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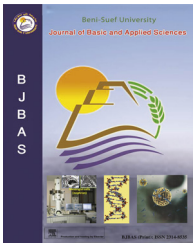

Full Length Article

Isolation of bergenin from *Peltophorum pterocarpum* flowers and its bioactivity

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ABSTRACT

Increasing interest in biological activity of natural products outlined the necessity of isolation of bergenin from *Peltophorum pterocarpum* (*P. pterocarpum*) flowers. The present investigation comprises isolation of bergenin accompanied by important in vitro antioxidant assays such as DPPH, hydroxyl radical and nitric oxide scavenging activities, and ferric reducing power. The aim includes DNA protection and cleavage. Bergenin showed 4.47, 13.03, and 8.67% of DPPH, hydroxyl and nitric oxide scavenging activities at 100 µg/ml. It showed less ferric reducing power. The result indicates that bergenin is a mild antioxidant. Bergenin did not protect the DNA but it cleaved the DNA at a low concentration of 10 µg/ml. *P. pterocarpum* flowers are a cheap source of bergenin and they could be used as a natural antioxidant.

1. Introduction

The *Peltophorum pterocarpum* tree is readily available and widely distributed in Asia. The bark of trees is used in dysentery, for gargles, tooth powders and externally (as lotions) in eye diseases, muscular pains and sores (Matthew, 1999). Antibacterial, antifungal, antioxidant activity and wound healing properties of the flowers of *P. pterocarpum* have been reported (Duraipandiyan et al., 2006; Sohail et al., 2007). During the flowering seasons, *P. pterocarpum* bears large amount of flowers. These flowers could not be consumed even by cattle. Utilization of such flowers is cost effective and environmentally benign. Antibacterial activity of aqueous and ethanolic extracts of *P. pterocarpum* has been reported (Voravuthukunchai et al., 2004). This plant is well known for its biomolecules. Flavanol glucoside from *Peltophorum africanum* (El-Sherbeiny et al., 1997) and *Peltophorum dubium* (Salvat et al., 2004), and novel cyanomaclurin from *P. africanum* (Bam et al., 1988) have been reported. *P. pterocarpum* is a yellow flame tree native of tropical Southeastern Asia. The wood has a wide variety of uses, including cabinet-making and the foliage is used as a fodder

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crop (Ethelbert and Walter Samuel, 1977). Upon the review of literature, it was found that the plant is traditionally used in the treatment of unhealthy skin, ringworm, constipation, insomnia and stomatitis. The leaves, bark and wood of *P. pterocarpum* contain tannins. Tannins are natural antioxidants possessing radical scavenging activity (Ciddi and Kaleab, 2005).

Plants contain a wide variety of antioxidants such as phenolic acids, flavonoids, quinines, tannins and ascorbic acid (Cai et al., 2004). Antioxidants are distributed in different parts of the plants such as wood, bark, and stems, pods, leaves and flowers (Chanwitheesuk et al., 2005). Antioxidants reduce or prevent the oxidation process which happens in the human body due to various environmental factors. Antioxidants have the potential to neutralize the harmful effects of free radicals in tissues and thus are believed to protect against cancer, arteriosclerosis, heart disease, and several other diseases (Bandyopadhyay et al., 2007).

2. Materials and methods

2.1. Chemicals

1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) was purchased from Sigma-Aldrich, Bangalore, India. Trichloroacetic acid (TCA), Sodium nitroprusside, potassium ferricyanide, ferric chloride, methanol, sodium nitrite, ammonium acetate, acetyl acetone, glacial acetic acid, ascorbic acid, ferrous ammonium sulphate, ethylenediaminetetraacetic acid (EDTA), dimethyl sulphoxide (DMSO), butylated hydroxyl anisole (BHA) and potassium persulphate were procured from Merck, India. All chemicals used were of analytical grade and used as such without further purification.

