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MIMUSOPS ELENGI BARK AND FRUITS EXTRACTS AS POTENTIAL FREE RADICAL SCAVENGING AGENTS.

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ABSTRACT

Mimusops elengi (ME) Linn (Sapotaceae) commonly known as Bakul, is used as tonic, febrifuge, as gargle for odontopathy, inflammation, bleeding gums and healing of ulcers. *Mimusops elengi* is a rich source of tannin, saponin, alkaloids and glycosides which may be responsible for its therapeutic benefits. Aim of the present study was to evaluate antioxidant and free radical scavenging activity of various extracts of Bakul bark and fruits. Chloroform, acetone methanol and aqueous extracts of Bakul bark and fruits were prepared and evaluated for presence of phenolics and flavonoids. Extracts showing maximum phenolic content expressed as mg of GAE/g of dry extract and flavonoid content expressed as mg of RE/g of dry extract were further selected for various *in vitro* free radicals scavenging assays using DPPH scavenging, ABTS, peroxynitrite radicals, xanthine oxidase inhibition (XOI), superoxide scavenging and lipid peroxidation assay. Methanol and acetone extract of Bakul bark were found to contain higher amount of total phenolics. Presence of both these constituents in Bakul bark was highest in methanol extract followed by acetone extract, aqueous extract and least in chloroform extract. Acetone extract of Bakul fruits alone showed considerable amounts of these constituents as compared to other fruit extracts. The methanol extract of Bakul bark showed lowest IC_{50} values than acetone extract when evaluated by DPPH, ABTS, peroxynitrite inhibition, xanthine oxidase inhibition, superoxide scavenging and lipid peroxidation inhibition methods indicating good free radical scavenging activity. Acetone extract showed least free radicals scavenging activities with very high IC50 values against these free radicals.

KEYWORDS: *Mimusops elengi*, DPPH, ABTS, total phenolics, flavonoids, rutin.

INTRODUCTION

Oxidative stress is caused due to over production of free radicals such as superoxide, hydroxyl and peroxyl which can cause oxidative damage of all major groups of biomolecules (DNA, protein, lipids and small cellular molecules). Oxidative stress plays an important role in the pathogenesis of various diseases such as cancer, atherosclerosis, diabetes, neurodegenerative disorders cataracts, inflammation and aging.[1] The antioxidant defences systems including enzymes (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymes defences (gluthatione, vitamins C and E) play an important role in scavenging oxidants and preventing cell injury. Antioxidants can inhibit or delay the initiation or propagation of oxidative chain reaction and thus prevent or repair cell damage caused by reactive oxygen. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are extensively used as antioxidants in order to reduce the damage caused by free radicals. However, the possible toxicity as well as general consumer rejection led to decreasing use of these synthetic antioxidants. The use of plants as traditional medicine still represent a large source of natural antioxidants which might serve as leads for the development of novel drugs. Plant material and their products are rich sources of a variety of phenolic compounds which have antioxidant and radical scavenging activities. Therefore, consumption of a variety of phenolic compounds with high antioxidant effects may reduce the risk of serious health disorder and have beneficial effects on the prevention or progression of diseases related to oxidative stress. Thus, there is a great deal of interest in exploring newer natural antioxidant molecules with health promoting potential.[2]

Mimusops elengi (ME) Linn (Sapotaceae) commonly known as Bakul, is a small to large evergreen tree found all over the different parts of Bangladesh, Pakistan and India. It is cultivated in gardens as an ornamental tree.[3] Earlier report revealed that the fruits are used in chronic dysentery, constipations; flowers are used as snuff to relive headache, lotion for wounds and ulcers. Barks are used to increase fertility in women and known to have antiulcer activity. Bark is used as a tonic, febrifuge, as a

gargle for odontopathy, inflammation and bleeding of gums.[4] *Mimusops elengi* is a rich source of tannin, saponin, alkaloids and glycosides. Several triterpenoids, steroids, steroidal glycosides, flavonoids and alkaloids have been reported from this species which may be responsible for its therapeutic benefits. Phytochemical review shows the presence of taraxerol, taraxerone, ursolic acid, betulinic acid, α-spinosterol, β-sitosterol, lupeol, alkaloid isoretronecyl tiglate and mixture of triterpenoid saponins in the bark of *Mimusops elengi*. The present study aims at evaluating antioxidant potential of bark and fruits of *Mimusops elengi* (Bakul).[5]

