Phytopharmacology

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Identification of acteoside as the active antioxidant principle of *Premna serratifolia* root wood tissues

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Received: 11 December 2012, **Revised**: 22 December 2012, **Accepted**: 23 December 2012

Abstract

Premna serratifolia Linn. (syn: *Premna integrifolia*) is one the most widely used plant in the Ayurvedic system of medicine. Several pharmacological activities including antioxidant effects and phytochemical investigations have been previously reported for the various parts of plant, except the root woody tissues. In the present study, the antioxidant activity and active principle of the root woody tissues were investigated. Antioxidant effect was routinely monitored using the DPPH radical scavenging assay while phytochemical investigation was based on analysis using HPLC and Teledyne Isco flash chromatography system. Through the use of comprehensive spectroscopy studies, the isolated active antioxidant principle was identified as acteoside (verbacoside). Aceoside, which was about four times more active (18.3 ± 3.7 μg/ml; 11.4 ± 2.3 μM) than the crude root wood extract (73.8 ± 2.4 μg/ml), could account for most of the reported pharmacological activity on *P. serratifolia*.

Keywords: *Premna serattifolia;* Verbenaceae; antioxidant; root wood; acteoside; verbacoside

Introduction

The genus *Premna* (Verbenaceae) is widely distributed in tropical and subtropical regions of Africa, Asia, Australia and the Pacific islands (Kadareit, 2004). *Premna serratifolia* Linn. (syn: *Premna integrifolia*), is a small tree or shrub that occur in India, Srilanka and the Andaman. Various parts the plant especially the roots are extensively used for thousands of years in Indian traditional medicine formulations such as Dashmoola kwatha, Chyanprashavleh, Haritakiavleh, Ayushyavardhaak tel and Narayana taila (API, 2001; Jothi et al., 2010). Among the various medicinal uses reported for the roots are for treating diabetes, chyluria, gonorrhoea, inflammation, swelling, bronchitis, dyspepsia, headach, liver disorder, piles, constipation and fever (API, 2001). Some pharmacological studies have revealed that the plant possess anti-coagulant (Gopal and Purushothaman, 1984), anti-inflammatory (Karthikeyan and Deepa, 2011), anti-arthritic (Karthikeyan and Deepa, 2010; Rajendran and Krishnakum-

ar, 2010), antinociceptive (Karthikeyan and Deepa, 2010), hypoglycemic (Alamgir et al., 2001; Dash et al., 2005; Kar et al., 2011), gastrprotective (Jothi et al., 2010), antimicrobial properties (Rajendran and Basha, 2010) and cardioactivity (Rajendran et al., 2008).

Yadav et al. (2011) have recently studied the antioxidant activity of the stem bark of *P. serratfilia* along with its active constituents. It was reported that 4″-hdroxy-*E*-globularinin, 10-*O*-trans-p-coumaroylcatalpol, premnosidic acid, 10-*O*-trans-p-coumaroyl-6-*O*-α-l-rhamnopyranosyl catalpol and a new dimeric lignin were responsible for the antioxidant effect of the stem bark (Yadav et al., 2011). Similarly the leaves and roots of *P. serratifolia* have also been demonstrated to possess antioxidant effect (Gokani et al., 2011; Selvam et al., 2012). To date a number of phytochemical studies on the leaves (Otsuka et al., 1993), stem bark (Yuasa et al., 1993) and root bark (Yadav et al., 2010) of the plant are reported but the root wood that plays major role in the Ayurvedic medicine has not yet been studied. We herewith report the identification of the antioxidant principles of the root wood tissues.

Materials and Methods

General methodology

^{1}H NMR, ^{13}C NMR and 2D-NMR (COSY, NOESY, HSQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One bond ^{1}H–^{13}C connectivities were determined with HSQC while two- and three-bond ^{1}H–^{13}C connectivities were determined by HMBC experiments. Chemical shifts were reported in δ (ppm) using the solvent standard and coupling constants (*J*) were measured in Hz. A Waters Synapt G2 TOF mass spectrometer (Waters, UK) with an electrospray ionisation probe was used to acquire data over a mass range of 50-800u. A lock spray correction was applied to each acquired data set using Leu-Enk. The mass pectrometer was coupled with an Acquity UPLC system comprising of Acquity UPLC BEH C18 1.7 μm 2.1x50 mm column and Waters TUV at 220nm detector. Samples dissolved in methanol (0.1 mg/ml) were injected into the LC-mass spectrometer operating in a positive ion mode.

