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Antimalarial Potential of Fraction 5 from Ethanolic Leaves Extract of *Artocarpus Altilis*

Einstenia Kemalahayati[1], Hilkatul Ilmi[2], Agriana Rosmalina Hidayati[3], Marsih Wijayanti[1], Lidya Tumewu[2], Suciati[2,4], Achmad Fuad Hafid[2,4], Aty Widyawaruyanti[2,4*]

[1]Master Program of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
[2]Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
[3]Departement of Pharmacy, Faculty of Medicine, University of Mataram, Mataram, Indonesia
[4]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

*Corresponding author: aty-w@ff.unair.ac.id

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Abstract

Background*: Artocarpus altilis leaf extract (AAL.E) was separated by VLC, and six fractions were obtained. Fraction 5 (AAL.E.5) showed antimalarial activity with an IC_{50} value of 3.71 µg/mL.* ***Objective****: This study aimed to determine the antimalarial activity of AAL.E.5 subfractions against P. falciparum, the mechanism of action against Plasmodium Falciparum Malate quinone oxidoreductase (PfMQO), and the active substances.* ***Methods****: The AAL.E.5 was separated by open-column chromatography and eluted with chloroform-methanol gradient elution in order of increasing polarity. The antimalarial activity of all subfractions was assessed using a lactate dehydrogenase (LDH) assay against P. falciparum and the mechanism of action of the PfMQO enzyme. The profiles of the most active subfractions were analyzed using High-Performance Liquid Chromatography (HPLC).* ***Results****: The separation of fraction 5 (AAL.E.5) yielded 11 subfractions (AAL.E.5.1–AAL.E.5.11). Screening antimalarial activity at 10 µg/mL in this subfraction showed that only five subfractions (AAL.E.5.6-AAL. E.5.10) inhibited P. falciparum and two subfractions (AAL.E.5.6 and AAL.E.5.10) inhibited the PfMQO enzyme. Only subfraction 6 (AAL.E.5.6) inhibited both, with IC_{50} values of 6.609 µg/mL and 20.34 µg/mL. The thin layer chromatography profile of AAL.E.5.6 revealed reddish-orange spots, indicating the presence of flavonoid compounds, and was also presumed from the UV-visible to HPLC chromatogram for band I in the 300 – 400 nm range and band II in the 240–285 nm range.* ***Conclusion****: Subfraction 6 has antimalarial activity against P. falciparum and is thought to have a mechanism of action in PfMQO. Based on the TLC, HPLC, and UV-Vis spectra, subfraction 6 was assumed to be a flavonoid.*

Keywords*: antimalaria, Artocarpus altilis, flavonoid, lactate dehydrogenase (LDH), PfMQO*

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INTRODUCTION

Malaria is an infectious disease caused by the protozoan parasite *Plasmodium*, which is transmitted by the bite of female Anopheles mosquitoes. Malaria has the world's most extensive and endemic distribution, primarily in the tropical and subtropical climates. Malaria affected an estimated 229 million individuals in 2019, with an increase of 228 million in 2018 (WHO, 2020). An estimated 219 million malaria cases were reported in 2017 compared to 217 million (WHO, 2018). Globally, malaria deaths have decreased steadily from 451,000 in 2016 to 409,000 (WHO, 2020). Indonesia is malaria endemic, with approximately 180,000 confirmed cases of malaria from 26 malaria-endemic areas nationally (Sitohang *et al*., 2018). The province with the highest number of malaria cases in Indonesia was Papua (12.07%). West Papua (8.64%), East Nusa Tenggara (1.99%), North Maluku (1.36%), and Maluku (1.21%) (RISKESDAS, 2019).

Malaria is difficult to control owing to its widespread treatment resistance. Malaria control in Indonesia remains restricted in terms of treatment due to discrepancies in malaria case management at all levels of health care, as well as the rise of malaria parasite resistance to available commercial antimalarial drugs, such as chloroquine and pyrimethamine sulfadoxine (Cui *et al*., 2015). However, this therapeutic modification is considered ineffective. The lack of Artemisinin Combination Therapy (ACT) coverage is caused by numerous non-malaria-endemic locations, such as Jakarta, which are still relatively weak in dealing with malaria cases from endemic areas (Kinansi *et al*., 2021). As existing antimalarials are ineffective, a novel technique to prevent malaria transmission is required (Noronha *et al*., 2019). As malaria therapies have become increasingly resistant, new drugs derived from various plants are being explored.

