The Bioscan AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES 20(2): 111-121, 2025 www.thebioscan.com

BIOCHEMICAL CHARACTERIZATION OF ETHANOLIC CRUDE INK AND MUCUS OF DOLABELLA AURICULARIA, WEDGE SEA HARE (LIGHT FOOT 1786) TUTICORIN COAST

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DOI: 10.63001/tbs.2025.v20.i02.pp111-121

KEYWORDS
Dolabella Auricularia, Ethanolic extract, Invitro, Biochemical activity.

Received on:

20-03-2025

Accepted on:

25-04-2025

Published on:

30-05-2025

ABSTRACT

Significantly, the formative research evaluates the biochemical properties of ethanolic extracts of ink and mucus derived from *Dolabella auricularia* commonly known as wedge sea hare from Tuticorin coast. The quantitative and qualitative research focussed on concentration of bioactive molecules. The invitro analysis of ink and mucus of *D.auricularia* exhibited potent biochemical activities. The extracts also demonstrated α-amylase inhibition (ink: 50.41%, mucus: 52.01%), membrane stabilization activity (ink: 36.62%), protein denaturation inhibition (ink: 86.46%, mucus: 91.67%), metal chelation (ink: 83.94%, mucus: 80.31%), and antioxidant activity (ink: 72.22%, mucus: 61.11%). These findings suggest wedge sea hare, *Dolabella auricularia* as a potential source of therapeutic agents, requiring further investigation to isolate bioactive compounds for pharmaceutical applications.

INTRODUCTION

The sea is a treasure of life, which contains a wealth of varied organisms that yield new chemical compounds. These natural chemicals hold immense value for application in diverse industries like medicine, cosmetics, nutrition, and agriculture. In recent times, researchers have found numerous novel marine-derived compounds possessing strong medicinal properties. Even though only some of the marine-derived products are presently available on the market, some promising compounds are now in the process of being tested through clinical trials to be developed into drugs. While the marine world offers an extremely rich resource for novel compounds, it also represents a great challenge that requires multidisciplinary approach to bring the marine chemical diversity up to its therapeutic potential. Therefore, the marine environment with special reference to invertebrates that rely solely on innate immune mechanisms for host defence, is a spectacular resource for the development of new antimicrobial compounds (Maripandi *et al.,* 2010). The marine environment comprises of complex ecosystem with a plethora of organisms and many of these organisms are known to possess bioactive compounds as a common means of defense. The marine natural products have been investigated predominantly for their antimicrobial, cytotoxic, antitumour and anti-inflammatory properties (Anand *et al.*, 1997).

Recently, drug discovery programs have directed their attention to unusual sources like marine invertebrates (molluscs, sponges, sea cucumber, etc) hoping to identify more efficacious therapeutic tools with novel chemical structures and unique modes of action (Nocchi *et al.*, 2017). Sea hares are a group of molluscs, Gastropod; shell-free marine opisthobranchs, which includes several genera and many species (Derby, 2007) and recognized as a source of a diverse range of metabolites 5alpha,8alpha-Endoperoxides belong to a group of oxidized sterols commonly found in marine organisms and display several bioactivities, including antimicrobial, anti-tumour, and immunomodulatory properties. (Pereira et al., 2019). A huge number of molecules have been discovered from Aplysia species possess secondary metabolites (Abe Kawsar *et al.*, 2010), such as anti-cancer, anti-tumor and anti-viral compounds which are very useful in the pharmacological industry. In India, sea slugs have been used for the extraction of natural anti-cancer compounds like Cemadotin, Soblidotin, Kahalalide F and Synthadotin/ILX6512. (Sethi, 2019). The internal defence system of mollusks represents an efficient protection against pathogens and parasites, involving several biological immune processes, such

as phagocytosis, encapsulation, cytotoxicity and antigenic recognition of self and non-self. (Alesci, 2023). Several bioactive substances are found in the ink alone and some in opaline alone, and others are generated only when ink and opaline are co-secreted and mixed in the mantle cavity (Kicklighter *et al.,* 2005; Derby *et al.,* 2007).

The screening of marine organisms to explore their potential as a source of biologically active products is necessary. A deep understanding of chemical and biological structure of bioactive compounds from marine organisms will pave way for identification of novel drugs. Based on the above facts the present study has been initiated to test biochemical characterisation of ethanolic crude ink and mucus of marine gastropod sea hare, *Dolabella auricularia*.

MATERIALS AND METHODS

Collection of *Dolabella auricularia*

For the present study, wedge sea hare *Dolabella auricularia* were collected from Therespuram, located in the Gulf of Mannar coastal region of Tuticorin. The samples were obtained using a drift gill net launched from a traditional fishing craft. After collection, the sea hares were immediately washed with seawater at the sampling site to remove adhered sediments and impurities. Once after proper cleaning the samples were then placed in polythene bags for transportation. On the same day, the samples underwent a quick rinse with tap water in the research laboratory to get rid of remaining contaminants, guaranteeing the purity of the specimens for further analysis. (Hassan *et al*., 2020).

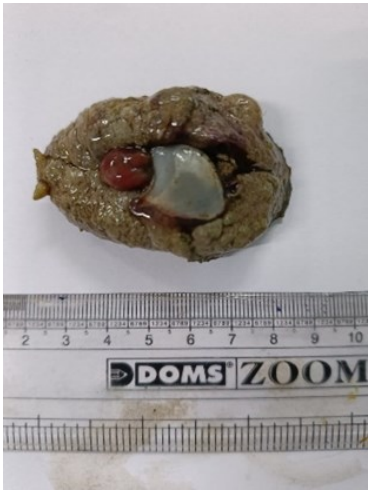

Fig 1: Ventral view of *Dolabella auricularia*

EXTRACTION OF CRUDE INK AND MUCUS

Under sterile conditions, the ink was harvested from the specimens following the careful dissection of *D. auricularia* ink sac. Crude ink was centrifuged prior to its use to remove any debris, diluted with the help of sterile distilled water, and stored at -20°C. Mucus of *D.auricularia* was dissected by gently stimulating the specimens to secrete their slime. The mucus secretions were collected, centrifuged, and filtered to obtain clear samples.

