ISOLATION AND *IN VITRO* ANTIMALARIAL ACTIVITY OF HEXANE EXTRACT FROM THAI *PICRASMA JAVANICA* B1 STEMBARK

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Abstract. The *in vitro* antimalarial activities against *Plasmodium falciparum* K1 of four extracts from the stembark of *Picrasma javanica* Bl; *ie* water, methanol, chloroform and hexane extracts were studied using a modification of the [^{3}H]hypoxanthine incorporation method. It was found that the hexane extract showed *in vitro* antimalarial activity with IC_{50} of 3.3 μg/ml. The extract was further fractionated using quick column chromatography, resulting in ten fractions. Fraction V was the most effective against *P. falciparum* K1 with IC_{50} of 4.4 μg/ml. Further isolation of fraction V using a column chromatographic technique provided six fractions. According to ^{1}H- and ^{13}C-NMR spectra, it could be concluded that the major compound in fraction V-3 was β-sitosterol. Unfortunately, the antimalarial activity of β-sitosterol could not be determined because of its low solubility in DMSO. However, fractions V-2 and V-4 still showed *in vitro* antimalarial activities with IC_{50} of 2.8 and 3.4 μg/ml, respectively. The further fractionation of these two active fractions could lead to promising candidates as antimalarial agents.

INTRODUCTION

Malaria is still the most prevalent and deadly parasitic disease in the world, infecting more than 300 million people and causing more than one million deaths each year (WHO, 1998). Human malaria is caused by four species of parasitic protozoa (i) *Plasmodium falciparum*, (ii) *P. vivax*, (iii) *P. ovale* and (iv) *P. malariae*. Of the four species, *P. falciparum* is responsible for the most severe and deadly form of malaria. However, there are only a few antimalarials, available for the treatment of *P. falciparum* infection. In addition, the resistance of *P. falciparum* to the classical antimalarials, such as quinine; chloroquine and mefloquine, has rapidly increased (Willems, 1991; Bray and Ward, 1993; Nosten and Price, 1995; Olliaro *et al*, 1996). These have triggered off a massive screening of new compounds from either synthesis, or natural products, for potential antimalarials.

Based on traditional medicine, artemisinin (qinghaosu) has been isolated from the Chinese herb *Artemisia annua* and its semisynthetic derivatives have been developed (Cumming *et al*, 1998). Although artemisinin is now effective in the treatment of malaria of both chloroquine-sensitive and resistant strains of *P. falciparum*. *P. falciparum* might rapidly develop resistance to the drugs. Therefore, it is necessary to search for new compounds as back-up antimalarials.

The bark of the medicinal plant *Picrasma javanica* Bl was reputedly used for the treatment of malaria in traditional medicine in Myanmar, Indonesia and Thailand (Ketusinh, 1948; Old Style Doctor Association, 1962; Pavanand *et al*, 1988). Pavanand *et al* (1988) demonstrated that the chloroform extract of the bark possessed a high level of *in vitro* antimalarial activity against asexual stage *P. falciparum*. Further isolation and purification of the chloroform extract resulted in the identification of two pure alkaloids in the class 1-substituted-4-oxygenated-β-carbolines, 4-methoxy-1-vinyl-β-carboline and 6-hydroxy-4-methoxy-1-vinyl-β-carboline. The first compound was effective against *P. falciparum* isolates with mean IC_{50} of 2.4 μg/ml, while the second one showed mean IC_{50} of 3.2 μg/ml.

In order to study the structure-activity relationships of 1-substituted-4-oxygenated-β-carbolines, we decided to reinvestigate the antimalarial activities and chemical constituents of Thai *P. javanica*. Herein, the results of isolation and *in vitro* antimalarial activity of the hexane extract from Thai *P. javanica* Bl stembark are reported.

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MATERIALS AND METHODS

Plant material

Stembark of *P. javanica* was collected from Queen Sirikit Botanical Garden, Chiang Mai, Thailand in July 2000, and was identified by comparison with the references deposited there, and at the Faculty of Pharmacy, Chiang Mai University, Thailand.

Preparation of crude extracts from *P. javanica* stembark

About 100 g of dried ground *P. javanica* stembark were separately macerated in 600 ml methanol, chloroform and hexane for three days or boiled with 1.5 ● of water for 10 hours. Then, they were filtered and evaporated to dryness under reduced pressure. The residue plant materials were extracted again using the same process. The second extracts were pooled together with the first corresponding extracts.

Isolation of hexane crude extract

2,206.36 g of dried ground stembark were macerated with hexane again, which resulted in a hexane crude extract of 12.152 g. It was mixed with kieseguhr (12 g) and chromatographed on a silica gel 60 (230-400 mesh, 300 g) column using hexane-ethyl acetate (9:1, 7:3, 5:5, 3:7, 1:9, 0:10) as eluent. Ten fractions were collected, based on their chromatogram on silica gel 60 GF_{254} TLC using hexane-ethyl acetate (7:3) as the solvent system. These were designated as fraction I, II, III, …, X.

