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IN VITRO ANTIOXIDANT POTENTIAL OF BLEPHARIS PERSICA (BURM F) O KUNTZE (SEEDS)

Vichitra Kaushik*[1], Gagandeep Chaudhary[2], Shoaib Ahmad[2] and Vipin Saini[1]

[1]MM College of Pharmacy, Maharishi Markandeshwar University, Mullana, Haryana, India.

[2]Rayat and Bahra Institute of Pharmacy, Sahauran, Kharar, Mohali, Punjab, India.

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***Correspondence for Author**
Vichitra Kaushik
MM College of Pharmacy, Maharishi Markandeshwar University, Mullana, Haryana, India.

ABSTRACT

Antioxidant activities of the ethanolic (90%) and aqueous extracts of seeds of Blepharis persica and total phenolic and flavonoids contents were investigated for free and peroxyl radicle scavenging activity, reducing capacity by in-vitro models. They were examined by a DPPH free radical scavenging and ferric reducing power (FRPA) as Non-site specific assays and lipid peroxidation (TBARS) as site specific assay. In Non-site specific assays showed significant scavenging activity for the ethanolic (90%) and aqueous extracts. Site-specific lipid peroxidation also confirms the peroxyl radical scavenging capacity of ethanolic (90%) and aqueous extracts results were compared with standard antioxidant (Butyl hydroxy toluene). In general, the ethanolic (90%) and aqueous extracts showed significant ($P < 0.05$) activity in all systems, such results might be attributed to the prominent antioxidant effect. The antioxidant activities of all the tested sample was concentration dependent. Based on the results obtained, it was conclude that the B. persica seeds ethanolic (90%) and aqueous extracts may be valuable natural antioxidant sources and as potentially applicable in both medicine and the healthy food industry.

KEYWORDS: Antioxidant activity; DPPH; FRPA; Phenolic compounds; TBARS.

INTRODUCTION

Antioxidants are substances that, when present at low concentrations, compared with those of an oxidizable substrate, significantly prevent or delay a pro-oxidant–initiated oxidation of the substrate. (Halliwell and Gutteridge, 1984) The imbalance of oxidants and antioxidants of the body leads to an oxidative stress resulting in destruction of unsaturated lipids, DNA, proteins

and other essential molecules. Increasing evidence suggests that oxidative damage to cell components has a relevant pathophysiological role in several types of human diseases. (Ames et al, 1993) Most of the potentially harmful effects of oxygen are due to the formation of reactive oxygen species (ROS). The uncontrolled production of ROS and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing. Free radicals are been reported to cause red blood cell lysis in patients with blood pathologies such as thalassemia. (Vives-Corrons et al., 1995) The erythrocytes are highly susceptible to oxidative damage due to the high polyunsaturated fatty acid content of their membrane and the high cellular concentration of oxygen and haemoglobin, all of which are powerful promoters of oxidative processes. (Clemens et al., 1987) Exposure of erythrocytes to free radicals leads to a number of membrane changes including lipid peroxidation (Koster et al., 1983), reduction in deformability (Kurata et al., 1994), changes in cell morphology (Shinar et al., 1989), protein cross-linking and fragmentation. (Vissser et al., 1994) These are the most common configuration damage leading to lysis of red blood cells.

Blepharis persica (Burm. f.) O. Kuntze. (Acanthaceae) commonly known as Uttingana, Sunishannaka, Chaupatia and Borahu is a soft grey-pubscent perenial herb. (Anonymous, 1986) It is indigenous to India (Punjab, Western Rajasthan, Malwa region of Madhya Pradesh), Pakistan, Iran, Africa (Thar). (API, 2007) It is used as purgative, tonic, aphrodisiac (Pande and Pathak, 2009), diuretic, expectorant and used in treatment of urinary discharges, leukoderma, ascites, disorders of liver and spleen. (Gupta et al., 2004) It contains saponin, tannins, flavonoids and glycoside (blepharin). (Chatterjee et al., 1990; Mohaddese et al., 2013) It has delayed seed dispersal and rapid germination property. (Gutterman, 1972) The traditional formulations are available in the name of Kumaryasava in Ayurvedic system and Majoon-e-Bandkushad in Unani medicine system. (API, 2007).

