Antioxidant and DPP-4 Inhibitory Potential of n-Hexane, Ethyl Acetate, and Aqueous Fractions From *Avicennia marina* Fruit

Retno Sari Utomo[1*], Kyky Herlyanti[1], Dwi Hadi Setya Palupi[2], M. Noufal Hafidh[2]
[1] Magister's Program in Pharmacy, STIFAR Yayasan Pharmasi Semarang, Indonesia
[2]Undergraduate Program in Pharmacy, STIFAR Yayasan Pharmacy Semarang, Indonesia
[*]email : utomo.retno83@gmail.com

Abstract

Avicennia marina is one type of mangrove plant fruit that can be utilized for medical purposes through natural ingredients,one of which is the treatment of diabetes mellitus. *A. marina* contains secondary metabolites such as flavonoids, alkaloids, tannins, saponins, and terpenoids that have antioxidant and antidiabetic activities. The aim of this study is to determine the potential of n-hexane, ethyl acetate and water fractions of *A. marina* fruit as antioxidants using DPPH method and their antidiabetic activity through the inhibition of the enzyme dipeptidyl peptidase-4 (DPP-4) in vitro. The sample used fruit of *A. marina*, which was extracted using maceration methode with 96% ethanol for 3x24 hours. The obtained extract was fractinationated stepwise using n-hexane, ethyl acetate, and aqoeous as solvents. The test results on the n-hexane fraction showed antioxidant activity with an IC_{50} value of 77.74 ±0,96 ppm and a DPP-4 inhibition of 18.07±1,82%; the ethyl acetate fraction had an IC_{50} value of 45.27 ±2,49 ppm and a DPP-4 inhibition of 64.04%±0,23%; while the aqueous fraction had an IC50 of 95.65 ±0,25 ppm with a DPP-4 inhibition of 43.99±0,99%. Based on these results, the ethyl acetate fraction of the A. marina fruit shows the highest potential both as an antioxidant with vitamin C as a positive control with IC_{50} value 8.66 ±0,14 ppm and as a DPP-4 inhibition with sitagliptin as a positive control with DPP-4 inhibition 98,38±.0,60%.

Keyword : *Avicennia marina*, DPP-4 inhibitor, DPPH, diabetes mellitus

1. INTRODUCTION

Mangroves are a type of plant that grow in the transitional areas between land and sea, and are influenced by the tidal conditions of the sea. As an archipelagic country, Indonesia has abundant biodiversity, including various types of plants that have the potential to be utilized as medicinal materials. One type of mangrove plant that has medicinal properties is *A. marina*. *A. marina* belongs to the Acanthaceae family, and is one of the mangrove species that has high tolerance to salinity. This plant is capable of surviving and thriving in tidal environments, and is often found growing in clusters, forming its own communities in coastal areas near the sea (Erwin, 2020).

The fruit of *A. marina* is a mangrove fruit that is widely used by the community for snacks and to manage blood sugar levels. In addition, the fruit of *A. marina* is also used to treat other diseases such as fever reduction, antiplasmodial, antitumor, antidiabetic, anticancer,

antifungal, antibacterial, and antioxidant (Sabdaningsih dkk., 2024). The previous study, methanol extract of *A. marina* fruit has strong antioxidant activity (Anam dkk., 2020). The bioactive compounds contained in the leaves and fruits of *A. marina* include flavonoids, polyphenols, steroids, terpenoids, alkaloids, saponins, tannins, cardiac glycosides, and anthraquinones, which are considered responsible for its pharmacological activities (Fernandes dan Noor'an, 2019; Rozirwan dkk., 2022; Vasanthakumar dkk., 2019).

The fruit of *A. marina*, which has potential as an antidiabetic, has been extensively researched in the form of extracts. Setiawati's research (2020) found that ethanol extract of *A. marina* fruit can increase the levels of the enzyme catalase, which functions as an antioxidant that can lower blood sugar levels. The extract of *A. marina* leaves and fruits can lower serum glucose in streptozotocin-induced diabetic rats (Kamaei dan Moghadamnia, 2019). Based on this background, this study aims to evaluate the potential of n-hexane, ethyl acetate, and water fractions from *A. marina* fruit as antioxidants based on IC_{50} values using the DPPH method, as well as their antidiabetic activity through the inhibition of the dipeptidyl peptidase-4 (DPP-4) enzyme *in vitro*.

2. METHOD

Tools and Material

The main material in this research is the *A. marina* fruit sourced from Mororeja Village, Kaliwungu, Kendal Regency, Central Java, Indonesia. The chemicals used are Aquabidest, 96% ethanol, n-hexane, ethyl acetate, aquadest, DMSO, Dipeptidyl Peptidase-4 (DPP-4) Inhibitor Screening Assay Kit® from Elabscience (United States), 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Merck-Germany, Vitamin C (Merck-Germany), methanol.

