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In Vitro and In Silico Evaluation of Antioxidant Activity of Muscle and Endoskeleton from *Sepia officinalis*

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ABSTRACT

Sepia officinalis, commonly known as cuttlefish, is one of the diverse marine organisms consumed for its high protein content. Amino acids, as the building blocks of proteins, can function as antioxidant compounds. The high amino acid content in cuttlefish makes it a potential natural source of antioxidants. However, the use of cuttlefish in medicine, particularly as an antioxidant source, remains limited in Indonesia, where it is primarily utilized as a dietary protein source. This study aimed to evaluate the antioxidant activity of muscle and endoskeleton extracts from *S. officinalis* using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay in vitro, and to analyze their interaction with myeloperoxidase, an oxidant-producing enzyme, through an in silico approach. The results demonstrated that both muscle and endoskeleton extracts were capable of scavenging free radicals, showing DPPH inhibition ranging from 46.19% to 48.42%. The highest inhibition was observed at a concentration of 200 ppm for both extracts. Furthermore, in silico analysis revealed that tyrosine and phenylalanine were the key amino acids with antioxidant potential, as they inhibited myeloperoxidase activity and could reduce oxidant production by the enzyme. These novelty the first combined in vitro and in silico evidence of the antioxidant potential of *S. officinalis* muscle and endoskeleton extracts, representing a novel contribution to the exploration of marine-derived bioactive compounds in Indonesia. The study not only demonstrates the biochemical potential of cuttlefish as a natural antioxidant source but also supports its future application as a functional food ingredient and a prospective natural antioxidant for pharmaceutical development.

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INTRODUCTION

Free radicals are an unstable molecule due to the existence of unpaired electrons at their valency position in the constituent atoms of the molecule (Wang *et al*., 2020). This molecule can be produced either from organic or inorganic reaction and can be sourced from internal and external organisms. Free radicals might interrupt the other stable molecules during metabolism process to stabilize their electron. Therefore, it may cause damage to the structure and function of molecules, cells, and organs. The occurred damage can cause various type of diseases such as aging, cancer, and other degenerative disease (Halliwell, 2020). However, in the cell metabolism reactions, free radicals are required to homeostasis process, cellular signal regulation, and apoptosis (Harris & DeNicola, 2020). In addition, the presence of free radical inside the cell can be one of indicator of the infection manifestation due to pathogens, because at the time being infected, cell will produce free radical as a defensive means against the pathogen (Wu, 2020). In the microbicides immune systems, there are oxidoreductase enzymes, namely myeloperoxidase (MPO) which catalyzes the biosynthesis process of the oxidant product (HOCl) for phagocytosis pathogens. Even though it is important for human immune system, these oxidant products can interact with lipids, proteins, and nucleic acids which can be detrimental for cellular metabolism. For example, *low density lipoprotein* (LDL) which interacts with MPO products cannot be recognized by LDL receptors dan can trigger the emergence of metabolic disorders (Frangie & Daher, 2022). Therefore, it is important to maintain the free radical level in sufficient amount in accordance with the metabolism needs.

Healthy lifestyle by consuming balanced nutritious food is a one way to suppress the increase free radical level that can prevent the degenerative diseases emergence. This is supported by the high diversity of food source in Indonesia, more preferably food source from the sea (Nikawanti & Aca, 2021). One of the sea food sources, commonly consumed by the Indonesian people is cuttlefish (*Sepia officinalis).* This animal belongs to Cephalopod group that can be found in almost Indonesian ocean (Muchlisin *et al*., 2014). In the general anatomy of *Sepia sp.* consists of head, mantle, and endoskeleton. The head and mantle are the most commonly consumed parts. Both parts typically contain 78.5% protein, 16.2% fat, and 1.8% carbohydrates and the mineral content such as Ca, P, Mg, Fe, and Mn. The other compounds such as oxalate, phytate and trypsin inhibitors, also can be found (Lawal-Are *et al*., 2018; Sykes *et al*., 2009). In the endoskeleton, there are chitin and minerals in the form of Ca, Mg, Na, Br, K, Fe, and Sr (Florek *et al*., 2009). Cuttlefish ink is a metabolite produced from the metabolism of the organism which plays an important role not only in food but also in medicine. The main content of the ink is melanin and the other compounds are amino acids (Derby, 2014; Xie *et al*., 2021).

