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Original Research Article

Antiplasmodial and onset speed of growth inhibitory activities of *Tithonia diversifolia* (Hemsley) A Gray leaf fractions against *Plasmodium falciparum*

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Abstract

Purpose: *To investigate the antiplasmodial and onset of growth inhibitory activities of T. diversifolia fractions against Plasmodium falciparum FCR3 strain.*
Methods: *Seven fractions of T. diversifolia (F1-F7) were used in this study. Phytochemical analysis was conducted to identify the major compounds in the fractions. Various concentrations of fractions ranging from 2.5 – 100.0 µg/mL were exposed to P. falciparum FCR3 strain for 60 h and the growth inhibition was then calculated. The fraction which exhibited the best antiplasmodial activity was tested further to determine the growth inhibition onset against P. falciparum FCR3 strain. This was achieved by examining the inhibitory activity of the fraction when it was added at the beginning of the experiment and assessing subsequent parasite growth after 8, 16, 24, 32, and 40 h incubation.*
Results: *The major compounds found in the fractions were terpenes. Fraction six (F6) had the best antiplasmodial activity (IC_{50} 13.63 ± 1.43 µg/mL). During the first 32 h of incubation, F6 inhibited the growth of parasites and this increased with longer incubation time; 32 h incubation provided the highest growth inhibition (99.23 ± 0.05 %). After 32 h the inhibition activity began to decrease, and resulted in < 50 % inhibition at 48 h incubation. This result suggested that F6 is a rapid-onset antiplasmodial agent.*
Conclusion: *Fractions of T. diversifolia, especially F6, are promising antimalarial agents and require further development for clinical application.*

Keywords: *Tithonia diversifolia, Plasmodium falciparum, Onset speed, Antimalarial, Antiplasmodial*

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INTRODUCTION

Mortality and morbidity caused by malaria are still a major health problem worldwide especially in tropical and subtropical countries. The most frequent causes of malaria are *Plasmodium vivax* and *Plasmodium falciparum*, but most death cases are caused by the latter [1]. An effective treatment is an essential part of malaria control, unfortunately there has been an increasing number of reports that chloroquine and its substitute, sulfadoxine-pyrimethamine, are not working against falciparum malaria in many places, for instance in parts of South-East Asia

and South America [2]. This situation encourages the development of new antimalarial drugs, for which one of the efforts is by utilizing natural resources empirically used as antimalarial treatments, such as *Tithonia diversifolia* (Hemsley) A. Gray.

Many studies have investigated the pharmacological effects of *T. diversifolia,* such as the antioxidant [3,4], antidiabetic [5], antimicrobial [6], antileishmanial [7] and other biological activities. The plant is a member of the genus *Angiospermae.* The phytochemical analysis showed that phenols, polyphenols [8], monoterpenes and sesquiterpenes [9] were found to be present in the plant. Ethnobotanical surveys have shown that ether extracts of aerial parts of *T. diversifolia* exhibit good antiplasmodial activity (IC_{50} of 0.75 µg/mL against the FCA strain) [10]. Other researchers discovered that the antiplasmodial effects of methanol extract (IC_{50} = 8.12 µg/mL) were better than chloroform (IC_{50} = 10.64 µg/mL) on *P. falciparum* FCR3 strain [11]. Another study separated the compound of the methanol extract by ether, and found that the ether-soluble extract of *T. diversifolia* had better antiplasmodial activity against the FCR3 strain of *P. falciparum* than the ether-insoluble extract [12].

The antiplasmodial activity of drugs is affected by the drug concentration and drug exposure duration [13]. The percentage of growth inhibition at different times of incubation (exposure) may determine the onset of the antiplasmodial action of the drug candidate. So far, studies on the effect of fractions made from the ether-soluble extract of *T. diversifolia* leaves against *P. falciparum* FCR3 strain have not been investigated, as well as the effect of different exposure duration on the growth of parasites. Therefore, this study aimed to investigate those important considerations. The fraction which revealed the best antiplasmodial activity was tested further on the growth of parasites at different durations of incubation to examine the onset speed of antiplasmodial activity of the fraction.

