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Evaluation of biochemical and antioxidant properties of crude extracts from the Indian cattle leech *Poecilobdella granulosa*

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Oxidative stress is one of the factors influencing the structure and function of cells, tissues, and their components. Natural antioxidants have emerged as prophylactic and therapeutic agents for diseases like cancer and cardiovascular disease. The objective of the current study is to assess the biochemical composition and antioxidant potential of crude extracts from various organs of the Indian cattle leech *Poecilobdella granulosa*. The biochemical constituents and protein profiles of various crude samples were determined by Native and SDS polyacrylamide gel electrophoresis. Moreover, four different crude samples were subjected to check free radical scavenging activity. In the biochemical analysis, protein and carbohydrate content were found to be higher in whole-body extract and several major and minor bands were found ranging from 22 to 110 kDa during qualitative analysis. Interestingly, among the four crude samples, saliva alone showed antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl and superoxide anion radical scavenging activity. This study concluded that the crude saliva extract from *P. granulosa* is a highly selective and effective antioxidant for human welfare.

[**Keywords**: 2, 2-diphenyl-1-picrylhydrazyl, Antioxidant, Free radical, *Poecilobdella granulosa*, Saliva, Superoxide]

Introduction

A free radical is referred as any unstable molecular species that can exist independently and has an unpaired electron in an atomic orbital[1]. These unstable molecules induce oxidative damage to biomolecules such as proteins, lipids, carbohydrates and nucleic acids. There are several diseases including heart disease, cancer, stroke, diabetes, and ulcer which are caused by free radicals[2]. Antioxidants are the molecules which inhibit the free radicals[3]. These antioxidants delay or prevent cellular damage primarily through their ability to scavenge free radicals[4]. Many diseases are prevented by antioxidants, including ageing, allergies, asthma, arthritis, algesia, atherosclerosis, autoimmune diseases, bronchopulmonary dyspepsia, and cancer. It also protects against cataracts, cerebral ischemia, eczema, diabetes, inflammatory gastrointestinal diseases, and genetic disorders. Antioxidants are majorly classified into enzymes, proteins and small molecules[5]. They are classified as enzymatic or non-enzymatic antioxidants based on their activity. Enzymatic antioxidants work by reducing and eliminating free radicals. Non-enzymatic antioxidants, on the other hand, work by disrupting free radical chain reactions[6].

Antioxidants, both synthetic and natural, are frequently used in foods and medicines. Particularly, it has been noted that natural protein extracts or purified proteins are used as antioxidants[7]. The current global strategy is to find natural antioxidants that are both inexpensive and close to nature[8]. Particularly, animal sources are employed for pharmacological applications[9]. In this study, leeches are used as an experimental animal. Mostly, leeches are assumed as nasty bloodsucking creatures with little or no worth, but this perception is wrong. The usage of leech has occupied an important place in the Unani, Sidha and Ayurveda systems of medicine to treat various ailments. Recent scientific research on salivary components has restored its lost reputation. Leech saliva contains a variety of biologically active substances with anti-inflammatory, analgesic, and anaesthetic properties, as well as a possible antioxidant effect[10]. Not only the salivary components, the whole animal and its secretion has bioactive components, mostly peptides and proteins. Numerous research has been carried out in order to isolate and characterize Leech Saliva Extract (LSE)[11-12]. The LSE research has been expanded and developed by obtaining a novel approach for collecting salivary protein without killing leeches. A high concentration of protein was seenin leech saliva[13]. Many studies revealed the usage of leeches for various diseases like

blood-clotting disorders, varicose veins, diabetes, infectious diseases, skin disorders, osteoarthritis, ear abnormalities and also in dentistry[14]. The present investigation aims to assess the biochemical and antioxidant attributes of crude extracts from freshwater leech *Poecilobdella granulosa* for biomedical application.

Materials and Methods

Collection and maintenance of animal

Freshwater leech was collected from Jyothi Hospital, Tambaram, Chennai and identified as *Poecilobdella granulosa* based on its colour, pattern, size, etc., with the help of the Zoological Survey of India Manual[15]. Leeches were kept separately and maintained in well-aerated plastic containers filled with un-chlorinated tap water. The leeches were kept under a 12:12 light-dark cycle at 25±1 °C.

Preparation of crude extracts

About three-week starved animals were taken and washed with tap water followed by turmeric water and distilled water. Then the animal was kept at 4°C for complete paralysis. Afterwards, the animal was dissected to remove the ingested blood leftover using tap and distilled water. The dissected animal was cleaned and homogenised with a triple volume of sodium phosphate buffer (20 mM, pH 7.4) before centrifugation at 16000 g for 20 min at 4 °C. The crude extract (whole-body) of interest was obtained by collecting the supernatant[16]. Similarly, about 10 – 15 leeches were decapitated and their heads were homogenized in the same way to obtain the head extract.