Antioxidants can protect against the damage induced by free radicals acting at various levels. Dietary and other components of plants form major sources of antioxidants. The relation between free radicals, antioxidants and functioning of various organs and organ systems is highly complex and the discovery of ‘redox signalling’ is a milestone in this crucial relationship. Recent research was carried out for various strategies to protect crucial tissues and organs against oxidative damage induced by the free radicals. Therefore, the objective of the present study was to investigate the total phenolic content and antioxidant properties of its ethanolic (90%) and aqueous extracts of B. persica seed by DPPH, FRPA as non-site specific

assays and TBARS as Site specific assay. The present study would offer basic data on the natural antioxidant potential of seeds for the food or pharmaceutical industries and also provides scientific reference for the large scale usage and exploitation of the plant as a resource.

MATERIALS AND METHODS

Plant material

The seeds of the plant were collected in the month of April from Patiala, Punjab and authenticated by Dr. Sunita Garg, Chief Scientist, NISCAIR, New Delhi, India (Ref. No. - NISCAIR/RHMD/Consult/2013/2311/91 dated13/09/2013)

The seeds were shade dried, coarsely powdered and stored in an air tight container till use.

Extraction

Seeds powder was defatted with n-hexane. The dried solvent freed marc was extracted by cold maceration with ethanol (90%) and distilled water till exhausted completely. The ethanolic (90%) and aqueous extracts so obtained were freed of solvent under vacuum and the extracts were preserved in a refrigerator till further use.

Determination of total phenolic content by colorimetric method (Singleton et al., 1999)

Preparation of standard: Gallic acid was used to make the calibration curve. 10 mg of gallic acid was dissolved in 100 ml of 50% methanol (100 µg/ml) and then further diluted to 1, 2, 4, 6, 8 and 10 µg/ml. 1 ml aliquot of each dilution was taken in a test tube and diluted with 10 ml of distilled water. Then, 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. 4 ml of 20% (w/w) Na_2CO_3 were added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the standard was measured at 765 nm. Distilled water was taken as a blank.

Preparation of sample: 1 g of sample (seed powder) was added to 15 ml of methanol (50%) and extracted for three times by maceration of 2 h. Then filtered and make up the volume with methanol (50%) in volumetric flask up to 50 ml. 1 ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then, 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. 4 ml of 20% (w/w) Na_2CO_3 were added, adjusted with distilled water up to the mark of 25 ml, agitated and left to

stand for 30 min at room temperature. Absorbance of the sample was measured at 765 nm. Three parallel determinations were recorded. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as mg gallic acid equivalents (GAE) and percentage w/w. (Singleton et al., 1999).

Determination of total flavonoids content by colorimetric method (Woisky and Salatino, 1998)

Preparation of standard: Rutin was used to make the calibration curve. 10 mg of rutin was dissolved in 100 ml of 80% methanol (100 µg/ml) and then further diluted to 10, 20, 40, 60, 80 and 100 µg/ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with Shimadzu UV-Visible spectrophotometer. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in blank.

Preparation of sample: About 1 g of ethanolic (90%) and aqueous extracts were dissolved in 25 ml of 80% methanol. Similarly, 0.5 ml of ethanolic (90%) and aqueous extracts were reacted with aluminium chloride for determination of flavonoids content. Three parallel determinations were recorded. Quantification was done on the basis of a standard curve of rutin. Results were expressed as mg rutin equivalents (RE) and percentage w/w. (Woisky and Salatino, 1998).

Screening for antioxidant activity

Chemicals and reagents

1,1-Diphenyl-2-picryl hydrazyl (DPPH) and Butyl hydroxyl toluene (BHT) were obtained from Sigma–Aldrich Co., St. Louis, USA. Rutin was obtained from Acros Organics, NJ, USA. Ascorbic acid was from SD Fine Chemicals Ltd., Mumbai, India. All chemicals used were of analytical grade.

DPPH radical scavenging activity (Non-site specific) (Blois, 1958; Singh et al., 2008)

Preparation of sample dilution: 50 mg of each of ethanolic (90%) and aqueous extracts were weighed separately and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (10, 20, 40, 80, 160 µg/ml) of these solutions were prepared by diluting serially with methanol.

