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Full Length Research Paper

Bioactivity of the phytoconstituents of the leaves of *Persea americana*

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Isolation of the bioactive phytoconstituents of the leaves of *Persea americana* (Lauraceae), yielded, isorhamnetin (1), luteolin (2), rutin (3), quercetin (4) and apigenin (5). Compound 1 has been fully characterized. Compounds were evaluated for their ability to scavenge free radical using $DPPH^{\bullet}$ and H_2O_2 systems. The IC_{50} (mg/ml) of quercetin was 4.82×10^{-5}; rutin, 1.37×10^{-4}; luteolin, 3.34×10^{-4} and isorhamnetin, 4.41×10^{-4}. H_2O_2 scavenging activity of the compounds was quercetin > rutin > isorhamnetin > luteolin > apigenin > BHA ($p > 0.05$). From this study it could be concluded that the leaves of *P. americana* contain antioxidant activity which may be helpful in preventing the progress of various oxidative stress related diseases.

Key words: *Persea americana*, Lauraceae, flavonols, antioxidant activity, oxidative stress, radical scavenging.

INTRODUCTION

Persea americana Mill. (Lauraceae) has been greatly appreciated in recent times and researchers have found scientific support for its use in folk medicine. The aqueous extract of *P. americana* has been shown to produce vasorelaxation of the rat aortic ring and lower blood pressure (Owolabi et al., 2005; Adeboye et al., 1999). The work of (Ekor et al., 2006) reported the protective effect of *P. americana* against hepatotoxicity induced by paracetamol, (Adeyemi et al., 2002) determined its anti-inflammatory and analgesic properties and (Ojewole and Amabeoku, 2006) investigated its anticonvulsant property. The seeds of *P. americana* has been reported to lower blood pressure in normotensive and hypertensive rat models (Anaka et al., 2009; Imafidon and Amaechina, 2010) with reduction in the total cholesterol, LDL and triacylglycerol in the plasma, kidney, liver and heart of the hypertensive rat model at high dose of the seed extract (Imafidon and Amaechina, 2010). In our earlier study, phytochemical screening of the leaf extract of *P. americana* revealed the presence of flavonoids which were powerful antioxidants capable of scavenging free radicals (Rice-Evans, 1995; Rice-Evans and Miller, 1996; Rice-Evans et al., 1996; Owolabi et al., 2007) by donating a hydrogen atom or electron to stabilize the radical species (Figure 1). The metabolic study of the aqueous leaf extract of *P. americana* in rat model showed the presence of phenolic acids (Owolabi et al., 2007) which were metabolites of flavonol degradation by intestinal microflora (Havsteen, 2002).

To our knowledge, there is still limited literature on the radical scavenging activities of the leaves of *P. americana.* Additionally, compounds of the Nigerian specie of this plant have not been reported in the literature. Thus in continuation with our study of the leaves of *P. americana*, and as part of our on-going search for biologically active compounds, this study investigated the antioxidant activity of the compounds isolated from the leaves of *P. americana*. The results of this study would help in understanding the use of this plant in the treatment of various free radical mediated diseases.

MATERIALS AND METHODS

Collection of plant material

The leaves of *P. americana* previously identified (No. - FHI 106099)

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was collected in June, 2002 from the University of Lagos, Akoka, Lagos, Nigeria. The plant material was dried at 40°C for 5 days, powdered and stored in an impervious amber bottle until used.

Chemicals and instrumentation

All reagents were of analytical grade purchased from Sigma Chemical Co., London except stated otherwise. Silica gel 60 (70 - 230 mesh, Merck) was used for open column chromatographic separation. Silica Gel 60 F_{254} from Riedel-de Haeen was used to coat TLC plates in our laboratory. Compounds were detected under ultra-violet (UV)-light at 254 nm or spraying with anisaldehyde reagent and heating at 120°C for 3 min. The ultraviolet spectra were obtained using Hewlett-Packard Agilent 8453 UV-visible spectrometer (Germany). The Mass spectra were recorded on a Fisons Trio 100 quadrupole mass spectrometer. NMR spectra were recorded in DMSO-d_6 on Varian VXR 300s spectrometer at 300 MHz for ^{1}H and 75.5 MHz for ^{13}C using TMS as internal standard. Infra red spectra were recorded on Beckmann spectrophotometer. The elemental compositions were done on a Carlo Erba 1106 elemental analyzer.