Therefore, this study investigated the *in vitro* effect of fractions of *T. diversifolia* leaves on the growth of *P. falciparum* FCR3 strain and examined the onset speed of growth inhibition of *T. diversifolia* fractions against the *P. falciparum* FCR3 strain.

EXPERIMENTAL

This was an experimental study with posttest-only control group design using the *P. falciparum* FCR3 strain as the subject. The preparation of *T. diversifolia* (Hemsley) A. Gray fractions was conducted in Pharmacology and Therapy Department and the Plasmodium culture was conducted in Parasitology Laboratory, Faculty of Medicine, Universitas Gadjah Mada. The plant was identified by a taxonomist, Djoko Santosa, at the Pharmaceutical Biology Department, Faculty of Pharmacy, Universitas Gadjah Mada. One week prior to testing, fresh leaves of *T. diversifolia* (Hemsley) A. Gray were collected from Sleman, Special Region of Yogyakarta, Indonesia. Study protocols were approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine of Universitas Gadjah Mada (no. KE/FK/18/EC).

Fractionation of plant material

Leaves of *T. diversifolia* were oven-dried at 50 °C and ground to coarse powder using clean laboratory blender. Powdered leaves (500 g) were extracted with 1 L of methanol by cold maceration and left for 72 h. The solvent was renewed every 24 h. The extract was filtered and evaporated to dryness at room temperature. Methanol extract was partitioned in separating funnel with ether to yield ether-soluble and insoluble extract [12]. The ether-soluble extract (20.80 g or 4.16 % of the powdered leaf weight) was then fractionated by using vacuum liquid chromatography with GF254 silica gel as the stationary phase, and mobile phases used were as follows: n-hexane (100 %), n-hexane : ethyl acetate (9:1 v/v), n-hexane : ethyl acetate (8:2 v/v), n-hexane : ethyl acetate (7:3 v/v), n-hexane : ethyl acetate (6:4 v/v), n-hexane : ethyl acetate (5:5 v/v), ethyl acetate (100 %) and chloroform : methanol (1:1 v/v). The resulting fractions were evaporated to dryness at room temperature and then identified with thin layer chromatography (TLC). Fractions with similar spots on TLC were united to become seven fractions (F1 - F7). The chromatogram was observed under UV light at wave length 254 and 366 nm and sprayed with cerium sulfate to detect terpenoids in those fractions. All of those fractions were used as samples for antiplasmodial assay.

Plasmodium culture

Plasmodium falciparum FCR3 strain (chloroquine-resistant) obtained from the Parasitology Laboratory were maintained according to modified methods of Tragen and Jensen [14]. Parasites were cultured in human O^+ erythrocytes suspended in RPMI 1640 medium supplemented human O serum. To get early ring stages, cultures were synchronized using 5 % sorbitol [15].

Antiplasmodial assay

Each fraction was dissolved in DMSO and further diluted with RPMI 1640 medium (the final concentration of DMSO did not exceed 1 % and of fraction were 2.5, 12.5, 25, 50 and 100 µg/mL). Antiplasmodial assays were performed in 96-well culture plates as described by Desjardins *et al* [16]. Each well was filled with 100 µL of medium (RPMI 1640 with HEPES, $NaHCO_3$, 10 % human serum) which contain 2 % of synchronized parasitemia and 3 % of hematocrit. Test compounds of 100 µL (from all fractions) were added to each well.

All tests were performed in triplicate and infected erythrocytes without tested compounds (contain RPMI medium) was used as negative control. The culture was placed in a candle jar and incubated in a 5 % CO_2 incubator at 37 °C for 60 h. Thin smears were made from infected erythrocytes with or without fractions. Parasitemia was calculated by counting the number of parasites per 1000 Giemsa-stained erythrocytes using light microscopy.

Determination of IC_{50} values

Percent growth inhibition (H) was calculated as in Eq 1.