Preparation of standard dilution: 10 mg of rutin was weighed separately and dissolved in 100 ml of methanol get 100 µg/ml concentration. Lower concentrations (2, 4, 6, 8, 10 µg/ml respectively) of these solutions were prepared by diluting serially with methanol.

Procedure: The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts. The 0.1 mM solution of DPPH in methanol (22.2 mg in 1000 ml) was freshly prepared. Different concentrations of extract were added at an equal volume to methanolic solution of DPPH. After 30 min. at room temperature, the absorbance was recorded at 517 nm. Rutin was used as standard control. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals. Radical scavenging activity was calculated by the following formula % Free radical scavenging power = Abs. C - (Abs. S – Abs. B) / Abs. C × 100 IC_{50} value was determined from the plotted graph of scavenging activity against the different concentrations of B. persica ethanolic (90%) and aqueous extracts, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. The measurements were carried out three times and their scavenging effect was calculated based on the percentage of DPPH scavenged. (Blois, 1958; Singh et al., 2008).

Ferric reducing Power Assay (Non-site specific)

(Oyaizu, 1986; Tenpe et al., 2008) Preparation of sample dilution: 50 mg of each of ethanolic (90%) and aqueous extracts were weighed separately and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of these solutions were prepared by diluting serially with methanol.

Preparation of standard dilution: 50 mg of ascorbic acid was weighed and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of the solution were prepared by diluting serially with methanol.

Procedure:1 ml of the sample (25–400 µg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50ºC for 30 min and 2.5 ml of 10% trichloro acetic acid was added to the mixture and centrifuged for 10 min at 3000 rpm. About 2.5 ml of the supernatant was diluted with 2.5 ml water and is shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. L-Ascorbic acid was

used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations. (Oyaizu, 1986; Tenpe et al., 2008).

Lipid peroxidation (TBARS) (Site-specific)

(Halliwell and Gutteridge, 1989; Arora and Singh, 2009) Preparation of sample dilution: 50 mg of each of ethanolic (90%) and aqueous extracts were weighed separately and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of these solutions were prepared by diluting serially with methanol.

Preparation of standard dilution: 50 mg of Butyl hydroxyl toluene (BHT) was weighed and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of the solution were prepared by diluting serially with methanol.

Procedure: Normal male rats (250 g) were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% (w/v) homogenate was prepared with homogenizer at 0-4°C with 0.15 M KCl. The homogenate was centrifuged at 8000 rpm for 15 min and clear cell-free supernatant was used for the study with in vitro lipid peroxidation assay. Different concentrations (50-700 µg/ml) of extract dissolved in methanol and in test tubes. 1 ml of 0.15 M KCl and 0.5 ml of rat liver homogenates were added to the test tubes. Peroxidation was initiated by adding 100 µl of 0.2 mM ferric chloride. After incubation at 37°C for 30 min, the reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% TBA, and 0.5% butyl hydroxy toluene. The reaction mixtures were heated at 80°C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. (Halliwell and Gutteridge, 1989; Arora and Singh, 2009).

The percentage inhibition of lipid peroxidation is calculated by the formula

Inhibition of lipid peroxidation (%) = 1 – (Abs. S/Abs. B) × 100.

STATISTICAL ANALYSIS

Results were reported as means ± S.D. of three determinations. IC_{50} values were determined by interpolations. One Way ANOVA was used to evaluate differences between groups. The differences among the means were analysed by Tukey-Kramer test for multiple comparisons using computerized program at 95% ($P < 0.05$) confidence level. (Hosseinzadeh et al., 2005).

RESULTS AND DISCUSSION

The total phenolic content was found to be in ethanolic (90%) (2.05%w/w) and aqueous extracts (0.45%w/w). The calibration curve of standard gallic acid is given in **Fig 1** and the results are depicted in **Table 1**. The total flavonoids content estimation from ethanolic (90%) and aqueous extracts of seeds estimated to be 8.234%w/w and 1.375% w/w respectively. The calibration curve of standard rutin is given in **Fig 2** and the results are depicted in **Table 2**.

