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In vitro Antioxidant and free radical scavenging potential of *Parkinsonia aculeata* Linn.

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ABSTRACT

Parkinsonia aculeata is found most exclusively in the drier regions of India. Leaves from this plant contain orientin, iso-orientin, vitexin, iso-vitexin, lucenin-II, vicenin-II, diosmetin 6-C-B-glucoside, apigenin, luteolin, kaempferol, chrysoeriol, epi-orientin, parkinsonin-A, parkinsonin-B, and parkintin. In folk medicine, the plant leaves are used to treat jaundice. Due to the presence of flavonoids and its widespread use to treat jaundice, our aim with this study was to evaluate the antioxidant properties of *P. aculeata*. The antioxidant activity of the 70% hydroalcoholic extract of *Parkinsonia aculeata* was evaluated *in vitro* by various experimental parameters such as DPPH radical scavenging activity, nitric oxide scavenging, ß-carotene-linoleic acid module system, hydroxyl radical scavenging activity, and lipid peroxidation. Also, ferric ion reduction capability of the evaluated extracts, total antioxidant capacity, and total phenolic content, were also determined. Our results showed that *Parkinsonia aculeate* displayed potent antioxidant properties, supporting the ethnomedical use given to this plant to treat jaundice.

KEY WORDS: Antioxidant; DPPH; Free radical scavenging; Hydroxyl radical scavenging; *Parkinsonia aculeata*.

INTRODUCTION

The role of free radicals and reactive oxygen species (ROS) in the pathogenesis of human diseases such as cancer, aging, inflammatory response syndrome, respiratory diseases, liver diseases, and atherosclerosis, has been widely recognized (1). Electron acceptors, such as molecular oxygen, readily reacts with free radicals to become free radicals themselves, also referred as ROS, with chemical species such as superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl free radicals ($^{\bullet}OH$), which is known to induce damage to biomembranes, proteins, and DNA (2). Prominent manifestation of free radical activity in biological systems is lipid peroxidation and it is involved in the development of different diseases. The primary targets of free radical attack on lipids are polyunsaturated fatty acids (PUFA). Lipid peroxidation usually proceeds as a chain reaction: alkyl radicals are formed during the initiation step by the attack of lipids by free radicals or other reactive species, followed in the propagation phase by the formation of alkylperoxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals, and terminating with the formation of lipid hydroperoxides (ROOH) (3). However, these products readily decompose to other relatively more stable substances such as aldehydes (malondialdehyde, hydroxynonenal, dienals, etc.) or isoprostanes, which have been used to assess lipid peroxidation *in vivo*. Among the numerous analytical approaches for the estimation of oxygen radical mediated damage in biological systems determination of malondialdehyde (MDA), as one of the major aldehyde species, has been employed most frequently. The main method utilized is the reaction of MDA with thiobarbituric acid (TBA). The reaction of TBA with MDA and linked chromogens to lipoperoxides in biomaterials, resulted in the well known method, "Thiobarbituric Acid Reactive Substances" (TBARS). Malondialdehyde is a three-carbon molecule that constitutes one of the major secondary decomposition products of peroxidised PUFA. MDA is inferred to be cytotoxic and it has been found at elevated levels in various diseases thought to be related to free radical damage (3-5). Therefore, research has focused on the use of antioxidants, with particular emphasis on naturally-derived antioxidants, which may inhibit ROS production and may display protective effects. Plant phenolics, in particular phenolic acids (6-8), tannins (9, 10) and flavonoids (11) are known to be potent

antioxidants and occur in vegetables, fruits, nuts, seeds, roots and barks (2). In addition to their antioxidant properties, these compounds display a vast variety of pharamacological activities such as anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, anti-bacterial or antiviral activities (12-15), which may explain, at least in part, its use as alternative or supportive treatments in various degenerative diseases.

