J Bangladesh Agril Univ 17(4): 466–475, 2019 https://doi.org/10.3329/jbau.v17i4.44607

ISSN 1810-3030 (Print) 2408-8684 (Online)

Journal of Bangladesh Agricultural University

Journal home page: http://baures.bau.edu.bd/jbau, www.banglajol.info/index.php/JBAU

Concordance of antioxidant and anti-Inflammatory activity in *Xylocarpus granatum* (Koen)

Md. Emdadul Islam[1]✉, S.M. Mahbubur Rahman[1], Md. Hossain Sohrab[2], Rana Biswas[1], Mohammad Shaef Ullah[3], Kazi Didarul Islam[1]

[1]Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna 9208, Bangladesh
[2]Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka 1205, Bangladesh
[3]Department of Entomology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

ARTICLE INFO

Article history:
Received: 02 August 2019
Accepted: 21 September 2019
Published: 31 December 2019

Keywords:
Inflammation,
ROS,
Antioxidant, Secondary metabolites, Flavonoid

Correspondence:
Md. Emdadul Islam
✉: emdad950711@yahoo.com

ABSTRACT

Xylocarpus granatum, a common mangrove plant is traditionally used for the treatment of diarrhoea, cholera, fever, dyslipidemia, inflammation, etc. The present study was carried out to evaluate the antioxidant and anti-inflammatory potential of the ethanolic extract of *X. Granatum* by various *in vitro* methods such as 1,1-diphenyl-2-picryl-hydrazil (DPPH) free radical scavenging assay, reducing power assay, ferric reducing antioxidant power (FRAP) and β-carotene bleaching inhibition assay. Total phenolic and flavonoid content were determined. Anti-inflammatory activity was evaluated by *in vitro* human RBC membrane stabilizing assay and *in vivo* mice paw edema test. Ethanolic leaf extract (S2) of *X. granatum* showed significant scavenging effect on DPPH scavenging with a value of IC_{50} 165.95 µg/ml. In addition, it showed significant reducing potential with a value of 59.04 mM of ferrous equivalent per ml in FRAP assay and in reducing power assay the EC_{50} value was determined as 241.61 µg/ml. The ethanolic leaf extracts exhibited 72.3% β-carotene bleaching inhibition. The total phenolic and flavonoid content of the extract were 66µg/ml gallic acid equivalent and 47.66 µg/ml quercetin equivalent per gram of dry extract, respectively. The extract also exhibited 52.63% and 51.05% protection of RBC membrane in hypotonicity and heat induced lysis inhibition, respectively. Significant reduction of mice paw edema (36.34% in 20 µg/kg bw concentration) was observed in the extract. The results revealed that the leaf extract of *X. granatum* possesses strong antioxidant and anti-inflammatory potential.

Introduction

Metabolism implies oxidative processes which are vital for cell proliferation and survival. In the course of metabolism and stepwise oxygen reduction series, reactive oxygen species (ROS) are generated as the natural by product. ROS play an important role in numerous physiological and pathological processes including inflammatory diseases (Droge, 2002). High level of ROS is also involved in carcinogenesis and other diseases related to oxidative damage. Appropriate level of ROS is outmost necessity for cell proliferation, survival, growth and apoptosis. Therefore, maintaining the appropriate level of ROS is essential for healthy condition where antioxidant play crucial role through neutralizing ROS. The increment of intake of exogenous antioxidant would ameliorate the damage caused by oxidative stress. Besides, the demand of various antioxidants is increasing in food industries for functional food and fortification of processed food products. In addition, antioxidants are very useful active ingredients for the manufacturing of cosmetics including dermatological protection of skin from photo damage and aging.

The exogenous antioxidants are mainly derived from food and medicinal plants, such as fruits, vegetables, cereals, mushrooms, beverages, flowers, spices and traditional medicinal herbs (Fu *et al.*, 2011). These natural antioxidants from plant materials are mainly polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamin E and C) (Manach *et al.*, 2004). Generally, these natural antioxidants, especially polyphenols and carotenoids exhibit a wide range of biological effects, such as anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer (Xu *et al.*, 2017; Zhou *et al.*, 2016).

Living in extreme environment, mangrove plants often produce chemical classes of compounds those are often rich in chemical diversity. *Xylocarpus granatum* is a

Cite this article

Islam, M.E., Mahbubur Rahman, S.M., Sohrab, M.H., Biswas, R., Ullah, M.S., Islam, K.D. 2019. Concordance of Antioxidant and Anti-Inflammatory Activity in *Xylocarpus granatum* (Koen). *Journal of Bangladesh Agricultural University*, 17(4): 466–475. https://doi.org/10.3329/jbau.v17i4.44607

common plant of the Sundarbans which is traditionally used in cholera, fever, malaria, inflammation, dysentery, diarrhoea and other abdominal problems (Simlai and Roy, 2013). *X. granatum* has been reported to show central nervous system (CNS) depressant and DPPH radical scavenging activity (Alamgir *et al.*, 2006). Although DPPH free radical scavenging activity has previously been reported, a complete assay panel aiming towards antioxidant activity is yet to be reported in *X. granatum*. Furthermore, antioxidant metabolites often manifest anti-inflammatory activity. Hence, *in vitro* and *in vivo* analysis of the anti-inflammatory potential of *X. granatum* is of prime importance. Besides, a novel insight might emerge if this study could unreveal any concordance between antioxidant and anti-inflammatory activity.

Materials and Methods

Chemicals and reagents

DPPH (1, 1-Diphenyl-2-pycrylhydrazyl), quercetin, ascorbic acid, gallic acid, Folin- Ciocalteu reagent, β-carotene, linoleic acid and carragenan was purchased from Sigma Chemical Co. Ltd, (St. Louis, MO, USA). Merck, Germany. Other chemicals and solvents were purchased from Fisher scientific (Walham, MA 02454, USA) and Loba Chemie Pvt Ltd. (Mumbai, India). Solvents and all other reagents were of analytical grade. The standard drug diclofenac sodium and indomethacine were collected from Square Pharma Ltd, Bangladesh and used for anti-inflammatory activity study.