MATERIALS AND METHODS

Procurement of Plant Material

Stem bark and just ripe fruits of Bakul plant were collected from SNDT University Juhu Campus Santacruz, Mumbai, India and authenticated at Agarkar Research Insitute, Pune, India.

Chemicals and Instruments

Gallic acid, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), Allopurinol, Bovine Serum Albumin (BSA), Bovine Brain Extract (BBE), were purchased from Sigma Chemical Co. (USA). Nitroblue tetrazolium chloride (NBT) xanthine oxidase and xanthine were purchased from Himedia Ltd. Mumbai, India. Pyrogallol Red, Folin-Ciocalteu solution, potassium dihydrogen phosphate and dipotassium hydrogen phosphate, anhydrous sodium carbonate, ascorbic acid, ethylene diamine tetra acetic acid (EDTA), ferrous ascorbate, trichloroacetic acid, thiobarbituric acid, butylated hydroxytoluene (BHT) potassium persulfate, were purchased from S.D.Fine Chemicals, Mumbai, India. All other chemicals and solvents used were of analytical grade.

The instruments used for the study were UV spectrophotometer (Jasco, V-630), laboratory centrifuge (Remi motors, R4C) and digital pH meter (Equip-tronics, EQ-610).

Preparation of extracts

Bakul bark was dried, then powdered to 40 mesh and stored in an air tight container. Fruits of Bakul which are just ripe were collected, washed and dried. The seeds were removed from the fruits. Then the fruits were dried in sunlight, powdered, stored in air tight containers. The plant material was extracted in a Soxhlet extractor using various solvents such as chloroform, acetone, methanol and water. All extracts were filtered individually and evaporated to get dry extracts. The extractive values were determined. After drying, crude extracts were stored in desiccator till further use.

Phytochemicals Screening

Extracts were qualitatively tested for the presence or absence of active principles such as phytosterols, tannins, flavonoids, saponins, alkaloids, glycoside, triterpenoids and proteins.

Quantification of total phenolics – by Folin-Ciocalteu method

The total phenolic content in various extracts of the plants were measured using Folin-Ciocalteu reagent. 1 ml of extract solution was added to 0.5 ml of Folin Ciocalteau reagent and 5 ml of distilled water. The mixture was incubated at room temperature for 10 min. Then 1.5 ml of anhydrous sodium carbonate solution (10% w/v) was added and the final volume was made upto 10 ml. The final mixture was allowed to stand at room temperature for 2 h with intermittent shaking. Then the absorbance of the dark blue colour that developed was measured at 725 nm using UV-Vis spectrophotometer. Gallic acid was used as standard for preparing the standard curve (10 µg/ml - 100 µg/ml). The concentration of total phenolic compounds in the extracts was calculated using the following linear equation based on the calibration curve: $y = 0.010x + 0.01$ with $R^2 = 0.994$. The total phenolic content in the plant extract was expressed as mg of gallic acid equivalent per g of dry weight (mg GAE/g) of extract. All the tests were carried out in triplicate and the results are expressed as mean ± SD.[6,7]

Quantification of total flavonoids

The total flavonoid content of all the extracts was determined by aluminium chloride colorimetric method. 0.5 ml of sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min of incubation, 0.15 ml of 10% $AlCl_3$ solution was added and then allowed to stand for 6 min, followed by adding 2 ml of 4% NaOH solution to the mixture. Immediately water was added to the sample to make the final volume to 5 ml, the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance was determined at wavelength 510 nm. The total flavonoid content was expressed in mg of rutin equivalent per g of extract (mg RE/g) of extract.[7]

In vitro antioxidant studies

The methanol and acetone extracts of bark and fruits of Bakul with high phenolic and flavonoid content were further evaluated for their free radical scavenging potential.