Plant material

The roots of *P. serratifolia* growing for several years in our experimental field (Karunya Lane, Manganam Kara, Kalathilpady, Kottayam-10, India) were used. The location of the plant specimen source lies between 09^{0} 34.978’ north latitude and 076^{0} 33.146’ east longitudes. A voucher specimen numbered 2517 was kept in the CMS Herbarium, C. M. S. College, Kottayam.

Processing and extraction of the plant material

The bark and woody tissues of the plant were separated, air dried and grounded. The root bark (190 g) and root wood (330 g) were extracted twice by soaking in methanol (2.5 l) for two weeks. Removal of the solvent under reduced pressure yielded 20 and 19 g yield for the root bark and root wood tissues respectively.

Isolation of acteoside from P. serratifolia root wood

The methanol extract of the root wood which displayed antioxidant activity when assessed by DPPH (1, 1-diphenyl- 2-picryl hydrazyl; Sigma-Aldrich, UK) radical scavenging assay was taken for isolation of the active constituents. RediSep Rf flash silica column (24g; Presearch, Hampshire, UK) attached to a Teledyne Isco flash chromatography system was used for fractionation and isolation of acteoside. Briefly, a linear gradient of methanol starting from 0% to 40% over a period of 30 minutes were established. The chromatogram was monitored by observing absorbance at dual wavelengths: 214 and 254 nm. Four major fractions were obtained: I (0-8 min), II (10-17 min), III (17-25 min) and IV (25-30 min). Fractions II and III that showed antioxidant activity were further purified using the same system to provide acteoside in the yield of 30% (w/w versus the crude extract).

HPLC analysis

An Agilent 1200 series gradient HPLC system composed of degasser (G1322A), quaternary pump (G1322A), auto sampler (G1329A), thermostat column compartment (G1316A) maintained at 25 ^{O}C and a diode array detector (G1315D) was used. Plant extracts dissolved in methanol (1 mg/ml) were injected (20 µL) onto a reverse phase column (Agilent – Eclipse Plus C18, 5 µm, 4.9 x 150 mm). The mobile phase was a mixture of water (A) and methanol (B). The composition of the mobile phase at a flow rate of 1 ml/min was rising from 10% to 90% B over a period of 50 minutes.

DPPH radical scavenging activity

The antioxidant activity of test samples was measured by using our established microtitre-based DPPH assay (Habtemariam, 2007). Briefly, DPPH solution (0.1 mM, in methanol) was incubated with varying concentrations of test compounds for 20 min at room temperature and the absorbance of the resulting solution was read at 550 nm against a blank using Multiscan EX Reader (Thermo Labsystems, Altrincham, UK).

Results and Discussion

The radical scavenging activity of the crude methanolic extracts of *P. serattifolia* root tissues was assessed by using the DPPH bleaching assay as described previously (Habtemariam, 2007). The typical concentration-dependent effect shown in Figure 1 was in agreement with previous studies that revealed antioxidant activity for the leaves and stem bark of *P. serratifolia* (Gokani et al., 2011; Selvam et al., 2012; Yadav et al. (2011). When the root bark and root woody tissues were separately assessed for their antioxidant activity, we observed a super-imposable activity profile (Figure 1). The IC_{50} values obtained from four independent experiments were 72.5 ± 6.3 and 73.8 ± 2.4 µg/ml for bark and woody tissues of the root, respectively. This statistically insignificant difference ($p > 0.05$, unpaired t-test), suggests that both the root wood and root barks must be considered for establishing the scientific evidence of the reputed medicinal uses of *P. serratifolia* roots. Since the chemistry of the root bark has been studied in recent years, emphasis was given in the present study on the root woody tissues that is obtained in similar yield as the root bark.

