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Studies on in vitro antioxidant potential of pod and seed parts of *Bauhinia malabarica* Roxb.

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ABSTARCT

Aim: In Indian traditional medicine, importance of Bauhinia species is well evidenced from earlier literature. In the present investigation, the antioxidant and free radical scavenging efficiencies of 70% acetone and 50% methanol extracts of *Bauhinia malabarica* pod and seed were examined.

Materials and Methods: The extracts were screened for different antioxidant assays such as reducing power, $DPPH^{\bullet}$, nitric oxide, hydroxyl radical scavenging, metal chelating, $ABTS^{\bullet+}$ scavenging activity, lipid peroxidation preventive property and anti-haemolytic activities.

Results: It contribute significantly to the total antioxidant activity of the test drug. In β - carotene-linoleic acid system all the extracts, exhibited good antioxidant activity and the values were comparably higher than the synthetic antioxidant, BHT and BHA. Among the samples analyzed acetone extract of *B. malabarica* seed determined significant ($p<0.05$) antioxidant activity in terms of reducing power, $DPPH^{\bullet}$, metal chelating and $ABTS^{\bullet+}$ activity.

Conclusion: On the basis of the results obtained, seeds of *B. malabarica* were found to be a potent source of natural antioxidants due to their marked antioxidant activity. Overall, the acetone was found to be the best solvent for the extraction of antioxidant compounds. The results presented here implies that the consumption of such a legume food would not only improve the nutrient utilization but also provide the potential source of nutraceuticals for human health.

Keywords: *Bauhinia malabarica*, seed, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, radical scavenging activity, lipid peroxidation.

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Introduction:

Constant formation of Reactive Oxygen Species (ROS) and free radicals are witnessed in the human body by normal metabolic actions, and are one of the major implications of various pathogenesis of different human diseases, including cancer, ageing, diabetes and atherosclerosis[1]. Naturally occurring biochemical compounds such as flavonoids, catechins, lignans, phenolic acids, vitamins, terpenoids, and some other endogenous metabolites, are rich in antioxidant activity [2,3]. Therefore intake of these dietary antioxidants can reduce the risk of diseases caused by free radicals[4]. It is considered that the administration of natural antioxidants with multiple components could offer protection and combat oxidative stress-induced physiological malfunctions[5]. Phenolic compounds are one among the most widely distributed plant secondary metabolites and are found in many plants used as food and for their medicinal properties. Recently, the ability of phenolic compounds to serve as antioxidants has been well recognized[6]. Small and high molecular weight phenolics including flavonoids, phenolic acids and tannins to act as antioxidants has been extensively investigated[7]. Hence, for the treatment or prophylaxis of various oxidative stress-related diseases, there is great interest in the use of naturally occurring antioxidants[8].

The genus, *Bauhinia* belongs to the family, *Caesalpiniaceae* has been studied extensively in recent years for its medicinal values. Phytochemical investigations of the genus have revealed the presence of a number of compounds including steroids, glycosides, triterpenes, lactones, and flavonoids[9]. Biological studies have confirmed that these plants exert several medicinal properties, especially antinociceptive, antimicrobial, and antidiabetic[9,10].

Bauhinia malabarica Roxb. is a small or moderate sized deciduous tree. It is distributed throughout India, mainly on the sub- Himalayan tracts, Bengal, Assam and in south India. It is also found in peninsular India and in the western sub-Himalayan forests, deciduous and semi-evergreen forests, areas receiving 1000 to 3000 mm annual rainfall. The leaves of the plant are consumed in India, Indonesia and Thailand, among others. It is used in traditional medicine for wound healing, as a diuretic, to fight dysentery, to treat headache, fever and as an emmenagogue[11]. Leaves are acrid and are considered to be a febrifuge; it is also used as a flavoring agent for meat and fish[12]. The mineral content of the leaves shows that they are an excellent source of calcium and a very good source of iron. Young shoots are also edible and are used to treat worm infestations, leprosy, wounds, menorrhagia, gout, scrofula, wasting diseases, cough, haemorrhage, urinary disorders, glandular swellings and goitre[13]. Seven flavonols, including 6,8-di-*C*-methylkaempferol 3-methyl ether, kaempferol, afzelin, quercetin, isoquercitrin, quercitrin, and hyperoside were isolated from the methanol extract of leaves[11]. An optically active isomer of tartaric acid has been definitely identified and quantitatively analyzed from leaves of *B. malabarica*[14].

