

Gallic Acid Derivatives (GADs) from *Loranthus micranthus* Linn. Parasitic on *Hevea brasiliensis* with Antioxidative Capacity

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ABSTRACT: Semi-preparative High Liquid Column Chromatographic separation of the ethyl acetate soluble fraction of a methanol extract of the leaves of *Loranthus micranthus* Linn. parasitic on *Hevea brasiliensis* (Willd. ex A. Juss.) led to the isolation of two gallic acid derivatives (**1-2**). The structures of the compounds were solved by using spectroscopic methods (1D and 2D NMR and mass spectroscopic data) as well as by comparison with literature values. The compounds were identified as 3,4,5-tri-*O*-methyl gallic acid (**1**) and methyl 3-*O*-methyl gallate (**2**). The antioxidant activity of the isolated compounds was evaluated by using DPPH assay. Compounds **1** and **2** showed strong antioxidative capacity with inhibitory concentration (IC₅₀) values of 10.0 ± 0.03 and 45.0 ± 0.02 μM, respectively.

Key words: *Loranthus micranthus*, Structure elucidation, Gallic acid, Antioxidant, Oxidative stress

INTRODUCTION

Oxidative stress which results in excessive oxidative metabolism means an imbalance between pro-oxidants and antioxidant in which the pro-oxidant outweighs the antioxidant.¹ This stress can be due to several environmental factors such as exposure to pollutants, alcohol, medications, infections, poor diet, toxins, radiation, etc. Oxidative stress causes injury to cells, induces gene mutation, and is involved in carcinogenesis by influencing intracellular signal transduction and transcription factors directly or indirectly via oxidants. Oxidative damage is caused by free radicals and reactive oxygen species, mostly generated endogenously.²

Free radicals and reactive oxygen species can be involved in a higher number of diseases via lipid peroxidation, protein peroxidation and DNA damage.³ Currently, there is great interest in finding antioxidants from natural sources to minimize oxidative damage to cells. Gallic acid derivatives (GADs) are naturally occurring polyphenols that act as antioxidants by efficiently scavenging free radicals.⁴ The antioxidant properties of these natural polyphenols are assumed to occur by hydrogen atom transfer and single-electron transfer mechanisms.⁴ *Loranthus micranthus* Linn is a hemi parasitic plant growing on different host trees and shrubs but depends on their host plant for water and mineral nutrition, despite the fact that they produce their own carbohydrates through photosynthesis.⁵ The isolation of polyphenols from *L. micranthus* parasitic on

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Hevea brasiliensis and *Kola acuminata* with antioxidative potentials has been reported.^{6,7} The present research aims at isolating and characterization of GADs from *L. micranthus* parasitic on *H. brasiliensis* and the evaluation of their antioxidant properties.

MATERIALS AND MATERIALS

General. Optical rotation of the isolated compounds was recorded on a Perkin-Elmer 241 MC polarimeter. 1D and 2D NMR spectra of the isolated compounds were acquired on Bruker ARX (500 MHz). Electrospray Impact Mass Spectrometric (ESI-MS) measurement was performed on a Thermofinnigan LCQ DECA mass spectrometer. Analytical High Performance Liquid Column Chromatography (HPLC) was performed with a HPLC system (Dionex, Munich, Germany). Semi-preparative HPLC was performed on a MERCK HITACHI system equipped with a UV Detector. Vacuum liquid chromatography (VLC) was conducted on Silica gel 60 (0.04-0.063 mm mesh Merck) using a glass column (3×30 cm). Gel permeation column chromatography (CC) was carried out on Sephadex LH-20 (Merck, Germany) using a glass column (3 ×110 cm).

Chemicals. Methanol, *n*-hexane, *n*-butanol, ethyl acetate, propyl gallate, spectral grade methanol (Sigma), DMSO-*d*₆ (Uvasol, Merck), methanol-*d*₄ (Uvasol, Merck), methanol LiChroSolv HPLC grade (Merck). All solvents used were of analytical grade and purchased from the Institute of Chemistry, Heinrich-Heine Universität, Düsseldorf Germany. The non-spectra grade solvents were distilled before use and special grades were used for spectroscopic measurements.

