Bioactivity Screening of *Erechtites valerianifolia* (Link Ex Wolf.) Less. Extracts as Antiplasmodium, Antibacterial, Antifungal and Cytotoxic Agents

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ABSTRACT: Indonesia's rich natural resources have encouraged many researchers to explore their potential for finding treatment of endemic diseases and malignancy. One of these resources is *Erechtites valerianifolia* (Link Ex Wolf.) Less. Ex DC or "Jonggolan" (Indonesian name), a member of the family Asteraceae was collected from Meru Betiri Forest. Empirical evidence reveals the use of this plant to treat fever, diarrhea, tonsillitis, wounds, and eczema. A study in 2015 reported that the family Asteraceae passed the test of antioxidant and antibacterial activity. Yet, scientific information about *E. valerianifolia* has not been optimally explored, and studies of this plant are even very limited. Considering the background, this study was aimed to conduct bioactivity screenings of *E. valerianifolia* extracts as antiplasmodial, antibacterial, antifungal and was anticancer/cytotoxic agents. Extract was obtained through percolation method using hexane, dichloromethane, ethyl acetate, and methanol as the solvents. Gram positive bacteria (*Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* and *Bacillus cereus*) and gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were used as the antibacterial testing microorganisms. *Candida albicans* was the antifungal testing microorganism. The results showed that extracts, at a concentration of maximum 1000 ppm, were less active than the positive-control amoxicillin for antibacterial activities and ketoconazole for antifungal agents. Inhibition zones of *E. valerianifolia* extracts were invisible when compared to the positive controls. The screening of antiplasmodial (*P. falciparum* FCR3) activity indicated that IC_{50} of ethyl acetate extract was 117.122 µg/ml and was considered the best among other extracts. The cytotoxicity study on MCF 7, WiDr and Hela cell lines showed that the four extracts tested had good cytotoxicity with $IC_{50} < 30$ µg/ml.

Key words: *Erechtites valerianifolia*, antiplasmodium, antibacterial, antifungal, cytotoxicity.

INTRODUCTION

Traditional medicine is a heritage that has been developed since ancient times, and Indonesia is one of the countries with potential medicinal plants, where the natural resources most widely used as medicine ingredients are herbs and have existed for centuries. Searching for potential plants in nature is one of the methods to obtain a new drug.[1] Even though processing drugs from nature takes time, their adverse effects are relatively small. Indonesia's richness of natural resources has led a lot of researchers to investigate the potential of medicinal plants for endemic and malignancy disease treatment. *Erechtites valerianifolia* (Link Ex Wolf.) Less. Ex DC or locally named "Jonggolan", a member of the family Asteraceae, from Meru Betiri Forest is one of them. Located in Jember Regency, East Java-Indonesia, the forest is home to a large number of medicinal plants that function as vegetables to local communities.[2] The local people are unaware that *E. valerianifolia*, the plant they consider as weed, is in fact a medicinal plant. However, empirical evidence confirms that this plant is commonly used to treat fever, diarrhea, tonsillitis, wounds, and eczema, and

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members of the family Asteraceae are generally efficacious as an antimalarial. The study reported that the extract of plants in Asteraceae family has antimalarial activity against various types of *Plasmodium*.[3-5] In addition, a study in 2015 found that the extract of the family Asteraceae exhibits antioxidant and antibacterial activities.[6,7] The mechanism of action of antibacterial drugs is slightly similar to that of antifungal drugs and antimalarial/antiplasmodium; therefore, it is hypothesized that *E. valerianifolia* is likely to possess both antifungal and antiplasmodium activities. The antioxidant activity conducted with anticancer/cytotoxic so it is hypothesized that *E. valerianifolia* is likely to possess cytotoxic activity.[7,8]

Infectious diseases remain to be the most common among the population of developing countries, including Indonesia. One of their causes is a bacterial infection. Pathogenic bacteria is more harmful and causes either sporadic or endemic infections, such as *Escherichia coli,* Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus, Bacillus subtillis,* or *Pseudomonas aeruginosa* that cause diarrhea.[9,10]