PREPARATION OF EXTRACT

Wedge sea hare *D.auricularia* was dissected with caution to obtain its ink and mucus. After dissection, the ink was drained out and kept in a vial, then the mucus was collected and kept in a different vial. For preparing the extracts, the ink and mucus samples were treated with 70% aqueous ethanol. Further, 1 mL of ink was poured into a sterile tube, and 9 mL of ethanol were added to it. The mixture was blended well for even distribution, creating a 10% (w/v) ink extract in ethanol. following the same, 1 mL of mucus was mixed with 9 mL of ethanol in another sterile tube and blended well, creating a 10% (w/v) mucus extract in ethanol. After the preparation, the extracts were filtered through Whatman No. 4 filter paper to ensure removal of contaminants. The resultant purified extracts were then screened for their activities.

ANTIDIABETIC ACTIVITY

Alpha amylase inhibition assay

In order to determine the Alpha inhibition assay, initially 1ml of starch was added to control and sample tubes to serve as a substrate for the enzyme reaction. Then, 0.5ml of amylase enzyme was added to all the tubes to catalyze starch hydrolysis and incubation of test tubes at 37ºC for 30 min to allow the enzyme to react on the starch. After that 1ml DNSA (3,5-dinitrosalicylic acid) reagent was added to all the test tubes that stop the enzymatic reaction and develop colour which is associated with the reducing sugars formed after hydrolysis of starch. Subsequently, the tubes were kept for 15 min at 95 ºC, ensures full reaction of DNSA with the reducing sugars. Then the tubes were allowed to cool at room temperature and with spectrometer, the absorbance of the sample was measured at 510 nm. The inhibition of alpha amylase inhibition assay was then determined using the formula given below:

% Inhibition = (Abs Control - Abs Sample) / Abs Control * 100

The above analysis determined the test sample ability to inhibit amylase activity.

MEMBRANE STABILIZATION ASSAY

To evaluate the Membrane stabilization assay, a series of step were undergone. First 3ml of blood collected in a heparinized tube in order to prevent clotting. The blood was centrifuged at 2000 rpm for 5 minutes and then the cells from plasma was separated. To maintain their viability, the supernatant was discarded, and the cells were resuspended in cold PBS (phosphate-buffered saline). Later, 0.1 ml of the blood sample was aliquoted into the test tubes which serve as control. The sample of different concentration (200, 400, 600, 800, and 1000 µg/ml) were then added to the tubes, using PBS the tubes filled with same volume and uniformity is maintained. Further, the tubes were heat-treated at 40ºC for about 10 minutes to activate haemolysis, Then the tubes were permitted to cool at room temperature and using spectrometer the absorbance of each sample was measured at 540 nm. The inhibition of haemolysis was then calculated using the formula given below:

% Inhibition = (Abs Control - Abs Sample) / Abs Control * 100

The above analysis determined the test sample ability to inhibit haemolysis compared to the control.

PROTEIN DENATURATION INHIBITION ASSAY

Protein denaturation inhibition assay was done according to the methodology described by Gambhire *et al*., (2009) The reaction mixture of 5 mL was prepared consisted of 0.2 mL of 1% bovine albumin; 4.78 mL of phosphate buffered saline (PBS) at pH 6.4) to which 200 -1000 µg/ml of sample were added with varying concentration. The mixture was mixed thoroughly and was incubated in a water bath at 37 ◦C for 15 minutes and then the reaction mixture was heated at 70 ◦C for 5 minutes to initiate denaturation. After cooling, the Lowry reagent was added to the mixture and absorbance was measured at 660 nm using a UV/VIS spectrometer. A Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:

% Inhibition = [Absorbance sample/Absorbance standard] ×100

The assessment of the sample effectiveness in this assay against protein denaturation provided profound observation into its potential bioactive properties.

METAL CHELATION ACTIVITY

The ferrous ion chelating potential of the extracts was evaluated using the methodology described by Dinis *et al.*, (1994). The reaction mixture contained 1.0 ml of various concentrations of the extracts (2-10 .mg/ml) and 0.05 ml of 2 mM FeCl3. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine which initiated the complex formation with ferrous ions. The reaction mixture was shaken vigorously and left standing at room temperature for 10 min for proper reaction and the absorbance of the reaction mixture was measured at 562 nm against a reagent blank. A lower absorbance of the reaction mixture indicated a higher ferrous ion chelating ability. The control contained all the reagents except the sample. The inhibition of ferrous ion chelation percentage was calculated using the formula:

% Inhibition = [(Control- Test) / control] ×100

Metal chelation assay provides profound observation into the extracts, which is decisive for understanding their potential as antioxidants and their role in reducing oxidative stress.

TOTAL ANTIOXIDANT ACTIVITY BY PHOSPHOMOLYBDENUM METHOD

Determination of Total antioxidant activity

The total antioxidant activity of the extracts was evaluated by phosphomolybdenum method described by Prieto *et al.*, (1999) 1.0 ml of the extract was mixed with 1.0 ml of the standard reagent solution, which constituted 0.6M sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate. The tubes were capped and incubated in a thermal block at 95°C for 90 min to ease the appearance of green phosphomolybdenum complex, which indicate the antioxidant activity. After the mixtures was allowed to cool at room temperature, the absorbance was measured at 695 nm using a UV/VIS spectrophotometer, against a reagent blank which act as a control. The percentage of Antioxidant activity was calculated using the formula:

% Antioxidant activity = Sample Absorbance/Standard Absorbance × 100

The above method provides an authentic measure of the total antioxidant capacity of the extracts, which give consideration regarding the potential to nullify reactive species and ease oxidative stress.

