Evaluating the antioxidant activity of epidermal mucus extract in marine fishes *Anguilla anguilla* and *Brachirus orientalis*

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Abstract

Marine environment having an exceptional reservoir of bioactive products globally. Our research intended to explore the antioxidant potential of fish protein hydrolysates of *Anguilla anguilla* and *Brachirus orientalis*. The mucus extracts of the marine fishes were determined for their antioxidant activities such as DPPH radical scavenging activities, Nitric oxide radical inhibition assay (NO°), Hydrogen peroxide radical inhibition assay (H2O2), Superoxide radical inhibition assay (O2-), Assay for *in vitro* Ferric Reducing Antioxidant Power (FRAP assay) and Reducing power. *Brachirus orientalis* showed maximum percentage of activity against DPPH radical scavenging (62.12±0.51) and Nitric oxide radical inhibition assay (NO°) (61.12±0.31) compare to that of Anguilla *Anguilla,* Whereas the FRAP assay (59.22±0.43), Hydrogen peroxide radical inhibition assay (H2O2) (66.41±0.24), and reducing power assay (1.64±0.24) in *Anguilla anguilla* shows maximum activity compare to that of *Brachirus orientalis*. The Superoxide radical inhibition assay (O2-) was recorded same in both the fish species. This study indicates that the epidermal mucus of *Anguilla anguilla* and *Brachirus orientalis* pave way to develop novel natural antioxidants or new therapeutic agents.

Keywords: *Anguilla anguilla, Brachirus orientalis,* Nitric oxide assay, DPPH, Reducing power assay

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Introduction

An exceptional reservoir of bioactive natural products was found in marine environment, many of which exhibit novel structural and chemical characteristics not found in terrestrial natural products. The richness of the Diversity provides a great opportunity for new bioactive compounds. The availability of natural products isolated from aquatic animals is growing exponentially, with hundreds of new products coming into existence. Compounds that are found last year (Faulkner, 2002; Proksch and Muller, 2006). A layer of mucus is secreted by specialized goblet cells found in the epidermal layer (Najafian and Babji, 2012). Mucus are highly multifunctional substance plays a keyrole in respiration, ionic and osmotic control, reproduction and excretion, resistance to diseases, contact, feeding, nesting and defense (Shephard, 1994). Skin is the primary site for pathogen entry, the mucosal surface in fish having living cells which attracts organisms in the living environment. Skin mucus plays a very important role in preserving the health of fish, especially in intensive farming, where stress and infection levels can be high. A number of immune factors, including lectins, lysozymes, calmodulin, immunoglobulins, supplements, C-reactive proteins, proteolytic enzymes, anti-microbial peptides and proteins, are found in fish skin mucus (Esteban, 2012).

A reliable source of animal protein is fish it contains complete amino acid and it is consumed by all age groups. One of the main fishing opportunities with high nutritional level price is Eel (*Anguilla* sp.). Indonesian is recognized as the *Anguillid eel's* world's distribution center (Sugeha and Suharti, 2008).

In biological structures, free radicals derived from metabolism or environmental sources communicate continuously and their unregulated production closely correlates with the molecular level of several diseases (Huang *et al*., 2005). In antioxidative protection, radical scavenging antioxidants are especially essential in protecting cells from free-radical injury (Youwei *et al*., 2008). Antioxidant activity of Protein and peptide benefited from the scavenging particular radicals produced during peroxidation, the scavenging of oxygen-containing compounds or the chelating potential of metals (Kristinsson and Rasco, 2000). There have been several reports explaining the Antioxidant function in animal forms of protein hydrolysates, such as egg yolk (Park *et al*., 2001). This research was intended to explore the antioxidant function of fish protein hydrolysates.

Materials and methods

Sample collection

Fish samples were collected from Annankovil landing centre, Parangipettai, Cuddalore, and transferred to the laboratory and identified the trout. The specimen was reported to be *Anguilla anguilla* and

Brachirus orientalis on the basis of morphology and zoological taxonomy.

Preparation of mucus extract

The marine fishes were held in a tub containing fresh water to maximize the development of mucus. The mucus was removed from the surface of the fish body using a sterile spatula and transferred into a sterile container and stored in at 4^{o}C for further analysis. 5mL of mucus samples were with equivalent volume of physiological sterilized saline (0.85% NaCl) and the mixture was centrifuged for 15 minutes at 5000 rpm. The supernatant was analyzed for antioxidant activity.