Extraction, isolation and structure elucidation

The pulverized leaves (1.5 kg) were successively defatted with petroleum ether (60 - 80°C) then extracted with aqueous EtOH (70 %). Concentration of the 70% EtOH solution using rotary evaporator yielded 308.39 g residue which was 88% active in lowering blood pressure (Figure 2). The extract residue was chromatographed on silica gel column (70 - 230 µm, 3.5 x 60 cm) eluted with toluene then EtOAc in toluene, up to 100% and MeOH in EtOAc, up to 100% to give 15 fractions (F1-F15) on the basis of their tlc distribution and directed at each step by blood pressure lowering activity. Repeated cc and PTLC of F6 (435.3 mg) using EtOAc: MeOH (6:4) gave 2 frs., (fr. I, 101.2 mg and fr. II, 83.5 mg). Fr. II on acid hydrolysis afforded compound 1 (36.1 mg) and rutinoside (glucose and rhamnose) as the sugar portion as revealed by its co-chromatography with reference standards. Fr. I was further separated into 3 frs., Ia-Ic on PTLC using EtOAc-MeOH (8:2). Hydrolysis of fr. Ia afforded 2 (15.3 mg) and glucose as the sugar portion as determined by co-paper chromatography with reference standard in BuOH: AcOH: H_2O (4:5:1).

Purification of fr. Ic using MeOH: H_2O (8:2) afforded 3 (31.6 mg) and on acid hydrolysis gave 4 (8 mg) and glucose as the sugar portion. Fr. F7 (668.2 mg) was subjected to repeated column chromatography eluting with MeOH to give 4 (28.1 mg). Repeated cc of fr. F8 using $CHCl_3$: EtOAc (4:1) afforded 5 (7.7 mg). Compounds 2, 3, 4 and 5 were identified by comparison of the UV shift data and the NMR spectra data with those reported in the literature (Kumarasamy et al., 2005; Markham et al., 1978; Harborne, 1975). The structure of compound 1 was determined by detailed spectroscopic analyses using UV shift reagent, NMR, DEPT, IR and MS. Thereafter, free radical scavenging activity of each compound was evaluated.

Radical scavenging activity - DPPH

Compounds 1 - 5 were investigated for free radical scavenging activity using the method of (Geckil et al., 2005), butylated hydroxyl anisole (BHA) was used as standard. One ml each of different concentrations (1 to 20 x 10^{-4} mg/ml) of the compounds and BHA were separately added to 1 ml of 2 x 10^{-4} M DPPH$^{\bullet}$. The reaction mixtures were left in the dark for 30 min and the resulting colour was measured spectrophotometrically at 520 nm against the control.

Antioxidant activity – hydrogen peroxide scavenging

Hydrogen peroxide scavenging ability was determined according to the method of (Ruch et al., 1989). One ml of each compound at different concentrations of 10 and 20 x 10^{-4} mg/ml was added to 0.6 ml, 40 mM H_2O_2 solution prepared in phosphate buffer (pH 7.4). Absorbance of the reaction mixture was recorded at 230 nm after 10 min against a blank solution (phosphate buffer, pH 7.4). BHA was used as standard. The percentage of H_2O_2 scavenged by each compound or standard was calculated as % scavenged H_2O_2 = $[(A_0-A_1)/A_0] \times 100$ where A_0 is absorbance of control, and A_1 is absorbance of each compound or BHA.

Data and statistical analysis

Data are expressed as mean ± SD (standard deviation). Statistical analyses were performed with Student's t-test using SPSS 11 statistical package. Values were considered to differ significantly if the P value was less than 0.05.

RESULTS AND DISCUSSION

Initial screening of the 70% EtOH extract of the leaves of *P. americana* showed very good antioxidant properties evident by the increasing reduction of the stable DPPH radical (Table 1), and lowering blood pressure by 88% (Figure 2), hence, the extract was subjected to bioactivity-directed fractionation. Chromatographic separation of the 70% EtOH extract over silica gel using cc followed by continued purification on preparative TLC afforded five compounds.

