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ISOLATION AND STRUCTURE ELUCIDATION OF POLYPHENOLS FROM *LORANTHUS MICRANTHUS* LINN. PARASITIC ON *HEVEA BRASILIENSIS* WITH ANTIINFLAMMATORY PROPERTY

Matthias Onyebuchi Agbo[1,2*], Chukwuemeka Sylvester Nworu[3], Festus Basden Chiedu Okoye[4], Patience Ogoamaka Osadebe[2]

[1] Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Düsseldorf, Universitätstrasse 1, Geb. 26.23, 40225 Düsseldorf, Germany
[2] Department of Pharmaceutical and Medicinal Chemistry, University of Nigeria, Nsukka, Enugu State, Nigeria
[3] Department of Pharmacology & Toxicology, University of Nigeria, Nsukka, Enugu State Nigeria
[4] Department of Pharmaceutical and Medicinal Chemistry, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria

*** Corresponding author:** e-mail: matthias.agbo@unn.edu.ng

ABSTRACT

The present study was carried out to evaluate the anti-inflammatory activities of polyphenols isolated from the leaves of mistletoe (*Loranthus micranthus* Linn.) parasitic on *Hevea brasiliensis*. The anti-inflammatory properties of the isolated compounds were evaluated on the basis of their ability to inhibit the production of nitric oxide (NO) and tumuor necrosis factor-α (TNF-α) in lipopolysaccharide (LPS) activated RAW 264.7 mouse macrophages. Semi-preparative HPLC separation of the ethyl acetate (EtOAc) and butanol (*n*-BuOH) fractions of the leaves of mistletoe (*Loranthus micranthus* Linn) parasitic on *Hevea brasiliensis* led to the isolation of four polyphenols: 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG) (**1**); (-)-epicatechin-3-O-(3″-O-methyl)-gallate (ECG3″Me) (**2**); rutin (**3**) and peltatoside (**4**). Compounds **1-4** were isolated for the first time from this plant while **1** was isolated for the first time in nature. These compounds (**1-4**) were readily identified by comparison of their spectroscopic data with those reported in the literature. The polyphenols proved to have anti-inflammatory activity as evidenced by the suppression of inducible nitric oxide (iNO) and cytokine (TNF-α) levels in the culture supernatant of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages. However, the study showed that the quercetin diglycosides showed stronger inhibition of proinflammatory mediators than the epicatechin derivates. These data provide evidence that polyphenolic compounds isolated from the mistletoe parasitic on *Hevea brasiliensis* may contribute to its anti-inflammatory properties by inhibiting the expression of inducible nitric oxide and proinflammatory cytokines such as tumour necrosis factor-α.

Keywords: Mistletoe, structural elucidation, polyphenols, inflammation, inducible nitric oxide, tumour necrosis factor-α (TNF-α), RAW 264.7 murine macrophages

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INTRODUCTION

Mistletoes are hemiparasitic plants growing on different host trees and shrubs. They depend on their host plant for water and mineral nutrition, even though they produce their own carbohydrates through photosynthesis (Ali et al., 2005). Mistletoe grows on many host trees like *Kola acuminata*, *Baphia nitida*, *Persia americana*, *Irvingia gabonensis*, *Citrus simensis*, *Pentacletra macrophylla*, *Treculiar africana*, and *Ficus exaperata* (Ali et al., 2005; Osadebe et al., 2012). The leaves of mistletoes are traditionally used in folkloric medicine of Nigeria for the treatment of diarrhoea, epilepsy, hypertension and rheumatism (Griggs, 1991). The leaves of African mistletoe have been reported to have anti-inflammatory activity *in vivo* on Wistar albino rats (Patrick-Iwuanyanwu et al., 2010). Inflammation is a bodily response to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed physiological functions (Black and Berman, 1999). It is the body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. Inflammation is triggered by the release of chemical mediators from injured tissue and migrating cells (Sangita et al., 2012). A number of chemical mediators have been postulated to play important roles in the inflammatory process. The most common sources of chemical mediators include neutrophils, basophil, mast cells, platelets, macrophages and lymphocytes. Activated macrophages secrete a number of different inflammatory mediators, including interleukin-1β (IL-1β), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), nitric oxide (NO), and tumour necrosis factor-α (TNF-α) (Boscá et al., 2005; Lawrence et al., 2002; Kaplanski et al., 2003). However, if inflammation is not treated it leads to onset of diseases like vasomotor rhinorrhoea, rheumatoid arthritis and atherosclerosis (Henson and Murphy, 1989). In this study four polyphenolic compounds isolated from *Loranthus micranthus* parasitic on *Hevea brasiliensis* were tested, *in vitro*, for anti-inflammatory activity. The compounds were tested for their ability to inhibit inducible nitric (iNO) oxide and tumour necrosis factor-α (TNF-α) in murine macrophage cell line (RAW264.7). Inhibition of these two important mediators of inflammation is important in our understanding of the possible mechanism through which these compounds may be involved in mediating any anti-inflammatory effect shown by mistletoe (Nathan, 1992; Nworu et al., 2012; Lee et al 2012).

