

Author version: *Aquaculture*, vol.434; 2014; 100-107

Evaluation of antioxidant activities in captive-bred cultured yellow seahorse, *Hippocampus kuda* (Bleeker, 1852)

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Abstract

Globally, seahorses and other Syngnathid fishes are in great demand as they form an important ingredient in traditional Chinese medicine (TCM). As a consequence of dwindling supplies from natural sources, TCM traders are now looking for alternatives. Seahorse aquaculture has a great potential in bridging the gap between the demand and supply. An apprehension over the potency of cultured vis-à-vis wild caught seahorses, however remains unresolved. The yellow seahorse, *Hippocampus kuda* (Bleeker, 1852) is one of the heavily traded and expensive materials in TCM. In the present study, the antioxidant potential of methanolic extracts from three body portions (head, trunk and tail) in both genders of captive-bred *H. kuda* were evaluated. Mean measured values of different antioxidant tests such as total phenolic content (TPC), reducing power (RP), metal chelating activity (MCA), DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP), hydroxyl radical scavenging activity (HRS) and inhibition of lipid peroxidation (LPX) from both genders of *H. kuda* were, 16.08±4.23 mg gallic acid g⁻¹ dried extract, 0.32±0.05 Abs, 39.98±13.70%, 24.04±7.24%, 94.44±2.83 mg ascorbic acid g⁻¹ dried extract, 86.23±1.24% and 74.72±13.18%, respectively. The degree of antioxidant activity was observed to be directly proportional to the extract concentration in all body portions tested. Significant correlation between TPC and other tests such as RP, MCA, DPPH, FRAP, HRS and LPX ($P<0.05$) indicated the antioxidant potential of cultured *H. kuda*. Extracts from female *H. kuda* showed relatively higher levels of TPC and antioxidant activity compared to those from males. The essential antioxidant properties exhibited by cultured *H. kuda* were at par with those reported from wild *H. kuda*. The result of this study has the potential to allay apprehensions over the potency of cultured seahorses and may help in reducing the exploitation pressure of natural populations.

Keywords: Antioxidant activity, TCM, cultured, yellow seahorse, *Hippocampus kuda*, total phenolic content, DPPH

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1. Introduction

Syngnathid fishes, mainly seahorses and pipefishes form an ingredient in traditional medicine, particularly in Southeast Asia where traditional Chinese medicine (TCM) and its derivatives (e.g. Japanese and Korean traditional medicine) are practised (Scales, 2010; Sreepada et al., 2002; Vincent, 1996). In TCM, seahorses are credited with having a role in increasing and balancing vital energy flows within the body, as well as a curative role for ailments such as impotence and infertility, asthma, powerful general tonic, high cholesterol, goitre, kidney disorders, and skin afflictions such as severe acne and persistent nodules (Kumaravel et al., 2012; Moreau et al., 1998; Rosa et al., 2013; Shi et al., 2006; Sreepada et al., 2002; Vincent, 1995, 1996; Zhang et al., 2003). Further, the use of seahorse as a supplementary ingredient in TCM has been studied many years for its various bioactive properties including appetite enhancement, antioxidant, antitumor, anti-ageing, anti-fatigue and Ca^+ channel blocking (Hu et al., 2000; Zhang et al., 2001, 2003).

It has been estimated that over 20 million seahorses are traded annually by more than 45 countries worldwide for TCM (Woods, 2000). Developing countries such as Brazil, India, Indonesia, Malaysia, Mexico, Philippines, Thailand and Vietnam are the major exporters of wild caught seahorses for TCM trade (Koldewey and Martin-Smith, 2010). Dwindling natural supply of seahorses (as a consequence of large-scale exploitation) coupled with strict imposition of legal restrictions on wild collections in many countries have forced TCM traders to look for alternate sources to meet the demand (Koldewey and Martin-Smith, 2010). At this juncture, seahorse aquaculture has a great potential to integrate both conservation and sustainable development goals by providing an alternative livelihood option for fisher-folk communities who, in the absence of alternatives to fishing, continue to exploit declining seahorse populations (Sreepada et al., 2002). Seahorses are currently being cultured in some countries for TCM trade and tonic products (e.g., seahorse wine), as live aquarium fish, and in curios and souvenirs (Koldewey and Martin-Smith, 2010; Vincent and Koldewey, 2006). Although members of the TCM community have shown a willingness to accept the aquacultured animals, the apprehension over the lower potency of these compared to the wild-caught, remain unresolved (Moreau et al., 1998).

Many naturally occurring antioxidant compounds in main ingredients used for the preparation of TCM have been identified as free radical or active oxygen scavengers (Duh, 1998; Kumaravel, et al., 2012; Pan et al., 2007). Considering the pharmaceutical and therapeutic importance, the antioxidant potential in wild and cultured seahorses has been evaluated (Hung et al., 2008; Qian et al., 2008, 2012; Sen, 1998; Toan and Hanh, 2013). Except for the three reports (Qian et al., 2008, 2012; Toan

and Hanh, 2013), most of other studies are in regional languages (Chinese and Taiwanese) with incomplete methodology thus preventing the wider application of the obtained results.

The yellow seahorse, *Hippocampus kuda* (Bleeker, 1852) is one of the heavily traded in many Southeast Asian countries mostly for TCM (Job et al., 2002) and is one of the most expensive seahorse species in TCM (Qian et al., 2012). Given their importance as main ingredient in TCM and dwindling natural seahorse populations in the exporting countries, it is worth assessing the antioxidant properties of captive-bred cultured seahorses.

