Flavonoids from *Vernonia amygdalina* and Their Antioxidant Activities

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Flavonoids occurring in *Vernonia amygdalina* leaves have been studied. Three flavones were identified with chemical and spectroscopic techniques. They were identified as luteolin, luteolin 7-*O*-β-glucuronoside, and luteolin 7-*O*-β-glucoside. The most abundant compound was luteolin 7-*O*-β-glucuronoside. The antioxidant activity of the three flavones was determined by measuring the coupled oxidation of β-carotene and linoleic acid. It was shown that luteolin was a significantly more potent antioxidant than the synthetic antioxidant butylated hydroxytoluene (BHT) at the same concentration (15 mg/L). The two glycosides showed similar activities but significantly lower activities than luteolin or BHT.

Keywords: *Vernonia amygdalina; Compositae; flavones; luteolin; antioxidant activity*

INTRODUCTION

Vernonia amygdalina (Compositae) is a small tree that grows throughout tropical Africa. In some parts of West Africa, e.g. Nigeria, the plant has been domesticated and is locally known as "bitter-leaf", while the Yoruba tribe call it ewuro. The leaves are used as a leafy vegetable for preparing the popular bitter-leaf soup and the juice or extract serves as a tonic drink. It contains 18% protein, 8.5% fiber in a dry matter, and a good composition of macroelements and microelements (Igile et al., 1994). Moreover, *V. amygdalina* has been used in traditional medicine as an antihelmint, an antimalarial, and a laxative herb. It was observed that an apparently sick wild chimpanzee chewed this plant to extract bitter juice and after a while it seemed to return to its normal activity (Ohigashi et al., 1991).

These observations stimulated research on the chemical principals of *V. amygdalina*. Several stigmastane-type saponins such as vernoniosides A_1, B_1 (Ohigashi et al., 1991), A_1, A_2, A_3 (Jisaka et al., 1992), B_2, B_3, A_4 (Jisaka et al., 1993), and C (Kamperdick et al., 1992) have been identified in the leaves. It was shown that the A series of these saponins were bitter. Mixtures of saponins as well as vernonioside A_1 were shown to affect body and liver weights, urine and fecal output, and plasma and liver cholesterol concentrations in mice fed diets amended with these compounds (Igile et al., 1994). The antiplasmodial activity of some sesquiterpene and steroidal constituents of *V. amygdalina* was tested, and some were proved to be active against *Plasmodium falciparum in vitro* (Phillipson et al., 1993).

The current search for potential antioxidative principles to replace suspected tumor-causing synthetic analogues such as BHT (Ito et al., 1985) necessitated this investigation. Antioxidative principles have been implicated as useful compounds in food preservation and as parts of anticancer formulations and patents (Loliger, 1991). Although luteoline has been reported to be a strong antioxidant (Torel et al., 1986), no report has so far been given on the antioxidative potentials of its glycosidic derivatives.

The present paper characterizes flavonoids of *V. amygdalina* leaves and describes their antioxidant activities compared with BHT. The work was aimed at furthering our understanding of the relationships between the chemical composition of this plant and its nutritional and pharmaceutical significance.

MATERIALS AND METHODS

Plant Material. The leaves of *V. amygdalina* were harvested fresh from a horticultural garden in Ibadan, Nigeria, and were air-dried. The plant was botanically identified by Dr. J. Lowe of the Botany Department, and a voucher sample was deposited in the Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria.

Extraction. The dried material was finely powdered, and a 600 g portion was macerated with 3 × 4 L of 30% aqueous MeOH. The mixture was centrifuged and the supernatant evaporated (40 °C) until MeOH was almost removed. This solution was then loaded on a LiChroprep RP18 column (3 cm × 5 cm, 25–40 μm, Merck) preconditioned with water. The column was washed with water to remove sugars (Oleszek et al., 1988) and then successively with 200 mL of 30% MeOH (fraction 1), 200 mL of 40% MeOH (fraction 2), and 100 mL of 50% MeOH (fraction 3).

Separation of Flavonoids. Fraction 1, showing one flavonoid spot on TLC, was loaded on the LiChroprep RP18 column (40 cm × 3 cm, 25–40 μm) preconditioned with water. The column was washed with 30% MeOH, and 10 mL fractions were collected with a fraction collector and monitored with TLC. Fractions showing the presence of flavonoid were combined, MeOH was removed *in vacuo*, and the flavonoid was extracted into EtOAc. Solvent was evaporated, and solid residue was dissolved in MeOH and loaded on a polyamide column (20 cm × 1.2 cm, Woelm TLC, Germany) preconditioned with water. The column was washed with 100 mL of water followed by 200 mL of 30% MeOH and then 100 mL of EtOH. The EtOH eluate, showing a single spot on TLC, was evaporated, yielding a yellow solid that was redissolved in MeOH and crystallized. This yielded pure flavonoid **1** as yellow needles (240 mg).

