Total polyphenol content, anti-oxidative and anti-bacterial properties of seahorses traded as traditional medicine

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Abstract
The present study examined the total polyphenol content (TPC), anti-oxidative and anti-microbial properties of various extracts of male and female seahorses, Hippocampus barbouri. The TPC was assessed by the Folin-Ciocalteu’s method, while the anti-oxidative activities were determined by two different methods; scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals and ferric reducing ability plasma (FRAP) assays. The antimicrobial properties of H. barbouri extracts were evaluated using disc diffusion method. The TPC content extracts of H. barbouri ranged from 2.99 to 5.00 mg GAE/g. The ability to reduce DPPH was strongest in ethanol extract and lowest in ethyl acetate extract. Contradictory, the antioxidant property of H. barbouri in ethanol extracts was much lower than in ethyl acetate and methanol extracts. The values of TPC, DPPH and FRAP assays showed the existence of a significant negative relationship between DPPH and FRAP assays (r = 0.909, p<0.01). Only male ethyl acetate extract of H. barbouri showed anti-microbial activity against both Bacillus cereus and Staphylococcus aureus. These findings indicated that H. barbouri is a potential source of natural anti-oxidant and possess some anti-microbial activities against the harmful bacteria strain.

Keywords: seahorse, Hippocampus barbouri, traditional medicine, antioxidant, antimicrobial

1.0 Introduction
Seahorses are traded in the Asia Pacific region for two major purposes: as ornamental fish and traditional medicine. In Sabah, Malaysia, at least 10 seahorse species were recorded so far [1] and Hippocampus barbouri is one of the most commonly available seahorse species traded as traditional medicine. These marine fishes are unique species with the male having specialized brood pouch for egg incubation and exhibit paternal care. Locally, seahorse products are believed to be able to cure a variety of diseases with maximum benefits when used in pair. This local perception may has some basis because several studies have reported the nutritional value of seahorses which are characterized by high protein content, good amino acid and fatty acid profiles, with various bioactivities such as anti-tumor, anti-fatigue, anti-oxidant, anti-microb and anti-inflammatory[2,3,4,5,6,7,8]. Since there are more than 35 seahorse species were recorded globally [9], previous studies on different subjects are only limited to certain species only. The full potential of seahorses as food source and application in pharmaceutical industry must be carefully examined to make sure these valuable fish species are not further exploited without thorough scientific evaluation. If they are proven to be of high value for the benefit of human health, aquaculture of seahorse is seen as the most viable alternative to capture fisheries of seahorses. Therefore, the present study was conducted to evaluate the anti-oxidation and anti-microbial activities from male and female H. barbouri traded in the local market as traditional medicine. To our knowledge this is the first report on the anti-oxidation and anti-microbial activities of H. barbouri.

2.0 Materials and Methods

2.1 Seahorse samples
Dried products of male and female seahorse (H. barbouri) were purchased from the local market in Kota Kinabalu Sabah, Malaysia. Species identification was done following [10]. The total length and weight of seahorse were measured individually. They were then homogenized using a grinder and stored in a zip-lock plastic bag in a
refrigerator until needed.

2.2 Crude Extraction for Anti-oxidative and Anti-microbial Properties
The dry samples (61.54 g for male; 89.93g for female seahorses) were placed into a 1000 mL beaker, added with 300 mL ethyl acetate and was mixed using an orbital shaker for 4 hour. Samples were then filtered by vacuum filtration and the residue was re-extracted with 95% ethanol and methanol. The filtrate was then evaporated with a rotary evaporator at the boiling point temperature of the solvents to concentrate the compounds and freeze dried to remove the water and was kept at -40°C in the refrigerator until further use.