Parkinsonia aculeata (Leguminosae) is a small tree found almost throughout the drier parts of India. Previous investigations showed that the leaves from the plant contains orientin, iso-orientin, vitexin, iso-vitexin, lucenin-II, vicenin-II, diosmetin 6-C-B-glucoside, apigenin, luteolin, kaempferol, chrysoeriol, epiorientin, parkinsonin-A, parkinsonin-B, and parkintin (16-19). It was also reported that the leaves are considered as diaphoretic, abortive (20, 21), and anti-microbial (22). The leaves are given with fresh cow milk to treat jaundice. Given the fact of the presence of flavonoids and its widespread use to treat jaundice, our aim in this work was to evaluate its antioxidant properties in several *in vitro* models.

MATERIALS AND METHODS

Plant material and extraction

The leaves of the plant were collected during September 2004 in Chitradurga district of Karnataka state, India and authenticated by Dr. Shivashankar Prasad, Prof. & Chairman, Dept. of Botany, University of Mysore, Mysore. The voucher specimen (KMPA01) of the plant is preserved in Department of Pharmacognosy. Leaves dried under shade, powdered and extracted with 70% aqueous ethanol by cold maceration. The extraction was done for 72 hours. After extraction the extract was separated from marc by filtration through filter paper. The marc was pressed in muslin cloth to remove the solvent which is left in the marc after filtration. Filtrate was preserved in a well closed container. Marc left after extraction was extracted by cold maceration for 3 more days with same amount of fresh solvent and the process was repeated for one more time. i.e. the drug was extracted 3 times with a gap of 3 days each. On the 10^{th} day the filtrates were pooled and concentrated to a syrupy liquid under reduced pressure using Superfit Rotary vacuum evaporator, dried and stored in a dessicator. Same is used for below mentioned experiments.

Chemicals

α,α-diphenyl-ß-picryl hydrazyl (DPPH), egg phospatidylcholine, ß-carotene, and γ-linoleic acid were obtained from Sigma Chemical Co. (St.Louis, MO, USA). BHT (butylated hydroxy toluene), ascorbic acid, Tween-40, deoxy-*D*-ribose, trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were obtained from Hi-Media Labs (Mumbai, India). 1,10-*o*-phenanthroline, ferric chloride ($FeCl_3$), ammonium molybdate, and sodium dithionite were obtained from Ranbaxy Fine Chemicals (New Delhi, India). Phenylhydrazine and Folin-Ciocalteau's phenol reagent were obtained from BDH Products (UK). Silymarin was a kind gift from Dr. Chidambaramurthy K.N., CFTRI, Mysore, India. All other chemicals used were of analytical grade. The solvents used for extraction were from Ranbaxy Fine Chemicals (New Delhi, India). The UV-Visible spectrophotometric values were recorded in JASCO UV-500 Spectrophotometer.

Preliminary phytochemical investigations

The preliminary phytochemical screening of the extract was carried out to know the different constituents present in it as per the standard procedures. The extracts were tested for alkaloids (23, 24), sterols (25), triterpenes (26), saponins (27), flavonoids (28, 29), tannins (24, 29), carbohydrates (30), cardiac glycosides, lactones and amino acids (24, 31).

DPPH radical scavenging activity

Free radical scavenging potentials of the extracts were tested against a methanolic solution of α,α-diphenyl-β-picryl hydrazyl (DPPH) (32). 9.09 µg/mL to 181.82 µg/mL of extract, 4.54 µg/mL to 27.27 µg/mL of BHT and ascorbic acid as standards in 500 µL ethanol were taken and added with 5 mL of 100 µM DPPH in methanol. The mixture was allowed to stand at room temperature for 20 minutes. The control was prepared as above without extract. The readings were read at 517 nm using methanol as blank. The absorbance of control was first noted at 517 nm. The change in absorbance of the samples was measured. Scavenging activity is expressed as the inhibition percentage calculated using the following equation: % Anti radical activity = {(Control Abs.- Sample Abs.)/Control Abs.}x 100. Each experiment was carried out in triplicate and results are expressed as mean % antiradical activity ± SD.