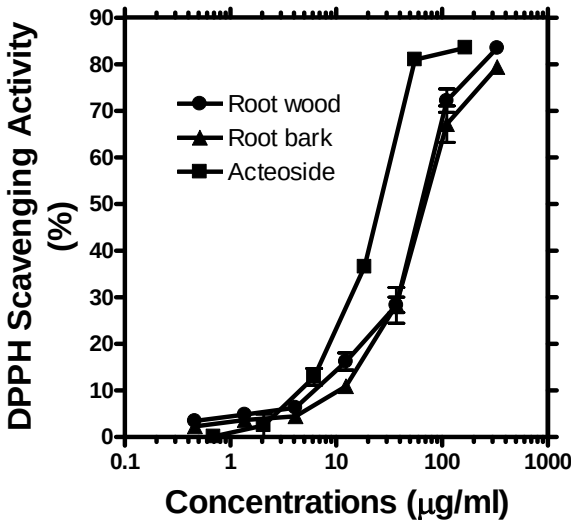

Figure 1. Antioxidant activity of the root barks and root wood extracts of *P serratifolia*

HPLC analysis of the antioxidant woody tissue extract revealed one prominent peak (retention time of 23.9 min) within the 50 min analysis time (Figure 2). It appears from this figure that the extract was made of one major constituent in an incredibly pure form instead of being complex mixture as expected in crude extracts. NMR analysis, however, revealed that the extract is a crude mixture suggesting that there are lots of compounds that were not picked up by the UV detection system. Further purification was done by a fast Combiflash ISCO chromatography system that allows the processing of large scale separations. Repetitive purification using this system afforded the antioxidant principle that is shown as a major HPLC peak in the HPLC chromatogram (Figure 2). Antioxidant activity study of the isolated

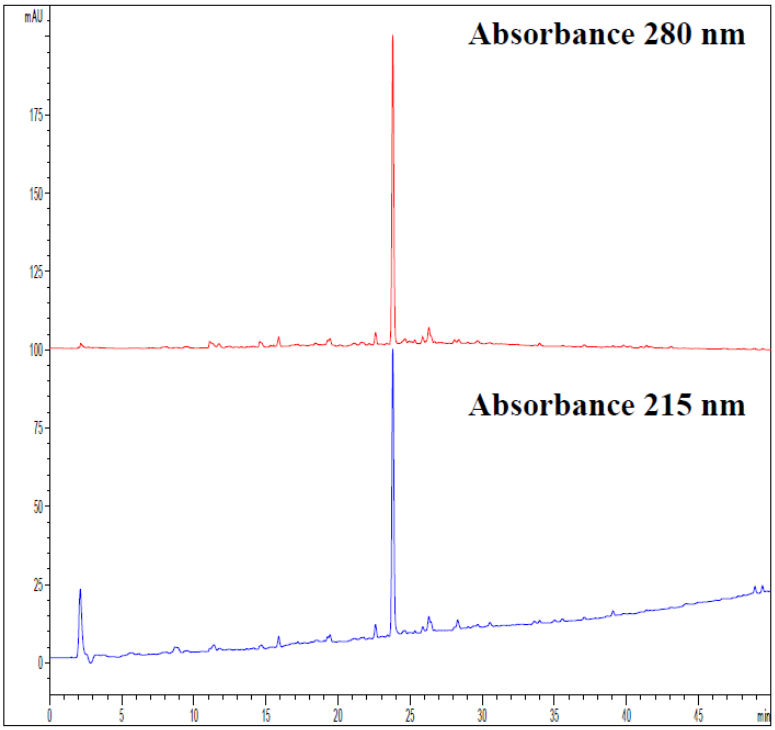

Figure 2. HPLC profile of the crude root wood extract of *P. serratifolia*

active compound revealed a better activity profile than the crude extracts (Figure 2). The IC_{50} value of the compound was also 18.3 ± 3.7 µg/ml and was about 4-times more potent the crude extracts.