2.2. Extraction and isolation of bergenin

Flowers of *P. pterocarpum* were collected from Periyar University Campus on March 2011 and authenticated by Dr. S. Karuppusamy, Professor of Botany, Madura College, Mudarai. Shade dried flowers of *P. pterocarpum* (1.0 kg) were chopped and defatted with (3 × 3L) hexane. Defatted material was extracted with methanol (3 × 5L) using soxhlet apparatus. The methanol extract was concentrated under reduced pressure yield a gummy crude extract (100 g). This crude was dissolved in ethyl acetate. Crude compound was obtained and was repetitively washed with fresh ethyl acetate. Crystalline substance (1.0 g) was crystallized from methanol. Purity of the compound was tested using hexane and ethyl acetate at the ratio of 2:8.

2.3. Characterization of bergenin

Colourless crystals, recrystallized in MeOH, mp-236 °C, UV (MeOH) λ_{max} (nm): 229 and 274. IR (KBr) ν_{max} (cm^{-1}): 2926, 1728, 1673 and 1471, 1093 and 790. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.98 (1H, m, arom, 7-H); 5.63 (1H, d, H-10b); 4.97 (1H, dd, H-4a); 4.00 (1H, dd, H-4); 3.80 (2H, d, H-11); 3.98 (3H, s, H-12); 3.76 (2H, m); 3.66 (1H, m, H-2); 3.52(1H, dd, H-3). ^{13}C NMR (100 MHz, DMSO-d_6): δ163.3 (C-6); 150.3 (C-8); 148.0 (C-10); 140.5 (C-9); 118.0 (C-6a); 115.9 (C10a); 112.6 (C-5); 109.4 (C-7); 81.7 (C-2); 79.7 (C-4a); 73.6 (C-4); 72.1 (C-10b); 70.3 (C-3); 61.1 (C-11); 59.8 (C-12). ^{1}H NMR and ^{13}C NMR spectral data are given in Table 1.

Table 1 – ^{1}H NMR and ^{13}C NMR spectral data of bergenin.

Position	^{1}H NMR	^{13}C NMR
2	3.66 (1H, m, H-2)	70.3
3	3.52 (1H, dd, H-3)	72.1
4	4.00 (1H, dd, H-4)	73.6
4a	4.97 (1H, dd, H-4a)	79.7
5	–	–
6	–	163.3
6a	–	118.0
7	6.98 (1H m, H-7)	109.4
8	9.70 (OH)	150.3
9	–	140.5
10	8.43 (OH)	148.0
10a	–	115.9
10b	5.63 (1H, d, H-10b)	81.7
11	3.76 (2H, m)	61.1
OCH_3	3.98 (3H, s, H-12)	59.8

Antioxidant properties of bergenin were evaluated by four different assays such as (i) DPPH radical scavenging activity; (ii) Hydroxyl radical scavenging activity; (iii) Ferric reducing power (Fe^{3+}/Fe^{2+}); (iv) Nitric oxide scavenging activity.

2.4. DPPH radical scavenging activity

Different concentrations (10, 50 and 100 μg/ml) of sample were taken in different test tubes. The volume was adjusted to 500 μl by adding methanol. Five millilitres of a 0.1 mM methanolic solution of DPPH were added to these test tubes and shaken vigorously. A control without the test compound, but with an equivalent amount of methanol was maintained. The test tubes were allowed to stand at room temperature for 20 min. The absorbance of the samples was measured at 517 nm. DPPH radical-scavenging activity was calculated by the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = ((A_{DPPH} - A_s)/A_{DPPH}) \times 100$$

where A_{DPPH} is the absorbance without samples and A_s is the absorbance in the presence of the samples. A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity (Subramanian et al., 2013).

2.5. Hydroxyl radical scavenging activity

This radical scavenging activity of the compound was determined by employing the method outlined in the literature (Klein et al., 1991). Different concentrations (10, 50 and 100 μg) of sample were taken in different test tubes and made up to 250 μl with 0.1M phosphate buffer. Ascorbic acid (AA) was used as standard for comparison. One millilitre of iron–EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of EDTA (0.018%), and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. These

reaction mixtures were incubated at room temperature for 15 min. The reaction was terminated by the addition of 1 ml of ice-cold TCA (17.5% w/v). Three millilitres of Nash reagent (150 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 l with distilled water) was added to all of the test tubes and left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured at 412 nm against blank. The percentage hydroxyl radical scavenging activity was calculated by the following formula:

$$\text{Hydroxyl radical-scavenging activity (\%)} = ((A_c - A_e)/A_c) \times 100$$

where A_c is the absorbance without samples and A_e is the absorbance in the presence of the sample.

