DNA barcoding for seahorse identification and its potential as antioxidant and stimulant indicator

M Nurilmala¹*, E M Sari¹, A Abdullah¹, H H Hizbullah¹, N A Butet² and A O Sudrajat³

¹Department of Aquatic Products Technology, Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB University), Indonesia
²Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB University), Indonesia
³Department of Aquaculture Technology, Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB University), Indonesia

*E-mail: mnurilmala@ipb.ac.id

Abstract. Seahorse (Hippocampus spp.) is a species of unique marine biota showing male pregnancy. The purpose of this study was to identify seahorse species (Hippocampus spp.) through molecular approach by DNA barcoding using COI gene marker, determine the potential of seahorse as an antioxidant and an indicator of immunomodulatory properties. The research results showed the nucleotide sequences of DNA sample H1, H2, and H6 identified as H. kuda, while H3, H4, and H5 were identified as H. comes with the level of identity (homology) as much as 98%-99%. In general, the results of antioxidant activity in seahorse samples can be used as a source of natural antioxidants, namely in the form of seahorse hydrolysates and seahorse ethanol extract (IC₅₀ 50-100 ppm). The highest cell proliferation activity was from the seahorse powder group at a concentration of 250 ppm with the value of OD (Optical Density) of 0.227±0.002 and SI (Stimulant Index) of 120.74%. These results indicated that seahorses potentially have indicators of immunomodulators properties.

Keywords: antioxidant, DNA barcoding, immunomodulator, seahorse

1. Introduction

Indonesia has 9 species of seahorse, namely H. barbouri, H. bargibanti, H. comes, H. histrix, H. kelloggii, H. kuda, H. spinosissimus, H. trimaculatus, and Hippocampus sp. Nov (Lourie et al 2004). Some of these species can be sustained by aquaculture. The seahorse species which are cultured by fishermen Pulau Badi, South Sulawesi, Indonesia are H. barbouri, H. comes, and H. kuda (Dwiputra 2013). Those species are commonly utilized by the local society. The presence of seahorse would eventually be extinct if the utilization is out of control and not being watched properly.

Several regional organizations and IUCN have registered a few species into Appendix II list of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Foster and
Vincent 2004). The Chinese community and some others in Asia, including Indonesia more often use seahorses as traditional medicine or in China known as Traditional Chinese Medicine (TCM) in dry form (Chang et al 2013). Utilization of seahorses has not yet been identified with certainty and accuracy. The identification of seahorse species is important for additional information and policymakers regarding the status of the species which most commonly used.

Species identification method using DNA is the most effective and accurate method of species identification. The main purpose of this method is to identify the molecular markers of organisms using DNA markers (DNA barcodes) and can create a specific database that will be more taxonomically accurate. It will also have more valid data compared to databases in GeneBank (Teletchea 2009). Genetic markers originating from mitochondria have been extensively researched to help solve authentication problems or certainty in species identification, even to the level of populations of the same species. Some mitochondrial gene markers that are frequently used are Cytochrome oxidase I (COI), Cytochrome b (Cyt b) markers, 16S rRNA, and control region (Chang et al 2013, Panithanarak 2015).

The fast mutation rate that occurs in the COI gene can identify different species. The COI gene has been widely used for species identification, some research results show that COI contains enough variation so that it can accurately identify a variety of animals, including marine aquatic organisms and freshwater organisms (Ward et al 2005). Identification of the COI gene is expected to be able to determine the species of seahorses and further analysis can be done to see the genetic relationship with phylogenetic analysis. This phylogenetic analysis can be used to determine the genetic relationships between species from mitochondrial DNA sequencing. This study also conducted a potential analysis related to the benefits of seahorses as the immune system (immunity).

Seahorses have some potential as raw materials for natural medicines for enhancing stamina (immunomodulators) (Zhang and Lei 2008), in addition, other benefits can be used as antitumor (Huang et al 2009), antioxidants (Qian et al 2012), antibacterial (Kumaravel et al 2015), and suppress neuroinflammatory (Ryu et al 2010). It has been known that there are benefits of seahorse as a stamina enhancer (immunomodulator) based on empirical experiences. The various benefits of seahorses cannot be separated from the role of the content of the bioactive compounds. More people use seahorses as medicine, because of their belief that seahorses can be used as medicine to heal the body (Himaya et al 2012). Another potential for seahorses is as antiaging which can inhibit the pigmentation process caused by UV-B radiation (Qian et al 2012). Other than pharmaceutical benefits, seahorses are also a source of protein and have many nutrients for human consumptions.

