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Antibacterial and *in vivo* Antiplasmodial Activities from *Garcinia lasoar*

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Cycloartane type-triterpenoid was isolated from ethyl acetate of *Garcinia lasoar* stem bark extract. The structure was elucidated by extensive IR, ^{1}H and ^{13}CNMR techniques as 3-acetyl-3-oxo-cycloart-24-en-26-oic acid (OC). Antibacterial activities of extract and OC were also evaluated for *Staphylococus aureus* and *Escherichia coli* with chloramphenicol and ampicillin as positive control. The extract and OC showed antibacterial activities against *S.aureus* with IC_{50} values of 32.36 µg/mL and 0.0005 µM, respectively. The *in vivo* antiplasmodial activity of stem bark extract also analysis use four groups of *Mus musculus* were infected by *Plasmodiumberghei*. The animal treated with extract showed suppression of parasitemia to 62.72±0.01% compared with the *P. berghei* infected-mice (negative control) with ED_{50} value of 68.98 mg/kg BW. The dose of extract in 200 mg/kg BW was more potential to reduce parasitemia of infected mice with *P.berghei*. The ethyl acetate extract of the stem bark *G.lasoar* and OC have antibacterial and antiplasmodial properties therefore, investigation is necessary to evaluate their mechanism of action.

Keywords: antibacterial; antiplasmodial; cycloartane*; Garcinia lasoar; M.musculus, P.berghei*

I. INTRODUCTION

Garcinia species are known to contain secondary metabolites xanthones, benzophenones, flavonoids, stilbene, triterpenoid and depsidone (Nilar *et al.,* 2005). The components showed that various of bioactivities, e.g. freeradical scavenging, anti-inflammatory, antibacterial, antimalarial, and antidiabetes (Minami *et. al.,* 1994; Vlietinck *et. al.,* 1998; Merza *et. al.,* 2006; Cos *et. al.*, 2006; Chin *et. al.,* 2008; Elfita *et. al.,* 2009; Fatmawati *et. al.,* 2014; Sukandar *et. al.,* 2016; Kainama *et. al.,* 2019; Kainama *et al.,* 2020). Several flavonoids have been reported active as anti-HIV and anticancer (Gustafson *et. al.,* 1992; Williams *et al.,* 2003).

Garcinia lasoar (locally named "manggustang utang" in Ambon-Indonesia) can be found in primary forest of Moll. In some Maluku communities, the stem bark of *G. lasoar* used in the treatment of malaria as endemic area, tumour, antibacterial and diabetes but this has not been scientifically verified. This study reports the result of isolation of major compound from ethyl acetate of stem bark *G.lasoar*. Furthermore, have been done *in vitro* antibacterial activity of the major compound and antimalarial potential of the extract stem bark *G.lasoar in vivo* at *Plasmodium berghei* (*Pb*) infected mice model.

II. MATERIALS AND METHOD

The *G.lasoar* was collected from Hatu Village Maluku, Indonesia. Taxonomical identification of plant with voucher

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specimen number is 51 has been done in Laboratory of Biology, Universitas Pattimura, Indonesia.

A. Preparation of extract and isolation OC

The 5.2 kg dried stem bark of *G.lasoar* was extracted with 3 × 7 L of ethyl acetate by maceration for 3 x 24 hours. The solvent was evaporated to yield 192.0 g of brown crude extract. Furthermore, the crude extract separated into vacuum liquid chromatography (VLC) on silica gel (300 g) using *n*-hexane, CH_2Cl_2, EtOAc, and MeOH, respectively. The separation for each 3 × 1.5 L to get *n*-hexane (HxF), dichloromethane (DF), ethyl acetate (EAF), and methanol (MF) fractions. DF (20.49 g) separated then through VLC-2 on silica gel (100.0 g) with gradient of ethyl acetate: *n*-hexane (1:99, 3:97, 5:95, 7:93, 100:0, v/v, each 500 mL) to obtain R,S and T fractions. The fraction R (13.5 g) was separated by VLC-3 on silica gel (150.0 g) with gradient of ethyl acetate: *n*-hexane (5:95, 10:90, 15:85, 50:50, 70:30, 100 v/v, each 500 mL) to obtain four fractions (R1-R4). Sub fraction R2 (3.7g) was separated to column chromatography (CC) on silica gel (15.0 g) with a gradient of ethyl acetate and dichloromethane (1:99,5:95,30:70,50:50,100 v/v, each 100 mL) to obtain R2.1-R2.10. Purification subfraction R2.9 with *n*-hexane in hot condition (3 x 20 mL) to yield OC (316.1 mg) (white powder, m.p 154-155^{o}C) dissolved in $CHCl_3$.

