

## Total phenolic content and *in-vitro* antioxidant activities from methanolic extract of alligator pipefish, *Syngnathoides biaculeatus* (Bloch, 1785)

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Alligator pipefish, *Syngnathoides biaculeatus* (Bloch, 1785) is one of the heavily traded and expensive ingredient in traditional Chinese medicines and there were no reports on its antioxidant activities. Total phenolic content (TPC) and *in-vitro* antioxidant activities in terms of reducing power (RP), metal chelating activity (MCA), 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP), inhibition of lipid peroxidation (LPX) and hydroxyl radical scavenging (HRS) activity were evaluated at extract concentrations ranging between 200 and 800 µg. Levels of TPC, RP, MCA, DPPH scavenging, FRAP, LPX and HRS activity measured was 19.81±1.10 to 64.04±0.45 mg gallic acid g<sup>-1</sup> dried extract, from 0.16 to 0.31 Abs, from 6.20±0.21% to 29.33±1.73%, from 16.59±0.01 to 40.21±0.02%, from 75.46±0.90 to 167.25±0.41 mg ascorbic acid g<sup>-1</sup> dried extract, from 54.59±1.5% to 84.28±3.5% and from 84.89±0.26 to 91.02±0.01%, respectively. Significant correlation ( $P<0.05$ ) between TPC and other antioxidant properties confirm the antioxidant potential of *S. biaculeatus*. Results of this study would form a baseline information on the antioxidant potential of alligator pipefish.

[**Keywords:** Antioxidant, alligator pipefish, *Syngnathoides biaculeatus*, total phenolic content, DPPH]

### Introduction

According to WHO<sup>1</sup>, millions of people worldwide depend on traditional medicine or complementary and alternative medicine for healthcare. Many Syngnathid species, mainly seahorses and pipefish constitute main ingredient in traditional Chinese medicine (TCM). They are considered as potential pharmacological mine for various diseases including cancer and impotence<sup>2</sup>. Many naturally occurring antioxidant compounds in TCM sources have been identified as free radical or active oxygen scavengers<sup>3,4</sup>. The antioxidants scavenge free-radicals or potentially harmful reactive oxygen species (ROS) formed during peroxidation and compounds containing oxygen or the chelating capacity of metals<sup>5</sup>.

The use of alligator pipefish, *Syngnathoides biaculeatus* (Bloch, 1785) as an ingredient in the TCM has a history of over 600 years, known as Hailong<sup>6,7</sup>. In spite of heavy exploitation of *S. biaculeatus* for TCM trade, there are no precise estimates of trade volumes. According to Vincet<sup>8</sup>, the import of dried pipefishes into Taiwan during the period 1983-1993 was estimated to be 1600–16500 kg year<sup>-1</sup> while, Martin-Smith *et al.*<sup>9</sup> place this figure around 7500–21300 kg year<sup>-1</sup> into Hong Kong from 1998 to 2000. It is believed that the trade has possibly occurred from tropical countries, particularly India, Malaysia, Philippines and Thailand<sup>9,10</sup>. Although antioxidant

properties from seahorse species have been documented<sup>11-13</sup>, the antioxidant potential of the alligator pipefish, the most expensive ingredient in TCM is lacking.

The alligator pipefish, *S. biaculeatus* has wide geographic distribution throughout the Indo-Pacific region in seagrass meadows<sup>14</sup>. It is the most common pipefish species in the Palk Bay and Gulf of Mannar regions on the east coast of India<sup>15</sup>. Due to its economic importance in TCM and lack of baseline information on the antioxidant potential, the total phenolic content and *in-vitro* antioxidant activities of *S. biaculeatus* collected from Palk Bay were evaluated in the present study.