The purpose of this study was to identify molecular seahorse species using COI gene markers, to determine the effect of seahorse (ethanol extract, powder, and hydrolysates) on antioxidant activity, and determine its immunomodulatory properties on mice lymphocyte cell proliferation activity. immunomodulatory properties.

2. Materials and methods

2.1. Tools and materials

The tools used in this study included micropipette (Thermo scientific, Vantaa-Finland), centrifuge perfect spin 24 plus (Peqlab Biotechnologie GMvH, Erlanger-Jerman), PCR Thermocycler Biometra T1 machine (Biometra GMbH, Göttingen-Germany), vertical electrophoresis TU100YK (Scie-plus Ltd, Cambridge-England), and horizontal electrophoresis, evaporators, and other glassware.

The main material used was seahorse samples from the catches of North Sumatra Sibolga fishermen, Bintan Island, and Pramuka Island. The total number of samples used was 45. The body parts used for
molecular were the meat, while for other analyzes all parts of the body except the contents of the stomach and gills. The chemicals used were DNA DNeasy Blood and Tissue kit (Qiagen Vaenlow-Netherland), DNA DNeasy Mericon Food kit (Qiagen Vaenlow-Netherland), universal primers, ethanol 96%, separating gel (12.5%), stacking gel (3%), RPMI 1640, Bovine Serum phosphate (PBS), and MTT. The software used was the MEGA 6 (Molecular Evolutionary Genetics Analysis) program, BLASTn (Basic Local Alignment Search Tool nucleotide).

2.2. DNA extraction, amplification, and electrophoresis (Chang et al 2013)
DNA extraction process aimed to destroy cells and separate DNA in the sample. The extraction process used the standard protocol Blood and Tissue kit and Mericon Food kit (Qiagen). The DNA extraction process consisted of three stages, namely destruction of the cell wall (lysis), separation of DNA from other materials, and DNA purification.

The extracted sample was then amplified. Amplification used PCR machines with the temperature condition of 94°C for 3 minutes, denaturation of 94°C 45 seconds, annealing 57.5°C for 1 minute and 45 seconds, extensions 72°C for 1 minute, post extensions 72°C for 6 minutes, storage at 15°C for 10 minutes. The process was carried out as many as 40 cycles of DNA Amplification used Universal primers.

Visualization of PCR used electrophoresis. Electrophoresis is a technique for separating charged molecules, which aims to determine whether there is an amplification of DNA or PCR products. The electrophoresis results were then observed with the help of UV light.

2.3. Sequencing and phylogenetic tree
The amplified DNA sample was then sequenced through the sequence service company, which was 1st base in Malaysia. The nucleotide base sequence was then performed using the MEGA 6 (Molecular Evolutionary Genetics Analysis) program. Species identification was carried out by BLAST, by comparing the DNA sequence database on GeneBank (http://blast.ncbi.nlm.nih.gov).

Phylogenetic tree was constructed to determine the genetic relationship and genetic distance of a species. Genetic distance is a measure of genetic differences between populations due to mutations, selection, random crosses and gene drift that will cause evolution. Sequential data for outgroup species were obtained from GeneBank NCBI.

2.4. Antioxidant activity DPPH (1,1-diphenyl-2-picrylhydrazyl) (Salazar-Aranda et al 2009)
Measurement of antioxidant activity in seahorses was carried out on 3 different sample forms namely ethanol extracts, powder, and hydrolysate seahorses taken from all parts of the body of the seahorse except the innards. The ethanol solution concentrations used were 250 ppm, 600 ppm, 950 ppm, and 1300 ppm. The comparative antioxidant used as ascorbic acid (Vitamin C) which is a synthetic antioxidant with a concentration of 1 ppm, 2 ppm, 3 ppm, 4 ppm, 5 ppm, and 6 ppm. Antioxidant activity in this study was obtained from the measurement of absorbance using a UV-Vis spectrophotometer which was then used to calculate the value of percent inhibition of antioxidant compounds against DPPH. The results of this percent inhibition test will be forwarded in the form of a linear equation to the concentration of the solvent that can be used as a basis in IC$_{50}$ calculations.

