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Antioxidant and anti-inflammatory efficacy of functional proteins obtained from seven edible insects

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Abstract
Edible insects are alternate approaches for the manufacturing of cost-effective, energy-competent protein-rich diet. These alternative diets and foodstuffs are immediate requirements for the exploiting global population. Hence, the present study aimed to prepare the functional proteins obtained from seven edible insects using gastrointestinal digestion and absorption mechanisms. Furthermore, these functional proteins were undergone to determine antioxidant and anti-inflammatory efficacy. Seven insects' proteins were isolated and determined by SDS-PAGE followed by gastrointestinal enzymatic (α-amylase, pepsin, pancreatin, and bile) digestion. The concentration of functional proteins was quantified using trinitrobenzene sulphonic acid method. Antioxidant efficacy of functional proteins was determined by using radical scavenging assay (DPPH and ABTS), Fe^{2+} chelating assay, and ferric reducing power. Anti-inflammatory efficacy was determined using the inhibitory activity of lipoxygenase and cyclooxygenase-2. Based on the findings, the results suggest that the consumption of edible insects and foods enriched with insect proteins that may be potential therapeutic effects on the human body.

Keywords: edible insects, enzymatic digestion, functional proteins, antioxidant, anti-inflammatory activity

Introduction
According to the FAO reports in 2050, the total human population is expected to be 9.5 billion people worldwide, which elevates food scarcity up to 75% compared with our existing food supplies [1]. The traditional sources of protein might be inadequate for the exploiting population, and hence substitution foods are urgently required. In this context, insects are promising sources of protein-rich diet and can be replaced all agricultural as well as animal-derived protein food. Presently, about 870 million people are undernourished globally [2], which will enhance rapidly due to the industrialization of the world. Edible insects are recognized as human food that can aid emerging nations to sustenance their requirements for basic components of regimes [3], but might also offer complimentary food for established nations' populations.

Normally, insects are a readily offered the main sources of carbohydrates, lipids, protein, vitamins (A, D, E, K, and C), and various minerals including K, Na, Ca, Cu, Fe, Zn, Mn and P [4-7]. The energy composition of insects is generally average when compared with the meat excluding pork, which chiefly contains high fat [8]. In addition, insects are a noble source of essential amino acids and polyunsaturated fatty acids, which are required for the synthesis of protein as well as host growth and development [9]. Normally, food products are augmented with protein obtained from pulses and however insect protein is superior based on their nutritive properties since insect protein comprises all the essential amino acids. Moreover, insects are richer in protein than soybean (41.1%), lentils (26.7%) and beans (23.5%) [5].

According to the extensive range of edible insect species, the nutritious value of edible insects is extremely different. Even within the same insects' species, the nutritional value may vary based on the insect's metamorphic stages, habitat, adaptation, and its food [10]. Ramos-Elorduy *et al.* [6]. Analyzed around 78 classes of insects and calculated their calorific value in the range of 293- 762 kcal/ 100 g of dry matter. The consumption of insects is nowadays very common in many populaces around the world. It is projected that about 1,900 species of insects are eaten by over two billion people in about 113 countries across Asia, Africa, and the Americas [10, 11].

Insects provide cultured delicious dishes, which offer in various food courts, having totally altered the appearance and bestowing them as much more pretty and flavorful [12]. Entomophagy refers to the use of insects as food, termed as a human insectivore, which is seriously influenced by cultural and religious practices. Various studies showed that the impact of insect breeding on the environment [13, 14], and about safety features of ingesting of insects [15, 16]. Containing a good source of nutrients. However, there is a lacking of investigation elucidating the effect of the thermal process (e.g., boiling, baking) on the digestibility of edible insects. Hence, in this present study seven species of insects (Table 1) were chosen, which are well identified and easy to breed in Tamil Nadu. The aim of present investigation was to determine the effect of heat treatment of edible insects (raw, boiled, baked) on antioxidant and anti-inflammatory activity of functional proteins acquired under using gastrointestinal digestion and absorption conditions.

Table 1: List of common edible insect species

English name	Order	Family	Binomial name	Consumption stage
Bamboo Worms	Lepidoptera	Crambidae	*Omphisa fuscidentalis*	Larvae
Crickets	Orthoptera	Gryllidae	*Gryllodes sigillatus*	Adult
House fly	Diptera	Muscidae	*Musca domestica*	Larvae
Locusts	Orthoptera	Acrididae	*Schistocerca gregaria*	Adult
Mealworms	Coleoptera	Tenebrionidae	*Tenebrio molitor*	Larvae
Silkworm	Lepidoptera	Bombycidae	*Bombyx mori*	Larvae
Weaver Ants	Hymenoptera	Formicidae	*Oecophylla smaragdina*	Adults

2. Materials and Methods

2.1 Insects samples

The following seven common insects' species were examined (Table 1): Bamboo worms (Larvae), crickets (Adult), house fly (Larvae), locusts (Adult), mealworms (Larvae), silkworm (Larvae), and weaver ants (Adults). These insects' specimens were acquired from the local commercial dealer. The appropriate food will be provided for individual insects.

