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ANTIMALARIAL ACTIVITY AND TOXICITY EVALUATION OF THE ALKALOID-RICH FRACTION OF *MOMORDICA CHARANTIA* FRUITS

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ABSTRACT: Introduction: We investigated to an *in-vitro* and *in-vivo* evaluation of antimalarial and toxicity of the alkaloid-rich fraction of *Momordica charantia* fruits. **Materials and Methods:** *In-vitro* and *in-vivo* antimalarial activity tests of *Plasmodium falciparum* strain 3D7 culture were performed on *Plasmodium berghei*-infected mice using suppressive and prophylactic methods, and evaluation of fraction toxicity was conducted on zebrafish (*Danio rerio*). **Results and Discussion:** The study found that IC_{50} value of the *in-vitro* test was 0.17 ± 0.12 µg/mL and anti-malaria test using a suppressive and prophylactic method at a dosage of 100 mg/kg/day was found to inhibit parasites by 74 and 73%, respectively. The evaluation of toxicity by the use of zebrafish revealed abnormalities in the formation of the heart (51.28%) and yolk sac (51.28%). **Conclusion:** Alkaloid rich-fractions have powerful *in-vitro* and *in-vivo* antimalarial activity, but they have teratogenic effects that can inhibit the formation of the heart and yolk sac.

INTRODUCTION: Malaria is caused by parasites of the *Plasmodium* family and is transmitted by female *Anopheles* mosquitoes. There are four different species of human malaria (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*), of which *P. falciparum* and *P. vivax* are the most prevalent and *P. falciparum* is the most dangerous.

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Plasmodium knowlesi is a zoonotic plasmodium that is also known to infect humans. Between 2000 and 2015, the incidence of malaria among populations at risk fell by 37% globally; during the same period, malaria mortality rates among populations at risk decreased by 60%. It is estimated that 6.2 million deaths from malaria have been prevented globally since 2001. [1]

In 2011, the number of malaria cases in Indonesia was 256,592 people from 1,322,451 cases of suspected malaria blood preparations being examined, with annual parasite incidence (API) of 1.75 per thousand populations. This means that for every 1,000 persons in endemic areas, there are 2 people who have been infected with malaria.

The greatest burden of malaria is in the provinces of eastern Indonesia, where malaria has become endemic [2]. Currently, Indonesia Ministry of Health announced that malaria pre-elimination stage should be reached by 2020 and be free of malaria transmission by 2030 to achieve the goal of an Asia-Pacific free of malaria by 2030. [3]

Natural products and derivatives of the natural product have been a good source of new biologically active compounds for centuries. The first effective treatment for malaria was the natural product quinine, an alkaloid isolated from the bark of the South American *Cinchona* tree in 1817. [4] Alkaloids are one of the major classes of natural products that exhibit antimalarial activity.

Indeed, quinine, the first antimalarial drug, belongs to this class. Over 100 alkaloids from higher plants were reported to demonstrate significant antimalarial activity in studies published between 1990 and 2000; some of which are more potent than chloroquine [5]. The research was conducted to determine alkaloid compounds in bitter melon (*Momordica charantia*). Previous studies have found that extracts of bitter melon have the most powerful properties among any other plant extracts, which have been used as antimalarials in Sei. Kepayang area, Asahan, North Sumatra [6].

The objective of the Study: The objective of the study was to characterize the influence of alkaloid rich-fractions of bitter melon (*Momordica charantia*) of *in-vitro* and *in-vivo* evaluation of antimalarial and toxicity.

MATERIALS AND METHODS:

Preparation of Alkaloid-rich Fraction: Three hundred grams of dried *Simplicia* was alkalinized in 150 ml of 25% NH_4OH, then extracted using ml of methanol for 2 days, and then stored under room temperature. The liquid extract was filtered; the filtrate was thickened using a rotary evaporator. The thick extract was added with 15 ml of H_2SO_4 in aquadest and then filtered. The filtrate was extracted using petroleum ether. The liquid phase was added with 25% NH_4OH to obtain pH 9-10 and then extracted using chloroform. The chloroform was partitioned using aquadest up to normal pH (pH 7); the chloroform phase was taken and to obtain the alkaloid fraction.

