Hepatoprotective and Antioxidant Activity of Two Iridoids from *Mussaenda 'dona aurora'*

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Mussaenda 'dona aurora' (sepals) has been investigated for its hepatoprotective and antioxidant activities. The highest activity was observed in the ethyl acetate fraction. The separation of the ethyl acetate fraction gave two iridoids, sanshiside-D and lamalbide. Sanshiside-D exhibited a hepatoprotective activity greater than silimarin as was evidenced by significant reduction of ALT and AST in the serum enzyme levels.

Key words: Mussaenda, Iridoids, Sanshiside-D, Antioxidant

Introduction

The *Mussaenda* genus has been instrumental in the discovery of medicinal natural products. The plants are members of the Rubiaceae (madder or coffee family) and are native to the Old World tropics, from West Africa through the Indian sub-continent, South-East Asia and to Southern China (Huxley *et al.*, 1999). There are more than 200 species of *Mussaenda* known. Some species of *Mussaenda* have been used in Chinese and Fijian traditional medicine. Triterpene glycosides from the stem bark of *M. macrophylla* have been shown to be active against oral pathogens (Kim *et al.*, 1999). *M. frondosa* possesses antibacterial activity (Jayasinghe *et al.*, 2002). Glycosidic iridoids like mussaein are not cytotoxic (Dai *et al.*, 2002). *M. pubescens* exhibited anti-RSV activity with 50% inhibition (Li *et al.,* 2004). The sepals of *M. phillipica* cultivars are active (Vidyalakshmi *et al.*, 2007).

Mussaenda 'dona aurora' and *M. 'queensirikit'* are the white and pink varieties of the *M. phillipica* cultivar. The white variety is a large shrub with medium-sized oval leaves and large velvety sepals surrounding small star-shaped leaves (Huxley *et al.*, 1999). A preliminary screening exhibited good antioxidant properties of *M. 'dona aurora'* (Vidyalakshmi *et al.*, 2007). A new iridoid glycoside, sanshiside-D, has been identified (Vidyalakshmi *et al.*, 2009). The abundance of the iridoids and its wide availability makes the plant a potential target for studying the activity of this drug. Hence it has been ventured to study the antioxidant activity of active fractions and the isolated compounds. The ethyl acetate extract has been fractionated and studied for its hepatoprotective and antioxidant properties.

Material and Methods

Animals

Swiss albino mice of either sex weighing 22–25 g (Central Animal Facility, IISc, Bangalore, India) were held under controlled temperature [(25 ± 2) °C] and illumination (12 h light cycle starting at 06:00 am). Animals were maintained on laboratory chow and free water.

Plant material

The sepals of *Mussaenda 'dona aurora'* were collected in and around Thanjavur in December 2005. The plant has been authenticated (CARISM 0018) by botanists at Rabinad Herbarium, St. Josephs College, Trichy, India.

Extraction and isolation

The fresh sepals (5 kg) were extracted with 85% methanol. After concentrating under reduced pressure, the extract was dissolved in water and extracted with petroleum ether (500 mL), Et_2O (3 × 500 mL) and EtOAc (5 × 2.5 L) successively.

The EtOAc extract was evaporated under reduced pressure at 30–35 ºC to dryness. The extract was purified by preparative HPLC [bondapak RP C-18, 4 μm, 21.20 × 250 mm and UV detection at 254 nm), H_2O/MeOH (70:30), 15 min → H_2O/MeOH (50:50), 20 min → H_2O/MeOH (20:80), 50 min] to obtain fractions F1 (below Rt = 10.00 min) and F2 (above Rt = 30.00 min). They were concentrated *in vacuo*. The major compounds in F1 were the iridoids sanshiside-D and lamalbide, while F2 contained isoquercitrin, rutin and quercetin trisaccharide (Vidyalakshmi *et al*., 2009).

Sanshiside-D: White powder, m.p. 65–67 °C. – $[\alpha]^{30}$ –30º (*c* 0.40, MeOH). – UV: λ (MeOH) = 283.18, 328.26 nm; (+NaOMe) 268.55, 375.70 nm. – NMR: see Table I. – QToF MS: *m/z* = 529, 345, 327, 313, 285, 267, 253, 225, 211, 207, 193, 163, 149, 145.

Preparation of samples

The fractions F1 and F2, the ethyl acetate extract (MEE), the isolated compounds (sanshiside-D and lamalbide) and the standard antioxidants [ascorbic acid, rutin, butylated hydroxytoluene (BHT) and α-tocopherol] were dissolved in distilled dimethyl sulfoxide (DMSO) separately and used for the *in vitro* antioxidant assays applying five different methods. The stock solutions were serially diluted with the respective solvents.