In vitro-assay antioxidant activity

DPPH radical scavenging activity

The assessment of DPPH radical scavenging assay was conducted according to the method Barros *et al*. (2007). About 0.3 mM solution of DPPH in 100% ethanol was prepared and 1 mL of this solution was added to 3 mL of the supernatant (mucus) dissolved in ethanol at different concentrations (20–100 µg/mL). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer. The radical scavenging operation was measured using the following equation as a percentage of DPPH discoloration**:**

DPPH radical scavenging % = [(A0 – A1)/A0] × 100

Where A0 is the absorbance of the DPPH solution and A1 is the absorbance of the sample.

Ferric Reducing Antioxidant Power (FRAP assay)

The FRAP assay was conducted with minor modifications by the system defined by Benzie and Strain (Benzie, 1996). It is based on the Fe3+-TPTZ to Fe2+-TPTZ complex reduction theory at low pH, which gives blue colour and can be calculated at 593 nm.

Preparation of the FRAP working reagent: The 300 mM concentration and pH 3.6 acetate buffer are prepared using sufficient quantities of anhydrous sodium acetate, glacial acetic acid and purified water. In 40 mM hydrochloric acid, TPTZ solution of 10 Mm concentration was prepared. Using anhydrous ferric chloride, an aqueous ferric chloride solution of 24.998 mM concentration was prepared. To prepare the FRAP functioning reagent, acetate buffer, TPTZ solution and freshly prepared ferric chloride solution were combined in 10: 1: 1 proportion. 3.0 mL of FRAP working reagent was applied with 0.1 mL of PG, blended well and absorbance was assessed after 10 minutes. As normal, freshly formulated aqueous ascorbic acid solution (0.1 mg/mL) was used.

Nitric oxide radical inhibition assay (NO°)

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen

to produce nitrite ions, which was measured by Griess reagent (Garrat, 1964). The reaction mixture (3 mL) containing 10 mM sodium nitroprusside in phosphate buffered saline, and the fractions or the reference compound (curcumin) at different concentrations (50–800 µg/mL) were incubated at 25°C for 150 min. About 0.5 mL aliquot of the incubated sample was removed at 30 min intervals and 0.5 mL Griess reagent was added. The absorbance of the chromophore formed was measured at 546 nm. Inhibition of the nitric oxide generated was measured by comparing the absorbance values of control, fractions and curcumin (50–800 µg/mL).

Nitric oxide radical scavenging = A blank _ A Sample × 100 / A blank

Superoxide radical inhibition assay (O_2^-)

To assess the O_2^- radical scavenging behaviour of samples, the approach defined by Jing *et al*., 1995. In short, an extract of 1 mL was applied to 9 mL of the 5 mM Tris HCl buffer (pH 8.2). 40 µl was applied to the mixture of 4.5 mM pyrogallol. The mixture was shaken and only a drop of ascorbic acid was applied to it after 3 minutes (0.035 percent). The absorbance of the reaction mixture after 5 min was measured at 420 nm (Similar concentration extract was used as the blank to eliminate interference). In contrast with that of the monitor, the O2-radical scavenging behaviour was expressed by the oxidation degree of the test group. The scavenging impact percentage was calculated using the equation below:

O2- radical scavenging % = [A0-(A1-A2)/A0] ×100

Where A0 is the pyrogallol absorbance of the TrisHCl buffer, A1 is the extract addition absorbance, and A2 is the blank extract absorbance.

Hydrogen peroxide radical inhibition assay (H_2O_2)

To assess the H_2O_2 scavenging potential of extracts, the technique stated by Ruch *et al*., 1989. Hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the fractions (20–100 µg/mL) in distilled water was added to 0.6 mL of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity at different concentrations of the fractions was determined and the IC_{50} values were compared with the standard.

H_2O_2 extract scavenging capacities have been estimated using the formula:

H_2O_2 radical scavenging % = [(A Blank – A Sample)/A Blank] × 100

Reducing power

The diminishing strength of epidermal proteins Samples of mucus have been measured according to the procedure With Garcia *et al*. (2014). This originally adapted from Oyaizu, 1998. One mL of the fraction (50–800

µg/mL), 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide were incubated at 50°C for 30 min and 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000g. About 2.5 mL of the supernatant was diluted with 2.5 mL of water and shaken with 0.5 mL of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm compared with the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

Results

In both aqueous and lipid components in intracellular and extracellular environments, free radicals are generated; so, it is important for the body to provide a mixture of water soluble and lipid soluble antioxidants to obtain the maximum spectrum of safety. The body synthesizes certain forms of antioxidants while others are derived from external sources such as food, nutraceuticals (Sharma, 2009).