RESULT AND DISCUSSION

Antidiabetic activity-Alpha amylase inhibition assay

During this experiment, a set of test tubes was prepared to study the effect of different concentrations of an unknown substance on both chemical or biological system. Test tube 1 acted as control, giving a simple standard for comparison. Test tubes 2 to 6 had differing concentrations, 200, 400, 600, 800, and 1000 µg/ml, respectively. To evaluate each of these test tubes, study aim to find out the concentration influences on the response, which helped to assess the potency and efficacy of the substance. This systematic process is required for knowing the dose-response relationship, under varying conditions.

Fig: 2 Effect of Alpha amylase inhibition assay: A sample set with a control and a series of concentrations

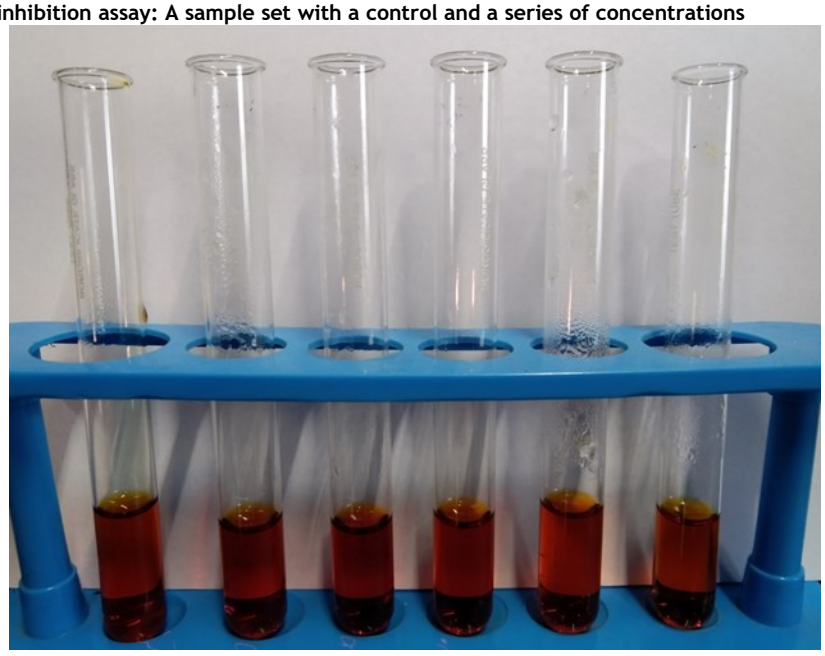

Table: 1 Sample concentration on absorbance and % of inhibition at 510 nm

Sample µg/ml	Absorbance at 510nm	% Inhibition
Control	1.21	
Ink 200	1.17	3.31
400	1.06	14.15
600	0.90	25.62
800	0.76	37.19
1000	0.60	50.41
Mucus 200	1.03	14.88
400	0.95	21.49
600	0.90	25.62
800	0.75	38.01
1000	0.58	52.07

The control sample absorbance is 1.21 at 510nm. Once the concentration of ink and mucus increases, the absorbance decreases and therefore the percentage of inhibition increases based on the dosage effect. Comparatively, mucus showed higher

percentage of inhibition at higher concentration of 800 µg/ml and 1000 µg/ml when compared to ink sample.

Our study confirmed both ink and mucus sample showed inhibition effect at increased concentration of samples. Therefore, it indicated the concentration-dependent effect. The result also suggested that higher concentration resulted in greater percentage of inhibition. Regarding Alpha amylase inhibition, Graciela *et al.*, (2022) study proved inhibition activity indicates that the extract contained bioactive metabolites, which have an inhibitory effect on the enzyme α-amylase. However, extracts if had no effect on the enzyme activity, suggesting that extracts do not contain inhibitors of the enzyme α-amylase. So, it is concluded the extracts of both ink and mucus contain inhibitors/bioactive metabolites of the enzyme alpha amylase

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Fig: 3 Antidiabetic activity of ink at different concentration of sample, µg/ml

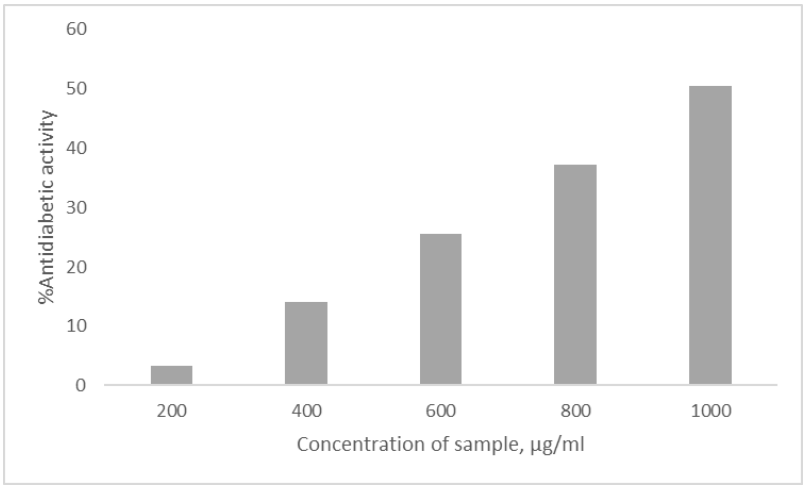
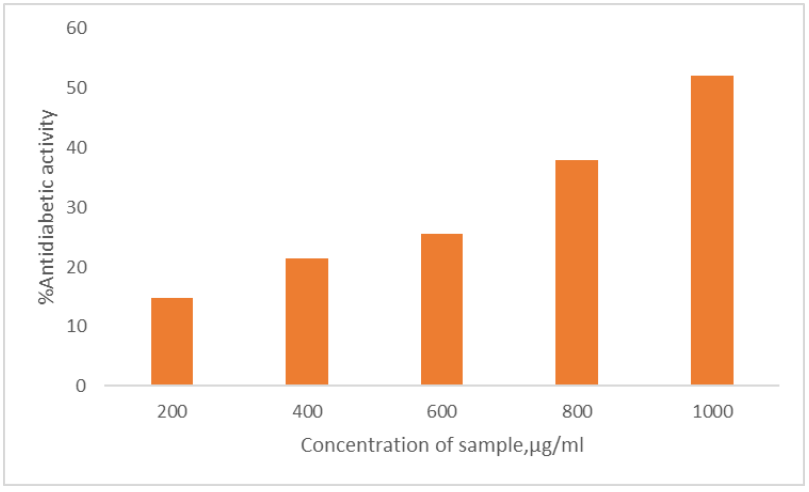


