Vol 10, Special Issue 1, 2018

INTERNATIONAL JOURNAL OF APPLIED PHARMACEUTICS

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

ISSN - 0975-7058

Research Article

ANTIOXIDANT ACTIVITY OF THE ASCIDIAN MARINE INVERTEBRATES, *DIDEMNUM* SP.

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Received: June 2018, Revised and Accepted: October 2018

ABSTRACT

Objective: This study aimed to determine the antioxidant activity of samples of the ascidian *Didemnum* sp. collected from Seribu Islands, Jakarta.

Methods: Antioxidant activity was tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. *Didemnum* sp. was extracted into methanol and then fractionated using *n*-hexane, ethyl acetate, and water. Fractions with the highest antioxidant activity were further fractionated using accelerated column chromatography.

Results: The concentration of sample that reduced the DPPH radical by 50% (IC_{50}) in a methanol extract of *Didemnum* sp. was 105.10 µg/mL. The ethyl acetate fraction had the highest antioxidant activity (IC_{50} of 90.804 µg/mL). The most active fraction obtained from accelerated column chromatography fraction had an IC_{50} of 86.35 µg/mL. The compounds contained in the most active fractions were alkaloids, saponins, steroids/triterpenoids, and glycosides.

Conclusion: The methanol extract of the ascidian *Didemnum* sp. exhibited antioxidant activity. Fractionation of the *Didemnum* sp. extract showed that the ethyl acetate fraction had the highest antioxidant activity. Further, fractionation of the ethyl acetate fraction by accelerated column chromatography showed that fraction VI had the highest antioxidant activity. The most active fraction contained alkaloids, steroids/triterpenoids, saponins, and glycosides.

Keywords: 1,1-Diphenyl-2-picrylhydrazyl, Ascidiacea, *Didemnum* sp., Seribu Islands.

 DOI: http://dx.doi.org/10.22159/ijap.2018.v10s1.17

INTRODUCTION

Indonesia has a highly diverse marine biota system. This diversity provides an opportunity to utilize marine biota as a source of natural medicines. However, the utilization of this potential has not been optimal. According to some studies, marine organisms are a source of new potential drug compounds. Collaborations between researchers from various institutions with pharmacologists have led to major advances in the discovery of medicines from marine biota. As an example, >10,000 bioactive compounds have been successfully isolated from marine biota and approximately 300 patents of these compounds were successfully published from 1969 to 1999 [1,2].

Some experts have reported the discovery of various compounds with certain activities such as antimicrobial, antitumor, anti-inflammatory, antiviral, antitoxin, anticancer, anticoagulant, antifungal, immunostimulant, and antioxidant activities, from marine biota, including algae, corals, sponges, and tunicates. Examples include bryostatin-1 (polypeptide isolated from bryozoa *Bugula neritina*), which has anticancer and immunostimulant efficacies; dolastatin-10 (polypeptide isolated from *Dolabella auricularia* mollusk), which is an antitumor agent; and detachable B isolated from the tunicate *Trididemnum solidum*, which shows antiviral, antitumor, and activities in addition to antioxidant activity of Phyrophophytin, found in *Eisenia bicyclis* [3,4].

Recently, the use of antioxidant compounds has developed rapidly for both food and medicine. Drug use is growing as the knowledge of free radical activity increases against some degenerative diseases such as heart disease and cancer [5]. Free radicals are relatively unstable molecules and have unpaired electrons in their outer orbitals, so they search for electron pairs and are able to react with proteins, lipids, or DNA. Reactions between free radicals and those molecules may cause disease. The reactivity of these free radicals can be reduced by antioxidant compounds [6].

Antioxidants are compounds that are capable of inhibiting the oxidation of other molecules. These antioxidant compounds will deliver one or more electrons to free radicals, which can stop the damage they cause. In the body, there is an endogenic antioxidant mechanism, but if the amount of free radicals in the body is excessive, external (exogenic) antioxidants are required [7]. There are two classes of antioxidants characterized by the source: Natural antioxidants and artificial/synthetic antioxidants. Synthetic antioxidants might have harmful side effects for human health. The concerns about possible side effects of synthetic antioxidants have increased interest in natural antioxidants as potentially better alternatives [8,9].