Meanwhile, fungus thrives in tropical regions with high humidity like Indonesia. One of the pathogenic fungi causing an infection named vulvovaginal candidiasis in women is *Candida albicans*; its incidence rate reached 75% among Indonesian women. In addition, fungus can invade such other organs as the mouth, skin, nails, lungs, gastrointestinal tract, urinary tract, heart and brain membrane that cause fever.[11]

Malaria is a tropical disease widely spread in several regions in Indonesia. Most malaria endemic areas are outside Java, in which many malaria-prone tropical forests grow. During the period 2000-2004, the endemic rate of malaria in Indonesia indicated an increase. The Annual Parasite Incidence (API) in Indonesia from 1995 to 2008 per 1000 people rose rapidly, and in 2008 it even went up to 2.47. Extraordinary events of malaria in Indonesia attacked 15 provinces during the period 1998-2003. In 2011, the number of malaria deaths reported reached 388 cases.[12-15] Malaria is a disease caused by Plasmodium with *Plasmodium falciparum* as a parasite with the most resistance rate. Efforts are underway for the treatment of malaria patients as well as prevention through malaria elimination initiatives. The high rate of malaria in Indonesia has led to a new issue: resistance to chloroquine as the first-line treatment for malaria. In 1990, cases of *P falciparum* chloroquine resistance occurred in all provinces in Indonesia. Treatment of *P. falciparum* malaria, especially in Indonesia, initiated in 2012 according to the policy of The Indonesian Department of Health and WHO recommendation was required to use a new drug combination of artemisinin derivative known as artemisinin combination therapy (ACT). Artemisinin is an isolate of *Artemisia annua* (family Asteraceae). Treatment using a combination of drugs does not necessarily resolve malaria problems completely, considering that malaria epidemics spread faster.[14,16-18]

Cancer is malignant disease involving abnormal cell growth with the potential to invade or spread to other parts of the body. The development of cancer in the world is increasingly. Preliminary test of anticancer activity is cytotoxicity. The antioxidant activity conducted with anticancer/cytotoxic activity through reactive oxygen species (ROS) inhibition.[7]

A wide range of resistant antibacterial, antifungal, antiplasmodial and anticancer/cytotoxic drugs has recently been discovered. Scientific information and research about *E. valerianifolia* has not been optimally explored and highly limited so leading to obscure data about the bioactivity screening of *E. valerianifolia* as antiplasmodium, antibacterial, antifungal and cytotoxic activity.[6,19-28]

Based on empirical studies, this present research was aimed to conduct drug discovery through extraction of *E. valerianifolia* using a percolation method with hexane, dichloromethane, ethyl acetate, and methanol as the solvents followed by bioactivity screening against cancer cell lines, bacteria, fungus, and plasmodium. The plant *E. valerianifolia* can be seen in figure 1.

Figure 1. *Erechtites valerianifolia* (Link Ex Wolf.) Less. Ex DC.

MATERIALS AND METHODS

Materials: *Escherichia coli, MRSA* (*Methicillin*-resistant *Staphylococcus aureus*), *Staphylococcus aureus, Bacillus subtillis*, and *Pseudomonas aeruginosa*, amoxicillin, hexane, dichloromethane, ethyl acetate and methanol of *E. valerianifolia*, *C. albicans*, potato dextrose agar (PDA) with 4 grams of potato content, 20 grams of dextrose, 15 grams of agar, ketoconazole, alcohol, wipes, sterile cotton, sterile distilled water, physiological saline and Muller-Hinton agar, chloroquine, *P. falciparum* FCR3 (chloroquine-resistant plasmodium), hydrogen peroxides in methanol, RPMI 1640, HEPES, sodium bicarbonate 90%, hypoxanthine, gentamicine, aqua, 10% serum, 20% giemsa in aqua, physiological salt, AlbuMAX II (Invitrogen), ethanol, dimethyl sulfoxide (Fluka), giemsa stain, hydrogen peroxidase in methanol, normal goat serum, phosphate buffer saline (PBS) pH 7.4, Hela, WiDr, MCF-7 from Parasitology Laboratory Collection, Faculty of Medicine, Universitas Gadjah Mada, DMEM (Gibco), sodium bicarbonate (Sigma), hepes (Sigma), fetal bovine serum (FBS) 10% v/v (Gibco), penicillin-streptomycin 1% v/v (Gibco), fungisone 0,5% v/v (Gibco), MTT: MTT (3-(4,5-dimethyl thiazole- 2-il (-2,5-diphenyl tetrazolium bromide))) 20 mg, HCl 0.01 N (Merck), 96-well plateand H_2O buffer (pH 6.8) were collected from appropriate sources and used in the experiments.