Estimation of total phenolics

Antioxidant compounds generally contain phenolic group(s) and, hence, the amount of phenolic compounds in all the three extracts (F1, F2 and MEE) of the sepals was estimated by using the Folin-Ciocalteu reagent (Sadasivam and Manikam, 1992). The total phenolic compounds content was calculated and expressed as gallic acid equivalent in mg/g of extract.

Estimation of total flavonoids

The flavonoid content in the whole extract has been determined following the method of Chang *et al.* (2002). It is expressed as the flavonoid content obtained by the DNPH and $AlCl_3$ method.

Table I. ^{13}C (125 MHz, DMSO-d_6) and ^{1}H (500 MHz, DMSO-d_6) NMR data of sanshiside-D.

C	^{13}C	^{1}H	C	^{13}C	^{1}H
1	91.81	5.5 (d, 1.5)[a]	10″	20.38	2.0 (s)
3	151.17	7.4 (s)	1‴	125.56	
4	109.06		2‴	121.29	6.9 (dd, 8.5, 1.5)
5	32.88	2.9 (m)	3‴	144.86	7.4 (d, 1.5)
6	74.73	5.0 (m)	4‴	148.80	
7	77.28	4.8 (d, 3)	5‴	114.74	
8	76.47		6‴	115.70	6.7 (d, 2)
9	46.34	2.7 (d, 5)	7‴	114.26	6.3 (d, 16)
10	21.02	1.9 (s)	8‴	145.53	7.43 (d, 16)
1′	97.66	4.4 (d, 7.5)	9‴	165.69	
2′	73.06	2.9 (m)	6-CO$\underline{C}H_2$	70.40	5.1 (s)
3′	77.75	3.1 (t)	6-$\underline{C}OCH_2$	169.03	
4′	70.85	3.0 (t)	7-OCO$\underline{C}H_3$	21.02	
5′	69.99	4.5 (br s)	7-O$\underline{C}OCH_3$	169.60	
6′	61.05	3.4 (d, 2)	6′′-CO	174.90	
2′′	70.73	4.0 (br s)	3′′	36.28	
4″	68.10	5.1 (d, 4)	7″-CO	171.96	
5”	37.18	1.8 (m)	4-$\underline{C}OOCH_3$	166.20	
8″	21.6	1.7 (s)	4-COO$\underline{C}H_3$	51.62	3.5 (s)
9″	30.66	2.1 (d, 1)			

[a] Chemical shifts are in ppm, multiplicities and coupling constants in Hz are in parentheses.

The standard naringenin solution was diluted to 10, 25, 50, 100, 1000 μg/mL. 2.5 mL of sample solution were used for estimation. 1 mL of the diluted solutions reacted with 2 mL of 1% DNPH reagent and 2 mL of ethanol at 50 °C for 50 min. After cooling to room temperature the reaction mixture was mixed with 5 mL of 1% KOH solution and incubated at room temperature for 2 min. Then 1 mL of the mixture was taken, mixed with 5 mL of methanol and centrifuged at 1000 × g for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 mL. The absorbance of the supernatant was measured at 495 nm.

The standard quercetin solution was diluted in the range of 1–25 μg/mL. The diluted standard solutions and sample solutions (0.5 mL) were separately mixed with 95% ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After incubation of the reaction mixture for 30 min, the absorbance was measured at 415 nm.

Scavenging of ABTS radical cation

To 0.2 mL of the extracts with various concentrations, the compound or standard, 1.0 mL of distilled DMSO and 0.16 mL of ABTS solution were added and incubated for 20 min. The absorbance of these solutions was measured spectrophotometrically at 734 nm (Re *et al.*, 1999).

Nitric oxide radical inhibition assay

Nitric oxide was generated from sodium nitroprusside and analyzed by the Griess reaction (Green *et al.*, 1982). The reaction mixture (6 mL) containing sodium nitroprusside (5 mM, 4 mL), phosphate buffer saline (1 mL) and the extracts, the compounds and standard solutions (1 mL) were incubated at 5 ºC for 150 min. The samples were reacted with Griess reagent (1% sulfanilamide, 2% *o*-phosphoric acid and 0.1% naphthylethylenediamine hydrochloride). The absorbance was measured at 540 nm against the corresponding blank solutions using a Perkin Elmer UV spectrophotometer.

Lipid peroxidation inhibitory activity

The lipid peroxidation inhibitory activity tests of the extracts, the compounds and standard were carried out according to the method of Duh *et al.* (2001). Egg lectin (3 mg/mL, phosphate buffer, pH 7.4) was sonicated. The test samples of different concentrations were added to 1 mL of liposome mixture; control was without test sample. Lipid peroxidation was initiated by adding 10 mL ferric chloride (400 mM) and 10 mL ascorbic acid (200 mM). After incubation for 1 h at 37 ºC, the reaction was stopped by adding 2 mL of 0.25 M HCl containing 15% trichloroacetic acid and 0.375% thiobarbituric acid (Ohkawa *et al.*, 1979). The reaction mixture was boiled for 15 min, then cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm.

