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NUTRIENT COMPOSITION AND ANTIOXIDANT ACTIVITIES OF MUGA AND ERI SILKWORM PUPAE

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ABSTRACT
This study investigated the nutrient composition and antioxidant activity of pupae of the muga silkworm (*Antheraea assamensis)* and eri silkworm (*Samia ricini*). Antioxidant activity was determined by using 1-1 diphenyl 2 picryl hydrazyl (DPPH) assay radical, reducing power assay and lipid peroxidation assay. The methanolic pupae extract (MPE) of muga and eri showed good DPPH radical scavenging activity with IC_{50} value of 25.83 µg/mL and 18.71 µg/mL respectively. The methanolic pupae extract (MPE) of muga and eri showed good *in vitro* lipid peroxidation activity with IC_{50} value of 35.51 µg/mL and 25.2 µg/mL respectively. The MPE of muga pupae had phenolic (12.2 mg catechin/g) and flavanoid content (5.45 mg quercetin/g) ($p < 0.05$) and MPE of eri pupae had significant higher phenolic (17.69 mg catechin/g) and flavanoid content (3.47 mg quercetin/g) ($p < 0.05$). Therefore, pupae could be used as natural antioxidants on food products.

KEYWORDS: *Antheraea assamensis*, *Samia ricini*, Antioxidant, DPPH

INTRODUCTION

Insects have played a very important role as a source of food in the history of human nutrition, especially in developing countries (Bodenheimer, 1951). The consumption of insects has been documented in Japan, Thailand, Africa, Latin America, Australia, Mexico and other parts of the developing world where they represent a cheap source of good quality protein (De Foliart and Green, 1999, Mitsuhashi 1997, Yhoung Aree *et al.*, 1997). Among the insect, silkworms are well known as an efficient large scale producer of silk threads. The by-products of manufacturing silk include the unusable parts of the pupae and cocoon. Spent silkworm pupae are a waste material often discarded in the open environment or used as fertilizer (Wei *et al.*, 2009). The pupae can be sold for fertilizer; biogas (Viswanath and Nand, 1994), feed stuff (Nandeesha *et al.*, 1990, Rangacharyulu *et al.*, 2003) and other agricultural purposes. In China, human consumption of silkworm pupae has been practiced since the very earliest times and has been approved as a new source by the Ministry of Health of the Republic of China (Zhou and Han, 2006a). The indigenous population in northeast India uses a variety of insects as food, one of which is the pupae of eri silkworm (*Samia ricini*) and muga silkworm (*Antheraea assamensis)*. The consumption was highest for eri (87%), followed by muga (57.4%) and mulberry (24.6%)(Mishra *et al.*, 2003). Proximate analysis of pupae showed that it contains 55-60% protein, 25- 30% lipid, 4.96% fibre and other substances *e.g.* hormones, trace elements, and vitamins, thus indicating that it could be a good protein (Yang *et al.*, 2002). Pharmacological studies show that silkworm pupae are alimental for increasing immunity, protecting the liver and preventing cancer. Consumption of silkworm pupae could supplement Vitamin B_2 intake, which can be important to prevent the serious effects of Vitamin B_2 deficiency (Kwon *et al.*, 2012). In this study, the phenolic content, antioxidant and antityrosinase activity of eri and muga silkworm pupae is reported. Moreover, the diversity on physical and chemical parameters of eri and muga silkworm pupae was not studied earlier. Therefore, the study was design for the first time to evaluate the nutritional potential of pupae of the silkworm *Antheraea assamensis* and *Samia ricinii* based on its nutritional composition and antioxidant activities.

MATERIAL & METHODS

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl), thiobarbituric acid (TBA), catechin, quercetin, tyrosinase (1000 units/mL) were purchased from Sigma, St Louis, MO, U.S.A. Ascorbic acid, sodium dodeycylsulfate (SDS), n-butanol, pyridine, tris-HCl buffer, ferrous **s**ulfate, potassium ferricyanide, methanol, folin-Ciocalteu reagent, L-3,4-dihydroxyphenylalanine (DOPA) were purchased from Merck India.

Sample preparation: Preparation of pupae extract

Pupae of the silkworm *Antheraea assamensis* and *Samia ricini* were purchased from the Dhemaji district of Assam and authenticated by an entomologist from Gauhati University, Assam, India. The characteristics of the pupae conformed to the standard criteria. After the sample was washed with distilled water and ground in a meat grinder, it was centrifuged at 3000 rev/min for 20 min to get rid of deposits (mainly pupa shell). The liquid centrifugate was spray-dried and ground into fine powder. The pupae extract were prepared in methanolic solvent. About 10 g of the dried powdered materials were dissolved in 100 mL of solvent for 48 hours followed by filtration. The filtrate was then concentrated under reduced pressure using a rotary evaporator (Buchi R-124) at low temperature (< 40 °C) for

methanolic pupae extract (MPE) and kept in 4ºC until used.

