ORIGINAL ARTICLE

Phytochemical screening, antimalarial activities, and genetic relationship of 16 indigenous Thai Asteraceae medicinal plants: A combinatorial approach using phylogeny and ethnobotanical bioprospecting in antimalarial drug discovery

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ABSTRACT

Emergence of artemisinin resistance leads the people to discover the new candidate for antimalarial drug. Combinatorial phylogeny and ethnobotanical approach may be useful to minimize the expenditure and time in laboratory testing. Seven hundred and thirty-three ethnomedicinal plants were listed from literature search. Obtained 340 internal transcribed spacer (ITS) sequences of plant list which met criteria were retrieved from GenBank NCBI and analyzed by MUSCLE and maximum likelihood phylogenetic test to generate the phylogenetic tree. Interactive phylogenetic tree was generated by Interactive Tree of Life (ITOL, https://itol.embl.de) and showed strong clustered pattern on Asteraceae. Afterward, 16 species of Asteraceae were selected to investigate the antimalarial activity, phytochemical, and genetic diversity. The presence of phytochemical was determined by standard method. DNA fluorescence-based assay was performed to determine the antimalarial activity against 3D7 *Plasmodium falciparum*. IC_{50} µg/mL was used to categorize antimalarial activity. On the other hand, ITS universal primer was used to amplify and sequence the obtained extracted DNA of tested plant by cetyltrimethylammonium bromide method. Phylogenetic analyses were performed by MAFFT and RAxML with automatic bootstrapping. ITOL and Adobe Illustrator were used to generate interactive phylogenetic tree. All species tested showed the presence of phenolics and flavonoids, whereas alkaloids and terpenoids were shown vary among tested extracts. Among 16 species tested, 1 species exhibited good-moderate (*Sphaeranthus indicus,* IC_{50} 6.59 µg/mL), 4 weak (*Artemisia chinensis, Artemisia vulgaris, Tridax procumbens,* and *Blumea balsamifera*), and 3 very weak (*Eupatorium capillifolium, Wedelia trilobata,* and *Vernonia cinerea*). Generated phylogenetic tree by ITS data was able to separate the tested species into their tribal

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INTRODUCTION

Herbal medicine is still being important health-care system, especially in developing countries. This traditional medicine

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classification. In addition, new medicinal properties of *A. chinensis* were discovered. Combining phylogeny approach with ethnobotanical data is useful to narrow down the selection of antimalarial plants candidate.

Key words: Antimalarial, Asteraceae, ethnobotany, genetic relationship, phylogeny, phytochemicals

practice is passed down through generation based on the experience started long time before any written records.[1] Numerous drugs including artemisinin, quinine, morphine, aspirin, and many others are derived from traditional uses.[2,3] Afterward, directed ethnobotanical bioprospecting became vogue to discover new drugs. However, species extinction, loss of traditional medicine knowledge, and limited ethnobotanical databases were encountered by merely using this approach.[2] In addition, patient's conditions, culture, spirit, belief, and communication with ancestors may be involved in the therapy depending on the traditional healer. Consequently, placebo effect may be happened and resulted in less or inactive activity during laboratory testing. In a sum, ethnobotanical-directed bioprospecting can be time-consuming, spend more expenditure, and is struggling to keep the pace with the modern approach.[1,2,4,5]

On the other hand, phylogenetic mapping of numerous cross-cultural ethnomedicinal plants revealed that similar therapeutic activity was found to be concentrated in certain lineage.[5] Various cultures use similar species or plant family to treat similar diseases or symptoms.[3] Combining ethnobotanical bioprospecting data and phylogeny may become new prospective tool to predict the medicinal bioactivity of plants due to the chemical compounds can be gene governed as a necessity of defense mechanism. Hence, similar bioactivity may be shared between related species.[5-7] Drug-derived natural product also showed to be produced by preexisting prolific drug families.[8] Nevertheless, secondary metabolites synthesis is affected by environmental factors, hence over-simplify of the phylogeny approach prediction result should not be taking done carelessly. Other studies showed that stimulant chemicals were quite scattered in the phylogenetic tree.[9] Hence, prospective chance using this approach still needs to be explored.