Compounds 2 (luteolin), 3 (rutin), 4 (quercetin) and 5 (apigenin) were identified by comparison of the UV shift data and the ^{1}H and ^{13}C NMR spectra data with those reported in the literature. Compounds 1 (isorhamnetin) was obtained as a greenish yellow crystals, (m.p. 188-190°C) its empirical formula, $C_{16}H_{12}O_7$ was deduced from the elemental composition, C-62.41%, H-3.86% and O-33.73% and EIMS which showed the $[M]^+$ at m/z 316. Compound 1 gave positive Mg-HCl (reddish) and $FeCl_3$ (greenish brown); uv λmax (MeOH) nm: 260, 272(sh), 322(sh), 367; +NaOAc: 258(sh), 290, 375; +$AlCl_3$: 267, 298(sh), 439; +$AlCl_3$/HCl: 243(sh), 265, 334(sh), 423; EIMS fragmentation of 1 produced ions at 302 (M–14, base peak), 301 (M–15, demethylation), 285 (M–31, demethoxylation), 273 (M–43, flavonol ring contraction), 193 (M–3´-O-methyl-4´-hydroxyphenyl B ring), 153, 152, and 137 attributed to typical RDA fragmentation; IR υ_{max} cm^{-1} (film on KBr plate) 3400.3 (OH), 2980.7 (C-H), 1690.5 (C=O), 1640.8 (C=C).

The positive Mg-HCl and $FeCl_3$ tests observed with compounds 1 suggests the presence of flavonoid skeleton. The UV spectrum of compound 1 showed band II at 260 nm and band I at 367 nm being compatible with 4' hydroxylation (Mabry et al., 1970). Sodium acetate (NaOAc) and $AlCl_3$-HCl induced bathochromic shifts of band II indicated the presence of a free hydroxyl group at the C-5 and C-7. The ^{1}H-NMR (DMSO-d_6) showed five

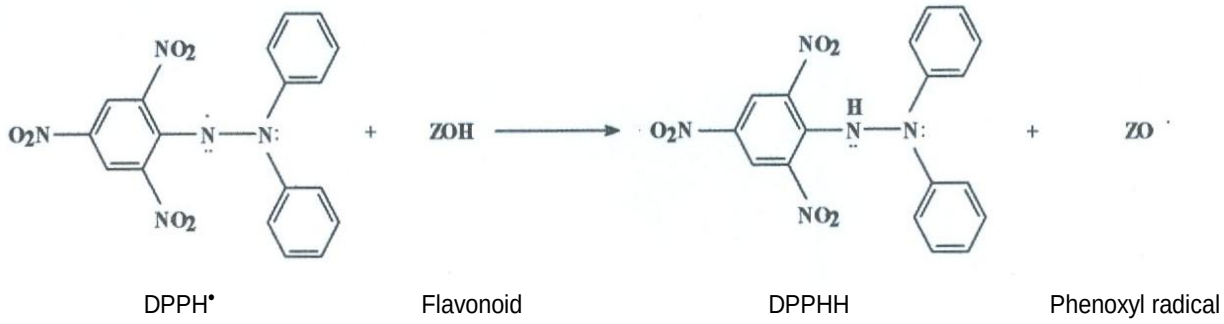


Figure 1. DPPH free radical scavenging by flavonoid.

Figure 2. Arterial blood pressure (BP) tracings of the 70% ethanol extract of the leaves of *P. americana* in anaesthetized normotensive rats.