Nitric oxide scavenging activity

Scavenging of NO was determined using sodium nitroprusside (SNP) as NO donor. SNP (10mM) in phosphate buffered saline was mixed with different concentrations of extract (100 µg/mL to 1000 µg/mL) in ethanol and incubated at 25^0C for 180 min. Ascorbic acid, instead of extract was used as positive control in

the concentration of 25 µg/mL to 125 µg/mL. Further an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphylethylenediamine dihydrochloride and 3% phosphoric acid) was added to the incubated solution. The absorbance was immediately measured at 546 nm (33). Nitric oxide scavenging activity was calculated with the following equation: NO scavenging activity = {(Abs of Control-Abs of sample)/Abs of Control}x100. All experiments were performed in triplicate. The results are expressed as mean % NO scavenging activity ± SD.

Antioxidant assay using β - Carotene Linoleate Model System (B CLAMS)

The antioxidant activity of the 70% hydroalcoholic extract was evaluated by the method of Hidalgo et al (34) with slight modifications. In brief, 5 mg β-Carotene, 40 mg γ-linoleic acid and 400 mg of Tween-40 were mixed in 1 ml chloroform. Chloroform was removed under vacuum using the flash rotary evaporator at 40^0C. The resulting mixture was added with 20 mL water and an emulsion was prepared. The emulsion was further diluted with 80 mL of oxygenated water (Oxygen was passed into the 1L of double distilled water for 5 min at the flow rate of 10 mL/min.). 100, 200, 300, 400 and 600 µg of BHT and 100, 200, 400, 600 and 800 µg of PA in alcohol were added in separate test tubes and volume was made up to 0.4 mL with ethanol. 0.6 mL of water and 3 mL of emulsion was added to each test tube. Absorbance of all samples were taken at 470 nm at Zero time and test tubes were placed at 50^0C in water bath. Measurement of Absorbance was continued at an interval of 30 minutes, till the color of β-Carotene disappeared in the control reaction (t = 180 min). A mixture prepared as above without β-Carotene emulsion served as blank and mixture without extract served as control. BHT, instead of extract was used as positive control in the concentration of 25 µg/mL to 150 µg/mL. Dose response of antioxidant activity of PA was determined at different concentrations. The antioxidant activity (%AA) was evaluated as the bleaching of β-Carotene with the following equation: % AA = $100\{1- (A^o - A^t)/A^o0 - A^t0)\}$. Where % AA = Antioxidant activity, A^o = absorbance of sample at Zero time, A^00 = Zero time absorbance of control, A^t = Absorbance of sample after incubation for 180 min, A^t0 = Absorbance of control after incubation for 180 min. All experiments were performed in triplicate. The results are expressed as mean ± SD.

Hydroxyl radical scavenging activity

The reaction volume contains different concentrations of PA (from 5 µg to 80 µg) or Gallic acid, which was employed as positive control (0.5 µg to 4 µg), 2.8 mM deoxy-D- ribose, and 0.2 mM phenylhydrazine (35). The mixture was incubated for 2 hours at 37^0C in a water bath. Hydroxyl radical scavenging was measured by TBARS method of Ohkawa)36). To each test tube, 1 mL of TCA (2.8%, %w/v), containing 1% TBA, was added. Further on, the test tubes were heated in a water bath for 30 min. and cooled at room temperature. A mixture prepared as above without deoxy-D- ribose served as blank. Absorbance was read at 532 nm. Hydroxyl radical scavenging activity was calculated with the following equation: percentage hydroxyl radical scavenging activity = {(C- S)/C} X 100, Where C is the absorbance of the control and S is the absorbance of the sample. All experiments were performed in triplicate and the results are expressed as mean ± SD.