Despite the many medicinal properties and being a well known natural source of food, meager information is obtainable regarding the *in vitro* antioxidant activity for this plant. Therefore, the present study was carried out to elucidate the *in vitro* antioxidant potential of pod and seed extracts of *B. malabarica*. The antioxidant activities were measured using ferric reducing antioxidant power, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $NO^{\bullet}$, hydroxyl radical scavenging, iron chelating, β-carotene- linoleate model system and antihemolytic activity.

Materials and Methods

Procurement and preparation of plant materials:

Pod and seeds of *B. malabarica* Roxb. were collected from the conservation management area of Siruvani hills, Coimbatore, Tamil Nadu, India. The authenticity of the selected plant materials were duly identified and confirmed by comparison with reference specimens preserved in the herbarium at Botanical Survey of India, Southern Circle, Coimbatore. The voucher specimens (vide no: BSI/SC/5/23/08- 09/Tech.-1718) were lodged in the departmental herbarium for further reference. The plant materials were cleaned, washed with copious amounts of distilled water, shade dried, chopped into bits, and coarsely powdered in a Willy mill (Nippon Electricals, Chennai, India) to 60-mesh size for extraction.

Preparation of crude plant extracts:

50 g of coarsely powdered plant samples were exhaustively extracted with acetone/ water (70/30, v/v), followed by methanol/water (50/50, v/v) using a round bottom flask with an attached reflux condenser for 3 h at a controlled temperature. The extracts were filtered and concentrated to dryness under reduced pressure using rotary vacuum evaporator (RE300; Yamato, Japan), lyophilized (4KBTXL-75; Vir Tis Benchtop K, New York, USA) to remove traces of water molecules and the lyophilized powders were stored at -20°C until used directly for the assessment of various *in vitro* antioxidant activities.

Determination of *in vitro* antioxidant activity:

Reducing power:

The Fe^{3+} reducing power of the extract was determined according to the method suggested by Oyaizu (1986)[15]. Various concentrations of the extracts (dissolved in the appropriate solvents) were dissolved in 1.0 ml of phosphate buffer and 5.0 ml of 0.2 M phosphate buffer to adjust the pH 6.6. Subsequently, 5.0 ml of 1% potassium ferricyanide was added. The mixture was incubated at 50°C for 20 min and then cooled. The reaction was terminated by adding 5.0 ml of 10% TCA solution (w/v), and the mixture was centrifuged (REMI, India) at 1000 rpm for 10 min. The upper layer of the supernatant (5.0 ml) was taken and mixed with 5.0 ml of distilled water and 1.0 ml of 0.1% (w/v) ferric chloride. The absorbance was read spectrophotometrically at 700 nm against water blank. Rutin, quercetin, BHA and BHT were served as positive controls for comparison. All the tests were carried out in triplicate. A higher absorbance indicates a higher reductive capability.

DPPH Radical Scavenging Activity:

The antiradical efficiency was assessed using the DPPH• method as described by Blois (1958)[16]. In this method commercially available methanol soluble, stable free radical DPPH was used. In its radical form, DPPH has an absorption band at 515 nm, which disappears upon reduction by an antioxidant compound or a radical species. For the photometric assay, different volumes of the extracts were taken in different test tubes. The volume was adjusted to 100 µl with methanol. 5.0 ml of 0.1 mM methanolic solution of DPPH• was added to these tubes and shaken vigorously. The tubes were allowed to stand for 20 min at 27 ºC. The control was prepared as above but without the test extract and methanol was used for the baseline correction. Changes in the absorbance of the samples were monitored at 517 nm. Results were compared with the activity of rutin, quercetin, BHA and BHT. The per cent of DPPH• discolouration of the samples was calculated using the following formula:

DPPH radical scavenging activity (%) = [(A_{517} of control - A_{517} of sample) /A_{517} of control] × 100.