Against this background, the antioxidant activity in methanolic extracts prepared from three body portions (head, trunk and tail) in both genders of captive-bred cultured *H. kuda* (F2 generation) were evaluated in terms of total phenolic content, reducing power, metal chelating activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, ferric reducing antioxidant power, hydroxyl radical scavenging activity and lipid peroxidation activity. It is expected that the outcome of this study would infuse confidence within TCM community in favour of aquacultured animals and may provide greater impetus and encouragement in developing large-scale commercial seahorse aquaculture operations. This would ultimately help in conservation of threatened seahorse populations.

2. Materials and methods

2.1. Chemicals

Folin—Ciocalteu reagent, Sodium Carbonate, Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT), Potassium Ferricyanide, Trichloroacetic acid (TCA), Ferric Chloride, Ascorbic acid (AA), Hydrogen peroxide, Potassium iodide, Ferrous chloride, Ferrozine and 2-deoxy-D-ribose were procured from Sigma Aldrich Chemicals (Milwaukee, WI, USA), while methanol and hydrochloric acid (HCl) were from Merck (India). All chemicals were of standard analytical grade and solvents were of HPLC grade.

2.2. Seahorse and experimental facilities

New born juveniles (pelagic phase) released from a single *H. kuda* spawner belonging to the F2 generation were reared in rectangular light blue background fibreglass reinforced plastic (FRP) tanks (capacity, 100 L) at a juvenile density of 2 fish L⁻¹ until they attained settlement phase. Thereafter, juveniles were reared in large FRP tanks (capacity, 500 L) secured with different types and sizes of holdfasts depending upon the growth of juveniles. Various husbandry practices as described in Pawar et al. (2011) were followed. The seawater used was treated by rapid sand filtration, bio-filtration and then passed through ultraviolet radiation. Adequate aeration was provided to the FRP tanks using air blowers. Juveniles were fed *ad libitum* thrice a day (0600, 1400 and 2200h) with different live prey organisms such as amphipods (*Grandidierella* sp.), mysids (*Mesopodopsis orientalis*), adult *Artemia*

(*Artemia salina*), and small fish larvae (*Ambasis* sp. and *Oreochromis* sp.). The proportion of individual prey organisms in the diet varied with progressive growth of juveniles (amphipods, 70–80%, mysids, 10–20%, adult *Artemia*, 5–10%, small fish larvae, 5–10%). The tanks were cleaned daily and important water quality parameters were measured twice a week. The measured physico-chemical parameters of seawater in the rearing tanks fell within the optimum levels recommended for culture of seahorses (Murugan et al., 2009; Pawar et al., 2011): temperature (27 ± 2 °C), salinity (30 ± 1 ppt), DO (6.1 ± 0.6 mg L⁻¹), pH (7.6 ± 0.3), NO₂-N (<0.02 mg L⁻¹) and NH₃/NH₄ (0 mg L⁻¹). Healthy adult males (height, 130 ± 2.65 mm; wet weight 15.06 ± 0.95 g) and females (height, 126 ± 2.26 mm; wet weight, 13.88 ± 0.67 g) of *H. kuda* of F2 generation were randomly selected from rearing tanks. Seahorses from two different FRP tanks were used to avoid a possible tank effect.

Healthy adult seahorses were netted and anaesthetized by immersion until sedate in clove oil (50 mg L⁻¹) solution (Pawar et al., 2011) and washed thoroughly before being euthanised. Considering the conservation status of seahorses, minimum numbers of cultured fishes (15 from each gender) were sacrificed for evaluating the antioxidant properties. Sexes were identified following Lourie et al. (1999). Seahorses were dissected separately sex-wise, using sterilized scalpel into different body portions such as head (H), trunk (TR) and tail (TL) regions according to Lourie et al. (1999). TL portion of male seahorses included brood pouch also. Different body portions (gender-wise) were separately dried in a hot air oven (40 °C) until a constant weight was obtained.

2.3. Sample extraction

Different body portions were grounded separately to a fine powder and passed through a 0.5 mm mesh sieve. Extracts were prepared in methanol only as previous study by Qian et al. (2008) reported higher activity in this extract compared to other extracts (water and ethanol). Powdered dried samples of individual body portions (5 g) 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) at 180 rpm for 24 h at room temperature (28 ± 2 °C). The mixture was centrifuged at 3500 rpm for 10 min at 4 °C and filtered with Whatman No. 1 filter paper. Methanol in the extract was removed by rotary evaporation (BUCHI Rotavapor R-200) at 45 °C. Extracts were prepared in duplicate and extraction yield is expressed in terms of percentage. Duplicate extracts were pooled together and stored at - 80 °C until analyses were conducted. Each dried extract was then re-dissolved in methanol (HPLC grade) at a concentration of 2 mg mL⁻¹ as a stock extract solution. This stock extract solution was then used for the estimation of various antioxidant activities.