DPPH (1, 1, diphenyl 2-picryl hydrazyl) assay

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined.[8] A 0.1 mM solution of DPPH in methanol was prepared. An aliquot of 1 ml of the extract solution in various concentration ranges was added to 3 ml of the DPPH solution. The decrease in absorbance was determined at

517 nm after 30 min. The percentage scavenging activity was calculated from $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/ standard. A blank is the absorbance of the control reaction (containing all reagents except the test compound). The % scavenging activity and IC_{50} value of extracts were calculated for the various concentrations. Ascorbic acid was used as standard antioxidant for comparison. All the tests were carried out in triplicate and the results were expressed as mean ± SD.

Inhibition of Pyrogallol Red bleach by peroxynitrite

Peroxynitrite (ONOO-) was prepared by reacting 2 M H_2O_2 in 2 M HNO_3 with 2 M $NaNO_2$, followed by stabilization of the product with 4 M NaOH. The solution was frozen at -70ºC. Peroxynitrite concentration was determined spectrophotometrically at 302 nm (ε=1670 M^{-1} cm^{-1}) and dilutions in 1M NaOH were made in order to achieve 200 µM solution. All the extract solutions were prepared by dissolving in methanol. Pyrogallol red solution (100 µM) was prepared in 100 mM phosphate buffer, pH 7.4. 1 ml of extract solution was added to 2 ml of 100 µM pyrogallol red solution.0.5 ml of 200 µM peroxynitrite solution was added to the mixture and immediately vortexed. This step is critical for the reproducibility of results, especially for peroxynitrite. After 15 min the absorbance was measured using UV-vis spectrophotometer at 540 nm. The % inhibition of pyrogallol red bleaching was determined using the formula $[(A_1-A_2)/A_1]$ X 100, where A_1 is the absorbance in presence of antioxidants and A_2 is the absorbance in absence of antioxidants. The IC_{50} values of extracts yielding 50% inhibition of pyrogallol red bleaching were estimated. Ascorbic acid was used as standard antioxidant for comparison. All the tests were carried out in triplicate and the results were expressed as mean ± SD.[9,10,11]

ABTS assay

The antioxidant activities of all the extracts in the reaction with the stable $ABTS^{+}$• radical cation were determined.[12,13] The reaction between ABTS and potassium persulfate directly generates the blue/ green $ABTS^{+\cdot}$ chromophore, which can be reduced by an antioxidant, thereby resulting in a loss of absorbance at 734 nm. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{+\cdot}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 hr. The $ABTS^{+\cdot}$ solution was diluted with a phosphate buffer (2 mM, PH 7.4) to achieve an absorbance of 0.8 ±0.014 at 734 nm. Extract solutions were mixed with $ABTS^{+\cdot}$ solution and after 1 min the absorbance was read using UV-vis spectrophotometer at 734 nm. Phosphate buffer solution was used as a blank. The % radical-scavenging activity of the samples was determined using the formula $[(A_{control}–A_{test})/ A_{control}]$ X 100, where $A_{control}$ is the absorbance of the control (ABTS+• solution without test sample) and A_{test} is the absorbance of the test sample (ABTS+• solution with extract). The IC_{50} values scavenging 50% of $ABTS^{+\cdot}$ were estimated. Ascorbic acid and trolox were used as standard antioxidants for comparison. All the tests were carried out in triplicate and the results were expressed as mean ± SD.