Determination of Total Alkaloid: Two grams of extracts were carefully weighed, then extracted using 20 ml of methanol and 2 ml of ammonia, heated above a water bath for 30 min, and then filtered. The extraction process was replicated until the positive reaction against the alkaloid was stopped (negative alkaloid). The extract was added with 10 ml of 1 N hydrochloric acid; the extract was evaporated to a volume of 5 ml, and then filtered in a separate funnel.

The filtrate was alkalinized using sodium hydroxide up to pH 10 and then filtered several times using chloroform until the alkaloid was perfectly extracted. The chloroform extract was evaporated at a temperature of 50 °C and then dried at a temperature of 100 °C to obtain a normal weight. The total alkaloid was weighed [7].

***In-vitro* Antiplasmodial Assay:** *P. falciparum* 3D7, obtained from Tropical Disease Center, University of Airlangga, Surabaya was maintained in our laboratory with 5% hematocrit (human-type O-positive red blood cells) in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 25 mM HEPES, 370 µM hypoxanthine, 40 µg/mL 1 gentamycin, 0.25 $\mu g ml^{-1}$ Fungizone and 0.5% [w/v] AlbuMax II) in 60 mm petri dish by modified candle jar method [8]. The culture was routinely monitored through geimsa staining of the thin smears. Standard drugs (artemisinin and chloroquine) and the fraction-rich alkaloid bitter melon (*M. charantia*) at different concentrations of 1, 10, 100 µg/mL were prepared in DMSO and then diluted to achieve the required concentrations.

The synchronized culture with parasitemia of 1.5 and 3% hematocrit was incubated in 96-well microtitre plate, predisposed to multiple concentrations of compounds/extracts for 48 h at 37 °C in a candle jar. Blood smears from each well were fixed in methanol, stained with giemsa's stain and the numbers of infected RBCs per 5000 cells were counted.

The antimalarial activity of the test fraction was expressed as 50% inhibitory concentration (IC_{50}) determined from the dose-response curve by non-linear regression analysis (curve-fit) using Graph Pad Prism (version 4) software [9].

***In-vivo* Antiplasmodial Assay:**

Suppression Test Method: Male Albino mice (6 to 8 weeks old, weighing 20 ± 2 g) were used in the experiment. The mice were housed in standard macrolon type II cages in air-conditioned rooms at 22 °C, 50 to 70% relative humidity, fed with the standard diet and received water *ad libitum*. *Plasmodium berghei* strain ANKA was maintained by serial passage of the infected blood through the intraperitoneal injection (ip). The test protocol was based on the 4-day suppressive test [10].

Repository Test: The prophylactic activity of the extract was tested using the residual infection procedure. Adult mice of both sexes were weighed and randomized into five groups of six mice each. Group I mice was given 1 ml of normal saline. Groups II, III and IV were given 1, 10 and 100 mg fraction/kg of body weight orally, respectively while Group V mice received 5 mg of chloroquine /kg of body weight orally daily for 5 days. On the fifth day, all the mice were inoculated with a standard inoculum of 0.1 x 107 *P. berghei* berghei NK 65 infected erythrocytes. A thick film of blood smears was then made from each mouse 72 h after treatment and examined microscopically for parasitemia level [11].

Embryo-toxicity and Teratogenicity Assay: The assay established by Dulay *et al*., (2012) was followed in this study. Embryos were exposed to the different concentrations (100, 200, 300, 400, 500, 600, 700, 800 and 900 ppm) of each fraction, and embryo water served as the control in the 12-well ELISA plate. Four embryos were assayed per treatment, and each treatment was replicated three times. The plates were incubated at 26 ± 1 °C [12].

Toxic and teratogenic effects were examined using a compound microscope every after 12 h after exposure to treatment. Mortality, hatchability, heartbeat rate, and malformations were recorded. Death was defined as coagulated embryos and an absence of visual heartbeat. Morphological endpoint evaluation of zebrafish was based on the established parameters: lethal (coagulation, tail not detached, no somites, and no heart-beat), teratogenic (malformation of the head, tail, and heart, scoliosis, deformity of yolk, and growth retardation), and normal [12, 13]. The validity of the test was determined. Data were analyzed using analysis of variance (ANOVA) and compared using the Duncan's Multiple Range Test (DMRT) at 5% level of significance. The Sirichai Statistics 6.07 program was used for analysis.