One of the potential marine resources of natural antioxidants is the ascidian *Didemnum sp*. These organisms belong to the Subphylum: Tunicata, Class: Ascidiacea, and Familia: Didemnidae. The habitat of *Didemnum* sp. is spread across the Indo-Pacific waters (including Indonesia) and can be found at depths of 1–20 m [10-12]. The results of Krishnaiah *et al*. [13] showed that the lamellarin (lamellarin γ and I) alkaloids isolated from *Didemnum obscurum* from India is a potentially highly potent antioxidant. In his research, Krishnaiah *et al*. [13] used the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The DPPH method provides reactivity information about a compound tested with a stable radical, provides strong absorption at 517 nm, and exhibits a dark purple color [14].

There has been no reported research on antioxidant activity testing of the ascidian *Didemnum* sp. found in Seribu Islands, Jakarta. The purpose of this study was to determine the antioxidant activity in samples obtained from the ascidian *Didemnum* sp.

METHODS

This study was conducted in the Phytochemical Laboratory of the Department of Pharmacy and the Animal Taxonomy Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, from August to November 2011. The following equipment was used: Blender (Waring), vacuum swivel evaporator (Buchi and Stuart), ultrasonicator (Vollrath), ultraviolet-visible (UV-visible) spectrophotometer (Shimadzu), water bath (Imperial IV), thin-layer chromatography (TLC) vessel, freeze dryer (Scanvac), UV lamp, vacuum column chromatography equipment, clamps and supports, analytical scales (Acculab and Acis), Eppendorf pipettes (Socorex), and glassware.

Samples of the ascidian *Didemnum* sp. were collected from Seribu Islands, Jakarta, in September 2011. The sample was identified by the Laboratory of Animal Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. The chemicals used were methanol p.a (Merck, Germany), anhydrous sodium sulfate (Merck), sulfuric acid (Merck), lead (II) acetate, boric acid powder (Merck), oxalic acid powder (Merck), acetic acid anhydrous (Univar, USA), iron (III) chloride, sodium chloride (Mallinckrodt Chemicals, USA), gelatin (Merck), and sodium hydroxide (Univar). The material used for a positive antioxidant activity comparison in this study was quercetin (Sigma-Aldrich).

Collection and provision of simplicia

Didemnum sp. was collected freely from the waters of the island of Pramuka, Seribu Islands, Jakarta, at a depth of 2–5 m by snorkeling. The ascidian samples were placed in a ziplock bag underwater. The samples were soaked in methanol in glass sample bottles. The *Didemnum* sp. samples were identified at the Laboratory of Animal Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia.

Preparation of reagent solutions

a. Sodium hydroxide 2 N
A 2 N sodium hydroxide solution was prepared by accurately weighing 80.02 g NaOH and dissolving it in aquadest to make 1 L.

b. Hydrochloric acid 2 N
A 2 N hydrochloric acid solution was prepared by accurately weighing 72.93 g of HCl and dissolving it in aquadest to make 1 L.

c. Sulfuric acid 2 N
A 2 N sulfuric acid solution was prepared by accurately weighing 98.08 g H_2SO_4 and dissolving it in aquadest to make 1 L.

d. Hydrochloric acid 10%
A 10% hydrochloric acid solution was prepared by accurately weighing 100 mg HCl and dissolving it in aquadest to make 1 L.

e. Bouchardat reagent solution
A Bouchardat reagent solution was made from a mixture of iodine and potassium iodide. A total of 2 g of iodine P and 4 g of potassium iodide P were dissolved in aquadest to make 100 mL.

