Berkala Ilmu Kedokteran
Vol. 39, No. 1, Maret 2007: 7-13

Antiplasmodial activity of fractions isolated from methanolic extract of meniran herb (*Phyllanthus niruri* L) traditionally used to treat malaria

Mustofa[1], Eti Nurwening Sholikhah[1], Subagus Wahyuono[2]
[1]Department of Pharmacology & Toxicology, Faculty of Medicine,
[2]Department of Biology of Pharmacy, Faculty of Pharmacy,
Gadjah Mada University

ABSTRACT

Mustofa, Eti Nurwening Sholikhah, Subagus Wahyuono - ***Antiplasmodial activity of isolated from methanolic extract fractions of meniran herb (Phyllanthus niruri* L.*) traditionally used to treat malaria***

Background: Meniran herb (*P. niruri*) has been traditionally used to treat malaria in Indonesia. Previous screening *in vitro* antiplamodial activity of several extracts of *P. niruri*. showed that methanolic extract was the most active extract against *Plasmodium falciparum*. However, which fractions of methanolic extract of *P. niruri* showing the most potent antiplasmodial activity have not been investigated.
Objectives: To know the antiplasmodial activity of active fractions of *P. niruri*.
Methods: Bioassay guided fractionation was performed using suitable solvents to separate active fractions from methanolic extract. Initially, the methanolic extract was separated with ethyl acetate to give ethyl acetate soluble and ethyl acetate insoluble fractions. The active fraction was then subjected to column chromatography using Sephadex LH-20 as stationary phase and n-hexane : methanol : acid acetic (7.5 : 12.5 v/v : 10 drops) as mobile phase to give four fractions (**F I F IV**). The *in vitro* antiplasmodial activity on *P. falciparum* strains was reflected by the concentration inhibiting 50% of the parasite growth (IC_{50}). Identification of active constituents in the fractions was carried out according to thin-layer chromatography (TLC) method.
Results: The results showed that the ethyl acetate insoluble fraction was more active against *P. falciparum* (IC_{50}, 2.2 to 2.4 μg/mL) than ethyl acetate soluble fraction (IC_{50}, 4.3 to 4.8 μg/mL). The fractionation of the ethyl acetate insoluble fractions gave 9 fractions that were grouped into 4 fractions according to TLC picture. Fraction **III** (**F III**) was the most active fraction (IC_{50}, 3.4 to 4.1 μg/mL). Identification of active constituents in the **F III** using TLC showed the existence of polyphenolic compounds. Further study will be conducted to isolate and purify of the polyphenolic compounds and to evaluate their antiplasmodial activity.
Conclusion: Fraction **III** containing polyphenolic compounds of *P. niruri* is the most active *in vitro* against *P. falciparum*.

Key words : *Phyllanthus niruri* – malaria - antiplasmodial activity - *in vitro* - polyphenol

ABSTRAK

Mustofa, Eti Nurwening Sholikhah, Subagus Wahyuono - ***Aktivitas antiplasmodium fraksi dari ekstrak metanol tanaman meniran (Phyllanthus ninuri) yang secara tradisional digunakan untuk mengobati malaria***

Latar belakang: Tanaman meniran (*P. niruri* L.) secara tradisional telah digunakan untuk mengobati malaria. Penelitian pendahuluan terhadap berbagai ekstrak tanaman meniran menunjukkan bahwa ekstrak metanol adalah aktif terhadap *P. falciparum*. Namun demikian, fraksi manakah dalam ekstrak metanol yang mempunyai aktivitas antiplasmodium belum pernah dikaji.
Tujuan: untuk mengetahui aktivitas antiplasmodium fraksi aktif tanaman meniran.