Based on previous studies, the hydrolysate of *S. officinalis* mantle protein has antioxidant activity. This is shown by the ability to inhibited free radical of 2,2-diphenyl-1-picryhydrazyl (DPPH) and lipid peroxidation (Balti *et al*., 2011). Moreover, pepsin extract from Sepia *sp.* mantle has antioxidant activity as measured by various antioxidant assays (Siahpoosh & Alikhani, 2016). Other studies have reported that *S. officinalis* ink showing potential as an antioxidant and anti-inflammatory agent that can reduced the gastric mucosal damage as well as reduced the gastric nitrate oxide levels in mice with ulcer stomach induced by ethanol (Sadek, 2022).

There have not been many scientific studies in medicine that utilizes *S. officinalis* in Indonesia since the utilization of cuttlefish is only known as a food source. Therefore, the objective of this study is to examine the antioxidant activity of *S. officinalis* especially from muscles and endoskeleton, based on their ability to scavenge free radical *in vitro. In silico* antioxidant studies was also conducted as a further approach to their antioxidant activities. The results of this study are expected to disclosed the medical benefits of *S. officinalis* especially in the development of functional food as natural antioxidant source based on marine biota.

MATERIALS AND METHOD

Materials. A total of 250 g *Sepia officinalis* obtained from the local fish market Sepatan, Tangerang, Banten, Indonesia (6° 10′ 17″ S, 106° 38′ 26″ E), transported to laboratory in frozen condition, and stored (-20°C) for one week. Identification of the taxonomy on the samples were conducted at Ecology Laboratory, Syarif Hidayatullah State Islamic University Laboratory Center. The chemical materials used in this study were absolute grade for analysis.

Methods.

Extraction. Extraction was carried out after the muscle and endoskeleton samples were separated, cleaned, and air-dried. A total of 32 g of refined muscle and endoskeleton samples were macerated in 150 mL of 90% ethanol (Merck, Germany) for 72 hours. The obtained filtrate was then evaporated at 60 °C using a rotary evaporator (Büchi R-100), yielding muscle extract (MSO) and endoskeleton extract (ESO). The extract yield was calculated based on the ratio of the extract weight to the initial sample weight.

Antioxidant activity assay. The DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich, USA) was used as the free radical (Alfarabi *et al*., 2022a). A total of 1 mL of samples (10, 50, 100, 150, and 200 ppm) were mixed with 1 mL of DPPH (0.1 mM). After incubation for 30 min at room temperature, the absorbance was measured at 517 nm (DLab SP-UV1100). In this assay, DPPH solution without sample was used as a negative control and ascorbic acid solution (Sigma-Aldrich, USA) was used as a positive control. The free radical reaction inhibition of DPPH from the samples and the positive control was expressed as a percentage inhibition and calculated based on the ratio of the difference in absorbance between the negative control and the sample to the absorbance of the negative control. The lower the absorbance value, the higher the percentage of free radical inhibition of the sample. In this assay, IC_{50} values were calculated from the percentage inhibition versus sample concentrations.