Xanthine oxidase assay

The inhibitory effect on xanthine oxidase was measured spectrophotometrically at 295 nm under aerobic condition.[14,15] The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.5), sample solution dissolved in distilled water or DMSO, freshly prepared enzyme solution (0.2 U/ml of xanthine oxidase in phosphate buffer) and distilled water. The assay mixture was pre-incubated at 37°C for 15 min. Then substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37^0C for 30 min. Next, the reaction was stopped with the addition of 0.5 M HCl. The absorbance was measured using UV-VIS spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced with the phosphate buffer. Another reaction mixture was prepared (control) having water/DMSO instead of test compounds in order to have maximum uric acid formation. Thus, XOI activity was calculated using the following equation in which α is the activity of XO without test extract and β is the activity of XO with test extract. % XO inhibition = $(1 – \beta/\alpha) \times 100$

Superoxide scavenging assay

Superoxide was generated by xanthine oxidase system.[16,17,18] The reaction mixture consisted of sodium phosphate buffer (pH 7.4), 3 mM Xanthine, 3 mM EDTA, 0.15%BSA, 15 mM Nitroblue tetrazolium chloride, sample solution The reaction mixture was incubated at 25^0C for 10 min. Then reaction was initiated by adding 1.5 U/ml of xanthine oxidase and incubated at 25^0C for 20 min.After 20 min the absorbance was measured at 560 nm using UV-Vis spectrophotometer. The inhibtion rate was calculated by measuring the amount of the formazan which was reduced from NBT by superoxide usin the following equation where, A1- Absorbance of control and A2- Absorbance in presence of test compounds: % Inhibition= [A1-A2/A1 X 100].

Ascorbate iron induced lipid peroxidation

10 mg of bovine brain extract was mixed with phosphate buffer pH 7.4 and sonicated in an ice bath until a milk-like suspension was obtained. The lipid suspension was mixed with 1 mM $FeCl_3$ and extract. The peroxidation was initiated by adding 1 mM ascorbate. The mixture was incubated at 37^0C for 60 min. After incubation, 10% trichloroacetic acid was added and centrifuged at 1800 rpm for 10 minutes. After centrifugation, 1ml of supernatant was collected and mixed with 1ml of 0.67% thiobarbituric acid (TBA). The mixture was vortexed and heated in boiling water bath at 100^0C for 20 min and then rapidly cooled and the extent of oxidation inhibition was estimated from the absorbance of the organic layer at 532nm. A tube containing all the reaction mixture except

the plant extract was used as control. Blank was phosphate buffer. The percent inhibition was calculated with the formula Percent inhibition (%) = (Abs of control – Abs of sample) X 100 / (Abs of control).[19,20]

RESULTS AND DISCUSSION

Extractive Values

The extractive values for Bakul bark were highest for methanol extract (12.80% w/w) followed by aqueous extract (9.80% w/w), acetone extract (8.70% w/w) and chloroform extract (3.93% w/w) respectively.

The extractive values for Bakul fruits were highest for methanol extract (9.56% w/w) followed by acetone extract (5.61% w/w), chloroform extract (4.31% w/w) and aqueous extract (2.80% w/w) respectively.

Quantitation of Total Phenolics and Total Flavonoids

Methanol extract and acetone extract of the Bakul bark were found to contain higher amount of total phenolics (160.3±3.20, 133.3±5.16 mgGAE/g of extract) and total flavonoids (165.69±9.13, 149.75±6.82mg RE/g of extract) as compared to other extracts, Fig. 1, Fig. 2. Acetone extract of Bakul fruits were found to contain high amount of total phenolics 44.98±0.95 mg GAE/g of extract) and total flavonoids 40.83±6.55 mg RE/g of extract as compared to other extracts, Fig. 3, Fig. 4. Therefore, these extracts were selected for various *in-vitro* antioxidant assay methods.