f. Mayer reagent solution
The Mayer reagent was made from a mixture of mercury (II) chloride P 2.266% w/v and potassium iodide P 50% w/v. A mercury (II) chloride solution was prepared by accurately weighing 1.3596 g of mercury (II) chloride P and dissolving it in aquadest to make 60 mL; the potassium iodide solution was prepared by accurately weighing 5 g of potassium iodide P and dissolving it in aquadest to make 10 mL. Both solutions were mixed, and aquadest was added to make 100 mL.

g. Dragendorff reagent solution
The Dragendorff reagent was made from a mixture of bismuth nitrate P 40% w/v in nitric acid and potassium iodide P 54.4% w/v. The bismuth nitrate solution was prepared by weighing 8 g bismuth nitrate P and dissolving it in nitric acid to make 20 mL; a potassium iodide solution was prepared by weighing 27.2 g of potassium iodide P and dissolving it in aquadest to make 50 mL. Both solutions were mixed. A clear solution was taken and aquadest added to make 100 mL.

h. Molisch reagent solution
The Molisch reagent was a 3% w/v α-naphthol solution in 0.5 N nitric acid. The preparation was made by weighing 1.5 g α-naphthol P and dissolving it in 50 mL of 0.5 N nitric acid.

i. Bromine reagent solution
Bromine reagents were prepared by mixing 9.6 mL bromine P with 30 mL potassium bromide and then diluting to 100 mL with aquadest.

Extract manufacture

All of the samples of the ascidian *Didemnum* sp. were removed from sample bottles using tweezers, drained, and then smoothed using a laboratory grinder (laboratory blender). Approximately 1110 g of *Didemnum* sp. as a wet mass was weighed, divided into four parts (each weighed to approximately 250 g), placed into 1000 mL glass beakers, and then mixed with 500 mL of methanol. Samples that were mixed with methanol were stirred to achieve homogeneity using an ultrasonicator for 1–2 h, macerated for 3 days, and then filtered. The maceration and filtration were repeated until the colored filtrate became colorless. Then, the methanol in the filtrate was evaporated using a rotary evaporator at a temperature of 40°C to obtain a viscous extract that could still be poured, and then, the extract was evaporated again using a water bath until a viscous extract was obtained. The extracts were weighed to determine the yield.

Extract fractionation

Extract fractionation with n-hexane

50 grams of methanol evaporated extract was added to 200 mL of water and homogenized. A liquid extraction was performed using a separation funnel with *n*-hexane at a volume equal to the amount of water to form two layers. The liquid was shaken gently, and then, the *n*-hexane layer was collected. The extraction was repeated until the *n*-hexane layer was colorless. The layers were combined and making another two layers, that is, the water layer and the *n*-hexane layer. Then, the *n*-hexane layer was evaporated using a water bath and was referred to as the *n*-hexane fraction.

Fractionation with ethyl acetate

The water layer obtained in the procedure was added to the same amount of ethyl acetate to form two layers. The liquid was shaken gently, and then, the coating of ethyl acetate was collected. The extraction was repeated until the ethyl layer was colorless. The layers were combined, and two coatings were obtained, that is, the water layer and ethyl acetate coating. Then, the ethyl acetate coating was evaporated using a water bath and was referred to as the ethyl acetate fraction.

Water fraction

The water layer obtained in the procedure was thickened using a freeze dryer and was referred to as the water fraction.

Identification of antioxidant compounds

A 10 mg extract was dissolved in 10 mL of the solvent used in the previous extraction. Approximately 20 µL was spotted onto a silica gel F_{254} TLC plate using a capillary pipe. After the spot was dry, the silica plate was sprayed with DPPH solution. After a few minutes, the spots were observed and characterized. A positive result was a yellow zone with a purple background.

Antioxidant activity test using the DPPH method [15]

The antioxidant activity in each extract was tested using the Blois method. The concentration of sample that reduced the DPPH radical by 50% (IC_{50}) value was calculated from a regression equation, y= ax+b, where, y=concentration and x=absorbance.

Developing DPPH solutions

A total of 10 mg DPPH were weighed and dissolved in 100 mL methanol p.a to give a DPPH concentration of 100 µg/mL.