Nutrient analysis

Moisture, ash, crude fibre and fat contents were assayed by different method (Sadasivam and Manickam 1996). Protein content was determined by the Bradford method. Carbohydrate was determined by Anthrone method.

DPPH· Assay

Relatively stable organic radical DPPH· has been widely used in the determination of antioxidant activity of extract following the method of Brand William method (Brand-William *et al.*, 1995). The solution of DPPH· in methanol (6×10^{-5} M) was prepared just before UV measurements. Samples were added to DPPH· solution in 1:1 ratio followed by vortex. The reaction was allowed to take place in the dark at room temperature under nitrogen atmosphere. The absorbance at 517 nm was measured at different time intervals. A decreasing intensity of the purple colour was taken as increasing scavenging activity. Ascorbic acid served as a standard. The inhibition percentage of radical scavenging activity was calculated using the following equation.

$$\text{Inhibition (\%)} = [(A_0\text{-}A)/A_0] \times 100$$

Where A_0 is the absorbance of DPPH· in the absence of the sample and A is the absorbance of DPPH· in the presence of sample.

***In vitro* lipid peroxidation Assay**

Lipid peroxidation, induced by Fe^{2+} ascorbate system in rat liver homogenate by the method of Bishayee and Balasubramanian (1971) was estimated as thiobarbituric acid reacting substances (TBARS) by Okhawa *et al.*, (1979). The reaction mixture contained rat liver homogenate 0.25 mL (10% w/v in 0.05 M phosphate buffer, pH 7.4), 0.1 mL Tris-HCl buffer (150 mM, pH 7.2), 0.05 mL ascorbic acid (0.1 mM), 0.05 mL $FeSO_4 .7H_2O$ (4 mM) and 0.05 mL of MPE of pupa. The mixture was incubated at 37 ºC for 1 hr and then 1.5 mL 2-thiobarbituric acid (TBA, 0.8% w/v), 1.5 mL acetic acid (20%) and 0.2 mL sodium dodecylsulfate (SDS, 8.1% w/v) were added to the reaction mixture. The mixture was made up to 4.0 mL with distilled water and heated at 95˚C for 60 min. After cooking with tap water, 1.0 mL distilled water and 5.0 mL of mixture of n-butanol and pyridine (15: 1 v/v) were added. The mixture was shaken vigorously and centrifuged at 5000 g for 10 min. After centrifugation, the optical density of the butanol layer was measured at 532 nm in a spectrophotometer.

Tyrosinase inhibition activity

Tyrosinase activity was determined by spectrophotometry with minor modifications (Masamoto *et al.*, 2003). Tyrosinase from a mushroom solution was prepared at a concentration of 100 units/mL in 0.2 M phosphate buffer solution (pH 6.5). Tyrosinase mushroom solution (400 µL) and phosphate buffer solution at pH 6.5 (300 µL) were mixed with or without the MPE. The mixture was then pre-incubated at 25ºC for 5 min before adding 400 µL of 1.25 mM L-3,4-dihydroxyphenylalanine solution, and the reaction was monitored at 475 nm. The percentage of inhibition of tyrosinase activity was calculated as Inhibition, (%) = [(A − B)/A] × 100

Where, A represents the difference in the absorbance of the control sample between incubation time periods of 0.5 and1.0 min, and B represents the difference in the absorbance of the test sample between the same incubation times. Each result is presented as the mean from three concurrent readings. Kojic acid was used as a positive control.

Reducing power Assay

Reducing power was determined according to the Oyaizu (1998). 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferricyanide were added to 1 mL sample solution and mixed gently. The mixtures were incubated at 50˚C in a water bath for 20 min. Reaction was stopped by adding 2.5 mL of 10 % trichloroacetic acid (TCA) and the mixtures were centrifuged at 4000 rpm for 10 min. From the top layer, 2.5 mL was transferred into tubes containing 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3.6H_2O$). The resulting solutions were mixed well and after 5 min the absorbance was measured at 700 nm against blanks.

Total polyphenol content

Total phenolic compounds were determined by Folin–Ciocalteu method (Singleton and Rosssi 1965). 0.5 mL of extract was mixed with Folin-Ciocalteu reagent (2.5 mL, diluted 10 times) and incubated for 2 min at room temperature followed by addition of sodium carbonate solution (2 mL, 7.5% w/v). The mixture was then allowed to stand for 30 min at room temperature and absorbance was measured at 765 nm. The amount of total polyphenol was calculated and results were expressed as catechin equivalent i.e., expressed as mg cathechin/g dry weight of sample. All measurement was done in triplicate.