### Material and Methods

Total of 30 dead specimens of alligator pipefish (*Syngnathoides biaculeatus*) caught as by-catch in wind-driven country trawl or 'Vallams' mainly operated for crab/shrimp fishery collected from Mullimunnai village (9°39'24.48"N, 78°58'14.05"E) Tamil Nadu, India (Fig.1) during October 2012 served as material for the present study. However, no fish was specially caught or killed for this study. Mean wet weight and total length of the fishes collected were 4.22±0.47 g and 18.5±3.51 cm, respectively. After collection, the fishes were washed with ice cold distilled water and then immediately frozen

in liquid nitrogen and preserved at  $-80\text{ }^{\circ}\text{C}$  until taken up for analyses. Lyophilised and powdered fishes were used for the preparation of extracts.

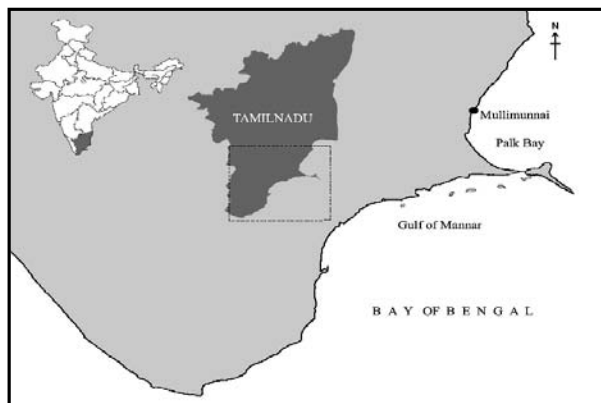


Fig. 1— Collection site of alligator pipefish, *S. biaculeatus* specimens at Mullimunnai village marked with (●) along Palk Bay, Tamil Nadu, India.

Folin-Ciocalteu reagent, sodium carbonate, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), potassium ferricyanide, trichloroacetic acid (TCA), ethylenediamine tetracetic acid (EDTA) disodium salt, ferric chloride, ascorbic acid (ASA), hydrogen peroxide, potassium chloride, ferrous chloride, ferrous sulfate, ferrozine, 2-deoxy-D-Ribose were procured from Sigma Aldrich, India while methanol and hydrochloric acid (HCl) were of analytical grade from Merck, India.

Freeze dried pipefishes were ground to a fine powder and passed through a 0.5 mm mesh sieve. Five grams of the powder were mixed with 100 mL of concentrated HPLC grade methanol (w/v), incubated in a platform shaker (Remi Orbital shaking incubator Model: RIS - 24 BL, India) for 24 hrs at 180 rpm at room temperature ( $28\text{ }^{\circ}\text{C}$ ). The mixture was centrifuged at 3500 rpm for 10 min at  $4\text{ }^{\circ}\text{C}$  and filtered with Whatman No. 1 filter paper. Methanol in the extract was removed by rotary evaporation (BUCHI Rotavapor R-200) at  $45\text{ }^{\circ}\text{C}$ . Each extraction was conducted in duplicate. Extraction yield was expressed as percentage of dried sample. The dried duplicate extracts were pooled together and stored at  $-80\text{ }^{\circ}\text{C}$  until analyzed. Dried extract was then re-dissolved in methanol (HPLC grade) at a concentration of  $2\text{ mg mL}^{-1}$  as a stock extract solution. The stock extract solution was used for the determination of antioxidant activities. Three different extract concentrations, 200  $\mu\text{g}$ , 400  $\mu\text{g}$  and 800  $\mu\text{g}$  were tested in triplicate.

Total phenolic content (TPC) was quantified following the method suggested by Slinkard & Singleton<sup>16</sup>. The reaction mixture contained 0.1, 0.2 and 0.4 mL of sample extract; 1 mL of Folin-Ciocalteu reagent (1:5 dilutions swirl to mix with distilled water and incubated for one to eight min at room temperature,  $28\text{ }^{\circ}\text{C}$ ) added and mixed thoroughly. 3 mL of 2% sodium carbonate solution was added to the mixture and was allowed to stand for 2 hrs, with intermittent shaking. Absorbance was measured on spectrophotometer (UV 1800, Shimadzu, Japan) at 760 nm, and compared with a standard curve of gallic acid ( $10\text{ } \sim 100\text{ }\mu\text{g mL}^{-1}$ ). The amount of TPC is expressed as mg gallic acid  $\text{g}^{-1}$  dried extract.