2.2. Chemicals

COX and LOX activity assay Kit, α-amylase from hog pancreas, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile extract, and the molecular marker were procured from Sigma-Aldrich, St. Louis, MO, USA. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, trinitrobenzene sulphonic acid, 2,2-diphenyl-1-picrylhydrazyl, and glacial acetic acid, were all purchased from Chemico Glass & Scientific Company, Erode, Tamil Nadu, India. All the current study chemicals used were of analytical grade.

2.3. Edible Insect Preparation

The listed seven species of insects were fasted for about 24 h prior to eliminating residual food in their gastrointestinal tract. Each insects' species were randomly divided into 3 groups based on the raw and thermal treatment namely boiling, and baking. The first group of insects was used for the experiment as raw without any thermal treatment. The second group of tested insects were added into boiling water and heated for 15 min at 100°C. The third groups of insects were baked in a heated oven for 15 min at 150°C. Finally, all these groups of insects (about 500 g) of were ice-covered, lyophilized, and kept at −20°C for further investigation.

2.4. Preparation of protein from listed edible insects

The proteins were prepared from the listed edible insects' species based on the method of Girón-Calle *et al*. [17]. Concerning the pH of the solution. Concisely, three individual groups of insects were pulverized using Waring blender and added with NaOH (0.2%) in a proportion of 1:10 (*w/v*). The protein extraction was carried out under stirring for 1 h at room temperature. Later, the samples were centrifuged at 10000 rpm for 15 min at 4°C. The pH of the achieved supernatants containing insects' proteins was modified using HCl (0.1 M). Precipitated proteins were centrifuged at 4°C for 15 min at 10000 rpm and washed using double distilled water. The obtained proteins were lyophilized and maintained at −20°C for further examination.

2.5 Protein profile determination by SDS-PAGE

The protein profile determination was performed using SDS-PAGE. A Mini-PROTEAN BioRad Tetra Cell electrophoresis system (BioRad, Hercules, CA, USA) with 20 mA was used. The samples were mixed with buffer samples (BioRad, 1:1, *v/v*) and were run at 50-70 V for 5 min prior to the electrophoresis start. The gels were stained with Coomassie Brilliant Blue R-250 using the molecular marker in the sort of 10-200 kD molecular weight.

2.6. *In vitro* enzymatic digestion and absorption

In vitro digestion of lyophilized materials obtained from whole insects were performed according to the technique pronounced by Jakubczyk *et al*. [18]. With minor alteration. Concisely, the samples were kept (4%, *w/v*) with stimulated saliva solution. The enzymatic hydrolysis was performed at 37°C in a dark environment with the treatment of succeeding gastrointestinal enzymes viz., α-amylase, pepsin, pancreatin, and bile. The enzymatic reaction was ceased by using a water bath at 90°C for 5 min. The hydrolysates were immediately segregated by centrifugation at 10000 for 5 min followed by dialysis using a membrane tube. The mechanism of absorption was also performed in the dark at least an hour in the room temperature. Finally, the obtained soluble hydrolysates so-called functional proteins were lyophilized and stored at −20°C until further practice.

2.7 Determination of the functional protein concentration

The quantities of soluble proteins obtained from seven edible insects were determined by the method of trinitrobenzene sulphonic acid with slight modifications [19].

2.8. Determination of Antioxidant activity

The antioxidant activity of functional protein hydrolysate was assayed by two free radical-scavenging methods namely the DPPH$_{\bullet}$free radical scavenging assay and the ABTS$_{\bullet}^{+}$radical cation decolorization assay (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) [20, 21], and by two reducing power methods, namely total reducing power assay (TRP) as modified iron (III) to iron (II) reduction assay, and the ferric reducing antioxidant power assay (FRAP) [20, 21].

2.8.1 DPPH$_{\bullet}$assay: Briefly, 10 µl of functional protein hydrolysate was added with 1 ml of DPPH$_{\bullet}$ and made up to a final volume of 4 ml using methanol. The mixtures were mixed thoroughly and kept in the room temperature for 1hr. The absorbance of the color was measured using a spectrophotometer at 517 nm.