The Mulberry family (Moraceae) is found worldwide, with the majority of species found in Asia, while the Indo-Pacific Islands have 60 genera and 1,400 species (Berg, 2001). *Artocarpus* is the largest genus of Moraceae, is high in phenolic compounds, and is widely used in traditional medicine. Widyawaruyanti (2007) revealed that heteroflavone C, a prenylflavonoid compound isolated from the stembark of *Artocarpus champeden*, had an IC_{50} antimalarial activity of 0.001 – 1 µmol/L. A prenyl chalcone, morachalcone A, was isolated as an active antimalarial agent from the ethanol extract of *A. champeden* stem bark by Hafid (2012) and had an IC_{50} of 0.18 µg/mL. Bourjot (2010) examined an ethyl acetate extract from the bark of *Artocarpus styracifolius*, which has antimalarial activity against *P. falciparum* with an IC_{50} of 0.5 µg/mL - 6.9 µg/mL and succeeded in isolating prenylated flavonoids. From a dichloromethane extract of *Artocarpus altilis* root, Boonphong (2007) isolated nine prenylated flavones with antimalarial activity ranging from 1.9 - 4.3 µg/mL. Ethanol extracts from *A. altilis* leave suppressed the development of *P. falciparum in vitro* with an IC_{50} value of 1.32µg/mL and *P. berghei in vivo* with an ED_{50} of 0.82 mg/kg body weight, according to Hafid (2016). Lactate dehydrogenase (LDH) assay revealed that fraction-6 of *Artocarpus sericicarpus* dichloromethane exhibited an IC_{50} value of 1.53 ± 0.04 µg/mL against *Plasmodium falciparum* (Tumewu *et al*., 2020).

Syah (2006) isolated two acylated flavonoid derivatives from *Artocarpus altilis* leaves: 2-geranyl-2, ’ 4, ’ 3,4-tetrahydroxidihidroxcone and 8-geranyl-4', 5,7-trihydroxy flavanone. According to Fajriah *et al.* (2013), the ethyl acetate fraction of *A. altilis* leaves produces prenylated flavonoids, specifically the 1-prenylated flavonoid (2,4-dihydroxy phenyl) -3- (8-hydroxy-2-methyl-2-ethane-2-ethane-2-ethane-2-e (4-methyl-3-pentenyl) -2H-1benzopyranyl] -1-propanone. Nguyen *et al.* (2013) obtained the auron flavonoid altilisin H-J from a methanolic extract. Nguyen *et al.* (2013) isolated eight geranylated dihydrochalcones from the leaves of the methanolic extract of *A. altilis*, designated sakenins A-H.

Based on a previous study, fractionation of ethanol extract of *A. altilis* leaves obtains six active fractions (AAL.E.1-AAL. E.6). The microscopic antimalarial results showed that AL. E.2, AAL.E.4, and AAL.E.5 were active as antimalarials (Hidayati, 2020). AAL.E.2 isolated 1-(2,4-dihydroxy phenyl)-3-[8-hydroxy-2-methyl-2-(4-methyl-3- pentenyl)-2H-1-benzopyran-5-yl]-1-propanone, and AAL.E.4 isolated geranyl-2′, 4 ′, 3, 4-tetrahydroxy-dihydrochalcone. Both inhibit *P. falciparum* and *Pf*MQO enzymes (Hidayati *et al*., 2023). Further studies are needed on fraction 5 (AAL.E.5) to determine the active substance that acts as an antimalarial agent. This study aimed to identify the active substance that functions as an antimalarial agent in AAL.E.5 of *A. altilis*.

MATERIALS AND METHODS

Materials

Plant material

Fresh *Artocarpus altilis* leaves were obtained from the Purwodadi Botanical Garden, East Java, Indonesia. A licensed botanist identified this plant in the Purwodadi Botanical Garden in East Java (No: B-

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107/IPH.06/AP.01/II/2020). The specimens were stored in the herbarium of the Natural Product Medicine Research and Development (NPMRD) at the Institute of Tropical Diseases, Universitas Airlangga, Indonesia.