H (%) = Pc - (Pt/Pc)100 …….. (1)

where Pt and Pc are parasitemia in test compound and control, respectively. The concentration at which growth was inhibited by 50 % (IC_{50}) was estimated by probit analysis SPSS program software. The fraction which presented the best antiplasmodial activity (lowest IC_{50}) was assessed for the onset speed of growth inhibition against the *P. falciparum* FCR3 strain.

Evaluation of antiplasmodial action speed of onset of *T. diversifolia* fractions

Erythrocyte suspensions at 2 % parasitemia and 3 % hematocrit with ring-stage synchronized parasites were distributed in triplicate into 96-well plates. The fractions were added at final concentrations of 1.5, 1,5 and 150 µg/mL, and incubated at 37 °C in a candle jar for 8, 16, 24, 32, and 48 h. After the incubation, aliquots were removed for assessment of *Plasmodium* growth using the same method above.

Statistical analysis

SPSS program was used for statistical analysis. The IC_{50} of 7 fractions were compared with that of controls using one-way ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS

Fractions of *T. diversifolia* were produced by separating the ether-soluble extract (4.16 % of the dried powder weight) using vacuum liquid chromatography with GF_{254} silica gel as the stationary phase and mixture of n-hexane and ethyl acetate as the mobile phases. The method of fractionation results 11 fractions. Fractions containing similar compounds on TLC were combined to get 7 fractions (F1 - F7). The weights of the dried F1 – F7 were 0.03, 0.59, 0.81, 0.3, 1.02, 1, 0.48 g and their *rendements* were 0.006, 0.12, 1.16, 0.06, 0.204, 0.2 and 0.096 % respectively. *Rendement* of fraction is a comparison between the final weight (weight of fraction produced after evaporation) with the initial weight (weight of biomass used) multiplied by 100%.

In this study, to detect the organic compounds contained in fractions, ultraviolet (UV) light (254 and 366 nm) was used, then followed by spraying the chromatogram with cerium sulfate. The polarity of fractions from F1 until F7 were different; F1 was more nonpolar than F2, F2 more nonpolar than F3, and so on (Figure 1). The 7 fractions obtained were tested for antiplasmodial activity.

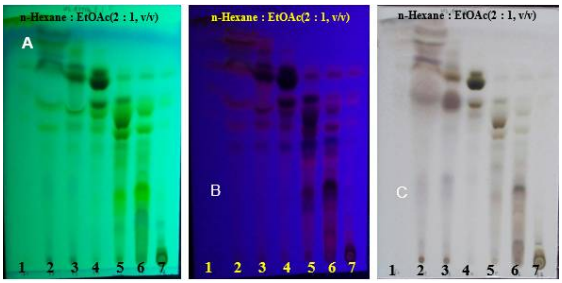

Figure 1: Chromatogram of the 7 fractions of *T. diversifolia* (Hemsley) A.Gray. Stationary phase = silica gel GF254, mobile phase = n-hexane:ethyl acetate (2:1; v/v); A = UV 254; B = UV 366; C = CeSO4

The fractions of *T. diversifolia* inhibited *Plasmodium* growth *in vitro*. Table 1 displays the IC_{50} values attained after 60 hours of fractions exposure. The fractions showed a wide range of antiplasmodial potencies with IC_{50} values ranging from 13.63 to 2755.36 µg/mL against a chloroquine-resistant strain (FCR3) of *P. falciparum*. The best antiplasmodial activity was F6 with the IC_{50} value 13.63 ± 1.43 µg/mL followed by F7, F4, F5, F3, F2, F1 with the IC_{50} values 23.27 ± 2.07, 34.73 ± 5.73, 36.54 ± 2.16, 42.44 ± 0.93, 1029.75 ± 561.97, and 2755.36 ±

277.84 µg/mL, respectively ($p < 0.05$). Fraction 6, which showed promising activity, then was exposed to *P. falciparum* strain FCR-3 at different incubation durations.