Reducing power (RP) of alligator pipefish extracts was determined as suggested by Oyaizu<sup>17</sup>. Reaction mixtures were prepared by adding 2.5 mL of phosphate buffer (0.2 M, pH 6.6), 2.5 mL potassium ferricyanide (1%) and pipefish extracts (0.1, 0.2 and 0.4 mL). Reaction mixtures were incubated at  $50\text{ }^{\circ}\text{C}$  in water bath for 30 min and allowed to cool at room temperature ( $28\text{ }^{\circ}\text{C}$ ). To each reaction mixture, 2.5 mL of 10% TCA was added and centrifuged at 2000 rpm for 10 min. The supernatant (2.5 mL) was added with 2.5 mL of distilled water and 0.5 mL ferric chloride (1.0%), allowed to react for 10 min at room temperature ( $28\text{ }^{\circ}\text{C}$ ) and then absorbance (Abs.) was measured at 700 nm. Ascorbic acid (ASA) solution ( $100\text{ }\mu\text{g mL}^{-1}$ ) was used as standard (Table 1). Absorbance values of reaction mixture are directly proportional to the levels of reducing power.

Method suggested by Dinis *et al.*<sup>18</sup> was used for measuring the metal chelating activity. Briefly, different volumes of the extract (0.1, 0.2 and 0.4 mL) were added to 0.2 mL of 2 mM ferrous chloride. Reaction mixture was initiated by addition of 0.2 mL of ferrozine. Mixture was shaken vigorously and allowed to stand for 10 min at room temperature ( $28\text{ }^{\circ}\text{C}$ ). The absorbance was measured at 562 nm on spectrophotometer. Metal chelating activity of the extract was compared with the standard EDTA ( $100\text{ }\mu\text{g mL}^{-1}$ ). Reaction mixtures without ferrous chloride and ferrozine or complex formation molecules were served as control. Metal chelating activity (%) of pipefish extract was expressed as follows:  

$$\text{MCA (\%)} = \frac{[A_0 - A_1/A_0] \times 100}{A_0}$$
 where,  $A_0$  — absorbance of the control, and  $A_1$  — absorbance with the test compound.

The free radical scavenging potential in alligator pipefish methanol extract was measured

following the method suggested by Blois<sup>19</sup> using 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Initial absorbance, and absorbance after 30 min of the reaction mixture (incubated in dark) [2.5 mL of DPPH solution (0.1mM in methanol)] and sample extract (0.1-0.4 mL), was measured at 517 nm. Butylated hydroxytoluene (BHT, 100 µg mL<sup>-1</sup>) was used as standard. The DPPH radical scavenging activity (%) was calculated using the

(TBARS) in the incubation mixture was measured at 532 nm with ascorbic acid (100 µg mL<sup>-1</sup>) as standard (Table 1). Reaction mixture without any extract and standard was used as control. The lipid peroxidation inhibition (%) was calculated as

$$\text{LPX inhibition (\%)} = [1 - (A_0 - A_1 / A_2)] \times 100,$$

where, A<sub>0</sub> is the absorbance in the presence of

Table 1. Values of standards at different concentrations used in present study

Standard concentrations	Reducing power (Abs.)	Metal chelating activity (%)	DPPH radical scavenging activity (%)	Hydroxyl radical scavenging activity (%)	Lipid peroxidation inhibition (%)
10 µg	0.2910±0.009	41.7732±0.069	38.8620±0.17	90.4324±0.14	85.7691±0.004
20 µg	0.4185±0.002	82.4216±0.023	43.4371±0.05	92.8879±0.182	89.0881±0.013
40 µg	0.6373±0.001	87.6205±0.12	45.5448±0.021	93.0248±0.009	96.1237±0.15
80 µg	1.0846±0.15	97.2627±0.10	52.2698±0.013	93.1911±0.10	98.0518±0.008
100 µg	1.2781±0.01	98.7138±0.03	64.8362±0.08	96.9230±0.08	98.6509±0.019

formula: DPPH radical scavenging activity (%) =  $[A_0 - A_1 / A_0] \times 100$ ,

where, A<sub>0</sub> — is initial absorbance (0 min) and A<sub>1</sub> — is final absorbance (after 30 min).