Mustofa, Eti Nurwening Sholikhah, Department of Pharmacology & Toxicology, Faculty of Medicine. Subagus Wahyuono, Department of Biology of Pharmacy, Faculty of Pharmacy, Gadjah Mada University Yogyakarta, Indonesia

Bahan dan cara: Fraksinasi yang mengacu pada bioassay dilakukan dengan menggunakan pelarut yang sesuai untuk memisahkan fraksi aktif dari ekstrak metanol. Pada awalnya, ekstrak metanol dipisahkan dengan etil asetat sehingga diperoleh fraksi larut etil asetat dan tidak larut etil asetat. Fraksi tidak larut etil asetat selanjutnya dipisahkan dengan kolom kromatografi dengan fase diam Sephadex LH-20 dan fase gerak n-hexan: metanol : asam asetat (7,5 : 12,5 v/v : 10 tetes) sehingga diperoleh 4 fraksi untuk diuji aktivitasnya. Aktivitas antiplasmodium pada strain P. falciparum ditunjukkan dengan nilai IC_{50} yaitu kadar fraksi aktif yang mampu menghambat pertumbuhan parasit hingga 50%. Identifikasi kandungan senyawa dalam fraksi aktif dilakukan dengan metode kromatografi lapis tipis (KLT).
Hasil: Hasil penelitian menunjukkan bahwa fraksi tidak larut etil asetat lebih aktif (IC_{50}, 2,2 – 2,4 μg/mL) dari pada fraksi larut etil asetat (IC_{50}, 4,3 – 4,8 μg/mL). Dan pemisahan fraksi tidak larut etil asetat diperoleh 9 fraksi yang dapat dikelompokkan menjadi 4 fraksi berdasarkan gambaran KLTnya. Fraksi **III** (**F III**) merupakan fraksi paling aktif dengan IC_{50}, 3,4 – 4,1 μg/mL. Identifikasi kandungan aktif **F III** dengan KLT menunjukkan adanya senyawa polifenol. Penelitian lanjut akan dilakukan untuk mengisolasi dan memurnikan senyawa polifenol dan mengkaji aktivitas antiplasmodiumnya.
Simpulan: Fraksi **III** dari ekstrak metanol meniran yang mengandung senyawa polifenol merupakan fraksi paling aktif secara *in vitro* terhadap *P. falciparum.*

INTRODUCTION

Malaria remains the most widespread parasitic disease in sub sub-tropical and tropical countries.[1] Various drugs are available and extensively used to treat malaria such as chloroquine, sulfadoxine-pyrimethamine, quinine and artemisinin. However, malaria parasites resistant to these drugs are developing now and widespread in many countries in the world. Particulary, chloroquine-resistant strains of *P. falciparum* have become a major health problem in all malaria endemic areas around the world.[2] Hence, the need to find new antimalarial drugs with novel actions is high-priority to control the disease. Medicinal plants are expected to play a seminal role in this regard, especially in view of the success with two antimalarial drugs quinine and artemisinin, both of which are isolated from medicinal plants and are used clinically.

Phyllanthus niruri, locally named meniran, is a herb that grows in tropical areas like the rain forest of the Amazon, South America, Bahamas, China, Southern India and South East Asia like Indonesia. *Phyllanthus niruri* has a long history in herbal medicine systems worldwide. The plant is employed extensively in traditional medicine for medical purposes for hundred years, i.e. gallbladder stones, hepatitis, cold, flu, hypertension, diarrhea, cancer and various infection such viral infection, urinary tract infection and fever in malaria.[3] For the last reason, we identified *P. niruri* as potential candidate for antimalarial drug.

In our preliminary study, three extracts of *P. niruri* i.e. aqueous, methanolic and chloroformic extracts have been evaluated for their *in vitro* antiplasmodial activity. Among these three extracts tested, methanolic extract exhibited the highest antiplasmodial activity against *P. falciparum* chloroquine-resistant (FCR-3) and –sensitive (D10) strains with the IC_{50} ranging from 2.3 to 3.9 μg/mL. The aim of the present study is to know which fractions of methanolic extract of *P. niruri* showing the most potent antiplasmodial activity.