B. Antibacterial Activity

The *Escherichia coli* (MTCC 443) and *Staphylococus aureus* (MTCC 737) used in this study. Preparation of inoculums have followed Nzogong *et al.* (2018) with a slight modification. To find out antimicrobial activity then according to the dilution method by Arias *et al.* (2004). Each sample of 50µL suspension of *S. aureus E.coli* with a concentration of 10^4 CFU/mL were added to the 5 µL sample in the Falcon tube which contained 445 µL NB media. Then the sample was incubated in an incubator shaker for 18 hours at 37^{o}C. Antimicrobial activity was calculated at a wavelength of 630 nm with 96 microplates well readers in which positive controls were ampicillin and chloramphenicol, negative controls in the form of DMSO and blanks in the form of a mixture of samples and media. The percent of inhibition according to Equation (1):

$$\% \text{ inhibition} = \frac{OD_{630} \text{ negative control} - OD_{630} \text{ sample control}}{OD_{630} \text{ negative control}} \times 100 \quad (1)$$

C. In vivo Antiplasmodium Assay

M.musculus weighing 20-24g were obtained from the Zoology Laboratory Universitas Pattimura, Indonesia. The animal housed, fed and given water following the method by de Souza *et al.* (2017). All ethical protocols used for the study were approved by the Department Ethics Committe of Universitas Airlangga-Indonesia. The acute toxicity evaluation, induction of the experimental malaria model, determination of parasite density and parasitemia suppression following the guidelines of method by de Souza *et al.* (2017). The percent of parasitemia according to Equation (2).

$$\% \text{ parasitemia} = \frac{\text{quantity of parasitised erythrocytes}}{500} \quad (2)$$

III. RESULT AND DISCUSSION

A. Structure Elucidation of OC

The isolation of OC reported for the first time from stem bark *G.lasoar*. White amorphous solid showed data of IR (KBr): υ_{max}: 3431, 2968,1728,1652, 1261,1033 cm^{-1}. The structure of OC was establish using IR, 1D and 2D NMR (HMQC, HMBC, dqf QOSY, DEPT) spectroscopy techniques. The analysis HMQC and DEPT of ^{13}C-NMR resonances of OC showed 32 carbon signals into seven methyl, eleven methylene, six methine and eight quaternary carbons. The presence of a non-conjugated carbonyl (216.0), a carboxyl (171.89), a double bond (CH-group at 142.26, and chemical shift 127.99 with quaternary carbon), and a cyclopropyl part (quaternary carbons at 20.76 and 25.97; a CH_2-group at 28.22) were clearly explained the cycloartenone-like structure. The data on the above (Table 1) showed that backbone of OC to be a cycloartane-type triterpene (Souza *et al.,* 2017). In the ^{1}H-^{1}H QOSY and HMQC experiments for this compound showed, four fragments were established according to ^{1}H-^{1}H spin system of H-1-H2/ H-5, H-6/ H-6, H-7/ H-7, H-8/ H11- H-12/ H17,H20/ H20,H21/ H-20, H22/H-22, H-23/ H-23, H-24 represent in bold lines (Figure 2). The HMBC correlation of CH_3-28 and CH_3-29/ C-5,H5/. Olefinic proton (δH-24 6.71) and two olefinic carbons (δC-24 142.26 and δC-25 127.99)

have shown existence of trisubstituted olefin group. The HMBC correlations between methyl protons (δH-27 1.27) and the olefinic carbons together with the carboxylic carbons (δC-26 171.89) has proven it is subtitution a methyl on C-25 (Figure 1). The above result, the presence of an olefinic absorption band in its IR spectrum. The data of ^{1}H and ^{13}C NMR spectrum similar to literature is triterpenes type cycloartane that is compound 23-acetyl-3-oxo-cycloart-24-en-26-oic acid (OC) from *Mangifera indica* (Anjaneyulu *et al.,* 1999).