Maximum absorption wavelength determination of DPPH

The absorption spectrum from 400 to 700 nm of the 100 µg/mL DPPH solution was measured using a UV-visible spectrophotometer. The wavelength corresponding to the maximum absorption was determined.

Blank solution

The blank solution was prepared by mixing 3.0 mL methanol p.a with 1.0 mL of DPPH in a test tube that was shaken until homogeneous. The solution was incubated at 37°C for 30 min. The absorption was measured at a wavelength of 517 nm using a UV-visible spectrophotometer.

Test solutions

Preparation of stock solutions (concentrations: 1000 µg/mL).

A total of 10 mg of extract were weighed and dissolved in 10 mL of methanol p.a until homogeneous.

a. Preparation of a series of working solutions (concentrations 25, 100, 150, and 200 µg/mL)

Stock solution aliquots of 0.25, 1.0, 1.5, and 2.0 mL were added to separate 10 mL volumetric flasks and adjusted to volume with methanol p.a.

b. Testing

A 1.0 mL aliquot of each working solution was placed into separate test tubes, and then, 1.0 mL of the 100 µg/mL DPPH solution was added to 2.0 mL of methanol. The tube was shaken to achieve a homogeneous mixture and then incubated at 37°C for 30 min. The absorption at 517 nm was measured using a UV-visible spectrophotometer.

Preparation of quercetin solution for comparison

a. Preparation of stock solution (concentration=1000 µg/mL)

A 10 mg portion of quercetin was dissolved in 10 mL of methanol p.a.

b. Preparation of a series of working solutions (concentrations of 1, 2, 4, and 5 µg/mL)

Stock solution aliquots of 0.01, 0.02, 0.04, and 0.05 mL were added to separate 10 mL volumetric flasks and adjusted to volume with methanol p.a.

c. Testing

A 1.0 mL aliquot of each test solution was placed into a separate test tube, 1.0 mL of 100 µg/mL DPPH was added to 2.0 mL of methanol, and the mixture was shaken until homogeneous. The mixture was then incubated at 37°C for 30 min, and the absorption at 517 nm was measured.

Calculation of IC_{50}

For each concentration of sample solution, the IC_{50} values reflecting the concentration that produced 50% inhibition of DPPH radicals were calculated by dividing (Blank Absorbance – sample absorbance) with blank absorbance, then times 100%. The equation y=a+bx was determined from linear regression, where, x was the concentration (µg/mL) and y was the percentage inhibition (%). Antioxidant activity was expressed as the IC_{50}, previously defined. The IC_{50} was obtained from the x value after setting y=50. From the equation y=a+bx, we calculated the value of IC_{50} by dividing (50-a) with b.

Chromatography

Most active accelerated column chromatography fraction

The separation was performed on the fraction with the lowest IC_{50}, that is, the ethyl acetate fraction. The stationary phase was 60 g of silica gel (Merck) and the mobile phases employed were distilled technical solvents ranging from 100% *n*-hexane to hexane: ethyl acetate mixtures of 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 35:65, 60:40, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95, and 100% ethyl acetate followed by the mixtures of ethyl acetate: methanol of 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 35:65, 60:40, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95, and 100% methanol (100 mL of each mixture).

Sample preparation was performed as follows: A total of 2.5 g of the ethyl acetate fraction was dissolved in several milliliters of ethyl acetate, and then, a small amount of silica gel was added. Subsequently, the sample and silica gel were stirred until the mixture was homogeneous, and then, the solvent was allowed to evaporate at room temperature until a dry powder was obtained.

The separation was performed on columns. The columns were packed dry by gradually adding silica gel into columns 3 cm in diameter and 15 cm long. During the filling process, the columns were tapped on all sides slowly to obtain a uniform layer. The height of the stationary phase was approximately 3/5 of the column height. The columns were connected to vacuum pumps, which ran for a while to make the stationary phase denser. Then, n-hexane was added to wet the silica gel. After the adsorbent was fully wetted and homogeneous, n-hexane was added to fill just above the surface of the stationary phase. The sample was added. Then, the solvent was allowed to pass through the column to elute the adsorbed components and was collected as the solvent fraction.