2.6. *Ferric reducing antioxidant power*

Various concentrations of the sample (10, 50 and 100 μg) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Next, 2.5 ml of 10% (w/v) trichloroacetic acid were added. Five millilitres of the above solution were mixed with 5 ml of distilled water and 1 ml of 0.1% of ferric chloride. The absorbance was measured at 700 nm (Barreira et al., 2008).

2.7. *Nitric oxide radical scavenging activity*

Nitric oxide scavenging activity was determined according to the method of (Kumar et al. (2008). Various concentrations (10, 50 and 100 μg) of the sample were taken in different test tubes and made up to 3 ml with 0.1M phosphate buffer (pH 7.2). Sodium nitroprusside (5 mM) prepared in buffered saline (pH 7.2) was added (1 ml) to each tube. The reaction mixture was incubated for 30 min at room temperature. A control without the test compound was maintained. After 30 min, 1.5 ml of the above solution was mixed with 1.5 ml of Griess reagent (1% Sulphanilamide, 2% phosphoric acid and 0.1% N-1-Naphthylethylenediamine dihydrochloride). The absorbance of the samples was measured at 546 nm. Nitric oxide radical scavenging activity was calculated using the following formula:

$$\text{\% NO radical scavenging activity} = (A_{control} - A_{sample}) \times 100/A_{control}$$

2.8. *Statistical analysis*

Triplicate analyses were performed by excel sheet. The results were presented as the mean ± S.D. Statistical analysis was performed using student's t-test and a $P < 0.05$ was regarded to be significant.

3. Results and discussion

3.1. *Characterization of bergenin*

Compound PP-1 was isolated as white crystals. The melting point of 236 °C is consistent with the published melting point of bergenin 235 °C (Nasser et al., 2009). UV–Visible spectrum of bergenin showed λ_{max} at 229 and 274 (S-1). Its IR absorption band showed the presence of hydroxyl (3429 cm^{-1}), methyl group (2926 cm^{-1}), carbonyl (1728 cm^{-1}), benzene ring (1673, 1471 cm^{-1}) and substituted phenolic groups (1093 and 790 cm^{-1}) (S-2). The ^1H NMR spectrum exhibited a signal for one aromatic proton (s, 6.98, 1H) and a signal for methoxy protons (s, 3.98, 3H) (S-3). The ^{13}C NMR spectrum exhibited fourteen carbon signals including carbonyl carbon δ_C (163.38), one methoxyl carbon at δ_C (59.82), olefinic carbons δ_C 109.4, 118.07, 150.36, 140.59, 148.08 and 115.95 (S-4). The spectral data of PP-1 were compared with the literature and confirmed that the structure of PP-1 was bergenin as shown in Fig. 1 (Caldas et al., 2002; Da Silva et al., 2009; Jahodar et al., 1992; Nunomura et al., 2009).

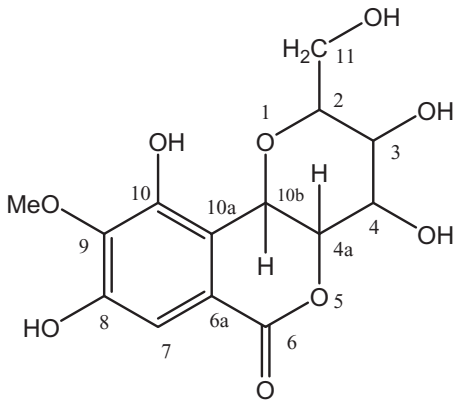

Fig. 1 – Structure of bergenin.