MATERIALS AND METHODS

Materials

The *P. niruri* herb was collected from its natural habitat in Sleman, Yogyakarta and were identified by comparison with authentic specimens in Laboratory of Pharmacognosy, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta. The chloroquine-resistant *P. falciparum* strain, FCR-3 was obtained from the laboratory stock at the Life Science Laboratory collection, Gadjah Mada University, Yogyakarta.

Plant fractions preparation

The *P. niruri* herb were air dried and powdered. The powdered material (1 kg) was macerated with chloroform (5 L) at room temperature for 24 hours and then filtered to separate residue from filtrate. The process was repeated 3 times. The residue (960.15 g) was then further macerated 3 times with methanol (5 L) as above. The filtrates were combined and evaporated under vacuum to dryness to give the methanolic

extract (50.25 g). The methanolic extract was extracted with ethyl acetate to give ethyl acetate soluble (10.52 g) and ethyl acetate insoluble (39.73 g) fractions. The ethyl acetate insoluble fraction showing more active than that of the ethyl acetate soluble fraction was subjected to column chromatography (CC) using Sephadex LH-20 (Pharmacia) as stationary phase and n-hexane : methanol : acid acetic (7.5 : 12.5 v/v : 10 drops) as mobile phase. The CC provided 9 fractions (fraction 1-9). The fractions were combined on the basis of their thin layer chromatography (TLC) picture [SiO_2 F_{254} (E Merck), n-hexane : methanol : acid acetic (7.5 : 12.5 v/v : 10 drops) to give 4 fractions (F I – F IV). .

***In vitro P. falciparum* culture**

A strain of *P. falciparum*, FCR-3 chloroquine resistant strain (with IC_{50} for the chloroquine at 151 ng/mL) was used and cultured continuously according to Trager & Jensen with modifications described by Van Huyssen & Rieckmann.[4,5] The parasites were maintained *in vitro* in human red blood cells ($O^{\pm}$), diluted to 1% hematocrit in RPMI 1640 medium supplemented with 25 mM HEPES and 30 mM $NaHCO_3$ and complemented with 10% human serum. Before used, parasite cultures were synchronized by a D-sorbitol lysis in order to obtained ring stage of *P. falciparum* as reported by Lambros & Vanderberg.[6]

In vitro *antiplasmodial activity testing*

The antiplasmodial activity of *P. niruri* fractions was evaluated by a radioactive method described by Desjardins *et al*.[7] The fractions were tested in a triplicate of 96-well culture plates. Parasites mostly at ring stages were suspended in RPMI 1640 medium containing 30 mM $NaHCO_3$ to 1% parasitemia and 2 % hematocrit and distributed into wells of a 96-well micro plate (100 mL per well). The fractions were tested with a series concentration (0.01 to 100 μg/mL) in 100 μL RPMI 1640 medium. The micro plate containing parasite culture and fractions were incubated at 37^0C in candle jar incubator for two intervals, 24 and 72 hours. The control, parasite cultures freed from any fractions were referred to as 100% growth. After 18 or 60 hrs incubation, 50 μL of culture medium containing 0.25 μCi [3H]-hypoxanthine was added into each well and the parasites were incubated for another 6 or 12 hrs. At the end of the incubation period, the content of each well was harvested on glass fiber filter papers with a Cell Harvester and dried for 2 hrs at 60^0C. The radioactivity of each well was measured by a liquid scintillation counter. Parasite growth was estimated by [3H]-hypoxanthine incorporation. The net incorporation of radioactivity was obtained after subtraction of the non-specific incorporation measured in uninfected erythrocytes, and expressed as a percentage of the incorporation by the positive controls. The IC_{50} values, concentration required to inhibit parasite growth by 50%, were determined by linear interpolation from the growth inhibition curves generated for each extract-parasite combination.

Identification of active fractions

Partial identification of constituents in the active fraction was carried out by TLC. Silica gel F_{254} was used as stationary phase and ethyl acetate was used as mobile phase of TLC system. The TLC chromatogram was visualized under UV lights (254 and 366 nm), and visualized upon specific reagents.

