

Short Communication

Antioxidant content and activity of the Indian fresh-water pearl mussel in the prevention of arthritis in an experimental animal model

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Abstract

The flesh of the Indian fresh-water mussel, *Lamellidens marginalis* (LM; Lamarck, 1819), is the byproduct of pearl culture and a cheap protein source. The present study investigated the antioxidant content of this ethnomedicinally cited species to outline its importance in food security and disease prevention. LM was found to be rich in polyphenol antioxidants with good correlation with its reducing capacity. LM also showed a significant free-radical-scavenging activity, H₂O₂-scavenging activity and Fe-chelating activity. To study the effect of this dietary antioxidant against oxidative stress, we took inflammatory arthritis as a model. LM-treated arthritis rats showed a higher antioxidant defence system with elevated superoxide dismutase, total thiol, glutathione *S* transferase, glutathione peroxidase, total antioxidant status and catalase concentration of haemolysate. Oxidative stress markers like serum thiobarbituric acid-reacting substances, methyl glyoxal, NO and total oxidant status levels were decreased in LM-treated arthritis rats. Hence, the dietary antioxidants of LM were found to be effective in the prevention of oxidative stress in inflammatory arthritis. In conclusion, LM, the cash-crop byproduct, provides a rare opportunity for income and nutrition, not only by providing cheap and available energy, protein and dietary factors, but also by providing antioxidants effective against chronic inflammatory disease.

Key words: *Lamellidens marginalis*: Antioxidants: Arthritis: Nutrition

In developing countries, where commercial farming is essential for income, food security is being compromised as a consequence of deviation from subsistence production⁽¹⁾. The low-income-group population has therefore come to rely more on cheap energy from foods devoid of antioxidants and other dietary factors⁽²⁾. Developing countries have thus become the cohabitat of diseases like malnutrition and obesity⁽³⁾, cancer and chronic diseases. Hence, to address this problem, we should look for foods which can help generate income as well as provide proper nutrition and food security.

In the present paper, we have studied the antioxidant content and activity of a commercially important fresh-water pearl mussel, the *Lamellidens marginalis* (LM; Lamarck, 1819). This species can be considered as an important food source to solve the contradiction between commercial farming and

food security, as the energy- and protein-rich flesh of the mussel is a byproduct of pearl culture. Substantiated by the antioxidant content, rarely found in animal protein, the flesh can be a complete package of energy and dietary factors besides being cheap, ethnic and easy to culture.

LM is available in the densely populated food-insecure inlands of India, Bangladesh, Burma, Sri Lanka and Nepal. The edible foot portion has been reported to have ethnomedicinal usage⁽⁴⁾. Mussels are unique in their content of phenol proteins⁽⁵⁾ and have anti-inflammatory effects in experimental models and clinical studies⁽⁶⁾. Hence, we studied the antioxidant content of mussel and explored the effect in *in vitro* and in an adjuvant-induced arthritis model. The antioxidant defence system of marine mussels was reported in the perspective of pollution and heavy-metal toxicity⁽⁷⁾.

Abbreviations: ~ SH, total thiol; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; FCA, Freund's complete adjuvant; GPx, glutathione peroxidase; GST, glutathione *S* transferase; LM, *Lamellidens marginalis*; SOD, superoxide dismutase; TAS, total antioxidant status; TOS, total oxidant status.

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However, the dietary significance of the antioxidant has so far not been reported. This information is a rare example of integration of commerce and nutrition, both for energy and disease prevention, as a perfect piece to fit in the jigsaw of integrated nutritional approach.

Materials and methods

Chemicals

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium (ABTS) salt, EDTA, reduced glutathione, metaphosphoric acid, Folin-Ciocalteu reagent, 1-chloro-2,4-dinitrobenzene, potassium ferricyanide, ferrozine, pyrogallol and sodium nitroprusside were purchased from SRL. Thiobarbituric acid, NADPH, *tert*-butyl hydroperoxide, ferric chloride, Freund's complete adjuvant (FCA) and indomethacin, Trolox, *O*-dianisidine dihydrochloride, xylenol orange and 1,2-diamino benzene were purchased from Sigma.

Animals

Wistar strain male albino rats, about 9–12 weeks old (120 (SE 10) g), were used for the *in vivo* experiments. The animals were collected and housed in a controlled environment (room temperature: $23 \pm 2^\circ\text{C}$, relative humidity: 60 (SE 5)%, 12 h day–12 h night cycle) and fed *ad libitum* with a balanced diet and water. All animal experiments were approved by the Departmental Animal Ethical Committee and were in accordance with the guidelines of the committee for the purpose of control and supervision of experiments on animals, Government of India.