2.4. Total phenolic content (TPC)

The amount of TPC was determined according to Slinkard and Singleton (1977). The reaction mixture containing 0.1, 0.2 and 0.4 ml extract volumes was mixed with 1 ml of Folin–Ciocalteu reagent (1:5 dilution swirl to mix with distilled water and incubated for 1 to 8 min at room temperature, 28 ± 2 °C) and mixed thoroughly. A volume of 3 ml sodium carbonate solution (2%) was added to the mixture and was allowed to stand for 2 h, with intermittent shaking. The absorbance was measured on spectrophotometer (UV 1800, Shimadzu, Japan) at 760 nm and compared with a standard curve of gallic acid ($10\text{--}100\ \mu\text{g mL}^{-1}$). Values of TPC are expressed as mg gallic acid g^{-1} dried extract.

2.5. Antioxidant activities

Antioxidant activities measured in this study included reducing power (RP), metal chelating activity (MCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP), hydroxyl radical scavenging (HRS) activity and lipid peroxidation (LPX) inhibition assay.

2.5.1. Reducing power (RP)

RP of seahorse extracts was determined by a modified method of Oyaizu (1986). Reaction mixtures were prepared by mixing 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%) and varying volumes of extracts (0.1, 0.2 and 0.4 ml). Ascorbic acid (ASA) solution ($10\text{--}100\ \mu\text{g mL}^{-1}$) was used as a standard. Reaction mixtures were incubated at 50 °C in a water bath for 30 min and allowed to cool at room temperature (28 ± 2 °C). To each reaction mixture, 2.5 ml of 10% trichloroacetic acid (TCA) was added and centrifuged at 2000 rpm for 10 min. A volume of 2.5 ml of distilled water and 0.5 ml ferric chloride (1.0%) were added to the 2.5 ml supernatant, allowed to react for 10 min at room temperature (28 ± 2 °C), and then the absorbance was measured at 700 nm. Absorbance values of reaction mixture are directly proportional to the levels of reducing power.

2.5.2. Metal chelating activity (MCA)

MCA was determined according to the method of Dinis et al. (1994). 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 solution. The reaction mixture was initiated by the addition of 0.2 ml of ferrozine (5 mM). The mixture was shaken vigorously and allowed to stand for 10 min at room temperature (28 ± 2 °C). The absorbance was measured spectrophotometrically at 562 nm. The ability of the extract to chelate ferrous ions was compared with the standard EDTA ($10\text{--}100\ \mu\text{g mL}^{-1}$) solution and expressed as follows:

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

2.5.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging potential was measured following the method of Blois, (1958) using DPPH. To 2.5 ml of 0.1mM DPPH solution (prepared in methanol), sample extracts (0.1–0.4 ml) were added separately. The initial and final absorbance (after 30 min of the reaction; incubated in dark) were measured at 517 nm. Butylated hydroxytoluene (BHT) was used as a standard (10–100 $\mu\text{g mL}^{-1}$). DPPH radical scavenging activity (%) was calculated using the formula:

$$\text{DPPH radical scavenging activity (\%)} = [A_0 - A_1/A_0] \times 100$$

Where, A_0 – initial absorbance (0 min) and A_1 – final absorbance (after 30 min)

2.5.4. Ferric reducing antioxidant power (FRAP) assay

FRAP assay, which measures the reduction of ferric tripyridyltriazine (Fe^{+3} -TPTZ) complex to the ferrous tripyridyltriazine (Fe^{+2} -TPTZ) by a reductant at low pH, was performed according to the method described by Benzie and Strain (1996). For the determination of FRAP activity, working solution was freshly prepared using 2.5 ml of acetate buffer (300 mM, pH 3.7), 2.5 ml TPTZ solution (10 mM TPTZ in 40 mM hydrochloric acid) and 2.5 ml ferric chloride solution (20 mM). FRAP assay was performed by mixing 1.5 ml freshly prepared working solution and different sample volumes (0.1–0.4 ml) using ASA (100–1000 $\mu\text{g mL}^{-1}$) as a standard. The mixture was incubated for 10 min and the intensity of blue colour complex was measured at 593 nm. The results were expressed as mg ASA g^{-1} of dried extract.

2.5.5. Hydroxyl radical scavenging (HRS) activity

The ability of the extracts to inhibit hydroxyl radical mediated degradation of 2-deoxy-D-ribose was determined spectrophotometrically (Chung et al., 1997). The reaction mixture containing 0.2 ml of 10 mM ferrous sulphate, 0.2 ml of 10 mM EDTA, 0.2 ml of 10 mM 2-deoxy-D-ribose, sample extracts (0.1 to 0.4 ml) and 1 ml of phosphate buffer solution (0.2 M, pH 7.4) was mixed. Then, 0.2 ml of 10 mM hydrogen peroxide 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% thiobarbituric acid (TBA) were added to the test tubes. TCA was added to stop the reaction. The 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 a cold water bath. Ascorbic acid solution (10–100 $\mu\text{g mL}^{-1}$) was used as standard. The absorbance was read at 532 nm. HRS activity (%) was calculated using the equation below.

HRS activity (%) = [(Absorbance control – Absorbance sample) / Absorbance control] × 100

2.5.6. Lipid Peroxidation (LPX) inhibition assay

LPX inhibition assay was performed following the method described by Jena et al. (2010). Fresh sheep liver was obtained from local slaughter house washed with ice cold potassium chloride (1.15%) and homogenized (10% w/v) with phosphate buffer (50 mM, pH 7.4) in Teflon Potter-Elvehjem homogenizer. Homogenate was then filtered through four-layered cheese cloth and centrifuged at 10000 rpm for 10 min, at 4 °C and the supernatant was used for LPX assay.