RESULTS AND DISCUSSION

In our continuing study for finding new antimalarial agent from Indonesian medicinal plant that could provide alternative medicine to chloroquine, we have investigated the *P. niruri* herb. Our previous study showed that methanolic extract of *P. niruri* exhibited the best antiplasmodial activity against both *P. falciparum* chloroquine-resistant (FCR-3) and –sensitive (D10) strains with the IC_{50} ranging from 2.3 to 3.9 μg/mL. In order to know which fractions of the methanolic extract showing the most potent antiplasmodial activity, bioassay-guided fractionation was applied to monitor the antiplasmodial activity of the fractions obtained from the methanolic extract. In this study, the methanolic extract was subjected to partition with ethyl acetate to give ethyl acetate soluble and ethyl acetate insoluble fractions. The two fractions were then determined their antiplasmodial activity against *P. falaciparum* chloroquine-resistant strain (FCR-3). The ethyl acetate insoluble fraction turned out to be more active than that of ethyl acetate soluble

fraction (TABLE 1). The growth inhibition of the ethyl acetate insoluble at concentration of 0.2 µg/mL and up was higher than the ethyl acetate soluble fraction, observed at 24 and 72 hour incubation as well. Both fractions at 10 µg/mL completely inhibited the *Plasmodium* growth (FIGURE 1).

TABLE 1. The growth inhibition (%) of *P. falciparum* (FCR-3 strain) *in vitro* in the presence of ethyl acetate soluble and ethyl acetate insoluble fractions of *P. niruri* at various concentrations after 24- and 72-h incubations.

Concentration (µg/mL)	Ethyl acetate soluble fraction (%)		Ethyl acetate insoluble fraction (%)	
	24 h	72 h	24 h	72 h
0.01	0.0 ± 0.0	5.1 ± 1.0	5.1 ± 0.9	9.1 ± 1.0
0.02	8.5 ± 1.9	15.3 ± 1.2	2.5 ± 0.9	10.8 ± 1.1
0.1	19.8 ± 2.6	25.5 ± 2.8	9.8 ± 3.6	15.5 ± 2.8
0.2	8.5 ± 1.8	26.7 ± 1.5	18.1 ± 2.5	26.7 ± 1.5
1	9.9 ± 1.6	32.9 ± 2.6	46.7 ± 1.6	50.5 ± 2.1
2	40.5 ± 3.5	52.7 ± 3.1	99.1 ± 2.7	95.7 ± 3.2
10	99.8 ± 1.2	100.0 ± 0.0	99.1 ± 1.4	100.0 ± 0.0
20	95.6 ± 2.5	99.6 ± 2.8	97.6 ± 1.3	99.8 ± 1.1
50	97.8 ± 1.8	99.8 ± 3.8	100.0 ± 0.0	100.0 ± 0.0
100	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