2.8.2 ABTS$_{\bullet}^{+}$radical assay: Briefly, 1ml of ABTS solution along with 1 mL of functional protein hydrolysate was mixed with equal volume of potassium persulfate and the mixture was kept under dark for 24 hrs incubation to generate the ABTS$_{\bullet}^{+}$radical cation. The absorbance was measured using a spectrophotometer at 734 nm.

2.8.3 TRP assay: Shortly, 10 µl of the functional protein hydrolysate was mixed with 1 ml of potassium ferrocyanide

and 1mL of phosphate buffer and the mixture was kept in the water bath at 50 ^{0}C for 30 minutes. Then the mixture was added with 1ml of trichloroacetic acid and the mixture was centrifuged at 3000 rpm for 10 min. Take 1ml of the supernatant added with 1 ml of distilled water and 0.5ml of $FeCl_3$.The mixture was mixed thoroughly and The absorbance was measured using a spectrophotometer at 700 nm.

2.8.4. FRAP assay: Briefly, 1 mL of functional protein hydrolysate was added to 1 ml of FRAP reagent, and makeup to the final volume of 4 ml by distilled water. The mixture was kept in the water bath at 37 °C for 30 minutes. The absorbance was measured using a spectrophotometer at 734 nm.

2.9 Determination of Anti-Inflammatory Activity

2.9.1 Assay of lipoxygenase inhibitory activity: Lipoxygenase (LOX) inhibitory activity was determined based on the method of Axelroad *et al*. [22]. with minor modifications. Briefly, 1 ml of functional protein hydrolysate was added with 1 ml of phosphate buffer (1/15 M, pH 7.0), followed by 10 µL of a lipoxygenase solution (167 U/mL), and 50 µL of an inhibitor solution and kept for incubation for 3 min at 25°C. Then, the mixture was added with 40 µL of linoleic acid (2.5 mmol/L). The same concentration of the enzyme without an inhibitor was kept as control. The absorbance of lipoxygenase activity was determined spectrophotometrically at 234 nm.

2.9.2. Assay of cyclooxygenase inhibitory activity: cyclooxygenase 2 (COX-2) inhibitory activity was determined based on the adaptation of previously Zielińska *et al*. [19] with slight modifications. Briefly, about 10 µl of functional protein hydrolysate was added with 150 µl of Tris-HCl buffer (0.1M, pH 8.0), 10 µl of heme, 20 µl of arachidonic acid, and 10 µl of enzyme were kept at room temperature for 5 min. This method assays the peroxidase component of COXs. The peroxidase activity was determined spectrophotometrically at 590 nm.

2.10 Statistical Analysis

The given data are Mean ± S.D of three independent replicates. The results were compared by one-way analysis of variance (ANOVA) and the significant differences among the test means were done by Tukey's method. The differences among the means at 5% level ($P < 0.05$) were considered statistically significant.

3. Results and Discussion

3.1 Electrophoretic profiles of insects' protein

The protein profile of the heat-treated insects was determined by SDS-PAGE by using Mini-PROTEAN BioRad Tetra Cell electrophoresis system, BioRad, Hercules, CA, USA. The heat treatment of insects resulted in the appearance of bands with high molecular mass and disappearance of bands with lower mass. The raw, boiled, and baked proteins obtained from seven different classes of edible insects exhibited bands in a range of 14-220 kDa (Figure 1). It should be noted that the SDS-PAGE profile in the raw crickets and silkworms showed 60.0 kDa bands. The major bands were identified in a range between 29.0 and 44.0 kDa and at 60.0 kDa.

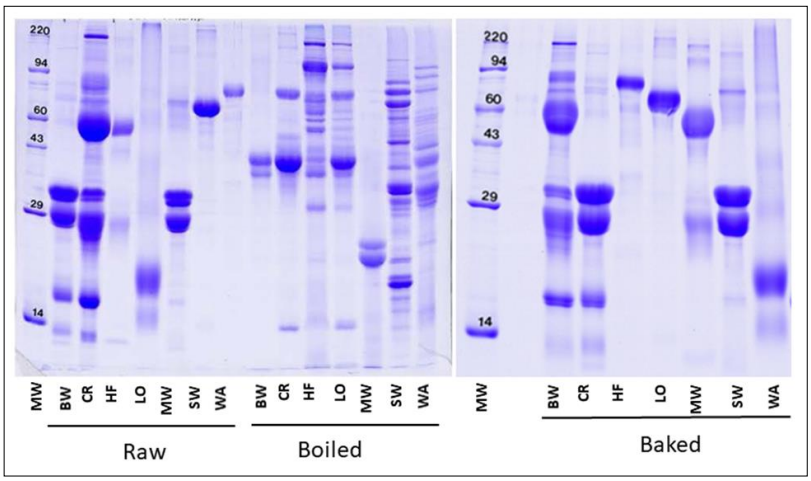


Fig 1: Electrophoretic pattern of functional protein obtained from various insects. Lane: MW- molecular weight; BW- Bamboo Worms; CR- Crickets; HF-House fly; LO-Locusts; MW- Mealworms; SW-Silkworms; WA- Weaver Ants