Lipid peroxidation assay

Egg phospatidylcholine (20 mg) in chloroform (2 mL) was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5 mL) with a vortex mixer. The mixture was sonicated to get a homogeneous suspension of liposomes. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid into a mixture containing liposome (0.1 mL), (37) 150 mM potassium chloride, 0.2 mM ferric chloride, PA (20 to 80 µg/mL), or silymarin (1 to 10 µg) in a total volume of 1 mL. The reaction mixture as incubated at 37°C for 40 min. After incubation, the reaction was stopped by adding 1 mL of ice-cold 0.25 M sodium hydroxide containing 20 % TCA (w/v), 0.4 % TBA (w/v), and 0.05 % BHT (w/v). After incubating in a boiling water bath for 20 min, the samples were cooled to room temperature. The blank was prepared in the same manner but without liposomes. The pink chromogen was extracted with 1 mL of n-butanol. The absorbance was read at 532 nm. Anti-lipid peroxidation activity was calculated with the following equation: % Anti lipid peroxidation activity = {(C- S)/C} X 100, Where C is the absorbance of the control and S is the absorbance of the sample. All experiments were carried out in triplicate, and results are expressed as mean ±SD.

Reduction of Ferric Ions

The reaction mixture containing *o*-phenanthroline (0.5 mg), 0.2 mM ferric chloride, PA (100 to 1000 µg) or ascorbic acid (10 to 50 µg), previously dissolved in ethanol was made up to a final volume of 5 mL. Then,

it was incubated at room temperature for 15 – 20 min. In another set, 0.3 mM sodium dithionite was added instead of the extract. In all cases, the absorbance was read at 510 nm. In the case of sodium dithionite, the absorbance was considered as 100% reduction of all ferric ions present (38, 39). All experiments were carried out in triplicate, and results are expressed as mean ± SD.

Total antioxidant capacity

Total antioxidant capacity was measured according to the method previously reported by Prieto (40), with slight modifications. In brief, 100 µg of extract and 100 µg of BHT and Silymarin (as standards) were taken in 0.1 mL of alcohol, combined separately in an eppendroff tube with 1.9 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1.9 mL of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. For samples of unknown composition, water-soluble antioxidant capacities are expressed as equivalents of ascorbic acid. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid. The experiment was conducted in triplicate and values are expressed as ascorbic acid equivalents in µg per mg of extract (mean ± SD).

Total Phenolic Content

PA was diluted with the alcohol 95% to a suitable concentration for analysis. TPC of PA was assessed approximately by using the Folin-Ciocalteau Phenol reagent method (41). To 100 µL of the extract (200 µg) was added 0.5 mL of Folin–Ciocalteau reagent and 1mL of sodium carbonate (4% w/v) and the volume made upto to 2 mL, contents were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured in a UV–Vis spectrophotometer. The total phenolic content was expressed as Gallic acid equivalents (GAE) in micrograms per mg of sample, using a standard curve generated with Gallic acid. The experiment was conducted in triplicate and values are expressed in mean ± SD.

RESULTS

Preliminary phytochemical investigation - Preliminary phytochemical screening of PA showed the presence of sterols, flavonoids, carbohydrates and glycosides.

DPPH radical scavenging activity

As shown in Table 1, PA strongly scavenged, in a dose-dependent manner, the DPPH radical with an IC_{50} value of 105.90 ± 1.36 µg/mL. IC_{50} values of ascorbic acid and BHT were found to be 4.91 ± 0.36 µg/mL and 21.88 ± 2.12 µg/mL, respectively.

Nitric oxide scavenging activity

PA moderately inhibited NO in a dose-dependent fashion (Table 2). IC_{50} value was determined as 617.0 ± 12.5 µg/mL, which is greater than the obtained with ascorbic acid (83.80 ± 3.75 µg/mL).

Antioxidant assay using β – Carotene Linoleate Model System

The antioxidant activity of PA as measured by the bleaching of ß-Carotene is presented in Table 3. IC_{50} value of PA and BHT were found to be 197.**7**±4.04 µg/mL and 68.51±2.5 µg/mL respectively.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using phenylhydrazine. Phenylhydrazine in solution has been shown to produce hydroxyl radicals . Hydroxyl radical scavenging was measured by studying the competition between deoxy-d-ribose and sample extracts for hydroxyl radicals produced by Phenyl hydrazine. The extent of deoxy-d-ribose degradation is measured as TBARS by method of Ohkawa et al . PA scavenged the hydroxyl radicals strongly with an IC_{50} value of 62.67±2.08 µg/mL. IC_{50} value of GA is 3.3±0.2 µg/mL (Table 4).