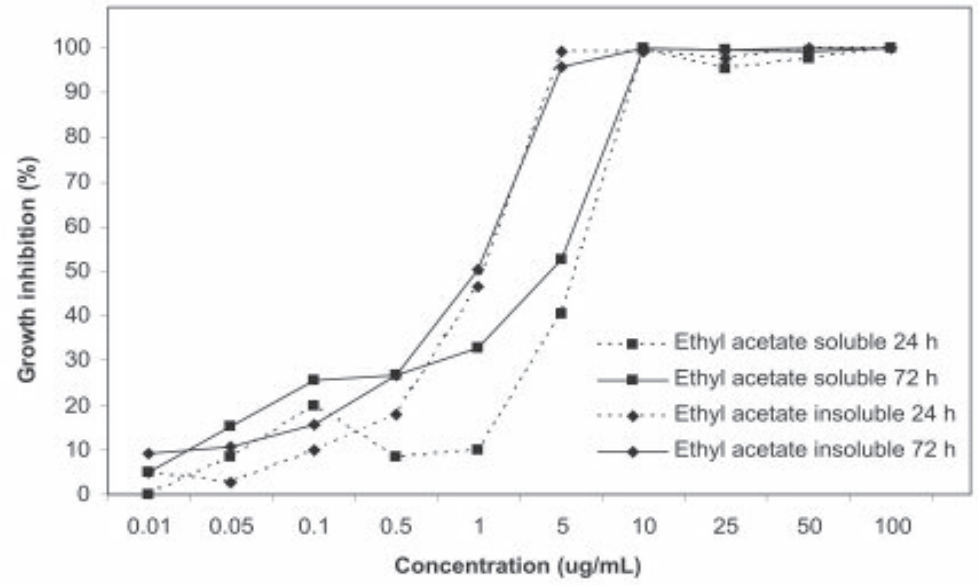

FIGURE 1. The growth inhibition (%) of *P. falciparum* (FCR-3 strain) *in vitro* in the presence of ethyl acetate soluble and ethyl acetate insoluble fractions of *P. niruri* at various concentrations after 24-h and 72-h incubations

The ethyl acetate insoluble fraction showed higher antiplasmodial activity (IC_{50}, 2.2 to 2.4 µg/mL) than ethyl acetate soluble fraction (IC_{50}, 4.3 to 4.8 µg/mL) as showed in Table 3. Based on these results, the ethyl acetate insoluble fraction was subjected to CC to give four combined fractions and then evaluated for their *in vitro* antipalsmodial activity. The effect of these fractions on the growth inhibition of the *Plasmodium* is shown in TABLE 2 and FIGURE 3.

The IC_{50} values of the different fractions ranged (F I – F IV) from 3.4 to 17.1 µg/mL after a 24 h incubation (Table 3). Although, these IC_{50} values were not different with that of after 72 h incubation (IC_{50}, 4.1 – 18.1 µg/mL) indicated that there was no cumulative effect after contact

TABLE 2. The growth inhibition (%) of *P. falciparum* (FCR-3 strain) *in vitro* in the presence of fractions (**F I – F IV**) of *P. niruri* at various concentrations after 24-h and 72-h incubations.

Concentration (µg/mL)	Fraction **I**		Fraction **II**		Fraction **III**		Fraction **IV**	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
0.1	15..2 ± 2.5	17.1 ± 2.1	10.0 ± 0.9	15.8 ± 2.2	19.8 ± 2.5	7.8 ± 2.1	16.8 ± 0.1	15.8 ± 1.5
0.5	15.0 ± 2.1	28.5 ± 3.5	15.5 ± 1.8	20.5 ± 3.3	28.5 ± 1.9	10.2 ± 1.4	10.0 ± 0.0	15.0 ± 2.2
1	12.6 ± 3.2	15.2 ± 3.5	20.5 ± 3.3	25.8 ± 3.9	42.8 ± 2.1	35.8 ± 3.3	15.5 ± 2.1	20.5 ± 3.1
5	26.1 ± 2.1	35.1 ± 3.2	25.0 ± 2.5	32.9 ± 3.5	55.2 ± 3.5	65.2 ± 3.2	25.9 ± 2.5	25.9 ± 2.9
10	37.4 ± 3.6	35.4 ± 4.0	45.5 ± 3.1	60.4 ± 3.1	100.0 ± 0.0	97.4 ± 1.5	37.5 ± 1.2	28.5 ± 1.2
25	65.4 ± 2.4	68.1 ± 3.2	95.5 ± 2.9	90.1 ± 2.3	100.0 ± 0.0	95.4 ± 1.2	70.5 ± 2.0	65.7 ± 2.3