Antioxidant activities of the extracts were expressed as $IC_{50,}$ (the microgram of extract to scavenge 50% of the DPPH radicals) and were obtained by interpolation from linear regression analysis. A lower IC_{50} value indicates greater antioxidant activity.

Nitric oxide scavenging activity:

Nitric oxide scavenging activity was determined according to the method suggested by Sreejayan and Rao (1997)[17]. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which can be estimated using the Griess reagent. Scavengers of nitric -oxide act against oxygen, leading to reduced production of nitrite ions. In brief, 3.0 ml of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with different concentrations of the extract and incubated at 25°C for 150 min. 0.5 ml of the incubated solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% N- 1-naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylenediamine dihydrochloride was measured at 546 nm and percentage scavenging activity was measured with reference to standards. IC_{50}, an inhibitory concentration was estimated from the % inhibition plot.

Hydroxyl radical scavenging activity:

The scavenging activity for the sample extracts on hydroxyl radical was measured according to the method of Klein *et al*. (1991)[18]. 20 µg concentration of the extract was added with 1.0 ml of iron – EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1.0 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) sequentially. The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80- 90ºC for 15 min in a water bath. The reaction was terminated by the addition of 1.0 ml of ice – cold TCA (17.5% w/v). Then, 3.0 ml of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2.0 ml of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at laboratory temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm against reagent blank. Results were compared with the activity of standard antioxidants viz., rutin, quercetin, BHA and BHT. The % hydroxyl radical scavenging activity (HRSA) was calculated using the following formula:

HRSA (%) = [$A_{control} - A_{sample} / A_{control}$] × 100.

Chelating ability for ferrous ions:

The ferrous chelating potential of the extracts were assessed according to the method suggested by Yamaguchi *et al*. (2000)[19]. The reaction was initiated with the sequential addition of 250 µg of sample extract, 0.25 ml of 1 mM $FeSO_4$ solution, 1.0 ml of 0.2 M Tris–HCl buffer (pH 7.4), 1.0 ml of 2, 2’ bipyridyl solution, 0.4 ml of 10% hydroxylamine hydrochloride and 2.0 ml of ethanol. The final volume was made up to 5.0 ml with deionized water and the absorbance was determined at 522 nm. EDTA was used to benchmark the chelating abilities. Lower absorbance of the reaction mixture indicated higher ferrous ion chelating ability. Results were expressed as mg EDTA equivalent/ g sample extracts.

Trolox equivalent antioxidant capacity (TEAC) assay:

Antioxidant activity was performed using an improved $ABTS^{\bullet+}$ method proposed by Siddhuraju and Manian (2007)[20]. The ABTS radical cation ($ABTS^{\bullet+}$) was generated by a reaction of 7 mmol/L ABTS and 2.45 mmol/ L potassium persulfate after incubation for 16 h at laboratory temperature in dark. Blue – green $ABTS^{\bullet+}$ was formed at the end of this period. Prior to assay, the solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to obtain an absorbance of 0.700 ± 0.02 at 734 nm, the wavelength of maximum absorbance in the visible region. The stock solution of the sample extracts in ethanol was diluted such that, after introduction of a 10 µl aliquot of each dilution into the assay, they produced between 20-80% inhibition of the blank absorbance. After the addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution to 10 µl of sample extracts or Trolox standards (final concentration 0-15 µM) in ethanol, absorbance was recorded at 30ºC, exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicates were maintained for the experiments and the per cent inhibition of the blank absorbance at 734 nm was plotted as a function of Trolox concentration[21]. The unit of total antioxidant activity (TAA) was defined as the concentration of Trolox having the equivalent antioxidant activity expressed as µmol/ g sample extracts on dry weight basis.

Inhibition of β – carotene bleaching:

The antioxidant capacity of the extract was evaluated using β – carotene-linoleate model system[22]. 1.0 mg of β – carotene was dissolved in 10 ml of chloroform and mixed with 20 mg of linoleic acid and 200 mg of Tween – 40 emulsifier mixture. Chloroform was completely removed at 45 °C under vacuum using a rotary vacuum evaporator. 50 ml of oxygenated distilled water was added slowly to the semi-solid residue with vigorous agitation, to form an emulsion. A 5.0 ml aliquot of the emulsion was dispensed into tubes containing 100 µg/ ml of the sample extract. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. Subsequent absorbance readings were recorded at 15 min intervals by keeping the sample tubes in a water bath at 50 °C until the visual colour of β – carotene in the control sample disappeared (about 120 min). A blank, devoid of β – carotene was prepared for background subtraction. Rutin, quercetin, BHA and BHT were used as standards. All determinations were performed in triplicate and averaged.