The ^{1}H and ^{13}C NMR data clearly indicated the presence of a caffeic acid, phenyl ethanol and two sugar moieties (Table 1). Direct H-C connectivites were established by HSQC studies while ^{1}H NMR signals were identified through the use of COSY NMR studies. The observed NMR data were in general in good agreement with those previously published for acteoside (Li et al., 2005; Otsuka et al., 1993). Further evidence corroborating the sites of linkage of the different structural moieties as shown in Figure 3 came from the HMBC studies (Table 1). The key feature of this studies (Table 1) were the observation of the expected ^{3}J coupling (Table 1) as depicted in the structure of acteoside. In the MS study, the molecular ion appeared as a sodium adduct of acteoside ($[M+Na]^{+}$) at m/z 647.1958. The classical fragmentation at m/z 535, 461, 325 and 163 were further consistent with those previously reported for acteoside (Li et al., 2005). On the basis these spectroscopic data, the antioxidant principle isolated from the root wood tissues was identified as acteoside (Figure 3). The compound was previously isolated from the leaves of *Premna corymbosa* that is considered to be the same (synonym) as *P. serratifolia*.

Table 1. ^{1}H, ^{13}C and HMBC NMR data for acteoside. All experiments were run in CD3OD– δ values in ppm and coupling constant (*J*) in parenthesis are shown.

Moiety	Position	^{1}H NMR (δ_H)	^{13}C NMR (δ_C)	Major HMBC Correlations (^{2}J, ^{3}J)*
Phenyl ethanol	1	-	131.4	
	2	6.61 d (2.2)	117.1	C-1, C-4,
	3	-	146.1	
	4	-	144.7	
	5	6.60 d (8.1)	116.1	C-1, C-3
	6	6.48 dd (8.1, 2.2)	121.3	C-2, C-4
	7	2.78 m	36.5	C-1, C-2, C-6, C-8
	8	3.7 and 4.04 m	72.3	**C-1''**
Caffeic acid	1'	-	127.6	
	2'	6.98 d (2.2)	115.2	C-4', C-6'
	3'	-	146.8	
	4'	-	149.8	
	5'	6.69 d (8.3)	116.5	C-1', C-3'
	6'	6.85 dd (8.3, 2.2)	123.2	C-2', C-4'
	7'	7.50 d (15.8)	148.0	C-6'
	8'	6.19 d (15.8)	114.7	C-9'
	9'	-	168.3	
Glucose	1''	4.49 d (7.8)	104.2	**C-8**
	2''	3.39 d (9.1)	76.2	C-3'
	3''	3.84 t (9.0)	81.7	**C-1''',** C-2'', C-5''
	4''	4.90 t (9.3)	70.4	**C-9', C-3''**, C-2''
	5''	3.52 m	76.0	
	6''	3.63 and 3.52 br d (11.5)	62.3	
Rhamnose	1'''	5.18 d (1.6)	103.3	**C-3''** , C-2/C-3, C-5'''
	2'''	3.91 dd (3.1, 1.8)	72.3	C-4''', C-3'''
	3'''	3.57 br d (7.9)	72.0	C-5'''
	4'''	3.30 br d (8.2)	73.8	C-5'''
	5'''	3.54 m	70.5	
	6'''	0.99 d (6.2)	18.4	

*3*J* connectivities showing the linkage of sub-structural moieties are shown in bold red fonts.