Docking analysis. The antioxidant activities of MSO and ESO were analyzed by docking was conducted with AutoDock Vina in PyRx software, then the results were evaluated and visualized using Discovery Studio Visualizer (BIOVIA Discovery Studio Visualizer V21.1.0.20298). The purpose of this study was to examine the antioxidant activity. Myeloperoxidase protein was used as a receptor in this analysis. The 3D crystal structure of the protein (1DNU) was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (https://www.rcsb.org). The ligands used for this analysis is metabolites that generally contained in *Sepia sp.* (muscle, endoskeleton, and ink) and obtained based on the literature study **(Table 1)**. The 3D structure of the ligands was sourced from the PubChem chemical database (https://pubchem.ncbi.nlm.nih.gov/). The ligands were evaluated by Physicochemical ADME analysis and evaluated drug-likeness parameters using the free web tool SwissADME (Absorption, Distribution, Metabolism, and Excretion) (http://www.swissadme.ch/) (Abdel-Malek *et al*., 2024). Receptor preparations such as removal of water molecules and ligands that are not required for docking were analyzed using PyMOL 2.5.4 software. The ligand molecules were subjected to energy minimization using a Merck Molecular Forcefield (MMFF94) to prevent interference during the docking simulation. This process was executed through Open Babel in PyRx software (PyRx-Phyton Prescription 0.8) (Kudatarkar *et al*., 2021). The gridbox position is in the center of the selected amino acid residues (Gly90, Gln91, Asp94, His95, Asp98, Phe99, Thr238, Arg239, Glu242, and Met243) with coordinates X: 23.7230, Y: -16.677, and Z: -4.3486 **(Figure 1)**. This configuration obtained based on literature studies and predictions using PrankWeb: Ligand Binding Site Prediction web service (https://prankweb.cz/) (Blair-Johnson *et al*., 2001; Jakubec *et al*., 2022).

Table 1. Metabolites in *Sepia officinalis* (muscle, endoskeleton, and ink).

Metabolites	Source
Glucuronic Acid Iduronic Acid Galacturonic acid Mannose Xylose Rhamnose	Muscle (Jridi *et al*., 2019)
Octopus	Muscle (Storey & Storey, 1979)
Chitin Aragonite	Endoskeleton (Florek *et al*., 2009)
Arabinose Rhamnose Xylose Mannose	Endoskeleton (Okutani & Morikawa, 1978)

Galactose Glucosamine Galactosamine	
Eumelanin	Ink (Derby, 2014)
Eumelanin Aspartic acid Threonine Serine Glutamic acid Glycine Alanine Cysteine Valine Methionine Isoleucine Leucine Tyrosine Phenylalanine Lysine Histidine Arginine Proline	Ink (Xie *et al*., 2021)

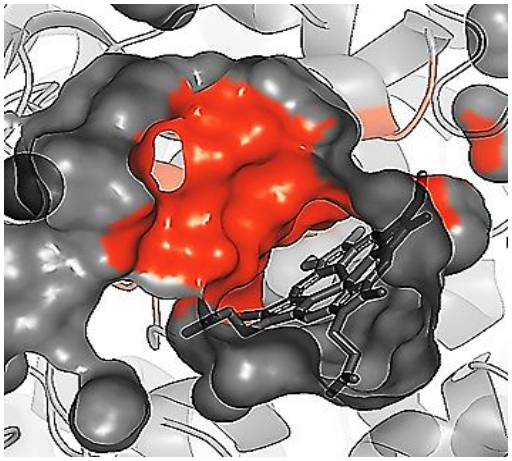

Figure 1. The Grid box position (red surface) in the protein structure (1DNU).

RESULTS AND DISCUSSION

MSO and ESO Yields

The yield obtained from both extracts had different values. MSO had yield of 6.26% and ESO of 0.78% **(Table 2)**. The difference in the resulting yield might have occurred because the source objects of the extracted samples were two different organs of *S. officinalis*. Hence, there are some differences not only in the content, but also in the metabolite types in both organs. Muscle from the cuttlefish contains protein and carbohydrates, meanwhile the endoskeleton contains a large number of chitins, minerals, and some carbohydrate compounds (Florek *et al*., 2009; Sykes *et al*., 2009; Lawal-Are *et al*., 2018; Jridi *et al*., 2019; Okutani & Morikawa, 1978; Storey & Storey, 1979). In addition, differences in the yield values might occur because of the extraction methods and solvents are used during the extraction. The more appropriate method and solvents used to separate the target metabolites of the sample, the more metabolites that can be extracted. Therefore, it is necessary to determine the physical and chemical characteristics of the target metabolites, such as polarity properties and the effect of temperature on metabolites (Bachtler & Bart, 2021;

Dong *et al*., 2021). The extraction method used in this study was maceration which is a simple conventional extraction method and is not specific to certain metabolites. This method conducted by soaking the samples with solvent in certain time (Alfarabi et al., 2022b).