MATERIALS AND METHODS

Plant material

Fresh leaves of mistletoe (*Loranthus micranthus* Linn.) parasitic on *Hevea brasiliensis* were collected from Enugu-Ezike in Enugu State, Nigeria in January 2012. The plant material was identified and authenticated by Mr. A. O. Ozioko of the Bioresources Conservation and Development Program (BDCP), Nsukka. A voucher specimen (LM1610) was deposited at the herbarium of the Institute.

Preparation of extract

The dried leaves (500 gm) of *Loranthus micranthus* Linn. parasitic on *Hevea brasiliensis* were macerated with 3.0 L of 100 % methanol (MeOH) and extracted at room temperature for 48 h with agitation. The resulting methanol extract was concentrated *in vacuum* at 40 °C to obtain the dry methanol extract.

Isolation of the polyphenols

The dry methanol extract (50 gm) was dissolved in 400 mL of 10 % methanol-water and the resulting mixture (i.e., the aqueous layer) partitioned with 3.0 L *n*-hexane (6 x 500 mL), 3.0 L ethyl acetate (6 x 500 mL) and 1.0 L *n*-butanol (2 x 500 mL) using separating funnel to obtain *n*-hexane (HF, 3.90 gm, 7.8 %), ethyl acetate (EF, 13.8 gm, 27.6 %), *n*-butanol (BF, 12.6 gm, 25.2 %) and water (WF, 1.02 g, 2.04 %) fractions respectively. The ethyl acetate fraction (5 gm) was purified by vacuum liquid chromatography using silica gel (230 – 400 mesh,

3.0×30 cm, 500 gm) as the stationary phase and eluted with a gradient of *n*-hexane in ethyl acetate (10:0, 8:2, 6:4, 4:6, 2:8, 0:10, each 500 mL) and of dichloromethane (DCM) in methanol (9:1, 7:3, 5:5, 3:7,1:9, each 1000 mL) to afford 11 sub-fractions (EF1-EF11). Fraction EF6 (348.5 mg) was further fractionated on Sephadex LH-20 (100 % MeOH) to afford seven sub-fractions (EF6A–EF6G). Fraction EF6E (36.3 mg) was purified using semi-preparative HPLC with MeOH–H_2O as the mobile phase to yield **TMECG** (2.9 mg). Similarly, fractions EF6F (39.0 mg) were purified in a similar way to afford **ECG3″Me** (15.8 mg). Also fraction EF9 (600 mg) was subjected to Sephadex LH-20 column chromatography (3×110 cm) eluted with 100 % MeOH to give nine sub-fractions (EF9A–EF9I).

Rutin (5.6 mg) was obtained from sub-fractions EF9B (77.3 mg) by semi-preparative HPLC. The *n*-butanol fraction (10 g) was purified by vacuum liquid chromatography over silica gel (230 – 400 mesh, 3.0×30 cm, 800 g) and eluted with a gradient of DCM in MeOH (9:1, 8:2, 7:3, 6:4, 5:5,4:6, 3:7, 2:8, 1:9,0:10, each 1000 mL) to give 10 fractions (BF1-BF10). Fraction BF3 was subjected to Sephadex LH-20 column chromatography eluting with 100 % MeOH (3×110 cm) to afford seven sub-fractions (BF3A–BF3G). Sub-fraction BF3A (65.6 mg) was purified by semi-preparative HPLC (MeOH–H_2O) to give **peltatoside** (2.6 mg).

