Amoebicidal Activities of Indonesian Medicinal Plants in Balikpapan, East Kalimantan

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Abstract: *Entamoeba histolytica* is a protozoan agent causing human amoebiasis, which is responsible for 100,000 deaths annually throughout the world. The recommendation in the treatment of amoebiasis using metronidazole has been reported to be less effective, because of the drug resistance effect by *Entamoeba histolytica*. Therefore, the search of new drugs with amoebicidal activity is important. The natural substances from medicinal plants are potentially a good object to be studied. The aim of this study was to evaluate Indonesian medicinal plants for their anti-amoebic activities. The hexane, dichloromethane and methanol extracts of 114 samples derived from 22 species of medicinal plants explored in the Balikpapan forest, East Kalimantan had been tested. Their anti-amoebic activity was determined by *in vitro* cell-based assay against *Entamoeba histolytica* HM-1:IMSS (clone 6) strain. According to cell-based assay, five of 114 samples tested showed anti-amoebic activities. The highest anti-amoebic activity was obtained from the dichloromethane extract of *Cratoxylum sumatranum* stembark with 50% inhibitory concentration (IC_{50}) of 22.07 ± 0.84 µg/ml. Subsequently, the dichloromethane extract of leaves and the dichloromethane extract of stem from *Garcinia parviflora* with IC_{50} of 38.59 ± 9.46 µg/ml and 68.34 ± 0.4 µg/ml, respectively. The hexane extract of stembark and the dichloromethane extract of stem from *Cratoxylum sumatranum* had IC_{50} of 69.79 ± 16.58 µg/ml and 118.49 ± 15.26 µg/ml, respectively. The dichloromethane extracts of *Cratoxylum sumatranum* stembark and *Garcinia parviflora* leaves are the most potential candidates in the development of anti-amoebic drugs.

1 INTRODUCTION

Amoebiasis is an infection of the gastrointestinal tract in humans caused by the protozoa *Entamoeba histolytica* (*E. histolytica*). Protozoa parasites are able to attack the intestinal mucosa and can spread to other organs especially the liver. When the amoeba infection has reached the liver it will cause an amoebic liver abscess [1]. Amoebiasis infection is responsible for 100,000 deaths annually throughout the world. It is therefore considered to be the third most medically important parasitosis after malaria and schistosomiasis [2].

At present the types of antiamoebic drugs used in medical treatment are divided into two classes: luminal and tissue amoebicides. Iodoquinol and paromomycin are used for the treatment of luminal amoebicides [3], while the medications used for the treatment of tissue amoebicides is metronidazole [4]. However, several studies have reported that drug resistance is cause by *E. histolytica* [5,6], thus requiring increased doses to treat infection [7] and thereby increasing the risk of discomfort from the drug's side effects [8,9]. Other studies have also reported that metronidazole is less effective against infections occurring in the intestinal lumen tissues [10].

Since humans are among the main hosts that place this parasitic life cycle, then proper treatment for amoebiasis infection is necessary to stop the development of the parasite. The search for an effective new drug for anti-amoebic activity with small side effects is needed at this time. In this case the selection of natural ingredients as a drug has advantages based on its long-term use by humans. The natural substances obtained from medicinal

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plants are potentially a good object to be studied and are expected to have low toxicity on humans [11].

According to the WHO (World Health Organization) report, around 80% of community in less developed countries almost completely rely on traditional medicine for their health treatment. [12]. Extracts from various plants have been isolated and explored for their anti-amoebic activity [13]. A wide variety of active phytochemicals, such as flavonoids, terpenoids, polyphenols, coumarin, saponins, alkaloids, xanthone and thiophenes, had been identified as inhibiting the growth of various protozoa [14]. Moreover, a number of bioflavonoid compounds, such as apigenin, galangin, kaempferol, narigenin, pinocembrin and quercetin showed biological activity against *E. histolytica* and *G. lamblia* [15]. Indonesia is said to have the second largest biodiversity in the world, with around 40,000 species of endemic plants including 6,000 medicinal plants [16]. A further study aimed at finding new anti-amoebic agents for the treatment of amoebiasis was conducted identifying of 22 medicinal plants obtained from forest exploration in Balikpapan, East Kalimantan, Indonesia. The selected plants were evaluated for the activity of their crude extract in inhibiting the growth of *E. histolytica* according to *in vitro* cell-based assay. The selection of these plant species is primarily based on the follow-up of the use of ethnobotany for the treatment or relief of symptoms of infectious diseases.

2 EXPERIMENTAL

2.1 Plants Materials

The plants used in this study were the results of Balikpapan's forest exploration (East Kalimantan, Indonesia). The plants used have been verified by licensed botanists at the Balikpapan Botanical Gardens, Balikpapan, Indonesia. The plant species, botanical names, families, and parts of plants used to obtain the extract are presented in Table 1.

2.2 Extraction of Medicinal Plants

The dried plant materials (100 g) were pulverized and then subjected to solvent extraction with different polarities sequentially in ascending order starting with hexane, dichloromethane (DCM) and ultimately methanol. The extraction process was carried out by using an ultrasonic system for each solvent. The filtrates were evaporated using an evaporator at a temperature not more than 40 °C. The extracts for bioactivity assay were dried in vacuum before being used.