Fraction 2, showing the presence of one flavonoid spot, was concentrated and loaded on the LiChroprep RP18 column

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preconditioned with water. The column was washed with water (100 mL) followed by 30% MeOH (isocratic) and a gradient of 40–50% MeOH. Eluates were collected as 10 mL fractions with a fraction collector, and those showing the presence of flavonoid were combined and condensed. This was loaded on a polyamide column preconditioned with water. The column was washed with water and then isocratically with 30% MeOH. Alcohol was removed and flavonoid was extracted into EtOAc. Evaporation of solvent provided a yellow solid, which was crystallized from MeOH yielding flavonoid **2** as pale yellow leaflets (48 mg).

Fraction 3, showing one flavonoid spot together with some steroidal saponins, was evaporated *in vacuo* to remove MeOH, and flavonoid was extracted into EtOAc. Solvent was evaporated to dryness, redissolved in 30% MeOH (3 mL), and loaded on a cellulose column (42 cm × 2 cm, Cellulosepulver MN 300, Macherey, Nagel & Co., Germany). The column was subsequently washed with water (100 mL), 30% MeOH (100 mL), and 40% MeOH (100 mL). The 40% MeOH eluate containing a single compound was evaporated to remove alcohol, and flavonoid was extracted into EtOAc. Solvent was removed and the obtained yellow solid was crystallized from MeOH, yielding flavonoid **3** in the form of crystalline, deep yellow needles (8 mg).

Analysis of Hydrolysis Products. Glycosides were hydrolyzed in 1 N HCl at 100 °C for 4 h. After cooling, the resulting aglycon was filtered off with a glass filter funnel and dried at 60 °C. Each was crystallized from MeOH. Their identities were confirmed by cochromatography (TLC and HPLC) with the authentic standards (Sarsyntex, Merignac, France).

Thin Layer Chromatography. Glycosides and aglycons were chromatographed on precoated plates of cellulose (DC-Fertigplatten, Merck) using 15% AcOH in H_2O (S1) or butanol–acetic acid–water (BAW, 5:1:4) as S2. Compounds were monitored under UV (at 366 nm) after exposure to ammonia vapors or after spraying with $AlCl_3$ reagent.

Sugars were chromatographed on cellulose using 1-butanol–pyridine–water–benzene (5:3:3:1) as solvent system (S3). Sugars were made visible by spraying the plates with aniline–phtalate followed by heating at 105 °C.

Analysis of Glycosides and Aglycons by Analytical HPLC. The purity of the separated compounds and their identities with available standards were analyzed with an analytical HPLC unit (Knauer, Germany) equipped with a computer system to monitor chromatographic parameters and to process the data and a variable-wavelength UV detector operating at 280 nm. Separations were performed on a 5 μm (250 × 4.5 mm i.d.) Eurospher 80 C_{18} column (Saulentechnik, Germany). Chromatographic runs were carried out using a gradient of solvents A (1% H_3PO_4 in H_2O) and B [1% H_3PO_4 in acetonirile–H_2O (4:6)]. The following gradient was used: linear from 20% B to 100% B during 80 min followed by isocratic 100% B over 5 min. Solvent was delivered at a 1 mL/min flow rate.

Spectral Analyses. The spectra and bathochromic shifts were recorded with a spectrophotometer (Beckman DU-68) according to the method of Mabry et al. (1970).

Fast atom bombardment (FAB) mass spectra were recorded with a Finnigan MAT 95 spectrometer using NBA as a matrix. Electron impact (EI) mass spectra were recorded with the same machine operating at an ionization energy of 70 eV.

Antioxidant Activity Tests. Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was used as the antioxidant activity test model (Pratt, 1992). Linoleic acid (0.02 mL) and Tween 20 (0.2 mL) were placed in Erlenmeyer flasks. One milliliter of β-carotene (0.2 mg/mL; Sigma, Deisenhofen, Germany) was added to each flask. Each mixture was then dosed with 0.2 mL of the corresponding concentration of tested flavonoids or butylated hydroxytoluene (15 mg/L). Samples without dosed compounds were used as standard. Each sample was taken to dryness using N_2 in the dark. Fifity milliliters of distilled water, saturated with air (0.5 L/min, 15 min), was added to each mixture and shaken. Their absorbances were read on a spectrophotometer (Beckman DB-T) at 470 nm. The samples were then subjected to autoxidation, thermally induced with a constant oven temperature of 50 °C for 2 h. Absorbances were taken at 10 min intervals to monitor the rate of bleaching of β-carotene. Each experiment was performed in three replicates.