Collection of sample and preparation of extract

Live adult fresh-water mussels were collected from the local market of Kolkata, India, and the species was identified as LM (voucher specimen no.: M26322/5) from the Mollusca Section of Zoological Survey of India, New Alipore, Kolkata, India. Aqueous extract of LM was prepared and expressed as per μg dry weight for *in vitro* experiments, and as per mg wet weight for *in vivo* experiments.

Determination of total phenol, reducing power, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium radical-scavenging activity, total antioxidant capacity, hydrogen peroxide-scavenging and metal-chelating activity

The total phenol content of the extract was determined by Folin-Ciocalteu's reagent, according to the method of Taga *et al.*⁽⁸⁾. The phenol content was evaluated from gallic acid standard curve (5–200 μg) and the value was expressed in terms of gallic acid equivalents.

Reducing power of the extract was determined according to Oyaizu⁽⁹⁾ and expressed in terms of ascorbic acid equivalent.

The ABTS⁺ radical-scavenging activity of LM was assessed according to Re *et al.*⁽¹⁰⁾ and total antioxidant capacity was

determined by comparing the ABTS⁺ radical-scavenging activity with trolox (0–2 mM) standard.

H₂O₂-scavenging activity of the extract was determined according to Nabavi *et al.*⁽¹¹⁾ and expressed as percentage of H₂O₂ scavenged.

The Fe²⁺-chelating activity of the extract was determined according to Ebrahimzadeh *et al.*⁽¹²⁾. The percentage inhibition of Fe²⁺-ferrozine complex formation by the extract was calculated according to the formula:

$$\text{Inhibition (\%)} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / (\text{Abs}_{\text{control}}) \times 100,$$

where Abs_{control} and Abs_{sample} are the absorbance of control and absorbance of sample, respectively. The metal-chelating activity of LM at the dose 100 mg/ml was expressed in terms of ascorbic acid equivalents.

Induction of oxidative stress associated with inflammatory arthritis and evaluation of oxidative stress defence system and pro-oxidant markers

Arthritis was induced in rats and the experiment was carried out as described by Chakraborty *et al.*⁽¹³⁾. Experimental animals were divided on the next day after adjuvant injection into the following five groups, I–V, for respectively, the saline-injected normal control, FCA-injected arthritic control, arthritic animals supplemented with LM 1 (500 mg/kg per d, per oral), LM 2 (1 g/kg per d, per oral) and indomethacin (1 mg/kg per d, per oral). Treatment was given from 1 to 13 d. On the 15th day, blood was collected, and serum (as per mg of protein⁽¹⁴⁾) and haemolysate (as per mg of Hb) markers of oxidative stress were assessed.

Serum superoxide dismutase (SOD) activity was estimated by measuring the percentage inhibition of the pyrogallol auto-oxidation by SOD according to the standard method⁽¹⁵⁾. Here, one unit of SOD was defined as the enzyme activity that inhibits the auto-oxidation of pyrogallol by 50%. Catalase activity of haemolysate was estimated using the method of Beers & Sizer⁽¹⁶⁾. Serum sulphhydryl group content was assayed as described by Elman⁽¹⁷⁾. Serum glutathione peroxidase (GPx) activity was estimated using the method of Paglia & Valentine⁽¹⁸⁾. Serum glutathione transferase (GST) activity was estimated using the method of Habig & Jakoby⁽¹⁹⁾. Serum total antioxidant status (TAS) value was estimated using the method of Re *et al.*⁽¹⁰⁾, Serum lipid peroxidation level by Buege & Aust⁽²⁰⁾ and serum NO synthase activity by the method of Granger *et al.*⁽²¹⁾. Total oxidant status (TOS) was assessed using the method of Erel⁽²²⁾ and oxidative stress index, the ratio of TOS and TAS, was measured by Harma *et al.*⁽²³⁾ and expressed as arbitrary units. To perform the calculation, the result unit of TAS was changed to μM Trolox equivalents, and oxidative stress index value was calculated as follows: oxidative stress index = ((TOS, μM -H₂O₂ equivalent) \times 100 / (TAS, μM -Trolox equivalents)).

Statistical methods

All the results were expressed as means with their standard errors, *n* 6. The level of significance was determined by

one-way ANOVA followed by Tukey's *post hoc* test. A value of $P < 0.05$ was considered as significant. Pearson's correlation coefficient (r) was evaluated between total phenol content and reducing power of LM. All statistical analyses were performed using Origin 7 and MS-Office Excel 2007 software packages.