Malaria is still a major public health problem which causes 405,000 deaths in 2018.[10] The emergence of currently available drug resistance has caused the effort to find a new drug becoming a critical priority.[11] Internal transcribed spacer (ITS) showed the high authentication which is able to distinguish at genus and species level.[12,13] This study is the first attempt which aimed to investigate the antimalarial activity, phytochemistry, and genetic relationship-based ITS region of selected indigenous Thai medicinal plants generated by purposive selection from phylogenetic mapping of ethnomedicinal plants used by various cultures around the world for treatment of malaria and its associated symptoms.

MATERIALS AND METHODS

Phylogenetic mapping of ethnomedicinal plants

A ethnomedicinal plant list has been obtained through literatures search from Scopus, PubMed, ScienceDirect, and Google Scholar. Data extraction was performed according to Alrashedy and Molina study[9] with some modification: (1) plants used in remedies were excluded and (2) congeneric taxa were presented once to avoid visually bias (e.g., *Artemisia afra*, *Artemisia annua*, *Artemisia brevifolia,* and *Artemisia gmelinii* were presented as *Artemisia* spp.).

Obtained ITS sequences from GenBank NCBI were aligned by using MUSCLE followed by maximum likelihood phylogenetic test in Mega-X software (https://www.megasoftware.net/). Creating datasets, annotation and made up the interactive tree were performed in Interactive Tree of Life (ITOL, https://itol.embl.de) and Adobe Illustrator 2020. The result of phylogenetic mapping of ethnomedicinal plants was used as a guide for plant selection for further laboratory testing.

Extraction of selected medicinal plants

Sixteen Thai medicinal plants were collected during November 2019–January 2020 from various geographical areas in Thailand, as shown in Table 1. All plant samples were authenticated by a botanist (Dr. Orawan Theanphong) and then compared with the herbarium specimens at Forest Herbarium, Thailand (BKF). Dried powder plant's material was extracted by maceration with ethanol.

Phytochemical screening

The phytochemical screening was performed by standard method to detect the presence of alkaloid (Dragendorff's and Wagner's test), phenolics (ferric chloride), flavonoids (alkaline), triterpenes and steroids (Salkowski), diterpenes (copper acetate), and lactones (Baljet).

In vitro antimalarial activity

DNA fluorescence-based method was performed at Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand. 3D7 *Plasmodium falciparum* was cultured and maintained at 1%–2% level of parasitemia in RPMI 1640 media-contained erythrocytes supplemented

Table 1: Antimalarial activity of selected Asteraceae medicinal plants

Number	Species (location)	Traditional uses			Part used	IC_{50} (μg/mL)	Category
		Treatment	Culture	Reference			
1	*Artemisia vulgaris* (Tak)	Malaria-fever	Northern America Latin	[14,15]	AP (F-L-S)	13.37	Weak
2	*Artemisia lactiflora* (Bangkok)	Heat clearing	Chaoshan China	[16]	AP (L-S)	>100	Inactive
3	*Artemisia dracunculus* (Nakhon Pathom)	Fever	India	[17]	AP (F-L-S)	>100	Inactive
4	*Artemisia chinensis* (Nonthaburi)	-	None	-	AP (F-L-S)	18.30	Weak
5	*Ageratum conyzoides* (Nakhon Pathom)	Fever	Asia, South America and Africa	[14,18]	AP (F-L-S)	>100	Inactive
6	*Blumea balsamifera* (Chiang Mai)	Malaria-fever	Malaysia, Vietnam	[19,20]	L	19.19	Weak
7	*Bidens pilosa* (Chiang Mai)	Malaria	Africa, China, Northern America Latin	[14,21]	AP (F-L-S)	>100	Inactive
8	*Vernonia cinerea* (Chiang Mai)	Malaria-fever	Cambodia, India, China	[22-24]	AP (F-L-S)	29.17	Very weak
9	*Eupatorium capillifolium* (Nonthaburi)	Fever	Native American	[25]	AP (L-S)	31.30	Very weak
10	*Eupatorium odoratum* (Chiang Mai)	Malaria	South western and eastern Nigeria	[26,27]	L	>100	Inactive
11	*Gynura divaricata* (Bangkok)	Fever	China	[28]	AP (L-S)	>100	Inactive
12	*Gynura pseudochina* (Bangkok)	Fever	Indonesia	[29,30]	L	>100	Inactive
13	*Tridax procumbens* (Chiang Mai)	Malaria-fever	Ghana, Guatemala, India	[31-33]	AP (F-L-S)	14.93	Weak
14	*Sphaeranthus indicus* (Mukdahan)	Fever	Ayurveda	[34]	AP (F-L-S)	6.59	Good-moderate
15	*Wedelia trilobata* (Chiang Mai)	Malaria-fever	Vietnam, Indonesia	[35]	AP (F-L-S)	29.12	Very weak
16	*Acmella oleracea* (Nakhon Sithumarat)	Malaria	India, Africa	[36]	AP (F-L-S)	N/D	Unstable
Artemisinin						19.91 nM	