Cell viability assay

The cytotoxicity effects of the compounds were evaluated in RAW 264.7 cells using MTT assays (Mosmann, 1983). Laboratory stock of RAW264.7 mouse macrophage cell line (ATCC, MD, USA) was cultured in R-10 medium, consisting of RPMI 1640 medium (Corning cellgro® RPMI; Mediatech Inc., Manassas, VA, USA) supplemented with 10 % heat-FBS, 50 µM 2-mercaptoethanol (Gibco, Invitrogen, USA), 100 U/ml penicillin (Gibco, Invitrogen, USA), and 100 µg/ml streptomycin (Gibco, Invitrogen, USA) in a 5 % CO_2 humidified atmosphere at 37 °C. The effect of TMECG, ECG3″Me, rutin and peltatoside on the viability of RAW264.7 mouse macrophages was determined using a modification of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay originally described by Mosmann (1983). The cells were seeded in triplicate into 96-culture well plates at a density of 10^4 cells/well in 100 µL. After 24 h of seeding, the cells were treated with graded concentrations (5, 25, 100, 250, and 500 µM) of the compound**s**. R-10 medium was used as the "no-drug" control. After 48 h of incubation at 37 °C under 5 % CO_2, a solution of MTT (20 µL per well of 5 mg/ml solution) was added and further incubated for 4 h to allow formazan formation in viable cells. Thereafter, the MTT-containing media were removed, and the reduced formazan dye was solubilized by adding 150 µL of dimethyl sulfoxide (DMSO) to each well. The plate was agitated for 15 min on a shaker. Then the optical density (OD) was determined at 570 nm using a multi-well plate reader (Bio-Kinetic Reader-E312e®; Bio-Tech Instruments, Winooski, VT). Each value of the triplicate determination was expressed as a percentage of the mean of the no-drug control wells.

Nitrite determination

Nitric oxide production and release by RAW264.7 cells was measured indirectly by determining nitrite accumulation in culture supernatant using the Griess reaction as described by Kim *et al*., (2013). RAW 264.7 cells ($5×10^5$ cells/well) were seeded in 24-well plate for 24 h. The cells were pretreated for 2 h with 0, 5, 25, and 100 µM of TMECG, ECG3″Me, rutin and peltatoside. Thereafter, lipopolysaccharide, LPS (1 µg/mL) (serotype 0128: B 12, L 4255; Sigma, St Louis, MO, USA) was added into each well and incubated for an additional 24 h. The quantity of nitrite generated was measured using the Griess reagent system. Equal volumes of cell-free culture supernatants and freshly prepared Griess reagent were added

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to the 96-well plate and incubated at room temperature for 10 min. The absorbance was measured on a microplate reader at 570 nm (Bio-Kinetic Reader-E312e®; Bio-Tech Instruments, Winooski, VT). Nitrite concentrations were extrapolated from a standard $NaNO_2$ curve included within each assay plate.

Measurement of TNF-α

The concentration of TNF-α in the conditioned culture supernatants from macrophage cultures was measured by cytokine enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA) according to the method described by Ravipati et al., (2012). RAW 264.7 cells (5×10^5 cells/well) were seeded in 24-well plate for 24 h. The cells were pre-treated for 2 h with 0, 5, 25, and 100 µM of TMECG, ECG3″Me, rutin, and peltatoside respectively before LPS (1 µg/mL) was added to the wells. After incubation, cell-free supernatant was harvested and stored at -20 °C for future analysis. The concentration of TNF-α was calculated from a standard curve of the standard mouse TNF-α included ELISA assay.

RESULTS

Semi-preparative HPLC separation of the ethyl acetate (EtOAc) and butanol (*n*-BuOH) fractions of the leaves of mistletoe (*Loranthus micranthus* Linn.) parasitic on *Hevea brasiliensis* led to the isolation of four polyphenols **1-4** (Figure 1).

3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (**1**) was obtained as a yellow amorphous powder; $[\alpha]_D^{20}$ = -106.9 ° (c 0.10, MeOH); UV (MeOH) λ_{max} 271.1 and 211.8 nm; HR-ESIMS: *m/z* 485.14423 $[M+H]^+$; ^1H and ^{13}C NMR spectroscopic data (Table 1).

