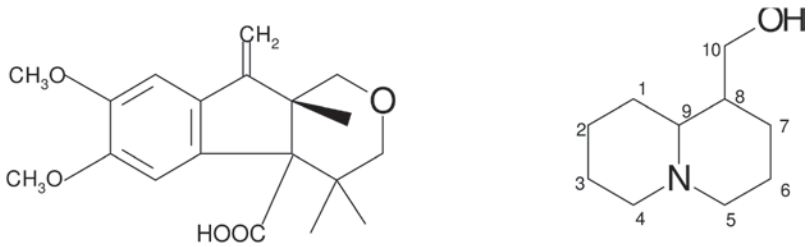
Similarly, the effects of the compounds on the expression of CD69 (early activation marker) of spleen cells of C57BL/6 mice was determined by the method described recently (Omeje et al., 2011). Briefly, single-cell suspension of spleen cells of C57BL/6 mice was prepared by dispersion straining and suspending 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 above compounds (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 (analyzed by SPSS version 11 (SPSS Inc., Chicago, IL, USA)), 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 Student's *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 $p < 0.05$.

Results

The phytochemical evaluation of partially purified CF indicated the presence of steroids in triterpenoids in large quantities, while alkaloids, glycoside, flavonoids, and resins occurred in appreciable amounts. The detailed chromatographic separation of the fraction led to the isolation of the two main constituents, lupinine and loranthoic acid in reasonable yields. The spectral data of the isolated compounds are shown below. The proposed structures are illustrated in Figure 1. Tables 1 and 2 depict the activities of these compounds on the C57B/L mice splenocytes and the early activation marker, CD69



(I) **Loaranthoic acid** (II) **Lupinine**

Figure 1. Structures of loranthoic acid and lupinine isolated from *Loranthus micranthus* leaves.

Table 1. Effects of the compounds on C57BL/6 mice splenocytes.

	Percentage proliferating cell population (%) by the compounds	
Treatment	Loranthoic acid	Lupinine
10 μg/mL	18.34 ± 1.56*	34.92 ± 1.22*
25 μg/mL	20.08 ± 0.83**	65.37 ± 1.71**
100 μg/mL	24.44 ± 2.58***	69.84 ± 0.19***
Control vehicle	7.69 ± 0.41***	7.69 ± 0.42***
LPS2 μg/mL	34.61 ± 0.44	34.61 ± 0.44
ConA 10 μg/mL	34.01 ± 0.32	34.01 ± 0.32

The compounds were tested at 10, 25, and 100 μg/mL and were compared to unstimulated control, LPS and ConA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$ values significantly different from control vehicle.

Table 2. Increased expression of CD69 expression by isolated compounds.

	Mean fluorescence intensities (MFI) of CD69 expression	
Treatment	Loranthoic acid	Lupinine
10 μg/mL	2.45 ± 0.07*	2.37 ± 0.13*
25 μg/mL	2.71 ± 0.06**	2.99 ± 0.10***
100 μg/mL	3.16 ± 0.06***	2.97 ± 0.16***
Control vehicle	1.69 ± 0.05	1.69 ± 0.05
ConA2 μg/mL	5.42 ± 0.11*	5.42 ± 0.11*
LPS10 μg/mL	5.27 ± 0.08*	5.27 ± 0.08*

The compounds were tested at 10, 25, and 100 μg/mL and were compared to unstimulated control, LPS and ConA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$ values significantly different from control vehicle.

molecules, respectively. The compounds were comparatively and potently stimulatory. The sesquiterpenoidal acid was isolated as a white amorphous powder; yield (43 mg), m.p.: 114°C. The UV λ_{max} (methanol) nm (ε): 278 (14,800), 316 (489), 330 (235). IR νmax^{KBr} 3527–3396 (OH), 3099 (-CO-CH_3), 3028 (aryl-H), 2929 (CH stretching, alkanes), 2850 (CH stretching, -CHO or -O-CH_3), 2484 (OH from acid), 1707 (C = O), 1624 (C = C), 1500 (m) (aromatic ring), 1329–1276 (-CH_3), 1031–1118 (C-O-C), 956–786 (substituted aromatic finger print); ESIMS (relative int.), M^+ (*m/z*): 332.1 ($C_{19}H_{24}O_5$). M^+ + H (333), 332 (100), M^+ – 18, M^+ – 36 (loss of H_2O), M^+ – 59 (CH_3COO^+), M^+ – 85 ($C_4H_9CO^+$). HREIMS (relative int.), calculated; M^+ (*m/z*): 332.1404 (100), HREIMS (relative int.), found; M^+ (*m/z*): 332.1401; HREIMS (relative int.), M + H^+ (*m/z*): 333.1697 (100) indicating a formula of $C_{19}H_{24}O_5$. ^{1}HNMR (CD_3OD, 500 MHz): δ 8.89 (1H, s, COOH), δ 7.89 (1H, s, J = 0Hz, H-1 aromatic), δ 7.77 (1H s, J = 0Hz, H-4 aromatic), δ 3.85 (3H s, -CO-OCH_3), δ 3.70 (6H s, -OCH_3 X 2), δ 3.50 (6H s, OCH_2), δ 1.45 (3H s, -CH_3), δ 1.25 (3H s, CH_3). ^{13}CNMR (CD_3OD, 500 MHz): δ 9.9 (CH_3), δ 37.5 (CH_2), δ 178 (C = O), δ 169 (C = CH_2). The DEPT-135 showed three signals for CH_2 (methylene), five signals for CH_3 (methyl) units, and two signals for CH (methine) units. The 1H-H COSY experiment revealed proton correlations supporting proposed structure. The sesquiterpene acid's probable structure is elucidated as 2, 3-dimethoxy-benzo [a, b] cyclopentenyl-3′, 3′, 5′-trimethyl pyran-4-carboxylic acid.