Fig: 4 Antidiabetic activity of mucus at different concentration of sample, µg/ml



Membrane stabilization assay

Membrane stabilization assay, includes a set of test tubes to study the effect of different concentrations of an unknown substance on both chemical or biological system. Test tube 1 acted as control, giving a simple standard for comparison. Test tubes 2 through 6 had differing concentrations, 200, 400, 600, 800, and 1000 µg/ml, respectively. To evaluate each of these test tubes, study aim to find out the concentration influences on the response, which helped to assess the potency and efficacy of the substance. This systematic process is required for knowing the dose-response relationship, under varying conditions.

Fig: 5 Effect of Membrane stabilization assay: A sample set with a control and a series of concentrations

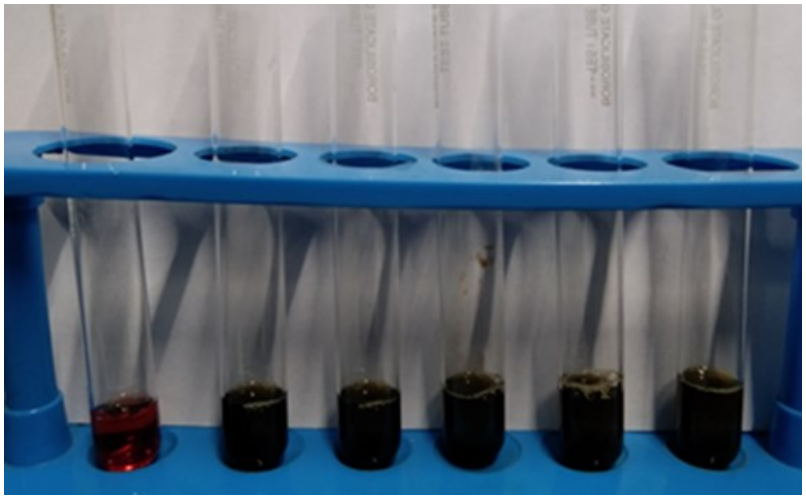


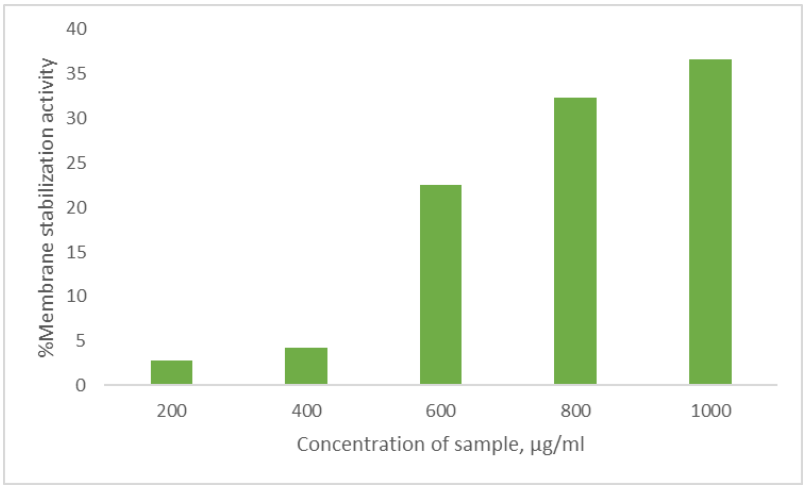
Table: 2 Sample concentration on absorbance and % of inhibition at 510 nm

Sample µg/ml	Absorbance at 510nm	% Inhibition
Control	1.42	
Ink 200	1.38	2.82
400	1.36	4.22
600	1.10	22.54
800	0.96	32.39
1000	0.90	36.62
Mucus 200	2	No activity
400	2	-
600	2	-
800	2	-
1000	2	-

The control of absorbance is 1.42. The absorbance decreases as the concentration of the ink (µg/ml) increases, it shows the ink has an inhibitory effect at 510 nm on the light absorption. The concentration of the ink and its inhibitory effect showed a positive association. Yesmin *et al.*, (2020) correlates our study that membrane stabilization assay is a concentration-dependent process and protection increased with increase in the concentration of the sample. All mucous concentrations for absorbance are at 2.00, which indicated no inhibition effect under the tested concentrations whereas ink showed remarkable inhibition above 600. µg/ml whereas mucous has no inhibitory activity at the tested concentrations.

Membrane stabilization used to prevent swelling caused by fluids in body tissues and organs. The leakage of serum protein and fluids into the tissue can be prevented by membrane stabilization. This process goes on by inflammatory intermediators where there is an increase in permeability of membrane (Chaitanya *et al.*, 2011) and measures the ability of a drug to stabilize the cell membrane by preventing its disruption and subsequent release of intracellular contents (Ameena *et al.*, 2023).