Among all fractions obtained, 41 were analyzed by TLC, and fractions having the same chromatographic pattern were combined to obtain nine combined fractions. The activities of the antioxidants were tested to identify the most active fraction in the combined fractions.

Chemical group identification of the most active fraction

The chemical content in the most active fraction was examined using several chemical reagents to test for alkaloid reagents, glycosides, saponins, flavonoids, tannins, anthraquinones, and steroids/ triterpenoids, among others.

Alkaloid identification

A 50 mg extract was dissolved in 9 mL of distilled water and 1 mL of 2 N HCL, heated over a water bath for 2 min, and then cooled. The further filtered filtrate was used as the experimental solution to be used in the following test:

a. To 1 mL of filtrate was added 2 drops of Bouchardat LP. Positive results were indicated by the presence of black chocolate deposits.
b. To 1 mL of filtrate was added 2 drops of Mayer LP. Positive results were indicated by the formation of white or yellow deposits that dissolved in methanol P.
c. To 1 mL of filtrate was added 2 drops of Dragendorff LP. Positive results were shown by the formation of a brown-orange precipitate.

Glycoside identification

A total of 50 mg of extract plus 15 mL of 10% HCl was heated until boiling, cooled, and filtered. The filtrate was washed with 10 mL of ether 3 times. Then, the filtrate was collected and evaporated, added to anhydrous sodium sulfate, filtered, and evaporated at 50°C. The remaining 2 mL of methanol P was added. This solution was used as the experimental solution.

a. The experimental solution of 1 mL was evaporated to dryness, the remaining 5 mL of anhydrous acetic acid P and 10 drops of sulfuric acid P. The positive result was characterized by blue or green formation.
b. The experimental solution of 1 mL was evaporated to dryness, and the residue was dissolved in 2 mL of water and 5 drops of Molisch LP. Then, 2 mL of sulfuric acid P was added carefully. Positive results were indicated by the formation of a purple ring at the liquid boundary (Molisch reaction).

Identification of saponin
A total of 50 mg of extract were placed into a test tube, 10 mL of hot distilled water was added, and the tube was cooled and shaken for 10 s. Positive results were indicated by the formation of a steady foam in <10 min as high as 1–10 cm. After the addition of 1 drop of 2 N hydrochloric acid, the foam did not disappear.

Identification of flavonoids
A total of 50 mg of extract dissolved in 5 mL of 96% ethanol were then subjected to the following procedure:

a. A total of 2 mL of an extract solution were mixed with 0.5 g of zinc powder, and then, 2 mL of 2 N HCl was added and left for 1 min. 10 drops of concentrated HCl were added, shaken slowly, then left for 2–5 min. Positive results were indicated by the formation of an intense red color.
b. A total of 2 mL of the extract solution were mixed with 0.1 g of magnesium powder. Then, 10 drops of concentrated HCl were added and gently mixed. Formation of a red-orange to red-purple indicated positive flavonoids. An orange-yellow color indicated the presence of flavones, chalcones, and aurones.
c. A total of 1 mL of the extract solution were mixed with acetone. Then, small amounts of boric acid and oxalic acid powder were added, and the mixture was heated carefully. The residue obtained was mixed with 10 mL of ether. Color changes were observed under 366 nm UV light. Intense yellow fluorescence indicated the presence of flavonoids.

Tannin identification [16]
A total of 50 mg of extract were added to 50 mL of hot water. The mixture was heated until boiling for 5 min and then filtered. Filtrates of 1 mL each were worked up as follows:

a. 3 ml of 10% gelatin solution was added, and the presence or absence of sediment was recorded.
b. Two drops of 3% $FeCl_3$ solution were added, and the occurrence or absence of color change to green-violet was recorded.
c. 3 ml of NaCl-gelatin solution (1% gelatin solution in 10% NaCl solution) was added, and the presence of precipitate was recorded.