Sample collection, identification and extraction

Fresh leaves and bark *of X. Granatum* were collected from Chadpai Range, the Sundarbans East Division, Bangladesh. After collection, it was taxonomically identified at Bangladesh National Herbarium, Mirpur, Dhaka (Accession no.: DACB 12789) and the voucher specimen was deposited. The bark and leaves were dried adequately under shade. The chopped and dried bark and leaves were then grinded into fine powder using a mechanical grinder.

Cold extraction was preferred to prepare the extracts from *X. granatum.* For this purpose, the plant materials (bark and leaf) were separated from each other and then cleaned by gentle washing with distilled water followed by air drying for several weeks under shade. The dried material was ground into coarse powder with a motorized plant grinder. The powder was kept in a dry, cool and dark place in a suitable airtight container until analysis commenced. About 400 gm of grinded powder was soaked into 900 ml of ethanol in flat bottomed glass container for a period of 15 days with frequent stirring and shaking. The whole mixture contents were filtered off with clear cotton plug to eliminate plant derbies and the extract was then filtered through Whatman No.1 filter paper. The solvent (ethanol) was evaporated with the electric fan at room temperature and the dried crude extract (yield value 12.5%) was finally obtained. The bark and leaf extract of *X. granatum* was designated as S1 and S2, respectively. The dried crude extract was stored in refrigerator at 4 °C for further study.

Test animal

Swiss-Albino mice of either sex (22 to 27 gm) were used for *in-vivo* ant-inflammatory study. The mice were collected from Animal Resources Department of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The mice were acclimatized by keeping in the polypropylene cages by providing with right rodent foods under standard laboratory condition (room temperature 25±2 °C, relative humidity 55±5%, and 12 hours light). Ethical ideologies and rules for Scientific Experiments on Animals originated by the Swiss Academy of Sciences and the Swiss Academy of Medical Sciences (1995) and the Animal Ethics and Regulation of Khulna University (Research Ref No. KUAEC-2017/08/15) were strictly followed. To draw human blood (RBC membrane lysis and stabilizing assay) procedure and rules of Bangladesh Medical Research Council was strictly obeyed.

In vitro antioxidant activity test

DPPH radical scavenging assay

The free radical scavenging activity of the extract was estimated *in vitro* by DPPH free radical scavenging assay (Afrin *et al.*, 2016). Aliquots of nine concentrations (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml) for plant extract and quercetin (standard) were made by serial dilution technique. Two ml of DPPH solution was applied on each test tube containing 1 ml of plant extract or standard. The final volume of the solution was 3 ml. After 30 minutes of incubation at room temperature, absorbance of each test tube was taken at 517 nm. Inhibition of free radical scavenging activity was calculated using the following equation:

$$\%\,\text{Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100 \quad \text{..... (i)}$$

IC_{50} value (µg/ml) is the inhibitory concentration at which 50% of DPPH radicals are scavenged. A calibration curve of quercetin was developed and IC_{50} value of the sample extracts and standard was calculated.

Reducing power assay

The assay was performed according to the method as described by Chen *et al.* (2014). Five different concentrations (25, 50, 100, 200 and 400 µg/ml) for plant extract and standard ascorbic acid were made.

Phosphate buffer (1.25 ml) and potassium ferricyanide (1.25 ml) were applied on each test tube. The reaction mixture was kept at 50 °C in water bath for 20 minutes and then cooled to room temperature. 1.25 ml of 10% trichloro-acetic acid (TCA) was then added to stop the reaction and centrifuged at 3000 rpm for 10 minutes. The upper layer of centrifuged solution (1.25 ml) was taken in test tube and mixed with 1.25 ml of distilled water. Finally, freshly prepared 250 µl ferric chloride solution was added and mixed by vortexing. The absorbance was measured at 700 nm. A blank was prepared in a similar manner excluding test sample or standard. OD values were plotted against concentrations, slope and intercept of the line was calculated and EC_{50} value was determined.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to Szollosi and Varga (2002). Acetate buffer (300 mM, pH 3.6), 20 mM ferric chloride solution, TPTZ solution (0.031 gm TPTZ in 10 ml 40 mM HCL) and FRAP working reagent (25 ml 300 mM acetate buffer + 2.5 ml TPTZ solution + 2.5 ml H_2O) was prepared. Then, 100 µl plant extract (100 µg/ml) or ascorbic acid solution (250 µM), 900 µl distilled H_2O and 2 ml FRAP working reagent were added to prepare reaction mixture. The reaction mixture was mixed well and kept at 37 °C in water bath (dark condition) for 30 minutes. After cooling, the absorbance was measured at 593 nm. Standard curve of $FeSO_4.7H_2O$ of different concentrations (0, 5, 10, 20 and 40 µM) was plotted and the FRAP value of sample and ascorbic acid was measured from the standard curve straight line equation.

β-Carotene bleaching inhibition assay

The prevention of β-carotene bleaching by plant extracts was assessed according to procedure of Ueno *et al.* (2014). One ml of β-carotene solution (0.2 mg/ml in chloroform) was pipetted into a round bottom flask (50 ml) containing 0.02 ml of linoleic acid and 0.2 ml of 100% Tween-20. The mixture was then evaporated at 40 °C for 10 minutes followed by dilution with 100 ml of distilled water. Then, 5 ml aliquots of the emulsion were transferred into different test tubes containing 0.2 ml of 1 mg/ml plant extracts dissolved in 70% ethanol. The mixture was gently mixed and placed in a water bath at 50 °C for 2 hours. Absorbance of the sample was measured at 0 hour and every 30 min for 2 hours at 470 nm. Inhibition of β-carotene bleaching was calculated using the following equation:

$$\%\,\text{Inhibition} = \frac{1-(\text{Absorbance of the control at time zero})}{\text{Absorbance of the sample after 2 hours}} \times 100 \quad \ldots\ldots \text{(ii)}$$

Quantitative determination of antioxidant

Total phenolic content determination

Total phenolic content was assessed according to Rebaya *et al.* (2014) using Folin–Ciocalteu (FC) reagent. Five concentrations (50, 100, 150, 200 and 250 µg/ml) of gallic acid solution were prepared. The plant extracts were prepared at concentration of 100 µg/ml. Then, 9 ml distilled water and 1 ml FC reagent was added to each tube of gallic acid and extract solution. Then, 10 ml of 7% sodium carbonate was added to the mixture and mixed gently. Distilled water was added to make the volume 25 ml. After incubation at room temperature for 60 minutes, the absorbance was measured at 750 nm. The standard calibration curve of gallic acid was plotted.