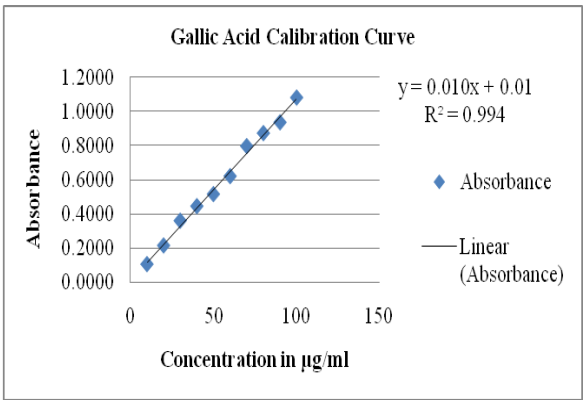

Figure 1. Calibration Curve for Standard Gallic Acid

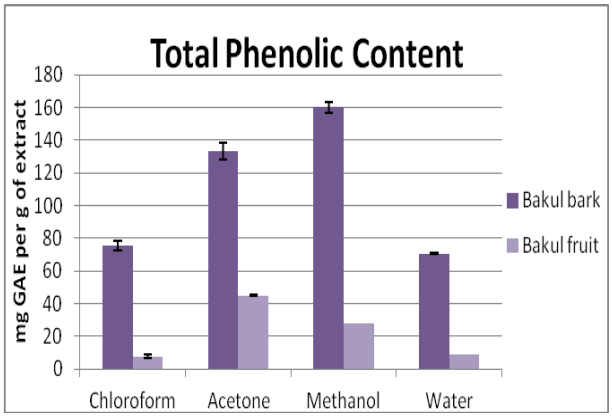

Figure 2. Gallic Acid Equivalent of Bakul bark and Bakul fruits

Values are presented as mean ± SD (*n* = 3).

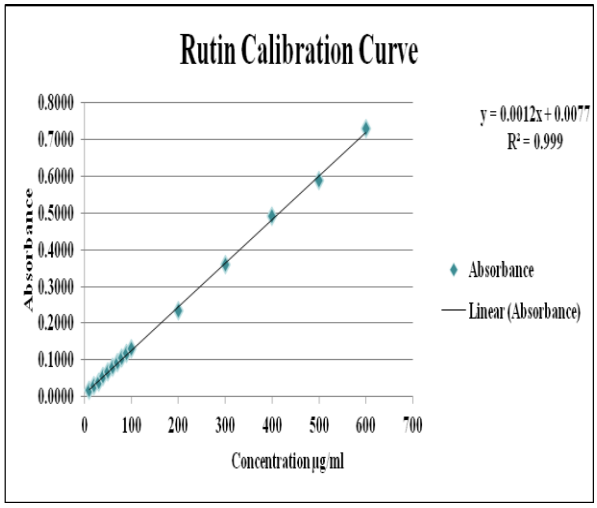

Figure 3. Calibration Curve for Standard Rutin

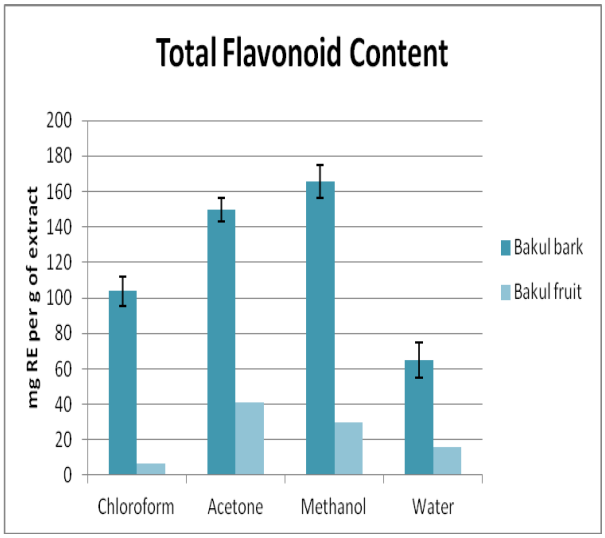

Figure 4. Rutin Equivalent of Bakul bark and Bakul fruits

Values are presented as mean ± SD (*n* = 3).