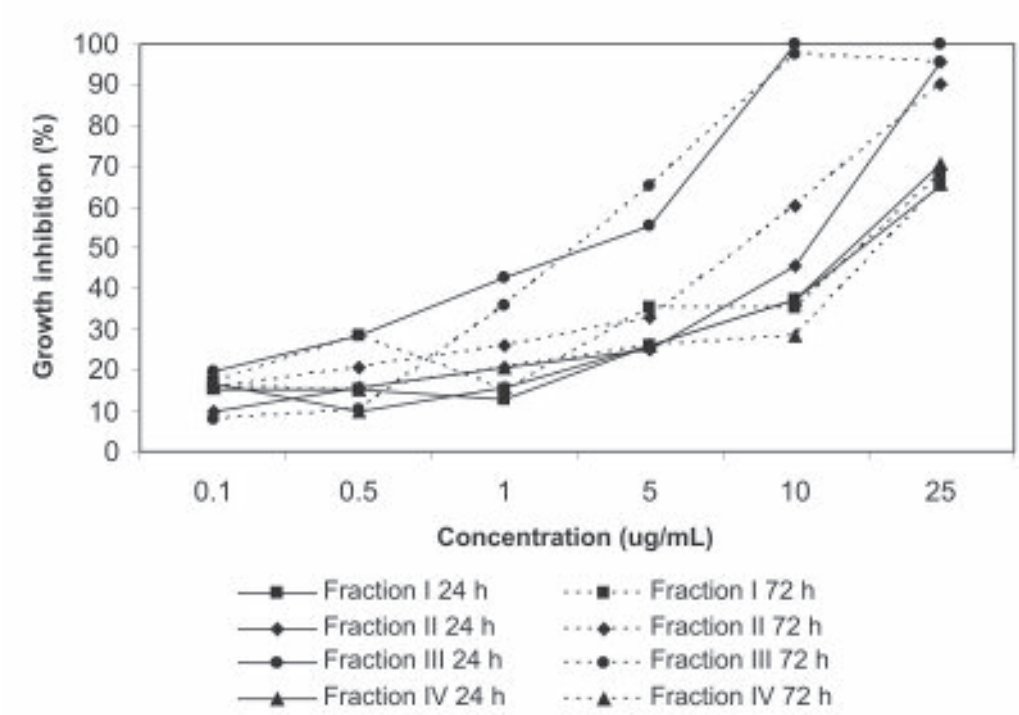

FIGURE 2. The growth inhibition (%) of *P. falciparum* (FCR-3 strain) *in vitro* in the presence of fractions (**F I – F IV**) of *P. niruri* at various concentrations after 24 h and 72 h incubations.

between parasite and fractions. Among 4 fractions tested, F III showed the strongest antiplasmodial activity (IC_{50}, 3.4 ± 0.5 µg/mL after 24 hrs incubation and 4.1 ± 0.7 µg/mL after 72 hrs) indicating that active compounds present in this fraction. However, the antiplasmodial activity of F III was lower than ethyl acetate insoluble fraction (IC_{50}, 2.2 ± 0.2 µg/mL after 24 h and 2.4 ± 0.3 µg/mL after 72 h) as the fraction where the F III was obtained before. It should be noted that the ethyl acetate insoluble fraction comprised of a mixture of many active compounds, some of which may give an additive or a synergic antiplasmodial effects with active compounds in F III.

TABLE 3. *In vitro* antiplasmodial activity (IC_{50} ± SD µg/mL) *P. niruri* herb fractions on *P. falciparum* strain (FCR-3) after 24 h and 72 h incubations.

Strain / Fractions	IC_{50} in µg/mL on FCR-3	
	24 h	72 h
Ethyl acetate soluble fraction	4.3 ± 0.5	4.8 ± 0.6
Ethyl acetate insoluble fraction	2.2 ± 0.2	2.4 ± 0.3
Fraction **I**	17.1 ± 0.4	15.9 ± 0.7
Fraction **II**	11.3 ± 0.6	8.0 ± 0.5
Fraction **III**	3.4 ± 0.5	4.1 ± 0.7
Fraction **IV**	15.8 ± 1.1	18.1 ± 1.3