Plant material. *Loranthus micranthus* Linn. leaves parasitic on *H. brasiliensis* were collected from Enugu-Ezike in Enugu State, Nigeria. The leaves were identified by Mr. A.O. Ozioko of the International Center for Ethnobotanical and Ethnomedicine Development (InterCEED) Nsukka, Enugu State, Nigeria. A voucher specimen (LM1610) was deposited at the herbarium of the Institute. The

leaves were air dried in the laboratory and pulverized using Thomas Willey Mill (England).

Extraction and solvent-solvent partitioning. Three hundred gram (300 g) plant powder was extracted with 2.5 L of methanol at room temperature for 48 hrs by cold maceration. The extract was concentrated *in vacuo* at 40°C using rotary evaporator (Büchi Rotavapor R-200, Germany). The methanol extract (42.0 g, 6.3%) was suspended in 400 ml of 10% methanol-water mixture and successively fractionated with *n*-hexane (5 x 500 ml), ethyl acetate (6 x 500 ml) and *n*-butanol (2 x 500 ml) to yield *n*-hexane (HF, 4.45 g, 11.12%), ethyl acetate (EF, 5.23g, 13.08%), and *n*-BuOH (BF, 1.02 g, 2.55%) soluble fractions, respectively. Part of the ethyl acetate soluble fraction (3.5 g) was purified further by vacuum liquid chromatography using silica gel (500 g, 230–400 mesh, Merck) as the stationary phase. The column was then eluted with a gradient of *n*-hexane-ethyl acetate (10:0 →0:10, each 500 ml) and dichloromethane-methanol (9:1 →1:9, each 1000 mL) to afford ten sub-fractions (EF1-EF10).

Isolation of the compounds. Fraction EF4 (44.60 mg) was purified by semi-preparative HPLC using MeOH-H₂O as mobile phase to afford compound **1** (2.90 mg). Also, fraction EF6 (348.5 mg) was further purified by fractionation on Sephadex LH-20 (100% MeOH) to provide seven sub-fractions (EF6A-EF6G). Fraction EF6B (38.20 mg) was purified by semi-preparative HPLC using MeOH-H₂O as mobile phase to yield compound **2** (9.20 mg).

Properties of 3,4,5-tri-*O*-methyl gallic acid (1). White amorphous solid; retention time (*t*_R) of 19.4 min; [α]_D²⁰ - 40.7 (c 0.10, MeOH); UV_{max} (MeOH) 216.6, 275.7 nm; ¹H NMR (DMSO-*d*₆) δ 3.87 (s, 3H, CH₃ -4-OMe), 3.88 (s, 6H, 3/5 -OMe), 7.32 (s, 2H, H-2/6). MS (ESI-MS) *m/z* 213.0 [M+H]⁺ (C₁₀H₁₂O₅+H), 181 [M-32+H]⁺ (loss of -OCH₃), 447 [2M+Na]⁺.

Properties of methyl 3-*O*-methyl gallate (2). White amorphous solid; retention time (*t*_R) of 15.0 min; [α]_D²⁰ -9.0 (c 0.02, MeOH); UV_{max} (MeOH) 215.8, 274.4 nm; ¹H NMR (δ values, CD₃OD), and

^{13}C (δ values, CD_3OD) NMR data: see table 1. MS (ESI-MS) m/z 199 $[\text{M}+\text{H}]^+$ ($\text{C}_9\text{H}_{10}\text{O}_5+\text{H}$).