3.2 Quantification of functional protein

The edible insects were digested under simulated gastrointestinal enzymatic conditions followed by absorption mechanisms. During absorption, all these tested samples containing functional proteins, which were considerably reduced (**Table 2**), due to the practice of dialysis of hydrolysates using membrane tube. The highest quantity of functional proteins after digestion were noted in the respective baked bamboo worms (25.9 mg/ml) and silkworms (27.9 mg/ml); boiled mealworm (28.9 mg/mL). The highest quantity of functional proteins after the absorption was found in the fraction obtained from the silkworm (3.18 mg/ml). Normally, the highest amount of proteins was found in hydrolysates obtained by digestion of all boiled insects and the however the lowest quantity of protein was noted in backed Locusts (6.9 mg/ml). The functional protein fractions obtained after the absorption mechanism, the lowest amounts of proteins also were found in the backed Locusts (0.83 mg/ml), while the samples obtained from raw insects exhibited the highest values showed in silkworm (3.25 mg/ml). Hence, the heat treatment usually produced a minor reduction in the quantities of proteins. These results are similar to that of previous studies, which showed the quantities of proteins

after the heat treatment was optimum when compared with the quantities of proteins in insect hydrolysates stage [23]. Similarly, the following edible insects contain respective protein quantity in *Gryllodes sigillatus* (8.5 mg/ml), *Tenebrio molitor* (8.16 mg/ml), *Schistocerca gregaria* (8.6 mg/ml), *Locusta migratoria* (5.88 mg/ml), *Zophobas morio* (1.88 mg/ml), and *Amphiacusta annulipes* (1.68 mg/ml) [23]. The quantities of proteins in insects after enzymatic digestion was generally higher than the protein quantity of fermented pea seeds that showed only 3.01 mg/ml after enzymatic hydrolysis [18].

Table 2: Functional protein concentration after digestion and absorption mechanism

Edible insects	Type of heat treatment	Functional protein concentration (mg/ml)	
		After digestion	After absorption
Bamboo Worms	raw	17.4 ± 1.25^{f}	1.56 ± 0.04^{d}
	boiled	12.3 ± 0.89^{g}	2.35 ± 0.05^{b}
	baked	25.9 ± 0.98^{c}	2.11 ± 0.03^{b,c}
Crickets	raw	21.9 ± 1.13 ^{d}	3.14 ± 0.06 ^{a}
	boiled	21.8 ± 0.71 ^{d}	2.04 ± 0.05 ^{c}
	baked	17.5 ± 0.57 ^{f}	2.24 ± 0.02 ^{b,c}
House fly	raw	24.5 ± 1.89^{c}	2.09 ± 0.06^{b,c}
	boiled	21.8 ± 0.98^{d}	2.28 ± 0.04^{b}
	baked	18.9 ± 1.25^{f}	1.62 ± 0.02^{d}
Locusts	raw	27.5 ± 0.99 ^{b}	1.3 ± 0.02 ^{e}
	boiled	8.4 ± 0.85 ^{h}	1.28 ± 0.02 ^{e}
	baked	6.9 ± 1.13 ^{i}	0.83 ± 0.02 ^{f}
Mealworms	raw	5.3 ± 0.42 ^{j}	1.57 ± 0.04 ^{d}
	boiled	28.9 ± 1.41 ^{a}	1.59 ± 0.04 ^{d}
	baked	24.5 ± 1.27 ^{c}	2.29 ± 0.06 ^{b}
Silkworm	raw	25.6 ± 2.13^{c}	3.25 ± 0.06^{a}
	boiled	21.6 ± 1.58^{d}	3.18 ± 0.05^{a}
	baked	27.9 ± 1.79^{b}	3.02 ± 0.04^{a}
Weaver Ants	raw	12.6 ± 1.37^{g}	1.35 ± 0.02^{e}
	boiled	18.9 ±1.26^{f}	1.29 ± 0.01^{e}
	baked	19.6 ± 0.89^{f}	0.98± 0.02^{f}

Means ± SD in triplicate. Different letters indicate significant difference ($p < 0.05$).