The DPPH solution used was made by dissolving DPPH crystals in 1 mM methanol solvent. Then the sample and comparison were transferred to a 100 µl microplate using a micropipette and 100 µl DPPH were added.
The mixture was incubated at 37°C for 30 minutes, then absorbance was measured using Elisa Reader. The antioxidant activity of each sample was expressed as percent inhibition, which was calculated by formula 1.

\[
\text{Antioxidant Activity (\%) = \frac{A \, \text{Blanko} - A \, \text{Sample}}{A \, \text{Blanko}}} \times 100\%
\]  

(1)

Notes:
A blanko = absorbance of the reaction results between deionized water and DPPH;
A sample = absorbance of the reaction results between samples and DPPH.

The extract concentration and inhibition values were plotted respectively on the x and y axes in the linear regression equation. The equation of the line obtained in the form of \( y = b \, (x) + a \) was used to find the value of inhibitor concentration (IC), by declaring a value of y as 50 and the value of x as the IC_{50}. IC_{50} value states the concentration of the sample solution needed to reduce DPPH by 50%.

2.5. Antioxidant activity ABTS (2'-azino-bis (3-ethylbenzothiazoline-6-Sulfonate Acid) (Thaipong et al. 2006 modified by Kusumaningtyas et al. 2015)

ABTS stock solution was made by dissolving ABTS in deionized water until the concentration reached 7.4 mM. Potassium persulfate stock solution made with a concentration of 2.6 mM. The solution to be reacted was made by mixing ABTS stock solution with potassium persulfate stock solution in a ratio of 1:1 (v/v). The oxidation reaction was carried out under dark conditions for 16-18 hours. The solution was further diluted with deionized water to obtain an absorbance of 1.1 ± 0.02 units at a wavelength of 405 nm. A total of 100 µL of the sample was mixed with 200 µL of ABTS radicals in a microplate and placed at room temperature for 10 minutes. Absorbance measurements were carried out at a wavelength of 405 nm. Antioxidant activity was calculated based on formula 2.

\[
\text{Antioxidant Activity (\%) = \frac{A \, \text{Blanko} - A \, \text{Sample}}{A \, \text{Blanko}}} \times 100\%
\]  

(2)

Notes:
A blanko = absorbance of the reaction results between deionized water and ABTS;
A sample = absorbance of the reaction results between samples and ABTS.

2.6. Lymphocyte cell proliferation activity test

The lymphocyte proliferation activity test used ethanol, powder, and protein hydrolysate extracts of sea horses were carried out in an aseptic manner. There are 4 treatment groups, namely: 3 groups of lymphocyte cell cultures each extract of seahorse, seahorse powder, and seahorse protein hydrolysate with concentrations of 250, 600, 950, 1300 ppm, and 1 group as a negative control (only lymphocyte cell culture). Each treatment was put in culture cell plates with 96 holes. Then stored in an incubator at 37°C. Every day 1 plate was taken to determine the activity of lymphocyte proliferation by observing its absorption (optical density) using a microplate reader on \( \lambda = 595 \) nm uptake, after each hole was dropped with 40µl of MTT 3- (4,5-dimethylthiazol-2-yl) absorption - after each hole was dropped with 40µl of MTT 3- (4,5-dimethylthiazol-2-yl) - 2,5 terminated methylrazolium bromide.

3. Results and discussion

3.1. Extraction and DNA electrophoresis

The results of the DNA extraction from 6 samples showed good quality (figure 1). Determination of the quality was carried out through electrophoresis using 1.2% agarose. The good DNA quality is indicated
by thick and bright DNA bands. These conditions would be suitable for further step as a template for COI gene amplification using the PCR technique.

### 3.2. DNA amplification

DNA Amplifications of seahorse specimens were carried out using the PCR method. PCR method is an enzymatic amplification process to multiply DNA (Chen and Janes 2000). PCR allows multiplying a DNA fragment quickly. In addition, PCR method could be successfully multiply using small amounts of components (Yuwono 2006). The results of amplification of DNA seahorse specimens (figure 2) show that all DNA seahorses were successfully amplified at 655 bp. This is consistent with the targeted DNA.

![Figure 1](image1.png)

**Figure 1.** DNA Electrophoregram of a seahorse on agarosa 1.2% (H1 and H2: seahorse from Sibolga-North Sumatera; H3 and H4: seahorse from Bintan Island; H5 and H6: seahorse from Pramuka Island).

![Figure 2](image2.png)

**Figure 2.** Electropherogram of PCR products on seahorse specimens (H1 and H2: seahorse from Sibolga-North Sumatera; H3 and H4: seahorse from Bintan Island; H5 and H6: seahorse from Pramuka Island).