The antioxidant activity (AA) was measured in terms of reduction in β – carotene bleaching activity using the following formula:

$$AA\ (\%) = [1- (A_s^0 - A_s^{120}) / (A_c^0 - A_c^{120})] \times 100$$

Where,

A_s^0 is the absorbance of sample at 0 min, A_s^{120} is the absorbance of sample at 120 min, A_c^0 is the absorbance of control sample at 0 min and A_c^{120} is the absorbance of control sample at 120 min.

Antihemolytic activity:

The preparation of erythrocyte membrane ghost and the subsequent determination of the antioxidant activity of the extracts on the chemically induced lipid peroxidation were performed according to the method set forth by Naim *et al*. (1976)[23]. The erythrocytes from cow blood were separated by centrifugation (2000 rpm for 10 min) and washed with saline phosphate buffer (0.9 g of sodium chloride dissolved in 100 ml of 0.2 M phosphate buffer of pH 7.4) until the supernatant becomes colourless. The erythrocytes were then diluted with saline phosphate buffer to give 4% (v/v) suspension. 500 µg of extract/ ml of saline phosphate buffer was added to 2.0 ml of erythrocyte suspension and the volume was made up to 5.0 ml with saline phosphate buffer. This mixture was pre-incubated for 5 min and then 0.5 ml of H_2O_2 solution of appropriate concentration in saline buffer was added. The concentration of H_2O_2 in the reaction mixture was adjusted so as to bring about 90% hemolysis of blood cells after 240 min. After the incubation time, the reaction mixture was centrifuged at 1500 rpm for 10 min and theextend of hemolysis was determined by measurement of the absorbance (at 540 nm) corresponding to haemoglobin liberation. Natural and synthetic standards at the same concentration as sample extract were used for comparison.

The percent hemolysis inhibition was calculated using the formula:

$$\text{Inhibition percentage} = [A_{\text{control}} - A_{\text{Sample}} / A_{\text{control}}] \times 100.$$

Statistical analysis:

For *in vitro* antioxidant activity of the extracts, the results were recorded as mean ± standard deviation (SD) (n = 3) and subjected to one- way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test using SPSS (version 9, SPSS Inc., Chicago, USA). $p< 0.05$ was chosen as the criterion for statistical significance.

Results and Discussion

Determination of *in vitro* antioxidant activity:

Reducing power:

Plants are generally reported to contain substantial amounts of polyphenols which are an important class of defense antioxidants. They possess ideal structural chemistry for free radical scavenging activity, and are shown to be more effective antioxidants *in vitro*[24]. Gordon (1990) reported that the antioxidant action of reductones is based on the breaking of free radical chain by donating a hydrogen atom[25]. Some studies have reported that the reducing capacity of a

compound may serve as a significant indicator of its potential antioxidant activity[26,27]. Fig. 1 shows the dose- response curves of *B. malabarica* pod and seed extracts. Their reductive abilities displayed an apparent linear relationship with concentration. The activity increased exponentially with increasing concentration of the test drug. It must be emphasized that the acetone extracts of *B. malabarica* seed exerted stronger reducing abilities than their corresponding solvent extracts and it even surpassed the efficiency of natural and synthetic antioxidants used. At the same concentration (20-100 µg/ml) tested, the reductive values of BHA was higher than the standards tested. Highly significant relationship between total phenolics and antioxidant activity were well reported in many plant species[28]. Chen and Ahn (1998) found that natural phenolics including flavonoids acts as Fe^{2+} chelators[29]. Therefore it is speculated that the presence of flavonoids, especially flavonols reported in this species might in turn contribute a big way for the effectiveness of phenolics-induced reducing power[11].