DPPH radical scavenging activity

DPPH is a free radical compound that has been commonly used to assess the ability to scavenge free radicals at different concentrations. The study findings of DPPH radical scavenging are presented in Figure 1. The extraction of both fishes *Anguilla anguilla* and *Brachirus orientalis* fishes shows high scavenging activity. The extract of *Brachirus orientalis* mucus showed maximum scavenging activity of 52.31% at low concentration of (20µg/mL) and 62.12% maximum concentration of (100 µg/mL) at different concentrations whereas *Anguilla anguilla* mucus showed minimum inhibition of 48.72% at low concentration (20µg/mL) and 59.12% at the maximum concentration (100 µg/mL).

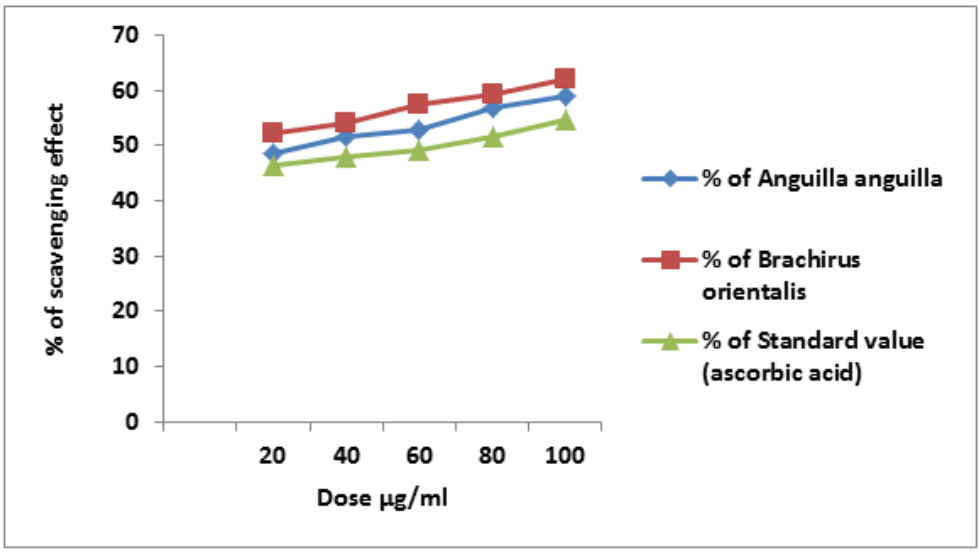

Figure 1: % of the DPPH radical scavenging activity.

Ferric Reducing Antioxidant Power (FRAP assay)

High Hydroxyl radical scavenging activities were observed in Ferric Reducing assay of fish epidermal mucus (Fig. 2). The scavenging assays were determined at increasing the concentration of the epidermal mucus. The extract of *Anguilla anguilla* mucus showed maximum inhibition 51.03% at low concentration (20µg/mL) and 57.22% at maximum concentration (100µg/mL) whereas *Brachirus orientalis* mucus showed maximum activity of 49.12% at low concentration (20µg/mL) and 54.73% at highest (100µg/mL) the inhibition activity was concentration dependent.