Extraction of *E. valerianifolia.* A total of 30 kg *E. valerianifolia* herb freshly obtained from Meru Betiri was dried at a low temperature (50^0-70^0C) to produce simplicia. Dry powder/simplicia was extracted using methanol, ethyl acetate, dichloromethane, and n-hexane by percolation method and evaporated using a rotary evaporator to obtain crude extract.[29]

Antibacterial activity. A total of 23 grams of Muller-Hinton agar powder was dissolved in sterile distilled water as much as 1000 ml. It was then heated to dissolve in an Erlenmeyer flask, plugged with fatty cotton and covered with aluminum foil and then sterilized by autoclaving at 121^0C for 15 minutes. Potato dextrose agar (PDA) was weighed as much as 39 g then dissolved into 1 L of distilled water and heated on a hotplate-stirrer until boiling to form agar. As much as 5 ml of the solution was put

into test tubes for agar slopes and 15 ml into sealed glass bottles for antifungal testing. The test tubes and sealed glass bottles containing agar were sterilized using an autoclave at 121^0C for 20 minutes. *E. coli*, MRSA (Methicillin-resistant *Stap. aureus*), *Stap. aureus, B. subtillis*, and *P. aeruginosa* were planted in a nutrient agar (NA) growth mediumand incubated at 37^0C for 24 hours. The bacteria to be tested were suspended by growing them in a liquid medium (physiological saline) and then incubated for 24 hours at 37^0C. Test fungi were cultured in prepared agar slopes and incubated at 30^0C for 1 day. *C. albicans* was suspended in 3 ml of physiological saline. In the antibacterial and antifungal activity test, the agar well diffusion method was used. A total of 200 ml of each suspension of bacteria and fungi was added to 20 ml of Nutrient Agar (NA) medium for bacteria and fungi. The mixture was rotated to be homogeneous, cooled and solidified in a sterile petridish. Wells with ± 6 mm diameter were formed using a perforator and 50 ml of each extract (sample) and positive control dissolved in sterile distilled water were added into the wells. Preincubation was performed for 30 minutes at room temperature before the test. Incubation was carried out at 37^0C for 48 hrs for bacteria and fungi. Zone of inhibition was observed and measured using a slide caliper at the end of incubation period. The positive control used was amoxicillin for antibacterial and ketoconazole for antifungal screening. MIC determination was undertaken using agar dilution method. Test samples with different concentrations were added into 19 ml of diluted agar media in a sterile petridish. The solution was mixed to obtain a homogeneous mixture and cooled to reach a solid state. A total of 1 dose of bacteria and fungi suspension was then inoculated on a surface of solid agar and then incubated at 37^0C for 48 hours for bacteria and fungi.[9,10,30]