Antioxidant assay. The antioxidant assay of the compounds was performed according to the procedure described by Agbo *et al.*⁸ Compounds **1-2** were dissolved in methanol to give a concentration of 1 mg/1000 μl stock solution. Ten microliter (10 μl) of these solutions was added to 490 μl DPPH solution (4.5 mg/100 mL) in an ependorf vial. The mixture was incubated for 5 min and the color change from deep violet to light yellow of the DPPH free radical was measured by recording the absorbance using a UV/Visible spectrophotometer (Perkin Elmer, Lambda 25) at 517 nm. Prior to the measurement, the difference in absorption between a DPPH blank solution and the positive control (propyl gallate, 76 μM) was determined. This difference was then taken as 100% antioxidative activity. The percent antioxidative activity was determined from the difference in absorption between the samples at 76 μM and the DPPH blank as follows:

$$aA (\%) = \frac{AB - Ap}{AB - Apos} \times 100$$

Where, aA = % antioxidative activity compared to the positive control, AB = absorption of the DPPH blank solution, Ap = absorption of the sample, and $Apos$ = absorption of the positive control (propyl gallate). Measurements were done in triplicates, and the IC_{50} values were determined by linear regression.

RESULTS AND DISCUSSION

The methanol extract of the leaves of *Loranthus micranthus* Linn leaves parasitic on *H. brasiliensis* was partitioned with *n*-hexane, ethyl acetate and *n*-butanol respectively. The ethyl acetate fraction was purified and two compounds were obtained (Figure 1). By means of spectroscopic analysis, they were identified as 3,4,5-tri-*O*-methyl gallic acid (**1**) and methyl-3-*O*-methyl gallate (**2**). These secondary metabolites were isolated from the leaves of *L. micranthus* leaves parasitic on *H. brasiliensis* for the first time.

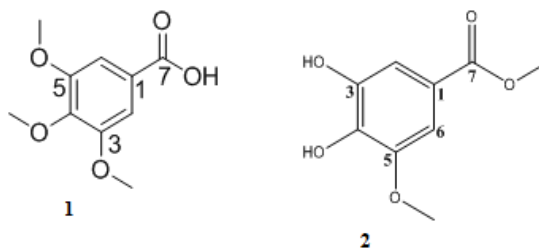


Figure 1. Chemical structures of compounds **1-2**.

Antioxidant assay. The antioxidative potentials of the isolated compounds were assessed using DPPH assay and propyl gallate as a positive control.

Compound **1** was obtained as white amorphous solid. The ESI-MS exhibited molecular ion peaks at m/z 213.0 $[\text{M}+\text{H}]^+$ which is consistent with the molecular formula of $\text{C}_{10}\text{H}_{12}\text{O}_5$. UV spectra displayed characteristic absorption bands for the benzoid nucleus and $\text{C}=\text{O}$ chromophores at 217 and 276 nm respectively (Figure 2). The ^1H -NMR spectrum of compound **1** indicated a singlet at δ_H 7.32 ppm integrating as 2H, suggesting the presence of tetra-substituted benzene ring. The proton signals at δ_H 3.88 ppm (6H, s) suggested the presence of two equivalent aromatic methoxyl groups (3/5-OMe) and another three proton singlet at δ_H 3.67 revealed the presence of another methoxyl at C-4, respectively. The mass spectrum showed fragments at m/z 181 $[\text{M}-32 + \text{H}]^+$ (loss of $-\text{OCH}_3$ unit); m/z 447 $[2\text{M}+\text{Na}]^+$ (Figure 3). The NMR data of compound **1** agreed with well published values for methyl 3,4,5-tri-*O*-methyl gallate.⁹ Therefore, compound **1** was characterised as 3,4,5-tri-*O*-methylgallic acid.

Compound **2** was obtained as a white amorphous solid. The ESI-MS of compound **2** exhibited molecular ion peak at m/z 199.1 $[\text{M}+\text{H}]^+$ suggesting that the molecular mass is 190 g/mol with a molecular formula of $\text{C}_9\text{H}_{10}\text{O}_5$ and having five degree of unsaturation. The ^1H NMR spectrum showed a singlet at δ_H 7.32 ppm integrating for 2H, suggesting the presence of tetra-substituted benzene ring. The proton signals at δ_H 3.87 ppm (3H,s) and δ_H 3.84 ppm (3H,s) in the ^1H NMR spectrum and the carbon signals at δ_C 52.8 ppm and δ_C 57.2 suggested the presence of an ester methoxyl and an aromatic

