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DNA scission and LDL cholesterol oxidation inhibition and antioxidant activities of *Bael* (*Aegle marmelos*) flower extracts

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ABSTRACT

Phenolic compounds and other antioxidants have been implicated in protection against non-communicable diseases (NCDs) in which oxidative stress is a main contributor. The extracts of *Bael* (*Aegle marmelos*) flower were examined for their phenolic content, free radical scavenging efficacy and inhibition of low density lipoprotein (LDL) cholesterol oxidation and DNA scission activities. The extracts of *Bael* flowers were prepared using different solvent systems and their total phenolic content (TPC) and total flavonoid content (TFC) determined. Selected extracts which showed high TPC were subsequently used to determine their efficacy in scavenging hydroxyl, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, using electron paramagnetic resonance (EPR) spectroscopy. The corresponding peroxyl radical scavenging activity was measured using oxygen radical absorbance capacity (ORAC) assay. The potency of the extracts in inhibiting hydroxyl and peroxyl radical-induced supercoiled DNA scission and inhibition of LDL cholesterol oxidation was also evaluated. The chemical identity of phenolic compounds present in the extracts was tentatively unraveled using HPLC-MS. Phenolic extracts of *Bael* flowers effectively inhibited hydroxyl, and peroxyl radicals. Phenolic extracts demonstrated notable inhibitory activity against hydroxyl and peroxyl radical-induced DNA scission and LDL oxidation. Vanillic, *p*-coumaric, chlorogenic, caffeic, and gentisic acids were identified as major phenolic acids, along with flavonoids, mainly catechin, and quercetin. The knowledge gained here may help better use of *Bael* flower extracts as functional herbal beverage ingredients in the prevention of NCDs.

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1. Introduction

Herbal beverages play a significant role in the traditional dietary habits in many countries, especially among health-conscious consumers. They also penetrate into an emerging niche market along with other beverages. A majority of herbal beverages are prepared from natural ingredients using different morphological parts, namely leaves, stems, roots, fruits, buds and flowers of plants. The type of herbal beverages popular in different regions of the world could be different and may be affected by local habits and availability of certain plants. Herbal teas/beverages are rich sources of natural bioactive compounds such as carotenoids, phenolic acids,

flavonoids, coumarins, alkaloids, polyacetylenes, saponins and terpenoids, among others. Health Canada categorizes herbal beverages under natural health products (NHPs).

Excessive generation of reactive oxygen species (ROS) in the body causes oxidative stress, a detrimental cascade of events leading to the oxidation of biomolecules such as proteins, lipids, carbohydrates and DNA in organisms. Oxidative stress is now well known for its pivotal role in the etiology of several chronic diseases such as cardiovascular diseases, arthritis, and diabetes, different types of cancers, autoimmune diseases and neurodegenerative disorders.

Studies have shown that major cardiovascular risk factors are associated with a significant increase of vascular production of ROS and lipid oxidation¹ In addition to the oxidative modification of low-density lipoprotein (LDL), there are a number of additional, likely important, oxidative events in cardiovascular disease (CVD). For instance increased ROS production has been identified as a major cause of endothelial dysfunction which is associated with a rapid loss

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of anti-atherogenic and anti-inflammatory properties of endothelium-derived nitric oxide ($\cdot\text{NO}$) in part due to its inactivation by superoxide. In addition, ROS are involved in signaling cascades leading to vascular pro-inflammatory and pro-thrombotic gene expression.² It is known that some dietary constituents may provide protection against cancer by eliminating/neutralizing certain ROS that damage DNA or initiate cancer. Therefore, external sources of antioxidants are required to prevent oxidative damage in the human body once internal antioxidant defense systems are challenged by over exposure to free radicals and other ROS. Phenolic compounds which are ubiquitous in plant foods and beverages as well as spices and herbs are known for their antioxidant and other bioactivities such as antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic and vasodilatory action, as well as anti-mutagenicity, anti-carcinogenicity and anti-aging effects.^{3,4}

Aegle marmelos is a tropical plant belonging to the family Rutaceae and is grown in Southeast Asian countries, including India, Sri Lanka, Pakistan, Bangladesh, Burma, and Thailand. It is also known as Bengal quince, Indian quince, holy fruit, golden apple in English, and *Bael* or *Bilva* in Hindi. Different parts of *Bael* tree are used for the treatment of a number of ailments in traditional Ayurvedic medicine. The fruit is a rich source of vitamin C, and riboflavin. The bark as well as fruit are used as Ayurvedic medicine for dysentery and various intestinal complaints. A number of bioactivities, namely microfilarial, radioprotective, analgesic, anti-hyperglycemic, anti-dyslipidemic, anticancer and antidiabetic activities have been reported.^{5–14} Coumarins (marmelosin, marmesin, imperatorin), alkaloids (aeglin, aegelenine), tannins (skimmianine), and other phenolic compounds are some of bioactive classes of compounds reported in different parts of *Bael* tree.¹⁵

The flowers of *Bael* tree are fragrant and are found in clusters along the young branches (plate 1). Herbal tea prepared using dried *Bael* flower in boiling water serves as a popular beverage in Sri Lanka since ancient times. In traditional medical systems distillation of flowers has been used to formulate a drug which is used as tonic for stomach and intestine. Further, anti-dysenteric, antidiabetic, and diaphoretic properties of *Bael* flower have also been reported.^{5,12} However, information on antioxidant activities of *Bael* flower extracts is limited. Thus, the present study was aimed to determine the antioxidant activity as well as the biological activities, reflected in the inhibition of DNA scission and LDL cholesterol oxidation activities, of the extracts of dried *Bael* flower.