Table 1: Antiplasmodial activity of *T. diversifolia* (Hemsley) A. Gray fractions against *P. falciparum* FCR3 strain

Fraction	IC_{50} ± SD (µg/mL)
1	2755.36±277.84
2	1029.75±561.97
3	42.44±0.93
4	34.73±5.73
5	36.54 ±2.16
6	13.63±1.43
7	23.27±2.07

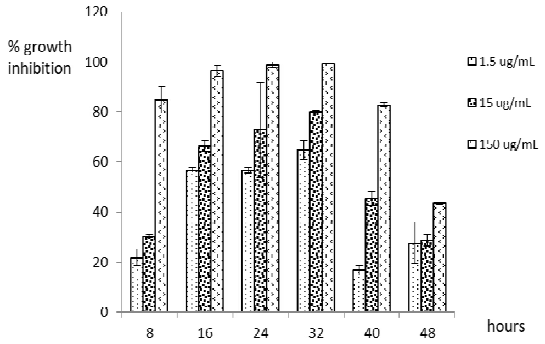

Figure 2: Growth inhibition of *P. falciparum* strain FCR3 in different exposure durations of F6 of *T. diversifolia*

Based on the exposure duration of the fractions in Figure 2, the growth inhibition of parasites was concentration-dependent. F6 (150 µg/mL) inhibited the growth of *P. falciparum* more than 1.5 and 15 µg/mL. Exposure to F6 in 8-h during the first 32 h of the experiment showed an increasing of the inhibition effect in line with the length of the exposure, from 20 to 99 %. The highest growth inhibition was achieved at 32 h incubation, with the inhibition from the lowest to highest concentrations of F6 were 64.78 ± 3.71; 79.86 ± 0.53; and 99.23 ± 0.05 %, respectively. On the other hand, the growth inhibition began decreasing after 32 hours exposure to F6, and resulted in no more than 50 % for the 48 h incubation.

DISCUSSION

Thin layer chromatography was used to detect the characteristic type of organic compounds in the fraction whether polar or non-polar and groups of the compounds by separating them on silica gel GF_{254} plate as the stationary phase using specific solvent or eluent as the mobile phase. Silica gel are silicon dioxides used for the stationary phase to represent adsorption-active surface centers that are able to interact with compounds of fraction. Silica gel GF_{254} plate means that it is mixed with a fluorescent powder and will fluoresce bright green under UV light of wavelength 254 nm. The eluent used can be polar or non-polar. Non-polar solvents will force non-polar compounds to the top of the plate, because the compounds dissolve well and do not interact with the polar stationary phase [17].

This study discovered that F1 was more non-polar than all of the others because the eluent used for F1 was also the most non-polar. In this study the eluent used for the seven fractions was a mixture of non-polar (n-hexane) and polar (ethyl acetate). Among those fractions, F1 had the highest concentration of n-hexane. On TLC, detection of substances in the fractions was viewed in daylight (not showed) and the colorless substances was excited to produce fluorescence by short wave (254 nm) and long wave 366 nm UV light. This visual detection method was chosen because it only requires simple equipment [18]. Under 254 nm UV light the substances in the fractions showed aromatic rings, conjugated double bonds, and unsaturated substances. Fluorescence under 366 nm showed that the fractions contained long chain conjugated double bonds**.** Analysis continued to chemical detection by spraying chromatogram with cerium sulfate resulting in finding the fractions contained terpenes that were indicated by black color spots on the white background. Terpenes, also referred to as terpenoids, such as diterpenes and sesquiterpene are phytoalexins involved in the direct defense of plants against herbivores and microbial pathogens [19], whereas in humans, terpenes may have biological activity against microbial pathogen.

This study discovered that F6, which contains terpenes, is a promising antiplasmodial agent [20]. According to WHO guidelines [20], antiplasmodial activity was classified as follows: highly active at $IC_{50} < 5$ µg/mL, promising at 5 – 15 µg/mL, low at 15 - 50 µg/mL and inactive at > 50 µg/mL. Based on the number of carbon atoms and isoprene units terpenes may be monoterpenes (C10), sesqueterpenes (C15), diterpenes (C20), or sesterpenes (C25) [21]. Interestingly, sesquiterpenes lactone is one member of the sesqueterpenes commonly found in compositae plants, such as *T. diversifolia* [9].