The total antioxidant capacity of alligator pipefish extract was determined by FRAP assay, which depends upon the reduction of ferric tripyridyltriazine (Fe<sup>+3</sup>— TPTZ) complex to the ferrous tripyridyltriazine (Fe<sup>+2</sup>— TPTZ) by a reductant at low pH. Method described by Benzie *et al.*<sup>20</sup> was used to perform FRAP assay. For the determination of FRAP activity, working solution was prepared using 2.5 mL acetate buffer (300 mM, pH 3.7), 2.5 mL TPTZ solution (10 mM 2,4,6-tripyridyl-striazine (TPTZ) in 40 mM HCl.) and 2.5 mL (20 mM) ferrous chloride solution. The working solution was prepared freshly. Five different concentrations of ASA (100~1000 µg mL<sup>-1</sup>) were used as standard. FRAP assay was done by mixing 1.5 mL freshly prepared working solution and different sample concentration. The mixture was incubated for 10 min and then intensity of blue coloured complex was recorded at 593 nm. The results were expressed as mg ASA g<sup>-1</sup> dried extract.

*In-vitro* lipid peroxidation inhibition assay was performed by the method described by Jena *et al.*<sup>21</sup>. Fresh sheep liver was obtained from local slaughter house, washed with ice cold potassium chloride (1.15%) and homogenized (10% w/v) with Teflon Potter-Elvehjem homogenizer. Homogenate was filtered through cheese cloth and centrifuged at 10000 rpm for 10 min, at 4 °C. Supernatant was used for LPX assay.

Peroxidation of liver homogenate was induced by ferrous sulfate solution. Liver homogenate was incubated with 100 mM of ferrous sulfate for 30 min at 37 °C; the formation of thiobarbituric acid reactive substances

extract, A<sub>1</sub> — absorbance without sheep liver homogenate and A<sub>2</sub> — absorbance of the control (without extract or standard).

The ability of the extract to inhibit hydroxyl radical-mediated 2-deoxy-D-ribose degradation was determined spectrophotometrically by the method described by Chung *et al.*<sup>22</sup>. The reaction mixture containing 0.2 mL of ferrous sulphate (10 mM), 0.2 mL of EDTA (10 mM), 0.2 mL of 2-deoxy-D-ribose (10 mM), 0.1-0.4 mL of extract sample and 1 mL of phosphate buffer solution (0.2 M, pH 7.4) was mixed. Then 0.2 mL of hydrogen peroxide (10 mM) was added to the reaction mixture and incubated at 37 °C for 4 h. Thereafter 1 mL of 2.8% TCA and 1 mL of 1% TBA were added to the tubes. Samples were mixed and heated in a water-bath at 100 °C for 15 min. The mixture was cooled by immersion for 5 min in an ice/water bath. The absorbance was read at 532 nm with ascorbic acid (100 µg mL<sup>-1</sup>) as standard. Reaction mixture without hydrogen peroxide served as control. The inhibition of deoxyribose degradation was calculated using below equation.

HRS activity (%) =  $[A_0 - A_s / A_0] \times 100$ , where A<sub>0</sub> is absorbance of control, A<sub>s</sub> is absorbance of sample.

Analytical data was tested statistically and expressed as means ± standard deviation. The Pearson correlation analysis was performed between variables and *P*-values lower than 0.05 i.e. (*P*<0.05) were regarded as significant. All statistical calculations were performed using statistical package Statistica 8.0 programme (Stat Soft 8.0).

## Results and Discussion

Extraction yield is one of the comparative indicators of antioxidant activity. Extraction yield obtained from alligator pipefish after evaporation

Table 2. Total phenolic content and antioxidant activities of alligator pipefish, *S. biaculeatus* at different extract concentrations.