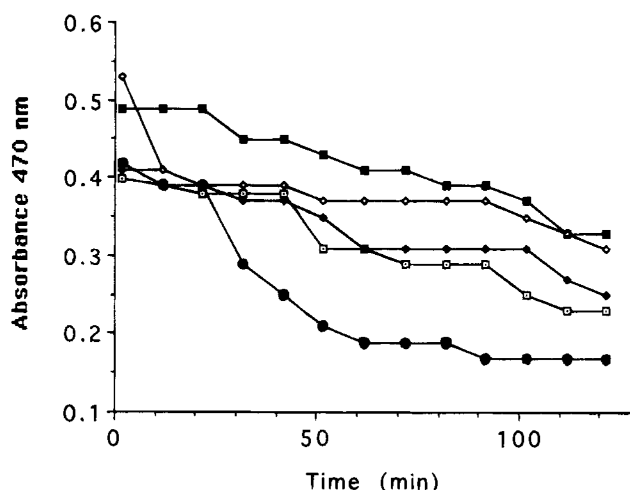

Figure 1. Antioxidant activity of *V. amygdalina* flavons: luteolin-7-GlcA (□), luteolin-7-Glc (◆), luteolin (■), BHT (◇), control (●).

RESULTS

Separation of the flavonoid constituents of *V. amygdalina* on RP18 and polyamide column chromatography provided three compounds.

Compound 1: (yellow needles) R_t (analytical HPLC) 21.6 min; R_f 0.08 (S1), 0.36 (S2); UV, λ_{max} (MeOH) 254, 266, 349, (NaOMe) 264, 400, ($AlCl_3$) 272, 298 sh, 428, ($AlCl_3$/HCl) 272, 294 sh, 360, 388, (NaOAc) 259, 402, (NaOAc/H_3BO_3) 260, 373 nm; FAB-MS m/z (rel intensity) (negative ion mode) 461 (57) $[M - H]^-$, 285 (14) $[M - H - \text{uronic acid}]^-$. Acid hydrolysis yielded glucuronic acid [TLC, R_f 0.07 (S3)] and aglycon [R_t 38 min; R_f 0.03 (S1), 0.8 (S2)].

Compound 2: (pale yellow leaflets) R_t (analytical HPLC) 20.4 min; R_f 0.06 (S1), 0.44 (S2); UV, λ_{max} (MeOH) 255, 267, 348, (NaOMe) 263, 349, ($AlCl_3$) 274, 299 sh, 329, 423, ($AlCl_3$/HCl) 273, 294 sh, 358, 387, (NaOAc) 259, 266 sh, 365 sh, 405, (NaOAc/H_3BO_3) 259, 372 nm; FAB-MS m/z (rel intensity) (negative ion mode) 447 (11) $[M - H]^-$, 285 (2) $[M - H - \text{hexose}]^-$. Acid hydrolysis provided glucose [TLC, R_f 0.44 (S3) and aglycon [R_t 38 min; R_f 0.03 (S1), 0.8 (S2)].

Compound F3: (deep yellow needles) R_t (analytical HPLC) 38 min; R_f 0.03 (S1), 0.8 (S2); UV, λ_{max} (MeOH) 253, 267, 291 sh, 348, (NaOMe) 266, 329 sh, 401, ($AlCl_3$) 274, 300 sh, 328 sh, 426, ($AlCl_3$/HCl) 266, 275, 294 sh, 355, 385, (NaOAc) 269, 326 sh, 384, (NaOAc/H_3BO_3) 259, 370, 430 nm; EIMS m/z (rel intensity) 286 (100) $[M]^+$, 285 (7) $[M - H]^+$, 258 (12) $[M - CO]^+$, 153 (23) $[A_1 + H]^+$, 152 (5) $[A_1]^+$, 134 (6) $[B_1]^+$.

Antioxidant Activities. The rates of discoloration of β-carotene in the presence of isolated flavonoids in comparison to BHT are shown in Figure 1. The three isolated compounds exhibited antioxidant activity but to varying degrees when compared to the control or BHT. Compound **3** appeared to possess greater antioxidant activity ($p < 0.05$) than the other compounds, including BHT. Compounds **1** and **2** inhibited the bleaching of β-carotene during the first 40 min of experiments to the same degree as BHT, and thereafter their inhibitory effect was significantly lower than that

of BHT but still evident. There was no difference in activity between compounds **1** and **2**.