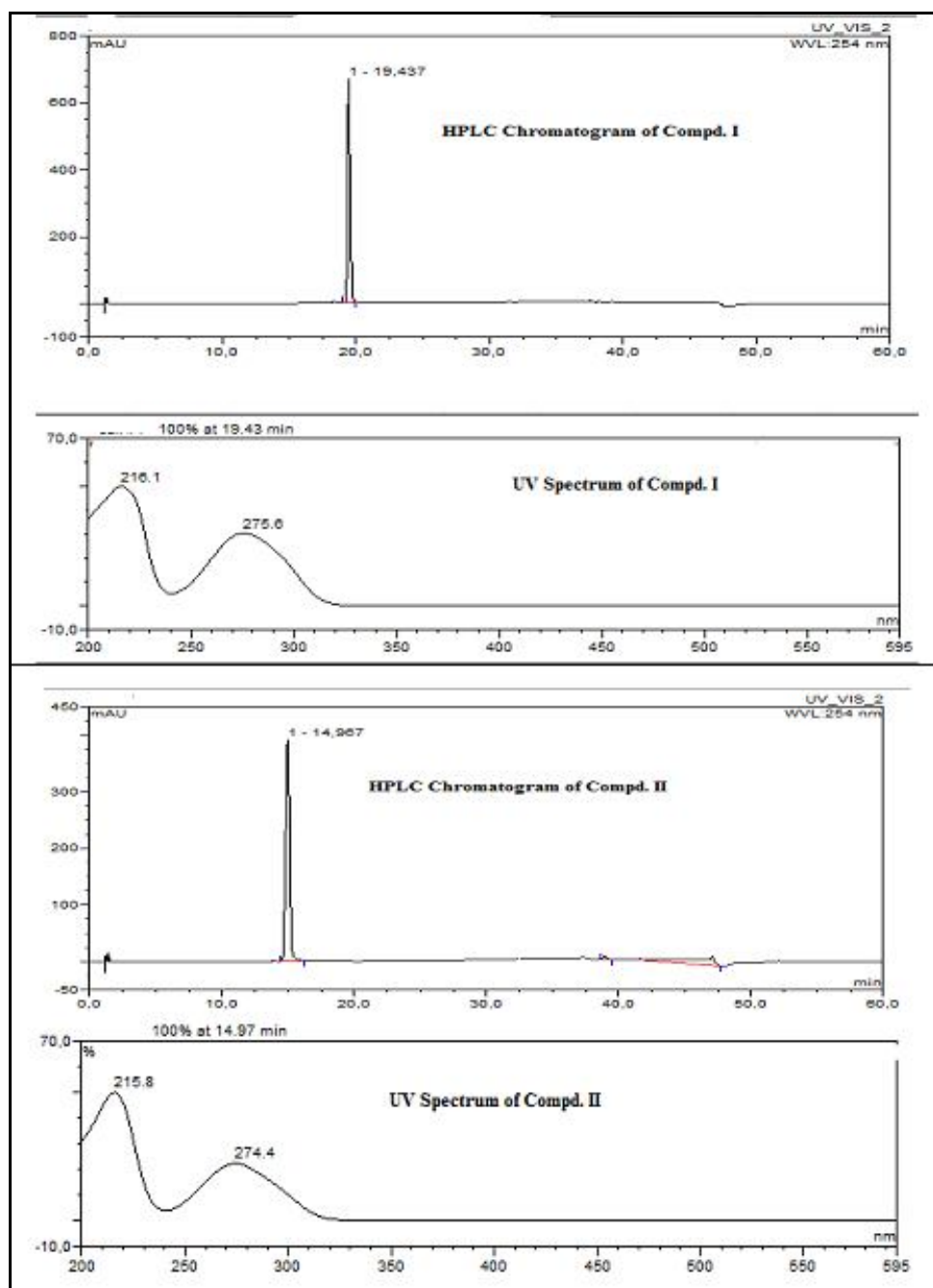


Figure 2. HPLC and UV analysis of Compounds **1** and **2**. HPLC column: C18 (5 μ m) 3000x8mm; gradient elution: methanol (solvent A) and nano pure H₂O (solvent B), detection wavelength: 254 nm. Flow-rate: 5.0 mL/min. The ethyl acetate fraction was purified by semi-preparative HPLC to afford compounds **1** and **2**.