### 3.3. Identification of species and nucleotide sequences

The amount of molecularly identified samples were 6 specimens, which two samples obtained from Sibolga North Sumatera, two samples were Bintan island, and others two samples were Pramuka island. These samples could be amplified with a base length of 655 bp (figure 3).

The results of determining the DNA nucleotide sequence in samples coded H1, H2, and H6 were identified as *H. kuda*, while H3, H4, and H5 were identified as *H. comes*. The results of molecular identification on H1, H2, H3, H4, H5, and H6 samples were identified with a level of identity (homology) of 98%-99% (table 1). The sample that will be used for further research is the *H. comes* species. The result of identification of seahorses using DNA is an accurate method for identifying species, this is confirmed by the report of Nicole *et al* (2012) that DNA-based barcoding method is a fast and accurate technique to identify crustaceans, molluscs, and fish genetically.
Table 1. BLAST analysis on species identification of seahorse.

| Samples | Species Identified | Query Cover | Identity Score | Accession number |
|---------|--------------------|-------------|----------------|-----------------|
| H1      | H. kuda            | 100%        | 99%            | AP005985.1      |
| H2      | H. kuda            | 100%        | 99%            | AP005985.1      |
| H3      | H. comes           | 100%        | 99%            | JX970973.1      |
| H4      | H. comes           | 100%        | 99%            | JX970973.1      |
| H5      | H. comes           | 100%        | 99%            | JX970973.1      |
| H6      | H. kuda            | 100%        | 98%            | AP005985.1      |

3.2. Phylogenetic tree
Construction of phylogenetic tree (figure 4) was carried out through Neighbor-joining method with bootstrap value of 1000 times. Phylogenetic tree construction was built using ingroup of genus *Hippocampus* for 38 sequences of mitochondrial DNA COI gene with an average sequence length between 655-683 bp, while outgroup was chosen from non Genus *Hippocampus*. The results of the phylogenetic tree showed two large clades, namely the first clade of 36 individuals composed of 6 individuals from this study and 30 individuals were from GeneBank and another clade consisted of *Hippocampus* obtained from GeneBank individuals downloaded from GeneBank.

One species of *Holothuria edulis* was obtained from GeneBank which was used as an outgroup species. Outgroup species used are species that have significant differences in the DNA sequence. However, there is a taxonomic correlation with the determined species. The phylogenetic tree shows that the results of the BLAST analysis conformed with the characteristics of the branch formed by the phylogenetic tree.

The length of a branch between species shows the difference in nucleotide bases and their rate of evolution. The longer branches indicated the species has a greater DNA base difference than other species. Conversely, the shorter a branch shows the difference in nucleotide bases and the less evolutionary rate.

3.4. Antioxidant activity
3.4.1. Antioxidant activity DPPH method. Antioxidant activity was demonstrated using the DPPH method. DPPH is a free radical that is stable and will be activated by allocating free electrons in a molecule. Antioxidant compounds will react with DPPH radicals through the donation of hydrogen atoms which causes DPPH color decay measured at a wavelength of 517 nm (Sanaye et al 2014). The DPPH method is a simple, easy method and uses small amounts of samples and in a short amount of time, where the results of antioxidant testing are determined by the IC50 value.

The figure illustrates the relationship between the concentration (ppm) of samples and the average % inhibition. It shows that the level of inhibition of free radical activity in the hydrolysate sample is higher than the others. Whereas the lowest inhibitory level is shown in seahorse powder samples. This is because that sea horse hydrolysate sample has a higher protein content compared to other samples. In accordance with the statement of Belleville-Nabet (1996) that one of the natural food compounds that have good free radical inhibitory activity is a protein compound, besides that, there is also the presence of flavonoid compounds in a material.

The relationship between concentration (ppm) and % inhibition in both powder and extract samples shows the value of R2 obtained from the curve above 0.96. This value indicates that there is a linear relationship of 96% between concentration and % inhibition. This value is greater than the R2 value of ascorbic acid and hydrolysate which shows a relationship of ±80% between the concentration and the average percent immersion (% inhibition). Based on the simple linear regression equation obtained, the
IC$_{50}$ value can be calculated by determining the concentration of the test sample which causes a reduction percentage of 50%. The results of the calculation of antioxidant activity can be seen from the IC$_{50}$ values presented in Table 2.

| Sample Form | IC$_{50}$ Value |
|-------------|-----------------|
| H1          | 120             |
| H2          | 118             |
| H3          | 115             |
| H4          | 113             |
| H5          | 112             |
| H6          | 110             |

Figure 3. Aligned nucleotide sequences on amplified seahorse DNA using COI gene fragment (H1 and H2: seahorse from Sibolga-North Sumatera; H3 and H4: seahorse from Bintan Island; H5 and H6: seahorse from Pramuka Island).