Lipid peroxidation assay

PA prevented lipid peroxidation strongly but less than the standard (Silymarin). IC_{50} values of antilipid peroxidation of PA and Silymarin were 49.33±1.53 µg/mL and 6.0±0.2 µg/mL respectively. The effect of PA to prevent lipid peroxidation is shown in Table.5. In biological systems, malondialdehye (MDA) is very reactive species and takes part in the cross linking of DNA, protein and also damaging the liver cells.

Reduction of Ferric Ions

Fe^{+2} reacts rapidly with 1, 10-*O*-phenanthroline and forms red colored complex which is exceptionally stable. This complex has a strong absorption in the visible spectrum at a wavelength of 510 nm. Extracts reacts with Fe^{3+} to reduce and convert it to Fe^{2+}. The degree of coloration indicates the reduction potential of the extracts. The change in the absorbance produced at 510 nm has been used as a measure of Ferric ions reducing activity. As illustrated in the Table

Table 1. *Free radical scavenging activity of PA, BHT and Ascorbic acid in DPPH method*

Conc. in µg/mL	% Free radical scavenging of PA	Conc. in µg/mL	% Free radical scavenging of BHT	% Free radical scavenging of Ascorbic acid
09.09	7.69±1.48 [a]	4.54	32.05±2.62[a]	49.19±1.52[a]
18.18	9.47±1.02	9.09	36.96±1.56	76.30±2.65
36.36	18.22±0.57	13.63	40.06±0.95	80.01±1.98
54.54	25.37±1.52	18.18	47.03±0.89	86.03±1.21
72.73	40.37±1.67	22.73	51.19±3.21	93.90±2.36
109.09	51.13±7.62	27.27	59.98±1.65	99.38±1.87
145.45	66.84±0.96			
181.82	89.43±0.39			
IC_{50}	105.90±1.36 [a] µg/mL	IC_{50}	21.88±2.12[a] µg/mL	4.91±0.36[a] µg/mL

Conc. – Concentration
[a] – Values are mean± Standard Deviation.

Table 2. *Nitric Oxide scavenging activity of PA and Ascorbic acid*

PA		Ascorbic acid	
Concentration in µg/mL	% NO scavenging activity	Concentration in µg/mL	% NO scavenging activity
100	17.09±2.78[a]	25	33.53±2.17[a]
200	26.65±4.55	50	42.56±2.58
300	34.21±3.89	75	46.23±2.10
400	41.97±3.66	100	59.76±4.16
600	48.79±3.42	125	69.56±0.53
800	53.61±2.37		
1000	64.17±0.98		
IC_{50}	617.0±12.5[a] µg/mL		83.80±3.75[a] µg/mL

[a] –Values are mean± Standard Deviation

Table 3. *Antioxidant property of PA and BHT in β – CLAMS method*

Concentration in µg/mL	% antioxidant activity of PA	% antioxidant activity of BHT
25	4.95±1.98[a]	20.71±3.34[a]
50	10.23±1.14	36.17±2.39
75	--	54.73±0.39
100	22.77±1.98	61.58±1.43
150	38.61±1.98	71.47±0.33
200	51.15±3.03	**---**
IC_{50}	197.7±4.04[a] µg/mL	68.51±2.5[a] µg/mL

[a] –Values are mean± Standard Deviation

Table 4 : Hydroxyl radical scavenging activity of PA and Gallic acid (GA)

Hydroxyl radical Scavenging activity of PA		Hydroxyl radical Scavenging activity of GA	
Concentration in µg/mL	% OH· radical scavenging	Concentration in µg/mL	% OH· radical scavenging
5	6.87±1.18[a]	0.5	20.91±6.51[a]
10	11.69±1.45	1	25.81±5.65
20	31.16±2.32	2	36.54±3.97
50	40.65±1.72	3	46.41±2.81
80	60.80±2.24	4	58.39±2.62
IC_{50}	62.67 ± 2.08 [a] µg/mL	IC_{50}	3.3±0.2 [a] µg/mL