Table 2. The yields from MSO and ESO.

Sample	Yield (%)
MSO	6.26
ESO	0.78

Antioxidant Activity

The analysis results showed that both samples have the ability to inhibit free radicals. The inhibition was proportional with the increase of concentration except for MSO **(Figure 2)**. Both extracts have antioxidant activities as free radical scavengers. The highest DPPH inhibition of ESO and MSO occurred at concentration of 200 ppm by 48.42% and 47.79%, respectively. The DPPH inhibition range of ESO was 46.19-48.42%, while the MSO in the range of 47.02-47.79%. The results showed that the samples inhibition does not reach 50%. The highest inhibition of ESO at 200 ppm was significantly different from that at low concentrations. In contrast, in MSO, inhibition at a concentration of 100 ppm was statistically different from the other concentrations. The antioxidant activities from the sample were lower than ascorbic acid as the positive control. From the analysis result, ascorbic acid has the IC_{50} value of 1 ppm.

DPPH is a free radical stable at room temperature. The purple color of the molecule exhibited suitable absorption at 517 nm. These antioxidant molecules, which reacted with DPPH, changed color to yellow. The possible reactions that might occur under these conditions are that antioxidant molecules give their electrons directly to the DPPH molecules, resulting in antioxidant molecules becoming a new free radical because they lose their electrons. Another possible reaction is shared electron by both molecule (Blois, 1958). Therefore, the measurement of antioxidant activities using DPPH is a suitable method for an initial study to determine the antioxidant activities from natural products. It is known that *S. officinalis* organ extracts have antioxidant activity (Balti *et al*., 2011; Siahpoosh & Alikhani, 2016; Sadek, 2022). This is due to the high protein content in the organ which play roles as a free radical scavenger. This activity is supported by the amino acids constituting the protein. Amino acids that contain sulfur, such as methionine and cysteine, have good antioxidant activity as radical superoxide molecule scavengers. In addition, the chain side from amino acid residues also affected antioxidant activities against reactive oxygen species (ROS) and reactive nitrogen species (RNS) so that proteins or amino acids can be very reactive to reacted with free radical molecules (Elias *et al*., 2008; Kim *et al*., 2017; Matsui *et al*., 2018).

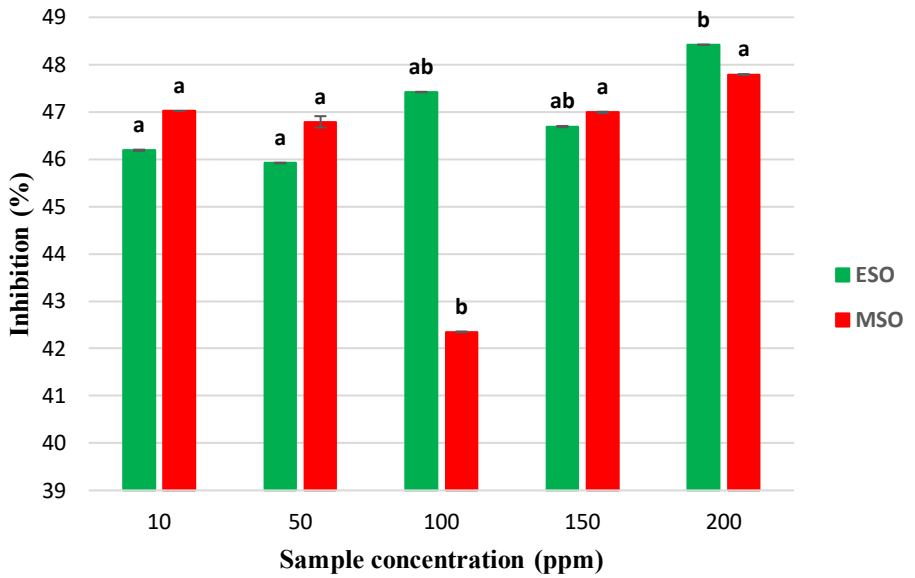

Figure 2. Antioxidant activity of *Sepia officinalis* (muscle and endoskeleton) extract. ESO: endoskeleton extract; MSO: muscle extract. The results of triplicate experiments presented with standard deviation. Different letter notations imply significant differences ($p < 0.05$).