Extract conc.	TPC (mg gallic acid g <sup>-1</sup> dried extract)	Reducing power (Abs.)	Metal chelating activity (%)	DPPH radical scavenging activity (%)	FRAP (mg ASA g <sup>-1</sup> dried extract)	HRS activity (%)	LPX inhibition (%)
200 µg	19.81±1.10	0.16±0.07	16.59±0.01	6.20±0.21	75.46±0.90	84.89±0.26	54.59±1.5
400 µg	36.09±0.83	0.23±0.05	29.50±0.03	11.56±0.57	100.8±1.00	87.83±0.11	70.34±7.9
800 µg	64.04±0.45	0.31±0.06	40.21±0.02	29.33±1.73	167.25±0.41	91.02±0.01	84.28±3.5

of methanol was estimated. Extraction yield obtained from *S. biaculeatus* (30.59±0.57%) is higher than those reported earlier from its close

The estimated TPC measured at 200 µg to 800 µg extract concentrations ranged between 19.81±1.10 to 64.04±0.45 mg gallic acid g<sup>-1</sup> dried extract (Table 2). TPC values obtained in present study are higher than reported from wild caught *H. kuda* (17.43±1.30 mg g<sup>-1</sup>) by Qian *et al.*<sup>11</sup>. Hydrogen or electron donation, metal ion chelation, neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides are the reactions exhibited by phenolic compounds during free radical scavenging activities<sup>23,24</sup>. A good correlation between TPC from different sources and their antioxidant potential has been documented by several workers<sup>25-28</sup>. In the present investigation, significant positive correlation ( $P<0.05$ ) between TPC and reducing power ( $r=0.9876$ ), metal chelating activity ( $r=0.9784$ ), DPPH radical scavenging activity ( $r=0.9852$ ), ferric reducing antioxidant power ( $r=0.9936$ ), inhibition of lipid peroxidation ( $r=0.9220$ ) and hydroxyl radical scavenging ( $r=0.9913$ ) activity was observed. The levels of TPC recorded in the present study reflect that *S. biaculeatus* may possess high degree of antioxidant property.

Reducing capacity of an extract may serve as an indicator for potential antioxidant activity. A significant correlation ( $P<0.05$ ) between RP and other antioxidant activities indicates the antioxidant potential of *S. biaculeatus*. RP of extracts (0.16 to 0.31 Abs.) was found to be concentration-dependent (200-800 µg). Standard ascorbic acid (at 100 µg) showed higher reducing power (Table 1). Our results agree with RP values reported by Qian *et al.*<sup>11</sup> in *H. kuda*. However, relatively higher RP values obtained by Hung *et al.*<sup>12</sup> in *H. kuda*, might be due to higher concentration of extract (40 mg mL<sup>-1</sup>). Reducing agents can reduce the oxidized intermediates such as metal ions during lipid peroxidation process<sup>29</sup>. The positive correlation between RP and MCA ( $r=0.9930$ ), DPPH radical scavenging activity ( $r=0.9520$ ), LPX ( $r=0.9384$ ) and HRS ( $r=0.9925$ ) activity indicates the greater ability of *S. biaculeatus* extracts to provide hydrogen ions for

relative wild syngnathid species, the yellow seahorse, *Hippocampus kuda* (6.9% w/w) in methanol by Qian *et al.*<sup>11</sup>. electrons to reduce metal ions and subsequently involved in scavenging of free radicals.

Metal chelating activity (MCA) of *S. biaculeatus* extract varied between 16.59±0.01 and 40.21±0.02% (Table 2). Standard EDTA showed higher metal chelating activity than extract (Table 1). On the other hand, Hung *et al.*<sup>12</sup> observed very low MCA (4.89±0.53 to 8.35±0.05% at 40 mg mL<sup>-1</sup> extract concentration) in cultured *H. kuda*. MCA plays an important role in antioxidant mechanisms due to its capability to reduce the concentration of the catalyzing transition metal in lipid peroxidation process<sup>30</sup>. In addition, it has been reported that some phenolic compounds exhibit antioxidant activity through the chelation of metal ions<sup>31</sup>. A positive high correlation between TPC and MCA ( $r=0.9784$ ) as well as between MCA and lipid peroxidation ( $r=0.9450$ ) was recorded. Furthermore, a high degree of positive correlation ( $P<0.05$ ) between MCA and other antioxidant activities (RP,  $r=0.9930$ ; DPPH radical scavenging activity,  $r=0.9343$ ; and HRS,  $r=0.9937$ ) suggests the greater chelating ability of the extracts from *S. biaculeatus*.