Antiplasmodium activity. *P. falciparum* FCR3 (chloroquine-resistant plasmodium) was obtained from Eikjman Jakarta-Indonesia. The ampoule containing falciparum strains was taken from a nitrogen tube and then heated in a water bath at 37^0C to obtain liquid form. The ampoule content was transferred to a conical tube and 3.5% NaCl was added drop wise with a ratio of 1:1 and shaken. As much as 10 ml of RPMI was added and the mixture was centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded, and RPMI and human serum type O were added and then incubated for 72 hours at 37^0C for the growth of *Plasmodium* in a candle jar. Parasite-infected red blood cells were cultured in a culture flask containing 10 ml complete medium (10% serum) with 1.5% final hematocrit. Parasite culture was carried out in LAF (Laminar Air Flow) under a sterile condition and then incubated in CO_2 environment at 37^0C. The medium was replaced every 24 hours. Synchronization was undertaken to obtain a ring stage. Parasite culture was added with 5% sorbitol and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded, and parasite sediment was soaked in sterile 5% sorbitol as much as threefold parasite volume and left at room temperature for 10 minutes. The parasite was then washed through centrifugation, and after addition of complete medium with fivefold volume, parasite sediment containing only ring-stage parasites was obtained and returned to the culture flask. Extracts of *E. valerianifolia* was mixed with 100µlof DMSO and RPMI 1640 to acquire 1 ml volume (stock) and then diluted with RPMI. The selected microculture plate had 96 wells, and each well was filled with 100 µl of complete medium containing parasites with 1.5% hematocrit and 2% parasitemia. Sample solution was prepared using a 100 µl micropipette, and each concentration was transferred into the wells from low to high respective concentrations. Microculture was placed in a vacuum desiccator with a candle jar. The desiccator was sealed exactly at the time of extinguished flame and then incubated in CO_2 incubator at 37^0C for 72 hours. After incubation, the microplates were removed from the candle jar. The test preparation was homogeneously mixed and centrifuged, the filtrate was removed, and the concentrated part was made into smear preparation. The smear was dried at room temperature, and fixation was conducted with 80% methanol. After drying, the smear was stained with 10% giemsa solution in distilled water for 30 minutes.

% Parasitemia = control parasitemia – drug parasitemia/ control parasitemia x 100%

IC_{50} values were obtained through probit analysis (5 inhibitions vs log dose). Here, chloroquine was used as a positive control.[31-33]

Cytotoxic activity. This colorimetric assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of viability of the cells culture. Prior to use in the assay, the cells were grown to 80-90% confluence and synchronized by incubation in the assay media for 4 hrs. Cell suspensions ($5x10^3$ cells/100 µl/well) were then incubated with various concentrations (12.5, 25, 50, 100, 200, 400 µg/ml) of plant extract solutions for 24 hrs with a final DMSO concentration of 0.1%. The cells were seeded in 96-well plates. Each concentration was seeded and triplicate plates were used the cell line. Then, the cells were incubated at 37°C. After 24 hrs incubation, cell viability was determined by adding (Sigma) tetrazolim salt as cytotoxicity indicator, so after 24 hrs of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4 hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. Tetrazolium salts are cleaved to formazan dye by cellular enzymes (only in the viable cells).[34] The percent viability was calculated by using formula:

% viability = (treatment cell count- blank /no treatment cell count-blank) ×100%

The concentration that results in 50% inhibition (IC_{50}) value of the extracts were calculated from the equation of the logarithmic line determined by fitting the best line to the curve formed from the data using Microsoft Excel and probit.

RESULTS AND DISCUSSION

Extraction of *E. valerianifolia.* The yield of *n*-hexane, dichloromethane, ethyl acetate and methanol extracts was as much as 42.9 gm (1:43% w/w), 21.4 gm (0.71% w/w), 29 gm (0.97% w/w) and 135.8 gm (3:53% w/w), respectively. The highest amount was found in the polar methanolic extract.

Antibacterial and antifungal activities. Gram positive bacteria (*Stap. aureus*, MRSA and *B. cereus)* and gram negative bacteria *(E. coli* and *P. aeruginosa)* were used as antibacterial test microorganisms, while *C. albicans* became the antifungal test microorganism. The results of antibacterial and antifungal activities at 1000 ppm showed that the four extracts did not have activity more than the positive control amoxicillin for antibacterial and ketoconazole for antifungal activity against tested microorganisms. No zone of inhibition appeared in extracts when compared with the positive control through diffusion method (Table 2). Minimum Inhibitory Concentration (MIC) indicates that the administration of maximum concentration of 1000 ppm in *E. valerianifolia* extracts were invisible in inhibition zones. In addition, the limited number of research on *E. valerianifolia* could not confirm which compound is responsible for antagonism in antibacterial and antifungal test. The most recent study of the genus *Erechtites* revealed that *E. hieracifolia* contains hieracifoline and jacobine alkaloids yet failed to provide antibacterial and antifungal effects.[35,36]

Antiplasmodium activity. Probit analysis was used to determine the IC_{50} values using chloroquine as the positive control. IC_{50} is the concentration at which 50% of *P. falciparum* is inhibited then classified as having high activity for concentration of $IC_{50} \leq 10$ µg/ml, moderate activity for 10-50 µg/ml, and low activity for > 50 µg/ml.[37,38]