Using the feeding apparatus, the saliva extract was collected from starved leeches. In brief, leeches were fed through a glass funnel covered in UV-sterilized parafilm membrane with the phagostimulatory solution (PHS) of 0.001 M arginine in normal saline. The phagostimulatory solution was maintained at 37 °C so that the leeches can suck the solution in the funnel through the membrane until satiation. Directly after leeches drop down from the parafilm membrane, they were immobilized by putting them in a plastic container surrounded by ice for 10 – 15 min[17]. This makes the leeches vomit whatever they have sucked. Only the colourless saliva (bloody fluids were discarded) was pooled and centrifuged for 10 min at 9000 rpm in 4 °C. Leech Saliva Extract (LSE) was obtained by filtering the supernatant using 0.45-µm Sartorius® nitrocellulose membrane.

Similarly, mucus was also collected from the same leech which was used for saliva collection. After saliva collection, the animal was completely washed with distilled water. The animal regainedits activity when kept in normal water (37 °C) and secrete mucus around its body. The mucus was collected, pooled and mixed with phosphate buffer saline (0.14 M Sodium chloride; 0.02 M Potassium phosphate, pH 7.2) in a 1:3 ratio and centrifuged for 20 min at 1000 rpm. The supernatant was collected and kept at -4 °C until use[18].

Biochemical estimations

The biochemical constituents like protein, lipid and carbohydrates were quantified using the standard methods[19-21].

Determination of protein profile

The protein profile of crude extracts from *P.granulosa* was analysed[22] by resolving in Native - Polyacrylamide Gel Electrophoresis (NATIVE-PAGE) and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)[23]. About 40 µg of protein was loaded on 10% gel and the resolved protein bands were visualized by staining with Coomassie brilliant blue (CBB R-250).

DPPH scavenging activity

The DPPH radical scavenging activity of leech extracts were examined by adding 2 ml of 0.16 mM DPPH solution (in ethanol) in to the tube containing 2 ml aliquots of samples in different concentrations (0.2 – 1 mg/ml)[24]. A separate control was also prepared using DPPH solution and water. Then the test tubes were thoroughly mixed using vortex for 1 min and left undisturbed in dark for 30 min at 30 °C. The absorbance was taken at 517 nm. Finally, the antioxidant potential was determined by calculating the inhibition percentage of the DPPH radical with the following formula:

DPPH scavenging effect (%) = $A_0 – A_1 / A_0 \times 100$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the test samples.

O_2 scavenging activity

The superoxide anions radicals were produced by adding 1 ml of 2.52 mM Nitro Blue Tetrazolium chloride (NBT) and 624 mM NADH (624 mM) stock solution into 1 ml of test samples. The scavenging reaction was activated by adding 1 ml of 120 µM Phenazine Methosulphate (PMS) solution. Then the tubes were incubated at 25 °C for 20 min and absorbance was taken at 560 nm against blank. Here

L-Ascorbic acid was served as a positive control. The decrease in the absorbance value indicates the superoxide anion scavenging activity[25]. The inhibition percentage of superoxide anion radicals was calculated using the following formula:

Inhibition (%) = $(A_0\text{-}A_1)/A_0 \times 100$

Where, A_0 is the absorbance without the sample and A_1 is the absorbance with the sample.

Statistical analysis

All analyses were performed with three or more independent experiments and the data are shown as the means ± standard deviation (SD) or means ± standard error (SE).

Results

Biochemical analysis

Analysis of various biochemical constituents in crude extracts of *P. granulosa* was estimated. The protein amount present in whole tissue, head, saliva and mucus are 20.12±1.05 mg/ml, 14.54±0.75 mg/ml, 2.84±0.20 mg/ml and 0.54±0.05 mg/ml, respectively. Also, the lipid content was found to be 1.92±0.04 mg/ml and 2.41±0.05 mg/ml in both whole tissue and head. In saliva and mucus, the concentration of estimated lipids was 0.89±0.02 mg/ml and 1.05±0.03 mg/ml, respectively. The carbohydrate content in the whole tissue, head, saliva and mucus were 2.90±0.10, 2.28±0.03, 0.16±0.00 and 0.02±0.00 mg/ml, respectively (Fig. 1). The maximum protein content was obtained in the whole-body extract (20.12±1.05 mg/ml).