2. Materials and methods

Dried flowers of *A. marmelos* purchased from a local market in



Plate 1. Flower and buds of *Aegle marmelose* used for preparation of herbal teas.

North Western Province in Sri Lanka were used in this study. Folin Ciocalteu's reagent, vanillic, *p*-coumaric, syringic, chlorogenic, caffeic, and gentisic acids, as well as catechin, quercetin, aluminium chloride, sodium nitrite, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), 2,2'-azobis-(2-methylpropionamide) dihydrochloride (AAPH), sodium chloride, trolox, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, ferrous chloride, ascorbic acid, ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), mono- and dibasic potassium phosphates, butylated hydroxyanisole (BHA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulphonic acid sodium salt (Ferrozine), were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Diethyl ether, ethyl acetate, hexane, acetone, methanol, chloroform, acetonitrile, formic acid, hydrochloric acid, sodium hydroxide and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada).

2.1. Sample preparation

Dried flowers of *Bael* (*A. marmelos*), cleaned to remove soil and other particles, were ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON, Canada) to obtain a fine powder which passed through mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH, USA).

2.2. Extraction of soluble phenolic compounds

Ground powder of *Bael* dried flower was used to extract soluble phenolic compounds under reflux conditions using different solvent systems. Briefly, for refluxing, 1 g of dried powder was placed in a round bottom flask, and extraction was carried out using 80% (v/v) methanol, 80% (v/v) ethanol, 50% (v/v) ethanol, 80% (v/v) acetone, or distilled water under reflux conditions in a thermostated water bath at 60 °C for 30 min. Meanwhile, another sample was extracted with distilled water in a thermostated water bath at 100 °C for 30 min under reflux conditions. After centrifugation of the resulting slurry for 10 min at $4000 \times g$ (IEC Centra MP4, International Equipment Co. Needham Heights, MA, USA), the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated in *vacuo* at 40 °C (Buchi, Flawil, Switzerland) and lyophilized for 72 h at -44 °C and 46×10^{-3} mbar (Freezone, Model 77530, Labconco Co. Kansas City, MO, USA). During all stages, extracts were minimally exposed to light by covering the containers with aluminium foil. Lyophilized crude phenolic extracts were stored at -20 °C until used for further analysis.

2.3. Determination of total phenolic content (TPC)

The total phenolic content of each extract was determined according to Singleton and Rossi¹⁶ with slight modifications as explained previously.¹⁷ The content of total phenolics in each extract was determined using a standard curve prepared for gallic acid and expressed as micromoles of gallic acid equivalents (GAE) per gram of crude extract.

2.4. Determination of total flavonoid content (TFC)

Total flavonoid content was determined using a colourimetric method described by Kim et al.¹⁸ and detailed by Chandrasekara and Shahidi.¹⁷ A standard curve prepared with catechin was used to calculate total flavonoid content which was expressed as micromoles of catechin equivalents (CE) per gram of crude extract.

2.5. DPPH radical scavenging activity

The effect of extracts on the scavenging of DPPH radicals was determined according to the method adapted from Chandrasekara and Shahidi.¹⁷ The phenolic extracts (0.5 mL) of *Bael* were added to 2 mL of methanolic solution of DPPH (0.19 mM), vortexed and allowed to stand at room temperature in the dark. The mixture was passed to the sample cavity of a Bruker E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker Biospin Co. Billerica, MA, USA) and the spectrum was recorded after 10 min. Methanol was used as the control in place of the extract. The parameters of Bruker E-scan EPR spectrometer were set as follows; 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.53 G centre field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. DPPH radical scavenging capacities of the extracts were calculated using the following equation. DPPH radical scavenging capacity (%) = $\{(EPR \text{ signal intensity for the control} - EPR \text{ signal intensity for the sample})/EPR \text{ signal intensity for the control}\} \times 100$.

2.6. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was determined according to Chandrasekara and Shahidi.¹⁷ Crude extracts of *Bael* flower were dissolved in deionized water and diluted appropriately. Extracts (100 μ L) were mixed with 100 μ L of H_2O_2 (10 Mm), and 200 μ L of DMPO (17.6 mM) and 100 μ L of $FeSO_4$ (0.1 mM). After 1 min the mixtures were introduced into the sample cavity of the EPR spectrometer and the spectrum was recorded. Deionized water was used as the control in place of the extract. Hydroxyl radical scavenging capacities of the extracts were calculated using the following equation. Hydroxyl radical scavenging capacity (%) = $\{(EPR \text{ signal intensity for the control} - EPR \text{ signal intensity for the sample})/EPR \text{ signal intensity for the control}\} \times 100$.