Lupinine was isolated as a white crystalline substance, yield (150 mg), m.p. = 238°C, showed positive reaction with Dragendorff's, Hager's, Mayer's, and Wagner's reagents. The UV λ_{max} nm (methanol) (ε): 288 (58.50), 337 (17.3), 372 (14.8). The IR ν_{max}^{KBr} 3419 (br, s OH), 3389 (CH_2-N-CH_2), 1406 (-CH_2-), 2820 (NCH_2 stretching). The ^{1}HNMR (CD_3OD, 500 MHz): δ 3.50 (2H,-CH_2-O-), δ 7.50 (1H, s, OH). ^{13}CNMR (CD_3OD, 500 MHz): δ 39.30(C-1), δ 39.97 (C-2), δ 39.89 (C-3), δ 40.39 (C-4), 40.37 (C-5), δ 39.80 (C-6), δ 39.70 (C-7), δ 39.60 (C-8), δ 39.23 (C-9), δ 41.65 (C-10). The DEPT-135 of lupinine revealed methylene groups close to oxygen or other electronegative atoms. The COSY spectrum indicated that the carbons and protons were magnetically equivalent. The spectroscopic data suggest, in strong terms, that the compound is identical with lupinine, an alkaloid, which also has been reported in mistletoes from other continents (Michael, 1998).

Discussion

The proposed structures of the novel compound I and the known compound II (lupinine) is as shown in Figure 1. Phytochemical analysis of compound I confirmed its sesquiterpenoidal nature and its behavior in alkaline medium (using sodium carbonate) suggested the presence of a carboxylic group. This finding is supported by the detection of alkaloids and triterpenoids in the partially purified CF. The sharp melting point of compound I is an indication of purity which was further confirmed by the spectral data. The UV absorption of compound I showed a high molar absorptivity of 14,800 at 278 nm indicating presence of conjugation in addition to two other distinct peaks at 316 and 330 nm. These UV absorption pattern is typical of terpenoids with some level of conjugations. The presence of a carbonyl functional group was confirmed by both the IR and ^{13}C-NMR signals at 1707 cm^{-1} and 178 ppm, respectively. Other observed functional groups (-OH, -C = C-, aromatic ring, CH stretching of alkanes, ether linkage) in compound I were accounted for in the IR spectrum and NMR spectra. The ESIMS (relative int.) of the compound I at M^+ (*m/z*) of 332.1 provided an evidence suggesting a reasonable molecular formula of $C_{19}H_{24}O_5$ for the compound. The fragmentation pattern were observed to include; M^+ + H (333), 332 (100), M^+ – 18, M^+ – 36 (loss of H_2O), M^+ – 59 (CH_3COO^+), M^+ – 85 ($C_4H_9CO^+$), all of which were accounted for in the mass spectral fragmentation pattern. The concrete evidence for the molecular structure and formula was provided by the HREIMS (relative int.), calculated; M^+ (*m/z*): 332.1404 (100), HREIMS (relative int.), found; M^+ (*m/z*): 332.1401; HRMS (relative int.), M + H^+ (*m/z*): 333.1697 (100) confirming a formula of $C_{19}H_{24}O_5$. The ^{1}HNMR (CD_3OD, 500 MHz) signal at δ 8.89 (1H, s) suggested the presence of carboxylic acid proton. Furthermore, evidence for this assumption was provided by the disappearance of the signal in deuteriated water. The signals at δ 7.89 (1H, s, J = 0Hz, H-1),