2.3 Sample Stock Preparations

Each of the dry extract was weighed for 10 mg and dissolved in 1 mL of dimethyl sulfoxide (Merck) to get stock solutions at a concentration of 10 mg/mL. The stock solutions were stored at-30 °C until being used.

2.4 Culture of Entamoeba Histolytica

The cells of HM-1:IMSS (clone 6) *Entamoeba histolytica* strain, were kindly provided by Prof. T. Nozaki, The University of Tokyo, cultivated in Bisate-Iron-Serum (BI-S) medium (Sigma) that was supplemented with 10% (v/v) bovine serum (Sigma) and 1% (v/v) Diamond Vitamin-Tweena solution (JRH Biosciences, USA) at 37 °C. The cell was conditioned for 2 days to reach a confluence 80%.

2.5 Analysis of Anti-amoeba Activities of Plant Extracts

The *Entamoeba histolytica* cells were seeded in 98-well plates. 200 µL of cells and BI-S medium were added into each well, then the wells were incubated 2 hours at 35.5 °C. After 2 hours of incubation, they were replaced with mixture of medium and extract (used 2.5 µL extract and 247.5 µL medium), then incubated 24 hours. The medium was replaced with 10 % WST-1 reagent (Roche, Germany) in warmed OPTI-MEM medium (Gibco-Life Technologies). After that they were incubated for 30 minutes at 37 °C and the absorbance at 560 nm was measured using Elisa reader. The percent inhibition of cells growth by the samples was calculated by comparing to the control by using probit analysis, and IC_{50} values were determined.

2.6 Cytotoxicity Assay

The cytotoxicity of the samples was assessed by MTT assay [17]. In brief, Huh7.it cells in 96-well plates were treated with serial dilutions of the medicinal plant extracts or control for 48 hours. The medium was replaced with MTT reagent containing medium and incubated for 4 hours. The MTT solution was removed and 100 µL/well of DMSO 100% was then put for dissolution. The absorbance at 560 nm was measured using Elisa reader. The percentages of cell viability was calculated by comparing to the control, and (CC_{50}) values were determined.

Table 1. Anti-amoebic activity against Entamoeba histolytica of Balikpapan medicinal plants tested in this study

No.	Plant Species	Family	% growth inhibition[a]								
			Leaves			Stem Bark			Stem		
			Hexane	DCM	Metha-nol	Hexane	DCM	Methanol	Hexane	DCM	Methanol
1	*Melicope glabra*	Rutaceae	0	4.36	0	16.09	12.78	0	-[c]	-	-
2	*Luvunga scandens*	Rutaceae	4.20	3.31	0	6.17	0.63	0.89	-	-	-
3	*Artocarpus sericicarpus*	Moraceae	13.14	22.79	0	0	0	21.36	-	-	-
4	*Artocarpus anisophyllus*	Moraceae	0	0.89	0	0	26.63	2.06	-	-	-
5	*Artocarpus dadah*	Moraceae	0	0.54	0	0	0	0	-	-	-
6	*Scorodocarpus borneensis*	Olaccaceae	0	0	0	0	28.87	0	-	-	-
7	*Eusideroxylon zwageri*	Lauraceae	0	1.07	0	-	-	-	-	-	-
8	*Fagraea racemosa*	Loganiaceae	0	6.43	12.78	-	-	-	16.89	16.09	**0**
9	*Pternandra galeata*	Melastomataceae	0	3.31	4.02	-	-	-	0.54	19.66	**6.97**
10	*Goniothalamus macrophyllus*	Annonaceae	0	10.36	12.96	-	-	-	32.71	40.66	**16.89**
11	*Fordia splendidissima*	Fabaceae	0	3.49	3.40	-	-	-	9.56	23.15	**11.17**
12	***Garcinia parviflora*** [b]	Clusiaceae	20.46	**53.71**[b]	10.99	-	-	-	49.33	**49.87**[b]	**7.33**
13	*Aglaia lawii*	Meliaceae	5.11	34.67	22.61	-	-	-	-	-	-
14	***Cratoxylum sumatranum*** [b]	Hypericaceae	0	41.20	12.78	**53.80**[b]	**97.23**[b]	29.67	2.40	**59.96**[b]	**27.61**
15	*Gonocaryum littorale*	Icacinaceae	0	0	26.72	-	-	-	-	-	-
16	*Orophea hexandra*	Lauraceae	5.99	23.68	30.56	-	-	-	-	-	-
17	*Alstonia angustiloba*	Apocynaceae	0	29.58	26.90	0	2.49	31.81	-	-	-
18	*Gymnacranthera farguhariana*	Lauraceae	0	29.13	0	-	-	-	-	-	-
19	*Alseodaphne elmeri*	Lauraceae	0	0	8.67	15.1	11.89	0	-	-	-
20	*Neolistsea cassiaefolia*	Lauraceae	0	0	0	-	-	-	-	-	-
21	*Vernonia arborea*	Asteraceae	6.34	2.75	0.48	7.30	15.08	0	-	-	-
22	*Ficus geocaris*	Moraceae	0	0	27.49	-	-	-	-	-	-