2.7. Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity measures scavenging activity of test compounds against peroxy radical generated by AAPH. The ORAC assay was based on the method explained by Chandrasekara and Shahidi.¹⁷ The samples, standards and other reactants were prepared in 75 mM phosphate buffer solution (PBS) (pH 7.0) and the final reaction mixture (295 μ L) contained 200 μ L (0.11 μ M) of fluorescein as oxidizable substrate, 20 μ L of extract or trolox and 75 μ L of AAPH (63.4 mM). The reaction was carried out in a Costar[®] 3695 flat bottom 96 well black microplates (Corning Incorporated, Corning, NY, USA). Determination of ORAC was carried out using a plate reader equipped with a built-in incubator (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany). Fluorescein (200 μ L) was manually pipetted into the wells containing the extract or standards (20 μ L) followed by incubation for 15 min at 37 °C. The injector pump was programmed to inject AAPH at the end of incubation during the first cycle. The plate was automatically shaken for 4 s after each addition and the microplate reader was programmed to perform additional shaking of the contents in wells before each reading was taken. A gain adjustment was performed before the beginning of the measurements to optimize the maximum sensitivity, by manually pipetting 200 μ L of fluorescein into the wells. Fluorescence recorded every minute for 25 cycles and each cycle was 210s. A control (phosphate buffer, fluorescein and AAPH) and different concentrations of trolox (6.25–50 μ M) as the standard were performed in each assay. All reactions mixtures were prepared in duplicate and three independent runs were performed for each sample. ORAC values of

extracts were expressed as micromoles trolox equivalents (TE) per gram of crude extract.

2.8. Ferrous ion chelating capacity

The ability of *Bael* extracts to chelate ferrous ions was measured according to the method described by Dinis et al.¹⁹ and as explained by Chandrasekara and Shahidi.¹⁷ The inhibition percentage of ferrozine-ferrous ion complex formation was calculated by the following equation. Metal chelating effect (%) = $\{1 - (\text{Absorbance of the sample}/\text{Absorbance of the control})\} \times 100$. The results were expressed as micromoles EDTA equivalents per gram of crude extract.

2.9. Inhibition of liposome oxidation induced by UVA light

The liposome suspension was prepared according to a previously described method²⁰ To evaluate the inhibition of liposome oxidation, 0.8 mL of α -phosphatidylcholine (PC) and 0.1 mL of extract (1 mg/mL) were mixed in an Eppendorf tube (1.5 mL) and total volume was made to 1 mL with PBS. The control was prepared with PC mixture and PBS. The mixtures were incubated for 5 min at 37 °C before exposing to UV light for 60 min²¹ using a Spectroline[®] UV lamp (364 nm, UVA) (Model ENF-240 C, Spectronics Co. Westbury, NY, USA) at room temperature. At the end of incubation conjugated diene hydroperoxides of a 100 μ L of sample diluted to 1 mL with methanol formed were measured (Model HP 8452A diode array spectrophotometer, Agilent Technologies, Palo Alto, CA, USA) at 234 nm. The results were expressed as percentage inhibition of oxidation.

2.10. Inhibition of copper-mediated human LDL peroxidation

Inhibitory activities of *Bael* flower extracts against human low density lipoprotein (LDL) cholesterol oxidation was determined by measuring conjugated dienes (CD) produced in the system using the method described by Andreasen et al.²² Human LDL cholesterol (in PBS, pH 7.4 with 0.01% EDTA), was dialyzed against 10 mM PBS (pH 7.4, 0.15 M NaCl) for 12 h under nitrogen at 4 °C and EDTA-free LDL cholesterol was subsequently diluted to obtain a standard protein concentration of 0.1 mg/mL in PBS. The diluted LDL cholesterol solution (0.8 mL) was mixed with 100 μ L of extract (0.5 mg/mL in PBS) in an Eppendorf tube. Oxidation of LDL cholesterol was initiated by adding 0.1 mL of 100 μ M $CuSO_4$ solution in distilled water. The mixture was incubated at 37 °C for 20 h. The initial absorbance ($t=0$) was read at 234 nm immediately after mixing and CD hydroperoxides formed were measured at 12, and 20 h. The concentration of CD formed was calculated using molar extinct coefficient of $29500 \text{ M}^{-1} \text{ cm}^{-1}$. The corrected absorbance at 12 and 20 h against 0 h was employed to calculate the percentage inhibition of CD formation using the following equation. Percentage inhibition of CD formation = $(\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{native}} \times 100$, where, $\text{Abs}_{\text{oxidative}}$ = absorbance of LDL mixture and distilled water with $CuSO_4$ only; $\text{Abs}_{\text{sample}}$ = absorbance of LDL with extract and $CuSO_4$; $\text{Abs}_{\text{native}}$ = absorbance of LDL with distilled water.