Identification of anthraquinone

A total of 50 mg of extract were dissolved in 5 mL of 2 N sulfuric acid, heated briefly, and then cooled. Then, 10 mL of benzene P was added, the solution was shaken and then was left for a while until the benzene layer separated. A yellow filtrate indicated the presence of anthraquinone. The benzene layer was shaken with 1–2 mL of 2 N sodium hydroxide and immobilized. An intense red water layer and a colorless benzene layer indicated the presence of anthrones.

Identification of phenol

A total of 20 mg of extract were added to 10 mL of water, stirred, and then, two drops of 1% $FeCl_3$ were added. Positive results were characterized by the formation of violet color. A total of 20 mg of extract were added to 10 mL of water and then filtered. Brom LP was added. A positive result was characterized by the formation of white deposits that dissolved immediately and settled when excess reagents were added.

Identification of steroids/triterpenoids [16]

A total of 50 mg of the extract were added to acetic acid anhydride and concentrated sulfuric acid (2:1). Positive results were characterized by the formation of red-green or violet-blue.

RESULTS AND DISCUSSION

Supply of materials

Observations during sampling showed that most of the samples were from organisms that lived on coral reef and concrete pole substrates. The samples collected in this study lived on a coral reef substrate. Successfully collected samples had a total wet weight of 1110 g.

Results of the identification of the marine animals performed in the Laboratory of Animal Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, UI, showed that the organism(s) used in this study was the ascidian *Didemnum sp*. The results of identification can be seen in Appendix 1.

Manufacture of the extracts from the ascidian *Didemnum* sp.

In this research, the first step was making the extract. The extraction method used to obtain the extract was maceration because this cold extraction method prevents metabolites from breaking down. The extraction process time will affect the amounts of the contents of the substance; therefore, the maceration was performed for 3 days to provide an optimal extraction process. The extraction was performed 7 times until the filtrate was colorless.

The wet mass of the *Didemnum* sp. used in this study was 1110 g. The evaporation of the extract used a vacuum swirl evaporator at a temperature of ≤40°C. It was intended that the compounds contained in the extract would not be damaged because the vacuum conditions allowed the solvent to evaporate at low temperatures. In addition, volatile solvents can be accommodated and reused for subsequent macerations.

The extract obtained from the evaporation using a vacuum swivel evaporator was evaporated using a water bath until a viscous extract was obtained. From the results of this methanol extraction, an extract that smelled fishy and dark green was obtained. The methanol extract of *Didemnum* sp. obtained from the 1110 g of sample weighed 50.394 g (4.72%). The percentage was included in the average range of percentage values of the crude extract in general. According to Schupp [17], the percentage of *Didemnum* sp. extract was in the range of 1.5%–15%. This research did not measure the water content because the sample was kept wet. In future research, the sample's water content should be measured.

Antioxidant activity test using the DPPH method (Blois, 1958) of a methanol extract of the ascidian, *Didemnum* sp.

Preliminary identification of antioxidant compounds in the methanol extract was performed by TLC. This identification showed a positive result, that is, a yellowish spot with a purple background. The antioxidant activity was then determined in a series of various concentrations of *Didemnum* sp. (25, 100, 150, and 200 µg/mL). The IC_{50} of *Didemnum* sp. was 105.10 µg/mL. These results showed that the methanol extract had potent antioxidant activity because the IC_{50} value was <200 µg/mL [15]. Our IC_{50} was higher than the IC_{50} of 41.21 µg/mL obtained in the research conducted by Hanani, Mun'im, and Sekarini [18] on sponge extracts of *Callyspongia* sp. from the Seribu Islands.