2.3 Determination of total polyphenols content
The total polyphenol content was determined by adapted method of Folin-Ciocalteu [11], using gallic acid with a standard phenolic compound as a reference [12]. For the preparation of calibration curve, 100 µL of 0.10, 0.15, 0.20, 0.25, and 0.30 mg/mL of gallic acid solution were mixed with 100 µL of Folin-Ciocalteu reagent (50%) and 2 mL sodium carbonate, Na₂CO₃ (2%). After 30 minutes, the absorbances were read at 760 nm with UV-Vis Spectrometer and the calibration curve was drawn. A total of 100 µL of the sample extract (0.30 mg/mL) was mixed with the same reagent as described above. The absorption was measured for the determination of total polyphenol content after 30 minutes. The total polyphenol content of the sample was expressed in milligrams per serving of gallic acid equivalents (GAE). The total polyphenol content in H. barbouri samples was calculated using the following equation:

\[
\text{Total polyphenol content, TPC (mg GAE/g)} = \frac{CV}{m} \\
\text{Where, } C = \text{Concentration calculated from the calibration equation (mg/mL),} \\
V = \text{Volume of the samples used (mL)} \\
m = \text{Mass of the samples used (g)}
\]

2.4 Antioxidative activity
The total antioxidative activity of H. barbouri extracts was determined using 1,1- diphenyl-2- picrylhydrazyl (DPPH) and ferric reducing ability plasma (FRAP) assay. The free radical scavenging activity of H. barbouri extracts was measured by using the 1,1- diphenyl-2-picryl-hydrazyl (DPPH) assay as described by Tepe et al. [13]. H. barbouri extract (0.1 mL) was mixed with DPPH radical in methanol (4 mL, 6 x 10⁻⁵ M). Deionized water was used as a control and contained all the reaction reagents except the extract. The reaction mixture was vortex-mixed and let to stand at room temperature in the dark for 30 minutes. Absorbance at 517 nm was measured using a spectrophotometer using methanol as a blank. The butylatedhydroxytoluene (BHT) was used as antioxidant reference and prepared in the same way without the extract. Antioxidative activity was expressed as a percentage inhibition of the DPPH radical and was determined using the equation described below [11]:

Inhibition,

\[
I (%) = \left( \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100
\]

Ferric reducing ability plasma (FRAP) assay was prepared on the day of experiment using three individually prepared solutions with following chemicals. Solution (a) 300 mM acetate buffer (pH 3.6), 3.1g sodium acetate trihydrate and 16 mL of glacial acetic acid were mixed then the solution was made up to 1 L with distilled water. Solution (b) 10 mM of 2,4,6-tripryridyl-s- triazine, TPTZ (molecular weight of 312.34 g/mol) was prepared in 40mM of HCl (molecular weight of 36.46 g/mol). Solution (c) 20 mM of FeCl₃·6H₂O (MW = 270.30 g/mol). Then, solution (a), (b) and (c) were mixed with ratio of 10:1:1. Besides, 1000 µM of ascorbic acid (molecular weight of 176.13 g/mol) was prepared as standard within 1 hour before use. After that, 3 mL of freshly prepared FRAP reagent was warmed to 37°C and reagent blank reading was taken at 593 nm. Then, 0.1 mL of H. barbouri extract was added along with 0.3 mL of distilled water. Absorbance at 593 nm was measured.

2.5 Antibacterial activities
The culture media used for bacteria cultures in this study was Nutrient Agar (NA). The media was prepared by adding 2.8 g of NA powder and dissolved with 70 mL distilled water in a 150 mL-Erlenmeyer flask. The media was adjusted to pH 7.2 and topped up to 100 mL with distilled water. After that, the medium was autoclaved at 121°C for 1 hour. The hot liquid agar was cooled approximately to 50°C and poured into sterilized petri dish and solidified at the room temperature. The targeted bacteria for anti-microbial screening were Bacillus cereus (Gram-positive bacteria) and Staphylococcus aureus (Gram-negative bacteria). Bacteria species were transferred from the stock broth medium onto NA plate by streaking. The plates are incubated at 37 ± 2°C for two days and were used as inoculum.