2.11. Supercoiled plasmid DNA strand scission inhibition

Inhibition activity of *Bael* extracts against supercoiled strand DNA scission induced by peroxy and hydroxyl radicals was evaluated according to a previously described method²⁰ Supercoiled plasmid DNA (pBR 322 from *Escherichia coli* RRI) was dissolved at a concentration of 50 μ g/mL in 0.5 M, pH 7.4 phosphate buffer solution (PBS). *Bael* extracts (0.25 and 0.5 mg/mL) were prepared in

PBS. In an Eppendorf tube (500 μ L), 2 μ L of a solution of supercoiled plasmid DNA, PBS, phenolic extract, H₂O₂ (1 mM) and FeSO₄ (0.5 mM) were added in the order stated to determine the inhibitory activity of millet extracts against hydroxyl radical induced DNA strand scission. The mixture was incubated at 37 °C for 1 h in the dark.²⁰ The loading dye (2 μ L), consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol in distilled water, was added to the reaction mixture at the end of the incubation period.

In another experiment, inhibitory effect of extracts against peroxy radical induced DNA scission was investigated. In this, AAPH was dissolved in PBS in order to attain a final concentration of 9 mM which was then mixed with DNA and the extracts to a final volume of 10 μ L. A control with DNA alone and a blank devoid of phenolic extracts were prepared with each set of phenolic extracts tested.

The samples were electrophoresed using a 0.7% (w/v) agarose gel prepared in Tris-acetic acid-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5). SYBR safe was added at a concentration of 100 μ L/L of TAE buffer as a gel stain. Submarine gel electrophoresis was run at 60 V for 5 h using a model B1A horizontal mini gel electrophoresis system (Owl Separation systems Inc. Portsmouth, NH, USA) and a model 300 V power supply (WMR International Inc. West Chester, PA, USA) at room temperature in TAE buffer. The bands were visualized under transillumination of UV light using Alphamager™ gel documentation system (Cell Biosciences, Santa Clara, CA, USA). The images were analyzed using Chemilmager 4400 software (Cell Biosciences, Santa Clara, CA, USA) to quantify DNA scission. The protective effect of phenolic extracts was calculated using retention percentage of the normalized supercoiled DNA as given below. DNA retention % = (Intensity of supercoiled DNA with the oxidative radical and extract/Intensity of supercoiled DNA in control) X 100.

2.12. HPLC-DAD analysis

Phenolic composition of phenolic fractions of *Bael* flower extracts was determined by HPLC analysis. The RP-HPLC analysis were carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1311A quaternary pump, a G1379A degasser and a G1329A ALS automatic sampler, a G1330B ALS Therm, a G1316A Colcom column compartment, a G1315B diode array detector (DAD) and a system controller linked to Chem Station Data handling system (Agilent Technologies, Palo Alto, CA, USA). Separations were conducted with a SUPERLICOSIL™ LC-18 column (4.6 × 250 mm, 5 μ m; Merck, Darmstadt, Germany). The mobile phase consisted of 1% formic acid (eluent A) and 100 % methanol (eluent B). Gradient elution was used as follows; 0 min, 5% B; 5 min, 25% B; 20 min, 50% B; 24 min, 55% B; 28 min, 60% B; 30 min, 70% B and 40 min, 95% B. The flow rate was adjusted to 1 mL/min and the detection of compounds was performed at 254, 280, and 320 nm. All samples were filtered through a 0.45 μ m PTFE membrane syringe filter (Whatman Inc. Florham Park, NJ, USA) before injection.

Phenolic acids, namely, vanillic, syringic, gentisic, chlorogenic, caffeic, and *p*-coumaric, were identified by comparing their relative retention times (RT), and UV spectra and ESI-MS spectra with authentic compounds. Flavonoids used as analytical standards included catechin, epigallocatechin, epicatechin, quercetin, and apigenin.

2.13. Statistical analysis

All experiments were carried out in triplicates and data were reported as mean \pm standard deviation. The Student's *t* test at $p \leq 0.05$ was used to determine significance of differences between different time intervals and concentrations. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc. Chicago, IL, USA).

3. Results and discussion

To the best of our knowledge this study is the first report on inhibition of LDL oxidation and DNA scission by *Bael* flower extracts. The results showed the potential of using herbal teas prepared from *Bael* flower as therapeutic dietary agents in attenuating NCDs in which oxidative stress plays a pivotal role. Table 1 presents the percentage yield of the crude extracts obtained using different solvent systems and their phenolic contents. The average yield of crude extract ranged from 6.8 to 24.7 % and the extraction obtained with water at 100 °C for 30 min gave the highest crude yield.