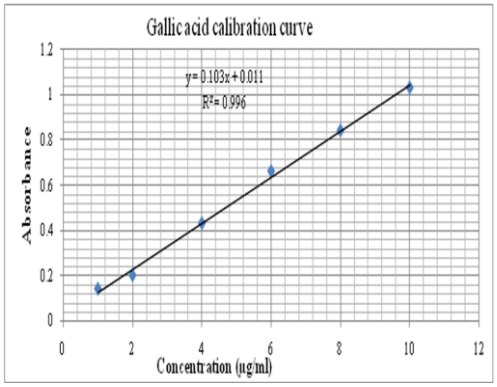

Fig 1: Caliberation Curve of Gallic acid

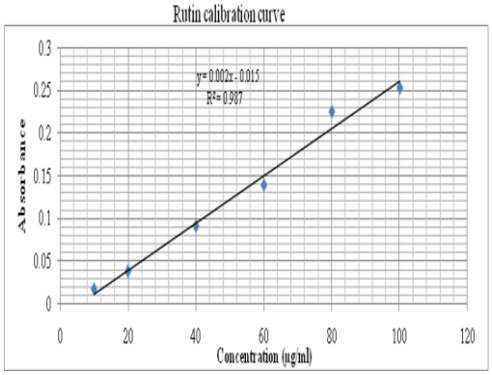

Fig 2: Caliberation Curve of Rutin

Table 1: Estimation of total phenolic compounds content

Test Sample	Extracts	Total phenolic content [µg gallic acid equivalents] (GAE)	Total phenolic content (% w/w)
B. persica (Seed Extract)	Ethanolic Extract (BPEE)	18.75	2.05
	Aqueous Extract (BPAE)	5.65	0.45

Table 2: Estimation of total flavonoids content

Test Sample	Extracts	Total flavonoids (mg rutin equivalents)	Total flavonoids (% w/w)
B. persica (Seed Extract)	Ethanolic Extract (BPEE)	1.254	8.234
	Aqueous Extract (BPAE)	0.078	1.375

Antioxidant activity

Free radicals are highly reactive molecules with an unpaired electron and are produced by radiation or as by-products of metabolic processes. They initiate chain reactions which lead to disintegration of cell membranes and cell compounds, including lipids, proteins and nucleic acids. Antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus reduce the level of oxidative stress and slow/prevent the development of complications associated with oxidative stress-related diseases.

DPPH method

The ethanolic (90%) and aqueous extracts of the B. persica seeds showed promising free radical scavenging effect of DPPH in a concentration dependent manner as shown in **Fig 3**. The ethanolic (90%) extracts showed more scavenging activity than the aqueous extract. Rutin was used as the reference standards are shown in **Fig 4**. The results were expressed as the dose required to cause 50% inhibition for extract (IC_{50}) and the results are depicted in **Table 3**. The present investigation has shown that the ethanolic (90%) and aqueous extracts of seeds of B. persica exhibited DPPH scavenging activity, the most effective being ethanolic (90%) extract which exhibited significantly higher DPPH scavenging activity (IC_{50} = 32.05±0.247µg/ml) followed by aqueous extract (IC_{50} = 45.35±3.625µg/ml) when compared with the IC_{50} value of the standard rutin (3.78±0.153 µg/ml).