[a] –Values are mean± Standard Deviation
OH· – Hydroxyl radical

Table 5. *Anti Lipid Peroxidation activity of PA and Silymarin*

Concentration in µg/mL	% Anti Lipid Peroxidation of PA	Concentration in µg/mL	% Anti Lipid Peroxidation of Silymarin
20	3.85±0.31[a]	1.0	3.13±3.65[a]
40	34.46±0.99	2.0	29.66±1.00
60	69.21±2.08	5.0	43.34±0.48
80	85.77±1.25	7.5	59.44±1.85
---	---	10.0	89.84±1.48
IC_{50}	49.33±1.53[a]	IC_{50}	6.0±0.2[a]

[a] *–Values are mean± Standard Deviation*

Table 6. *Ferric ion reduction activity of PA and Ascorbic acid*

PA		Ascorbic acid	
Conc. in µgs	% Ferric ion reduction	Conc. in µgs	% Ferric ion reduction
100	23.63±1.78	10	18.87±0.64 [a]
200	25.69±0.95	20	26.63±2.06
300	29.28±0.79	30	38.72±5.62
400	34.54±0.941	40	48.60±5.09
600	38.52±0.86	50	60.95±1.43
800	56.88±0.15	IC_{50}	39.15±3.65 [a] µgs
IC_{50}	726.67±11.5[a] µgs		

[a] *–Values are mean± Standard Deviation*

Table 7. *Total antioxidant capacity and Total Phenolic content of PA, Silymarin and BHT*

Extract/ Standard	Total antioxidant capacity[b]	Total Phenolic content[c]
PA	255.56+25.46 [a]	28.86±4.27 [a]
Silymarin	197.22±4.81	42.49±3.84
BHT	400.00±22.05	----

[a] *–Values are mean± Standard Deviation*
[b] *– Values are in µgs equivalent to Ascorbic acid per mg of extract.*
[c] *- Values are in µgs equivalent to Gallic acid per mg of extract.*

6, PA has shown IC_{50} value of 726.67±11.55 µg. IC_{50} of ascorbic acid is 39.15±3.65 µg.

Total antioxidant capacity

As shown in Table 7, Total antioxidant capacity of PA, Silymarin and BHT were 255.56+25.46, 197.22±4.81 and 400.00±22.05 µg equivalent to ascorbic acid/mg of PA, Silymarin and BHT respectively.

Total Phenolic Content

Total phenolic content (TPC) of PA and Silymarin were 28.86±4.27 µg and 42.49±3.84 µg equivalent to Gallic acid/mg of PA and Silymarin respectively. Total antioxidant capacity of PA is more than the Silymarin, where as TPC is less than the Silymarin. Total antioxidant capacity of PA and Silymarin are less than the BHT.

DISCUSSION

DPPH is stable nitrogen centered free radical that can accept an electron or hydrogen radical to become a stable dia-magnetic molecule. DPPH radicals react with suitable reducing agents then losing colour stiochiometrically with the number of electrons consumed which is measured spectrometrically at 517 nm. Ascorbic acid is a potent free radical scavenger and BHT is known antioxidant and is used as preservative (32, 41). So when compared to such pure components, IC_{50} value of 123.09±3.01µg/mL of PA is quiet high, and shows that PA is potent DPPH free radical scavenger. In very recent years, potent free radical scavengers have attracted a tremendous interest as possible therapeutics against free radical mediated diseases. Free radicals are constantly generated *in vivo* both by accidents of chemistry and for specific metabolic purposes. When an imbalance between free radical generation and body defense mechanisms occurs, oxidative damage will spread over all the cell targets (DNA, lipids, and proteins). It has been reported that a series of human illness such as cancer, atherosclerosis, cardio and cerebrovascular diseases, diabetes, immune system impairment, neurodegenerative diseases such as Parkinson's and Alzheimer's diseases and arthritis, as well as premature body aging, can be linked to the damaging

action of extremely reactive free radicals. Many phenolics, such as flavonoids, flavonols and flavones have potent antioxidant capacities (42). *P.aculeata* contains orientin, iso-orientin, vitexin, iso-vitexin, lucenin-II, vicenin-II, diosmetin 6-C-B-glucoside, apigenin, luteolin, 7-glycosyl kaempferol, chrysoeriol, epi-orientin, Porkinsonin-A, Porkinsonin-B and Parkintin (16-19). Kaempferol, luteolin, apigenin and vitexin reported to be potent antioxidants. Potent free radical scavenging capacity of PA can be attributed to different flavonoids present in the extract. So plant may be useful in the management of free radical mediated diseases.