AP: Aerial part, F: Flower, L: Leaves, S: Stem, N/D: Not defined

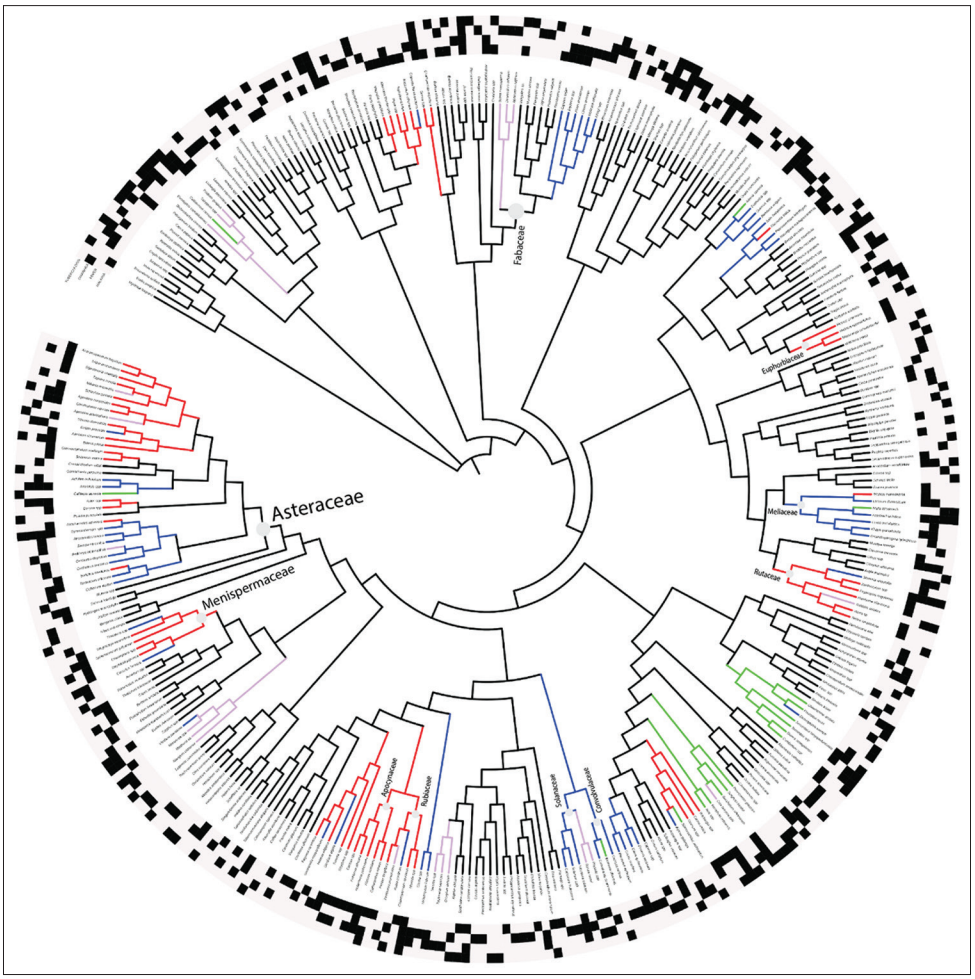

Figure 1: Phylogenetic mapping of ethnomedicinal plants used for four diseases (malaria, fever, diarrhea, and tuberculosis). Red and blue line colors indicate the therapeutic function for malaria and fever, respectively

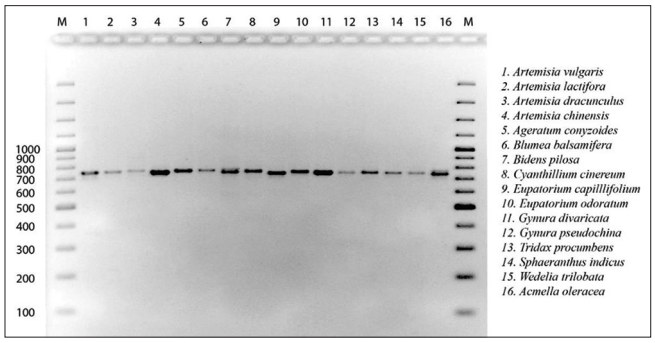

Figure 2: Amplified internal transcribed spacer of selected Asteraceae medicinal plants

with 4 mM hypoxanthine, 10% Albumax and 1M HEPES (4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid) buffer at 37°C, 5% CO_2, and 5% O_2. Synchronized parasites were obtained by treating with 5% D-sorbitol.