3.2. *DPPH radical scavenging activity*

Figure 2a illustrates a slight decrease in the concentration of DPPH free radical with various concentrations of the bergenin, due to their scavenging ability against the standard butylated hydroxyl anisole (Table 2). Concentrations of bergenin varied from 10 to 100 μg/ml. Scavenging activity (4.47%) of bergenin was significantly less as compared with that of BHA (85.49%) at 100 μg/ml. Plant extracts exhibit efficient antioxidant properties due to their bioactive molecules, including phenolics. It is believed that phenolic compounds contribute directly to the antioxidative effect of the extracts (Larson, 1988; Mariod et al., 2009). Natural antioxidants present in medicinal plants are responsible for inhibiting the harmful consequences of oxidative stress. DPPH radical is scavenged by polyphenols through donation of hydrogen, forming the reduced form of DPPH. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between bergenin and radical progresses, results in the scavenging of the radical by hydrogen donation. Though the compound contained hydroxyl groups, it showed very less scavenging activity against DPPH radical.

3.3. *Hydroxyl radical scavenging activity*

Hydroxyl radical inhibition of bergenin was investigated and these results are shown as relative activity against the BHA. Dose-dependent hydroxyl radical scavenging activity reveals

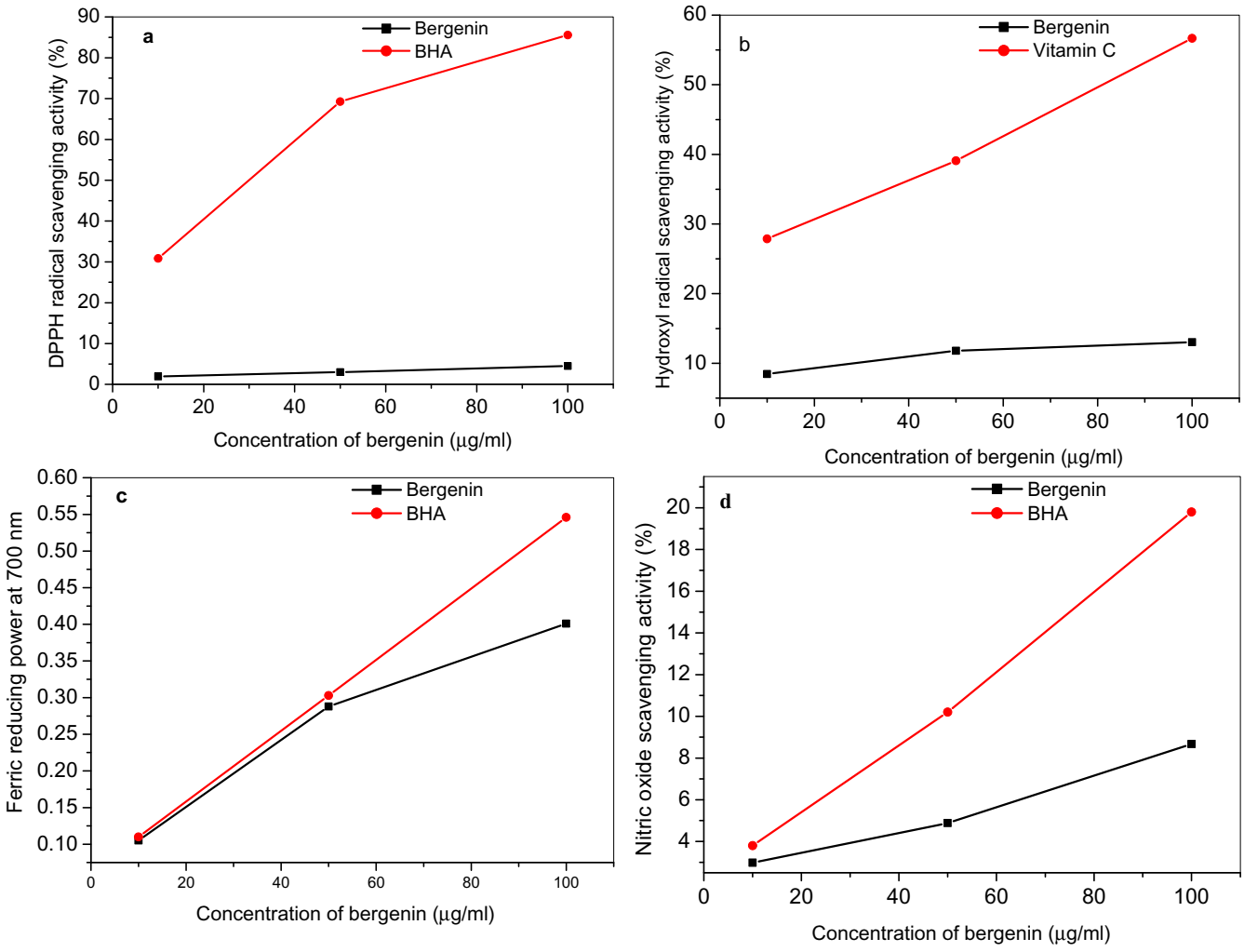

Fig. 2 – The antioxidant profile of bergenin by different in vitro methods. (a) DPPH radical scavenging; (b) Hydroxyl radical scavenging; (c) Ferric reducing power; (d) Nitric oxide scavenging activity (n = 3, mean ± SD).

that bergenin is a mild hydroxyl radical scavenger, acting as weak antioxidant. As can be seen in Fig. 2b, hydroxyl radical scavenging activities of bergenin and BHA at 100 µg/ml were 13.0% and 56.67, respectively. Like DPPH radical scavenging, hydroxyl radical scavenging is also significantly lower than that of BHA (Table 2).

3.4. *Ferric reducing power*