Table 1. ^{1}HNMR (500 MHz, $CDCl_3$) and ^{13}CNMR (125 MHz $CDCl_3$) data of OC (δ in ppm, *J* in Hz)

Position	δ_C	δ_H (mult,*J*)
1	33.39, CH_2	1.62, *m*
2	37.44, CH_2	2.31, *d* (2.4)
3	216.60, C	-
4	48.81, C	-
5	48.39, CH	1.56, *m*
6	21.48, CH_2	1.40, *m*
7	26.64, CH_2	1.68, *m*
8	50.22, CH	1.52, *m*
9	20.76, C	-
10	25.97, C	-
11	25.82, CH_2	1.34, *m*
12	32.80, CH_2	1.68, *m*
13	45.48, C	-
14	47.80, C	-
15	35.47, CH_2	1.35, *m*
16	29.48, CH_2	1.95, *m*
17	52.56, CH	1.52, *m*
18	18.28, CH_3	0.98, *s*
19	28.22, CH_2	0.61, *d* (4.0); 0.81, *d* (4.0)
20	32.65, CH	1.68, *m*
21	18.03, CH_3	0.75, *d* (22.4)
22	40.23, CH_2	1.56, *m*
23	68.94, CH	5.64, *m*
24	142.26, CH	6.71, *dd*(1.2;1.2)
25	127.99, C	-
26	171.89, C	-
27	12.53, CH_3	1.27, *s*
28	19.22, CH_3	1.21, *s*
29	21.08, CH_3	1.02, *s*
30	21.02, CH_3	0.91, *s*
$\underline{C}OCH_3$	170.57, C	-
$CO\underline{C}H_3$	22.17, CH_3	2.1, *s*

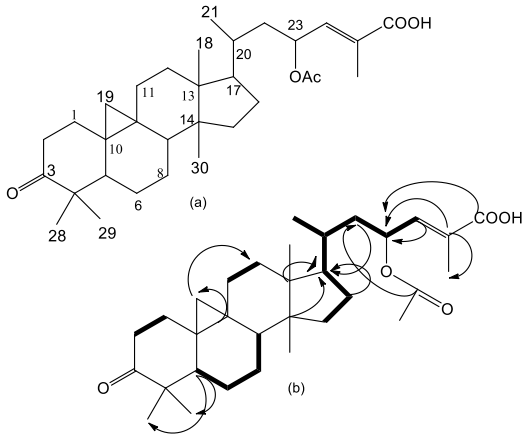

Figure 1. (a) Structural of OC; (b) Selected HMBC (H→ C) and ^{1}H- ^{1}H dqf COSY (━) correlation of OC

B. Antibacterial Activity of Extract and OC

The screening antibacterial activities of ethyl acetate extract (EaE) and OC were treated for both of *E.coli* and *S.aureus* by agar dilution method. The inhibition of both is compared to positive control. EaE, OC and positive control showed that similar to of activity inhibition of *S.aureus* in concentration of 500 µg/mL. The OC inhibits *S.aureus* (96.60±0.04%) at a concentration of 250 µg/mL higher than the EaE (Figure 2A). Conversely, at the same concentration, EaE was able to inhibit *E. coli* 57.73 ± 0.05%) (Figure1B). Furthermore, the lowest concentration (31.12 µg/mL) of inhibition EaE at *S.aureus* decreased to 50% from OC. The similar to condition occurs in OC for inhibition of *E. coli* (Figure 2A, Figure 2B). The values of the IC_{50} presented by substances against pathogenic bacteria *S.aureus* and *E.coli* are showed in Table 2. The IC_{50} values obtained for the tested substances in concentration 500-31.12 µg/mL. EaE and OC exhibited strong against *S.aureus*. Antibacterial capability of OC higher than EaE which is IC_{50} values 0.0005 µg/mL and 32.36 µg/mL, respectively. However, EaE and OC were low against *E.coli*, with IC_{50} > 100 µg/mL (Table 2).