Docking Analysis

There are 31 ligands which are metabolites from some *S. officinalis* organ based on literature. The results of ADME and drug-likeness physicochemical analysis showed that the ligand has good potential become drug molecule with varied gastrointestinal absorption parameters. However, eumelanin, which is the main ink compiler molecule in *S. officinalis*, does not meet the parameters of drug molecule candidates **(Table 3)**.

Table 3. The results of physicochemical ADME and drug-likeness analysis of *Sepia officinalis* metabolites.

Metabolites	Physicochemical					Pharmacokinetics		Drug-likeness	
	Mol. Wt. (g/mol)	Rotatable bonds	H-bond acceptors	H-bond donors	Molar Refractivity	Lipophilicity (Log *P*)	GI absorption	BBB permeant	Lipinski's violation	Bioavailability score
Glucuronic Acid	194.14	1	7	5	36.35	-2.45	low	no	0	0.56
Iduronic Acid	194.14	1	7	5	36.35	-2.57	low	no	0	0.56
Galacturonic acid	194.14	1	7	5	36.35	-2.12	low	no	0	0.56
Mannose	180.16	1	6	5	35.74	-2.24	low	no	0	0.55
Xylose	150.13	0	5	4	29.77	-2.00	low	no	0	0.55
Rhamnose	164.16	0	5	4	34.57	-1.36	high	no	0	0.55
Octopus	246.26	8	6	5	60.82	-1.88	low	no	0	0.55
Chitin	221.21	3	6	5	47.19	-1.78	low	no	0	0.55
Aragonite	100.09	0	3	0	6.77	-0.92	low	no	0	0.55
Arabinose	150.13	0	5	4	29.77	-1.80	low	no	0	0.55
Galactose	180.16	1	6	5	35.74	-2.16	low	no	0	0.55
Glucosamine	179.17	1	6	5	37.28	-2.54	low	no	0	0.55
Galactosamine	179.17	1	6	5	37.28	-2.32	low	no	0	0.55
Eumelanin*	544.6	8	11	7	138.38	-1.90	low	no	3	0.11
Aspartic acid	133.10	3	5	3	27.59	-2.04	high	no	0	0.56
Threonine	119.12	2	4	3	26.98	-1.73	high	no	0	0.55
Serine	105.09	2	4	3	22.18	-1.97	high	no	0	0.55
Glutamic acid	147.13	4	5	3	32.40	-1.68	high	no	0	0.56
Glycine	75.07	1	3	2	16.21	-1.69	high	no	0	0.55
Alanine	89.09	1	3	2	21.01	-1.46	high	no	0	0.55
Cysteine	121.16	2	3	2	28.94	-1.31	high	no	0	0.55
Valine	117.15	2	3	2	30.63	-0.78	high	no	0	0.55
Methionine	149.21	4	3	2	38.22	-0.59	high	no	0	0.55
Isoleucine	131.17	3	3	2	35.44	-0.39	high	no	0	0.55
Leucine	131.17	3	3	2	35.44	-0.38	high	no	0	0.55
Tyrosine	181.19	3	4	3	47.52	-0.48	high	no	0	0.55
Phenylalanine	165.19	3	3	2	45.50	-0.01	high	no	0	0.55
Lysine	146.19	5	4	3	38.14	-1.19	high	no	0	0.55
Histidine	155.15	3	4	3	37.65	-1.54	high	no	0	0.55
Arginine	174.20	5	4	4	44.54	-2.04	low	no	0	0.55
Proline	115.13	1	3	2	32.52	-0.92	high	no	0	0.55