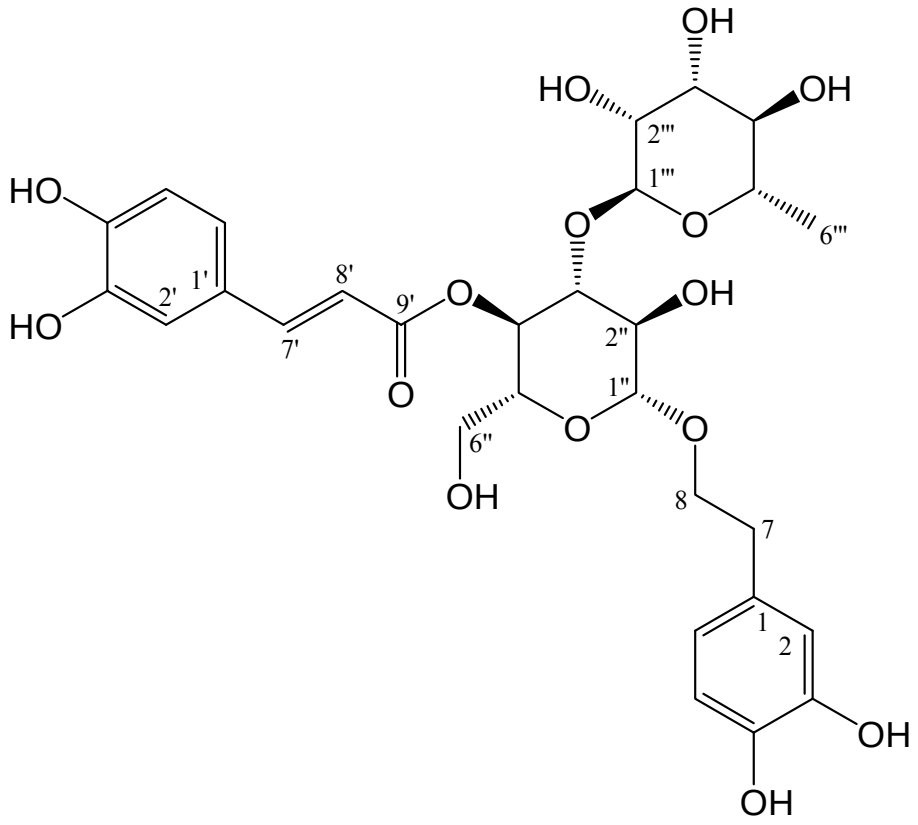

Figure 3. Structure of acteoside (verbacoside).

Acteoside isolated from several plants have been shown to possess potent antioxidant effects in a variety of *in vitro* assay models (Zaabat et al., 2011; Kim et al., 2009). Acteoside by its own or in crude plant extracts have also shown to display *in vitro* and *in vivo* anti-inflammatory (Akdemir et al., 2011; Kim et al., 2009; Korkina et al., 2007; Schapova et al., 1998; Speranza et al., 2010), wound healing (Akdemir et al., 2011; Korkina et al., 2007), antinociceptive (Akdemir et al., 2011; Backhouse et al., 2008) and hepatoprotective effects against carbon-tetrachloride (Lee et al., 2004) and D-Galactosamine/ lipopolysaccharide (Xiong et al., 1999). Protection of human neuroblastoma SH-SY5Y cells against β-amyloid-induced cell injury (Wang et al., 2009), 1-methyl-4-phenylpyridinium ion-induced neurotoxicity (Sheng et al., 2002) and rat cortical cells from glutamate-induced excitotoxicity (Koo et al., 2006) are among the various neurpotective effects of acteoside reported so far. Upregulation of heme oxygenase-1 by acteoside through ERK and PI3 K/Akt pathway has been shown to confer neuroprotection against beta-amyloid-induced neurotoxicity (Wang et al., 2012). The potential anticancer activity of acteoside is also evident from studies showing direct inhibition of human promyelocytic HL-60 leukemia cell proliferation (Lee et al., 2007) and PMA-induced invasion and migration of human fibrosarcoma cells (Hwang et al., 2011). Antimicrobial effects including activity against multiple-drug-resistant strains of *Staphylococcus aureus* (Nazemiyeh et al., 2008) have also been reported for acteoside. In view of acteoside's wide range of pharmacological effects, the finding of it being a major constituent of the root woody tissues could explain the reported medicinal value and pharmacological effects of the plant.

The present finding was also in good agreement with our previous reports that showed *Premna species* as good sources of pharmacologically active natural products (Habtemariam, 2003; 1995; Habtemariam et al., 1993; 1992; 1990). The identification of the active principles from these plants would allow the standardisation and modernisation of crude drug preparations like those comprising *P. serratifolia* in the Ayurvedic medicine.

In conclusion, the roots of *P. serratifolia* are extensively used in the Ayurvedic system of medicine for treating various disease conditions. As with the other plant parts, the roots have been shown to display antioxidant activities but the active principles have so far not identified. Our phytochemical analysis using antioxidant assay as a guide has resulted in the identification of acteoside as the active principle of the root woody tissues. This compound could account for most of the pharmacological activities reported for *P. serratifolia* roots.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors wish to thank the technical assistance of staff at the University of Greenwich analytical laboratories.

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