In the antibacterial assay showed that *E.coli* resistant to both of EaE and OC. It is was probably to result due to structural differences of OC and components in EaE (organic substances) at the outer membrane of bacteria *E.coli*. The Gram-positive *S.aureus* possess single peptidoglycan layer

structure which both of substances can penetrate the cell wall (Girma *et al.,* 2015). The increased hydrophobicity of membrane can lead to interaction with zwitterionic membranes (eukaryotic and Gram-negative membranes) (Mandal *et al.,* 2015). The OC with less hydrophobic and less bulky than EaE have hydrophilic face possibly more interactive between the negative charged *S.aureus* membrane.

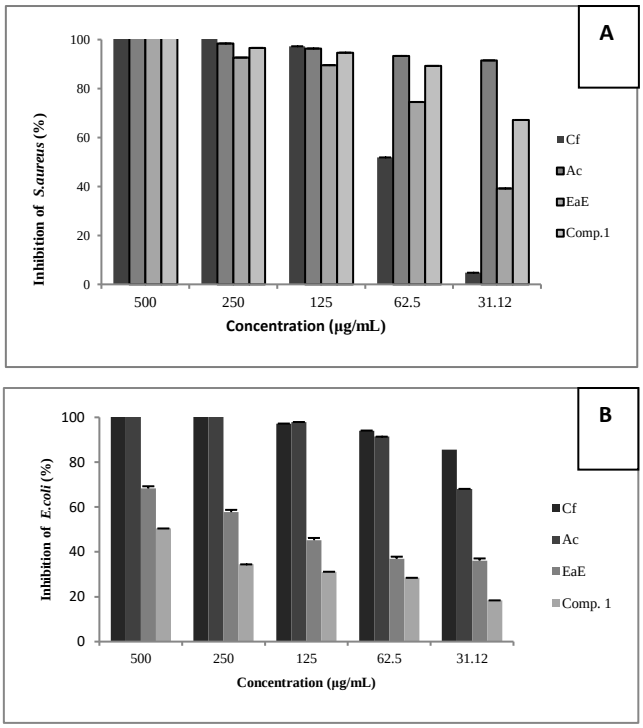

Figure 2. Inhibition (%) of bacterial (A) *E. coli* and (B) *S.aureus*. Each bar represents the mean ± SD inhibition of bacterial at different concentration (500-31.12 µg/mL). Significantly different (P<0.05). Abbreviation: Cf:Chloramphenicol; Ac:ampcilin; EaE: extract; comp 1: OC.

This can improve the electrostatic effect so as to increase activity. The structurally of this compound containing double bond at C-24, the C-26 carboxylic group and ketone function in A ring are effect important for antibacterial activity (Hsouna *et al.,* 2011). The OC isolated in this study can potentially acts as a lead compound in the development of more potent antibacterial against *S. aureus*.

Table 2. IC_{50} value of antibacterial activities of *G. lasoar* extract and OC

Sample/ Standard	Antibacterial activity IC_{50} (µg/mL*; µM**)	
	S.aureus	*E.coli*
EaE	32.36 ± 0.04*	>100
OC	0.0005±0.50**	>100
Ampicillin (Positive control)	0.00007±0.03*	3.25 ± 0.03*
Chloramphenicol (Positive control)	1.49 ± 0.05*	0.21 ± 0.07*

C. In vivo Antiplasmodial Activity of G.lasoar

In this study, the treatments of maximum dose EaE *G.lasoar* not cause significant symptoms acute toxicity. Observation of changes the body weight of mice were carried out related to amount of parasitemia in mice.