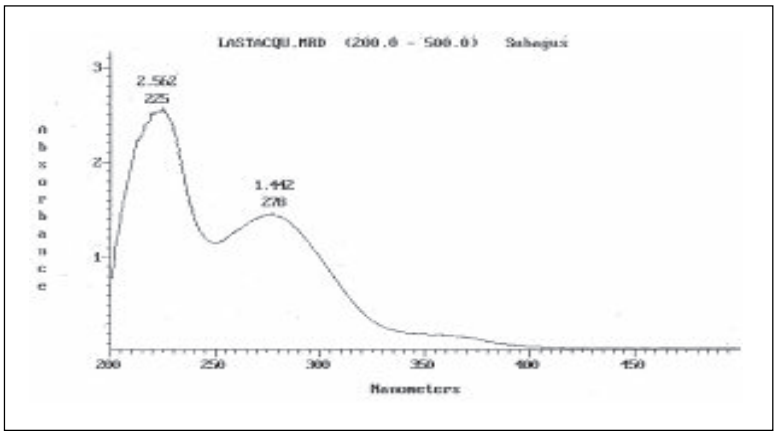

FIGURE 4. UV spectra of fraction **III** (MeOH).

The biological activities of *P. niruri* have been reported by some authors viz. anticancer[8,9], antimicrobial[10], antihypoglycemic[11,12], antiviral[13], antiHIV[14], analgesic effect[15,16,17]. However, little information about their antiplasmodial activity has been reported. The active compounds of *P. niruri* were also isolated and purified. This plant was reported contain lignan such as phyllanthine, hypophyllantine, isolintetralin[18], flavonoid such as quercetine, quercitrin, isoquercitrin, astragalin, rutine, eriodictyol-7-rhamnopyranoside, phisetine-4'-O-glycoside and nirurine[19,20], alkaloids such as chaempherol-4'-rhamnopyranoside, ENT-norsecurinine, and trans-phytol[21]. However, which of these active compounds showing antiplasmodial activity have not been reported.

In this study, identification approach of constituent present in the most active fraction (**F III**) were carried out by TLC. The TLC results showed that i) the spot absorbed UV light at 254 nm indicating the presence of chromophore (conjugated double bonds = -C=C-C=C-). In addition, visualization of the spot by ammonia did not provide yellow color indicating that active constituent is not a flavonoid class of compound but it was an aromatic compound; ii) the spot was dark fluorescence under UV light at 366 nm indicating that the chromopore is not long conjugated double bonds; iii) the detection with cerium (IV) sulfate tetrahydrate in sulphuric acid (heated at 110 ^{o}C for 3-5 minutes) provided bronze color and changed fastly to black color indicating that the constituent is an organic compound that destroyed by this reagent.

Based on these TLC results, it was predicted the existence of polyphenolic compounds in the **F III**. In order to prove this prediction, spot was visualized by ferri chloride reagent to give a dark blue color indicating the existence of polyphenolic compounds. In addition, UV spectra analysis

displayed a characteristic UV absorption (ë, in MeOH) at 225 and 278 nm strongly indicating the presence of aromatic compounds substituted by carbonyl (-C=O) and some hydroxyl (-OH) groups (Figure 4). To ascertain the presence of these carbonyl and hydroxyl groups, infrared (IR) spectra analysis must be performed.

Conclusion

The ethyl acetate insoluble fraction is more active against *P. falciparum* (IC_{50}, 2.2-2.4 µg/mL) than ethyl acetate soluble fraction (IC_{50}, 4.3-4.8 µg/mL). A CC fractionation of the ethyl acetate insoluble fraction gives four (**F I - F IV**) fractions of which **F III** is the most active having IC_{50} ranged 3.4-4.1 µg/mL. Identification approach of constituents in the **F III** using TLC shows the existence polyphenolic compounds.

ACKNOWLEDGEMENTS

This work received financial support from the QUE Project, Faculty of Medicine, Gadjah Mada University at 2002. The authors wish to thank Mr. Sukiran and Mrs. Rumbiwati for the excellent technical assistance.