3.1. Phenolic contents

The TPC of crude extracts were determined using Folin Ciocalteu's reagent and the values ranged from 1028 to 1615 μ mol GAE/g extract (Table 1). The order of TPC of extracts with different extraction conditions were 50% ethanol > 80% acetone > water at 100 °C > 80% methanol > 80 % ethanol > water at 60 °C. Crude extracts obtained with 50% ethanol, and water at 100 °C showed 1.5 times higher TPC than those obtained with 80% ethanol and water at 60 °C suggesting the compositional effect of extraction medium and temperature on TPC. The information generated in the present study lends further support on the preparation method of herbal teas from dried *Bael* flower to get the optimum utility as a functional beverage giving health benefits. Thus, boiling for 30 min produces *Bael* flower herbal beverages with higher TPC compared to those that can be prepared as infusions.

The TFC of crude extracts ranged from 564 to 1096 mg CE/g extract (Table 1). The crude extract obtained with 50% ethanol showed the highest TFC followed by those with 80% acetone and water at 100 °C. TPC ($r^2 = 0.837$; $p < 0.01$) of extracts positively and significantly associated with TFC suggesting their contribution to the TPC of crude extracts. The crude extracts which showed the highest TPC and TFC were selected to determine their antioxidant activities using different analytical methods. In agreement with the present study, Rajan et al.²³ reported that alcoholic extracts of *Bael*

Table 1

The yield, total phenolic (TPC) and total flavonoid (TFC) contents of phenolic extracts of *Bael* flower.

	Average yield of crude extract (%)	TPC (micromoles gallic acid equiv/g crude extract)	TFC (micromoles catechin equiv/g crude extract)
80% ethanol	6.8 \pm 1.3	1061.5 \pm 28.8d	678.7 \pm 26.5d
50% ethanol	22.3 \pm 2.3	1615.2 \pm 61.0a	1095.7 \pm 24.9a
80% methanol	15.2 \pm 1.9	1279.9 \pm 32.8c	891.0 \pm 19.6c
80% acetone	10.6 \pm 1.5	1522.8 \pm 29.4b	948.8 \pm 41.3b
Water at 60 °C	18.4 \pm 0.9	1027.7 \pm 41.6d	564.2 \pm 16.2e
Water at 100 °C	24.7 \pm 1.7	1496.7 \pm 42.8b	938.7 \pm 11.9b

Values in each column having the same letter are not significantly different ($p > 0.05$).

Table 2
Antioxidant activity of phenolic extracts of *Bael* flower.

Antioxidant activity	Extraction with 50% ethanol	Extraction with Water at 100 °C
DPPH radical scavenging capacity (%)	59.5 ± 3.3a	50.5 ± 2.5a
Hydroxyl radical scavenging capacity (%)	26.5 ± 3.9a	41.5 ± 3.4b
Inhibition of UVA induced liposome peroxidation (%)	77.3 ± 0.9a	81.1 ± 0.8a
Ferrous ion chelation activity (%)	16.9 ± 1.2a	20.4 ± 1.2a
Oxygen radical absorbance capacity (Trolox equivalents per g of crude extract)	930 ± 23a	942 ± 29a

Values in each row having the same letter are not significantly different ($p > 0.05$).

fruit pulp had higher levels of TPC and TFC than those of aqueous extracts. Furthermore, it was revealed that TPC and TFC of different parts of the plants varied.²⁴

3.2. Phenolic compounds identified

Table 2 presents individual phenolic compounds and their contents identified from *Bael* flower extracted in 50% aqueous ethanol. Hydroxycinnamic acids, hydroxybenzoic acids as well as some flavonoids were identified. Among hydroxycinnamic acids, chlorogenic, *p*-coumaric and caffeic acids were identified as major components. Gentisic, vanillic and syringic acids were among hydroxybenzoic acids identified. Catechin and quercetin were two major flavonoids identified in 50% ethanolic extracts of *Bael* flower and the contents were 5.80 and 1.35 mg/g crude extract, respectively. The results show that compounds belonging to flavonoids are predominant in the 50% ethanolic extracts of *Bael* flower compared to those of phenolic acids (Table 2).

3.3. Antioxidant activities

Antioxidant activities of phenolics extracted with 50% ethanol and boiling water at 100 °C are presented in Table 3. Except for hydroxyl radical scavenging activity, significant differences in the antioxidant activities were not observed between the aqueous-ethanol and aqueous *Bael* extracts used for the analysis in this study. Aqueous extracts showed 42% hydroxyl radical scavenging activity whereas aqueous-ethanol extract showed only 27%. Hydroxyl radical (OH) is extremely reactive and generates other radicals by reacting with biomolecules such as proteins, DNA and lipids. Further it plays a significant role as an initiator of lipid peroxidation.

DPPH, as a stable synthetic radical compared to highly reactive and transient peroxy and hydroxyl radicals, is widely used to evaluate free radical scavenging properties of antioxidant compounds. In the present study EPR spectroscopy was used to detect the signal intensity of DPPH radicals left upon reacting with phenolic compounds in the assay. Fig. 1 shows representative EPR spectra of DPPH radicals alone and in the presence of phenolic extracts of *Bael* flower. DPPH radical scavenging capacities were 51 and 60% for aqueous and aqueous-ethanol extracts of *Bael* flower extracts, respectively.