Parasite

P. falciparum was obtained and cultivated at the Natural Product Medicine Research and Development (NPMRD), Institute of Tropical Diseases, Universitas Airlangga, Surabaya, East Java, Indonesia.

Methods

Fractionation and HPLC

A total of 800 mg of AAL.E.5 was separated using silica gel in open-column chromatography and eluted with a chloroform-methanol gradient elution in order of increasing polarities (9.8:0.2 v/v) and obtained eleven subfractions (AAL.E.5.1- AAL.E.5.11). In future studies, the profile of the most active subfractions will be analyzed using High-Performance Liquid Chromatography (HPLC). The subfraction was eluted in the mobile phase acetonitrile-water (3:7 v/v) at a flow rate of 0.5 ml/min, a PDA detector, and an injection volume of 40 µL.

In vitro* cultivation of *Plasmodium falciparum

The chloroquine-sensitive *P. falciparum* culture was maintained using numerous modification approaches described by Trager and Jansen (1997) at the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. Fresh human erythrocytes of type O were suspended at 2% hematocrit in RPMI 1640 (Gibco) containing 25 mM HEPES buffer, 50 g/mL hypoxanthine, 2 mg/mL Natrium Bicarbonate, and 10 g/mL gentamycin to culture the parasites. The Incubation was carried out at 37°C in a gas mixture of 5% O_2, 5% CO_2, and 90% N_2.

Antimalarial LDH assay

The antimalarial assay was conducted using the LDH (Lactate Dehydrogenase) method. Samples were screened at a dose of 10 µg/mL. Briefly, a stock solution was prepared by dissolving 10 mg of the sample in 1000 µL DMSO. Several concentrations of the sample were prepared from 0.05 to 100 µg/mL. 0.4 µL sample from each concentration and 100 µL of parasites (ring stage) were added to the well plate. Adding (0.4 µL) were added to the well as a positive control (chloroquine diphosphate). The microplate was incubated for 72 h with a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. After incubation, 200 µL PBS was added to the well plate and centrifuged at 1300 rpm for 10 min at room temperature. The supernatant (240 µL) was removed and the well plate was kept at -30°C for 24 h. Anti-malarial activity was measured using the LDH method. Ten milliliters of LDH-buffer (Tris-HCL, Sodium L-Lactate, Triton X-100, deionized water) was added to 2 mg NBT (10 mg/ml, Sigma), 50 µL APAD stock (10 mg/ml, Oriental Yeast Co., Ltd), and 200 µL diaphorase stock (50 units/mL, Sigma). The substrate was mixed gently and kept in the dark. Add 90 µL of substrate per well plate and shaker at 400 rpm for 30 min. The absorbance was then measured using an ELISA Reader at a wavelength of 650 nm, and the results were analyzed using the GraphPad Prism program.

***Plasmodium falciparum* malate quinone oxidoreductase (*Pf*MQO) enzyme assay**

The stock solution of the sample was prepared by dissolving 10 mg in 1000 µL DMSO to produce a final concentration of 10,000 µg/mL as stock solution. The *Pf*MQO enzyme test mixture solution consisted of 20 mL of Hepes 50 mM (pH 7.0), 200 µL of 12 mM dichlorophenolindophenol (DCIP), 12 µL of 100 mM decilubiquinone (duQ), and 25 µL of *Pf*MQO 13.2 mg/mL. The assay solution in the microwell plate was then supplemented with 1 µL sample, and mixing of the solution in the microwell plate was carried out in a well plate mixer at 650 rpm for 1 min. then, while maintaining the temperature at 250 °C for 3 minutes, read the absorbance at a 600 nm wavelength. Next, 5 µL of L-malate (enzyme) was added to each well (apart from well number 12) and shaken with a plate mixer for 20 s. The absorbance at a wavelength of 600 nm was then measured after the incubation at 25°C for 10 min. Samples exhibiting more than 50% resistance were analyzed using GraphPad Prism to determine their IC_{50} values.