In vitro antioxidant assays

DPPH Scavenging

Both methanol and acetone extracts of Bakul bark were effective in reducing the stable radical DPPH to the yellow colored diphenylpicrylhydrazine, indicating that these extracts were able to scavenge DPPH radical (Table.1). Standard ascorbic acid exhibited maximum free radical scavenging activity with IC_{50} =13.24 ±0.48µg/ml. Methanol extract of Bakul bark (IC_{50} = 18.97±0.39µg/ml) showed a stronger DPPH radical scavenging activity than acetone extract (IC_{50} = 22.52±0.17µg /ml). Acetone extract of Bakul fruits (IC_{50} = 33.35±0.33 µg/ml) showed DPPH scavenging at higher IC_{50} as compared to Bakul bark extracts.

Table.No.1 IC_{50} values of bakul bark and fruit extracts for various free radical scavenging activity

Extracts/ Standards	IC_{50} values of extracts (µg/ml) Rose fresh petals					
	DPPH	Peroxynitrite	ABTS	Xanthine oxdiase	Superoxide Scavenging	Lipid Peroxidation
Bakul bark Methanol	18.97±0.39	86.22±1.63	206.58±7.36	80.14±1.31	685.38±7.43	91.46±2.42
Bakul bark Acetone	22.52±0.17	91.52±1.33	216.21±5.46	84.55±2.63	717.10±9.80	116.43±0.31
Bakul fruits Acetone	33.35±0.33	114.55±3.55	714.95±4.28	168.29±1.42	1179.22±9.91	206.70±2.70
Ascorbic Acid	13.24±0.49	49.41±0.27	19.03±0.02	-	-	-
Trolox	-	-	9.34±0.08	-	-	-
Allopurinol	-	-	-	3.38 ± 0.16	6.00±0.23	-
BHT	-	-	-	-	-	33.11±1.44

Peroxynitrite inhibition

Pyrogallol Red is a dye which gets bleached by peroxynitrite radical. All the rose petal extracts moderately inhibited bleaching of peroxynitrite as compared to standard ascorbic acid (Table.1). Methanol and acetone extracts of Bakul bark exhibited moderate activity at IC_{50} values of 86.22±1.63µg/ml and 91.52±1.33µg/ml respectively as compared to standard ascorbic acid (IC_{50} = 49.41±0.27µg/ml). Bakul fruits showed DPPH scavenging at very high IC_{50} value of 114.55±3.55 µg/ml as compared to Bakul bark extracts and standard ascorbic acid.

ABTS radical scavenging assay

Standard ascorbic acid and trolox showed potent ABTS scavenging at IC_{50} values of 19.03±0.02 µg/ml and 9.34±0.08 µg/ml respectively. Methanol extract and acetone extract of Bakul bark exhibited ABTS scavenging activity at higher IC_{50} values of 206.58±7.36µg/ml and 216.21±5.46µg/ml respectively as compared to the standards. Acetone extract of Bakul fruits showed lowest ABTS scavenging activity at very high IC_{50} values of 714.95±4.28 in comparison ith the standards and Bakul bark extracts (Table.1).

Xanthine oxidase inhibition assay

Allopurinol is a potent inhibitor of xanthine oxidase. It inhibited the enzyme at IC_{50} value of 3.38±0.16 µg/ml. Methanol and acetone extracts of Bakul bark inhibited the enzyme at higher IC_{50} values of 80.14±1.31µg/ml to 84.55±2.63µg/ml respectively. Acetone extract of Bakul fruits showed least inhibition of the enzyme at very high IC_{50} value of 168.29±1.74 µg/ml (Table.1 and Table.2).