δ 7.77 (1H s, J = 0 Hz, H-4) were assignable to two *para*-positioned aromatic protons (indicating the presence of aromatic moiety in the structure), the zero-coupling constant being also contributory to the confirmation of the assignments. In addition to the above evidence, the IR spectrum showed signals at 3028 and 1500 (m) cm^{-1} confirming the presence of an aryl proton and aromatic ring, respectively, in the molecular structure. The two ortho-coupled methoxyl groups showed signals at δ 3.70 with six protons on integration. The presence of a methyl group closer configurationally, in position, to an oxygen atom of a pyran nucleus was suggested by the proton NMR signal at δ 3.85 (3H, s). There were two other higher shielded methyl signals at δ 1.45 (3H, s) and δ 1.25 (3H, s). The ^{13}C NMR (CD_3OD, 500 MHz) signals at δ 9.9, 37.5, 178, and 169 were assignable to methyl carbon, methylene carbon in an electronegative environment, a carbonyl functional group (from the acid), and an alkene-functional group, respectively. Further evidence for assignments was provided by a methylene signal in the DEPT-135 spectrum of the compound at δ 37.5, which appeared inverted. Other methylene signals in the DEPT spectrum were observed at the usual much lower chemical shifts. In consideration of all these data, compound I was elucidated as 2, 3-dimethoxy-benzo [a, b] cyclopentenyl-3′, 3′, 5′-trimethyl pyran-4-carboxylic acid. Compound I or loranthoic acid is being reported for the first time in the Eastern Nigeria mistletoe. Interestingly, it has not been reported in any other mistletoe from other parts of the world. This current finding is, therefore, striking. On the contrary, the presence of compound II or lupinine has been reported in mistletoes of other continents (Michael, 1998), but no report was found for its immunomodulatory potentials. The detailed phytochemical tests proved that it was an alkaloid. In addition, the recorded spectral data matched perfectly with already existing data for lupinine (Michael, 1998). Further spectral evidence at this point was not considered irrelevant but supportive. The immunomodulatory potentials of these compounds were assessed *in vitro* in C57B/L mice splenocytes and the early activation marker, CD69 molecules and results compared to unstimulated controls and standard immunostimulants; LPA and ConA. Tables 1 and 2 depict the activities of these compounds on the C57B/L mice splenocytes and the early activation marker, CD69 molecules, respectively. In all the assessments, these compounds performed far better than standard controls in terms of immunostimulation of the target cells. However, lupinine was evidently more stimulating on the C57B/L mice splenocytes than the loranthoic acid. Lupinine, at the concentrations of 25 and 100 µg/mL showed percentage mitogenic stimulations of 65.37 ± 1.71 and 69.84 ± 0.19, respectively, compared to 20.08 ± 0.83 and 24.44 ± 2.58 recorded for loranthoic acid at the same concentrations. The standard controls exhibited lower stimulatory values of 34.61 ± 0.44 and 34.01 ± 0.32% for the LPS and ConA, respectively. The stimulation of this cell type is an indication of immune-modifying potentials of the isolated test compounds. The compounds, also, showed statistically significant ($p < 0.05$) mitogenic stimulation of the early activation marker, CD 69 molecule when compared to the unstimulated control, although the standards were visibly more potent. The activation of T lymphocytes, both *in vivo* and *in vitro*, induces expression of CD69. This molecule, CD 69, 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 (Hamann et al., 1993; Testi et al., 1994; Borrego et al., 1999). The CD69 is also a stimulatory receptor for natural killer cells. The enhancement of expression of this early activation marker by molecules is, therefore, a direct measurement of the immunomodulatory potentials of such molecules. The strong link between expression of the CD69 molecule and immunomodulation has been variously discussed (Borrego et al., 1993, 1994). The effect of these compounds on this molecule as well as the mitogenic mice splenocytes could, therefore, be regarded as an immunomodulatory response. Furthermore, the proliferation of the mitogenic splenocytes correlates well with the enhanced expression of the CD69 molecule. This supports in strong term that the activation of CD69 molecule precedes immunomodulatory reaction. It is being postulated that if these compounds, in their present form, evoked this level of response, structural modifications may likely afford better congeners. These congeners may serve as lead compounds for the development of candidates with strong immunomodulatory abilities and by extension possible active drugs against diseases that influence or affect the immune system, cancer, inflammation, diabetes, and viral infections being prominent. In conclusion, these isolated compounds have demonstrated potent mitogenic stimulations of the target cells and molecules *in vitro* and the claim of the ethnomedicinal use of the Eastern Nigeria mistletoe is further strengthened by these findings.

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Declaration of interest

The authors declare no conflict of interests.

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