In vitro assay was performed by using Eppendorf epMotion® 5075. The 96-well plates were dosed with 100, 25, 6.25, 1.5625, and 0.390625 μg/mL of extract. After incubation, 100-μL fluorescence dye was added, followed by 1 h incubation under dark environment. Optical density (OD) was measured at the excitation and emission at 485 and 530 nm, respectively. Artemisinin was used as a positive control. Determination of antimalarial activity was done based on the IC_{50} (μg/mL) with these following categories: very good (<0.1), good (0.1–1), good-moderate (>1–10), weak (>10–25), very weak (25–50), and inactive (>100).

Phylogenetic analyses of tested plants

Extracted DNA by cetyltrimethylammonium bromide method was amplified using universal ITS primer by polymerase chain reaction with 95°C denaturation, 50°C annealing, and 72°C extension. Polymerase chain reaction products were sequenced at Apical Scientific Sdn Bhd, Selangor, Malaysia. The obtained sequences were aligned with MAFFT followed by RAxML automatic bootstrapping phylogenetic test in CIPRES portal (www.phylo.org). *Cannabis sativa* was used as an outgroup. Visualization of phylogenetic tree was done using FigTree v. 4.0. ITOL and Adobe Illustrator 2020 were used to create the interactive tree.

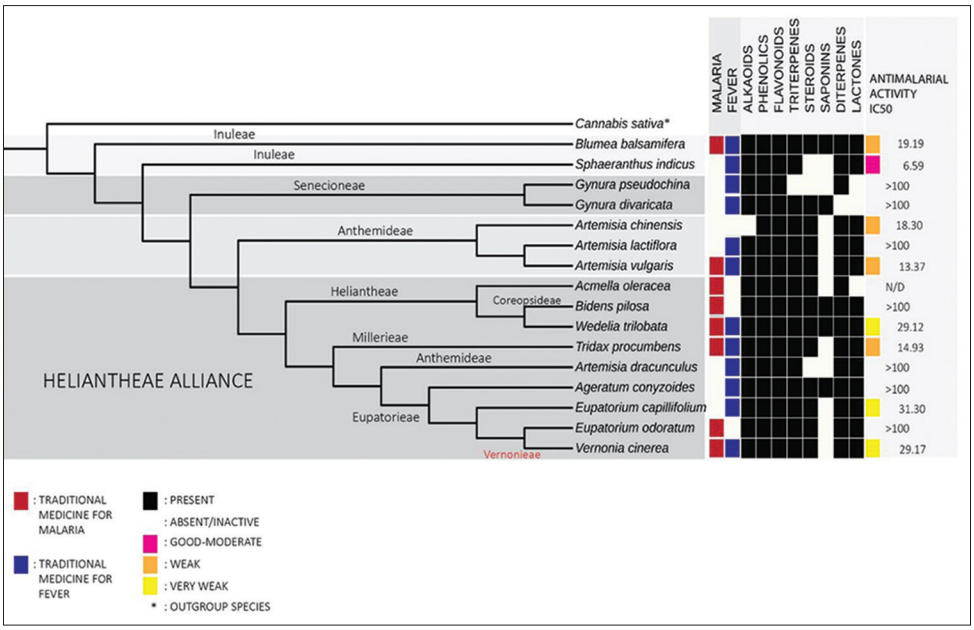

Figure 3: Phylogeny, ethnobotanical uses, phytochemical, and antimalarial activity of 16 Asteraceae medicinal plants

RESULTS AND DISCUSSION

Seven hundred and thirty-three plants used in various cultures including Africa (Zimbabwe, Uganda, West Bengal, Nigeria, Congo, Senegal, Ivory Coast, Kenya, Ghana, Madagascar, Limpopo, and Bizana) and Indomalaya (Nepal, Iran, India, Bangladesh, Pakistan, Malaysia, Thailand, and Indonesia) were obtained from literatures, nevertheless only 340 taxa were used for further analysis. Clustered pattern was majorly shown in Asteraceae for both diseases, hence this family was selected for further laboratory testing [Figure 1].