Table 3
Retention percentage of supercoiled pBR 322 plasmid DNA in peroxy radical-mediated and hydroxyl radical-mediated *in vitro* systems with phenolic extracts of *Bael* flower.

	Peroxy radical		Hydroxyl radical	
	0.5 mg/mL ^a	0.25 mg/mL ^a	0.5 mg/mL ^a	0.25 mg/mL ^a
Control		18±2		16±1
Gallic acid	98 ± 2a	99 ± 1a	90 ± 2a	41 ± 2a
Extraction with 50% ethanol	91 ± 1a	81 ± 1a	22 ± 3b	30 ± 1 b
Extraction with water at 100° C	92 ± 1a	83 ± 2a	23 ± 2 b	21 ± 3b

Values in each column having the same letter are not significantly different ($p > 0.05$).

^a Concentrations of extracts.

The oxygen radical absorbance capacity (ORAC) as trolox equivalents per gram of crude extract of aqueous and aqueous-ethanol extracts of dried *Bael* flower were 942 and 930, respectively. In the ORAC assay peroxy radicals are generated by thermal decomposition of the azo compound, AAPH. Fluorescein (FL) is employed as the probe and the fluorescence decay indicates its reaction with peroxy radical. In the presence of antioxidative compounds FL decay is inhibited and their intensity can be measured at 485 nm excitation and 525 nm emission. The protective effect of phenolic extracts against peroxy radicals is calculated from net integrated area under the kinetic curve (AUC) and compared to that of the trolox, a water soluble analogue of α -tocopherol.

The results in this study demonstrated that phenolic extracts of *Bael* flower scavenge peroxy radicals effectively (Table 3). This could be attributed to the high phenolic content in the crude extract of dried *Bael* flowers. Several *in vivo* studies have demonstrated the inhibition of lipid peroxidation by hydroalcoholic extracts of *Bael*. Jagetia et al.²⁵ reported that the intraperitoneal administration of the hydroalcoholic extract of the fruit pulp (20 mg/kg body weight) for five consecutive days before exposure to different doses of γ -radiation (6–9 Gy) could prevent radiation-induced lipid peroxidation in the liver, kidney, intestine and spleen of mice.

3.4. Liposome oxidation inhibition

Antioxidant activity of phenolics of *Bael* flower extracts were determined by their ability to inhibit conjugated diene hydroperoxide formation in phosphatidylcholine (PC) liposome membranes which were oxidized under UVA light. Ultraviolet radiation is known to damage lipid membranes directly or via a process mediated by ROS.^{26,27}

The results of the present study showed that *Bael* flower phenolics were effective against liposome peroxidation induced by UVA light at a concentration of 1 mg/mL of the extracts (Table 3). Phenolics of *Bael* flower extracts inhibited lipid oxidation ranging from 77 to 81%. The antioxidant activity of phenolic compounds may be related to both their structural characteristics as well as ability to interact with the liposome. It was reported that hydrophilicity of the phenolic compounds, degree of flavonol oligomerization and the number of hydroxyl groups in the respective