Peroxidation of liver homogenate was induced by ferrous sulphate solution. Liver homogenate was incubated with 100 mM of ferrous sulphate for 30 min at 37 °C; the formation of thiobarbituric acid reactive substances (TBARS) in the incubation mixture was measured spectrophotometrically at 532 nm. Ascorbic acid solution (10–100 µg mL⁻¹) was used as a standard. LPX inhibition (%) was calculated by following formula:

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

Where, A_0 – the absorbance in the presence of extract, A_1 – absorbance without sheep liver homogenate and A_2 – absorbance of the control (without extract or standard).

2.6. Statistical analysis

The Pearson correlation analysis was performed between variables (Zar, 1999). Significant difference was tested at a 5% level, represented as $P < 0.05$. All statistical calculations were performed using statistical package Statistica 8.0 programme (Stat Soft 8.0). The results are presented as mean ± s.d. of triplicate (n=3) analysis.

3. Results

3.1. Extraction yields

Pooled extraction yields (EY) from all body portions obtained from male *H. kuda* (12.56 ± 0.8% w/w) were significantly higher ($P < 0.05$) than those from female gender (7.21 ± 3.96% w/w). Profiles of EY (% w/w) obtained from different body portions of male *H. kuda* (MH=head, MTR=trunk and MTL=tail) were almost similar (MH, 12.72 ± 0.395; MTR, 11.16 ± 0.053; MT, 12.59 ± 0.176). A significant variation in EY from different body portions of female *H. kuda* (FH=head, FTR=trunk and FTL=tail) was discernible and followed the order FH (11.28 ± 0.014) > FTR (7.013 ± 0.213) > FTL (03.35 ± 0.034). Mean EY obtained from all the body portions in both genders of *H. kuda* was 9.68 ± 3.73% (w/w).

3.2. Total phenolic contents (TPC)

Values of TPC (mg gallic acid g⁻¹ dried extract) measured at extract concentrations between 200 and 800 µg ranged from 5.23±0.25 to 48.85±0.50 in both genders of *H. Kuda* (Fig. 1a). For comparative assessment purposes, values of TPC measured at highest extract concentrations (800 µg) were considered. Mean TPC were relatively higher in females when compared to males. Amongst female body portions, FTR extract showed higher TPC (48.85±0.50) compared to FH (29.08±0.27) and FTL (11.99±0.22). In contrast, the measured concentrations of TPC between different body portions in male *H. kuda* varied within a narrow range (MH, 22.4±0.32; MTR, 23.44±0.04; MTL, 22.07±0.03). Mean TPC obtained from all body portions of both genders was 16.08±4.23 mg gallic acid g⁻¹ dried extract.

3.3. Reducing power (RP)

The reducing power (RP) exhibited by extracts from different body portions of both genders of *H. kuda* was quite low when compared to the known standard antioxidant i.e. ascorbic acid, ASA (Table 1). The RP from three body portions in both genders of *H. kuda* followed the order FTR>MTR>MTL>FH>MH>FTL (Fig. 1b). Mean RP of 0.32±0.07 Abs was obtained from all body portions of both genders. A significant positive correlation between RP and TPC ($P<0.05$, $r=0.90$) was recorded.

3.4. Metal chelating activity (MCA)

Extracts from TR portions in both sexes of *H. kuda* showed relatively higher levels of MCA (MTR, 91.07±0.04%; FTR, 75.55±0.03%) compared to other body portions (MH, 34.69±0.05%; MTL, 45.72±0.02%; FH, 28.33±0.08%, FTL, 16.44±0.00%). Extracts from different body portions of male *H. kuda* showed higher degree of MCA when compared to those from female. (Fig. 1c). The pooled mean MCA from all body portions in both genders obtained was 39.98±13.70%. Standard EDTA showed higher metal chelating activity than all extracts (Table 1). A significant positive correlation between MCA and TPC ($r=0.81$) as well as HRS ($r=0.85$) was observed ($P<0.05$)

3.5. DPPH radical scavenging activity

Overall, relatively higher magnitudes of DPPH radical scavenging activity was recorded in extracts prepared from the females compared to the males (Fig. 1d). Extracts of *H. kuda* significantly reduced DPPH radicals in a dose-dependent manner and mean levels of DPPH radical scavenging activity from all body portions of both genders was determined to be 24.04±7.24%. In female *H. kuda*, highest radical scavenging activity was exhibited by FTR portion (71.80±1.18%) compared to other body portions (FH, 41.95±95%; FTL, 25.47±1.52%). In contrast, such variation in DPPH radical

scavenging activity between the different body portions of male *H. kuda* was not discernible (Fig. 1d). Standard BHT showed relatively higher DPPH radical scavenging activity compared to extracts (Table 1). Levels of DPPH radical scavenging activity significantly ($P<0.05$) correlated with TPC ($r=0.97$), RP ($r=0.98$) and MCA ($r=0.91$).

3.6. Ferric reducing antioxidant power (FRAP)

Measured FRAP activity (mg ASA g^{-1} dried extract) in *H. kuda* extracts significantly increased in a dose dependent manner ($P<0.05$) with a mean concentration of 94.44 ± 2.83 (Fig. 1e). In male and female *H. kuda*, the measured FRAP activity varied from 35.06 ± 0.13 to 173.01 ± 0.71 and from 42.75 ± 0.33 to 153.87 ± 1.13 , respectively. Activity levels of FRAP significantly correlated with TPC ($P<0.05$, $r=0.93$).