Fig: 6 Membrane stabilization activity of ink at different concentration of sample, µg/ml



Protein denaturation inhibition assay

The assay, comprises set of test tubes was prepared to study the effect of different concentrations of an unknown substance on both chemical or biological system. Test tube 1 acted as control, giving a simple standard for comparison. Test tubes 2 to 6 had differing concentrations, 200, 400, 600, 800, and 1000 µg/ml, respectively. To evaluate each of these test tubes, study aim to find out the concentration influences on the response, which helped to assess the potency and efficacy of the substance. This systematic process is required for knowing the dose-response relationship, under varying conditions.

Fig: 7 Effect of Protein Denaturation Inhibition: A sample set with a control and a series of concentrations

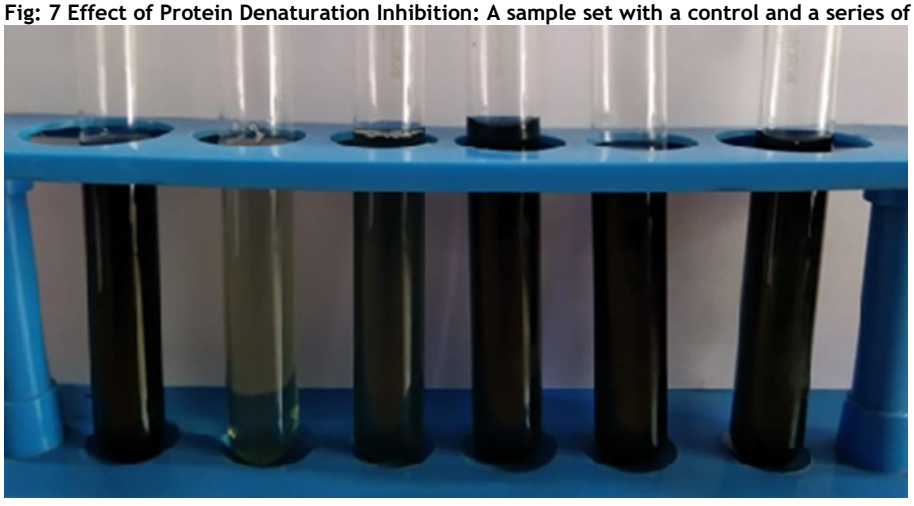


Table: 3 Sample concentration on absorbance and % of inhibition at 660 nm

Sample Concentration µg/ml	Absorbance at 660nm	%Inhibition
Aspirin	1.92	
Ink		
200	0.20	10.42
400	0.85	44.27
600	1.20	62.5
800	1.40	72.92
1000	1.66	86.46
Mucus		
200	0.17	8.85
400	0.76	39.58
600	1.17	60.94
800	1.38	71.88
1000	1.76	91.67

Protein denaturation occurs when a variety of physical and chemical agents alter the electrostatic force, hydrophobic bonds, disulphide and hydrogen bonds in proteins, rendering them insoluble (Sangeetha and Vidhya, 2016). Protein denaturation is mostly involved with chronic inflammation such as rheumatoid arthritis where denatured proteins act as auto-antigens leading to auto-immune disease (Karthik et al., 2013) The anti-inflammatory activity of a compound indicated by the percentage of protein denaturation assay. The Aspirin absorbance is 1.92 at 660nm, which act as a control. Aspirin, diclofenac, indomethacin, and ibuprofen are NSAIDs used for inflammation. They induce synthesis of a protein "lipocortin-1" which has the inhibitory effect on phospholipase A2 (Tripathi 2008). As the absorbance increases with the concentration of ink increases which indicated the inhibition of protein denaturation. As the percentage of inhibition increases with the concentrations there is a strong association between ink concentration and its capability to inhibit protein denaturation. Related to ink, mucus also had a same

effect on protein denaturation. Though both ink and mucus had the capability to inhibit protein denaturation, at lower concentrations ink showed greater effect of inhibition compared to mucous. NSAIDS such as aspirin are reported to inhibit protein denaturation (Karthik et al., 2013). Therefore, aspirin used as the standard for the protein denaturation inhibition assay and positive result is obtained.

Fig: 8 Protein denaturation of ink at different concentration of sample, µg/ml

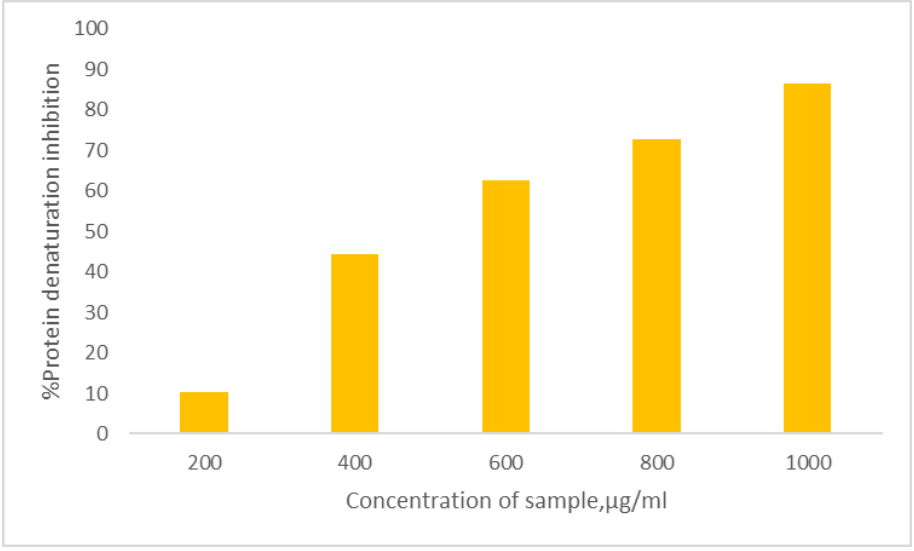
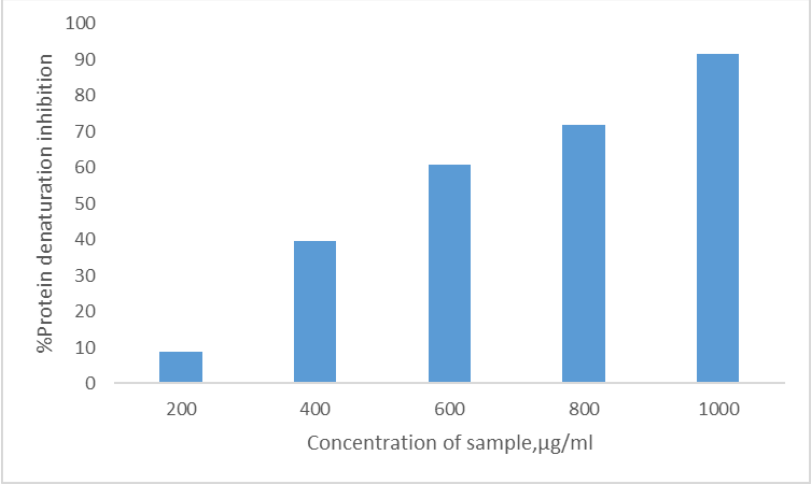