DPPH Radical Scavenging Activity:

Free radical scavenging ability of phenolic compounds is an important property underlying their various biological and pharmacological activities. DPPH•, a relatively stable organic radical with a characteristic strong absorption band at 517 nm in visible spectroscopy (deep violet color), was used to evaluate the free radical scavenging ability of the investigated samples and their respective solvent types (Table 1).

Plant parts	IC_{50} values (µg /ml)		Hydroxyl scavenging activity (%)	Metal chelating activity#	ABTS*
	DPPH•	NO•			
PA	18.6 ± 1[d]	32.7 ± 0.7[a]	28.3 ± 4.2[d]	268.1 ± 6.1[b]	6297.7 ± 192.9[b]
PM	24.2 ± 0.5[g]	78.5 ± 0.1[e]	14.8 ± 3.2[e]	95.3 ± 1.2[c]	4181.6 ± 185.9[d]
SA	8.8 ± 0.7[a]	39.7 ± 1.3[b]	32.1 ± 0.8[c]	361.7 ± 4.8[a]	6422.6 ± 5.8[a]
SM	10.6 ± 1.1[b]	83.2 ± 0.7[f]	27.2 ± 2.1[d]	91.6 ± 7.1[c]	4411.1 ± 978.8[c]
Ru	15.8 ± 0.01[c]	42.1 ± 0.03[c]	15.7 ± 0.8[e]	-	-
Qu	20.7 ± 0.1[e]	50.8 ± 4[d]	34. 9 ± 3.5[b]	-	-
BHA	21.4 ± 0.1[f]	52.9 ± 8[d]	35.5 ± 1.9[b]	-	-
BHT	34.7 ± 0.3[h]	38.5 ± 1[b]	45.6 ± 0.8[a]	-	-

Table 1: DPPH Scavenging, nitric oxide scavenging, hydroxyl scavenging, total antioxidant activity (TAA), and ferrous ion chelating ability of pod and seed extracts of B. malabarica.

PA – Pod Acetone, PM – Pod Methanol, SA – Seed Acetone, SM- Seed methanol, Qu – Quercetin, Ru – Rutin.

Values expressed in mg EDTA / g extract.

* Values expressed as TEAC (Trolox equivalent antioxidant capacity) in µ mol/g extract. Values are presented as the mean ± standard deviation of three independent experiments. Mean values not sharing a common letter in a column were significantly different ($p< 0.05$).

In the present study, all the assessed samples were able to interact intensively with DPPH and reduce the stable violet DPPH radical to the yellow DPPH-H, reaching their 50% reductive plateau ranging between 8.8 and 24.2 µg /ml. As reference, rutin taken as positive control recorded the highest scavenging efficiency towards DPPH radicals (15.8 µg /ml), followed by quercetin (20.7 µg /ml) and BHA (21.4 µg /ml). Similarly, the crude acetone extract of *B. malabarica* seed (8.8 µg /ml) possessed relatively the most effective DPPH radical quenching capacity with their IC_{50} values less than 10 µg /ml. These values were lower than the standard antioxidants analyzed. Not surprisingly, the methanolic extract of seed (10.6 µg /ml) and the acetone extracts of pod (18.6 µg /ml) also exhibited fairly outstanding antiradical capacity and their IC_{50} values were stronger or equal competing with the natural and synthetic standards tested. Therefore, the present study clearly indicates that the DPPH quenching ability of *B. malabarica* pod and seed could be related to the phytochemicals present in them which might possibly donate hydrogen from phenolic hydroxyl groups in order to discontinue the free radical chain reaction and prevent damage from free radicals[19,30].

Nitric oxide scavenging activity:

NO is one of the most widespread signaling molecule that participates virtually in every cellular and organ functions of the body. It is a free radical with a single unpaired electron. The plant / plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. In the present study, antioxidant ability of *B. malabarica* pod and seed extracts to scavenge nitric oxide generated *in vitro* by sodium nitroprusside were investigated (Table 1) and all the extracts inhibited nitric oxide generation in a concentration dependent manner. However their comparative data on nitric oxide scavenging activity fluctuated between plant parts and their solvent types. It exhibited excellent to fairly outstanding antioxidant activity with their IC_{50} values ranging between 32.7 and 83.2 µg/ml. However, it was observed that the acetone extract of *B. malabarica* pod (32.7 µg/ml) recorded markedly higher ability to scavenge NO and /or inhibits the production of nitric oxide radicals more actively than the different standard antioxidants tested. Interestingly, nitric oxide can be viewed as a radical itself and it was reported that nitric oxide molecules are directly scavenged by flavonoids[31]. Several flavonoids, including quercetin, result in a reduction in ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity[32]. When flavonoids were used as antioxidants, free radicals are scavenged and therefore can no longer react with nitric