RESULTS AND DISCUSSION

Separation of fraction 5 (AAL.E.5)

The ethanolic extract and fraction of *Artocarpus altilis* exhibited antimalarial activity. This study focused on *A. altilis*'s the antimalarial activity of A. altilis (AAL.E.5). Open column chromatography was used to isolate ethanol 5 from *A. altilis* (AAL.E.5) using a gradient elution of chloroform: methanol (98:2). Subsequently, 11 subfractions were generated (subfraction 1-11). All of–1-11 (AAL.E.5.1 - AAL.E.11) were examined using thin layer chromatography (TLC) with silica gel 60 RP-18 $_{F254}$ as the stationary phase and acetonitrile: water (7:3 v/v) as the mobile phase. The flavonoids were observed more clearly after spraying with H_2SO_4 reagent on the TLC plate. TLC spots indicate orange-yellow spots. Flavonol glycosides are indicated by orange-yellow hues (Gwatidzo *et al*., 2018). Subfractions (7 – 9) also

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performed well, as evidenced by their R_f values of 0.7 (AAL.E.5.7), 0.7 (AAL.E.5.8), and 0.68. (AAL.E.5.9) (Figure 1).

Antimalarial activity of subfraction *Artocarpus altilis*

Lactate dehydrogenase assay (LDH)

Antimalarial activity testing was performed to investigate the possible inhibition of *P. falciparum* growth by the samples. All subfractions (11 fractions) were screened for antimalarials at a concentration of 10 ug/ml, and only five subfractions (AAL.E.5.6 – AAL.E.5.10) showed inhibition of more than 50% (Figure 2). Therefore, IC_{50} calculations were performed on these subfractions.