3.3 Antioxidant assay

3.3.1 Free radical scavenging activity by DPPH$^{\bullet}$ and ABTS$^{\bullet+}$

The highest radical scavenging activity against DPPH$^{\bullet}$ was noted in the functional proteins obtained from the raw mealworm (IC_{50} value of 5.3 µg/ml) as well as the boiled and backed Locusts (8.4 µg/ml, 6.9 µg/ml) respectively (Figure 2). Similarly, the highest radical scavenging activity against ABTS$^{\bullet+}$ was found in the functional protein obtained from raw bamboo worms and boiled weaver ants at an IC_{50} value of 12.3, 29.5 µg/ml respectively. This outcome of the existing study showed that heat treatment of insects' proteins augmented the antiradical activity especially.

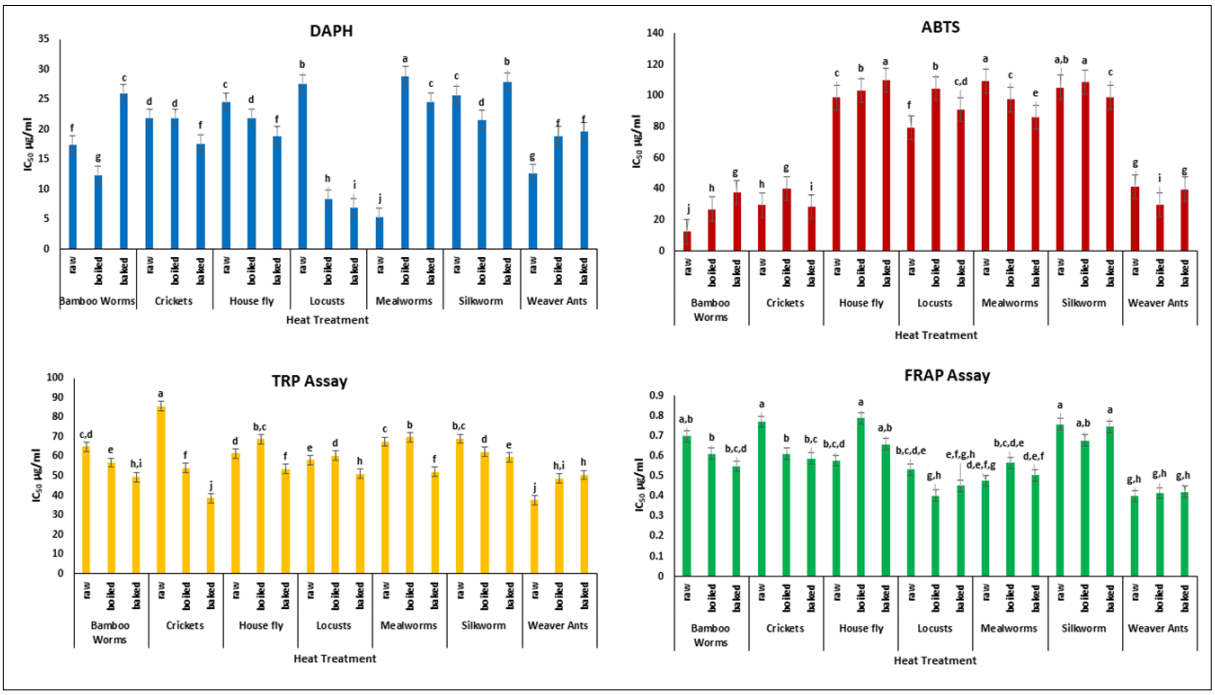


Fig 2: The antioxidant activities of functional protein after digestion.

Boiling and baking was considered by enhanced outcomes. During the heat treatment, the structure of insect proteins was altered and converted into smaller active fractions. [24] Hence, the outcome showed the mechanism of heat treatment aid to discharge larger proteins into smaller functional proteins with antioxidant potentials. The existing outcomes are similar to that of the previous experiment, who also suggested that heat treatment possess a positive influence on the antioxidant properties of chickpea seeds and beef meat [25, 26]. The lowest radical scavenging activity against DPPH and $ABTS^{\bullet+}$ were observed in the boiled meal worm (IC_{50} value 28.9 µg/ml) and backed house fly (IC_{50} value 109.8 µg/mL) (Figure 2). The maximum free radical scavenging activity against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ was observed in functional proteins obtained after the absorption process of boiled bamboo worms (IC_{50} value 10.5 µg/ml) and crickets (IC_{50} value 10.9 µg/ml) respectively (Figure 4). The outcomes are similar to that of previous studies, that $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were higher in the hydrolysate obtained after absorption process of *Amphiacusta annulipes* (IC_{50} value 19.1 µg/ml) and *Zophobas morio* (IC_{50} value 4.6 µg/mL) respectively [27, 28]. Serpen *et al*. [29]. described that the slight heat treatments might also upsurge the antioxidant potentials in beef, chicken, and pork, due to alterations in their tertiary and quaternary structures of functional proteins. Additionally, most often, the test samples undergo absorption were provided the greatest radical scavenging activity than normal enzymatic hydrolysis.