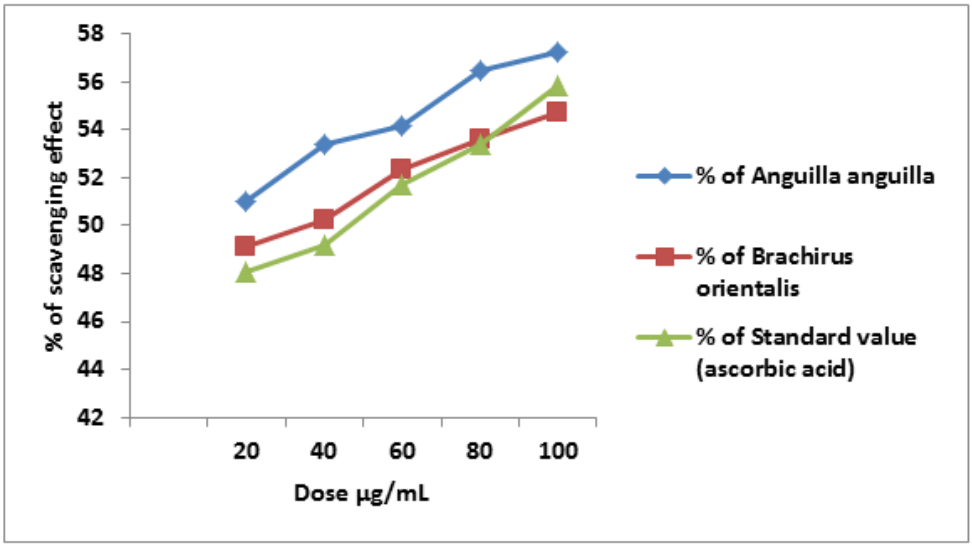

Figure 2: % of the Ferric Reducing Antioxidant Power (FRAP assay).

Nitric oxide radical inhibition assay (NO°)

The fish *Anguilla anguilla* and *Brachirus orientalis* mucus extract showed positive results in Nitric oxide radical inhibition assay. The scavenging activity was directly proportional to the increasing concentration of mucus in the fish. The mucus extract of *Anguilla anguilla* was 48.62% at low concentration (20µg/mL) 55.16% at maximum concentration (100 µg/mL) *Brachirus orientalis* wer 52.42% at minimum concentration (20µg/mL) and showed maximum inhibition 61.12% at highest concentration (100 µg/mL) (Fig. 3).

Superoxide radical inhibition assay (O_2^-)

Superoxide radical inhibition was recorded in fish epidermal mucus (Fig. 4). The scavenging activities were aiming at increasing the concentration of epidermal mucus. The *Anguilla anguilla* mucus extract showed 66.08% at minimum concentration (20µg/mL) 75.64% at maximum concentration (100 µg/mL) whereas *Brachirus orientalis* mucus showed 65.06% at minimum concentration (20µg/mL) 73.89% at maximum concentration (100 µg/mL) measured at various

concentrations of Superoxide radical inhibition assay.

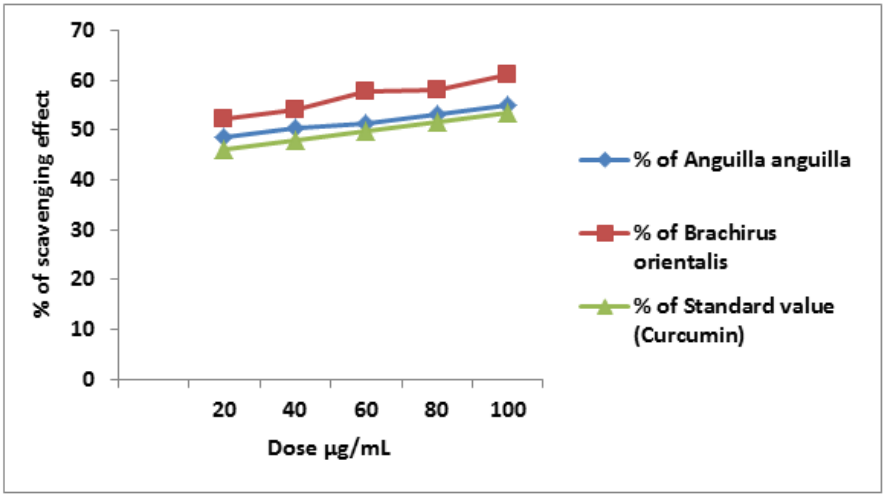

Figure 3: % of the Nitric oxide radical inhibition assay (NO°).

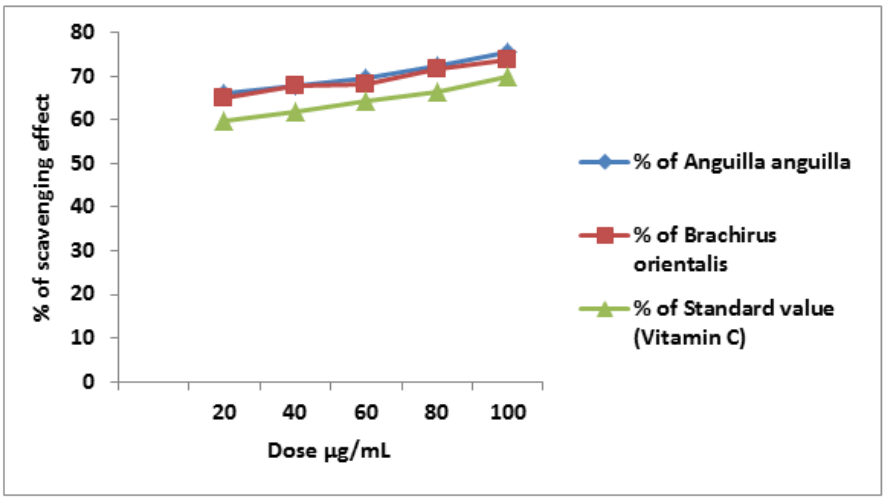

Figure 4: % of the Superoxide radical inhibition assay (O_2^-).