2.5.1 Fermentation of bacterial strains
The nutrient broth which consists of 0.8% nutrient broth powder. For preparing 100 mL of the fermentation medium, 1.3 g of nutrient agar powder were weighed and dissolved with 70 mL distilled water in a 150 mL Erlenmeyer flask. The media was adjusted to pH 7.0 and topped up to 100 mL with distilled water. After that, the medium was autoclaved at 121°C for 1 hour. The hot liquid agar was cooled approximately to 50°C and poured into sterile petri dish and solidified at the room temperature. The broth culture was then incubated at 37 ± 2°C for one day.

2.5.2 Preparation of discs
Filter paper discs of 6 mm diameter (Whatman No.3) were sterilized under UV light and applied with 20 µL of the seed extract and 20 µL of streptomycin with concentration of 1 mg/mL was used as a positive control. The discs were then dried in 37°C for 24 hours. The dried discs are kept in 4°C for storage.

2.5.3 Antibacterial screening assay
Antibacterial activities were screened using a disc diffusion method [14]. Bacteria from the broth culture were transferred (400 µL) onto Nutrient Agar (NA) with sterile pipette. Filter paper discs which were loaded with 20 µL of extract were placed on swabbed agar plates and pressed gently. The plate were inverted and incubated for 24 hours at 37°C. Streptomycin was used as positive control. Antibacterial activity was indicated by the presence of clear zone around the discs. Tests were repeated twice to ensure reliability of the result.
3.0 RESULT

3.1 Crude extract

The dried extract (g) and yield percentage (%) of both male and female samples of *H. barbouri* are shown in Table 1. The yield percentages for ethyl acetate extraction were lowest for both male (1.99 %) and female (1.93 %) *H. barbouri* samples if compared to extractions using ethanol (male, 2.08 % and female, 2.16 %) and methanol (male, 3.09% and female, 2.91%) as solvent. The order of the yields from high to low for the *H. barbouri* extracts were: methanol (male) > methanol (female) > ethanol (female) > ethanol (male) > ethyl acetate (male) > ethyl acetate (female).

3.2 Total polyphenol content

The total phenolic content was the highest in methanol extracts followed by the ethyl acetate and ethanol extracts. The total phenolic content of male and female extracts of *H. barbouri* ranged from 2.99 to 5.00 mg GAE/g (Figure 1). All male extracts showed higher phenolic contents than the female extracts.

3.3 Antioxidative Activity

3.3.1 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay

At concentration of 10 mg/mL, female *H. barbouri* extract (I % = 77.50 %; IC$_{50}$ = 0.01 mg/mL) with ethanol as solvent showed highest ability to reduce DPPH, followed by male ethanolic extract (I % = 74.89 %; IC$_{50}$ = 0.03 mg/mL), male methanolic extract (I % = 74.58 %; IC$_{50}$ = 0.0342 mg/mL), female methanolic extract (I % = 73.99 %; IC$_{50}$ = 0.02 mg/mL), ethyl acetate extract (I % = 65.15 %; IC$_{50}$ = 0.03 mg/mL) and male ethyl acetate extract (I % = 51.91 %; IC$_{50}$ = 0.09 mg/mL). The efficacy of ethanol and methanol extract of *H. barbouri* was higher than BHT with the IC$_{50}$ of 0.06 mg/mL and 65.88 % inhibition activity.

3.3.2 Ferric reducing ability plasma (FRAP) assay

The antioxidant capacity for male (0.92 mg FeE/g) and female (0.15 mg FeE/g) in ethanol extracts of *H. barbouri* are unexpectedly low and showing large gap values if compared to capacity in ethyl acetate extracts (male, 5.23 mg FeE/g and female, 3.70 mg FeE/g) and methanol extracts of *H. barbouri* (male, 2.83 mg GAE/g and female, 1.57 mg GAE/g) (Figure 4).