Total flavanoid content

The total flavonoids were estimated according to the aluminium chloride calorimetric assay (Yanping *et al.*, 2004). An aliquot (1mL) was added to 10 mL volumetric flask containing 4 mL of distilled water. To the above mixture, 0.3 mL of 5% $NaNO_2$ was added. After 5 minutes, 0.3 mL of 10% $AlCl_3$ was added. At 6th min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Results were expressed as quercetin equivalent i.e., expressed as mg quercetin/g dry weight of sample. All measurement was done in triplicate.

Statistical analysis

All the measurements were performed in triplicate. Mean values ± S.D were calculated. To determine the statistical significance, student's t-test was used and $p < 0.05$ were set as significant.

RESULTS

Nutrient Properties of eri and muga silkworm pupae

The nutrient properties of eri and muga silkworm pupae are given in the Table 1. There were statistically differences among eri and muga silkworm pupae in terms of moisture, protein, crude fat, total ash, crude fibre, carbohydrate ($p<0.05$).

TABLE 1. Proximate composition of pupae of the muga and eri silkworm

Component	Muga pupae	Eri pupae
Moisture g%	71.38 ± 2.56	72.48 ± 3.41
Protein g%	54.2 ± 1.25	52.53 ± 1.32
Fat g%	16.66 ± 1.45	20.54 ± 2.11
Ash g%	1.45 ± 0.03	1.01 ± 0.01
Crude fibre g%	3.05 ± 0.04	3.25 ± 0.04
Carbohydrate g%	1.62 ± 0.01	1.24 ± 0.02

DPPH· Assay

DPPH·, a stable free radical with characteristics absorption at 517 nm in methanol was used to study the radical scavenging effects of MPE of the muga and eri silkworm. As antioxidants donate proton to this radical, the absorption of scavenging activity of DPPH· decreases. The decrease in the absorbance at 517 nm is taken as a measure of the extent of radical scavenging. The IC_{50} values (the concentration with scavenging activity of 50%) of scavenging activities on DPPH· radical of MPE of muga and eri silkworm were found to be 25.83 µg/mL and 18.71 µg/mL respectively (Table 2).

TABLE 2. DPPH radical scavenging activity, *in vitro* lipid peroxidation (TBARS) activity and antityrosinase activity as IC_{50} (µg/mL) of the MPE of muga and eri silkworm

Methanolic extract	DPPH (µg/mL)	TBARS (µg/mL)	Antityrosinase assay (µg/mL)
Muga pupae	25.83 ± 3.12	35.51 ± 2.27	15.05 ± 1.78
Eri pupae	18.71 ± 1.72	25.2 ± 3.81	41.23 ± 3.12
Ascorbic acid	27.21 ± 2.52	-	-
Catechin	-	21.42 ± 1.48	-
Kojic acid	-	-	22.45 ± 1.51

***In vitro* lipid peroxidation Assay**

The MPE of the muga and eri silkworm showed a very strong lipid peroxidation inhibitory activity as shown in the Table 2, at a low concentration with an IC_{50} of 35.51 µg/mL and 25.2 µg/mL respectively. Catechin was used as control, with an IC_{50} of 21.42 µg/mL. The lipid peroxidation inhibitory activity of the MPE of muga and eri silkworm was not significantly different ($p > 0.05$) with the inhibitory activity of catechin.

Measurement of antityrosinase activity

The MPE of the silkworm produced dose dependent inhibition of tyrosinase activity. The MPE of the muga and eri silkworm showed a very strong antityrosinase activity with an IC_{50} value of 15.05 µg/mL and 41.23 µg/mL respectively. Kojic acid was used as a positive control with an IC_{50} value of 22.45 µg/mL (Table 2). We therefore examine whether muga and eri silkworm pupae inhibit tyrosinase activity. As expected, muga and eri silkworm pupae inhibit tyrosinase activity.

Reducing power Assay

The change of reducing power with different concentrations of MPE of the muga and eri silkworm is evaluated and is presented in Table 3. Trolox was used as control, and reducing power of MPE of muga at 80 µg/mL is 0.353. Higher absorbance indicates higher reducing power.