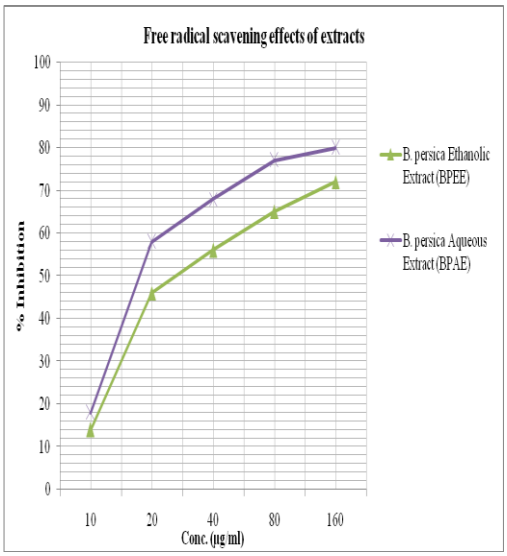

Fig 3: Effect of the plant extracts by DPPH

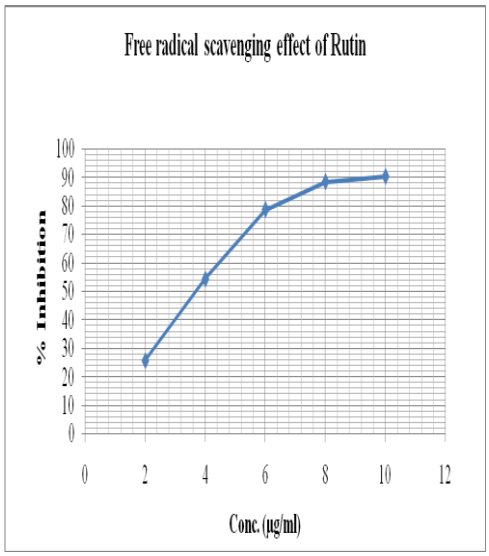

Fig 4: Effect of rutin by DPPH

Table 3: 50% inhibition for extract (IC_{50}) of B. persica by DPPH method

Test Sample	Extracts	IC_{50} (µg/ml) at 517nm
Blepharis persica (Seeds Extract)	Ethanolic Extract (BPEE)	32.05 ± 0.247
	Aqueous Extract (BPAE)	45.35 ± 3.625
Rutin	Reference Standard	3.78±0.153

Values are means ± SD of three determinations. IC_{50} values were determined by interplotations.

FRPA method

The reducing power of ethanolic (90%) and aqueous extracts of the B. persica seeds are shown in Fig 5. Ascorbic acid was used as the reference standards. The reducing power of ethanolic (90%) and aqueous extracts were found to increase in concentration dependent

manner up to a concentration of 400 µg/ml the values were remained lower compared to the ascorbic acid and the results are depicted in Table 4. The ethanolic (90%) extract showed more reducing power than the aqueous extract. The reducing capacity of a compound may serve as a like the antioxidant activity, the reducing power of the ethanolic (90%) and aqueous extracts increased with increasing the concentration. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample. The reducing power showed by the extract is statistically significant ($P < 0.05$). The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and free radical scavenging. (Sharma and Gupta, 2008).

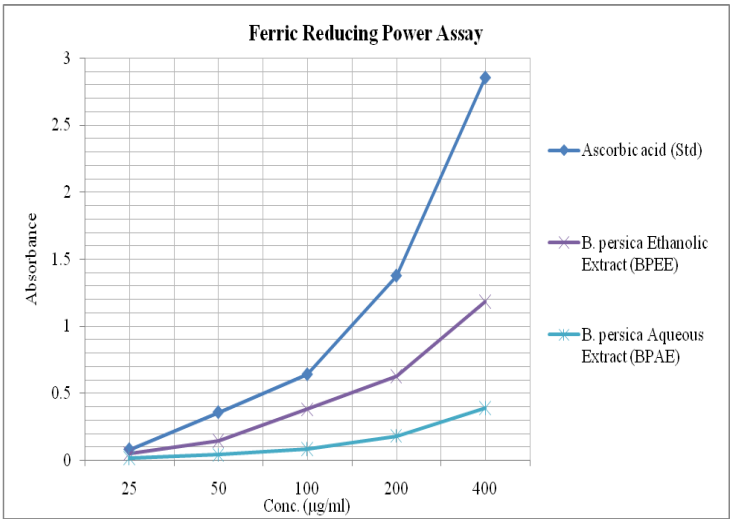

Fig 5: Total reducing power of the plant extracts in comparison of ascorbic acid as standard.

Table 4: Total reducing power of ethanolic (90%) and aqueous extracts

Conc. (µg/ml) (700nm)	B. persica (Seeds)		Ascorbic Acid
	Ethanolic Extract (BPEE)	Aqueous Extract (BPAE)	
25	0.046	0.016	0.078
50	0.144	0.042	0.355
100	0.377	0.085	0.638
200	0.623	0.182	1.374
400	1.182	0.391	2.852

Values are means ± SD of three determinations. IC_{50} values were determined by interplotations.