Hydrogen peroxide radical inhibition assay (H_2O_2)

Measurement of the activity hydrogen peroxide radical inhibition is considered to be one of the most effective ways to assess the ability of an antioxidant to suppress pro-oxidant levels, such as ferric oxide. The *Anguilla anguilla* and *Brachirus orientalis* showed positive results in this analysis (Fig. 5). 56.19% inhibition at minimum concentration (20µg/mL) and 66.41% at the maximum concentration (100 µg/mL) were observed in *Anguilla anguilla* and *Brachirus orientalis* showed on 54.91% at low concentration (20µg/mL) 63.72% at maximum concentration (100 µg/mL) for extraction were recorded and displayed.

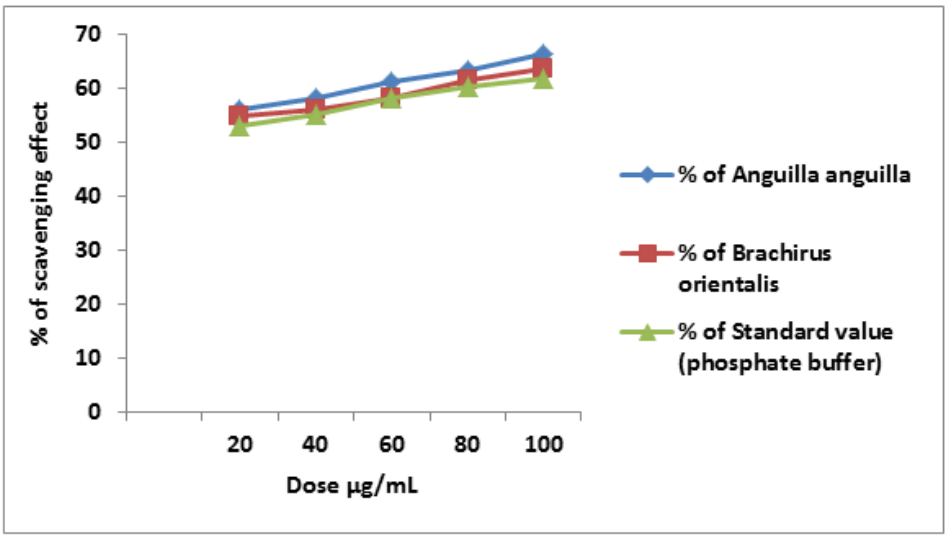

Figure 5: % of the Hydrogen peroxide radical inhibition assay (H_2O_2).

Reducing power assay

As a function of power reduction, the concentration dependence of anti-oxidant activity was explored (Fig. 6), as this provides a general view of the reductions. It was observed that the power reduction values were directly proportional to the concentration of fish mucus extracts. In this assay, *Anguilla anguilla* showed 0.72 at low concentration (20µg/mL) 1.64% at maximum concentration (100 µg/mL) and *Brachirus orientalis* showed 0.68% at minimum concentration (20 µg/mL) to 1.16 % at maximum concentration (100 µg/mL) were detected.

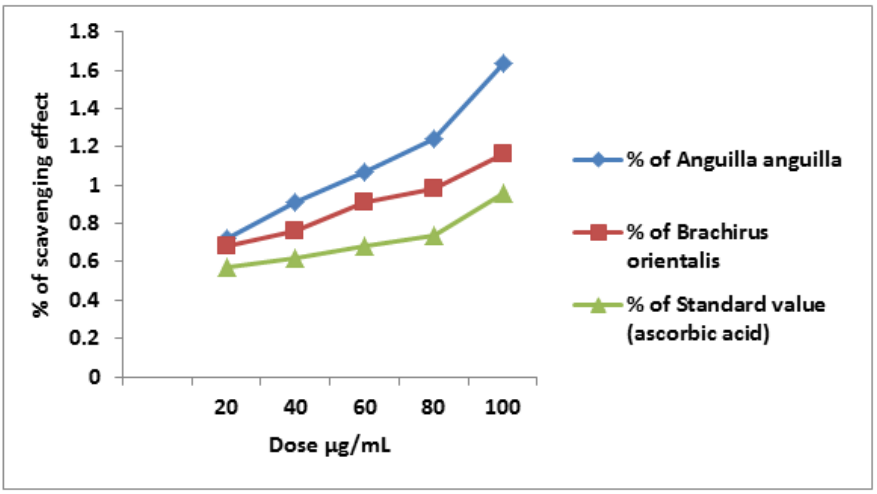

Figure 6: % of the Reducing power assay.