Total polyphenol content and total flavanoid content in the studied pupae

The polyphenol content of MPE of muga and eri silkworm was calculated using the Folin-Ciocalteu method. In the present experiment, the total phenolics content was determined and result were presented in Fig. 1. The result of the ANOVA analysis reveal a significant difference ($p < 0.05$) between total phenolic and flavanoid content of MPE of muga and eri silkworm. The total phenolic contents of muga and eri silkworm were 12.2 mg catechin/g and 17.69 mg catechin/g of dry pupae respectively. The flavanoids content of muga and eri silkworm were 5.45 mg quercetin/g and 3.47 mg quercetin/g of dry pupae respectively.

TABLE 3. Reducing power of MPE of muga and eri silkworm at different concentration

Concentration (µg/mL)	Muga pupae	Eri pupae	Trolox
5	0.019 ± 0.003	0.015 ± 0.003	0.035 ± 0.004
10	0.023 ± 0.002	0.028 ± 0.004	0.078 ± 0.004
20	0.029 ± 0.004	0.036 ± 0.003	0.142 ± 0.005
40	0.038 ± 0.004	0.042 ± 0.002	0.273 ± 0.007
80	0.043 ± 0.002	0.054 ± 0.003	0.353 ± 0.002

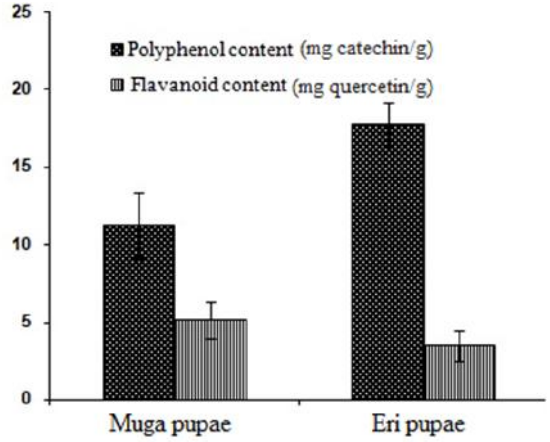

FIGURE 1. Total polyphenol and flavanoid contents of MPE of muga and eri silkworm

DISCUSSION

Crude protein content of eri silkworm and muga silkworm pupae was around 52% and 54% respectively which is higher than the 48.7% reported for spent silkworm pupae (Rao, 1994) and lesser than 71.9% reported from *A. pernyi*. Estimation of lipids is considered amongst the key factors for nutritional evaluation of any material (Ayaz *et al*., 2006). However, the presence of an appreciable content of lipids demonstrates the potential of any material to have dietary purposes with promising nutritional attributes. Crude fat content of eri silkworm and muga silkworm pupae was around 20% and 16% respectively. The fat content of eri and muga silkworm pupae in the present studies represent a good source of oil which is comparable with the fat content of 20.1% in pupae of the silkworm *A. pernyi* (Zhou and Han, 2006b).

However, the activity of antioxidant has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxide, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activities (Diplock, 1997). Reducing power is widely used to evaluate the antioxidant activity of polyphenols. The reducting power of a compound is related to its electron transfer ability and may, therefore serves as a significant indicator of its antioxidant activity (Ajila *et al*., 2007). The phenolic compounds were directly correlated with its antioxidant ability. The physiological effects of flavanoids include possible antioxidant activity, therefore, suggesting their role in prevention of coronary heart diseases including atherosclerosis (Sierens *et al*., 2002). Lipids, especially polyunsaturated fatty acids, are sensitive to oxidation, leading to the formation of malondialdehyde (MDA). The accumulation of MDA in tissues or biological fluids is indicative of the extent of free radical generation, oxidative stress and tissue damage. The potential of the MPE in inhibiting lipid peroxidation can be attributed to its phenolics and flavanoids contents. Antioxidants also assist in scavenging free radicals and preventing free radical chain reactions. Lipid peroxidation causes a decrease in membrane fluidity and in the barrier functions of the membranes. The many products of lipid peroxidation such as hydroperoxides or their aldehyde derivatives inhibit protein synthesis, blood macrophage actions and alter chemotactic signals and enzyme activity (Fridovich and Porter, 1981). Melanin is a natural and macromolecular pigment that is widely distributed in animals and plants. It is synthesised via dopaquinone, which is obtained from tyrosine by a tyrosinase. By controlling the enzymatic oxidation of a browning reaction, the shelf life of commercial products containing this pigment can be extended (Jo *et al*., 2008).

CONCLUSION

The preliminary results indicated that the muga and eri pupae have a great antioxidant potential with potent polyphenolic contents to scavenge free radicals and a good antityrosinase activity. Even though further investigations (*e.g*., *in vivo* and clinical studies) are required to in reducing the risk for several diseases, pupae might be a food product that are good sources of polyphenols have an impact on antioxidant potential and should be recommended for frequent consumption.

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