Based on the results of the data normality test according to the Kolmogorov-Smirnov Test shows the data were normally distributed and homogeneous so that the data can be further tested using ANOVA analysis (Analysis of Variance). ANOVA test results showed that the sample form factor significantly affected IC$_{50}$ values, so it could be continued with further tests using the Tukey test with a 95% confidence interval. This further test aimed to see the effect of IC$_{50}$ values on several sample forms.
Based on Tukey's further tests it was found that the IC$_{50}$ value of the powder form was significantly different (P < 0.05) from the other sample forms (extract, hydrolysate, and ascorbic acid), while the extract, hydrolysate and ascorbic acid forms were not significantly different (P < 0.05).

Antioxidant activity test results showed that sea horse powder showed inactive antioxidant strength with IC$_{50}$ values 1378.89±11.82 ppm. The highest strength of antioxidant activity in this study was very active which was shown in ascorbic acid, whereas in extract and hydrolysate samples showed the strength of active antioxidant activity. The highest antioxidant activity in this study was demonstrated in sea horse hydrolysate samples with an IC$_{50}$ value of 57.08±29.08 ppm. This value indicates that the smaller the IC$_{50}$ value, the higher the antioxidant activity. In accordance with Molyneux (2004), which states that the smaller the IC$_{50}$ value means the higher antioxidant activity. Several categories of antioxidant power are described by Jun et al. (2003) which states that IC$_{50}$ values $< 50$ ppm indicate very active antioxidant strength, IC$_{50}$ values 50-100 ppm indicate active antioxidant strength, IC$_{50}$ values 101-250 ppm indicate moderate antioxidant strength, IC$_{50}$ values 250-500 ppm indicate weak antioxidant strengths, and IC$_{50}$ values $> 500$ ppm indicate the antioxidant power of inactivity.

**Figure 4.** Phylogenetic tree construction on Hippocampus.
Table 2. Antioxidant activity by DPPH method.

| Sample forms | IC\(_{50}\) (ppm) | Antioxidant | Jun et al (2003) |
|--------------|-------------------|-------------|-----------------|
| Extracts     | 66.46±14.94\(^b\) | Active      | IC\(_{50}\) 50-100 ppm |
| Powder       | 1378.89±11.82\(^a\) | Inactive    | IC\(_{50}\) >500 ppm |
| Hydrolysate  | 57.08±29.08\(^b\)  | Active      | IC\(_{50}\) 50-100 ppm |
| Vitamin C    | 1.22±0.24\(^b\)    | Inactive    | IC\(_{50}\) <50 ppm |

Note: values with different superscripts show significant differences (Tukey P further test results <0.05).

In general, the results showed that seahorse has the antioxidant activity. Based on IC\(_{50}\) values showed that the best antioxidant activity was in the form of seahorse hydrolysate and seahorse ethanol extract, although the highest activity was shown in ascorbic acid. According to Amaliawati (2015) reported that ascorbic acid was classified as a very active antioxidant with an IC\(_{50}\) value of less than 10 which is 4,316 ppm. This is because ascorbic acid is easily oxidized by donating its hydrogen atom and forming a relatively stable ascorbil free radical.

3.4.2. Antioxidant activity ABTS method. Antioxidant tests on extracts, powder, and hydrolysate of seahorses were also carried out using the ABTS method. This method uses free radicals 2,2'-azino-bis (3-ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) produced when the ABTS substrate is oxidized with potassium persulfate. The results of antioxidant testing with the ABTS method for extracts, powder, and hydrolysate of sea horses can be seen in table 3.

Table 3. Test results of antioxidant activity by the ABTS method.

| Sample forms | IC\(_{50}\) (ppm) | Antioxidant | IC\(_{50}\) value |
|--------------|-------------------|-------------|------------------|
| Extracts     | 15143.529±862.89\(^b\) | Inactive    |                  |
| Powder       | 16802.791±708.13\(^b\)  | Inactive    |                  |
| Hydrolysate  | 15801.724±874.75\(^b\)  | Inactive    |                  |
| Vitamin C    | 10.93±0.00\(^a\)         | Very Active |                  |

Note: values with different superscripts show significant differences (Tukey P further test results <0.05).