3.7. Hydroxyl Radical Scavenging (HRS) activity

HRS activity, as a measure of the degree of inhibition of D-ribose degradation by hydroxyl radical, varied in a dose-dependent manner in both genders of *H. kuda* (Fig. 1f). The degree of HRS activity exhibited by trunk portion of female *H. kuda* was significantly higher ($128.20\pm 0.15\%$) than by male *H. kuda* ($95.68\pm 0.08\%$). Mean level of HRS activity measured from all body portions in both genders was $86.23\pm 1.24\%$. Standard ascorbic acid at $10\text{--}100\ \mu g\ mL^{-1}$ showed relatively similar HRS activity than all extracts except FTR (Table 1). HRS activity positively correlated with TPC ($r=0.84$), RP ($r=0.87$), MCA ($r=0.87$) and DPPH ($r=0.79$).

3.8. Lipid peroxidation (LPX) Inhibition assay

LPX inhibition activity measured in terms of thiobarbituric acid reactive substances (TBARS) measured from three body portions of *H. kuda* followed the order, TR>H>TL (Fig. 1g). Amongst two genders, relatively higher degree of LPX inhibition was observed in female *H. kuda* ($142\pm 1.20\%$) compared to male ($108.70\pm 0.20\%$). Mean LPX inhibition activity obtained from all body portions of both genders was $74.72\pm 13.18\%$. LPX inhibition activity shown by standard ascorbic acid at concentrations of $10\text{--}100\ \mu g\ mL^{-1}$ was quite similar to that of the sample extracts from all body portions except for FTR and MTR (Table 1). Levels of LPX inhibition activity were positively correlated with TPC ($r=0.61$), RP ($r=0.54$) and HRS activities ($r=0.83$).

4. Discussion

Although seahorses and other Syngnathids have been used in traditional Chinese medicine (TCM) for more than 600 years, the exploration for different bioactive compounds and antioxidant compounds has begun only recently (Hung et al., 2008; Li et al., 2008; Moreau et al., 1998; Qian et

al., 2008, 2012; Yang et al., 2012). Overexploitation of natural seahorse populations has led to the imposition of strict legal restrictions in many countries. Seahorse aquaculture offers an effective viable option in bridging the gap between the ever increasing demand dwindling natural supplies. Worried about the potency, many TCM traders are reluctant to accept seahorses produced from aquaculture (Moreau et al., 1998). Therefore, more comprehensive studies evaluating the bioactive and antioxidant potential from cultured seahorses are required for infusing confidence amongst TCM community.

Extraction yields obtained in the present study were relatively higher ($9.68 \pm 3.73\%$ w/w) than previously reported in wild *H. kuda* (6.9% w/w) by Qian et al. (2008).

4.1. Total phenolic content (TPC)

Mean values of TPC (16.08 ± 4.03 mg gallic acid g^{-1} of dried extract) obtained from captive-bred and cultured *H. kuda* were almost similar to those reported in wild *H. kuda* (17.43 ± 1.30 mg g^{-1}) by Qian et al., (2008). However, amongst different body portions, the TPC obtained from the trunk (TR) portion of both genders in *H. kuda* was relatively higher compared to other body portions (H= head; TL= tail). This might be due to activity of internal organs such as digestive tract, liver, gonads, kidneys etc in the trunk portion. Undoubtedly, a variety of enzymes, antioxidant activities and bioactive components used in biomedical applications are extracted from internal organs of marine fishes (Byun et al., 2003; Haard, 1998; Kim and Mendis, 2006; Kim et al., 1997, 2003; Trenzado et al., 2006). Further, higher TPC values recorded particularly in TR portion of female compared to the same portion in male could be attributable to the internal organs (most probably gonads). Furthermore, it has been documented that gonads serve as a reserve for a variety of fatty acids in fish (Alvarez et al., 2009) and this is true for seahorse species as well (Faleiro and Narciso, 2010; Planas et al., 2010). Phenols are known to be effective antioxidants for polyunsaturated fatty acids (Qian et al., 2008). The mean size of female *H. kuda* used in the present study (126 ± 2.26 mm Ht) was larger than the maturity size reported for this species in captivity (Thangaraj et al., 2006). Hence, presence of larger gonadal mass in females compared to the males may explain the higher levels of TPC in the former gender. The phenolic compounds exhibit considerable free radical scavenging activities through their reactivity as hydrogen or electron-donating agents, metal ion chelating properties, neutralizing capacity of free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Maqsood and Benjakul, 2010; Osawa, 1994; Rice-Evans et al., 1996). Significant correlation ($P < 0.05$) between TPC and other antioxidant activities such as reducing power (RP), metal chelating activity (MCA), DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP),

hydroxyl radical scavenging (HRS) activity and inhibition of lipid peroxidation (LPX) may explain the higher degree of observed antioxidant activities in the present study.