oxide, resulting in less damage[33]. Therefore, it has been speculated that polyphenolic phytochemicals such as flavonoids and tannins present in these extracts might play a pivotal role in scavenging of NO radicals.

Hydroxyl radical scavenging activity:

Hydroxyl radical is one of the most potent oxidant among the reactive oxygen species and is considered to be an initiator of lipid peroxidation. It induces severe damage to the adjacent molecules and can be generated in biological cells through the Fenton reaction. In biochemical system, superoxide radical and H_2O_2 react together to form a singlet oxygen and hydroxyl radical, which can attack and destroy almost all known biochemicals[34]. The hydroxyl radical produced cause sugar fragmentation, base loss and leakage of DNA strands[35], lipid oxidation and enormous biological damage[36]. In the present investigation, at a concentration of 20 µg/ml the species exhibited fairly moderate radical scavenging activities towards the hydroxyl radicals generated (14.8 and 32.1 %) in the reaction mixture (Table 1). Among the solvent types examined, acetone extracts of *B. malabarica* seed (32. 1%) revealed the highest hydroxyl radical scavenging activity and these values were found to be comparably higher than the natural antioxidant rutin (15.7%) (Table 1). It has already been discussed that naturally occurring phenolic compounds have the ability to scavenge free radicals due to their hydroxyl groups[37]. Furthermore, phenolic compounds are effective hydrogen donors, which make them a better source of antioxidants[38]. Siddhuraju (2007) stated that the potential scavenging abilities might be due to the active hydrogen donor ability of hydroxyl substitution[39]. Therefore it is apparent that *B. malabarica* pod, seed extracts not only scavenges off the free radicals but also inhibits the generation of free radicals.

Chelating ability for ferrous ions:

Analogous to hydroxyl radical scavenging property, metal chelation is also one of the important properties of antioxidant. The chelation of ferrous ion by *B. malabarica* pod and seed extracts were estimated by 2, 2'-bipyridyl competition assay and their values were expressed as EDTA equivalents on a dry weight basis (Table 1). It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion[40]. From our study, it was observed that *B. malabarica* pod and seed extracts displayed an apparent (p<0.05) antioxidant activities as they were able to chelate ferrous metal ion more efficiently with their values ranging between 91.6 and 361.7 mg EDTA/g of sample. In the investigated range of concentration (500 µg/ml), the acetone fractions exerted significant (p<0.05) metal chelating activity as in the case of reducing power (Fig. 1) and DPPH assay (Table 1). Among them, the acetone extract of *B. malabarica* seed was found to be the most active (361.7 mg EDTA/ g sample) ferrous ion chelator, while their methanolic fractions (91.6 mg EDTA/ g sample) exhibited correspondingly very low ability for iron binding. The ability of phenolic compounds to chelate metal ions depend on the availability of properly oriented functional groups[41] and we speculate that these endogenous chelating agents, mainly phenolics present in the plant samples might be responsible for the observed activity.

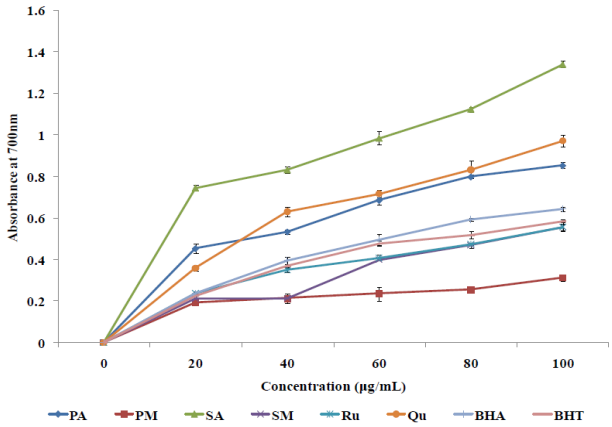

Figure 1: Reductive capability of pod and seed extracts of *B.malabarica* compared with standard antioxidants

PA – Pod Acetone, PM – Pod Methanol, SA – Seed Acetone, SM- Seed methanol, Qu – Quercetin, Ru – Rutin.