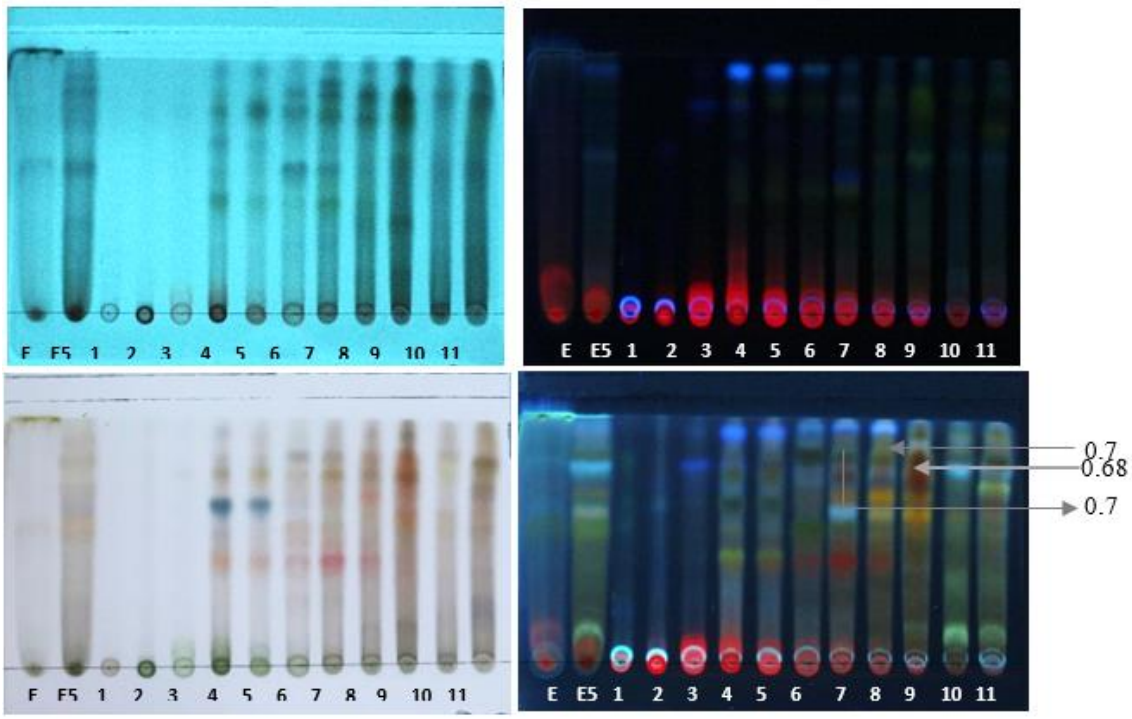

Figure 1. TLC profiles for detecting flavonoids in ethanol leaves of *Artocarpus altilis* using silica gel 60 RP-18 $_{F254}$ as the stationary phase and acetonitrile: water (7:3 v/v) as the mobile phase. The TLC spots were seen in the following conditions: UV 254 nm (a), UV 366 nm (b), white light after being sprayed with 10% H_2SO_4 and heated to 105° C for 5 min (c), and UV 366 nm after being sprayed with 10% H_2SO_4 and heated to 105° C for 5 (d)

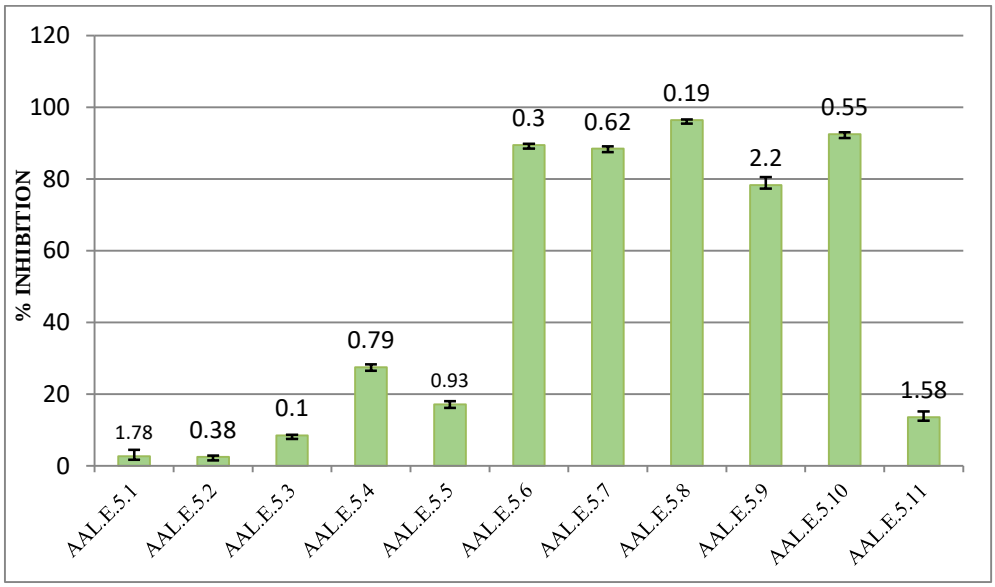

Figure 2. Inhibition of parasite growth by LDH assay at 10 µg/mL from AAL.E.5.1 – AAL.E.5.11 with duplicate measurement

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GraphPad Prism was used to calculate the IC_{50} values for the subfractions from 6 to 10 (AAL.E.5.6 to AAL.E.5.10), which had shown *P. falciparum* inhibitory activities of more than 50% (Table 1). The results showed that five subfractions (AAL.E.5.6 – AAL.E.5.10) were active as antimalarials. The criteria for the antimalarial activity of the extract, according to Basco *et al*. (1994) and Dolabela *et al*. (2008), were as follows: IC_{50} <10 µg/mL was the active extract, IC_{50} 10-50 µg/mL was included in the moderate criteria, IC50 50-100 µg /mL extract had low activity, and IC_{50} >100 µg/mL indicated that the extract was inactive. Subfraction 8 (AAL.E.5.8) has the highest antimalarial activity compared to the other subfractions, with the IC_{50} value of 6.01±0.03 µg/mL. Subfraction 6 (AAL.E.5.6) had an IC_{50} of 6.61 ± 0.03 µg/mL.

Table 1. The IC_{50} Values of subfractions from AAL.E.5.6 – AAL.E.5.10.