Total flavonoid content determination

This method is based on formation of flavonoid-$AlCl_3$ complex which is measured spectrophotometrically according to Rebaya *et al.* (2014). Five different concentrations (50, 100, 150, 200 and 250 µg/ml) of standard quercetin were prepared. The extract was prepared at 100 µg/ml concentration. Then, 5 ml distilled water and 0.3 ml of 5% $NaNO_2$ was added to each tube of quercetin and test extract solution. After five minutes of incubation at room temperature, 0.6 ml of 10% $AlCl_3$ and 2ml of 1 M NaOH was added to the reaction mixture. The mixture was allowed to stand at room temperature for 5 minutes. The absorbance was measured at 510 nm. The standard calibration curve of quercetin was plotted.

Anti-inflammatory assays

In vitro RBC membrane stabilizing assay

With the aim to investigate the anti-inflammatory activity of the plant extracts, effect on erythrocyte membrane was examined. This experiment was based on hypotonicity or heat induced lysis of human RBC cell and measuring of released hemoglobin at 540 nm (Shinde *et al.*, 1999).

Hypotonicity induced lysis inhibition

The packed RBC cells were washed three times with isosaline and 10% (v/v). Aliquots of 5 concentrations (50, 100, 200, 400 and 800 µg/ml) were made by serial dilution technique for plant extract as well as diclofenac sodium (standard). The assay mixture contains 2 ml hyposaline, 1 ml phosphate buffer saline (pH 7.4), 0.5 ml erythrocyte suspension and 1 ml of plant extract (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, and 800 µg/ml) or standard of various concentrations (12.5, 25, 50, 100 and 200 µg/ml). The control treatment was prepared with isosaline instead of the extracts. All the assay mixtures were incubated at room temperature for 10 minutes and then centrifuged at 5000 rpm for 10 minutes. The released hemoglobin content in the supernatant solution was measured at 540 nm.

Heat induced lysis inhibition

The heat induced lysis assay mixture contains 2 ml isosaline, 1 ml phosphate buffer saline (pH 7.4), 0.5 ml erythrocyte suspension and 1 ml of plant extract of different concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, and 800 µg/ml) or standard of various concentrations (12.5, 25, 50, 100 and 200 µg/ml). The assay mixtures were prepared in two sets. One set of tubes was incubated at 54 °C for 10 minutes in water bath and the other set was maintained at 0-5 °C in ice bath. The reaction mixtures were centrifuged at 5000 rpm for 10 minutes. The released hemoglobin content was measured at 540 nm. The percentage of inhibition or acceleration in hemolysis in RBC membrane stabilizing assay was calculated by the equation:

$$\%\,\text{Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100 \quad \text{..... (iii)}$$

In vivo rat paw edema test

Carrageenan-induced rat paw edema is used widely as a working model of inflammation in the search for new anti-inflammatory drug (Sarkhel *et al.*, 2016). Swiss albino mice (28±2 gm) were divided into 8 groups. Each group consisted of 3 mice. Group I mice were treated with carrageenan (1% w/v in normal saline) in the subplanter region of the right hind paw. Group II mice were given normal saline and treated as negative control. Group III mice were administered indomethacin (10 mg/kg, bw) and considered as standard.

Group IV to Group VIII were given increasing doses of ethanolic extracts (1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg bw and 40 mg/kg body weight). Acute paw edema was induced by injecting 0.1 ml of 1% (w/v) carrageenan solution. The linear paw circumference was measured up to 4th hour at hourly interval. The anti-inflammatory activity was measured by using the following equation, where T is thickness of paw in control group and T_0 is thickness of paw edema in the test group.

$$\%\,\text{Inhibition of edema} = \frac{T - T_0}{T} \times 100 \quad \text{..... (iv)}$$

Statistical Analysis

One-way ANOVA analysis followed by Benferroni test and correlation analysis was performed using GraphPad Prism 6 software.

Results

Antioxidant activity

One single method cannot precisely assess the antioxidant capacities due to the complex nature of different phytochemical classes present in plant extracts. In the present work, we included DPPH free radical scavenging, reducing power assay, Ferric reducing antioxidant power (FRAP) and β-carotene assays to test the antioxidant activities of the ethanolic extracts of *X. granatum* bark (S1) *and* leaf (S2).

DPPH free radical scavenging activity

The DPPH scavenging percentage of S1 and S2 was found 53.31% and 58.49%, respectively as compared to reference standard quercetin 72.73%. The S2 showed better DPPH scavenging potential with IC_{50} value of 128.24 µg/ml while quercetin possessed IC_{50} of 7.63 µg/ml (Figure 1b). DPPH scavenging effect (%) of the tested sample extracts was found to be concentration dependent (Figure 1a). Both S1 and S2 demonstrated significant DPPH free radical scavenging activity compared to standard quercetin (p value 0.0281).

Reducing power assay

The EC_{50} value of S2 was 241.61 µg/ml as compared to reference standard ascorbic acid with a value of 41.02 µg/ml (Figure 1c). Reduction of Potassium ferricianide to potassium ferrocianide of the tested sample extracts was found to be concentration dependent. Significant effect (p value 0.0342) in reducing ferric to ferrous by both S1 and S2 was observed as compared to standard ascorbic acid.

Ferric reducing antioxidant power (FRAP) assay

This method is based on the conversion of the ferric tripyridyltriazine [Fe (III)-TPTZ] complex to the ferrous tripyridyltriazine [Fe(II)-TPTZ] by the action of electron donating antioxidants. Figure 1d represents the standard curve of ferrous sulphate with known different concentrations which is used to calculate FRAP value of the test samples. The FRAP value of the S2 was found as 59.05 µM Fe^{+2}equivalent while the standard ascorbic acid showed the value of 86.66 µM Fe^{+2} equivalent (Figure 1e). Significant ferric reducing potential (p value 0.0214) was observed in both S1 and S2 as compared to standard ascorbic acid.