RESULTS AND DISCUSSION: To administer better drugs against resistant strains of *P. falciparum* (and recently *P. vivax*), there is a need to search for new molecules from new sources [15]. Medicinal plants have in the past been the source of some of the most successful antimalarial agents, such as the quinolines and artemisinin derivatives, and can still serve as a source for drug discovery [16]. Previous research, which was conducted in SeiKepayang area, Asahan, North Sumatera, using ethnopharmacological approach, found 16 species of plants, which had been used as antimalaria [6]. *In-vitro* antiplasmodial activity test found an IC_{50} value of 0.017 μg/mL, whereas *the in-vivo* test found an ED_{50} value of 19.61 mg/kg BWT. Extract of bitter melon was found to have more powerful activity than that of any other plant [17]. *In-vitro* antiplasmodial activities of some extracts and fractions of alkaloid are presented in **Table 1**.

TABLE 1: RESULTS OF *IN-VITRO* ANTIPLASMODIAL ACTIVITY TEST OF SOME EXTRACTS AND FRACTIONS OF BITTER MELON

Extract/ drug	Alkaloid concentration (%)	IC_{50} value (μg/mL)
Ethanol 96%	4.44 ± 0.75	30.26 ± 3.96
Ethyl acetate	9.51 ± 0.85	2.47 ± 0.22
n-hexane	6.51 ± 0.95	7.80 ± 0.79
Alkaloid rich-fraction	10.75 ± 0.86	0.17 ± 0.12*
Choloroquine	-	0.18 ± 0.15*

The alkaloid concentration test showed the best results for the alkaloid fraction (10.31%), followed by the thick extract of ethyl acetate (9.51%), the thick extract of *n*-hexane (6.51%), and thick 96% ethanolic extract (4.44%). *In-vitro* antiplasmodial activity test using *P. falciparum* strain 3D7 showed the most powerful alkaloid fraction compared to any other extracts and comparable to chloroquine.

In-vitro antiplasmodial activity assay on the culture of *P. falciparum* strain 3D7 showed that the alkaloid fraction of bitter melon (*M. charantia*) had an IC_{50} value of 0.17 ± 0.12 μg/mL, a value that was comparable to that of positive control, namely quinine and artemisinin, which had IC_{50} values of 0.38 ± 0.15 μg/mL and 0.15 ± 0.13 μg/mL, respectively. A study conducted by Yousif (2014)

showed a powerful antiplasmodial activity of the chloroform fraction of bitter melon (*M. charantia*) with an IC_{50} value of 1.83 ± 0.029 µg/ml in the culture of *P. falciparum* strain MRC-2 [18]. Chloroform is a semi-polar solvent that can attract alkaloid compounds. The alkaloid extracts obtained from medicinal plant species have a multiplicity of host-mediated biological activities, including anti-malarial, antimicrobial, antihyperglycemic, anti-inflammatory and pharmacological effects [19]. Some alkaloids have antiplasmodial activities, which have been successfully isolated, including alkaloids from *Cyclea barbata*, *C. atjehensis*, *Stephania pierrei*, *S. erecta*, *Pachygone dasycarpa*, *Curarea candicans*, *Albertisia papuana* (Menispermaceae), *Hernandia peltata* (Hernandiaceae), & *Berberisval diviana* (Berberidaceae) that exhibited a wide range of biological potencies in antiplasmodial assays, and the majority exhibited some degree of cytotoxicity against human KB cells. More than half of the tested compounds showed selective antiplasmodial activity, with an SI of > 100-fold greater toxicity towards one or both of the *P. falciparum* clones (D6 and W2), relative to KB cells [15].

In animal models of malaria, large experimental variability of the results is associated with drug, parasite and host interactions. For ethical reasons, the number of animals used may not be increased to more accurately characterize the antiparasitic effects. Despite this low experimental reproducibility, the murine malaria model used herein is an important tool in anti-malarial drug discovery and development programs [20]. The 4-day suppressive test, which primarily evaluates the antimalarial activity of candidate agents in early infections, and the prophylactic test, which evaluates the capability of candidate extracts in established infections, is commonly used for antimalarial drug screening. In both methods, the determination of the percentage of inhibition of parasitemia is the most reliable parameter.