Nitric oxide is produced by several different types of cells, including endothelial cells and macrophages. Although the early release of nitric oxide through the activity of constitutive nitric-oxide synthase is important in maintaining the dilation of blood vessels the much higher concentrations of nitric oxide produced by inducible nitric-oxide synthase (iNOS) in macrophages can result in oxidative damage. In these circumstances, activated macrophages greatly increase their simultaneous production of both nitric oxide and superoxide anions. Nitric oxide reacts with free radicals, thereby producing the highly damaging peroxynitrite. Nitric oxide injury takes place for the most part through the peroxynitrite route because peroxynitrite can directly oxidize LDLs, resulting in irreversible damage to the cell membrane. When flavonoids are used as antioxidants, free radicals are scavenged and therefore can no longer react with nitric oxide, resulting in less damage. Nitric oxide can be viewed as a radical itself and it is reported that nitric oxide molecules are directly scavenged by flavonoids (43). PA scavenged NO moderately and thus can be a potent anti-inflammatory drug. Further flavones reported to be present in the plant apigenin, luteolin and kaempferol found to cause down-regulation of iNOS expression and COX-2 expression in various cells. Apigenin and luteolin found to inhibit pro-inflammatory cytokine production in various cells. Inhibition of enzyme activities of such as cyclooxygenase (COX) and the nitric oxide (NO) producing enzyme, nitric oxide synthase (NOS) reduces the production of arachidonic acid (AA), prostaglandins (PG), leukotrienes (LT) and NO, crucial mediators of inflammation (13, 44). Thus, PA can be a potent antiiflammatory agent.

The mechanism of bleaching ß-Carotene is a free radical mediated phenomenon resulting from hydroperoxides formed from linoleic acid. ß-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radicals, upon the abstraction of hydrogen from one of its diallylic methylene group, attacks the highly unsaturated ß-Carotene molecules. As ß-Carotene, molecule looses their double bonds by oxidation, the compound loose its chromophore and characteristic orange color, which can be monitored spectrophotometrically at 470 nm. So in presence of antioxidants, ß-Carotene retains its color. Because antioxidants prevent abstraction of hydrogen from linoleic acid from its diallylic methylene group by donating hydrogen from itself. Thus prevents the oxidation of ß-Carotene (32). Here the IC_{50} values of BHT which is used as standard and PA are having near values. This shows that PA can donate hydrogen to linoleic acid free radicals as much as BHT can. PA can be used as natural antioxidant instead of BHT which is synthetic and use of BHT is said to unsafe and their toxicity is a problem of concern (45, 46).

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cell. This radical has the capacity to join nucleotide in DNA and cause strand breakage which contributes to carcinogenesis, mutagenesis, and cytotoxicity. In addition, this species is considered to be one of the quick initiator of lipid peroxidation process, abstracting hydrogen atom form the unsaturated fatty acids (32, 47, 48). PA has found to scavenge OH radicals but not as strongly as Gallic acid. Because GA is very potent OH radical scavenger (49). Thus PA may be useful as an antioxidant and may prevent damages that arise from OH radicals by scavenging them. This OH radical capacity of PA may be due flavonoids as found present in PA by qualitative chemical tests. It is found that Flavonoids are potent OH radical scavenging agents (42, 50).