4.2. Reducing power (RP)

Values of reducing power recorded from all body portions of cultured *H. kuda* are comparable with previous study by Qian et al. (2008) in wild *H. kuda* but are lower than those reported in cultured *H. kuda* by Hung et al. (2008). Higher RP recorded by Hung et al. (2008) may possibly be due to use of higher extract concentration (40 mg ml⁻¹). Amongst different body portions of cultured *H. kuda*, RP values from TR portion were relatively higher than those recorded from other body portions (H and TL). Similar observation with higher RP values from belly portion compared to other body portions in cultured *H. kuda* has also been reported by Hung et al. (2008). Internal organs associated with the respective body portions might explain the exhibited differential RP activities. A significant positive correlation observed between RP and DPPH radical scavenging activities as well as HRS and LPX inhibition activity ($P < 0.05$) implies that extracts from captive-bred and cultured *H. kuda* possess greater ability to donate hydrogen ions for electrons to capture metals ions.

4.3. Metal chelating activity (MCA)

Chelating of metal ions is often associated with redox active metal catalysis which prevents generation of reactive oxygen species (Ebrahimzadeh et al., 2008). Mean levels of MCA measured from different body portions in both genders of captive-bred cultured *H. kuda* was 39.98±13.70%. Similar MCA levels (mean, 28.77±10.24%) have been observed in extracts from a closely related seahorse species, the alligator pipefish (*Syngnathoides biaculeatus*) (Sanaye et al., unpublished). In contrast, significantly lower levels of Fe⁺ chelating activity in two different extracts (hot-water, 4.89±0.53%; ethanol, 8.35±0.05%) of cultured *H. kuda* has been reported by Hung et al. (2008). No plausible explanation, however could be offered for such low levels of MCA reported by Hung et al. (2008).

On the other hand, higher levels of MCA observed in this study might be due to the greater ability of the extracts to obstruct the formation of ferrous and ferrozine complex. This suggests that cultured *H. kuda* extracts are capable of reducing and/or capturing ferrous ion before ferrozine and thereby decreasing the rate of Fenton reaction (Ebrahimzadeh et al., 2008). Fenton reaction which is known to damage bio-molecules through LPX activity (Barbusinski, 2009; Ozsoy et al., 2009) and the reduction in the concentration of transition metals that catalyse lipid peroxidation is directly related to MCA (Chandra Mohan et al., 2012). Significant correlation between MCA and TPC and HRS activity suggests that extracts from captive-bred cultured *H. kuda* are involved in scavenging of

hydroxyl radicals which alternatively reduce the degree of lipid peroxidation. The antioxidant effect of several polyphenols acting as inhibitors of hydroxyl radical formation has been reported to correlate with iron chelating properties (Ohnishi et al., 1994).

4.4. DPPH radical scavenging activity

The mean levels of DPPH radical scavenging activity recorded in the present study were lower than those reported in wild (Qian et al., 2008) and cultured *H. kuda* (Hung et al., 2008). Vast observed differences in DPPH radical scavenging activity for the same species might be due to differences in methodology, size and gender. Optimum rearing conditions and supply of adequate quantities of nutritionally balanced feed in culture environment may limit the production of free radical scavenging activity. In contrast, the stress induced by environmental conditions and food may alter the production capabilities of antioxidants in a biological system in wild animals (Martinez-Alvarez et al., 2005). Thus the production of such antioxidant compounds and their DPPH scavenging effect expected to be highly variable. Such a large variability in the DPPH radical scavenging activity may possibly be due to struggle faced by wild animals for accessing food and acclimatizing to the dynamic environmental conditions.

Compared to other body portions, relatively higher levels of DPPH radical scavenging activity recorded in TR portion of *H. kuda* may have derived from the internal organs. Amongst genders, the activity levels were higher in females ($71.80 \pm 1.18\%$) than in males ($31.26 \pm 2.19\%$) and such a variation may be due to the higher gonadal mass in the former gender. These observations appears to be similar to those reported in cultured *H. kuda* by Hung et al. (2008) who found relatively higher scavenging activity in belly portions compared to other body portion. Furthermore, higher levels of DPPH activity in liver compared to other body tissues (muscles and skin) in four fish species have also been reported by Bhadra et al. (2004). The reducing action exhibited by *H. kuda* extract might have been mediated through the donation of hydrogen to a free radical thereby reducing to a non-reactive species. It has been reported that DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Matsukawa et al. 1997). Therefore, DPPH is often used as a substrate to evaluate antioxidant activity of natural antioxidants (Kim et al., 2002; Lee and Seo, 2006; Lu and Foo, 2000). Positive correlation between levels of DPPH radical scavenging activity and TPC, RP, MCA and HRS activity obtained in this study corroborates the antioxidant potential of cultured *H. kuda*.

4.5. Ferric reducing antioxidant power (FRAP)

The FRAP assay measures the reducing potential of an antioxidant that reduces ferric tripyridyltriazine (Fe^{+3} -TPTZ) complex to ferrous tripyridyltriazine (Fe^{+2} -TPTZ) (Benzie and Strain, 1996). In addition to being used as a tool to assess the potential biological antioxidants, the FRAP also measures chemical reductant (Griffin and Bhagooli, 2004). Mean FRAP values (94.44 ± 2.83 mg ASA g^{-1} dried extract) obtained in the present study are indicative of the antioxidant potential of cultured *H. kuda*. However, it must be kept in mind that depending on the assay system used, a test extract sample may show different results for antioxidative potential. Such apparent differences are due to the mechanism of antioxidative action which differs from one assay to another. For example, a good ferric reducer may not show a higher activity in radical scavenging assays (Samadi and Ismail, 2010; Samaranyaka and Li-Chan, 2011) and FRAP assay does not show reactions with some antioxidants such as glutathione (Guo et al., 2003).