3.4 Antimicrobial activity

Tables 2 and 3 show that *H. barbouri* extracts were not showing any antibacterial activity towards *B. cereus* while weak antimicrobial activity (ethyl acetate extracts) were shown towards *S. aureus*. The result shows that both female and male ethyl acetate extracts of *H. barbouri* yielded inhibition zones of 11.5 mm and 6.5 mm, respectively. No inhibition zones were observed for both female and male ethanol extracts towards *S. aureus*.
Table 1. Total extraction yield and yield percentage of H. barbouri sample.

<table>
<thead>
<tr>
<th>H. barbouri sample</th>
<th>Dry mass (g)</th>
<th>Solvent of extraction</th>
<th>Crude extract after freeze dried (g)</th>
<th>Yield percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>61.54</td>
<td>Ethyl Acetate</td>
<td>1.23</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>1.28</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>1.90</td>
<td>3.09</td>
</tr>
<tr>
<td>Female</td>
<td>89.33</td>
<td>Ethyl Acetate</td>
<td>1.72</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>1.93</td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>2.60</td>
<td>2.91</td>
</tr>
</tbody>
</table>

Table 2. Antimicrobial activities of H. barbouri against B. cereus.

<table>
<thead>
<tr>
<th>Samples (100 mg/mL)</th>
<th>Inhibition Zone (mm) Bacillus cereus (Gram-positive bacteria)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin (Positive Control)</td>
<td>i</td>
</tr>
<tr>
<td>Methanol (Negative Control)</td>
<td>11</td>
</tr>
<tr>
<td>Male Ethyl Acetate</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Ethanol</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Male Methanol</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Ethyl Acetate</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Ethanol</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Methanol</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Antimicrobial activities of H. barbouri against S. aureus.

<table>
<thead>
<tr>
<th>Samples (100 mg/mL)</th>
<th>Inhibition Zone (mm) Staphylococcus aureus (Gram-negative bacteria)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin (Positive Control)</td>
<td>i</td>
</tr>
<tr>
<td>Methanol (Negative Control)</td>
<td>22</td>
</tr>
<tr>
<td>Male Ethyl Acetate</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Ethanol</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Methanol</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Ethyl Acetate</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Ethanol</td>
<td>0</td>
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<td></td>
<td></td>
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<tr>
<td>Female Methanol</td>
<td>0</td>
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</tbody>
</table>

4.0 DISCUSSION

The total yield of extraction depends on the type of solvents with varying polarities, extraction time, pH and temperature as well as on the chemical compositions of the sample [15]. At constant pH and temperature, the yields normally increase significantly with time of extractions [16]. In the present study, total percentage yield for all extractions was 14.16% which indicated that not all the compounds had been successfully extracted from the sample. This may be due to the effect of extractions time of the sample which was conducted for 4 hour. Besides that, the extract yields are strongly dependent on the nature of extracting solvent due to the presence of different bioactive compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent [17]. These result also indicated that more bioactive compounds contained in the H. barbouri extract were likely soluble in methanol compared to ethanol and ethyl acetate.

The total polyphenol content of H. barbouri in the present study was considered a moderate level as higher yields up to 17.43 mg GAE/g was reported in other seahorse species, H. kuda [18]. Male extracts shows higher phenolic content in both ethanol and ethyl acetate solvents compared to female extracts. Male seahorse is responsible for incubating the eggs and nurturing their young inside the brood pouch which play important tasks in respiratory, osmoregulatory, excretory and provide nutrients to the embryo [19]. This paternal behavior and unique morphology of male seahorses could have some influence to the current findings. In addition, seahorse trade is relying on the supply from accidental catches in the trawl and these fish are sun-dried before being marketed to the local fish market which may also influence the quality of the samples. The female ethanol extracts had shown the highest ability to reduce DPPH inhibitory activity with lowest IC$_{50}$ value if compared to other extracts (Figures 2 & 3). IC$_{50}$ value was calculated as the amount sample causing a 50% inhibition of DPPH. It is negatively related to the antioxidant activity and expressed the amount of antioxidant needed to decrease its radical concentration by 50%. It was suggested that the lower IC$_{50}$ values the higher antioxidative capacity of the samples [20]. The present findings also indicate that polar solvents might be better extraction solvents for H. barbouri evaluation. Due to the lack of information available on the
antioxidant capacity of *H. barbouri* or any other seahorse species from the previous study, no comparison can be made.