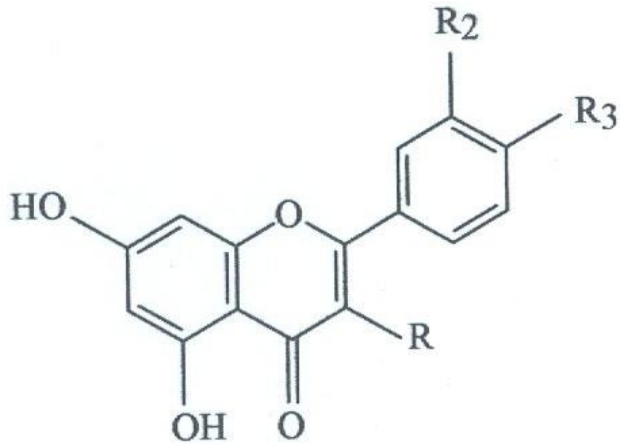
Table 1. Scavenging activity of 70% ethanol extract of the leaves of *P. americana* determined by the scavenging of DPPH radical.

Conc. of 70% ethanol extract (x 10^{-4} mg/ml)	% Scavenging activity
0	0.00 ± 0.00
0.1	33.17 ± 1.23
0.2	62.13 ± 1.18
0.4	87.23 ± 1.13
0.8	90.22 ± 0.98
1.0	92.11 ± 1.17
2.0	95.62 ± 1.21
4.0	98.34 ± 0.61

Values are mean ± SD. n=4, significance is set at $p \leq 0.05$.

one proton doublets at δ, 8.05 (J= 2.25 Hz H-2'); δ, 7.7 (J= 2.1 Hz, H-6'); δ, 7.1 (J= 4.5 Hz, H-5'); δ, 6.4 (J= 1.5 Hz, H-8); δ, 6. 2 (J= 1.5 Hz, H-6), a methoxyl signal at δ, 3.3 (3H, S, OMe-3') with typical signals of a chelated 3, 5, 7 OH groups appearing at δ, 9.2, δ, 10.6 and δ, 9.5 respectively. In the ^{13}C-NMR (DMSO-d_6), carbon resonances at lowest field are generally those of carbonyl carbon and the Ar-OCH_3 signals at about 55 - 60 ppm (Markham et al., 1978; Markham et al., 1982). Thus signal at 176.165 ppm was assigned to C-4 while the signal at 56.032 ppm was assigned to the methoxyl carbon on C-3'. The presence of a methoxyl group was evident by ion fragment at 285 [M-31], ^{1}H nmr signals at δ, 3.3 (3H, s) and ^{13}C nmr signals at 56.032. The position of a methoxyl group was confirmed through a cross peaks between C-3'/OMe3' in the HMBC spectra. The DEPT spectrum showed downward signals for the methyl and aromatic methine carbons and upward signals for the quaternary carbons which is in agreement with the work of (Silverstein and Webster, 1998). Based on these spectrochemical analyses, compound 1 was characterized as isorhamnetin (Figure 3).

The compounds (1 - 5) isolated from the leaves of *P. americana* clearly exhibited antioxidant activity in a concentration dependent manner using DPPH• (Figure 4) and H_2O_2 (Figure 5) assays. DPPH• is a free radical compound widely used to test the free radical scavenging ability of flavonoids (Figure 1). The scavenging of DPPH•



Quercetin (**4**)	$R=R_2=R_3=OH$
Rutin (**3**)	$R=O$-glucose; $R_2=R_3=OH$
Luteolin (**2**)	$R=H$; $R_2=R_3=OH$
Isorhamnetin (**1**)	$R= R_3=OH$; $R_2=OCH_3$
Apigenin (**5**)	$R=R_2=H$; $R_3=OH$

Figure 3. Structure of the flavoniods isolated from the leaves of *P. Americana.*