[a] Adjusted to a concentration of 100 µg/ml and positive control using cells in the BI-S medium
[b] The plant extracts with growth inhibition of ≥ 50 % and potentially high anti-amoebic activity
[C] Not computed

3 RESULTS AND DISCUSSION

A total of 22 species of medicinal plants from Balikpapan's forest exploration (East Kalimantan, Indonesia) were tested as anti-amoebic. The 22 samples were extracted using different polarity solvents, resulting in a total of 114 extracts being used in this study. In the screening, each extract was tested for inhibitory activity of *Entamoeba histolytica* HM-1:IMSS (clone 6) strain using concentration doses of 100 µg/mL with an incubation period of 24 hours. The results in the form of percent inhibitions of extract on cell-based assay against *E. histolytica* are presented in Table 1.

Among 114 tested extracts, only five extracts showed anti-amoebic activities higher or equal to 50% mortality. Five extracts were obtained from two plants species, namely *Garcinia parviflora* and *Cratoxylum sumatranum*. The highest anti-amoebic activity (% mortality = 97.23) was obtained from the dichloromethane extract of *C. sumatranum* stembark. This showed that the chemical compound from stembark of *C. sumatranum* had very strong amoebic cell inhibition activity according to cell-based assay.

At the end of the first screening, five extracts were obtained from the dichloromethane (DCM) extracts from leaf and stem of *G. parviflora*, the DCM extract from stem of *C. sumatranum*, and the hexane and DCM extracts from the stembark of *C. sumatranum*. For the five extracts tested for anti-amoebic activities and from cytotoxic test to obtain 50% inhibitory concentration (IC_{50}), 50% cytotoxic concentration (CC_{50}) and selectivity index (SI: CC_{50}/IC_{50}), the results are shown in Table 2.

According to the results of anti-amoebic activities, the DCM extract of *C. sumatranum* stembark showed the highest values of IC_{50} = 22.07 ± 0.84 µg/mL and SI = 1.35. Subsequently, the hexane

extract of stembark and the DCM extract of stem from *C. sumatranum* had IC_{50} of 69.79 ± 16.58 µg/mL and 118.49 ± 15.26 µg/mL, respectively.

The chemotaxonomy approach of plants from hypericaceae family, it had potential as an anti-amoebic. The methanol extract from *Harungana madagascariencis* (hypericaceae) has been reported to have good inhibitory activity against growth of *E. Histolytica* with IC_{50} of 82.05 µg/mL [18].

Furthermore, anti-amoebic activities of the DCM extract of leaves and the DCM extract of stem from *G. parviflora* gave IC_{50} of 38.59 ± 9.46 µg/mL and 68.34 ± 0.4 µg/mL, respectively. The ethanol extract from *G. mangostana* belongs to the genus Garcinia in the Clusiaceae family, within the same genus as *G. parviflora*, has been reported to possess minimal inhibitory concentration (MIC) against *E. histolytica* of 500 µg/mL [19].

Chemical compounds of *C. sumatranum* and *G. parviflora* that possess anti-amoebic activities have not yet been reported. The chloroform and acetone extracts from air-dried roots of *C. sumatranum*, was reported to possess antibacterial activities against *Staphylococcus aureus* and *Micrococcus luteus* [20]. The authors identified several compounds contained in the extract including xanthone and benzophenone compounds. Meanwhile, the methanol extract from twigs *G. parvifolia*, a plant genetically close to *G. parviflora*, has also been reported to have antibacterial activities against methicillin-resistant *Staphylococcus aureus*. The chemical compounds contained in the extract are phloroglucinol, depsidone and xanthone [21]. Therefore, further research is still required to isolate compounds that take a role in anti-amoebic activity, and the study is still under way.

Table 2. Anti-amoebic activity (IC_{50}) and cytotoxicity (CC_{50}) of *Garcinia parviflora* and *Cratoxylum sumatranum*

Plant Species	Parts	Solvent	IC_{50} (µg/ml)[a]	CC_{50} (µg/ml)[a]	SI
Garcinia parviflora	**Leaves**	**DCM**	**38.59 ± 9.46**	**39.29 ± 0.21**	**1.02[b]**
	Stem	DCM	68.34 ± 0.40	40.16 ± 0.39	0.59
Cratoxylum sumatranum	Stem bark	Hexane	69.79 ± 16.58	28.26 ± 0.16	0.41
	Stem bark	**DCM**	**22.07 ± 0.84**	**29.69 ± 1.57**	**1.35[b]**
	Stem	DCM	118.49 ± 15.26	25.93 ± 0.28	0.22

[a] Data represent mean ± SD of data from two repetitions experiment
[b] Plant extracts with the high SI values

CONCLUSION

The results obtained by dichloromethane extract from stembark of *Cratoxylum sumatranum* and leaves of *Garcinia parviflora* have better anti-amoebic activity than other extracts. This suggests that these plants are the most potential candidates in the development of anti-amoebic drugs, especially to confirm the correct amoebicidal activity and biochemical anti-amoebic inhibitory mechanism.

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