Values are presented as the mean ± standard deviation of three independent experiments.

Trolox equivalent antioxidant capacity (TEAC) assay:

The Total Equivalent Antioxidant Capacity (TEAC) was measured using the improved ABTS$^{\bullet+}$ radical decolorization assay. The decolorization of ABTS $^{\bullet+}$ cation radical is an unambiguous way to measure the total equivalent antioxidant capacity of test compounds or plants samples. Since, TEAC is a measurement of the effective antioxidant activity of the extract; a higher TEAC value would imply greater antioxidant activity of the sample. This assay was calibrated with the water-soluble á-tocopherol analogue, Trolox. In the evaluation of total antioxidant capacity by ABTS$^{\bullet+}$ method, all the sample extracts of *B. malabarica* were able to quench ABTS$^{\bullet+}$ radical more efficiently with their TEAC values ranging between 4181.6 and 6422.6 µmol trolox equivalent/g sample extract. The acetone extracts of *B. malabarica* seed (6422.6 µmol trolox equivalent / g) and pod (6297.7 µ mol trolox equivalent/g) provided higher TEAC values. The higher ABTS $^{\bullet+}$ radical scavenging activity in these extracts implies that polyphenolic compounds in these plants are capable of scavenging free radicals more efficiently by forming resonance -stabilized phenoxyl radicals[42]. Velioglu *et al*. (1998) reported a high correspondence between total phenolic content and antioxidant activity in fruits, vegetables and grain products[43].

Inhibition of β – carotene bleaching:

The antioxidant activity of the extracts was determined using β - carotene -linoleic acid coupled oxidation model system. As shown in Fig. 2, the addition of organic extracts (100 µg/ ml) of *B. malabarica* pod and seed extracts were found to be markedly effective (64.1 and 90.4%) in inhibiting the oxidation of linoleic acid and subsequent bleaching of â- carotene to various degrees. Apparently, the acetone extracts of *B. malabarica* seed recorded the highest antioxidative power (90.4%). Interestingly, it surpassed the performance of all the widely used natural and synthetic antioxidants tested and thereby strengthens their antioxidant power. These results postulated that the polyphenolic components were found to be comparably higher and had acted as an effective antioxidant in the β-carotene-linoleic acid model system. In similar lines, Su *et al*. (1988) reported that many hydrolysable tannins from *Osbeckia chinensis* were found to have potential antioxidative efficiency in the linoleic acid- thiocyante system which is also an experiment to assess the lipid peroxidation[44]. Nicoli *et al*., 1997 reported that medium dark roasted coffee brews had the highest antioxidant properties due to the development of Maillard reaction products[45]. Similarly, extract of roasted followed by defatted legume, peanut kernels, displayed most remarkable antioxidative activity on linoleic acid emulsions system[46]. Moreover, Siddhuraju and Becker, (2003) reported the hydrophobic antioxidants (phenolic acids, flavanones, flavonols) are very effective in oil-in-water emulsion system[47]. Almost congruous results were marked in the present study where the antioxidant activity could be related to the phytoconstituents such as flavonoids present in them.

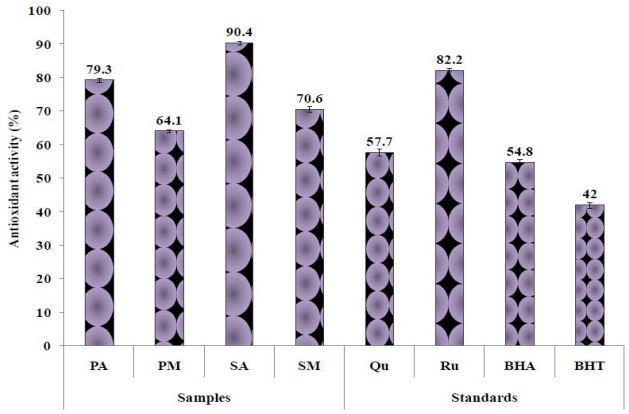

Fig 2: Lipid peroxidation preventive property of pod and seed extracts of *B. malabarica* in β-carotene-linoleic acid System

PA – Pod Acetone, PM – Pod Methanol, SA – Seed Acetone, SM- Seed methanol, Qu – Quercetin, Ru – Rutin.