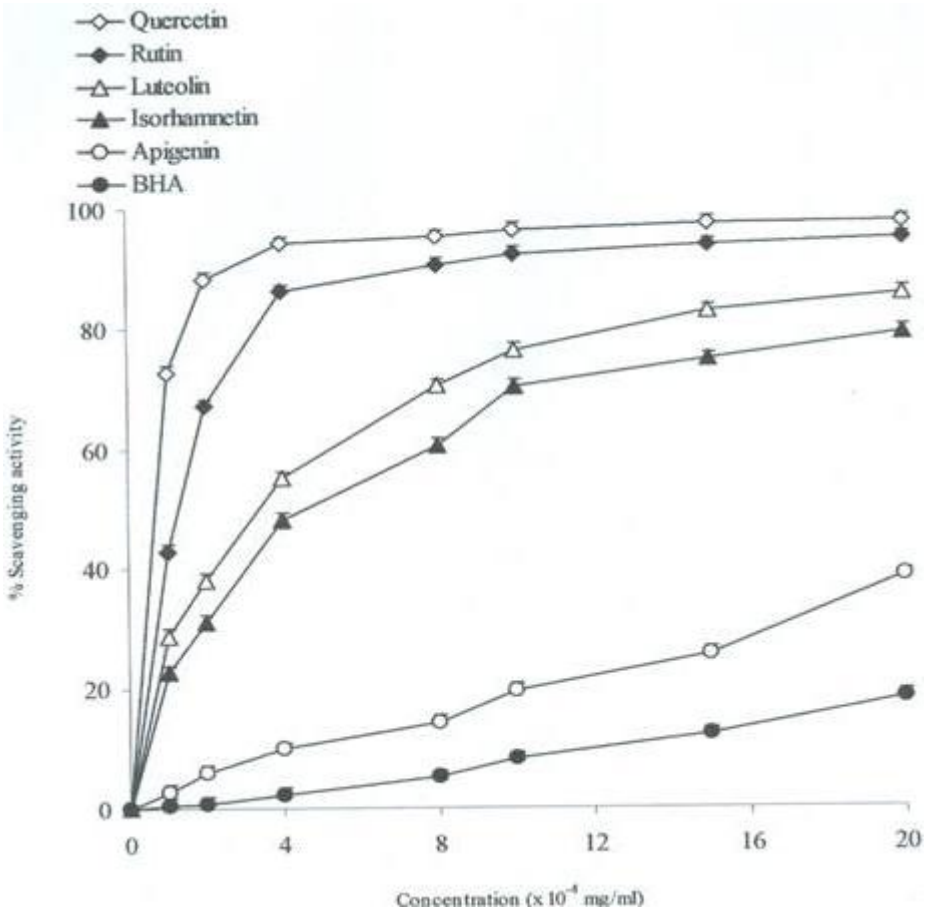


Figure 4. Scavenging activity of the constituents of the leaf of *P. americana* and BHA determined by the scavenging of 1,1-diphenyl-2-picrylhydrazyl radical. Values are mean ± SD. n= 4, significance is set at $p \leq 0.05$.