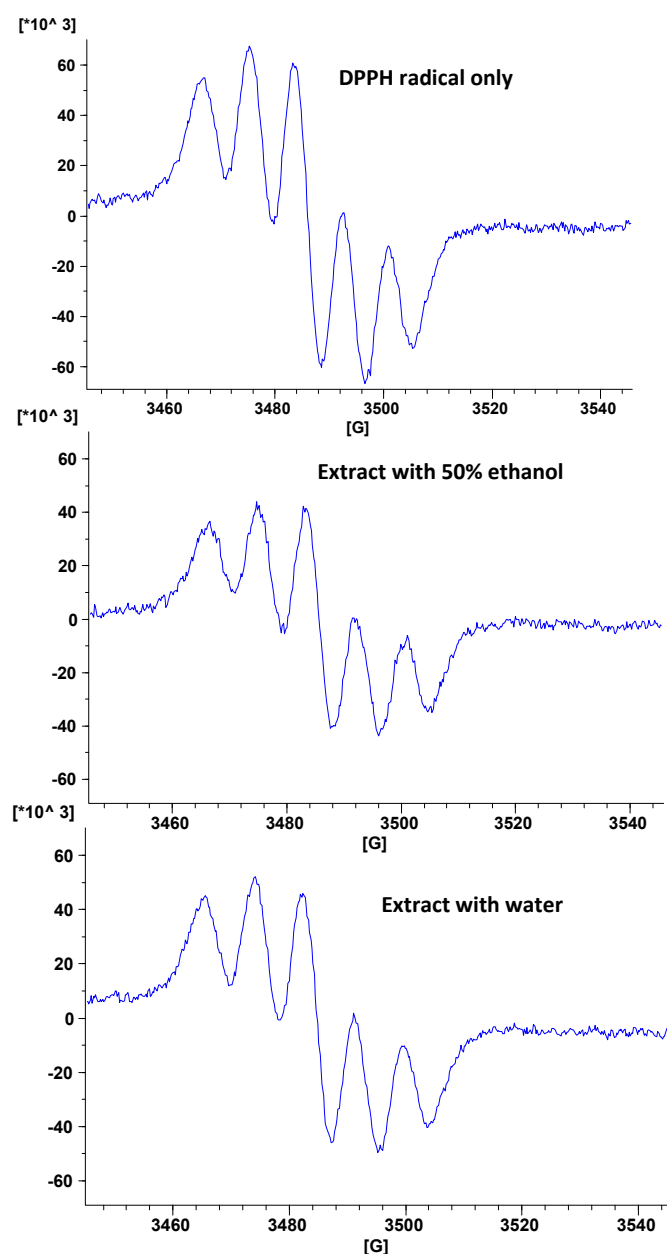


Fig. 1. Electron Paramagnetic Resonance signals of DPPH radicals alone and in the presence of *Bael* flower phenolic extracts.

molecule were associated with the protection against membrane oxidation.²⁸ Several authors have shown that flavonol and quercetin may protect against UV radiation through absorbance of UV radiation, antioxidant activity and interaction with signal transduction pathways.^{29,30} Furthermore, Fahlman and Krol²¹ showed that quercetin inhibited UVA and UVB radiation induced lipid oxidation mainly by scavenging radical species. Thus, peroxidation inhibition demonstrated by both aqueous and aqueous-ethanolic phenolic extracts in the present study could be due to their ability to scavenge free radicals generated during UV radiation.

3.5. Ferrous ion chelating activity

Ferrous ion contributes to lipid peroxidation as well as DNA oxidation by generating hydroxyl radical through Fenton reaction. Further, ferrous ion accelerates decomposition of lipid

hydroperoxides into peroxy and alkoxy radicals.³¹ In this study, *Bael* flower extracts showed chelating activity of ferrous ions in the range of 17–21%. It is noteworthy that phenolic extracts of *Bael* flower which displayed a relatively high ORAC and liposome oxidation inhibition activities (Table 3) demonstrated a low ferrous ion chelating activity. This could be due to deficit of compounds which are responsible for low ferrous ion chelating in the extract. In a previous study, Chandrasekara and Shahidi²⁰ showed that finger millet varieties which had high chelating activity were reported to contain flavan-3-ol monomers as well as dimers such as procyanidin B1 and B2.

3.6. Supercoiled plasmid DNA strand scission inhibition

Irreversible modification of DNA occurring as a result of oxidative damage has been identified as the first step in pathological conditions such as cancer and aging. The free radical mediated damage to DNA may vary and includes base modification, production of base-free sites, strand breaks, DNA-protein cross links and abnormal chromosomal arrangements, among others.³² Hydroxyl radical is able to abstract a hydrogen atom from the deoxyribose sugar moiety as well as pyrimidine and purine bases of DNA, producing single strands.³³ Double strand breaks occur near to each other on both strands, could be due to the multiple hydroxyl radical attacks and lead to lethal damage of the cells.³⁴ In the present study, free radical induced DNA strand scission inhibition was tested for two concentrations of crude phenolic *Bael* flower extracts, 0.25 and 0.5 mg/mL, and determined the percentage of the retention of supercoiled DNA. The results showed that both peroxy and hydroxyl radicals induced DNA strand scission.

Phenolic compounds can act as chemopreventive agents through antioxidative and a number of other mechanisms.³⁵ It is noted that phenolic extracts of *Bael* flower demonstrated effective inhibitory activity against peroxy radical induced DNA scission at both concentrations tested. Furthermore, both extractions with 50% ethanol and water at 100 °C showed more than 90% DNA scission inhibition and in this respect were close to that of gallic acid at concentrations of 0.25 and 0.5 mg/mL (Table 4). It is interesting to note that both extracts had high (81–83%) inhibition activity against peroxy radical-mediated DNA scission even after the extracts were diluted by a factor of two. The inhibition of DNA scission induced by peroxy radicals has previously been reported for a number of cereal phenolic extracts.^{20,36} Inhibition of peroxy radical induced supercoiled DNA scission of barley extracts ranged from 78 to 92% at a concentration of 4 mg/mL.³⁶ In a similar concentration schedule as in the present study, Chandrasekara and Shahidi²⁰ showed a 45–99% inhibition of peroxy radical induced supercoiled DNA scission of millet extracts. The observed scission inhibition could be due to the scavenging of peroxy radicals by phenolic compounds present in the extract. This was further confirmed by the results of the ORAC assay which showed superior peroxy radical scavenging activity in the present study.

For hydroxyl radical induced DNA scission, *Bael* flower extracts showed low inhibitory activity although gallic acid showed 90% inhibition at a concentration of 0.5 mg/mL (Table 4). The supercoiled DNA retention ranged from 21 to 30% against hydroxyl radical induced oxidation in the presence of *Bael* flower extracts. At a similar concentration of the extract (0.5 mg/mL), *Bael* flower phenolics inhibited DNA scission induced by peroxy radicals 3 to 4 times higher than that of hydroxyl radicals. The same pattern was observed at a lower concentration (0.25 mg/mL) of the extracts in this study. These results further suggest that phenolic compounds present in the crude extracts of *Bael* flower could be more effective against the scavenging of peroxy radicals compared to those of hydroxyl radicals.