Lipid peroxidation (TBARS)

The results of the TBARS assay for ethanolic (90%) and aqueous extracts are given in **Fig 6**. Both the samples showed anti lipid peroxidation activities, which are lower than that of butyl hydroxyl toluene (BHT). The percentage antioxidant activity of ethanolic (90%) extract, aqueous extract and BHT increased with increasing concentration as shown in **Fig 6**. These results were also expressed as the dose required to obtain 50% antioxidant index and the results are depicted in **Table 5**.

Table 5: Lipid peroxidation inhibition (TBARS) of extract and its fraction

Test Sample	Extracts	IC_{50} (µg/ml) at 517nm
B. persica (Seed Extract)	Ethanolic Extract (BPEE)	93.00±0.124
	Aqueous Extract (BPAE)	125.34±1.265
Butyl hydroxyl Toluene	Reference Standard	48.89±0.011

Values are means ± SD of three determinations. IC_{50} values were determined by interplotations.

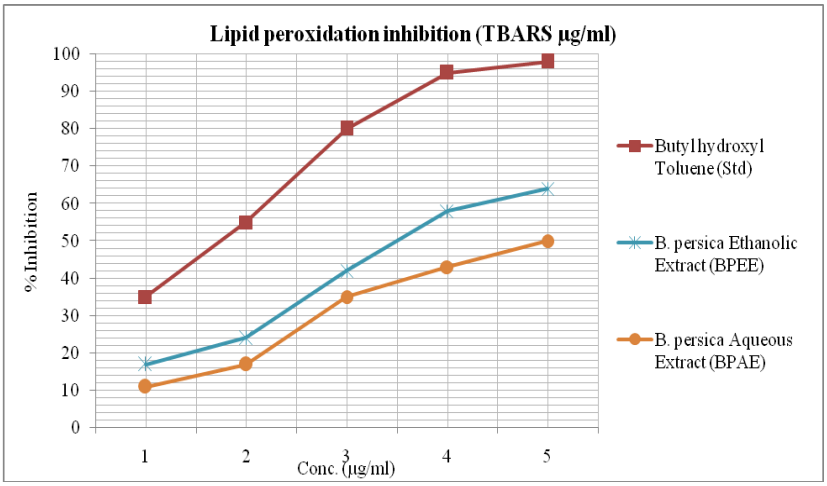

Fig 6: Antioxidant potential of the plant extracts in comparison of BHT as standard.

The ethanolic (90%) and aqueous extracts were tested to be effective in reducing the production of TBARS in a dose-dependent manner, thus allowing calculation of the concentration that would inhibit the TBARS production by 50% (i.e. the IC_{50}). Thus the decrease in the MDA levels in the presence of increased concentration of ethanolic (90%) and aqueous extracts indicates their role as antioxidants. TBARS assay was used to determine the anti lipid peroxidation properties of the ethanolic (90%) and aqueous extracts. Thus ethanolic (90%) and aqueous extracts inhibit the initiation of lipid peroxidation by scavenging the free radicals that form alkylperoxyl and alkoxyl radicals or can donate hydrogen atom to alkylperoxyl and alkoxyl radicals and thus stop chain propagation.

CONCLUSION

The ethanolic (90%) and aqueous extracts of B. persica seeds exhibited different levels of antioxidant activity in all the models studied. The results from various free radical scavenging systems revealed that the B. persica had significant antioxidant activity and free radical scavenging activity, with effective scavenging activity against free radicals such as DPPH and FRPA. In addition, B. persica inhibited peroxides in rat liver homogenate. These results clearly revealed that ethanolic (90%) extract of seeds of B. persica might act as a potential antioxidant for biological systems susceptible to free radical mediated reactions and therefore it might act as hepatoprotective, reduce the risk of aging related diseases and/or promote general human health. Based on these data, free radical scavenging property may be one of the mechanisms by which this drug is useful as a traditional medicine. However, additional studies are needed to characterize the bioactive compounds responsible for the observed in-vitro antioxidant in B. persica and different antioxidant mechanisms.

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