Sample	IC_{50} (µg/mL)
AAL.E.5.6	6.61 ± 0.03
AAL.E.5.7	6.97 ± 0.03
AAL.E.5.8	6.01 ± 0.03
AAL.E.5.9	9.39 ± 0.01
AAL.E.5.10	6.80 ± 0.02

Malate quinone oxidoreductase (*Pf*MQO)

All subfractions (AAL.E.5.1 – AAL.E.5.11) were screened for inhibitory potential against *P.falciparum* malate quinone oxidoreductase (*Pf*MQO) at 10 µg/mL (Figure 3).

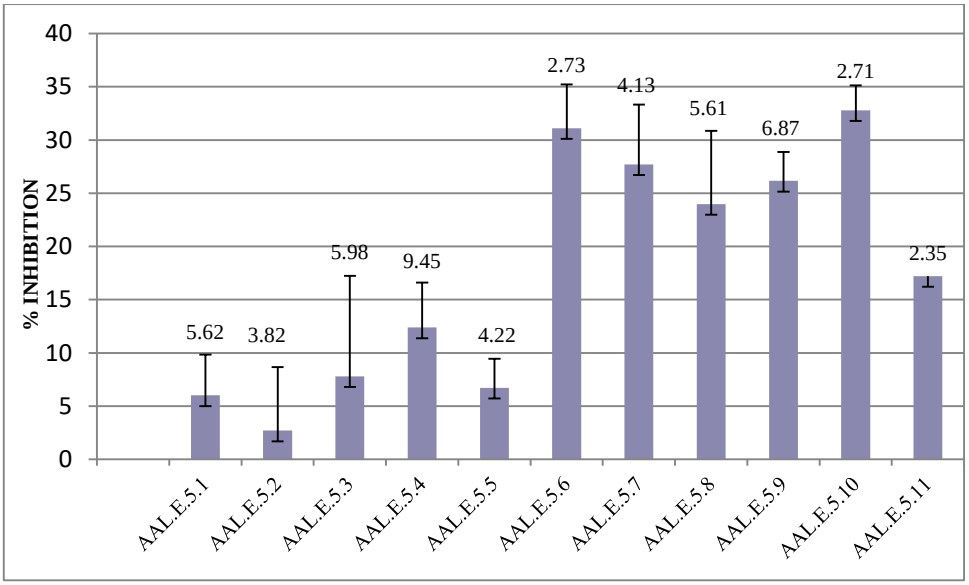

Figure 3. Inhibition of Parasite Growth by *Pf*MQO assay at 10 µg/mL from AAL.E.5.1 – AAL.E.5.11 with duplicate measurement

Therefore, the subfractions with the best inhibition were taken further to determine their IC_{50}. According to the findings, subfractions 6 (AAL.E.5.6) and 10 (AAL.E.5.10) exhibited medium IC_{50} values of 20.34 and 16.35 (g/mL), respectively. (Table 2).

Table 2. The IC_{50} value of AAL.E.5.6 – AAL.E.5.10

Sample	IC_{50} (µg/mL)
AAL.E.5.6	20.34 ± 0.99
AAL.E.5.10	16.35 ± 0.98

It can be claimed that the antimalarial activity was moderate when the IC_{50} was between 10 and 50 µg/mL. Subfraction 6 (AAL.E.5.6) and subfraction 10 (AAL.E.5.10) showed decreased MQO enzyme activity. Subfraction 10 displayed the highest inhibitory activity (AAL.E.5.10). These findings demonstrate that subfractions 6 (AAL.E.5.6) and 10 (AAL.E.5.10) displayed the best *Pf*MQO enzyme inhibition activity compared to the other subfractions because higher inhibition percentages resulted in lower enzyme activity, whereas lower inhibition percentages resulted in higher enzyme activity. The inhibitory process was performed by blocking the activity of *Pf*MQO. Hartuti *et al*. (2018) assumed that chemical ubiquinone binds to succinate dehydrogenase in mammals by inhibiting *Pf*MQO.