β-carotene bleaching inhibition assay

This method is based on the inhibitory potential of the bleaching of β-carotene by the antioxidants present in the test sample.

Oxidized linoleic acid was used to bleach β-carotene. The percentage of inhibition of β-carotene bleaching was found as 72.37% for S2 while the standard BHT exhibited 98.85% bleaching inhibition (Figure 1f). The β-carotene bleaching inhibition was found significant in both S1 (p value 0.0341) and S2 (p value 0.0082) compared to standard BHT.

Total phenolic content

The total phenolic content of plant extracts is shown in Figure 2a. S1 and S2 exhibited the total phenolic content (29.5 mg and 66 mg gallic acid equivalent/gm of dry extract, respectively). Both S1 (p value 0.0354) and S2 (p value 0.0021) demonstrated the presence of significant amount of Phenolic content compared to control group.

Total flavonoid content

The flavonoid content of S1 and S2 was 25.67 mg and 47.67 mg quercetin equivalent of dry extract, respectively (Figure 2b). Significant amount of flavonoid in both S1 (p value 0.0213) and S2 (p value 0.0021) compared to control group.

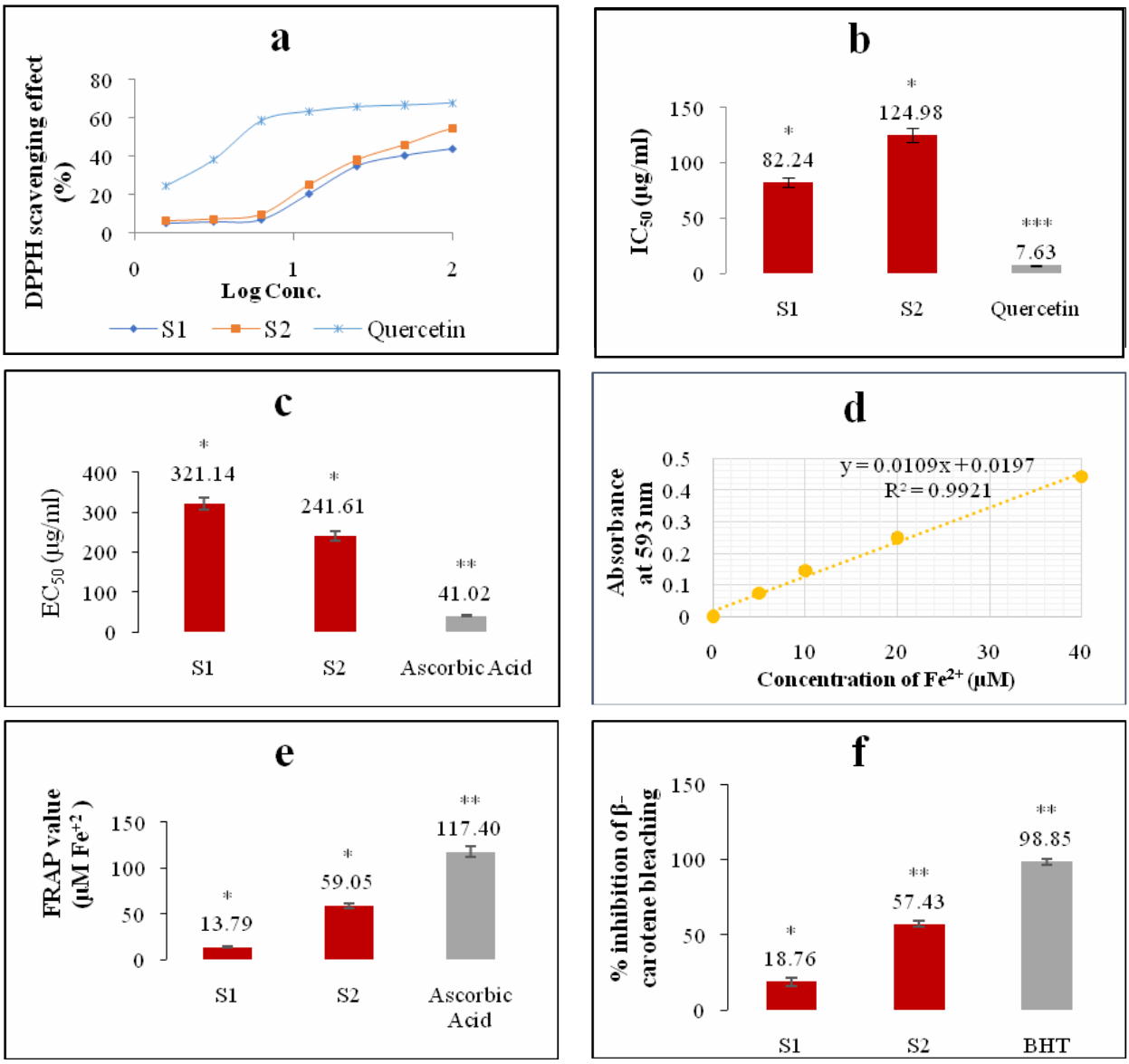

Fig. 1 Antioxidant assays of tested crude ethanolic bark and leaf extract of *X granatum* (S1 and S2, respectively): (a) DPPH free radical scavenging effect in percentage; (b) IC_{50} (µg/ml) value of DPPH free radical scavenging assay;(c) EC_{50} (µg/ml) value of reducing power assay; (d) Standard curve of Fe^{+2} (µM) of FRAP assay; (e) FRAP value (µM Fe^{+2}); (f) β-carotene bleaching inhibition (%). * denotes $p<0.05$, ** denotes $p < 0.01$ and *** denotes $p< 0.001$ compared to the respective control group