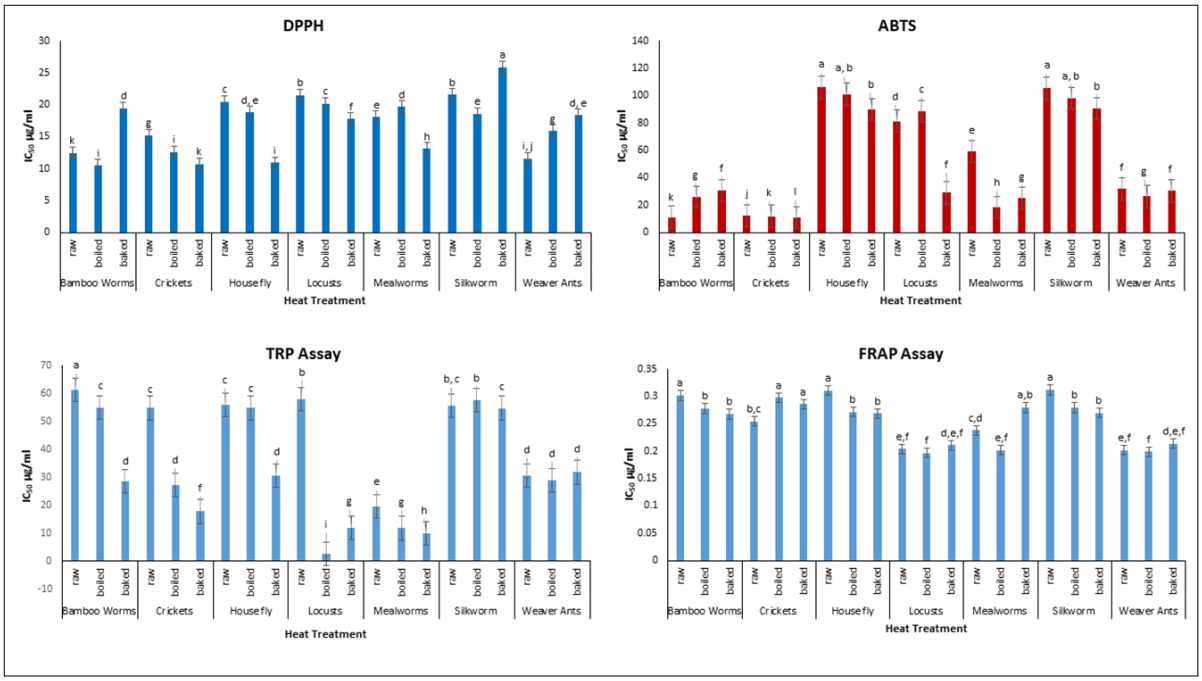


Fig 4: The antioxidant activities of functional protein after absorption.

3.3.2 TRP and FRAP assay

The maximum capacity to reduce total Fe^{2+} ions was reported in raw weaver ants' proteins (IC_{50} value 37.8 µg/ml) as well as baked cricket proteins (IC_{50} value 38.6 µg/ml) among all the enzymatic hydrolysis examined (Figure 2) and however, the functional proteins obtained after absorption from the boiled locusts showed the IC_{50} value 2.57 µg/ml (Figure 4). Usually, insects undergo heat treatment augments the ability of the functional protein fractions to chelate total Fe^{2+}. The use of heat treatment considerably impacts on the discharge of biologically-active proteins during digestion and absorption of proteins [25]. The maximum chelating activity of insect proteins could be connected to a definite protein organization as well as the functional groups of amino acids, which aids significant functions in terms of dismissing free radical cascade chain reactions and chelating metal ions [30]. Moreover, the maximum ability to chelate Fe^{2+} was found in the baked and boiled insects. These results agreed with a previous publication that total chelating Fe^{2+} ions were observed in raw chickpea (IC_{50} value of 81.6 µg/ml) and heat-treated chickpea (78.86 µg/ml). This outcome obviously demonstrated that the heat treatment of chickpea augmented the ability of functional proteins to chelate Fe^{2+} ions [25]. Likewise, silkworm protein hydrolysate was also chelating Fe^{2+} with an IC_{50} value of 2.03 mg/ml documented by Wu *et al*. [28]. A maximum value of ferric reducing power was observed in the raw weaver ants' protein hydrolysate among all the enzymatic hydrolysis examined (IC_{50} value of 0.399) (Figure 2). Similarly, the functional proteins obtained after absorption from the boiled locusts showed the IC_{50} value of 0.197 µg/ml (Figure 4). Largely, the lowermost results were observed for the functional protein among the hydrolysates after the absorption mechanisms. In this results, thermal treatment of insects provides mostly to augment reducing power among the protein fractions. The obtainable data agree with other results obtained for edible insects such as *Amphiacusta annulipes*, *Zophobas morio*, *Tenebrio molitor*, *Schistocerca gregaria*, and *Locusta migratoria*. [19, 24, 31, 32] Furthermore, the examined hydrolysates demonstrated higher reducing power than medicinal plants, leafy vegetables or legumes [33, 34].