Identification of subfraction 6 (AAL.E.5.6)

The UV spectra of AAL.E.5.6 were identified using high-performance liquid chromatography (HPLC). The

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maximum absorption was observed in the UV spectra for AAL.E.5.6, at wavelengths of 215 nm and 272 nm (Picture 4). The U.V. spectra of the majority of flavonoids exhibit two substantial absorption bands: band I in the range of 300 – 400 nm and band II at 240-285 nm (Markham & Mabry, 1975)

The typical absorption peaks of the flavan-3-ols, proanthocyanidins, and dihydrochalcone classes of flavonoid compounds were visible in the UV spectra of the primary peak. While flavanones and dihydroflavonols also have modest shoulders in band I at approximately 320 nm, proanthocyanidins, dihydrochalcones, and flavan-3-ols generally exhibit an absorption maximum in band II between 270 and 290 nm. This is because there is less conjugation between the B ring and other molecules, which results in decreased absorption of the I band (no double bonds other than the B ring). Except for anthocyanins and some aurons, the maximal absorption bands of most flavonoids I and II were below 400 nm. It can be inferred that the UV-VIS spectrum is frequently substituted when the term "UV flavonoids" is used (Santos-Buelga *et al*., 2003; Marston and Hostettman, 2006) (Figure 4).

Identification of subfraction 10 (AAL.E.5.10)

Then, using an HPLC Shimadzu LC-6AD, an analysis was carried out to determine the profile of the chromatogram compounds in subfraction 10 (AAL.E.5.10) using a stationary phase LiChrospher® 100 RP-18 (5 m) analytical column with a concentration of 1 mg/ml, flow rate of 0.5 mL/min, and isocratic solvent mixture of acetonitrile: water (3:7 v/v) (Figure 5). The HPLC chromatogram revealed a strong peak in subfraction 10 at a wavelength of 210 nm. *Pf*MQO is thought to be actively inhibited by this peak, which has a retention duration of 2.4 minutes. Flavonoids, especially dihydrochalcone compound as mentioned above, are said to have a UV spectrum with an absorption maximum at 280 nm (Figure 6 and 7).

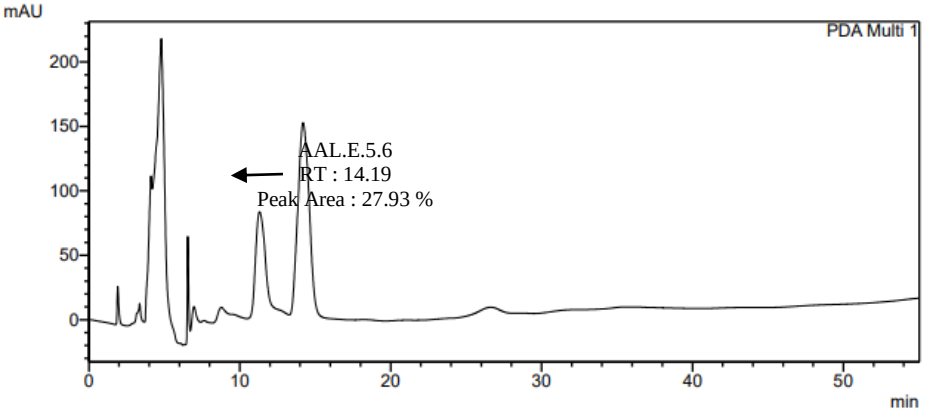

Figure 4. HPLC profile of AAL.E.5.6. The with acetonitrile: water (3:7 v/v) as mobile phase at a flow rate of 0.5 mL/min, injection volume 40 µL and the major peak was observed