DPPH radical scavenging activity measured at extract concentrations from 200 µg to 800 µg varied between 6.20±0.21% and 29.33±1.73% (Table 2). Standard BHT showed higher activity than extract (Table 1). DPPH radical scavenging activity observed in present investigation was found to be relatively lower than reported for *H. kuda*<sup>11,12</sup>. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule<sup>30</sup>. Therefore, DPPH is often used as a substrate to evaluate the degree of activity of natural antioxidants. Radical scavenging activity also attributed to phenolic content of extract<sup>32,33</sup>. The levels of DPPH radical scavenging activity exhibited by extracts often reflect their ability to provide hydrogen ion and electrons to bind or capture metal ions<sup>34</sup>. A significant positive correlation obtained between DPPH radical scavenging activity and TPC, RP and MCA ( $P<0.05$ ) reflect the antioxidant

capacity of *S. biaculeatus* with free radical scavenging properties.

FRAP assay reduces ferric complex (Fe<sup>+3</sup>-TPTZ) to ferrous (Fe<sup>+2</sup>-TPTZ) and measures the potentiality of biological antioxidants<sup>34</sup>. In the present study, FRAP values measured at three different concentrations varied between 75.46±0.90 and 167.25±0.41 mg ASA g<sup>-1</sup> dried extract (Table 2). Relatively high FRAP values and their significant positive correlation ( $P<0.05$ ) with DPPH radical scavenging activity ( $r=0.9954$ ), LPX ( $r=0.9060$ ) and HRS ( $r=0.9761$ ) indicate the high degree of antioxidant capacity of extracts prepared from *S. biaculeatus*.

The levels of LPX activity recorded at extract concentrations (200 to 800 µg) with standard ascorbic acid ranged between 54.59±1.5% and 84.28±3.5 % (Tables 1 and 2). Phenolic compounds exert their protective action in lipid peroxidation by scavenging the lipid derived radicals (R·, RO·, or ROO·)<sup>35</sup>. Lipids upon reaction with free radicals undergo the highly damaging chain reaction of lipid peroxidation leading to both direct and indirect effects<sup>36</sup>. Observed positive correlation between LPX and TPC, RP, MCA, DPPH scavenging activity, FRAP and HRS indicate that methanolic extract of *S. biaculeatus* extract has capacity to minimize lipid peroxidation by inhibition of free radicals and quenching of hydroxyl radicals in biological system.

Amongst all the oxidative radicals, hydroxyl radicals are the most reactive free radicals formed in biological system, which can almost react with all the substances in the living cells and induce severe damage to the cells<sup>37</sup>. In the present study, HRS activity of methanolic extracts of *S. biaculeatus* was compared with that of standard ascorbic acid (Table 1). HRS activity exhibited by methanolic extracts of *S. biaculeatus* ranged from 84.89±0.26% to 91.02±0.01%. These levels of activity are relatively higher than those reported for *H. kuda*<sup>11</sup>. Significant and positive correlation between HRS activity and TPC ( $r=0.9913$ ) recorded in the present study. It has been reported that the phenolic compounds donate electrons to hydroxyl radical thus neutralizing it to water<sup>38</sup>. Also significant positive correlation ( $P<0.05$ ) between HRS activity and other antioxidant activities shows the efficiency of *S. biaculeatus* extract in stabilizing lipid peroxidation through its hydrogen donating potential.

Results of the present study indicate that methanolic extract of *S. biaculeatus* showed high phenolic content and scavenging of free radicals such as DPPH, hydroxyl and reducing the ferric to ferrous ions. The results obtained in present

study are quite encouraging and highlights antioxidant potential of alligator pipefish. Further assessment of purified extracts prepared from *S. biaculeatus* using different cell lines may provide greater insights into their pharmacological importance in traditional Chinese medicines.

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