Hydroxyl radical scavenging potential

The ability of the extract to scavenge OH radicals was assessed using the classic deoxyribose degradation assay described by Halliwell and Gutteridge (1981). OH was generated in an 1 mL reaction mixture containing 0.1 mM phosphate buffer, pH 7.4, 2.8 mM deoxyribose, 20 mM $FeCl_3$, 100 mM EDTA (premixed immediately before its addition to the reaction mixture) and 500 mM H_2O_2 in the absence or presence of 5 mg to 1 mg of extract.

The reaction was commenced by adding 100 mM ascorbic acid. The samples were incubated for 60 min at 37 ºC in a shaking water bath followed by the TBARS assay. The extent of inhibition was compared with equivalent data obtained using 1 mM mannitol, a well-known hydroxyl radical scavenger.

Superoxide anion scavenging potential

The xanthine-xanthine oxidase (X-XO) enzymatic reaction is a suitable system to generate superoxide anions (O_2^{2-}) (Elizabeth and Rao, 1990). The reaction mixture contained 100 mM Na_2EDTA, 40 mM X and 40 mM NBT in 10 mM phosphate buffer, pH 7.4. The reaction was started by adding 10 mU/mL XO, and its rate was continuously monitored spectrophotometrically at $OD_{560\,nm}$ for 15 min at 25 ºC in the absence and presence of the extracts with different concentrations.

Hepatoprotective activity

To study the effect of the different extracts and the isolated compounds on CCl_4-induced liver injury in mice, animals were divided into 15 groups. Groups 1 (normal control) and 2 (induction con-

trol) received water for 7 d. Group 3 received picroliv (12 mg/kg, p.o.) for 7 d. Groups 4–15 were given F_1 (5, 10, 15 mg/kg body weight), F_2 (5, 10, 15 mg/kg body weight), MEE (5, 10, 15 mg/kg body weight) and sanshiside-D (2, 5, 10 mg/kg body weight).

All animals except for Group 1 were administered with 0.3% CCl_4 (10 mL/kg, i.p., dilution with olive oil). Animals in Groups 4–15 received drug for 7 d. The mice were then fasted for 24 h. On the eighth day all animals were sacrificed and blood was collected to prepare serum. 1.0 mL of blood was centrifuged at 3000 × *g* for 10 min to separate serum. ALT (alanine aminotransferase) and AST (aspartate aminotransferase) activities were evaluated with the Reitman-Frankel method using the reagent kit (Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China).

Statistical analysis

Data were expressed as mean ± S.D. The significant differences between the groups were assessed by standard or paired t-test using SPSS 10.0, and $p < 0.05$ was considered as significant difference.

Results and Discussion

The sepals of *Mussaenda 'dona aurora'* were successively extracted with petroleum ether, diethyl ether and ethyl acetate to obtain the corresponding extracts, which were tested for their antioxidant activity. The preliminary screening showed that the ethyl acetate extract (MEE) was highly active. The MEE had the highest phenolic content (44.70 mg/g), followed by F2 (24.2 mg/g) and F1 (23.71 mg/g). The total flavonoid content of F2 (56.82 mg/g) was highest. F1 did not contain any flavonoids. The MEE accounted for 18.95 mg/g flavonoids.

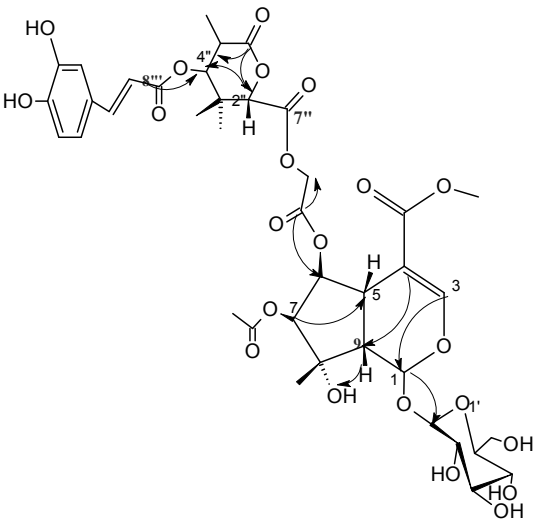

Fig. 1. Chemical structure of sanshiside-D.