3.4 Anti-inflammatory assay

3.4.1 LOX and COX-2 inhibitory activity

The activity of LOX and COX-2 were meritoriously inhibited by the edible insects' functional proteins obtained from digestion (Figure 3) and absorption (Figure 5). The functional proteins obtained after enzymatic digestion from baked weaver ants possess the most effective inhibitor of

LOX (IC_{50} value 0.98 mg/ml), while the raw locusts inhibited COX-2 most efficiently (IC_{50} value 10.91 µg/ml). The functional protein fraction obtained from the baked meal worm (IC_{50} value 3.13 µg/mL), raw meal worm (IC_{50} value 9.88 µg/ml) showed the best LOX and COX-2 inhibitory activity among the insects' proteins. The best inhibitory activity of the LOX and COX was determined for the functional proteins after digestion and absorption. Among them, the maximum inhibitory activity was observed for the functional proteins in the absorption process than the enzymatic digestion. Similarly, LOX inhibition by purple basil leaves containing anthocyanins which had an IC_{50} value of 41.0 mg/g [35], *Aronia melanocarpa* had an IC_{50} value of 30.3-91.0 µg/ml [36]. Additionally, in our present COX-2 inhibitory assay, the functional proteins obtained from raw locusts (IC_{50} value 5.05 µg/ml) outcomes were similar to that of previous studies, showed in the purple basil leaves (IC_{50} value 5.0 µg/ml) [37], *Rosa canina* (IC_{50} value 19.0-62.0 µg/ml) [38].

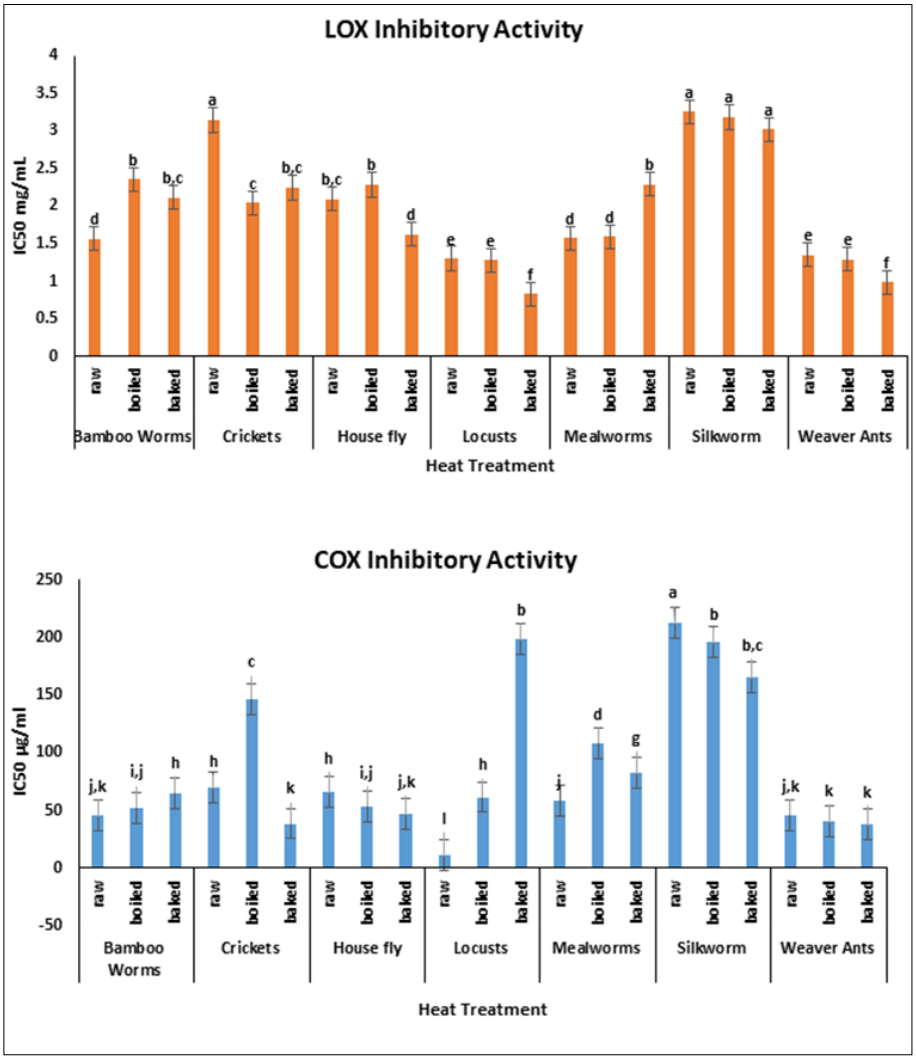
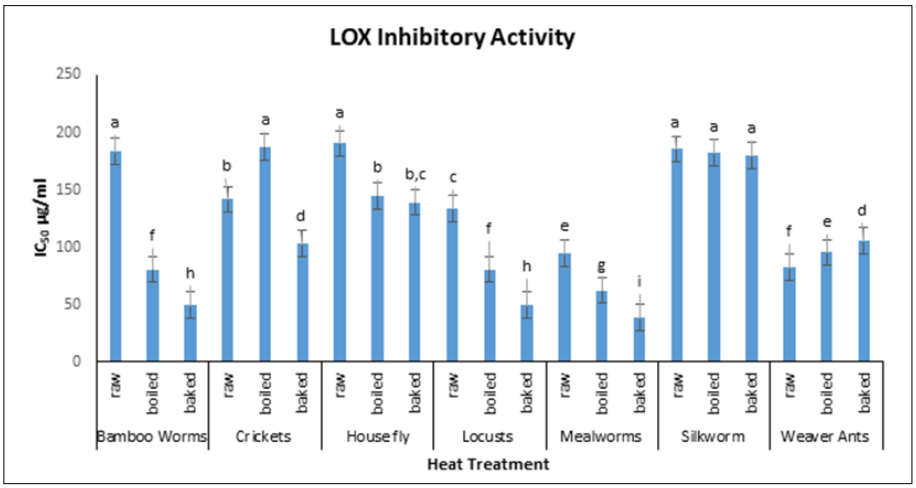