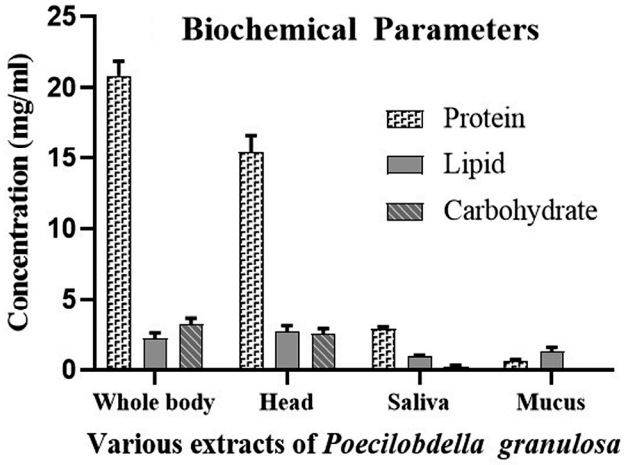

Fig. 1 — Biochemical constituents of various extracts of *Poecilobdella granulosa.* Each value was presented as the mean±SD; the values given in the above graph were expressed as mg/ml

Qualitative analyses of leech extracts

The qualitative protein profile of crude extracts prepared from various organs (whole-body, head, saliva and mucus) of *P. granulosa* was performed by Native and SDS-PAGE (Fig. 2). The major and minor bands of four crude protein extracts were identified using Native-PAGE. The SDS-PAGE analysis showed different polypeptide sub-units ranging from 22-110 kDa of the native protein samples (whole-body, head, saliva and mucus).

Assessment of antioxidant activity

The antioxidant potential of crude extracts was analysed by DPPH scavenging assay and superoxide anion radical scavenging assay. In both results, the percentage of free radical scavenging capacity of crude saliva was increased in a dose-dependent manner compared to L-ascorbic acid (standard). Here, various concentrations were used as 200, 400, 600, 800 and 1000 µg/ml and the inhibition percentage of scavenging activities of the saliva extract for DPPH radicals showed 82.8 % at 1000 µg/ml concentration (Fig. 3).

Besides, the decrease in the absorbance value at 560 nm with the leech saliva extract and reference standard L-ascorbic acid, their ability to quench superoxide free radicals showed maximum inhibition of 98.4 % at 1000 µg/ml of concentration. The superoxide radical scavenging activity was slightly enhanced with increasing concentrations. At higher concentration like 1000 µg/ml of saliva extract showed almost equal scavenging capacity to standard

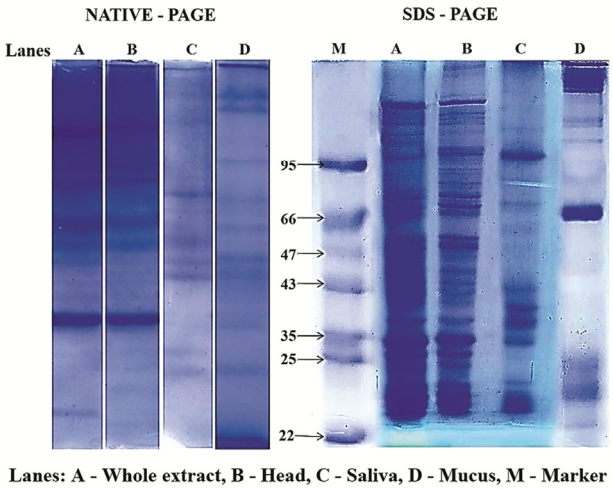

Fig. 2 — Qualitative analyses of crude proteins from various extracts of leech by Native and SDS PAGE. The proteins in the crude samples were resolved and stained with CBB and various Native proteins and subunits of native proteins were analyzed

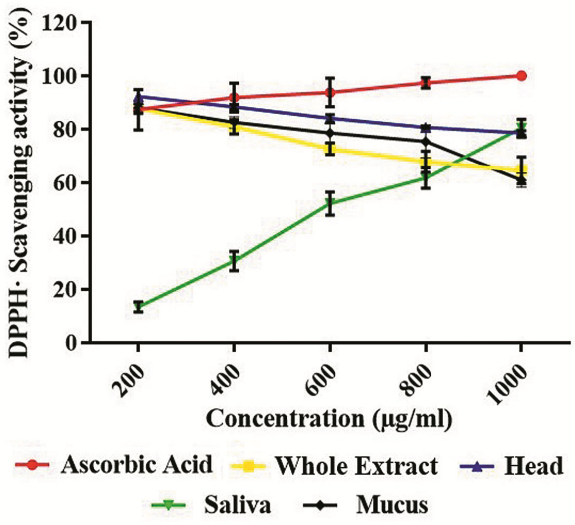

Fig. 3 — DPPH scavenging activities of various extracts of *Poecilobdella granulosa.* Each value was presented as the mean±SD; the values given in the above graph were percentage (%) scavenging activity; Ascorbic acid was only used as a reference sample in DPPH scavenging activity