The tools used are a beaker, blender, flannel cloth, separating funnel, rotary evaporator, water bath, analytical balance, maceration vessel, micropipette, multichannel pipette, Multimode reader (Synergy HTX), spectrophotometer UV-Vis (Shimadzu 1780).

Preparation of *A. marina* Fruit Extract

As much as 500 grams of *A. marina* fruit powder were extracted using maceration with 96% ethanol for 3 x 24 hours and occasionally stirred. The macerate was collected and concentrated using a rotary evaporator at a temperature of 40°C until a thick extract was obtained.

Preparation of the *A. marina* Fractions

Fifteen grams of *A. marina* fruit extract were added to 75 mL of distilled water, then placed into a separating funnel, 75 mL of n-hexane was added, and the mixture was shaken and allowed to stand until two separate layers formed. Then, drain the n-hexane fraction and evaporate it using a rotary evaporator and water bath until a thick n-hexane fraction is obtained. Then, add 75 mL of ethyl acetate to the separating funnel, and shake until two distinct layers are formed. Drain the aqueous fraction and the ethyl acetate fraction, then collect them in separate containers. The obtained ethyl acetate fraction and aqueous fraction are evaporated using a rotary evaporator and a waterbath until a thick ethyl acetate fraction and a thick aqueous fraction are obtained.

Antioxidant activity assay using the *1,1-diphenyl-2-picrylhydrazyl* (DPPH) method

Antioxidant activity assay using 1,1-diphenyl-2-picrylhydrazyl (DPPH). The test solution was prepared at concentrations of 40, 60, 80, 100, and 120 ppm dissolved in methanol. The determination of antioxidant activity was carried out by adding 0.2 mL of the sample solution into a test tube, followed by the addition of 4.0 mL of 0.7 mM DPPH for each concentration. Next, the mixture was homogenized by vortexing for 1 minute and incubated for 30 minutes. The absorbance of the solution is read at a wavelength of 515 nm. The calculation of the percentage of antioxidant activity can use equation 1 (Marinova dan Batchvarov, 2011).

$$\%\ antioxidant\ activity = \frac{Abs.control - Abs.sample}{Abs.control} x\ 100\% \qquad (1)$$

The concentration of the sample with % antioxidant activity was plotted in a linear regression equation to determine the sample concentration that can reduce free radicals by 50% (IC50). Vitamin C was used as a standart or positive control.

DPP-4 inhibition activity assay

The DPP-4 assay is used to measure the inhibitory activity of the enzyme peptidyl peptidase-4 using the Dipeptidyl Peptidase-4 (DPP-4) Inhibitor Screening Assay Kit® from Elabscience. A 96-well microplate was prepared and mapped in each well to facilitate the DPP-4 inhibition activity assay. The testing of DPP-4 enzyme inhibition activity was conducted individually as follows:

1. Blank well : Add 20 µL of buffer solution to the corresponding wells.

Control well : Add 20 µL of enzyme working solution to the corresponding wells.

Sample well : Add 20 µL of enzyme working solution to the corresponding wells.

2. Add 30 µL of 2% DMSO to blank well and control well. Add 30 µL of sample to sample well.
3. Mix fully with microplate reader for 3 s and incubate at 37℃ for 10 minutes.
4. Add 170 µL of reaction working solution into each well.
5. Incubate at 37℃ for 30 min. Measure the fluorescence intensity of each well at the excitation wavelength of 360 nm and the emission wavelength of 460 nm using a multimode reader.

The activity of the sample is expressed as a percentage of inhibition using the following formula in equation 2.

$$\%\ inhibition = \frac{(Fcontrol - Fsample)}{(Fcontrol - Fblank)}\ x\ 100\% \qquad (2)$$

Note : F control : The fluorescence intensity of control well

F sample : The fluorescence intensity of sample well (fractions sample/ positive control)

F blank : The fluorescence intensity of blank well.

The data can be collect as inhibiton percentage and analyzed use one way anova.