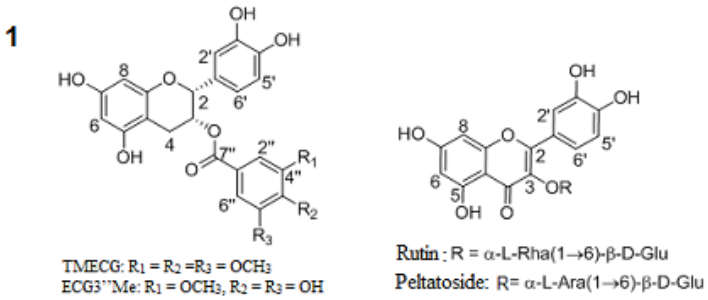

Figure 1: Structures of the isolated compounds **1-4**
The characterizations of the isolated compounds were done by comparison with reported values.

Table 1: ^1H-NMR (500 MHz) data of compound **1** in MeOH-d_4; J in Hz

	TMECG		
Pos.	δ_H (J in Hz)	δ_H [f] (J in Hz)	COSY (TMECG)
2	5.10,s	5.19,bs	3
3	5.51,s	5.48,m	2,4
4	3.02,m	3.10,m	3
	2.94,d (17.4)	3.05,m	
6	5.98,dd (12.6,2.3)	6.03,d (2.2)	
8	5.98,dd (12.6,2.3)	6.02,d (2.2)	
2'	6.98,d (2.0)	7.11,d (2.0)	
5'	6.71,d (8.2)	6.79,d (8.2)	6'
6’	6.80,m	6.90,dd (8.2,2.0)	5'
2"/6"	7.14,s	7.14,s	
3"/5"-OMe	3.81,s	3.80,s	
4"-OMe	3.78,s	3.73,s	

[f] Sánchez-del-Campo et al., 2008

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Epicatechin-3-O-(3″-O-methyl)-gallate (**2**) was obtained as a yellow amorphous powder; $[\alpha]_D^{20}$ = 181.8 ^{o} (c 0.10, MeOH); UV (MeOH) λ_{max} (MeOH) 216.4, 278.8 nm; ESI-MS: *m/z* 457 $[M+H]^+$; ^1H and ^{13}C NMR spectroscopic data (Table 2).

Table 2: ^1H (500 MHz) and ^{13}C-NMR (125 MHz), COSY and HMBC Data of compound **2**

ECG3″Me					
Pos.	**δ_C**	**δ_H**	**δ_H (Saijo 1982)**	**COSY**	**HMBC**
2	79.0	5.06,s	5.17,s	3	1'
3	70.8	5.51,s	5.53,s	2,4	
4	27.1	2.88,dd (17.4,2.4)	3.03,m	3	
		3.01,dd (17.3,4.6)			
5	157.7				
6	96.9	5.97,m	6.05,s		5
7	158.4				
8	96.2	5.97,m	6.05,s		5
9	158.3				
10	99.7				
1'	131.4				
2'	115.5	6.96,d (1.8)	7.11,d (2.0)		
3'	146.5				
4'	141.1				
5'	116.4	6.7,d (8.2)	6.77,d (8.0)	6'	
6'	119.6	6.81,dd (8.2,1.8)	6.92,dd (2,6)	5'	
1"	121.9				
2"	112.3	7.07,d (1.9)	7.10,dd (2,6)		7"
3"	146.5				
4"	144.8				
5"	149.5				
6"	106.6	7.01,d (1.8)	7.10,dd (2,6)		7"
7"	168.0				
3"-OMe	57.0	3.82,s	3.81,s		

Rutin (**3**) was obtained as a yellow amorphous powder; $[\alpha]_D^{20}$ = -11.8^{o} (c 0.10, MeOH); UV (MeOH) λ_{max} 203.0, 256.9 and 355.8nm; ESI-MS: *m/z* 611.0 $[M+H]^+$; ^1H and ^{13}C NMR spectroscopic data (Table 3).

Peltatoside (**4**) was obtained as a yellow amorphous powder; $[\alpha]_D^{20}$ = -15.0 ^{o} (c 0.10, MeOH); UV (MeOH) λ_{max} 203.0, 256.8 and 355.5 nm; ESI-MS: *m/z* 597 $[M+H]^+$; ^1H and ^{13}C NMR spectroscopic data (Table 3).

Cell viability MTT assay

The compounds were measured at treatment concentrations in the cell culture system.