DISCUSSION

The aqueous–methanolic extracts of *V. amygdalina* contained three major flavonoid constituents. They were successfully separated by the combination of reversed-phase C_{18} and polyamide preparative chromatography. Their structures were confirmed by chemical, chromatographic, and spectroscopic analyses.

Absorption spectra (UV–vis) performed in MeOH were nearly identical for the three compounds, indicating a flavone skeleton. The least polar compound, **3**, had a retention time (38 min) identical to that of luteolin. This identity was further supported by the EI mass spectrum, showing a molecular ion of 286, identical to the molecular weight of luteolin. Moreover, fragmentation patterns were characteristic for flavones possessing two OH groups in ring A and two in ring B. The locations of these groups were confirmed by UV spectra obtained with bathochromic shift reagents (Mabry et al., 1970). Hence, two absorption maxima in band II (255 and 267 nm), the bathochromic shift (23 nm) of band I with $NaOAc/H_3BO_4$, and the hipsochromic shift (32 nm) in band Ia with $AlCl_3/HCl$ indicated 3′,4′-di-OH conformation in ring B. The bathochromic shift (52 and 54 nm) in band I with NaOMe and NaOAc, respectively, proved the presence of an unsubstituted 4′-OH group. The absorption maxima of band II (267 nm) and its bathochromic shift (15 nm) with NaOAc supported the location of hydroxyls in positions 5 and 7 in ring A. Thus, both chromatographic and spectral analyses proved compound **3** to be the flavone luteolin.

Flavonoids **1** and **2** on acid hydrolysis yielded aglycons that were identical to luteolin (TLC, HPLC). However, these compounds yielded different sugars when acid hydrolyzed. Compound **1** yielded glucuronic acid, and compound **2** provided glucose (TLC). The FAB mass spectrum of compound **1** showed a strong molecular ion at m/z 461 and a second characteristic ion at m/z 285 corresponding to the loss of uronic acid (176 mu) from the parent ion. Similarly, the FAB mass spectrum of flavonoid **2** showed a molecular ion at m/z 447 and an ion at 285 indicating the loss of hexose (162 mu). Thus, chemical and spectral analyses confirmed compound **1** to be luteolin glucuronide, and compound **2** was identified as luteolin glucoside. A small bathochromic shift of band II (5 nm) with NaOAc indicated 7-O linkage of sugar components. Finally, these two compounds were identified as luteolin 7-*O*-glucuronoside and luteolin 7-*O*-glucoside.

As evaluated by the extraction efficiency, the most abundant compound in plant material was the glucuronide, followed by the glucoside of luteolin. The concentration of free luteolin was low.

The antioxidant activity of the three identified flavones was determined by measuring the coupled oxidation of β-carotene and linoleic acid. Significant differences were found between the activities of the three compounds, with the aglycon being a much more potent antioxidant than BHT and the glycosides being as potent as BHT during the first 40 min of experiments and significantly less active after that time.

Antioxidant activity of flavonoids is a well-documented phenomenon (Huang et al., 1992), and structure-dependent activities were reported. There is a general agreement that ortho-dihydroxylation of the B ring contributes markedly to the antioxidant activity of flavonoids, and all compounds with the 3′,4′-dihydroxy configuration studied thus far possessed antioxidant activity.

On the contrary, meta-5,7-hydroxylation of the A ring apparently had little, if any, effect on antioxidant activity (Pratt, 1992). The present studies seem to indicate that 5,7-hydroxylation may influence antioxidant activity. Glycosides with glucose or uronic acid linked at the 7-O position showed apparently lower activity than their aglycons having the 5,7-OH unsubstituted. It is not evident if this decrease of activity was the result of blockage of the 7-OH group or any other changes generated by glycosylation.

The flavones identified in the present work show antioxidant activity and are highly desired components of *V. amygdalina* leaves, elevating its nutritional quality due to its possible combating of carcinogenesis as well as aging processes (Huang and Ferraro, 1992). However, *V. amygdalina* leaves contain some antinutritional components such as stigmastane-type saponins. The presence of these compounds forced populations that utilize this plant to develop procedures that allow removal of most of the undesirable components prior to consumption. This is based on exhaustive washing of the leaves with water to completely remove bitterness. It is not clear if in this process flavones are washed out; it is highly probable, as the water solubility of the identified flavones is similar to the solubility of stigmastane saponins. This aspect needs further investigation.

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