The mechanism of the radical capture of DPPH by antioxidants involves proton donations to radicals as shown in Fig. 1. Proton donation caused purple DPPH radicals to become non-radical compounds, which lost their purple color as indicated by the decrease in absorption of DPPH at its maximum UV absorption wavelength. Measurement of the decrease in DPPH absorption in the test solution was compared with the absorption of blanks (DPPH solution and unsampled solvent). DPPH compounds reacted with antioxidant compounds by taking hydrogen atoms from antioxidant compounds to form electron pairs [19].

Fractionation of *n*-hexane, ethyl acetate, and water extracts of the ascidian *Didemnum* sp.

Methanol can extract and dissolve compounds with a wide range of polarities. Therefore, to narrow the scope of the analysis, the methanol extract was fractionated in solvents from non-polar to the most polar: n-hexane, ethyl acetate, and water.

The methanol extract was obtained from a sample weighing 50.394 g, but in the subsequent fractionation process, the sample only weighed 50 g because the rest of the extract was used to test the antioxidant activity and partly stored for backup in case the extract was needed

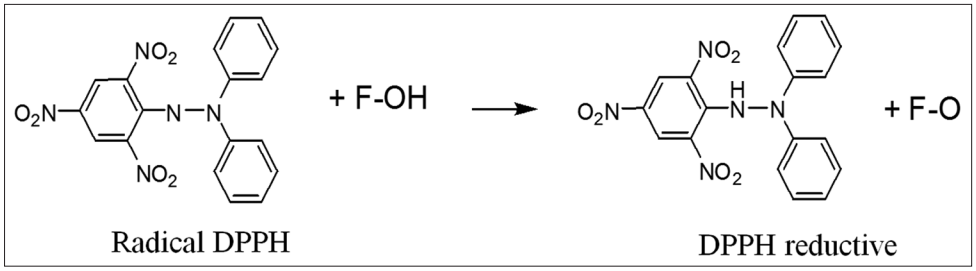

Fig. 1: The mechanism of 1,1-diphenyl-2-picrylhydrazyl radical capture by antioxidants involves proton donation

for additional testing. Then, the extract was added to 200 mL of water and fed into a separation funnel for successive liquid extraction into *n*-hexane and ethyl acetate. This liquid extraction separation was performed until the n-hexane and ethyl acetate layers were colorless. The fractionation was performed 8 times in *n*-hexane and 10 times in ethyl acetate. The water fraction had the highest yield of 43.3154 g (86.63%) compared with that of the *n*-hexane fraction (1.8802 g, 3.76%) and that of ethyl acetate (3.3347 g, 6.67%).

Antioxidant activity tests of the *n*-hexane, ethyl acetate, and water fractions using the DPPH method

The antioxidant activity of any viscous extract obtained was tested using the DPPH method. The identification of antioxidant compounds of each extract showed a positive result of yellow patches against a purple background. Antioxidant activity testing was then performed. Test solutions were prepared as a series of concentrations (25, 100, 150, and 200 µg/mL). The test results showed that the ethyl acetate fraction was the most active fraction because it gave the smallest IC_{50} of 90.804 µg/mL, whereas the IC_{50} was 109.3435 µg/mL for the *n*-hexane fraction and was 95.0997 µg/mL for the water fraction. These test results showed that the three extracts had strong antioxidant activity because the IC_{50} values were all <200 µg/mL [15].

Separation of chromatography the ethyl acetate fraction by accelerated column chromatography

The antioxidant activity test results showed that the ethyl acetate fraction had the highest activity, so it was selected for further study. Chemical separation was performed to obtain a purer fraction by accelerated column chromatography. Unlike conventional columns that use the force of gravity to drive the eluent flow, a vacuum was generated on the column chromatography in this study to accelerate the eluent flow.

Sample preparation used the dry method. Column packing was also performed in a dry manner. Column packing played an important role in the process of separation. The columns must be reasonably compressed with no trapped air because trapped air can cause a non-homogeneous stationary phase and result in poor separations.