Table 3: ^1H-NMR (500 MHz) data of compounds **3** and **4** in MeOH-d_4; J in Hz

	Rutin		Peltatoside	
Pos.	**δ_H (J in Hz)**	**δ_H [b] (J in Hz)**	**δ_H (J in Hz)**	**δ_H [e] (J in Hz)**
6	6.22,d (2.0)	6.20,d (1.6)	6.19,d (2,0)	6.19,d (1.5)
8	6.41,d (2.0)	6.39,d (1.6)	6.38,d (2.0)	6.38,d (1.5)
2'	7.67,d (2.1)	7.52,s	7.70,d (2,3)	7.67,d (1.5)
5'	6.88,d (8.4)	6.85,d (8.4)	6.87,dd (8.4)	6.87,d (7.8)
6'	7.64,dd (8.5,2.2)	7.55,d (8.4)	7.67,m	7.65,dd (7.8,1.5)
1"	5.11,d (7.7)	5.33	5.21,d (7.7)	5.18,d (7.5)
1"'	4.52,s	4.38	4.09,d (7.4)	4.05,d (7.3)
6"'-R	1.13,d (6.2)	1.11,d (6.0)	-	-
Sugar Protons	3.81-3.54		3.91-2.95	

[b] Biruk et al., 2012; [e] Clematis et al., 2011

Nitrite determination

The inhibitory effects of the compounds were evaluated on the production of nitrite, a stable metabolite of NO in RAW 264.7 cells that had been challenged with LPS in the presence or absence of the test compounds.

Measurement of TNF-α

The isolated polyphenols were evaluated and compared with regard to the production of TNF-α (major pro-inflammatory cytokines) in LPS-stimulated RAW 264.7 cells.

DISCUSSION

Compound 1 was obtained as a yellow amorphous powder (2.90 mg, t_R = 21.2 min). The UV spectrum showed absorption maxima λ_{max} (MeOH) 271.1 and 211.8 nm. The molecular formula $C_{25}H_{24}O_{10}$ was deduced from the HR-ESIMS with peak ion at *m/z* 485.14423 $[M+H]^+$, indicating fourteen de-

gree of unsaturation. The ^{1}H NMR (500 MHz, CD_3OD) spectral revealed an ABX-type aromatic protons at δ_H 6.71 (1H, d, J =8.2Hz, H-5'), 6.80 (1H,m,H-6') and 6.98 (1H, d, J =2.0Hz, H-2') and two-meta coupled aromatic protons signals at δ_H 5.98 (2H, dd, J = 12.6, 2.3Hz) attributed to the epicatechin protons in ring B. However, the galloyl moiety has a para methoxyl group with chemical shift δ_H 3.78 (3H, s, 4"-OMe) and two equivalent methoxyl groups with chemical shift δ_H 3.81 (6H, s, 3"/5"-OMe). In the ^{1}H-^{1}H COSY spectrum, H-3 proton (δ_H 5.10, s) was found to correlate to the two germinal protons (H_2-4) of C-4 and the aromatic proton (δ_H 6.71 d, H-5') was found to correlate with H-6' (δ_H 6.80, m). Compound **1** was isolated for the first time from a natural source. The NMR data of compound **1** agrees with literature values (Sánchez-del-Campo et al., 2008). Thus, the structure of compound **1** was established as 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG).

Compound 2 isolated from the EtOAc fraction as a yellow amorphous powder (15.80 mg, t_R =16.9 min). It showed UV absorbance at λ_{max} (MeOH) 216.4, 278.8 nm. Positive and negative ESI-MS showed molecular ion peaks at *m/z* 457 $[M+H]^+$ (base peak) and *m/z* 455 $[M-H]^-$ (base peak) respectively, indicating a molecular mass of 456 g/mol and a molecular weight of $C_{23}H_{20}O_{10}$. The ^{1}H NMR (500 MHz, CD_3OD) spectrum of ECG3″Me shows a 3,4-disubstitution on the ring B of the epicatechin unit with chemical shifts of δ_H 6.96 (d, J = 1.8 Hz, H-2'), 6.81 (dd, J = 8.2,1.8 Hz, H-6') and 6.70 (d, J = 8.2Hz, H-5') . The ^{1}H NMR also showed an AX-type aromatic protons at δ_H 7.02 (d, J= 1.8 Hz, H-2'') and 7.07 (d, J= 1.9 Hz, H-6') on the ring C, as well as one doublet of doublet at 5.97 ppm integrating as 2H suggesting that ring A is a tetra-substituted benzene ring. In the ^{1}H-^{1}H COSY spectrum, H-2 (δ_H 5.16, s) was found to correlate with H-3 (δ_H 5.51, s), which in turn correlates with H_2-4 (δ_H 2.88 and 3.01, each dd), in the ring A thus revealing a typical pattern of epicatechin nucleus. Also in the ring B, the aromatic proton H-5' (δ_H 6.7, d) was found to correlate with H-6' (δ_H 6.81, dd). The attachments of the methoxy group at position C-3" in the galloyl moiety was confirmed by the long range correlation of -OCH_3-3 to C-3" (δ_C 146.5) in the HMBC spectrum. The HMBC spectrum shows the correlation of H-2'' (δ_H 7.07) and H-6" (δ_H 7.01) with δc 168.0 (C-7"), as well as the correlation of 3"-OMe (δ_H 3.82) to C-7" (δ_C 57.0) suggesting that the oxymethyl group was connected to the benzene ring via C-3". The ^{13}C NMR (125 MHz, MeOH-d_4) spectrum showed 23 carbon signals, including one methylene (δ_C27.1), one oxymethyl group (δ_C 57.0), nine methines, 11 quaternary carbons and one ester carbonyl group (δ_C 168.0) as aided by the DEPT-135 experiment (Table 2). The NMR spectral data compares favourably with literature values (Saijo, 1982), thus compound **2** is named as (-)-Epicatechin 3-O-(3-O-Methyl) gallate (ECG3″Me).