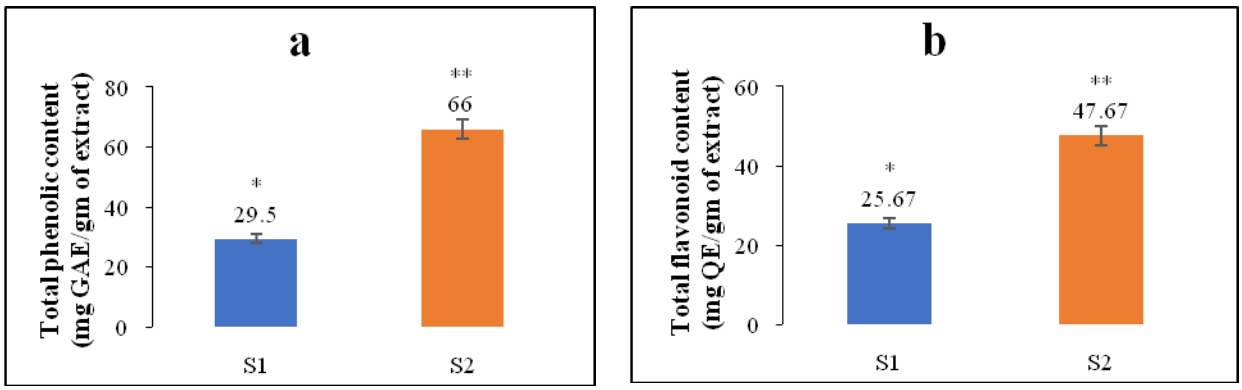

Fig. 2 Phenolic and flavonoid content of crude ethanolic extract of bark and leaf of *X. granatum* (S1 and S2, respectively) (a) Total phenolic content; (b) Total flavonoid content. Statistical analysis was performed using One way ANOVA followed by Bonferroni test, * denotes $p < 0.05$ and ** denotes $p < 0.01$ compared to the control group

Correlation between antioxidant activity and phenolic/ flavonoid contents

The linear correlation between the antioxidant capacity (measured by DPPH, reducing power, FRAP and β-carotene bleaching inhibition assay) and total phenolic content/total flavonoid content of the extracts (S1, S2) are shown in Table 1 and Figure 3. Strong and significant correlation was observed between total phenolic content and antioxidant activity was. Correlation between total flavonoid content and antioxidant activity was also significant. The results are in agreement with previous reports that showed a linear correlation between the antioxidant activity and total content of phenolic and flavonoid of some plant extracts (Ksiksi and Hamza, 2012).

Table 1. Linear correlations between antioxidant activities and the amount of total phenolic content/ total flavonoid content of the tested sample extracts (S1, S2, S3 and S4)

Assay	Correlation (R^2) (phenol vs assays)	Significance	Correlation (R^2) (flavonoid vs assays)	Significance
DPPH•	0.96	($P < 0.001$)**	0.92	($P < 0.01$)**
Reducing power	0.99	($P < 0.001$)**	0.99	($P < 0.001$)**
FRAP	0.93	($P < 0.01$) *	0.96	($P < 0.001$)**
â-carotene	0.91	($P < 0.01$)*	0.96	($P < 0.001$)**

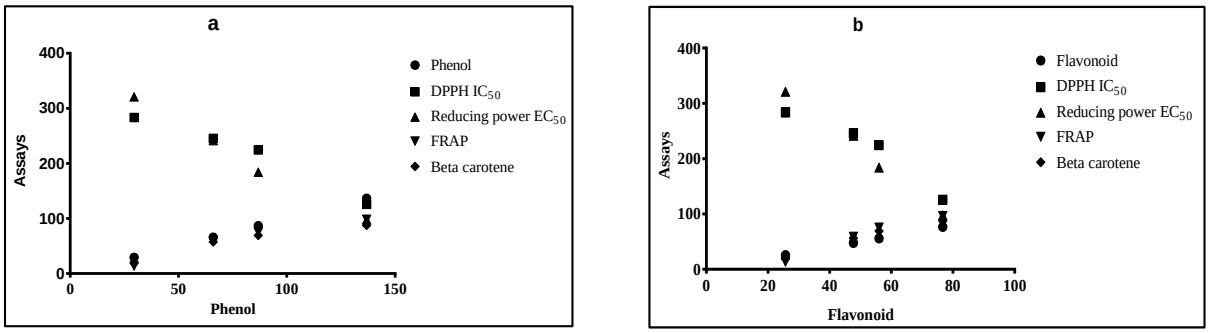

Fig. 3 Correlation between antioxidant content and antioxidant activities of *X. granatum* bark and leaf ethanolic extract: (a) Linear correlation between phenol content and antioxidant assays and (b) Linear correlation between flavonoid content and antioxidant assays.

Hypotonicity and heat induced lysis inhibition

The extracts were found to inhibit the haemolysis of erythrocytes induced by both hypotonic solution and heat (Table 2). The S2 showed better inhibition of erythrocyte lysis (52.63% at 800 µg/ml concentration with IC_{50} value of 586.44 µg/ml) while the standard diclofenac sodium exhibited 91.66% inhibition, with IC_{50} value of 69.33 µg/ml at 200µg/ml concentration. In heat induced lysis inhibition, 46.18 (%) inhibition of RBC lysis was observed in S2 while 69.52 (%) inhibitions were demonstrated by diclofenac sodium. Two-way ANOVA analysis revealed significant effect in inhibition of erythrocyte lysis for both S1 and S2 at $p < 0.001$ comparing with the control.

Mice paw edema reduction activity (In vivo)

The paw circumstances of carrageenan induced mice treated with plant extracts and reference standard indomethacine was measured up to 4th hour in hourly interval. The initial increase of edema thickness up to 30 minutes to 1 hour was observed due to carrageenan. The edema thickness subsequently reduced with time for the administration of extract and standard (Figure 4). It was found that 34.72% and 36.34% inhibition of edema thickness was achieved with S1 and S2 treated mice group at 20 µg/kg bw concentration, respectively while the standard indomethacine inhibited 54.1% at 10 µg/kg bw concentration. Two-Way ANOVA analysis also revealed significant effect in edema reduction by both S1 and S2 when compared to carrageenan control group at $p < 0.05$.