REFERENCES

1. WHO. Roll Back Malaria. A Global Patnership. Geneva., 1998.
2. Basco LK, Ruggeri C, Le Bras J. Molécules antipaludiques. Paris : Masson Ed., 1994.
3. Winarno MW, Sundari D, Paramita DI. Various research informations efficacy and phytochemistry of meniran herb (*Phyllanthus niruri*, L.), Indonesian Medecinal Plants Bulletin, 1993; 2(4): 24-25.
4. Trager W & Jensen J. Human malaria parasite in continuous culture. Science, 1976; 193: 673-75.
5. Van Huysenn W, Rieckmann KH. Disposible environment chamber for assesing the drug susceptibility of malaria parasites. Trop Med Parasitol, 1993; 44, 329-30.
6. Lambros C, Vanderberg JP. Synchronisation of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol, 1979; 64 : 418-20.
7. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. Antimicrob Agents Chemother, 1979; 16: 710-18.
8. Huang ST, Yang RC, Lee PN, Yang SH, Liao SK, Chen TY, Pang JH. Antitumor and antiangiogenic effects of Phyllanthus urinaria in mice Lewis lung carcinoma. Int Immunopharmacol 2006; 6(6): 870-79.
9. Giridharan P, Sumasundaram ST, Perumal K, Vishwakarna RA, Karthikeyan NP, Velmurugan R, Balakrishnan A. Novel substituted methylenedioxy lignan suppresses proliferation of cancer cells by inhibiting and activation of c-myc and caspases leading to apoptosis. Br J Cancer, 2002; 87: 98-105.
10. Mazumder A, Mahato A, Mazumder R. Antimicrobial potentiality of *Phyllanthus amarus* against drug resistant pathogens. Nat Prod Res 2006; (4):323-26.
11. Ali H, Houghton PJ, Soumyanath A. Alpha-amylase inhibitory of some Malaysian plants used to treat diabetes, with particular reference to *Phyllanthus amarus.* J Ethnopharmacol, 2006; 107(3): 449-55.
12. Khanna A, Rizvi F, Chander R. Lipid lowering activity of *Phyllanthus niruri* in hyperlipemic rats. J Ethnopharmacol, 2002; 82: 19.
13. Hukeri VI, Kalyani GA, Kakrani HK. Hypoglycemic activity of flavonoids of *Phyllanthus frametus* in rats. Fitoterapia, 1998; 59, 68-70.
14. Qian-Cutrone J, Huang S, Trimble J, Li H, Lin PE, Alam M, Klohr SE, Kadow KF. Niruriside, a new HIV RE/RRE binding inhibitors from *Phyllanthus niruri.* J Nat Prod, 1996; 59: 196-99.
15. Santos AR, Filho VC, Niero R, Viana AM, Moreno FN, Campos MM, Yunes RA, Calixto JB. Analgesic effects of callus culture extracts from selected spesies of *Phyllanthus* in mice. J Pharm Pharmacol, 199; 46: 755-59.
16. Santos AR, Filho VC, Yunes RA, Calixto JB. 1995. Analysis of the mechanisms underlying the antinociceptive effect of extracts of plant from the genus *Phyllanthus*. Gen Pharmacol, 1995; 26: 1499-506.
17. Santos AR, Filho VC, Yunes RA, Calixto JB. 2000. Antinociceptive properties of extracts of new species of plants of the genus *Phyllanthus* (Euphorbiaceae). J Ethnopharmacol, 200; 72(1/2): 229-38.
18. Huang YL, Chen CC, Ou JC. Isolintetralin: a new lignan from *Phyllanthus niruri*. Planta Med, 1992; 58:473.
19. Gupta DR, Ahmed B. Nirurine: a new prenylated flavonone glycoside from *Phyillanthus niruri.* J Nat Prod, 1984; 47(6): 958-63.
20. Keth Nara TJ, de Cervol JG, Stanisla E. Flavonoides de *Phyllanthus niruri* L., *P. urinaria* L., *P. orbiculatus*, L.C., Rich. Plants Medicinales et Phytotherapie, 1977; XI(2): 958-63.
21. Joshi BS. Isolation and structure (X-Ray analysis) of ENT-norsecurinine, an alkaloid from *Phyllathus niruri.* J Nat Prod, 1986; 49(4): 614-20.