Rutin (**3**) was isolated as a yellow amorphous powder (5.60 mg, t_R =20.2 min). The HPLC-ESIMS spectrum of **3** exhibited a peak ion at *m/z* 611.0 $[M+H]^+$, indicating a molecular formula of $C_{27}H_{30}O_{16}$, containing thirteen degrees of unsaturation. The UV spectrum showed absorbance maxima λ_{max} 203.0, 256.9 and 355.8 nm indicative of a quercetin diglycoside unit. In the ^{1}H NMR (500 MHz, CD_3OD) spectrum the aromatic protons exhibited an ABX coupling system at δ_H 7.70 (d, J=2.1Hz) for H-2', δ_H 7.67 (dd, J=8.5, 2.2Hz) for H-6' and δ_H 6.91 (d, J= 8.4 Hz) for H-5'. The other AX coupling system at δ_H 6.43 (d, J=2.0Hz) and δ_H 6.24 (d, J=2.0Hz) was assigned to H-8 and H-6 protons respectively, characteristic of quercetin moiety. From the mass spectrum data, fragments at *m/z* 465 $[M-146 + H]^+$ (loss of rhamnose); 303 $[M-146-162 + H]^+$ (loss of rhamnose and glucose), together with two anomeric protons at δ_H 5.11(d, J=7.7Hz) and δ_H 4.52 (1H, s) for glucose and rhamnose respectively, indicated the presence of two sugar residues in the molecule. The presence of a rhamnose moiety was further confirmed

by the presence of a methyl doublet at δ_H 1.13 (3H, d, J = 6.2 Hz) downfield in the ^{1}H NMR spectrum. The rest of the protons in the sugar moiety resonated between 3.57 and 3.84 ppm. The ^{1}H NMR data recorded on MeOH-d_4 were in good agreement with published values (Al-Sawi and Sleem, 2010; Biruk et al., 2012). Hence, the compound was identified unequivocally as rutin (3,3',4', 5,7-pentahydroxyflavone-3-rhamnoglucoside).

Peltatoside (4) was obtained as a yellow amorphous powder (2.60 mg, t_R =19.8 min). The HPLC-ESIMS spectrum of **4** exhibited a peak ions at *m/z* 597 $[M+H]^+$, and *m/z* 595 $[M-H]^-$ indicating a molecular formula of $C_{26}H_{28}O_{16}$, containing thirteen degrees of unsaturation. The UV spectrum showed absorbance maxima λ_{max} 203.0, 256.8 and 355.5 nm indicating a quercetin diglycoside unit. In the ^{1}H NMR (500 MHz, MeOH-d_4) three aromatic protons (δ_H 7.67, m); (δ_H 6.87, dd, J= 8.4 Hz) and (δ_H 7.70, d, J=2.3 Hz) assignable to H-6', H-5' and H-2' respectively. Also the ^{1}H NMR revealed two meta-coupled protons at δ_H 6.19, d (J =2.0 Hz) and δ_H 6.38(d, J= 2.0 Hz) for H-6 and H-8 respectively. From the mass spectrum data, fragments at *m/z* 465 $[M-132 + H]^+$ (loss of arabinose); 303 $[M- 132-162 + H]^+$ (loss of arabinose and glucose), together with the presence of arabinose and glucose moieties with the proton signals at δ_H 4.09, d, J=7.4 Hz and δ_H 5.21, d, J=7.7 Hz for the arabinose and glucose anomeric protons respectively, indicated the presence of two sugar residues in the molecule. The rest of the protons in the sugar moieties resonated between 2.95 and 3.91 ppm. The proton ^{1}H NMR data recorded in MeOH-d_4 were in good agreement with the published values (Clematis et al., 2011). Hence, the compound was identified as peltatoside.