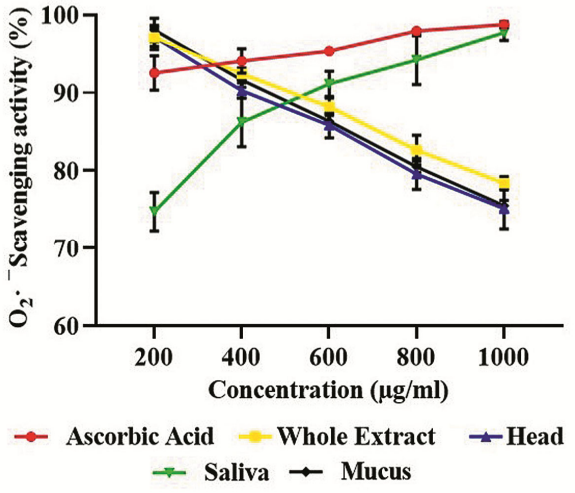

Fig. 4 — Scavenging effects of various extracts of leech *Poecilobdella granulosa* on superoxide anion radical ($O_2^{\cdot-}$). Each value was presented as the mean±SD; the values given in the above graph were percentage (%) scavenging activity; Ascorbic acid was only used as a reference sample in Superoxide anion radical scavenging activity ($O_2^{\cdot-}$)

ascorbic acid and the other extracts revealed no activity in all the concentrations (Fig. 4).

Discussion

Leeches have been used to treat a wide range of ailments since antiquity. Many literature reports revealed that LSE contains numerous bioactive compounds that aid in the treatment of various diseases[26]. Leeches contains more than 20 different bio molecules, each of which had a different mode of action. These included analgesic, anti-inflammatory, platelet function-inhibiting, anticoagulant, thrombin function-regulating, destructive of the extracellular matrix, antimicrobial, and anti-metastatic compounds[27]. The current study explores the biochemical and antioxidant activity of four different extracts (whole-body, head, saliva and mucus) from leech *P. granulosa.* The biochemical results demonstrated the highest content of protein in comparison to lipids and carbohydrates in the whole extract followed by other extracts. Similarly, the reports from the saliva of *Hirudinaria manillensis* showed 54.29 ± 2.58 µg/ml of protein[11]. In contrast, the protein (79.962 ± 3.213 µg/ml, 62.602±2.517 µg/ml) from Malaysian leech saliva varied according to the starvation time[17]. The electropherogram of separated proteins of crude extracts showed dissimilar protein bands in the proximal, mid and distal (Native and SDS-PAGE). Similarly, more than 25 types of peptides and proteins (10 to 170 kDa) were identified in leech *Hirudo medicinalis*[28]. Moreover, SDS-Tricine PAGE revealed the existence and absence of a few protein and peptide bands in saliva depending on the starvation period[29].

Antioxidants are also known as radical scavengers, which inhibit the oxidation process and eliminate free radical intermediates by oxidizing themselves even at very low concentrations[30,31]. Oxidative processes produce oxygen reactive species, which are a potent precursor of systemic cell and tissue damage[32]. In the present investigation, the leech extracts were evaluated for their antioxidant activity. When compared to the other three extracts, only the saliva extract displayed DPPH radical scavenging activity. Likewise, an extract of the saliva from the therapeutic Malaysian leech *Hirudinaria manillensis* revealed free radical scavenging activity, with an IC_{50} value of 7.282 µg/ml[33]. A previous report on saliva extract from *Aliolimnatis michaelseni* revealed high free radical scavenging activity with an IC_{50} value of 8.169 µg/ml[34]. Indonesian local leeches are also reported to possess an IC_{50} value of 47 – 108 µg/ml[35]. The liposomal leech saliva extract from *Hirudo medicinalis* exhibited anti-proliferation activity against the human breast adenocarcinoma cell line (MCF-7)[28]. Moreover, the saliva extract from *H. medicinalis* displayed anti tubercular activity[36].

Superoxide anion radical represent as one of the most powerful reactive oxygen species among free

radicals, and it is extremely harmful to cellular components[37]. In the PMS/NADH – NBT system, the superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT[38]. Despite the fact that protein samples generally have strong superoxide anion radical scavenging activity and this scavenging activity depends on the protein concentration[39]. Present results indicated that the radical scavenging effect slowly rose with increasing concentration, showing that the leech saliva extract is a more potent scavenger of superoxide radicals.

Conclusion

The present study revealed that the saliva extract of leech exhibited higher potent antioxidant activity than other extracts. As a result, the available data imply that saliva extract can be utilized as a reliable source of natural antioxidants in the medical field for the treatment of numerous diseases. Further investigation is desired to isolate the bioactive compounds to determine their pharmacological properties.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

AC & DP designed the work. DP & RGM performed the experimental work and animal maintenance. GJS drafted the work and interpretation of the data. KR contributed to the acquisition of data.

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