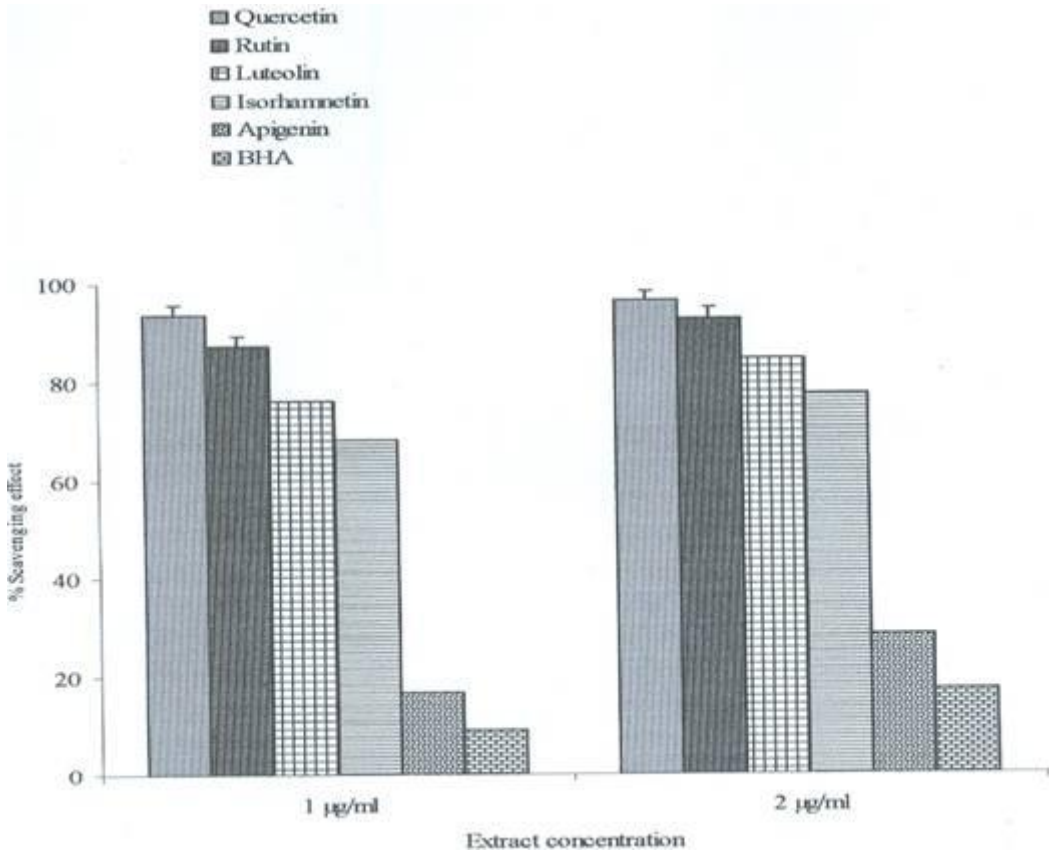


Figure 5. Effect of the constituents of the leaf of *P. americana* and BHA on hydrogen peroxide scavenging. Values are mean ± SD. n=6, significance is set at $p \leq 0.05$.

was deduced by decrease in absorbance of the reaction mixture as the purple $DPPH^{\bullet}$ changes to yellow in the presence of the compounds or standard. The antioxidant activity of the compounds was significantly higher ($p > 0.05$) than the reference standard, BHA with their 50% inhibitory concentration (IC_{50}) in increasing order of quercetin > rutin > isorhamnetin > luteolin > apigenin > BHA. The IC_{50} value of quercetin was 4.82×10^{-5} mg/ml; rutin, 1.37×10^{-4} mg/ml; luteolin, 3.34×10^{-4} mg/ml and isorhamnetin, 4.41×10^{-4} mg/ml. Hydrogen peroxide is a non-radical molecule generated *in-vivo* by several enzymes or by dismutation of two molecules of superoxide anions, a reaction catalysed by superoxide dismutase. H_2O_2 reacts with Fe^{2+} ion by Fenton reaction to form a highly reactive hydroxyl radical ($OH^{\bullet}$) which in turn reacts with organic molecules (Halliwell, 1991). It is therefore essential to remove H_2O_2 from biological system. The ability of the compounds or BHA to scavenge H_2O_2 increased with increase in concentration (Figure 5). The hydrogen peroxide scavenging ability of the compounds was significantly different ($p > 0.05$) from BHA exhibiting 96.45, 97.69, 81.52, 77.42, 47.33 and 23.53% for quercetin, rutin, isorhamnetin, luteolin, BHA and apigenin, respectively. The radical scavenging activity of flavonoids depends on the molecular structure and the substitution pattern of hydroxyl groups, that is, on the availability of phenolic hydrogen and on the possibility of stabilization of the resulting phenoxyl radicals through hydrogen bonding or by expanded electron delocalization (Rice-Evans et al., 1996). Hydroxyl groups at C-3 and C-3' are important in the antioxidant activity of flavonoids (Amić et al., 2002). Thus the very low antioxidant activity of apigenin may be due to the absence of free hydroxyl at C-3 and C-3´. That the activity of isorhamnetin is lower than quercetin indicates that free hydroxyl at C-3´ is essential for the antioxidant activity. It is generally accepted that glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Keharo and Adam, 1974; Kumarasamy et al., 2005).

Hence, glycosylation at C-3 of rutin may also have contributed to its low activity compared to quercetin. Since the compounds isolated from the leaves of *P. americana* are phenolics in nature, their scavenging activity may be as a result of their ability to donates electrons to H_2O_2 neutralizing it to water. The results of this study clearly showed that compounds of *P. americana* have powerful antioxidant activity. Therefore, the use of the leaves of *P. americana* might be helpful in

preventing or slowing down the progress of various oxidative stress-related diseases. Thus, further explaining its use in folkloric medicine.

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