TABLE 2: SUPPRESSIVE ACTIVITIES OF FRACTION ALKALOID OF *M. CHARANTIA* FRUITS DURING EARLY *P. BERGHEI* BERGHEI NK 65 INFECTION IN MICE (4-DAY TEST)

Drug/fraction	Dose (mg/kg/day)	Average % parasitemia	Average % suppression
The alkaloid fraction	1	28.45 ± 0.35*	37
M. charantia fruit	10	22.12 ± 0.45*	51
	100	11.65 ± 0.25*	74
Chloroquine	10	6.89 ± 0.58*	84
Control (water)	0.2 ml	42.90 ± 0.85	-

Data are expressed as mean ± SEM for five animals per group; *P< 0.05 when compared with control

TABLE 3: PROPHYLACTIC EFFECT OF FRACTION ALKALOID OF *M. CHARANTIA* FRUIT ON PARASITAEMIA IN MICE

Drug/fraction	Dose (mg/kg/day)	Parasite count (%)	% Prophylactic
The alkaloid fraction	1	6.35 ± 0.34	38
M.charantia fruit	10	5.70 ± 0.45	44
	100	2.75 ± 0.67	73
Chloroquine	10	0.98 ± 0.09	90
Control (water)	0.2 ml	10.25 ± 0.21	-

The alkaloid fraction of *M. charantia* fruit exerted a dose-dependent chemo-suppressive effect against *P. berghei* berghei NK 65. The fraction gave a significant (P<0.05) chemo suppression of 37% for the low dose of 1 mg/kg and 74% for the highest dose of 100 mg/kg when compared to the control. The observed higher efficacy of the standard drug, chloroquine (84%), which was higher than fraction treated groups, may in part be due to nonselectivity of the fraction or slow absorption and poor bioavailability of the fraction. In the prophylactic study, the alkaloid fraction of *M. charantia* fruit significantly (P<0.05) exerted a dose-dependent reduction in the level of parasitemia in the fraction treated groups, while the standard drug chloroquine gives the highest effect. As shown in **Table 3**, prophylactic activities were slightly lower than the suppressive activities for alkaloid rich-fraction. At 100 mg/kg/day, the alkaloid rich-fraction, *M. charantia*, exhibited a high (73%) parasite reduction. Chloroquine (10 mg/kg/day) was used as reference drugs and showed a parasite reduction of 90%.

The prophylactic effect of the alkaloid fraction M. charantia had shown antiplasmodial activity against *P. berghei* infected mice with maximum parasitemia chemo suppression of 74% at a dose of 100 mg/kg body weight dose. Unlike the 4-day suppression, chloroquine 5 mg/kg body weight did not show 100% parasitemia eradication; rather, it inhibited 90% **Table 2**. The high parasite count in the prophylactic test both in the alkaloid fraction *M. charantia* and in reference standard drugs treated groups might be attributed to the rapid metabolism of the administered alkaloid fraction *M. charantia* and reference standard drugs (before inoculation) to inactive products where the fraction and reference standard drugs were initially administered for four days (prophylactic test) before inoculation with *P. berghei* parasite [21].

The zebrafish is a vertebrate animal model that is increasingly used for *in-vivo* drug toxicity and efficacy screening, and for assessing chemical toxicity and safety [22, 23]. In contrast to other vertebrate models, the zebrafish completes embryogenesis in the first 72 h. Advantages of the zebrafish embryotoxicity test include the detailed knowledge available about embryogenesis in zebrafish and the independence of embryo development from maternal fish. Also, transparency of embryos makes it easy to monitor and evaluate. Furthermore, development takes only three days from fertilization until hatching, and at these stages, the zebrafish embryo is not considered a laboratory animal under European legislation. These advantages make the zebrafish embryo model suitable for relatively high-throughput tests [24].