Results

Antioxidant content of *Lamellidens marginalis* extract

The total phenol content of LM was found to be 82.81 (SE 0.75) μg gallic acid equivalent per mg of LM. Reducing power of per mg LM was comparable with 16.56 (SE 1.06) μg of ascorbic acid. Correlation between phenol content and reducing power of LM ($r = 0.98$) was found to be significant ($P = 0.003$).

LM showed ABTS⁺-scavenging activity and H₂O₂-scavenging activity dose dependently. The half maximal inhibitory concentration value of LM for ABTS⁺-scavenging activity was 7.81 mg/ml (1.299 mM-Trolox equivalents) and for H₂O₂-scavenging activity was 0.343 mg/ml.

LM showed 19.74% inhibition in Fe²⁺-ferrozine complex formation at the dose of 100 mg/ml concentration which was found to be 7.086 μg EDTA equivalents.

In vivo antioxidant activity of *Lamellidens marginalis* extract in Freund's complete adjuvant-induced arthritis model after oral supplementation

LM treatment significantly restored antioxidant defence systems. SOD, total thiol ($\sim\text{SH}$), GST level in serum, and catalase concentration of haemolysate were changed significantly in the arthritis group of animals, as shown in Table 1. Serum SOD, $\sim\text{SH}$, GST, GPx, TAS level and catalase concentration of haemolysate were found to be significantly decreased ($P < 0.05$) in adjuvant-injected arthritic rats when compared with normal rats after the 15th day of FCA injection. LM1,

LM2 and indomethacin-treated rats showed significant increases ($P < 0.05$) in serum SOD, $\sim\text{SH}$, GST, GPx level and catalase concentration on the 15th day after FCA injection when compared with arthritic rats.

The pro-oxidant markers, namely, serum thiobarbituric acid-reacting substance level, nitric oxide and TOS level, were found to be significantly increased ($P < 0.05$) in adjuvant-injected arthritic rats when compared with normal rats after the 15th day of FCA injection. LM1, LM2 and indomethacin-treated rats showed significant decreases ($P < 0.05$) in serum thiobarbituric acid-reacting substances, TOS and nitric oxide level on the 15th day after FCA injection when compared with arthritic rats (Table 1).

Discussion

An epidemiological association of decreased incidence of age-related diseases in humans with diets rich in polyphenols and antioxidants⁽²⁴⁾ has made antioxidant-rich plant-based foods more valued than antioxidant-scarce animal protein⁽²⁵⁾, which is a paradox in protein-energy malnutrition. Phenolic antioxidants of the Indian fresh-water pearl-producing mussel, LM, can address this problem and help in food security, as it is a more nutritious⁽²⁶⁾ alternative to meat, is cheaper, and is available as a byproduct of the cash-crop, pearl. Prabhakar & Roy⁽⁴⁾ showed that the foot portion of the mollusc is eaten for its ethnomedicinal benefits in North-Bihar, India. Polyphenol proteins have been reported to be secreted from the specialised phenol glands of the mussel foot⁽⁵⁾. Polyphenols were reported as the main antioxidants of mussels in pollution defence⁽²⁷⁾. Polyphenol antioxidants are found in plants and none so far has been reported in animals/meat consumed as food. These observations have led us to explore the antioxidant activity of the foot-pad of LM and to study its correlation with the phenol content of the foot-pad.

Thus, the experiments were designed to determine the total phenol content, its correlation with the free-radical-scavenging and antioxidant activity of LM extract in the

Table 1. Effect of *Lamellidens marginalis* (LM) and standard on serum antioxidant marker levels in Freund's complete adjuvant-induced arthritic animal model

(Mean values with their standard errors, $n = 6$)

Parameter	Control		Arthritis		Standard		LM1 (500 mg/kg per d, PO)		LM2 (1 g/kg per d, PO)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
TBARS (nmol MDA/mg protein)	0.09	0.01	0.17*	0.01	0.10†	0.01	0.09†	0.01	0.11†	0.01
$\sim\text{SH}$ ($\mu\text{mol}/\text{mg}$ protein)	1.24	1.23	0.39*	0.07	1.07†	0.09	0.82†	0.03	1.19†	0.09
GST ($\mu\text{mol}/\text{mg}$ protein)	1.39	0.02	0.43*	0.03	1.35†	0.05	1.21†	0.04	1.44†	0.09
GPx (mU/mg protein)	22.49	0.39	10.75*	0.32	23.98†	0.53	20.41†	0.64	19.48†	0.61
SOD (U/min per mg protein)	0.19	0.01	0.11*	0.01	0.18†	0.01	0.21†	0.01	0.18†	0.01
Catalase ($\mu\text{mol}/\text{mg}$ Hb)	7.87	0.54	4.88*	0.26	7.94†	0.26	8.00†	0.40	7.22†	0.24
NO (μM)	2.41	0.32	4.48*	0.19	2.73†	0.09	3.07†	0.12	2.50†	0.02
TAS (mM-Trolox equivalents)	2.54	0.05	2.17*	0.02	2.38†	0.03	2.54†	0.04	2.29†	0.08
TOS ($\mu\text{M}-\text{H}_2\text{O}_2$ equivalents)	5.04	0.59	15.77*	0.83	6.59†	0.40	5.98†	0.50	12.78†	0.84
OSI (arbitrary units)	0.20	0.03	0.725*	0.04	0.28†	0.02	0.23†	0.02	0.57†	0.54