The present study shows that the ethanol and methanol extracts of *H. barbouri* were effective anti-oxidant agent which acts as synthetic antioxidant (BHT). The ethanol extracts had the highest scavenging activity of radical whereas the ethyl acetate showed the opposite. Sim et al. [21] in their research mentioned that the one of the known mechanisms which inhibits cellular damage was a free radical scavenging mechanism. The DPPH free radicals scavenging method has been widely used in the determination and evaluation of the radical scavenging capacity of specific compounds or extract in a short time [22]. The method is based on the reduction of alcoholic DPPH solutions in the presence of hydrogen donating antioxidant. DPPH is a nitrogen-centred free radical, stable at room temperature and produces a purple solution in methanol [21]. In its radical form, DPPH solution shows a maximum absorption at 520 nm which visualized as a discoloration from purple to yellow [23]. Anti-oxidative activity is expressed as a percentage inhibition of the DPPH radical. As the concentration increases, the absorbance decreases due to the reduction of stable radical DPPH. Apart from that, the percentage of inhibition (I %) of all the *H. barbouri* extracts and BHT was increased with the increase of concentrations (0.02 - 0.1 mg/ml).

FRAP assay is a method used to determine the antioxidant capacity of a wide range of biological samples and pure compounds. The principle of this method is based on the reduction of ferric-tripyridyltriazine complex to its ferrous in the presence of antioxidant. It measures the change in absorbance at 593 nm owning the formation of blue colored Fe (II) tripyridyltriazine compound from colorless oxidized Fe (III) form by the action of electron donating antioxidants [24]. It was observed that the antioxidant capacity for both sexes *H. barbouri* samples in ethyl acetate extracts were higher if compared to ethanol extracts, with no definite trend on the seahorse sexes. Antibacterial activities of *H. barbouri* in the present study were measured by using a disc diffusion method [14], where extracts were subjected to screening by using two types of bacteria which were *Bacillus cereus* (gram-positive) and *Staphylococcus aureus* (gram-negative). Apart from that, streptomycin was used as a positive control whereas methanol was used as a negative control. The use of positive control and negative control in this study was to ensure the reliability of the result. The positive control should show clear inhibition zone around the targeted bacteria but the negative control should show no inhibition zone. The use of positive control was important because it was used to compare the effectiveness of the sample to kill the bacteria. Adjacent to, negative control was used to ensure the solvent used in dissolving the sample did not affect the extract that will have an effect on the result. The results revealed that *H. barbouri* had the bioactive compounds which were specific inhibit against *S. aureus* and can be recommended as an antimicrobial agent against the harmful bacteria strain in future. In a study on the antimicrobial effect of different seahorse species from Indian Coast, the inhabitation zones of less than 8 mm were recorded in *Klebsiella pneumoniae*, and *Vibrio cholera* in the case of *H. trimaculatus* and *Staphylococcus aureus* in the case of *H. kuda* in n-butanol extracts. Some meager activity was also observed in *H. kelloggi* towards *Streptococcus aureus* and *Salmonella typhi* [6].

In conclusion, *H. barbouri* shows some anti-oxidative and anti-bacterial activities. The present findings are considered important to support the alleged health benefit by the local consumer of dried seahorse product traded as traditional medicine. Due to its potential as food product and application in healthcare industry, aquaculture of this species should be considered to meet the demand for this product in the local and global markets and to reduce the dependency on wild seahorse resources.

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