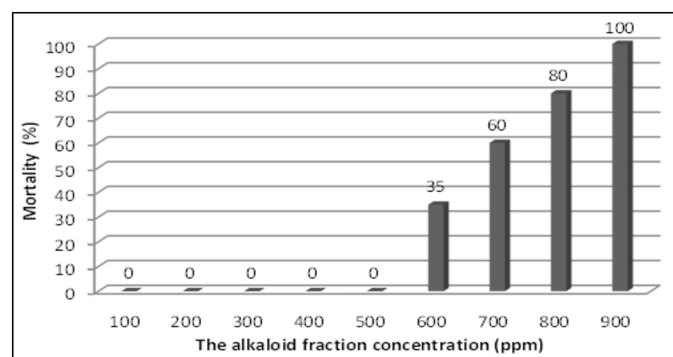

FIG. 1: MORTALITY OF *D. RERIO* EMBRYOS TREATED WITH ALKALOID RICH- FRACTION *M. CHARANTIA* FRUIT AT 96 h POST TREATMENT APPLICATION. THE MORTALITY OF EMBRYOS TREATED WITH 600 PPM OR HIGHER OF FRACTION WAS SIGNIFICANTLY HIGHER THAN THAT OF THE CONTROL EMBRYOS

Embryo-toxicity using *D. rerio* as a model is an important element for assessing the adverse effects of certain substance on cell structures and processes that are intrinsic to almost all cells. The toxic effect of the alkaloid fraction *M. charantia* fruit on *D. rerio* embryos was recorded, and the mean percentage mortality of embryos after 96 h of treatment exposure is shown in **Fig. 1**. It can be seen that the toxic effect of the alkaloid fraction *M. charantia* fruit was concentration-dependent, as evidenced by the increased mortality of embryos with a higher concentration of extract.

Fig. 1 shows alkaloid rich-fraction of bitter melon (*M. charantia*) fruits with high concentration (600-900 ppm). At a concentration of 600 ppm, embryos that hatched were only 65%; even at a concentration of 900 ppm, no embryo was hatched. Failure for the embryo to hatch might lead to the embryos' mortality. The highest mortality rate was seen at the concentrations of 700 ppm (60%), 800 ppm (80%), and 900 ppm (100%). **Table 4** and **Fig. 2** present the teratogenic effects on *D. rerio* embryo due to alkaloid rich-fraction exposure.

TABLE 4: TERATOGENIC EFFECT OF ALKALOID RICH FRACTION EXPOSURE ON ZEBRA FISH EMBRYO

Parts/ Body organ	Alkaloid rich fraction of *M. charantia* fruits	
	Σ^a	[%][b]
Axis	4	10.26
Brain	0	0
Caudal Fin	0	0
Circulation	12	30.77
Eyes	0	0
Heart	20	51.28*
Jaw	0	0
Otic	0	0
Pigmentation	0	0
Somite	0	0
Trunk	0	0
Yolk Sac	20	51.28*

[a] Number of embryos exposed to teratogenic effects at all concentrations and all treatment periods; [b], Number of embryos exposed to teratogenic effects /number of abnormal embryos at all concentrations and all treatment periods; *, Major malformations ($\geq$50%). An embryo may have more than one malformation.

Administration of alkaloid rich-fraction of *M. charantia* fruits induced teratogenic effects in the organs of the zebrafish embryo. This was evident with the malformation seen in the embryo. Mal-formation refers to the abnormal growth of an organ or tissue. Abnormality was seen in the

growth of pericardial area (51.28%) and yolk sac (51.28%), both of which were major malformations 50%, while the growth in the axial area (10.26%) and circulation (30.77%) were minor malformations. Major malformation began to be seen at a concentration of 600 to 800 ppm since 24 jpf.