Values were presented as the mean ± standard deviation of three independent experiments. Vertical bars labeled with different letters are significantly different (p< 0.05).

Antihemolytic activity:

Lipid oxidation of cow blood erythrocyte membrane mediated by H_2O_2 induces membrane damage and subsequently hemolysis. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell[48]. The protective effect of *B. malabarica* on hemolysis were investigated (Fig. 3) and it fluctuated between plant parts and solvent types. At the concentration of 500µg/ml in the reaction mixture, all the extracts of *B. malabarica* efficiently inhibited the hemolysis of erythrocytes and in most cases their results overhelmed the performance of natural and synthetic antioxidants used. Furthermore, the acetone extracts of *B. malabarica* seed (90%) and pod (84%) offered more protection against erythrocyte hemolysis when compared with the other studied components. Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals[49]. Dai *et al.* (2006) recorded that flavonols and their glycosides are competent antioxidants which are capable of protecting human red blood cells against oxidative hemolysis stimulated by free radical[50].

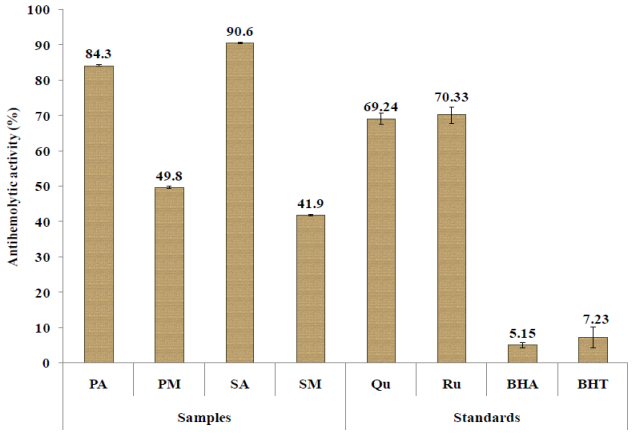

Figure 3: Antihemolytic property of pod and seed extracts of B. malabarica

PA – Pod Acetone, PM – Pod Methanol, SA – Seed Acetone, SM- Seed methanol, Qu – Quercetin, Ru – Rutin.

Values were presented as the mean ± standard deviation of three independent experiments. Vertical bars labeled with different letters are significantly different (p< 0.05).

Present study, thus proves that plant samples apart from having an excellent radical quenching property also have a good amount of protective capability against hemolysis due to the polyphenolic constituent in the extract. Similarly, in other previous analysis, a highly significant efficiency in inhibiting radical induced red blood cell hemolysis was also observed for *Oudneyna africanan*, *Artemisia arboresens* and *Globularia alpyum* whose activities were nearly similar to caffic acid[51]. Similarly, Barreira *et al*. (2008) reported that chestnut skins showed a high protective effect against erythrocyte hemolysis (outer skin: 95.7%, inner skin: 92.7%; at 1 mg/ml) when compared with the other studied components (flower: 75.8%, leaf: 59.0%, fruit: 23.3%; at 1 mg/ml)[52].

Conclusions

To conclude, *B. malabarica* pod, seed extracts presented a broad range of antioxidant activities but with different efficacies. Among the samples analyzed acetone extract of *B. malabarica* seed determined the highest range of antioxidant activity in terms of the assays used for the determination of antioxidant potential. In the present study, high contents of polyphenolics might be the major contributor of antioxidant capacities of these plant samples. However, the data pertaining to the bioactive compounds responsible for such activity will delineate the future use of this species in pharmaceutical and food industries. In addition, the observed *in vitro* activities suggest that all the investigated plant extracts could exert protective effects also in *in vivo* studies against oxidative and free-radical injuries occurring in different pathological conditions.

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