* Does not comply with Lipinski's rule; GI, gastrointestinal absorption; BBB, blood–brain barrier permeation

These analyses are the based on Lipinski's rules, large molecular weighted molecules will reduce the permeability of molecules in the intestine and into the central nervous system (< 500 Da), the higher log *P* value will reduce lipophilic properties (log *P* < 5), the more H-bond donors will reduce lipophilic properties (H-bond donors < 5), the more H-bond acceptors will reduce the lipophilic properties (H-bond acceptors < 10), and the molar refractivity (polarity of a molecule) value should be in the range of 40-130 (Pollastry, 2010).

The docking results showed that each ligand has varied binding energy from -3.7 to -6.2 kcal/mol. The interactions that occur between each ligand and receptor are H-bond, hydrophobic, and van der Waals bond. Since myeloperoxidase is a heme-containing enzyme, thus the interactions with heme occurred in this analysis. Thr100 and Glu102 were amino acid residues that have the most H-bond interacting with the ligand. While Phe99 was residue that has the most van der Waals interactions with the ligand. Phenylalanine and tyrosine were ligands that have binding energy below -6.0 kcal/mol. These ligands showed the strongest interactions with the receptor. Eight amino acid residues of the receptor interacted with tyrosine and phenylalanine. Meanwhile, in the interaction with heme, H-bond interactions occurred with tyrosine and Pi-Pi stacking bond interactions occurred with phenylalanine **(Figure 3 and Table 4)**.

The strength and weakness of the bond between the ligand and receptor can be influenced by the type of interaction. H-bond interaction has a stronger interaction than hydrophobic and van der Waals interactions. These interactions play an important role in biochemical reactions such as enzyme catalysis (Bulusu & Desiraju, 2019). Hydrophobic interactions occur between non-polar molecules in polar solvents. In proteins, these interactions occur between the chain sides of non-polar amino acid residues to stabilize the protein structure (Bogunia & Makowski, 2020). Van der Waals interactions can occur because of the transient dipole moment of atoms or molecules, which causes weak interactions between molecules with opposite transient charges. The strength of this interaction depends on the distance between the molecules, the longer the distance between the molecules, the weaker the interaction (Singh, 2016). Based on *in silico* studies, tyrosine and phenylalanine are the most potential ligands to become an inhibitor of myeloperoxidase. These amino acids can be used as antioxidants in biological systems.

MPO is a heme peroxidase-cyclooxygenase enzyme and its capable of oxidizing halide and pseudohalide ions (Cl(-), Br(-), I(-), and SCN(-)) with H_2O_2 to produce hypohalous acids (HOX) (Lazarevic-Pasti *et al*., 2015). This enzyme is found in neutrophil granules and plays a role in human atherogenesis, thus correlating with predicting cardiovascular disease events. MPO was predicted to be a major factor in inflammatory disorders, oxidative stress, and vascular dysfunction (Kim *et al*., 2022). Therefore, compounds that can inhibit MPO could be useful for antioxidant, anti-inflammatory and cardiovascular disease drug. *In silico* studies of inhibitor compounds for this enzyme, such as chalcones and their analogs, have shown good inhibition, making them promising candidates for new non-steroidal anti-inflammatory drugs (Dos Santos *et al*., 2021). The binding energy of ligand to enzyme in this study was weaker than amide group (−7.79 kcal/mol) and salicylhydroxamic acid as a positive control (−7.88 kcal/mol). However, the ligand and enzyme interaction were occurred at the same amino acid residues (Glu102, Glu242, Arg333, and Arg424) (Rajan *et al*., 2024). These results indicate that these amino acid residues are binding site inhibitors for MPO.

The findings of our study, which was conducted *in vitro* and *in silico*, indicate that the amino acid content of cuttlefish muscle and endoskeleton can function as a natural source of antioxidants. Moreover, these results can provide as a scientific foundation for the development of cuttlefish as a functional food with antioxidant benefits. However, further analysis is necessary to achieve this objective.