Cell viability assays of the isolated compounds were assessed using MTT assay. The compounds were measured at treatment concentrations in the cell culture system. The polyphenols did not cause significant loss in viability of RAW264.7 cells even at concentration up to 500 µM after 24 h of incubation. The result showed that at 24 h incubation, the viability of the RAW262.7 cells remained greater than 90 % at the highest treatment dose of the test compounds, showing that the test compounds have less toxicity on the mouse macrophage cells (Table 4).

The inhibitory effects of the compounds were evaluated on the production of nitrite, a stable metabolite of NO in RAW 264.7 cells that had been challenged with LPS in the presence or absence of the test compounds. The test compounds effectively suppressed LPS-induced nitrite production in a dose-dependent manner. The NO in LPS-stimulated RAW 264.7 cells exposed to 100 µM of the test compounds inhibited iNO production by 89.8, 96.5, 98.7 and 99.2 % (% of the control), for TMECG, ECG3″Me, rutin and peltatoside respectively (Figure 2).

Table 4: Effect of compounds **1-4** on viability of RAW264.7 mouse macrophage cells

	Percentage viability of RAW264.7 cells after 24 h			
Treatment	**TMECG**	**ECG3''Me**	**Rutin**	**Peltatoside**
Control	100.0±0.66	100.0±0.66	100.0±0.66	100.0±0.66
5 µM	105.9±0.83*	103.9±0.75*	107.3±0.65*	104.9±0.47*
25 µM	108.6±0.70*	106.4±0.59*	110.9±0.97*	109.6±0.60*
100 µM	109.7±0.85*	111.2±1.17*	117.7±1.15*	112.8±1.46*
250 µM	101.5±0.76	112.8±1.01*	110.8±1.09*	106.2±0.93*
500 µM	98.0±0.50	96.7±0.84	92.0±0.92*	96.3±0.60*

Values represent mean ± SEM of the results from three separate experiments done in triplicate, n= 6, $p < 0.05$ significant. Dunnett's *post-hoc* LSD (least significant difference) test.*$p < 0.05$, comparison with control group

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The IC_{50} values of the polyphenols indicate that rutin and peltatoside have a higher iNO inhibitory activity compared to the catechin gallates with values of 0.013, 8.05,14.67 and 11.18 µM for peltatoside, rutin, ECG3''Me and TMECG, respectively. These results show that these polyphenols could be exerting an anti-inflammatory effect by ameliorating the production of inflammatory mediators, including NO.

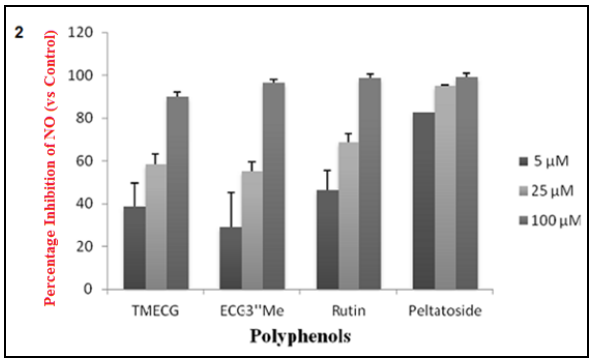

Figure 2: Suppression of LPS-induced NO production in RAW264.7 cells by the polyphenols of mistletoe parasitic on *Hevea brasiliensis*. Cells were treated for 24 h with 0, 5, 25, or 100 µM of the polyphenols in the presence of 1.0 µg/mL LPS. The normal group was treated with media only. The results are expressed as the mean ± SEM from three independent experiments.