Table 3. Effect of EaE *G. lasoar* on the mean body weight in *P.berghei* infected mice

Treatment	Body weight (g)		Changes in body weight (g)
	Initial	Final	
G1	20.17 ± 0.05	19.84 ±0.11	-0.32 ± 0.06
G2	20.58 ± 0.39	20.42±0.47	-0.16 ± 0.10
G3	21.25 ± 0.12	21.20 ±0.11	-0.05 ± 0.02
G4	21.93 ± 0.29	21.92±0.36	-0.01 ± 0.01

Infected only (G1); Infected + EaE dose 10 mg/kg BW (G2); Infected + EaE dose 100 mg/kg BW (G3); Infected + EaE dose 200 mg/kg BW(G4). Values are given as mean ± SD (n=3); Significantly different from normal (P < 0.05).

Table 3 showed that *P.berghei* infection in all groups of mice could cause significant weight loss (P<0.05) within the normal range. Thus, it can be said that the number of parasitemia or the number of red blood cells *P.berghei* infected can effect changes in body weight of mice.

The results showed that the growth of *P.berghei* was higher in the G1 than the treatment EaE *G.lasoar* group (G2 to G4). The parasites in blood samples post-inoculation (4^{th} day) showed level growth of parasite in the mean percentage of G1, infected only (48.3 ± 0.007%) higher than G2 (31.0 ± 0.01 %), G3 (29.30 ± 0.01%), G4 (16.20 ± 0.01%) (Figure 3). These data can show the effect of EaE on *P.berghei* infected with *M.musculus*. The inhibition growth of parasite activity (%) according to increasing dose EaE *G.lasoar*. Parasite growth inhibitory activitiy can be described as G4>G3>G2. The G4 can be classified as higher activity (62.72 ± 0,01 %) and G1 (negative control) showed that most active growth of parasite.

The results calculation ED_{50} value of EaE *G.lasoar* is 68.98 mg/kg BW which can provide information that extract classified as a moderate antiplasmodial.

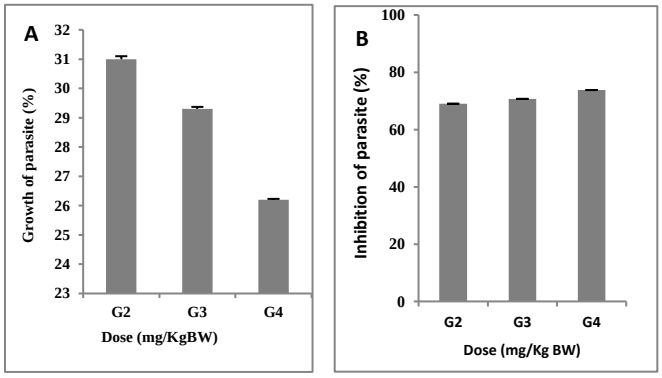

Figure 3. EaE *G.lasoar* Dose treatment (A) Growth of parasite (%) and (B) Inhibition parasite (%) in *M. musculus*. Significantly different (P < 0.001) in level parasitemia of G1 compared to G2-G4.

ANOVA test and LSD analysis (Figure 3 and Figure 4) showed that a significant differences in decrease of parasitemia level according to quantity of the dose. The levels (P<0.05) are G2 (-0.11± 0.02%), G3 (0.11 ± 0.04%), G4 (0.07 ± 0.05%) compared to G1 (0.01 ± 0.02%). The same condition was found among the treatment groups (G2 to G3). However, G3 and G4 showed no significant differences so that the use of a dose 200 mg/kg BW would have the same effect as 400 mg/kg BW.