Correlation among antioxidant, in vitro and in vivo anti-inflammatory activity

Linear correlation was observed between antioxidant and anti-inflammatory activity as well as *in vitro* anti-inflammatory activity and *in vivo* anti-inflammatory activity (Table 3, Figure 5). The R^2 value ranges from 0.82 to 0.99. Hypotonicity induced RBC lysis inhibition and mice paw edema reduction (%) demonstrated strong correlation (R^2 value 0.99 and 0.95, respectively) and heat induced RBC lysis inhibition exhibited moderate correlation (R^2 value = 0.82) with DPPH scavenging activity. Furthermore, hypotonicity induced RBC lysis inhibition showed strong correlation ($R^2 = 0.94$) and heat induced RBC lysis inhibition showed moderate correlation (R^2 = 0.86) against paw edema reduction activity.

Table 2. RBC membrane stabilizing assay: (%) inhibition of RBC lysis and IC_{50} values of sample extracts with hypotonicity induced and heat induced lysed RBC is shown

Sample	Conc. (µg/ml)	Hypotonicity induced lysis			Heat induced lysis		
		% inhibition of lysis ± SD	IC_{50} (µg/ml)	Two-Way ANOVA	% inhibition of lysis ± SD	IC_{50} (µg/ml)	Two-Way ANOVA
Diclofenac	12.5	10.84 ± 0.006			3.93 ± 0.014		
sodium	25	18.87 ± 0.004			25.35 ± 0.007		
	50	56.22 ± 0.024	69.33	S****	33.83 ± 0.013	121.79	S****
	100	89.95 ± 0.002		(P<0.001)	49.57 ± 0.002		(P<0.001)
	200	91.66 ± 0.004			69.52 ± 0.004		
S1	50	34.51 ± 0.005			2.84 ± 0.002		
	100	44.44 ± 0.001			19.10 ± 0.005		
	200	46.57 ± 0.001	621.72	S**	30.48 ± 0.006	804.57	S**
	400	48.46 ± 0.009		(P<0.001)	41.46 ± 0.003		(P<0.001)
	800	51.06 ± 0.004			43.49 ± 0.003		
S2	50	24.12 ± 0.002			16.31 ± 0.01		
	100	35.52± 0.003			29.16 ± 0.005		S**
	200	40.35± 0.001	586.44	S***	36.11 ± 0.007	797.62	(P<0.001)
	400	51.75 ± 0.005		(P<0.001)	42.70 ± 0.006		
	800	52.63± 0.005			46.18 ± 0.006		

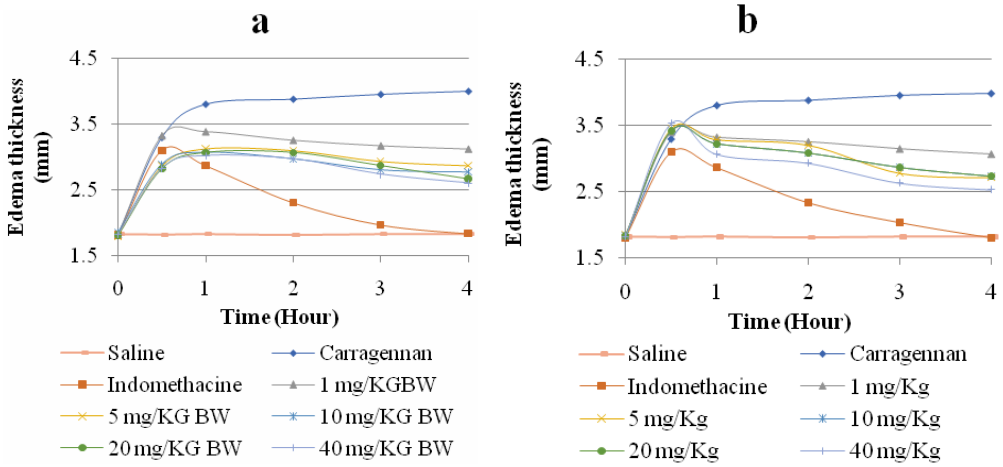

Fig. 3 A series of matched experiments demonstrating the dose-dependent inhibition of carrageenan-induced mice paw edema by 1-40 µg/ml sample extracts. Carrageenan: negative control; Indomethacine: reference standard (positive control); mg/KG BW: mg/KG body weight of mice (treatment). (a) Carrageenan induced edema thickness and reduction by S1; (b) Carrageenan induced edema thickness and reduction by S2.

Table 3. Correlation between antioxidant and anti-inflammatory activity; between in vitro anti-inflammatory and in vivo anti-inflammatory activity

	Assay	Correlation (R^2)	Significance
Antioxidant activity vs anti-inflammatory activity	DPPH vs hypotonicity induced RBC lysis inhibition	0.99	P < 0.01 (***)
	DPPH vs Heat induced RBC lysis inhibition	0.82	P < 0.05 (*)
	DPPH vs mice paw edema reduction %	0.95	P < 0.05 (*)
In vitro anti-inflammatory activity vs *In vivo* anti-inflammatory activity	Hypotonicity induced RBC lysis inhibition vs paw edema reduction	0.94	P < 0.05 (*)
	Heat induced RBC lysis inhibition vs paw edema reduction	0.86	P < 0.05 (*)