PO, per oral; TBARS, thiobarbituric acid-reacting substances; MDA, malonaldehyde; $\sim\text{SH}$, total thiol; GST, glutathione transferase; GPx, glutathione peroxidase; SOD, superoxide dismutase; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index.

* Mean values were significantly different from control ($P < 0.05$, one-way ANOVA).

† Mean values were significantly different from arthritis ($P < 0.05$, one-way ANOVA).

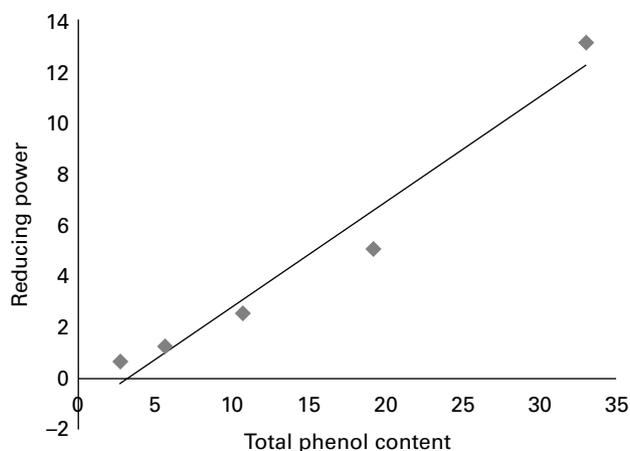


Fig. 1. Regression plot of total phenol content (μg gallic acid equivalents) and reducing power (μg of ascorbic acid equivalents) of *Lamellidens marginalis* extract (LME). Both the values were measured in same dose of LME expressed in mg dry weight ($y = 0.406x - 1.287$; $R^2 = 0.961$). x, Total phenol content; y, reducing power; r^2 , square of Pearson's correlation coefficient.

in vitro system, followed by its antioxidant efficacy in the adjuvant-induced arthritis model in rats.

Table 1 shows that LM can be considered as a food rich in antioxidants with a significant correlation between phenol content and reducing powers ($r = 0.98$; Fig. 1), indicating that the antioxidant property of LM is highly dependent on its phenol content. To study the association of this dietary antioxidant with the prevention of *in vivo* oxidative stress, we selected the FCA-induced inflammatory arthritis model that mimics human rheumatoid arthritis along with the feature of severe reactive oxygen species generation⁽²⁸⁾. The oxidative burst takes place within the activated macrophage to combat foreign antigens in arthritis. NO is a good indicator of this reactive oxygen species generation and thus the alteration of nitric oxide level was often estimated to determine macrophage activity⁽²⁹⁾. Grant *et al.*⁽³⁰⁾ showed that elevated NO level in FCA induced arthritis. Depletion of the body's antioxidant defence reserves takes place to combat the oxidative burst within activated macrophages. SOD, catalase and $\sim\text{SH}$ are the enzymatic and non-enzymatic antioxidant defence systems of the body, markedly altered during this process. GPx regenerates the reduced glutathione from its oxidative form to sustain the antioxidant status of the body. Significantly decreased GPx level in the arthritis animal indicated the over-usage of enzyme to regenerate reduced glutathione to resist against reactive oxygen species. GST reduces organic peroxides in the presence of reduced glutathione and converts it to oxidised form. Thus, alteration of this total system indicates alteration of the body's oxidative and antioxidant status as found in the present experiment with FCA-induced animals, which was restored due to LM treatment, thereby indicating LM to have antioxidant potential.

In summary, it could be concluded that the edible portion of LM, the pearl culture byproduct, contains antioxidants, particularly the unique phenolic proteins that are effective in the prevention of arthritis, a chronic inflammatory disease. Hence,

this makes LM a suitable candidate in the design of an integrated nutritional approach.

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