methoxyl groups, respectively (Table 1). The ¹³C NMR spectrum of compound **2** exhibited resonances for nine types of carbons comprising a C=O signal at δ_c 169.0 (indicating the presence of carbonyl ester),

two unsymmetrical oxygenated aromatic carbon signals at δ_c 146.4 (C-3) and δ_c 140.8 (C-4); one quaternary aromatic carbon signal at δ_c 121.6(C-1) and two unsymmetrical aromatic methine carbon

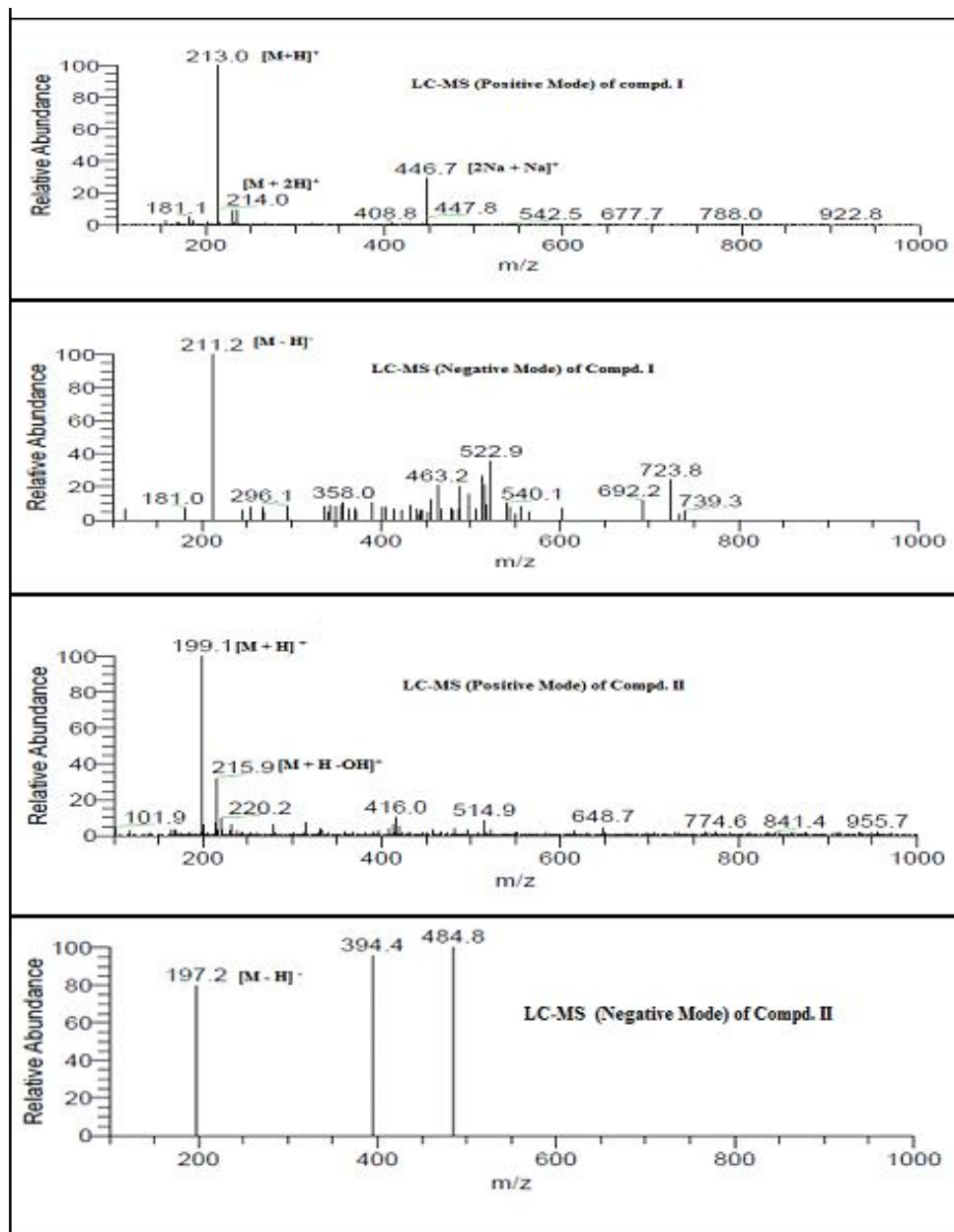


Figure 3. LC-MS Data of Compounds 1-2.