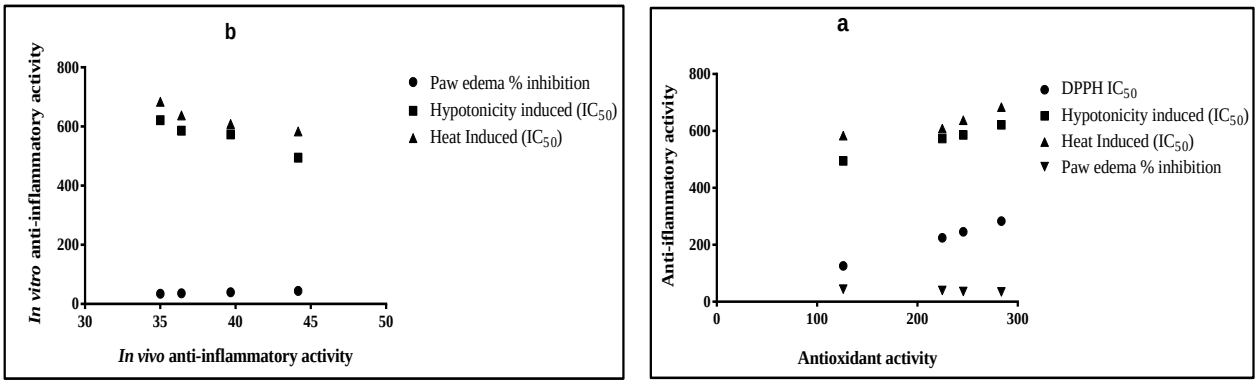

Fig. 4 Correlation among the activities of *X. granatum* bark and leaf ethanolic extracts: (a) Linear correlation between antioxidant activity assay (DPPH• scavenging) and anti-inflammatory assay (Hypotonicity and heat induced RBC lysis inhibition, mice paw edema % inhibition); (b) Linear correlation between *in vivo* anti-inflammatory activity (mice paw edema % inhibition and *in vitro* anti-inflammatory activity (hypotonicity and heat induced lysis inhibition of RBC)

Discussion

Oxidation and generation of reactive oxygen species (ROS) is a regular process in cellular metabolism. Antioxidant, the plant secondary metabolites are mostly phenolic compounds which play vital role in neutralizing free radicals of ROS by donation of electron or by inhibition of enzymes involved in oxidation and inflammation. Inflammation is currently treated by non-steroidal anti-inflammatory drugs (NSAIDs) which unfortunately have side effects with increasing risk of blood clot resulting heart attack. Therefore, development of potent natural products with compelling antioxidant and anti-inflammatory properties are now under consideration.

In our study, significant antioxidant activity was observed in leaf extract of *X. granatum* (S2) as compared to reference standards. The total phenol and flavonoid content were similarly higher in S2 (Figure 2). Linear correlation between total phenol and antioxidant activity and between total flavonoid and antioxidant reflecting the agreement of content and activity of antioxidant among the tested plant extracts. Zamani *et al.* (2015) reported plant antioxidant activity of *X. granatum* seed kernel with IC_{50} 10.61 µg/ml. Similarly, *in vitro* antioxidant activity has been reported by Das *et al.* (2019). The antioxidant potential of *X. granatum* revealed in this study is in close agreement with both above mentioned reports.

The *in vitro* anti-inflammatory assay revealed the potential of S1 and S2 as compared to standard diclofenac sodium in both hypotonicity and heat induced lysis inhibition (Table 2). Carrageenan, a polysaccharide used to induce inflammation, is one of the most suitable procedures to explore anti-inflammatory activity. Carrageenan paw edema is sensitive to cycloxygenase and lipoxygenase inhibition.

The enzyme cycloxygenase is directly involved in inflammation through production of prostaglandin and lipoxygenase is also indirectly causes inflammatory response (Pidgeon *et al.*, 2007). The extracts tested in

this study demonstrated significant percent inhibition ($p < 0.05$) of paw edma in 4 hours (S1: 35%, S2: 34.72% at 40 mg/kg bw). Although there is no report of anti-inflammatory activity of *X. granatum*, an indirect anti-inflammatory activity mediated by cycloxygenase, 5-lipoxygenase and acetylcholine esterase inhibition has been reported in *X. muluccensis* (Eldeen *et al.*, 2016). Jahan *et al.* (2012) also reported anti-inflammatory activity of the kernel root of another species of *Xylocarpus* genus namely *X. mekongensis* (Lamk.) M. Roem. These published reports indicate the presence of anti-inflammatory principles in the genus *Xylocarpus* and our finding further substantiates such claim. It is noteworthy to mention that we report for the first time the anti-inflammatory potential of *X. granatum* ethanolic bark and leaf extracts. From the panel of antioxidant and anti-inflammatory assays it can be inferred that both ethanolic bark and leaf extract of *X. granatum* possesses potent antioxidant and anti-inflammatory activity. The linear correlation between antioxidant activity and anti-inflammatory activity and between *in vitro* and *in vivo* anti-inflammatory activity indicate the anti-inflammatory manifestation which is executed through antioxidation. Linearity in correlation hence substantiates that the anti-inflammatory activity is contributed by the antioxidant molecules present in the sample plant extracts.

Conclusion

This study substantiates the presence of antioxidant metabolites of polyphenolic origin in *X. granatum* growing abundantly in the Sundarbans mangrove forest, the largest mangrove block in the world situated in the southern coastline of Bangladesh and India. The leaf extract of *X. granatum* exhibited strong anti-inflammatory activity that we infer due to the antioxidant metabolites. Besides, the significant presence of polyphenols, particularly flavonoids, along with their correlation with antioxidant and anti-inflammatory activity hence shed light on further extensive research to find out the active principle(s) responsible for the said activities. The bioactivity associated with *X. granatum* might find phrma-, neutra- and cosmeceutical as well as agrochemical implications.

Acknowledgements

Experimental facilities were provided by Biochemistry and Molecular Biology Laboratory, Khulna University, Bangladesh. We acknowledge the Sundarbans East Division, Ministry of Environment, Forest and Climate Change, Government of Bangladesh for necessary administrative and logistic support in collecting plant sample material.

Funding

The work was financially supported by Grant for Advanced Research in Education (2016-17/64), Ministry of Education, Government of Bangladesh.

References

Afrin, S., Pervin, R., Sabrin, F., Rony, S. R., Sohrab, M. H., Islam, M.E., Islam, K.D. and Billah, M. M., 2016. *In vitro* Antioxidant Activity, Antimicrobial and Preliminary Cytotoxic Activity *of Cynometra ramiflora*- A mangrove plant, *Journal of Microbiology, Biotechnology and Food Sciences*,6 (2): 844–850

Alamgir, M., Alam, S.M.S., Alaul, M., Rashid, M., Hasan, M. and Choudhuri, M.S.K., 2006. Preliminary evaluation of some medicinal plants of Sundarbans mangrove forest on central nervous system. *Oriental Pharmacy and Experimental Medicine*, 6(3): 215–220.