Table 4
Individual phenolic compounds identified in *Bael* flower extracted with 50% ethanol.

Phenolic compounds	Molecular weight	[M-H] ⁻ (m/z)	ESI negative ions (m/z)	Content (mg/g crude extract)
Gentisic acid	154	153	329	0.95 ± 0.04
Vanillic acid	168	167	123	0.94 ± 0.01
Syringic acid	198	197	153	0.07 ± 0.01
Chlorogenic acid	354	353	191	0.19 ± 0.01
Caffeic acid	180	179	135, 167	0.03 ± 0.01
<i>p</i> -Coumaric acid	164	163	119, 139	0.95 ± 0.01
Catechin	290	289	245	5.80 ± 0.21
Quercetin	302	301	121, 179	1.35 ± 0.04

It is demonstrated that both the quantity and the concentration of different phenolic compounds in the extracts may be responsible for the observed effects. In a previous study, Noroozi et al.³⁷ reported that flavonoids were effective against hydrogen peroxide-initiated oxidative DNA damage to human lymphocytes. In the present study, both extracts showed high inhibition ability against peroxy radical-mediated DNA oxidation, possibly due to their high content of flavonoids. HPLC analysis revealed that catechin and quercetin were the major flavonoids in *Bael* flower extract (Table 2).

3.7. Inhibition human LDL cholesterol oxidation

Fig. 2 shows the inhibitory activities of *Bael* flower extracts at a concentration of 0.5 mg/mL against human LDL cholesterol oxidation induced by cupric ion. Extracts of dried *Bael* flower showed more than 70% inhibition of oxidation of LDL cholesterol. Furthermore, both aqueous and aqueous-ethanol extracts showed effective inhibition of oxidation after 12 and 20 h and aqueous-ethanol extracts showed superior inhibition than that of aqueous extract.

Uptake of oxidized LDL by macrophages and smooth muscle cells leads to the formation of fatty streaks or vascular lesions which accumulate lipids further in the progression of atherosclerosis. Therefore, natural antioxidants from dietary sources that may inhibit LDL cholesterol oxidation are of great importance in the prevention of cardiovascular diseases.^{38,39}

Inhibitory effects of phenolic compounds against LDL cholesterol could be due to their free radical scavenging activity or metal ion chelating properties. Further, phenolic compounds may protect endogenous antioxidative compounds such as β -carotene, tocopherols, and ubiquinol of LDL cholesterol molecule. In addition, phenolics can inhibit enzymes such as xanthine oxidase involved in the initiation of oxidation or cell mediated LDL cholesterol oxidation. The ability of phenolic compounds to inhibit copper ion-

mediated LDL cholesterol oxidation may be attributed to their capacity to remove cupric ions from the LDL cholesterol.⁴⁰ It has been shown that copper mediated oxidation of tryptophan residues in apolipoprotein B could be a leading cause for initiating cholesterol oxidation in the LDL cholesterol.⁴¹

4. Conclusion

The phenolic extracts of dried *Bael* flower extracts serve as a natural source of antioxidants and effectively inhibit the DNA strand scission induced by peroxy radicals and human LDL cholesterol oxidation induced by cupric ions. Phenolic extracts from *Bael* flower showed high liposome peroxidation inhibition activity. However, they exhibited low hydroxyl radical scavenging and ferrous ion chelation activities, suggesting the differential effects of constituent phenolics of the extract in diverse assay systems. There is an increasing demand for natural sources of antioxidants which can be used for managing several pathological conditions and maintenance of health. Dried *Bael* flower has a great potential to be utilized as a functional beverage in health promotion and disease risk reduction.

Conflict of interest

The authors state that there is no conflict of interest to declare.

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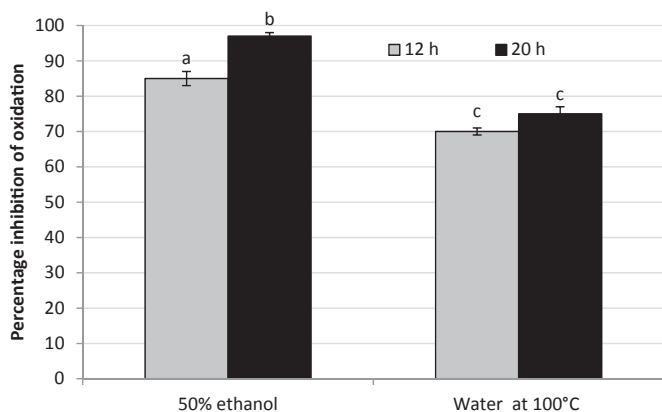


Fig. 2. Percentage inhibition of LDL cholesterol oxidation by phenolic extracts of dried *Bael* flowers.

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