The isolated polyphenols were evaluated and compared with regard to the production of TNF-α (major pro-inflammatory cytokines) in LPS-stimulated RAW 264.7 cells. At 24 h, the polyphenols at treatment dose of 5 to 100 µM produced significant dose-dependent decrease in TNF-α production. In particular, the inhibition in the production of TNF-α by the catechin gallate derivatives at treatment dose of 100 µM revealed comparable inhibition in the production of proinflammatory cytokine of 88.2 and 82.4 % (% of the control) for TMECG and ECG3″Me, respectively. This trend was also observed in the inhibition of the cytokine by the quercetin diglycosides at the same treatment dose with percentage of inhibition of 91.3 and 99.6 % (% of the control) for rutin and peltatoside respectively (Figure 3). Also, the quercetin diglycosides had a lower IC_{50} (11.84 and 7.75 µM) when compared to the IC_{50} of the catechin gallates (14.94 and 22.60 µM) (Table 5).

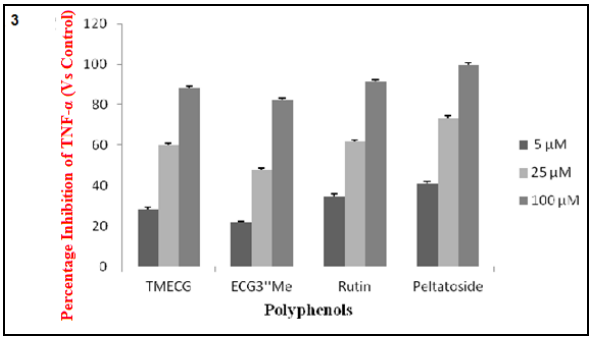

Figure 3: Suppression of LPS-induced cytokine production (TNF-α) in RAW264.7 cells by the polyphenols of mistletoe parasitic on *Hevea brasiliensis*. RAW264.7 cells were treated for 24 h with 0, 5, 25, or 100 µM of the polyphenols in the presence of 1.0 µg/mL LPS. The normal group was treated with media only. The cell culture media were then collected. The results are expressed as the mean ± SE from three independent experiments.

Table 5: IC_{50} [a] values of compounds **1-4**

	NO	TNF-α
Compounds	**IC_{50} (µM)**	**IC_{50} (µM)**
TMECG	11.18	14.94
ECG3''Me	14.67	22.60
Rutin	8.05	11.84
Peltatoside	0.013	7.75

[a] IC_{50} value was calculated from the least-squares regression equations in the plot of the % inhibition vs. logarithm of three graded concentrations.

STATISTICAL ANALYSIS

The data were expressed as mean ± SEM of at least triplicate determinations (n = 3). To demonstrate statistical significance of data, a One-way Analysis of Variance (ANOVA) using GraphPad Prism 5 software was performed followed by Dunnett's *posthoc* test. Differences between test and control treatments are considered significant at $P < 0.05$.

CONCLUSION

The four polyphenolic compounds isolated from mistletoe and investigated in this study showed potent inhibition of inducible release of important proinflammatory mediators, NO and TNF-α. Mistletoe is used in alternative and complementary medicine for a variety of indications including treatment of degenerative inflammation of the joints (Blumenthal, 1998). In a recent study, the anti-oxidative property of these polyphenols has been reported (Agbo et al., 2013).

Polyphenols can exert their anti-inflammatory properties through the modulation of mitogen-activated protein kinases (MAPK) signalling pathways (Kong et al., 2000; Wiseman et al., 2001) and NF-κB and AP-1 transcription factors (Manna et al., 2000), inhibition of the production of inflammatory cytokines and chemokines, suppressing the activity of cyclooxygenase (COX) (O'Leary et al., 2004) and inducible nitric oxide synthase (iNOS) (Donnelly et al., 2004) and thereby decreasing the production of reactive oxygen and nitrogen species (ROS/RNS). Previous studies have shown that quercetin inhibits lipopolysaccharide (LPS)-stimulated TNF-α production and NF-κβ activation in RAW 264.7 macrophage (Comalada et al., 2005; Cho et al., 2003; Wadsworth and Koop, 1999). Recently, a study showed that oral administration of rutin reduced, in a dose-dependent manner, the polymorphonuclear neutrophils chemotaxis to FMLP in a model of rat paw oedema (Sunita et al., 2011). Cytotoxicity profiles for the isolated polyphenols were assayed in parallel using the MTT protocol (Table 1). The result of the cytotoxicity assay showed that the inhibition of proinflammatory mediators caused by the compounds was not due to loss in viability of the treated murine macrophages at the concentration range used.

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