Chen, Y., Miao, Y., Huang, L., Li, J., Sun, H., Zhao, Y., Yang, J., and Zhou, W., 2014. Antioxidant activities of saponins extracted from Radix Trichosanthis: an *in vivo* and *in vitro* evaluation. *BMC Complementary and Alternative Medicine*, 14(1): 86. https://doi.org/10.1186/1472-6882-14-86

Das, SK, Samantaray D, Sahoo SK, Pradhan SK, Samanta L and Hrudayanath T., 2019. Bioactivity guided isolation of antidiabetic and antioxidant compound from *Xylocarpus granatum* J. Koinig bark. *3 Biotech*, 9 : 198–206. https://doi.org/10.1007/13205-019-1711-y

Droge, W., 2002. Free radicals in the physiological control of cell function. *Physiological Reviews*, 82(1): 47–95. https://doi.org/10.1152/physrev.00018.2001

Eldeen, I.M.S., Mohamed H, Tan W, Siong JYF, Andriani Y and Tengku-Muhammad TS., 2016. Cycloxygenase, 5-lipoxygenase and acetylcholine-esterase inhibitory effects of fractions containing ȧ-guaine and oil isolated from the root of *Xylocarpus moluccensis*. *Research Journal of Medicinal Plants*, 10 : 286–294. https://doi.org/10.3923/rjmp.1016.286.294

Fu, L., Xu, B.-T., Xu, X.-R., Gan, R.-Y., Zhang, Y., Xia, E.-Q. and Li, H.-B., 2011. Antioxidant capacities and total phenolic contents of 62 fruits. *Food chemistry*, 129(2): 345–350.

Jahan, I.A., Hossain, M.H., Nimmi, I., Islam, S., Kawsar, M.H. and Isalm, A. 2012. Evaluation of anti-inflammatory and antioxidant potential of the kernel root of *Xylocarpus mekongensis* (Lamk.) M. Roem. *Oriental Pharmacy and Experimental Medicine*, 12:181–188. https://doi.org/10.1007/13596-012-0068-0

Ksiksi, T. and Hamza, A.A., 2012. Antioxidant, Lipoxygenase and Histone Deacetylase Inhibitory Activities of *Acridocarpus orientalis* from Al Ain and Oman. *Molecules*, 17(11): 12521–12532. https://doi.org/10.3390/molecules171112521

Kumar, V. and Tyagi, D., 2013. Phytochemical screening and free-radical scavenging activity of *Bergenia stracheyi*. *Journal of Pharmacognosy and Phytochemistry*, 2(2): 175–180.

Manach, C., Scalbert, A., Morand, C., Remesy, C., and Jimenez, L., 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, 79(5): 727–747. https://doi.org/10.1093/ajcn/79.5.727

Pidgeon, G.P., Lysaght, J., Krishnamoorthy, S., Reynolds, J.V., O'Byrne, K., Nie, D. and Honn, K.V., 2007. Lipoxygenase metabolism: roles in tumor progression and survival. *Cancer and Metastasis Reviews*, 26(3-4): 503–524. https://doi.org/10.1007/s10555-007-9098-3

Rebaya, A., Belghith, S.I., Baghdikian, B., Leddet, V.M., Mabrouki, F., Olivier, E., Cherif, J. and Ayadi, M.T., 2014. Total phenolic, total flavonoid, tannin content, and antioxidant capacity of *Halimium halimifolium* (Cistaceae). *Journal of Applied Pharmaceutical Science*, 5(1): 52–57.

Sarkhel, S., 2016. Evaluation of the anti-inflammatory activities of *Quillaja saponaria* Mol. saponin extract in mice. *Toxicology Reports*, 3: 1–3. https://doi.org/10.1016/j.toxrep.2015.11.006

Shinde, U., Phadke, A., Nair, A., Mungantiwar, A., Dikshit, V. and Saraf, M., 1999. Membrane stabilizing activity—a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. *Fitoterapia*, 70(3): 251–257.

Simlai, A., and Roy, A., 2013. Biological activities and chemical constituents of some mangrove species from Sundarban estuary: An overview. *Pharmacognosy Reviews*, 7(14): 170–178. https://doi.org/10.4103/0973-7847.120518

Szollosi, R. and Varga, I.S. 2002. Total antioxidant power in some species of Labiatae (Adaptation of FRAP method). *Acta Biologica Szegediensis*, 46 (3): 125–127.

Ueno, H., Yamakura, S., Arastoo, R.S., Oshima, T. and Kokubo, K., 2014. Systematic Evaluation and Mechanistic Investigation of Antioxidant Activity of Fullerenols Using β-Carotene Bleaching Assay. *Journal of Nanomaterials*, 2014: 7. https://doi.org/10.1155/2014/802596

Wangensteen, H., M. Duong, G., Alamgir, M., Sarder, M., B. Samuelsen, A. and E. Malterud, K., 2006. Biological activities of limonoids, catechins, procyanidins and extracts from *Xylocarpus granatum*. *Natural Product Communications*, 1(11): 985–990. https://doi.org/0.1177/1934578X0600101113

Winter, C.A., Risley, E.A. and Nuss, G.W., 1962. Carrageenin-induced edema in hind paw of the rat as an assay for anti-iflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine*, 111: 544–547. https://doi.org/10.3181/00379727-111-27849

Xu, D.-P., Li, Y., Meng, X., Zhou, T., Zhou, Y., Zheng, J., Zhang, J.-J. and Li, H.-B., 2017. Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources. *International Journal of Molecular Sciences*, 18(1): 96. https://doi.org/10.3390/ijms18010096

Zamani, Nr., Gazali, M, and Batubara, I. 2015. The study of tyrosinase and antioxidant activity of *Xylocarpus granatum* Koenig seed kernel extract towards evidence based indigenous knowledge from Togean Archipelago, Indonesia. *Jounal of Marine Science Research and Development*, 5: 168–172. https://doi.org/10.4172/2155-9910./000168

Zhou, Y., Zheng, J., Li, Y., Xu, D.P., Li, S., Chen, Y.M. and Li, H.B., 2016. Natural Polyphenols for Prevention and Treatment of Cancer. *Nutrients*, 8(8): 515. https://doi.org/10.3390/nu8080515