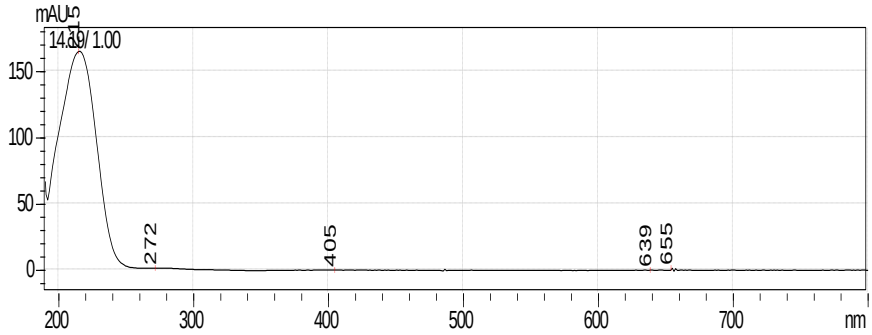

Figure 5. UV-Visible spectra of major peak of AAL.E.5.6

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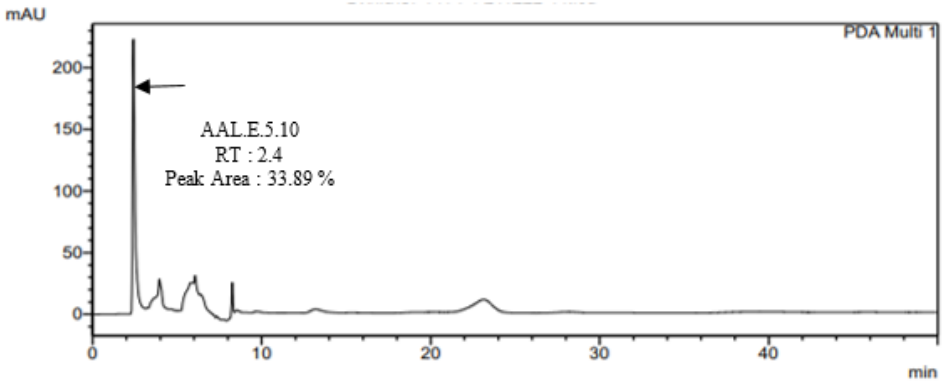

Figure 6. HPLC profile of AAL.E.5.10. The with acetonitrile: water (3:7 v/v) as mobile phase at a flow rate of 0.5 mL/min, injection volume 40 µL and the major peak was observed

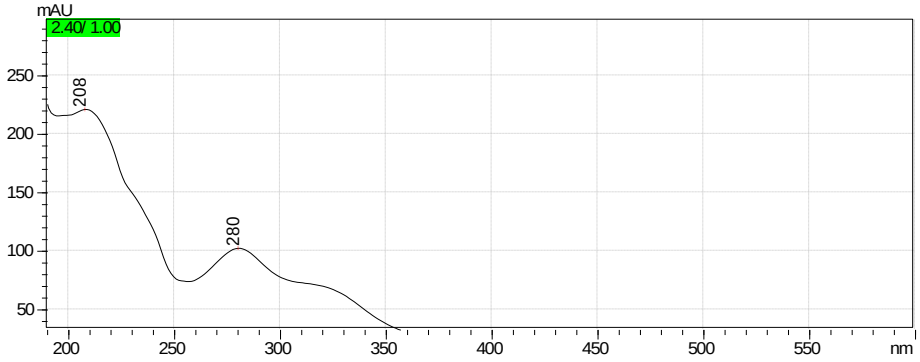

Figure 7. UV-VIS chromatogram of AAL. E.5.10

CONCLUSION

Artocarpus altilis subfraction 8 (AAL.E.5.8) had the most potent antimalarial activity among all the subfractions. However, subfraction 6 (AAL.E.5.6) showed antimalarial efficacy and *Pf*MQO enzyme inhibition. It may be assumed that the antimalarial activity against *P. falciparum* and *Pf*MQO enzyme inhibition may be due to the presence of important plant secondary metabolites, flavonoids. However, further research is needed to confirm this hypothesis. Flavonoid compounds in AAL.E.5.6 that were detected by yellow spots on TLC and UV profiles at a wavelength of 272 nm on HPLC are encouraged to proceed further for isolation to develop pure compounds that can subsequently serve as antimalarials.

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AUTHOR CONTRIBUTIONS

Conceptualization, A. W., S., A. F. H..; Methodology, A. W., E. K., H. I., L. T.; Software, H. I.; Validation, A. W., H. I.; Formal Analysis, E. K., H. I.; Investigation, A. R. H., E. K., M. W., L. T.; Resources, A. W., A. F. H.; Data Curation, E. K., H. I., A. R. H., M. W., L .T.; Writing - Original Draft, E. K.; Writing - Review & Editing, A. W., S., E. K., H. I.; Visualization, E. K., H. I.; Supervision, A. W., S.; Project Administration, A. W., A. F. H.; Funding Acquisition, A. W.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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