Ferric reducing power was seen to increase with increase in the concentration of the compound as shown in Fig. 2c. EC_{50} was calculated from the graph to determine the concentration of bergenin that provided 0.5 absorbance at 700 nm.

Table 2 – Radical scavenging activities of bergenin.

DPPH radical scavenging activity (%)			Hydroxyl radical scavenging activity (%)	
Concentrations (µg/ml)	Bergenin (%)	BHA (%)	Bergenin (%)	BHA (%)
20	1.95 ± 0.09	30.79 ± 0.15	8.48 ± 0.59	27.88 ± 0.40
50	3.07 ± 0.12	69.12 ± 0.07	11.82 ± 0.46	39.09 ± 0.20
100	4.47 ± 0.12	85.48 ± 0.15	13.03 ± 0.35	56.67 ± 0.92
Nitric oxide scavenging activity			Ferric reducing power (OD)*	
	Bergenin (%)	BHA (%)	Bergenin (%)	BHA (%)
20	2.98 ± 0.38	3.80 ± 0.60	0.105 ± 0.008	0.11 ± 0.006
50	4.88 ± 0.34	10.20 ± 0.52	0.288 ± 0.008	0.303 ± 0.001
100	8.67 ± 0.45	19.18 ± 0.39	0.401 ± 0.006	0.546 ± 0.009

* OD, Increasing the optical density.

Concentrations of bergenin and standard varied from 10 to 100 μg/ml. Reducing powers of bergenin and standard at 100 μg/ml were 0.401 and 0.546, respectively. The reducing power of compound may serve as an indicator of its antioxidant activity. In the reducing power assay, bergenin would result in the reduction of Fe^{3+}/ferricyanide complex to its form (Meir et al., 1995). The increased absorbance values of the compound at 700 nm indicate an increase in reductive ability. The reducing power of BHA was found to be significantly higher than that of bergenin (Table 2). In this assay, the yellow colour of the test solution was changed into various shades of green and blue depending on the reducing power of each compound. These data imply that bergenin has limited reducing capacity when compared with standard.

3.5. *Nitric oxide scavenging activity*

Scavenging activities of bergenin and standard were 8.67% and 19.80%, respectively. Scavenging activity of the compound was significantly lower than that of the standard as shown in Fig. 2d. Results of the nitric oxide, DPPH radical and hydroxyl radical scavenging activities are similar except that of ferric reducing power (Table 2).