signals at δ_c 112.4 and 106.2 (C-2 and C-6) (Table 1). Based on HMBC spectrum, 3J correlations were observed between methine aromatic protons signal at δ_H 7.17 with the carbonyl ester carbon signal at δ_c 169.0. Further analysis of HMBC spectrum showed correlation between the ester methoxyl proton signals at δ_H 3.87 with carbonyl ester carbon signal at δ_c 169.0 (Figure 4). Similar correlations were also

observed in methyl gallate structure elucidation as reported by Mohd *et al.*¹⁰ thus compound 2 is elucidated as methyl 3-O-methylgallate.

Gallic acid and its derivatives are widely present in the plant kingdom and are known to display antioxidant activity by the ability to counteract the damaging effects of free radicals in tissues and thus

are believed to protect against cancer, arteriosclerosis, heart disease, and several other diseases.⁹ Among polyphenols, gallic acid derivatives are a well-known group of naturally occurring compounds which have been found in many phytomedicines.¹¹ The antioxidant activity of plant extracts or isolated secondary metabolites can be determined using different methods. The commonest

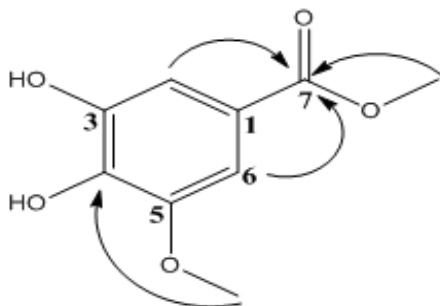


Figure 4 . Key HMBC (H→C) correlations of compound 2

Table 1. ¹H (500 MHz), ¹³C (125 MHz) NMR and HMBC data of compound 2.

Position	δ_c	δ_H (J in Hz)	HMBC
1	121.6	-	-
2	106.2	7.17,d (1.9)	4,5,7
3	149.5	-	-
4	140.8	-	-
5	146.4	-	-
6	112.4	7.17,d (1.9)	4,5,7
7	169.0	-	-
3-OMe	57.2	3.84,s	-
7-OOMe	52.9	3.87,s	7

Table 2. Antioxidant assay of compounds 1-2.

Compounds	Antioxidative potential
	IC ₅₀ (μM)
1	10.0 ± 0.03
2	45.0 ± 0.02
Chlorogenic acid	67.9 ± 0.10

method is the chemical assay, which is based on the ability, to scavenge various kinds of free radicals and uses DPPH (1, 1-diphenyl-2-picrylhydrazyl).¹² DPPH is scavenged by antioxidants either by donating a hydrogen atom or transferring of an electron to the odd electron of nitrogen to neutralize free radical

character, leading to the loss of its purple color to the yellow colour of a residual picryl group. This change in the colour can be quantified spectrophotometrically by a decrease in absorbance at wavelength 517 nm.¹³ The antioxidant activities of the isolated compounds were assayed using the DPPH assay with propyl gallate as positive control. The IC₅₀ isolated compounds (**1-2**) were found to be 10.0 ± 0.03 and 45.0 ± 0.02 μM, respectively. The isolated compounds were found to exhibit more antioxidative potentials than the reference compound (chlorogenic acid) with IC₅₀ value of 67.9 ± 0.10 μM (Table 2) in DPPH assay. The result indicates that gallic acid derivatives from Nigerian mistletoe could be used in the management of some ailments caused by oxidative stress.

CONCLUSION

The present study has revealed that gallic acid derivatives isolated from *L. micranthus* parasitic on *H. brasiliensis* possesses antioxidative potentials. Work is on-going with a view to compounding Mistletoe tea that could be used to scavenge free radicals from the body.

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