Based on the results of the data normality test according to the Kolmogorov-Smirnov Test shows the data are normally distributed and homogeneous so that the data can be further tested using ANOVA analysis (Analysis of Variance). ANOVA test results showed that the sample form factor significantly affected IC\(_{50}\) values, so it could be continued with further tests using the Tukey test with a 95% confidence interval. This further test aims to see the effect of IC\(_{50}\) values on several sample forms. Based on Tukey's further tests it was found that the IC\(_{50}\) value of ascorbic acid samples was significantly different (P<0.05) from other sample forms (extracts, hydrolysate, and powder), whereas between the extract, hydrolysate, and powder forms were not significantly different (P>0.05).

The results of extract, powder, and sea horse hydrolysate have antioxidant activity that is classified as very weak because it has an IC50 value greater than 0.20 mg/mL. Molyneux (2004) states that a substance has antioxidant properties when the IC50 value is less than 0.20 mg/mL. If the IC50 value obtained ranges from 200-2000 ppm, then the substance is less active but still has potential as an antioxidant.

3.5. Mice lymphocyte cell proliferation activity

The results of testing the mice lymphocyte cell proliferation activity in all treatments can be seen in table 4. The average value of the mice lymphocyte cell proliferation activity is expressed as a form of absorption (optical density / 595 nm) and stimulant index (IS).
Table 4. Mean activity of mouse lymphocyte proliferation activity as indicated by optical density (OD) value and stimulant index from various treatments of seahorse samples.

| Concentration (ppm) | Extracts OD | Powder OD | Hydrolysate OD | Control Cell OD |
|---------------------|-------------|-----------|----------------|----------------|
|                     | IS(%)       | IS(%)     | IS(%)          | IS(%)          |
| 250                 | 0.213±0.010a| 0.227±0.002a| 0.225±0.002a  | 113.30         |
| 600                 | 0.196±0.003b| 0.178±0.011b| 0.179±0.008b  | 94.68          |
| 950                 | 0.198±0.004b| 0.167±0.012b| 0.184±0.005b  | 88.65          |
| 1300                | 0.199±0.014b| 0.168±0.008b| 0.219±0.030a  | 89.36          |

Note OD (optical density); IS (Stimulant Index); values with different superscripts show marked differences (Duncan’s further test results P<0.05).

The results of the lymphocyte cell proliferation activity test showed that the average optical density (OD) and stimulant index (IS) values for the groups treated with seahorse extract, seahorse hydrolysate, and seahorse powder were higher than those in the control group, especially at a concentration of 250 ppm. The highest cell proliferation activity was shown in groups with 250 ppm seahorse powder concentrations with OD values 0.227±0.002 and IS 120.74% significantly different (P<0.05) with other concentrations. The higher the value of the stimulant index, the more the number of lymphocytes active in stimulation. According to Rohrer et al. (1999) the greater the number of active lymphocytes the greater the number of active ingredients in the stimulation process.

The results of this study can illustrate that seahorse extract, powder, and sea horse hydrolysate has potential as an indicator of immunomodulators in vitro as shown in increasing the activity of mice lymphocyte cell proliferation. This increase is reflected in the OD and IS values in the protein and extraction group.

4. Conclusion

The conclusions of the study are: a) DNA nucleotide sequences of H1, H2, and H6 samples were identified as *H. kuda*, while H3, H4, and H5 were identified as *H. comes* with an identity level of 98%-99%. b) In general, the results of antioxidant activity in seahorse samples can be used as a source of natural antioxidants. Based on IC50 values show that the best antioxidant activity both with DPPH and ABTS methods is in the form of sea horse hydrolysate and seahorse ethanol extract (IC50 50-100 ppm), but the highest activity is shown in ascorbic acid (vitamin C) as a positive control.c) Lymphocyte cell proliferation activity is shown by the average optical density (OD) and stimulant index (IS), where the treated sample has higher activity compared to the control group. The highest activity was the seahorse powder group at a concentration of 250 ppm with OD (Optical Density) value 0.227±0.002 and IS (Stimulant Index) 120.74%. These results indicate that seahorses have the potential to be an indicator of the nature of the immunomodulator.

Acknowledgment

This research was funded by the Ministry of Research, Technology and Higher Education of Indonesia through the Penelitian Unggulan Perguruan Tinggi (PUPT) research grant.

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