Fig 3: The anti-inflammatory activities of functional protein after digestion.



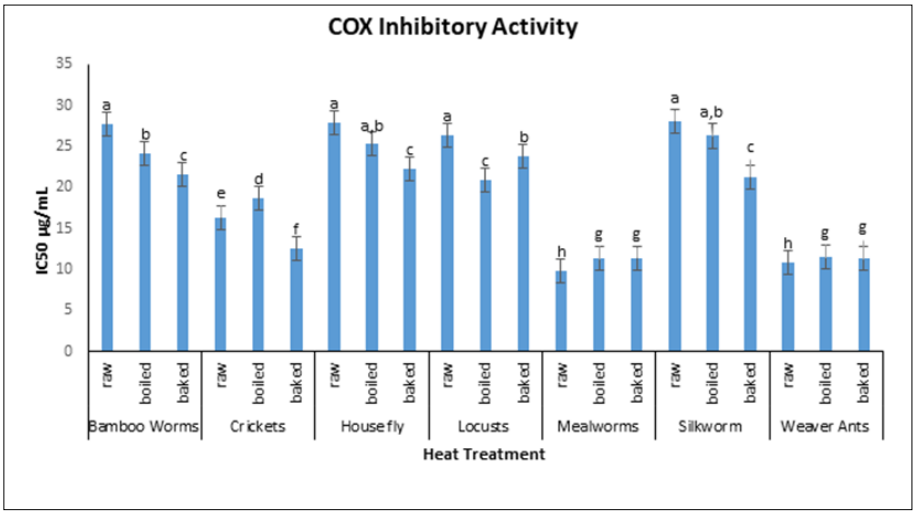


Fig 5: The anti-inflammatory activities of functional protein after absorption

Conclusion

Based on the findings, the results suggested that the edible insects encompass high content of functional proteins, which possess antioxidant and anti-inflammatory potential. The outcome of the investigation further demonstrated that after the digestion and absorption mechanism, the edible insects have maximum free radical scavenging activity and an ability to chelate total ions and can inhibit lipoxygenase and cyclooxygenase-2 activity. Furthermore, the practice of thermal treatment can positively influence the antioxidant properties of the proteins. These outcomes clearly specify that thermal treatment has an important effect on the availability of proteins for enzymatic digestion, leading to upsurge contents of antioxidant functional proteins. It has been observed that intake of edible insects and insects based foods enriched functional proteins that may be potentially favorable to human health. Entomophagy is well-known nutraceuticals proved to be a novel in averting human diseases, as edible insects may potentially avert oxidative stress associated diseases.

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