The results of *in vitro* antiplasmodium activity test with probit analysis showed that IC_{50} of ethyl acetate extract was 117.122 µg/ml and considered the best among other extracts. The activity of the four extracts of *E. valerianifolia* was lower than chloroquine as the positive control with IC_{50} of 1.114

$\times 10^{-3}$ µg/ml. The results of antiplasmodium test of the four extracts were lower than the positive-control (chloroquine) because the extract might consist of various components possibly encouraging a mechanism of antagonism compared to single compounds. The *in vitro* antiplasmodium test results were shown in table 1. In addition, since research on *E. valerianifolia* remains limited there has been no confirmation as to which compound causes antagonism in antiplasmodium test. The latest research on the genus *Erechtites* was on *Erechtites hieracifolia* that contains hieracifoline and jacobine alkaloids but still provided no information about antiplasmodium activitiy.[35,36] However, different species might contain different compounds.

Table 1. IC_{50} of *E. valerianifolia* extracts on *Plasmodium falciparum* FCR3.

Extract	IC_{50} (µg/ml)
n-Hexane extracts	183.979
Dichloromethane extracts	554.719
Ethyl acetate extracts	117.122
Methanol extracts	373.424
Chloroquine	$1.114x10^{-3}$

Table 2. IC_{50} of four extracts of *E. valeranifolia* against Hela, WiDr, and MCF-7 cell lines.

Extract	IC_{50} (µg/ml)		
	Hela	WiDr	MCF-7
n-Hexane	3,096	3,779	10,159
Dichlormetane	4,639	1,848	3,800
Ethyl acetate	12,281	11,762	0,015
Methanol	1,614	3,415	6,406
5-Flurouracil (control)	4,186	0,000	0,353

Cytotoxicity. These activities were analyzed by monitoring cell viability of treated and untreated cells by their reduction of the tetrazolium substrate, MTT. Cell line used were MCF-7, Hela and WiDr. Generally, cytotoxity test showed IC_{50} of extract for *E. valeranifolia* which was better activity than positive control (5-fluorouracil). Profiles obtained from cell viability MTT test were shown in table 2. Bioactivity screening of four extract *E. valeranifolia* showed to have potential chemopreventive activity (IC_{50} < 30 µg/ml). To be a good drug candidate, the IC_{50} value of such agent should be sufficiently low to avoid any possible unspecific effects. The American National Cancer Institute assigns a significant cytotoxic effect of promising anticancer product for future bioguided studies if it exerts an IC_{50} value < 30 µg/ml.[39] In this preliminary study, we have focused our interest on crude plant extracts for bioactivity screening, the cytotoxic activity could be due to the presence of active products that could probably have increasingly anti-growth effects. The IC_{50} of four extracts of *E. valeranifolia* were found to be significant in every cell line, when compared to positive control. This study provides an important basis for further investigation into the isolation, characterization and mechanism of cytotoxic compounds from the screened medicinal plant. Thus, this plant could be a source of new lead structures in drug design for cancer treatment. In addition, further studies are required to elucidate the precise molecular mechanisms and targets for cell growth inhibition which will allow the rationale design for more effective molecules for eventual use as cancer chemopreventive and/or therapeutic agents.

CONCLUSION

For gram positive bacteria (*Stap. aureus*, (Methicillin-resistant *Stap. aureus* and *B. cereus)* and gram negative bacteria *(E. coli* and *P. aeruginosa)* as test microorganisms as well as *C. albicans* as antifungal test microorganism, the four extracts at a concentration of maximum 1000 ppm, demonstrated no activity. The *in vitro* antiplasmodium FCR3 test with probit analysis demonstrated IC_{50} of ethyl acetate extract as 117.12 µg/ml and was considered the best among other extracts. Compared to the positive-control chloroquine with <50 µg/mlof IC_{50}, the four extracts of *E. valerianifolia* possessed lower activity. The results showed that the four extracts showed good cytotoxic activity on MCF 7, WiDr and Hela cell lines with IC_{50}<30 µg/ml, when they were compared with 5-fluorouracil as positive control.

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