3.6. *DNA protection and cleavage*

One of the objectives is to test the DNA protection and cleavage of bergenin. Thus the concentrations of the compound were varied from 10 to 500 μg/ml to evaluate the DNA protection. It did not protect the DNA at 500 μg/ml as shown in Fig. 3. From the gel picture (Fig. 4), it is evident that the bergenin has cleaved DNA partially at 10 μg/ml, but complete cleavage was observed in all other higher concentrations.

For in vitro antioxidant screening, DPPH, hydroxyl radical scavenging, nitric oxide scavenging and ferric reducing power are most commonly used. Antioxidant activity of a substance cannot be evaluated by using a single method due to oxidative processes. Therefore, four methods have been employed to evaluate the antioxidant capacity (Gulcin et al., 2005).

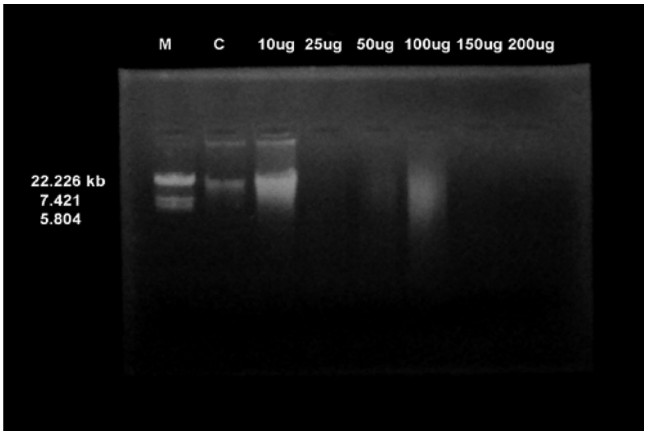

Fig. 3 – DNA protection of bergenin.

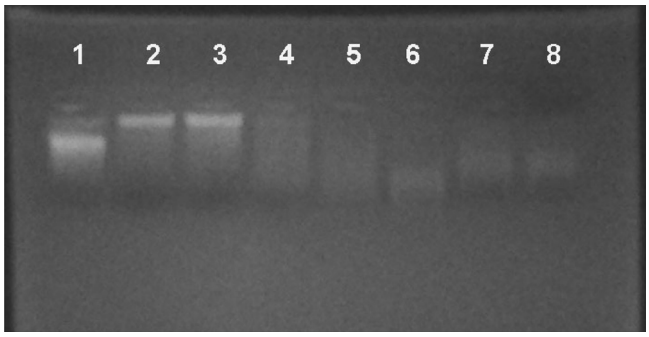

Fig. 4 – DNA cleavage of bergenin.

Bergenin, a coumarin derivative possessed many biological activities such as antibacterial, anti-inflammatory, anticancer and antifungal activities. Previously, bergenin has been isolated from many medicinal plants such as *P. africanum* and *Flueggea virosa* (Da Silva et al., 2009; Magalhães et al., 2007). Different investigators have used different methods to isolate bergenin. Various quantities of bergenin have been reported. Here, we have reported 1.0 g of bergenin from 1 kg of flowers. Moreover, column chromatography was not employed for isolation. This is a very simple and economical method with respect to usages of solvent. Every flowering season, large amount of flowers become waste since they could not be eaten by cattle too. Hence, utilization of such flowers to isolate natural bergenin is economically viable. With respect to the biological activity, limited studies were undertaken in *P. pterocarpum*. Also, it can be seen that methanol and ethyl acetate are the suitable solvents for the extraction and isolation of bergenin from *P. pterocarpum* flowers.

4. Conclusions

We have reported a simple isolation procedure for bergenin from *P. pterocarpum* flowers. Bergenin showed mild scavenging activity against DPPH, hydroxyl, nitric oxide radicals and ferric reducing power. Moreover it may act as a pro-oxidant. Bergenin can be explored for its applications in the form of pro-oxidant. Isolation of bergenin from the flower waste reduces the cost of chemical synthesis. Since it is a natural compound, it could be explored for some other food applications other than as antioxidant. In conclusion, *P. pterocarpum* flowers are a cheap source of raw materials for the isolation of natural bergenin.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.bjbas.2015.06.002.

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