Previous study discovered that methanol and chloroform extracts of *T. diversifolia* inhibit *P. falciparum* FCR3 strain (IC_{50} = 8.31 and 10.41 µg/mL, respectively) [11]. An almost similar result has been obtained by Goffin *et al.* [10] who studied the activity of *T. diversifolia* against *P.*

falciparum strain FCA (a chloroquine-sensitive strain), and discovered that from 30 fractions of *T. diversifolia,* fraction 17 had the best activity with IC_{50} 0.37 µg/mL and the compound inside was sesqueterpene lactone tagitinin C.

It is very likely that the potential antimalarial compound in F6 exposed to the *P. falciparum* FCR3 strain (a chloroquine-resistant strain) in this study is also tagitinin C and F6 has potential to be developed as a new antiplasmodium agent similar to the most renowned antimalarial drug, artemisinin isolated from *Artemisia annua*, which is also terpene-based drug. The lower antiplasmodial activity of F6 compared to Goffine *et al* may be caused by differences in the strain of parasites, the parts of the plant, the places where the plant grew, and the method of preparation of the fraction.

Understanding the onset of action and stage specificity of new substances for malaria treatment is considered to be important, especially in view of the increasing development of resistance in parasites to known antimalarial drugs. In this study, exposure duration of F6 in all concentrations against *P. falciparum* showed that during the first 32 h the growth of parasites was inhibited and the best growth inhibition was the highest concentration (150 µg/mL). It may be interpreted that F6 has cytocidal effect which can kill parasites at early rings (0 - 7 h), late rings (15 - 22 h), early trophozoites (20 - 27 h), and late trophozoites (25 - 32 h) stages [22] and those stages were more sensitive to F6. At the later time interval (after 32 h), the exposure to F6 showed a decreasing trend of growth inhibition.

This finding suggests that there is a recovery mechanism, and the inhibitory effect of F6 appears to be cytostatic. Whitehead and Peto [23] proposed that to determine cytocidal or cytostatic effect, observation and measurement of parasitic growth only conducted during the first cycle is not enough. There is optimal effect if F6 exposure is added during the first cycle and then the growth and morphological appearances of *P. falciparum* are measured in the second and third asexual cycles. Any decrease in growth of parasites between the second and third cycles can be interpreted as an index of the parasites' recovery from the cytotoxic effect. If there is no growth of parasites, the antiplasmodial effect of F6 is clearly cytocidal.

This research demonstrated that F6 of *T. diversifolia* revealed cytocidal effects at early rings to late trophozoites which is similar with eurycomanone, an isolate of *Eurycoma longifolia,* which can kill the ring stage of *P. falciparum* and inhibited the development of young schizont to mature schizont [24]. The contrary result was seen with *T. glaucescens which* inhibited the parasites at the transition from the trophozoite to the schizont stages [25]. Compared to chloroquine, the antiplasmodial effect of *T. diversifolia* and *E. longifolia* were faster because chloroquine was more sensitive to trophozoite and schizont than ring stages [26] whereas *T. glaucescens* had the same stage of activity as chloroquine. It leads us to conclude that different medicinal plants affect at different stages of the parasitic life cycle which may relate to the compound properties contained in the various plants.

CONCLUSION

Fraction 6 (F6) of *T. diversifolia* (Hemsley) A. Gray is a promising compound for the development of a new antimalarial agent due to its ability to inhibit *Plasmodium* growth. It provides a rapid antimalarial activity as demonstrated by the high growth inhibition in 32 h.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Syarif designed the study, conducted the experiments and drafted the manuscript. Wahyuningsih prepared the fractions of *T. diversifolia*. Mustofa and Ngatidjan critically reviewed the manuscript. All authors read and approved the manuscript.

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