Fig: 9 Protein denaturation of mucus at different concentration of sample, µg/m



Metal chelation activity

Metal chelation assay, includes a set of test tubes to study the effect of different concentrations of an unknown substance on both chemical or biological system. Test tube 1 acted as control, giving a simple standard for comparison. Test tubes 2 through 6 had differing concentrations, 200, 400, 600, 800, and 1000 µg/ml, respectively. To evaluate each of these test tubes, study aim to find out the concentration influences on the response, which helped to assess the potency and efficacy of the substance. This systematic process is required for knowing the dose-response relationship, under varying conditions. The percentage oi Inhibition allows to analyse the effect of the test substance to a control, reveals the relationship between concentration and inhibitory effect. This standardized evaluation drawn conclusions on the efficacy of the substance in the experiment.

Fig: 10 Metal chelation activity: A sample set with a control and a series of concentrations

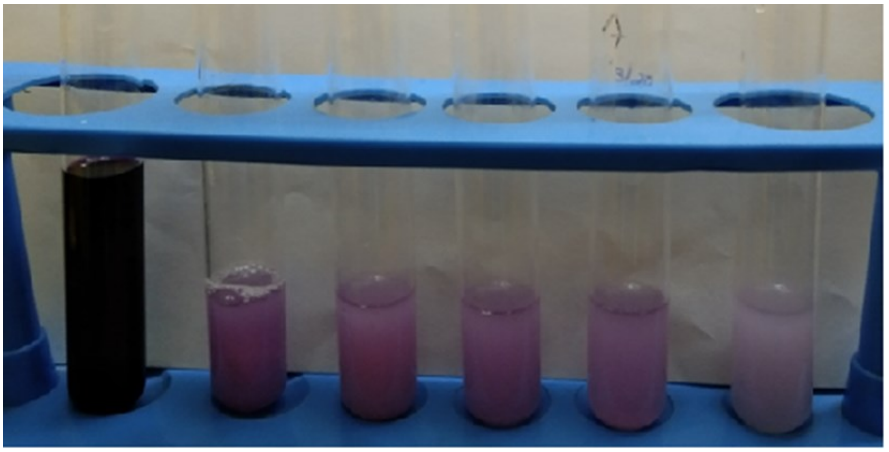


Table 4: Sample concentration on absorbance and % of inhibition at 660 nm

Sample Concentration µg/ml	Absorbance at 660nm	%Inhibition
Control	1.93	
Ink		
200	0.88	54.40
400	0.76	60.62
600	0.69	64.25
800	0.42	78.24
1000	0.31	83.94
Mucus		
200	0.82	57.51
400	0.78	59.59
600	0.71	63.21
800	0.44	77.20
1000	0.38	80.31

Metal chelating assays as widely used methods to determine antioxidant ability of compounds. The accumulation of heavy metals in humans can cause serious damage to different organs, especially respiratory, nervous and reproductive and digestive systems. Biologically, metal chelation therapy is often used to treat metal toxicity (Gulcin & Alwasel 2022). Ouahhoud *et al.*, (2022) strongly suggest that Crocus sativus by-products contain natural antioxidant, metal chelating and nonprotective compounds, which may be capable of reducing the risk of cancer and other diseases associated with daily exposure to genotoxic xenobiotics. Similarly, the metal chelating assay of *D.auricularia* ink and mucus contribute shield benefits. The notable presence of metal chelating compounds in ink and mucus could help to get protection over toxic substances and alleviate cellular protection against toxic substances. The absorbance is 1.93 for control sample at 660 nm. The value of absorbance decreases with increasing concentration of ink which indicated the metal chelation activity. Similarly, mucus also decreases in absorbance with increasing concentration. The metal chelatin activity of sample and standard at different concentrations were found in the order 1000µg/ml <800µg/ml <600µg/ml <400µg/ml <200µg/ml for ink and similar order were found for mucus. Though both ink and mucous showed the metal chelation activity, ink exhibited higher percentage in comparison with mucus particularly at the highest concentration of 1000 µg/ml.