3. RESULTS AND DISCUSSION

Antioxidants are compounds that can ward off free radicals that cause various diseases. The way antioxidant compounds work is by stopping free radical reactions from metabolism in the body or from the environment (Prasetyo dkk., 2021). Antioxidants come from synthetic and natural sources. In Indonesia, with its tropical climate, there are various types of plants, one of which is *A. marina*. *A. marina* is a type of mangrove fruit that is widely utilized by the community as food and medicine. The bioactive compounds contained in *A. marina* are believed to help treat various diseases, including fever reducers, antiplasmodial, antitumor, antidiabetic, anticancer, antifungal, antibacterial, and antioxidants (Sabdaningsih dkk., 2024). The ethanol extract of *A. marina* fruit showed an antioxidant activity with an IC_{50} 126,74±2,08 ppm, which is classiefied as moderat in inhibiting free radicals. It should be noted that this IC_{50} value indicates the concentration of extract required to reduce 50% of free radical activity in the DPPH assay. The lower of IC_{50} value, the stronger the antioxidant activity of the extract. Acoording to Anam, et.al (2020) the methanol extract of *A. marina* fruits reported has strong

antioxidant activity with IC_{50} value 85,246 ppm. The difference in results obtained due to differences in solvent, place of growth, soil salinity, temperature, humidity of A. marina plants. *A. marina* fruits contain bioactive compounds such as alkaloids, flavonoids, saponins, tannins dan steroids (Setiawati dkk., 2020).

Antioxidant assay were conducted quantitatively using the DPPH (*2,2-diphenyl-1-picrylhydrazyl*) method at a wavelength of 515 nm. The DPPH method is a method that can measure antioxidant activity quickly, simply, and without requiring high costs. The magnitude of antioxidant activity is indicated by the IC50 value, which is the concentration of the sample solution required to inhibit 50% of DPPH free radicals. Therefore, this study measured the antioxidant activity of the n-hexane fraction, ethyl acetate fraction, and aqueous fraction of the A. marina fruit. As a standart, Vitamin C (ascorbic acid) was used. The results of the antioxidant activity test of the three fractions of the *A. marina* fruit are presented in Figure 1.

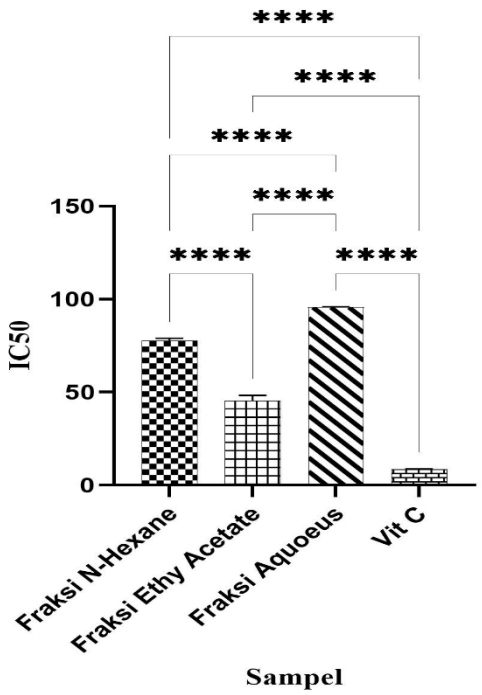

Figure 1. Antioxidant activity of the n-hexane, ethyl acetate and aqueous fraction of *A. marina* fruits

The test results of antioxidant activity on n-hexane, ethyl acetate, and aqueous fractions presented with IC_{50} value showed a significant difference ($P<0.0001$). The standard used ascorbic acid as antioxidant activity with an IC_{50} value of 8,66±0,14 ppm. The highest antioxidant activity of sample ethyl acetate fraction is 45,27±2,49 ppm, this indicates that the ethyl acetate fraction of *A.marina* has very strong antioxidant activity (less than 50 ppm). As for the n-hexane fraction and aqueous fraction, they have IC_{50} values of 77.74±0,96 ppm and

95.65±0,25 ppm, where the antioxidant activity of the n-hexane fraction and aqueous fraction is strong with a range of 50 ppm to 100 ppm. (Purwanto dkk., 2017). The antioxidant activity of a compound can be classified based on the IC_{50} value obtained. If the IC_{50} value of an extract is below 50 ppm then the antioxidant activity is very strong category, the IC_{50} value is between 50-100 ppm means the antioxidant activity is strong category, the IC_{50} value is between 100-150 ppm means the antioxidant activity is moderate category, the IC_{50} value is between 150-200 ppm means the antioxidant activity is weak category, while if the IC_{50} value is above 200 ppm then the antioxidant activity is categorised as very weak (Fitri, 2021).

The results showed that all fractions had antioxidant activity from polar, semipolar and non-polar solvents. The difference in activity obtained in each fraction may be due to differences in the content and amount of active compounds contained in the fraction, so that the antioxidant activity obtained is also different. The ethyl acetate fraction has higher antioxidant activity compared to the n-hexane and aqueous fractions, this is thought to be due to the content of active compounds from several antioxidant compounds that are semipolar more than those that are polar and non-polar contained in *A. marina* fruit.