It has been reported that the reducing capacities are often due to certain compounds present in the extracts which aid in breaking the free radical chain through donation of hydrogen atom (Duh et al., 1999). The positive correlation between FRAP and TPC ($r=0.93$) indicate that the redox potential of phenolic compounds play an important role in determining the antioxidant activity (Rice-Evans et al., 1996). Higher FRAP values are indicative of higher antioxidant capacity as this assay determines total antioxidants which act as reducing agents (Rabeta and Nur Faraniza, 2013). Based on the FRAP values recorded in the present study, it can be concluded that the magnitude of ferric complex reduction in aiding scavenging of free radicals appears to be highly significant in extracts prepared from cultured *H. kuda*. These results highlight the usefulness of FRAP assay in determining the antioxidant potential in seahorses.

4.6. Hydroxyl radical scavenging (HRS) activity

Hydroxyl radical is a powerful pro-oxidant particularly of lipids and the ability of quenching hydroxyl radicals by an antioxidant appears to be directly related to the prevention of propagation of lipid peroxidation process (Batista et al., 2010). In the present investigation, the HRS activity values obtained from all body portions in both genders of cultured *H. kuda* were quite high ($86.23 \pm 1.24\%$) but were marginally lower than those reported in wild *H. kuda* (Qian et al., 2008). Significant correlation between HRS and other antioxidant properties (TPC, RP, MCA, DPPH radical scavenging activity and FRAP) reflect on the higher ability of extracts from cultured *H. kuda* to scavenge or quench hydroxyl radicals efficiently and minimize lipid peroxidation. According to Smith et al. (1992) inhibition of deoxyribose degradation are caused by those molecules which are

able to chelate iron ions and hence prevent them from complexing with deoxyribose and keep them inactive in a “Fenton” reaction.

4.7. Inhibition of Lipid peroxidation

Lipid peroxidation is the most extensively studied manifestation of oxygen activation in biology which refers to oxidative degradation of lipids. Upon reaction with free radicals, lipids may undergo the highly damaging chain reaction of lipid peroxidation leading to both direct and indirect effects (Devasagayam et al., 2004). In present investigation, *H. kuda* extracts showed greater inhibitory capabilities of lipid peroxidation (74.72±13.18%). Cheng et al. (2003) reported that phenolic compounds offer protective action in lipid peroxidation by scavenging the lipid derived radicals (R^\cdot , RO^\cdot or ROO^\cdot) to arrest the chain reaction. In addition, it is also well known that free radicals cause autoxidation of unsaturated lipids (Kaur and Perkins, 1991). Positive correlation between LPX and other antioxidant activities such as TPC, RP, FRAP and HRS scavenging activity reflects the higher ability of captive bred and cultured *H. kuda* extract to minimize lipid peroxidation by inhibition of free radicals action and quenching of hydroxyl radicals in biological system.

5. Conclusions

One of the main bottlenecks identified for the establishment of biologically and economically viable commercial seahorse aquaculture is that of providing sufficient quantities of nutritionally-complete live food for different stages of rearing. High survival and growth rates by feeding various prey organisms during the captive breeding of different seahorse species have been reported (Job et al., 2002; Murugan et al., 2009; Otero-Ferrer et al., 2012; Payne and Rippangale, 2000; Woods, 2003). In addition to optimising the biological factors affecting the seahorse culture, it is also important to examine the subsequent transformation nutraceutical properties of offered feed into body composition. This aspect is of paramount importance as the cultured seahorses have to compete with the wild for traditional Chinese medicine market.

Results of the present study indicated that captive-bred and cultured yellow seahorse, *H. kuda* possess antioxidant activities at par with wild caught ones. The antioxidant properties exhibited by captive-bred and cultured *H. kuda* may not be representative of all cultured seahorse species, particularly those from different geographical areas and reared under varying feeding regimes. Nevertheless, the results obtained in the study may allay the apprehensions over the potency of cultured seahorses in TCM and may help in conservation of resources by reduced dependence on natural populations.

The proportion of amphipods and mysids in the diet was relatively higher than other prey organisms during captive rearing of *H. Kuda*. In fact, these are the most dominant prey organisms observed in the guts of wild seahorses (Do et al., 1998; Kendrick and Hyndes, 2005; Kitsos et al., 2008; Truong, 1998; Woods, 2002). The biochemical composition of fish is known to be strongly affected by the composition of their food (Henderson and Tocher 1987; Lin et al., 2009; Orban et al., 2003). Therefore, it is likely that the high levels of antioxidant activities recorded in captive-bred *H. kuda* may be related to the diet. Further research assessing the antioxidant potential of seahorses grown under different feeding regimes would enhance current understanding the interrelationship between feed and antioxidant profiles.

Acknowledgements

The authors are grateful to the Director, CSIR-National Institute of Oceanography, Goa (India) for the facilities and encouragement. Financial support came from the Department of Biotechnology (BT/PR-8271/AAQ/03/308/2006) and PSC0206 (Govt. of India). Thanks are due to the anonymous reviewers for the constructive criticisms which improved the quality of the manuscript greatly. Permission granted by the Ministry of Environment & Forests, Government of India for the collection of seahorses is gratefully acknowledged. This paper represents contribution No. 5627 of the CSIR—National Institute of Oceanography, Goa (India).