Fig: 11 Metal chelation activity of ink at different concentration of sample, µg/m

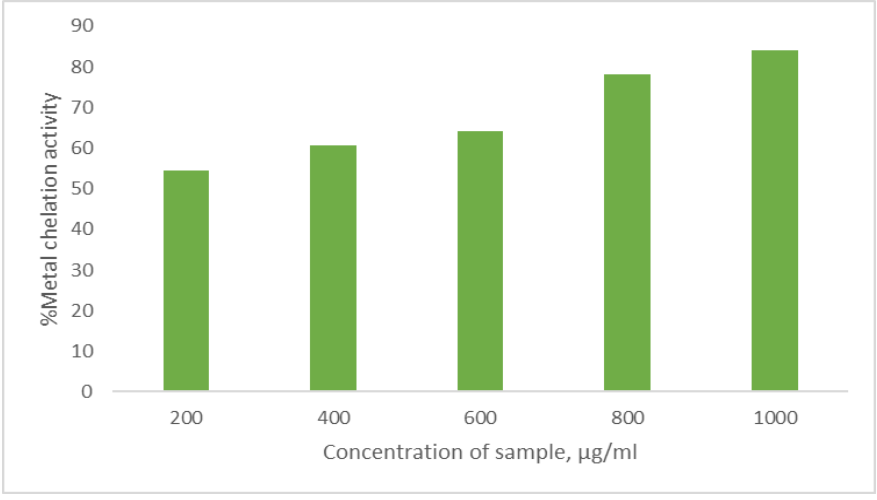
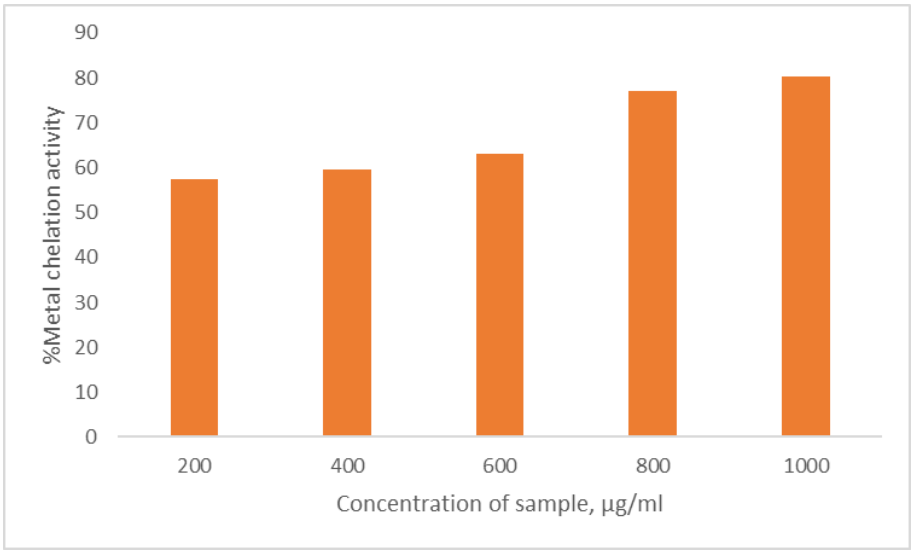


Fig: 12 Metal chelation activity of mucus at different concentration of sample, µg/m



Total Antioxidant activity by phosphomolybdenum method

Antioxidant activity by phosphomolybdenum experiment, used to assess the antioxidant properties of a test substance and compared to Ascorbic Acid, a standard antioxidant. Test tube 1 acted as control, giving a simple standard for comparison. Test tubes 2 through 6 had differing concentrations, 200, 400, 600, 800, and 1000 µg/ml, respectively. To evaluate each of these test tubes, study aim to find out the concentration influences on the response, which helped to evaluate the efficacy of the substance.

This analysis determined the inhibition of oxidant by test substances compared to the Ascorbic Acid standard. A higher percentage of inhibition at a particular concentration indicates a stronger antioxidant effect of the test substance. By plotting the results, we can evaluate the dose-response relationship to understand whether the antioxidant activity increases with concentration. This systematic approach will help us gain insights into the efficacy and potential applications of the test substance in fields such as biochemistry, pharmacology, or food science.

Fig: 13 Antioxidant activity by phosphomolybdenum method: A sample set with a control and a series of concentrations

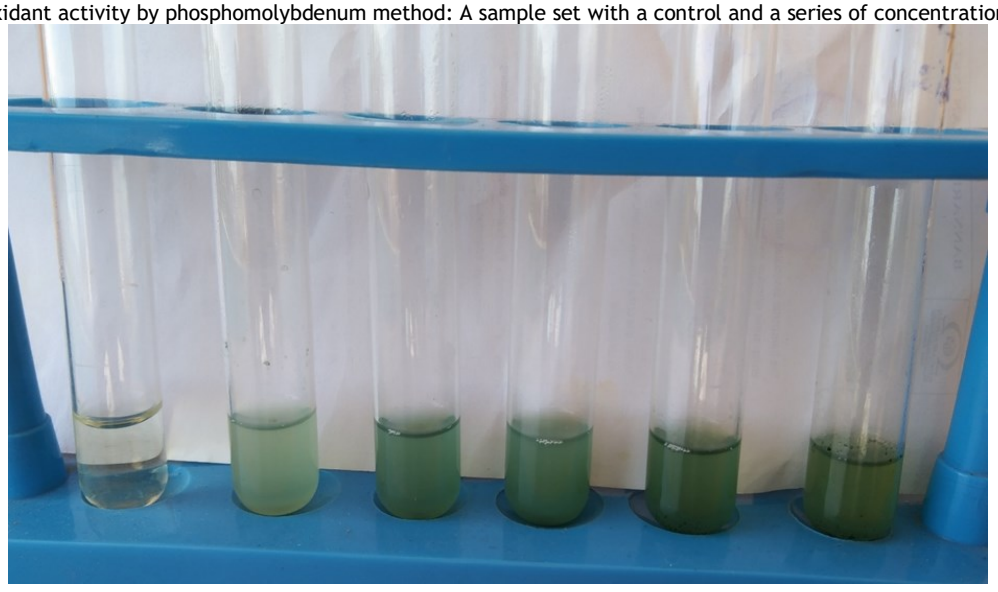


Table 5: Sample concentration on absorbance and % of inhibition at 695 nm

Sample	Sample concentration µg/ml	Absorbance at 695 nm	% Antioxidant activity = sample Absorbance /Standard Absorbance × 100
Ascorbic acid	1000	1.80	
Ink	200	0.50	27.78
	400	0.75	41.67
	600	0.92	51.11
	800	1.10	61.11
	1000	1.30	72.22
Mucus	200	0.48	26.67
	400	0.72	40
	600	0.88	48.89
	800	0.97	53.89
	1000	1.10	61.11