Generated phylogenetic tree by ITS sequence [Figure 2] was able to separate species into their tribal classification. However, several species including *Artemisia dracunculus, Bidens pilosa,* and *Vernonia cinerea* showed to be grouped in other clades instead of their own tribal clade based on current classification.[37] *A. dracunculus* has grouped in the clade of Heliantheae alliance instead of Anthemideae together with other *Artemisia* species. *V. cinerea* which is a member of Vernonieae-Cichorioideae has been grouped in Eupatorieae-Asteroideae [Figure 3]. On the other hand, *B. pilosa,* member of Coreopsideae, has been grouped in Heliantheae. A small number of samples and variation which can occur within species due to nonhomologous copies with the mutation may contribute the generated result.[38,39]

The four *Artemisia* were investigated for observing whether these closed-related species with *A. annua* will show the similar power of bioactivity or not. Our result showed that *Artemisia vulgaris* and *Artemisia chinensis* showed a weak antimalarial activity [Table 1] whereas *Artemisia lactiflora* showed inactive. On the other hand, our finding revealed that the new medicinal property of *A. chinensis* has been discovered by using the phylogeny approach. The antimalarial activity exhibited from *Artemisia* species can be caused by artemisinin which may act agonist or antagonist with other compounds and hence may show different powers of action. *Artemisia* species including *A. vulgaris* and *A. dracunculus* were reported to contain artemisinin even though the content was lower compared with *A. annua* [Table 2].[40]

According to our result, Inuleae and Anthemideae are worth to be investigated. Our study revealed that phylogeny approach is useful to narrow down the selection and hence will be helpful to minimize the expenditure and time in laboratory testing. However, various factors such as uneven number of tested tribe's member, part used, and typical compound in each tribe should be considered before undergoing the evaluation. Secondary metabolites can be gene governed (e.g., terpene synthesis has regulated by 8 TPS gene subfamilies) but known to be versatile caused by coevolution and environmental factor stimuli.[41,42] For example, antimalarial artemisinin content has shown to be widespread in *Artemisia* genus, and among 117 investigated *Artemisia* taxa, four clades have been highlighted due to the occurrence of artemisinin.[43]

CONCLUSION

Clustered pattern of medicinal plants used of malaria and its associated symptoms was shown in the phylogenetic tree with the strong clumping pattern in Asteraceae. Among 16 tested Asteraceae plants, *Sphaeranthus indicus* showed to be the best among others. Antimalarial properties of *A. chinensis* were discovered by using phylogeny approach.

Table 2: Preliminary phytochemical screening

Number	Species	Phenolics	Flavonoids	Alkaloids		Triterpenes	Steroids	Lactones	Diterpenes	Saponins
				Drag	Wag					
1	*Artemisia vulgaris*	++	+	−	+	+	+	+	+	−
2	*Artemisia lactiflora*	+	+	+	+	+	+	+	+	−
3	*Artemisia dracunculus*	++	+	−	+	+	−	+	+	−
4	*Artemisia chinensis*	++	++	−	−	+	+	+	+	−
5	*Ageratum conyzoides*	+	+	+	+	+	+	+	+	+
6	*Eupatorium odoratum*	++	+	+	+	+	+	+	+	−
7	*Vernonia cinerea*	++	+	+	+	+	+	+	+	−
8	*Wedelia trilobata*	++	+	−	+	+	+	+	+	−
9	*Tridax procumbens*	+	+	+	+	+	+	+	+	−
10	*Blumea balsamifera*	++	+	+	+	+	+	+	+	−
11	*Gynura divaricata*	++	+	−	+	−	+	−	−	+
12	*Gynura pseudochina*	++	+	+	+	−	−	−	+	−
13	*Bidens pilosa*	++	++	+	+	+	+	+	+	+
14	*Eupatorium capillifolium*	+	+	+	+	+	+	+	+	−
15	*Sphaeranthus indicus*	++	++	+	+	+	−	+	+	−
16	*Acmella oleracea*	++	+	+	+	+	+	−	+	−

++: present with intense color. +: present -: not present

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Conflicts of interest

There are no conflicts of interest.

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