Discussion

The skin mucosa of fish has different components such as proteins, carbohydrates, lipids, metabolites (Zaccone *et al*., 2001). Mucins typically possess repetitive regions rich in threonine, serine and proline (Rose and Voynow, 2006). The fish mucus also

contains few carbohydrate components. Although their functions are not well defined, some protective roles have been suggested (Esteban, 2012). Mucus composition varies amongst different fish species. The mucus cells and the composition of the mucus are influenced by various endogenous (e.g., sex, developmental stage) and exogenous factors (e.g., stress, hyperosmolarity, pH and infections) (Ellis, 2001). A variety of materials has been identified where glycosamino glycans, lysozyme, immunoglobulins, complement, carbonic anhydrase, a range of lectins and calmodulin are found in fish mucus (Shephard, 1994). Fishes' mucus contained to lipid from previous study, its revealed free fatty acids which may provide antioxidant agents and protection against bacterial and fungal attack (Lewis, 1970).

The present observation on DPPH scavenging activity of fish mucus extract have been observed in *Brachirus orientalis* at maximum concentration 100 µg/mL. Similar result was studied in water extract of *Clarias sp* epidermal mucus had the lowest DPPH scavenging activity, 38.28% at 100µg/mL. Among the different extracts of *C. gariepinus* and *Clarias sp* epidermal mucus, dichloro methane phase extract of *C. gariepinus* exhibited highest DPPH radical scavenging activity value at all concentrations tested (Theodore *et al*., 2008).

The scavenging activities of fish mucus samples were concentration dependent. The sample showed increasing Ferric Reducing Antioxidant Power (FRAP) as the concentration increased in both the extracts. However, all extracts showed lower reducing power and FRAP activities than ascorbic acid at the same concentrations (Nurul Mariam Hussin *et al*., 2017).

Nitric oxide interacts with oxygen to produce stable products, nitrite, and nitrate. Scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite. (Marcocci *et al*., 1994). The nitric oxide scavenging play an important role in the progression of many diseases and pathological conditions such as septic shock, atherosclerosis, ischemia reperfusion, neurodegerative disorders like Alzheimers, arkinsons diseases, cancer and diabetes (Vardarajan *et al*., 2000; Law *et al*., 2001).

Superoxide anion is also an initial free radical formed mitochondrial electron transport system. Mitochondrial generate energy using four electron chain reaction, reducing oxygen to water.it plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical or singlet oxygen in living systems (Kulisic *et al*., 2004).

Reducing power activity is a determination of ability of samples extracts to reduce iron (III). Reducing power of all samples was concentration-dependent. Increased absorbance of the reaction mixture indicated increased of reducing power. Higher reducing power have better

abilities to donate electron where the presence of reducers causes the reduction of the Fe3+/ ferricyanide complex to the ferrous form, therefore measuring the formation of Perl's Prussian blue at 700nm can monitor the Fe2+ concentration (Theodore *et al*., 2008).

Conclusion

From the study results indicates that the epidermal mucus of *Anguilla anguilla* and *Brachirus orientalis* has led to the possible application of a natural antioxidant or new therapeutic agent. *Anguilla anguilla* and *Brachirus orientalis* epidermal mucus fish are required for further analysis. In conclusion, the *Anguilla anguilla* and *Brachirus orientalis* epidermal fish mucus extracts has been showed good antioxidant activity DPPH assay, Nitric oxide radical inhibition assay (NO°)**,** Ferric Reducing Antioxidant Power (FRAP assay), Superoxide radical inhibition assay (O_2^-)**,** Hydrogen peroxide radical inhibition assay (H_2O_2) and, Reducing power assay. The extracts of *Anguilla anguilla* and *Brachirus orientalis* epidermal fish mucus extracts can be used as potential natural sources of antioxidant which pave way for pharmaceutical industry.

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