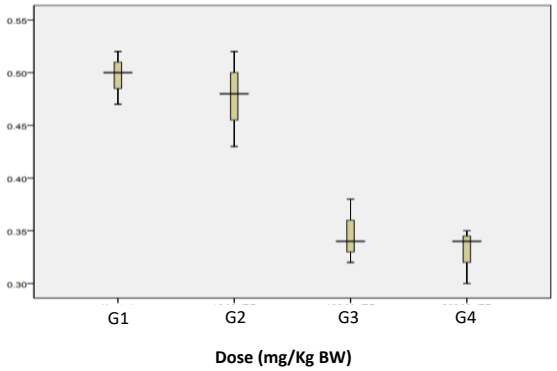

Figure 4. Reduce of parasitemia (%) of EaE *G.lasoar*

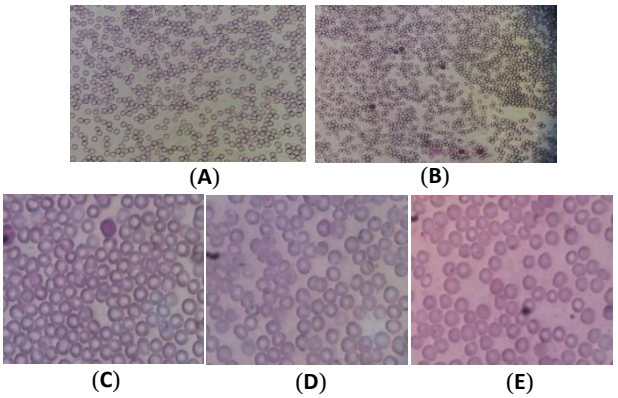

Figure 5. Microscopic evidence of erythrocytic (A) Negative control; (B) Positive control; (C) Dose 10 mg/kg BW; (D) Dose 100 mg/BW; (E) Dose 200 mg/BW

The number of trofozoites and schizons in erythrocytes can give an indication of the level parasitemia (Kainama *et al.,* 2019). Figure 5 shows the microscopic erythrocytes of *M.musculus* infected then EaE *G. lasoar* treatment. The dose of 100 mg/BW (Figure 5D) and 200 mg/BW (Figure 5E) gave better condition than the dose of 10 mg/kg BW (C).

D. Correlation between Phytochemical of Extract and Bioactivities

Phenolic and flavonoid compounds mostly found in plant are reported to have numerous biological effects including antibacterial and antiplasmodial. It is widely accepted that activities of plant materials are strongly linked to the concentration of total phenolic and total flavonoid. The phenolic and triterpenes in the EaE of *G.lasoar* (Kainama *et al.,* 2020) estimated be it against *P.berghei* at *in vivo* antiplasmodial which it was inhibition of mitochondrial activities (Bantie *et. al.,* 2014; Chander *et al.,* 2016).

The antibacterial and antimalarial activity were correlated with the presence of secondary metabolites in EaE (Nick *et al.,* 1995). The data both of total phenolic content (TPC) and total flavonoid content (TFC) of stem bark extract of *G. lasoar* not showed in this report but Pearson's correlation coefficient (*r*) can be made as in Table 4. There is positive correlation (*r*:0.519) between TPC and *S.aureus* antibacterial activity, statistically significant p<0.001. The high positive correlation between TFC and *S. aureus* antibacterial activity (*r*:0.989). Otherwise, there are weak correlation (*r*) between antibacterial activity with TPC even negative correlation of TFC-*E.coli*. The result study showed that positive correlation between TPC-antiplasmodial activity also TFC-

antiplasmodial activity of EaE. The significant contents of total phenolic and flavonoid in the extract, this would strengthen the evidence for a high of *S.aureus* antibacterial and antiplasmodium activities.

Table 4. Pearson's correlation cooficient (*r*) between phytochemical of extract and bioactivities

	TPC	**TFC**	***Ec***	***Sa***	**AP**
TPC	1*				
TFC	0.654***	1*			
Ec	0.283*	-0.317**	1*		
Sa	0.519*	0.989*	0,020*	1*	
AP	0.729*	0.654*	0.774*	0.562*	1*

TPC: Total phenolic content; TFC: Total flavonoid content; *Ec*:*E. coli*; *Sa*; *S. aureus*; AP: antiplasmodial Significant different *: P<0.001; **: P<0.01; ***: P<0.05.

Correlation coefficient can provide confirmation that triterpenoid and the phenolic compounds are the main constituents contributed to the antibacterial and antiplasmodial activity of these EaE. It is also possible that the synergistic effect among to the bioactive lead to increasing activity of the extract.

IV. CONCLUSION

The study investigated the potential significance of extract from *G.lasoar* and isolated major compound (OC) in the development of source antibacterial and antiplasmodium, following the widespread use of this species in traditional medicine. The discovery of active compound against *S.aureus* and *in vivo* method extract has good activities as antiplasmodial. Furthermore, that can be used as antibacterial antimalarial drugs alternative or as a combination with availability other.

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