Ascorbic acid is used as standard for phosphomolybdenum to evaluate the antioxidant activity. Total antioxidant capacity was determined by the phosphomolybdenum method (Prieto *et al.*, 1999). The phosphomolybdenum method is quantitative since the antioxidant capacity is expressed as the number of equivalents of ascorbic acid (Khan et al., 2012). The ascorbic acid absorbance is 1.80 at 1000 µg/ml. An increase in antioxidant activity was noted with respect to the increase in absorbance with concentration. Mashwani *et al.*, (2013) stated that assay concentrations of antioxidant in the sample were directly proportional to the reduction potential of the sample and standard thus showed higher absorbance and higher reduction potential at increased concentration. The above said statement agrees with our present study. At each series of concentration, the ink showed a moderate to strong antioxidant activity. Similarly, mucus also showed increase in absorbance with concentration. The antioxidant activity of sample and standard at different concentrations were found in the order 1000µg/ml >800µg/ml >600µg/ml >400µg/ml >200µg/ml for ink and similar order were found for mucus. Though mucus showed significant antioxidant properties, it is little lower in comparison to that of ink. Thus, ink had higher antioxidant activity compared to mucus at each concentration.

Fig: 14 Antioxidant activity of ink at different concentration of sample, µg/m

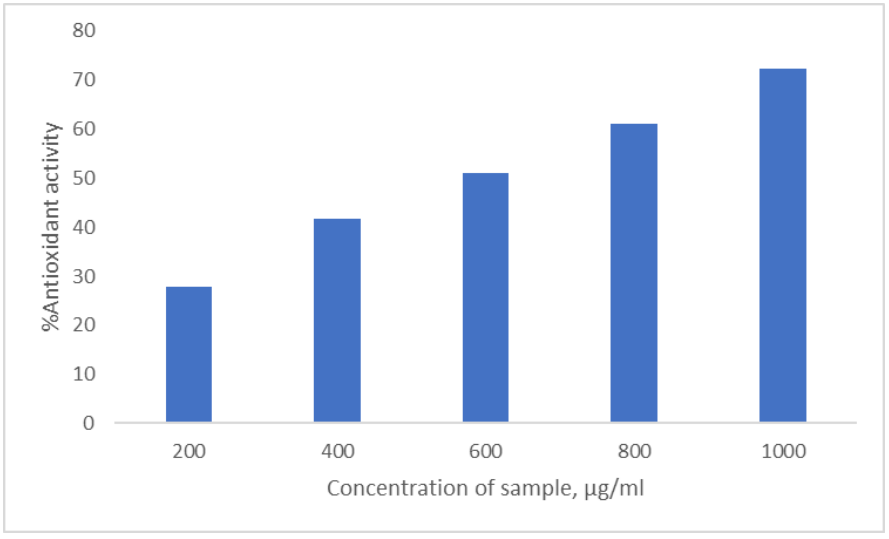
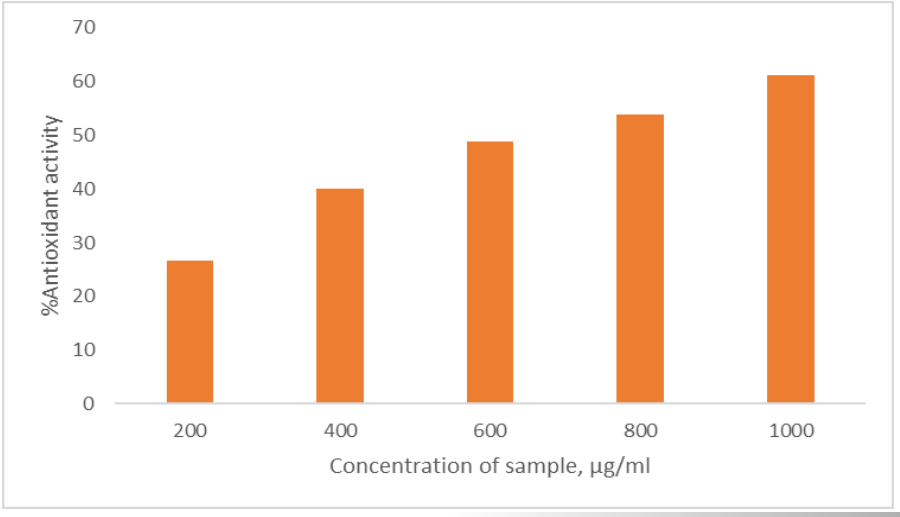


Fig: 15 Antioxidant activity of mucus at different concentration of sample, µg/m



CONCLUSION

In conclusion, the present study indicates, the ethanolic crude ink and mucus of *D.auricularia* showed good spectrum of biochemical activity. Biochemical activity of ink was more pronounced than mucus. The study proved as therapeutic agents in handling infections and diseases. However further investigations to separate and elucidate the bioactive compounds and mechanism of actions are needed for development of novel drugs. Moreover, present data of our study can collaborate with the defense mechanism of *D.auricularia*.

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