The mobile phases used were distilled technical solvents starting from 100% *n*-hexane followed by hexane: ethyl acetate mixtures of 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 35:65, 60:40, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, and 5:95 ending in 100% ethyl acetate and then ethyl acetate: methanol mixtures of 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 35:65, 60:40, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, and 5:95 ending in 100% methanol (100 mL each). The mixed mobile phases of various compositions allowed the separation of compounds of varying polarities. Elution was performed with gradual increases in the polarity of the mobile phases. Accelerated column chromatography gave 41 fractions, with a volume of 100 mL for each fraction.

The accelerated column chromatographic fractions were eluted with mixed hexane: ethyl acetate and ethyl acetate: methanol (7:3, v/v) mobile phases. TLC on silica gel F_{254} plates was used to select the fractions to combine. Selection of eluents was based on previous work. Fractions with the same spots were combined because they were considered to have almost the same chemical content. Of the 41 fractions separated by accelerated column chromatography, nine joint fractions were obtained. Fractions 1–6 were combined into fraction I, fraction 7 into fraction II, fractions 8–12 into fraction III, fractions 13–17 into fraction IV, fractions 18–20 into fraction V, fractions 21–25 into fractions VI, fractions 26–28 into fraction VII, fractions 29–32 into fraction VIII, and fractions 33–41 into fraction IX. The I–IX fractions had weights of 170.1, 82.0, 257.6, 218.2, 270.0, 486.9, 210.0, 260.3, and 507.9 mg.

Antioxidant activity test of fractions I–IX using the DPPH method

Antioxidant compounds in fractions I–IX were identified by a positive result indicated by a yellow spot with a purple background after spraying with DPPH. The highest antioxidant activity was observed in fraction VI, with an IC_{50} of 86.3507 µg/mL followed by fraction IX (103.5269 µg/mL), fraction VIII (104.17 µg/mL), fraction VII (104.8748 µg/mL), fraction III (115.1258 µg/mL), fraction V (117.6768 µg/mL), fraction II (210.0203 µg/mL), fraction IV (229.0921 µg/mL), and fraction I (312.0034 µg/mL).

Six fractions (III, V, VI, VII, VIII, and IX) had IC_{50} values of <200 µg/mL, and the other three fractions (I, II, and IV) had IC_{50} values of >200 µg/mL. The large IC_{50} values in fractions I, II, and IV may be due to the small amounts of antioxidant compounds in the fractions.

Quercetin was used as a positive antioxidant comparative compound. The antioxidant power of fraction VI was still below that of quercetin (IC_{50} of 1.6599 µg/mL) because quercetin is a pure substance and the fraction was not.

Identification of the compound groups in the most active fraction

The main groups of chemical compounds in the most active composite fraction (fraction VI) were identified using various chemical reagents. The most active fraction contained alkaloids, steroids/triterpenoids, saponins, and glycosides. These results were consistent with the results of Krishnaiah *et al*. [13] who stated that the lamellarin alkaloids (lamellarin γ and I) were isolated from ascidians. The Indian *D. obscurum* was a potential antioxidant compound. The research conducted by Hanani, Mun'im, and Sekarini [18] on the sponge extract *Callyspongia* sp. from the Seribu Islands also identified antioxidants including alkaloid groups. The compounds that donated a proton had sufficiently strong radical capture activity. These compounds were phenols, flavonoids, tannins, and alkaloids [20].

CONCLUSION

The methanol extract of the ascidian *Didemnum* sp. exhibited antioxidant activity. Fractionation of the *Didemnum* sp. extract showed that the ethyl acetate fraction had the highest antioxidant activity. The highest antioxidant activity was observed in fraction VI, with an IC_{50} of 86.3507 µg/mL. The result of antioxidant activity tests of the *n*-hexane, ethyl acetate, and water fractions using the DPPH method showed that the ethyl acetate fraction was the most active fraction because it gave the smallest IC_{50} of 90.804 µg/mL. Further, fractionation of the ethyl acetate fraction by accelerated column chromatography showed that fraction VI had the highest antioxidant activity. The most active fraction contained alkaloids, steroids/triterpenoids, saponins, and glycosides.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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