Previous research using ethanol extract of *A. marina* fruit can increase catalase enzyme activity, where catalase acts as an endogenous antioxidant that has a major role in controlling H_2O_2 by catalysing H_2O_2 into oxygen and water so that it is non-toxic. This increase in catalase enzyme can reduce blood sugar levels of alloxan-induced rats (Setiawati dkk., 2020). Research by Fernandes and Noo'an (2019) showed that the content of γ-sitosterol which is an antioxidant has the highest percentage in ethanol extract of *A. marina* fruit, where γ-sitosterol is a promising candidate as an effective antidiabetic.

The high antioxidant activity in the DPPH assay indicates the potential of the compound in counteracting oxidative stress, which also plays a role in the mechanism of DPP-4 enzyme inhibition as a therapeutic target for type 2 diabetes. DPP-4 is an enzyme that degrades incretin hormones, namely GLP-1 and GIP. DPP-4 inhibitors improve hyperglycaemia conditions by stabilising post-prandial blood sugar levels by increasing GLP-1 and GIP levels in patients with type 2 diabetes. The DPP-4 enzyme inhibitory activity assay was performed in vitro using the DPP-4 Enzyme Inhibition Assay Kit from Elabscience with a fluoroscence-based method.

Dipeptidyl peptidase-4 (DPP-4) is a type of hormone serine exopeptidase, can decompose the second place peptide N chain, peptide bond of alanine and proline residues. DPP-4 will degrade two intestinal increatin hormones, GLP-1 and GIP, causing the half-life of these hormones to be very short. Inhibition of DPP-4 will lead to an extension of the half-life of GLP-1 and GIP, thereby increasing plasma insulin levels and promoting a decrease in blood

sugar levels in the body (Sarian dkk., 2017). This assay uses the fluorogenic substrate Gly-Pro-Aminomethylcoumarin (AMC). DPP-4 releases free AMC groups through peptide bond cleavage resulting in fluorescence that can be analysed using excitation wavelengths and emission wavelengths. The addition of DPP-4 inhibitor can inhibit the activity of the enzyme. (Setyaningsih dkk., 2019). The DPP-4 inhibitory activity assay showed that the n-hexane, ethyl acetate and aqueous fraction of *A. marina* fruit had DPP-4 enzyme inhibitory activity as presented in Figure 2.

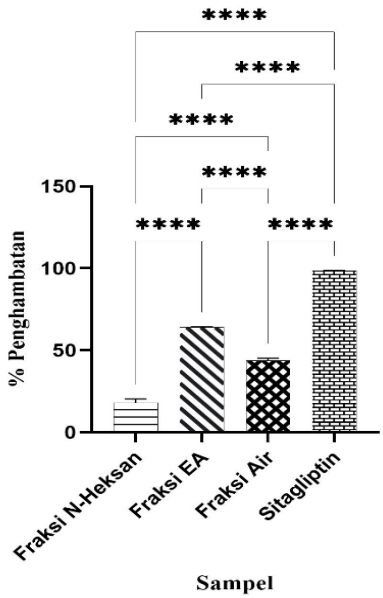

Figure 2. DPP-4 inhibibition activity of *Avicennia marina* fractions

The result DPP-4 inhibition of n-hexane, ethyl acetate, and aqueous fractions *A. marina* fruits showed a significans difference ($p<0,0001$). The test results showed DPP-4 inhibition of the n-hexane fraction was 18.07±1,82%, the ethyl acetate fraction 64.04±0,23% and aqueous fraction 43.99±0,99%. This means that the inhibition given by the ethyl acetate fraction is greater than aqueous fraction and the n-hexane fraction. The standard used sitagliptin which is a DPP-4 inhibitor class diabetes mellitus drug with DPP-4 inhibition 98,38±0,60% . Sarian's research (2017) also showed that bioactive compounds in plants contribute to DPP-4 inhibitory activity. This is the basis for the assumption that bioactive compounds in the ethyl acetate fraction of *A. marina* fruit are likely to have an inhibitory effect on the DPP-4 enzyme, so that the ethyl acetate fraction has the greatest DPP-4 inhibitory activity.

4. CONCLUSION

Based on the results obtained, it is concluded that the n-hexane, ethyl acetate and aqueous fractions of *A. marina* fruit have potential antioxidant activity and DPP-IV inhibitory activity. The highest antioxidant activity was obtained from ethyl acetate fraction with IC50 value of 45.27±2,49 ppm, n-hexane fraction with IC_{50} value of 77.74±0,96 ppm and aqueous fraction with IC_{50} value of 95.65±0,25 ppm. While the greatest DPP-IV inhibitory activity is the ethyl acetate fraction, aqueous fraction, and n-hexane fraction of 64.04±0,23%, 43.99±0,99% and 18.07±1,82% and has a potential effect as an antidiabetic drug.

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