The lipid peroxidation has been broadly defined as the oxidation deterioration of polyunsaturated lipids. Lipid peroxidation usually proceeds as a chain reaction: alkyl radicals ($R^{\cdot}$**)** are formed during the initiation step by the attack of lipids by free radicals or other reactive species, followed in the propagation phase by the formation of alkylperoxyl ($ROO^{\cdot}$) and alkoxyl radicals ($RO^{\cdot}$), terminating with the formation of lipid hydroperoxides (ROOH). A probable alternative fat of peroxy radicals is to form cyclic peroxidase; these cyclic peroxidase, lipid peroxidase and cyclic endoperoxidase fragment to aldehyde including MDA

and polymerization product. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process (3, 51). In biological systems, malondialdehye (MDA) is very reactive species and takes part in the cross linking of DNA, protein and also damaging the liver cells. Determination of lipid peroxidase content was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic condition (1). PA has strongly inhibited the lipid peroxidation as shown in the Table 5. IC_{50} value of PA is. 68.13±1.38 µg/mL and that of Silymarin is 6.0±0.2 µg/mL. This indicates that Silymarin is more than ten times potent than PA. Silymarin is a mixture of flavonolignan and is proven potent hepatoprotective and antioxidant agent (52). So when compared to such potent agent, the values obtained indicate that PA is good antilipid peroxidation agent. As shown in Table 1 and 2, PA has found to be good free radical scavenging agent and also found to contain diverse and potent antioxidant flavonoids (16-19). So PA can 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.

Both PA and Ascorbic acid have reduced the Fe^{+3} to Fe^{+2} moderately. The reducing properties are generally associated with the presence of reductones. Antioxidant action of the reductones is based on the breaking of free radicals chain by the donation of a hydrogen atom. The reductones also react with certain precursors of peroxide, thus preventing formation of peroxide (53).

As phenolics are responsible for antioxidant activity, generally, it is expected that extract/drug which contains high TPC would show highest total antioxidant capacity. But in this case though TPC of Silymarin is more than the PA and total antioxidant capacity is less than the PA. This may be due to nature of constituents of PA and Silymarin. Silymarin was used as a standard because it is a mixture of flavonolignans and is potent well known hepatoprotective and antioxidant agent. It contains only mixture of flavonolignans (52, 53). PA contains constituents like carbohydrates, sterols and glycosides apart from different flavonoids, and TPC is only due to different flavonoids. This may be the reason for high TPC of Silymarin and less TPC of PA. But the total antioxidant activity is due to all the components present in the extract. As stated earlier *P.aculeata* contains orientin, iso-orientin, vitexin, iso-vitexin, lucenin-II, vicenin-II, diosmetin 6-C-B-glucoside, apigenin, luteolin, 7-glycosyl kaempferol, chrysoeriol, epi-orientin, Purkinsonin-A, Purkinsonin-B and Parkintin. Kaempferol, luteolin, apegenin -7-glycoside and vitexin reported to be good antioxidants with radical scavenging activity of 93.5%, 87.6%, 34.8% and 21% respectively (42). Structures of epi-orientin, Parkinsonin-A, Parkinsonin-B orientin, isoorientin are closely related and these are derivatives of luteolin (17-19) . In few experiments, silybin one of the constituent of silymarin is found to be less active than the luteolin. Because of these reasons, though the TPC of PA is less than the Silymarin, PA showed more antioxidant capacity than Silymarin.

CONCLUSION

The data presented here indicate that the marked antioxidant activity of PA seems to be due to presence of flavonoids like flavones, flavanes, flavonols, which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction. The qualitative chemical tests also revealed the presence the flavonoids. Further total phenolic content of extract confirmed that PA contains good amount of phenolics. There is good scope in examining the leaves for its antioxidant and free radical scavenging activity in *in vivo* models and for hepatoprotective activity and thus establish the evidence for using this plant in treatment of jaundice in folk medicine. Free radicals and reactive oxygen species are involved in a variety of pathological events such as aging, inflammation, cancer, atherosclerosis, diabetes. The plant would be useful for the treatment of various diseases mediated by free radicals. The PA found to suppress lipid peroxidation, hydroxyl radical formation, through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination. Overall, the plant would be useful as an antioxidant and free radical scavenging agent and thus help in treatment of many diseases mediated by Reactive oxygen species.

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