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Table 1. Typical values of standards at different concentrations (mean±s.d) used for assessing antioxidant activities in captive-bred cultured yellow seahorse (*Hippocampus kuda*).

Standards	Ascorbic acid			EDTA	BHT
Concentrations	Reducing power (Abs.)	Hydroxyl radical scavenging activity (%)	LPX inhibition (%)	Metal chelating activity (%)	DPPH radical scavenging activity (%)
10 µg	0.29±0.01	90.43±0.14	85.77±0.01	41.77±0.07	38.86±0.17
20 µg	0.42±0.02	92.89±0.18	89.09±0.01	82.42±0.02	43.44±0.05
40 µg	0.64±0.01	93.02±0.01	96.13±0.15	87.62±0.12	45.55±0.02
80 µg	1.09±0.15	93.19±0.10	98.06±0.01	97.26±0.10	52.27±0.01
100 µg	1.28±0.01	96.92±0.08	98.66±0.02	98.72±0.03	64.84±0.08

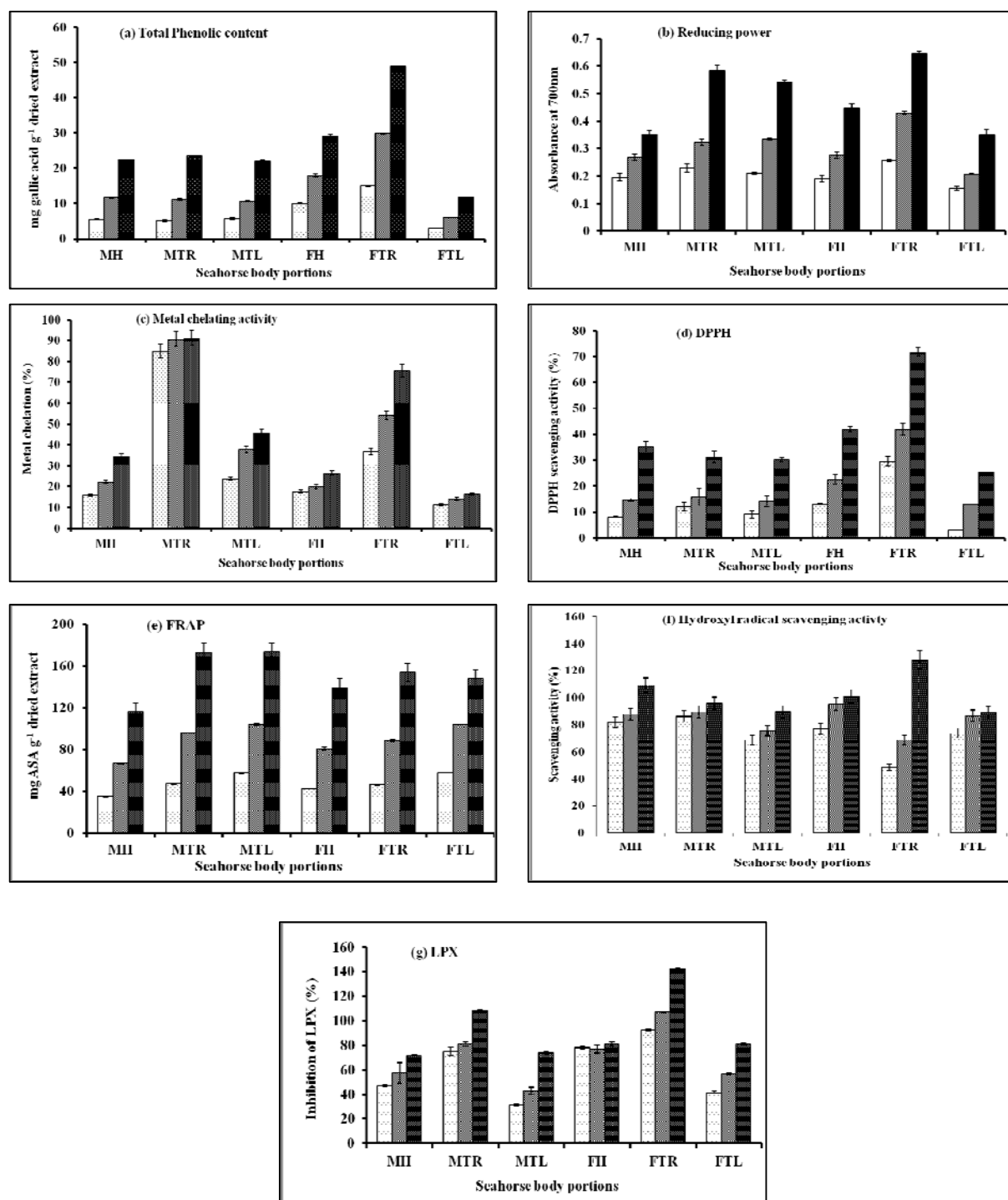


Fig. 1. Antioxidant activities in different body portions of in two genders of the yellow seahorse, *Hippocampus kuda*; (a) Total phenolic content (b) Reducing power (c) Metal chelating activity (d) DPPH radical scavenging activity (e) Ferric reducing antioxidant power (f) Hydroxyl radical scavenging activity (g) Lipid Peroxidation inhibition (MH = male head; MTR= male trunk; MTL= male tail; FH= female head, FTR = female trunk and FTL = female tail (- 0.200 µg ; - 400 µg and - 800 µg of extract).