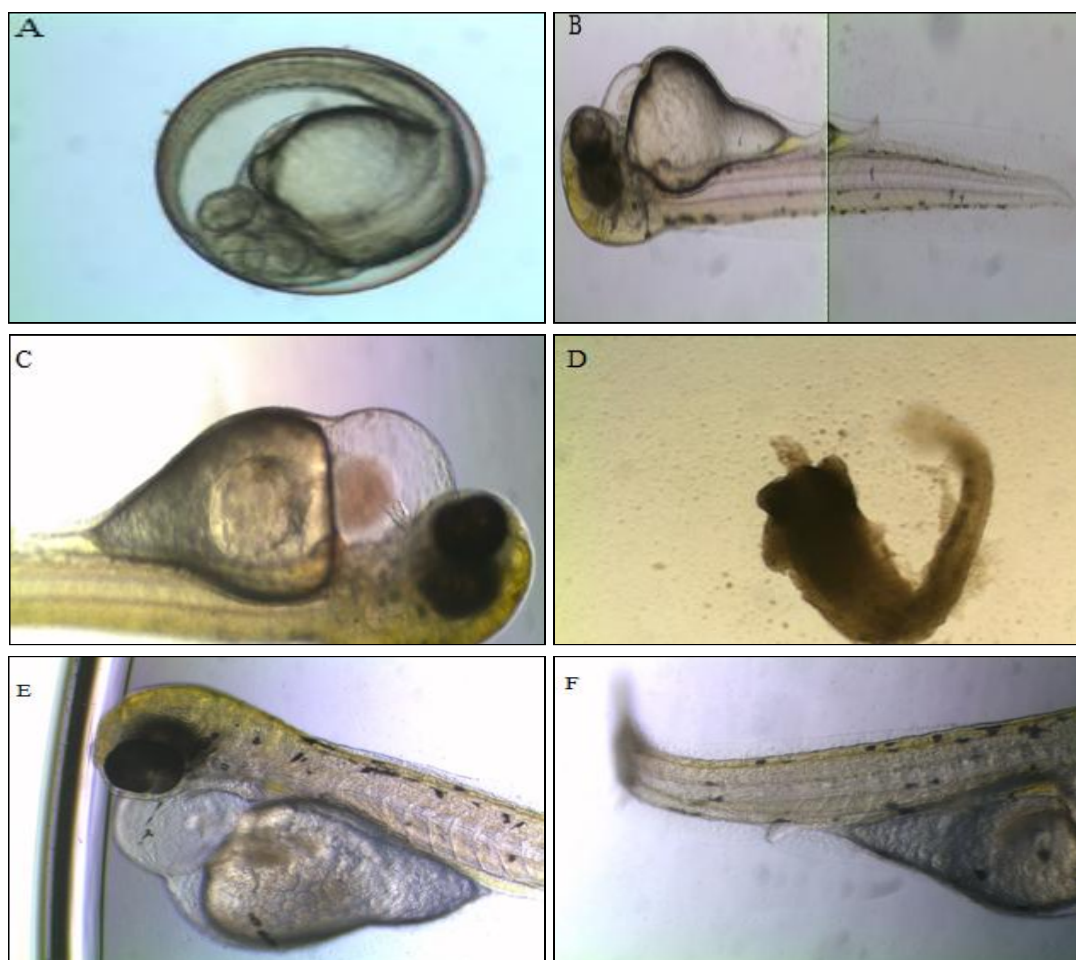

FIG. 2: MORPHOLOGY OF NORMAL ZEBRAFISH EMBRYO IN CONTROL GROUP, AND SOME ABNORMALITIES IN TREATMENT GROUP WITH ALKALOID FRACTION: (A) NORMAL EMBRYO K- 24 HPF, (B) K-48, (C) PERICARDIAL EDEMA (72 HPF, 800 PPM), (D) EMBRYO'S COAGULATION (96 HPF, 800 PPM), (E) PERICARDIAL EDEMA AND YOLK EDEMA (72 HPF, 800 PPM), (F) TAIL MALFORMATION (72 HPF, 800 PPM)

The present study provides the first examination of observable alkaloid fraction of bitter melon (*M. charantia*) toxicity on zebrafish development. Morphological developments such as the tail, head, embryonic movement, pigmentation, heart, yolk sac, and hatching were within the normal range when compared to the normal group. From the acute toxicity test, it was observed that the fraction alkaloid of *M. charantia* fish mortality rate increased with increasing concentration of the fraction alkaloid.

The zebrafish embryos that were exposed to sublethal concentrations of these extracts showed some developmental abnormalities. Most of the fraction alkaloid *M. charantia* induced abnormalities in developing heart and yolk sac in a dose-dependent manner. The embryos exposed to the extracts had cardiac edema with an enlarged cardiac chamber (cardiac hypertrophy) compared with the control. The major alkaloid in the betel nut, arecholine treated embryos, exhibited general developmental retardation in a dose-dependent manner by depletion of intracellular thiols; Birth Defects Research [23].

CONCLUSION: The result of this study showed that the fraction-rich alkaloid of bitter melon (*M. charantia*) have antimalarial potentials; therefore, the plant should be extensively investigated to isolate and identify their active antimalarial constituents.

This study also concludes that alkaloid fraction may lead to abnormal development of the heart and yolk sac in zebrafish.

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CONFLICT OF INTEREST: There are no conflicts of interest.

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