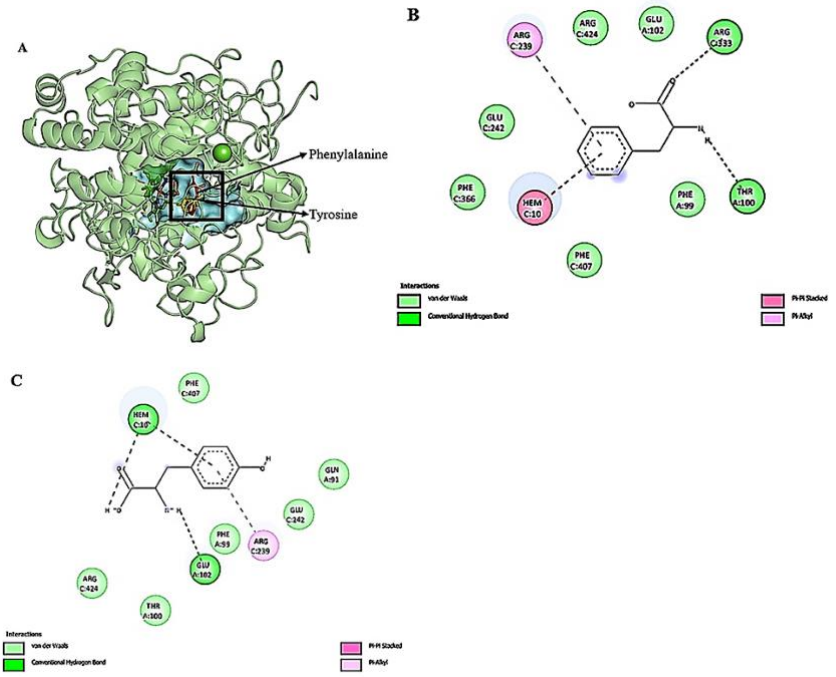

Figure 3. Ligands and proteins (1DNU) interactions. (A) Ligands position in the protein structure, (B) phenylalanine 2D interactions, (C) tyrosine 2D interactions.

Table 4. Docking analysis results.

Parameters	Ligand	
	Tyrosine	**Phenylalanine**
Binding Energy (kcal/mol)	-6.2	-6.1
H-bond interacting residue	Glu102	Thr100, Arg333
Hydrophobic bond interacting residues	Arg239	Arg239
van der Waals bond interacting residues	Gln91, Phe99, Thr100, Glu242, Phe407, Arg424	Phe99, Glu102, Glu242, Phe366, Phe407
Heme interacting	H-bond	Pi-Pi stacking bond

These novelty study the first combined in vitro and in silico evidence of the antioxidant potential of *S. officinalis* muscle and endoskeleton extracts, representing a novel contribution to the exploration of marine-derived bioactive compounds in Indonesia. The study not only demonstrates the biochemical potential of cuttlefish as a natural antioxidant source but also supports its future application as a functional food ingredient and a prospective natural antioxidant for pharmaceutical development.

CONCLUSION

Muscle and endoskeleton of *Sepia officinalis* extracts have the antioxidant activities with ability as a free radical scavenger. This can be attributed to the high protein content of cuttlefish, which produces the amino acids that make up the protein and acts as an antioxidant agent. Based on *in silico* studies, tyrosine and phenylalanine are amino acids that have potential as antioxidant by inhibiting the myeloperoxidase thus can reducing the oxidant product of the enzyme. However, antioxidants *in vitro* with different methods and *in vivo* from the muscles and endoskeleton extracts are needed for further study, especially related to the bioactivity of the total extract and hydrolysate of *S. officinalis* muscle protein.

AUTHORS CONTRIBUTION

M. Alfarabi and K. Daniella designed and conducted the study, analyzed and interpreted the data, and wrote a draft of the manuscript. R.A. Ismail and F. Fahrudin analyzed and interpreted the data. M. Alfarabi & J.M. Cing reviewed the draft manuscript, and supervised the entire process.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest, and will take full responsibility for the content of the article, including implications of AI-generated art.

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