Superoxide scavenging assay

Allopurinol is a potent scavenger of superoxide anions and showed excellent scavenging at IC_{50} value of 6.00±0.23 µg/ml. The superoxide scavenging effect for Bakul bark was found to be greater for methanol extract (IC_{50} 685.38±7.43µg/ml) as compared to acetone extract (IC_{50} 717.10±9.80µg/ml). Both these extracts showed moderate scavenging as compared to standard allopurinol. The scavenging effect for acetone extract of Bakul fruits was found to be least (IC_{50} 1179.22±9.91µg/ml) as compared to other extracts and standard (Table.1).

Lipid Peroxidation

Bakul bark methanol and acetone extracts inhibited lipid peroxidation at higher IC_{50} values ranging from 91.46±2.42µg/ml to 116.43±0.31µg/ml as compared to standard butylated hydroxyl toluene 33.11±1.44 µg/ml. However, acetone extract of Bakul fruits inhibited lipid peroxidation at very high IC_{50} value of 206.70±2.70 µg/ml (Table.1).

The present paper describes and compares different *in vitro* antioxidant and free radical scavenging activities of the extracts of Bakul bark and fruits. Bakul bark extracts showed good scavenging activity against various free radicals as compared to fruits. The wide range of free radical scavenging activity of the extracts indicates the potential of the Bakul bark as a source of natural antioxidants. It can have potential application to reduce oxidative stress along with health benefits.

DPPH solution is decolourised from deep violet to light yellow. The percent reduction in absorbance indicates the DPPH radical scavenging potential of the extracts. Both the methanol and acetone extract of Bakul bark exhibited good activity comparable to standard ascorbic acid. Acetone extract of Bakul fruits showed less DPPH scavenging as compared to bark extracts.

Pyrogallol red is a dye. Peroxynitrite radical bleaches this dye and the intensity of dark red colour decreases. Antioxidants scavenge the peroxynitrite radical thereby preventing the bleaching of pyrogallol red and retaining its colour intensity. In this assay, the increase in absorbance is proportional to inhibition of pyrogallol red bleach by peroxynitrite. Both the extracts of Bakul bark were found to have appreciable peroxynitrite scavenging activity as compared to Bakul fruit acetone extract.[21]

$ABTS^{\cdot+}$ radical is a chromophore having blue colour. The Bakul bark extracts were able to scavenge the radical cation effectively at high IC_{50} values as compared to standards. Bakul fruit acetone extract was found to be less effective in scavenging ABTS radical.

Xanthine oxidase-derived superoxide anion has been linked to various degenerative and metabollic disorders. Therefore superoxide radical scavenging by antioxidants has physiological implications. Allopurinol is a potent

inhibitor of xanthine oxidase. However chronic treatment with allopurinol has its own side effects. Hence, natural antioxidants which inhibit this enzyme and also have superoxide scavenging activity would be beneficial in preventing various complications of oxidative stress related disorders. Bakul bark extracts were found to have moderate xanthine oxidase inhibitory activity as well as superoxide scavenging activity though at higher IC_{50} values as compared to allopurinol. But the safety of this drug along with its antioxidant potential will be a better therapeutic approach. Acetone extract of Bakul fruits showed poor xanthine oxidase inhibition and superoxide scavenging in comparison with Bakul bark extracts.[22,25]

Oxidative stress is one of the main factors involving in the development of peroxyl radicals, which may play a role in DNA damage, glycation and protein modification reactions, and in lipid peroxidation. Lipid peroxidation in biological systems can lead to production of Malondialdehyde. Malondialdehyde is very reactive and takes part in cross-linking with DNA and proteins and also damages liver cells. Bakul bark extracts exhibited a better inhibitory action against peroxidation of Bovine brain extract, suggesting better protection against lipid peroxidation. Acetone extract of Bakul fruits showed lesser inhibition of lipid peroxidation as compared to other bark extracts.

CONCLUSIONS

In conclusion, the present study has showed that methanol extract of Bakul bark exhibit potent scavenging activity against different free radicals. The antioxidant activity can mainly be attributed to phenolic compounds such as flavonoids and tannins. Bakul bark can prove to be excellent antioxidant source for oxidative stress related disorders.

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