Among the three extracts and the isolated compounds, sanshiside-D (Fig. 1) and lamalbide, tested for their *in vitro* antioxidant activity, F1 and sanshiside-D exhibited potent antioxidant activity in ABTS, inhibition of lipid peroxidation and nitric oxide radical inhibition assays. The IC_{50} values (Table II) were found to be comparable to those obtained for the standards used. However, the MEE, F2 and lamalbide were found to inhibit lipid peroxidation and scavenge H_2O_2 moderately to low. The results showed that F1 is the most potent antioxidant fraction containing iridoids, lamalbide and sanshiside-D. Among

Table II. *In vitro* antioxidant activity of compounds isolated from *Mussaenda 'dona aurora'* sepals and standards.

Compound	IC_{50} ± S.E.M.[a] [μg/mL]					
	ABTS	LPO	NO	OH	SOD	H_2O_2
Lamalbide	1.7 ± 0.1	432.0 ± 13.9	23.8 ± 0.05	185.9 ± 14.5	>1000	61.1 ± 0.01
Sanshiside-D	0.3 ± 0.02	3.8 ± 0.3	1.4 ± 0.3	12.8 ± 0.1	44.3 ± 8.3	0.4 ± 0.04
Standards						
Ascorbic acid	11.2 ± 0.5	–	–	–	–	87.3 ± 3.5
Rutin	0.5 ± 0.26	–	88.4 ± 2.5	203.6 ± 3.25	121.2 ± 129.5	36.2 ± 0.25
BHT	–	–	–	74.7 ± 1.49	–	24.7 ± 1.53
α-Tocopherol	91.6 ± 1.67	–	–	–	–	

– Not done.
[a] Average of three determinations.

the two iridoids, the new iridoid sanshiside-D was highly active.

Sanshiside-D showed highest NO scavenging activity [90.43% (100 μg/mL)] and its IC_{50} value was less than 2 μg/mL. The caffeoyl moiety of sanshiside-D is responsible for its exceptionally high free radical scavenging properties. Caffeic acid and some of its derivatives such as caffeic acid phenethyl ester and octyl caffeate are potent antioxidants which present important anti-inflammatory actions (da Cunha *et al.*, 2004). In inhibiting lipid peroxidation, the highest activity was observed at a lower dose. At 5 μg/mL, sanshiside-D inhibited lipid peroxidation to an extent of 75% while at 100 μg/mL, the inhibition seemed to diminish. At higher concentration, the availability of this highly polar molecule decreased in the vicinity of phospholipid bilayers. In the case of rutin, chelation with Fe^{2+} has enhanced the activity inspite of its glycosylation. Rutin showed feeble activity at 10 μg/mL (12.45% inhibition) while sanshiside-D maintained maximum activity (79% inhibition). This might probably be due to the bulkiness of the sugar moiety which prevents the molecules from being completely conjugated due to the steric hindrance.

The activity of the iridoids determined by the deoxyribose method was higher than that of the flavonoids. In the case of flavonols (rutin), the 3-OH and the C2–C3 double bond is not involved in scavenging. This is not surprising as OH radicals are known to react quite indiscriminately. This is in accordance with the higher activity of rutin when compared to the *in vitro* assay of lipid peroxidation.

The scavenging of superoxide radicals was quite different from inhibition of malondialdehyde formation. Only moderate activity was found for all compounds. It is known that greatest activity is found among non-glycosidic flavonols and flavanols. Several flavonoids are also active superoxide scavengers in a non-enzymatic system as was evidenced by the inhibition of the nitro blue tetrazolium reduction (Huguet *et al.*, 1990). Picroliv, picroside-I and kutkoside, the iridoids from *Picrorhiza kurrooa*, inhibited the non-enzymatic generation of O^{2-} anions in a phenazine methosulfate-NADH system (Chander *et al.*, 1992). Sanshiside-D showed protection against superoxide radicals to an extent of 79% at 100 μg/mL while rutin to an extent of 61% at 100 μg/mL.

The AST and ALT levels of the MEE showed a dose-dependent decline compared with that of the CCl_4-induced group. Groups administered the F2 solution revealed no decrease in AST and ALT levels andthose administered. F1 showed an almost tantamount protective effect.

The obtained results demonstrated that some of the isolated compounds play an important role in the antioxidant activity of *M. 'dona aurora'*. One proposed mechanism of antihepatotoxic action of picroliv is the reactivation of superoxide dismutase to counteract the formation of free radicals and the subsequent production of lipid peroxides. The other possible action of protecting the liver by these drugs may be the correction of the NADH:NAD ratio and ATP concentration, and stabilization of the plasma membrane as it is expected from a typical hepatoprotective agent. The same mechanism could be proposed for the new iridoid sanshiside-D. Its caffeoyl moiety could account for its enhanced activity. Further studies are in progress to investigate the possible mechanisms. The hydromethanolic extract of *M. 'dona aurora'* could thus be considered as a source of potential antioxidants and will promote the reasonable usage of this plant in medicine.

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