Purification of fraction with antimalarial activity

Based on the antimalarial activity of each fraction, fraction V (0.7593 g) was further purified by column chromatography (SiO_2 60 g, eluents; hexane, hexane-ethyl acetate 9:1, hexane-ethyl acetate 8:2, chloroform-ethyl acetate 19:1, ethyl acetate, methanol) to give six isolated fractions, designated as fraction V-1, V-2,…,V-6. Fraction V-3 was crystallized in methanol to provide white crystal of 102.1 mg.

Structure elucidation of fraction V-3

The chemical structure of fraction V-3 was elucidated from ^{1}H- and ^{13}C-NMR spectra. These spectra were performed with a JEOL JMN-A 500 spectrometer. The solvent for these spectra was deuterated chloroform using tetramethylsilane as the internal reference standard. The chemical shifts were reported in the ppm scale: ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.66-0.98, 1.00-2.29, 3.50 (m), 5.33 (m); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ 11.83 (C-18), 11.96 (C-29), 18.76 (C-21), 19.01 (C-27), 19.37 (C-19), 19.79 (C-26), 21.06 (C-11), 23.04 (C-28), 24.29 (C-15), 26.04 (C-23), 28.22 (C-16), 29.11 (C-25), 31.61 (C-2), 31.87 (C-7 and C-8), 33.91 (C-22), 36.11 (C-20), 36.48 (C-10), 37.22 (C-1), 39.75 (C-12), 42.25 (C-4 and C-13), 45.80 (C-24), 50.10 (C-9), 56.02 (C-17), 56.73 (C-14), 71.78 (C-3), 121.69 (C-6), 140.71 (C-5).

In vitro antimalarial activity test

The antimalarial activity of extracts against *P. falciparum* K1 infected red cells was measured by using the [^{3}H]hypoxanthine incorporation method reported by Desjardins *et al* (1979) and modified by Komchonwongpaisan *et al* (1995). Briefly, extract was dissolved in dimethyl sulfoxide (DMSO) and diluted with the culture medium to the required concentration. A mixture of 25 μl of the medium containing a sample and 200 μl of 1.5% cell suspension with 1-2% parasitemia at ring stage was cultured for 24 hours, after which 25 μl of 0.25 μCi [^{3}H]hypoxanthine was added. After an additional 18 hours in culture, the cells were harvested onto glass-fiber filters (Unifilter®, Packard, USA). The filters were air-dried and 20 μl liquid scintillation fluid (Microscint, Packard) was added. The radioactivity on the filters was then measured using a microplate scintillation counter (Topcount, Packard, USA). The IC_{50}s, the concentrations required for 50% reduction of the radioactivity as compared to control without the sample, of the sample against these infected cells were obtained from dose-response curves.

RESULTS

Extraction, isolation and *in vitro* antimalarial activity

The extractions of dried ground stembark of Thai *P. javanica* provided 0.453 g of hexane crude extracts, as shown in Table 1. This extract was effective against *P. falciparum* K1 with IC_{50} of 3.3 μg/ml. The scale-up of the extraction provided about 13 g of crude extract. Further isolation of 12.152 g of crude extract obtained ten fractions. The *in vitro* antimalarial activity test of these fractions showed that fraction V was the most active, with IC_{50} of 4.4 μg/ml, as shown in Table 2. Purification of fraction V (0.7593 g) provided six fractions and their antimalarial activities are shown in Table 3. It was found that the white crystal from fraction V-3 did not dissolve in DMSO, and therefore its antimalarial activity was not determined. Fractions V-2 and V-4 showed antimalarial activities against *P. falciparum* K1 with IC_{50s} of 2.8 and 3.4 μg/ml, respectively.

Structure elucidation of pure compound

The major compound of fraction V-3 could be

Table 1
Crude extracts obtained from *P. javanica* stembark and their antimalarial activities against *P. falciparum* K1.

Crude extracts	Weight (g)	% yield	IC_{50} against *P. falciparum* K1(μg/ml)
Methanol crude extract	3.674	2.84	22.1
Chloroform crude extract	1.674	1.61	20.0
n-Hexane crude extract	0.453	0.38	3.3
Water crude extract	10.487	9.03	inactive

Table 2
Isolated fractions obtained from hexane crude extract of *P. javanica* stembark and their antimalarial activities against *P. falciparum* K1.

Isolated fractions	Weight (g)	% yield	IC_{50} against *P. falciparum* K1(μg/ml)
I	1.202	9.89	Inactive
II	0.340	2.80	Inactive
III	0.521	4.29	Inactive
IV	1.246	10.25	30.0
V	1.840	15.14	4.4
VI	0.723	5.95	20.0
VII	1.005	8.27	18.0
VIII	1.044	8.59	13.0
IX	0.292	2.40	21.0
X	0.188	1.55	12.0

Table 3
Isolated fractions obtained from fraction V and their antimalarial activities against *P. falciparum* K1.

Isolated fractions	Weight (mg)	% yield	IC_{50} against *P. falciparum* K1(μg/ml)
V-1	9.6	1.26	Inactive
V-2	70.6	9.30	2.8
V-3	102.1	13.45	Not dissolved in DMSO
V-4	25.5	3.36	3.4
V-5	17.5	2.30	11.8
V-6	4.7	0.62	12.5

assigned as a known steroid, β-sitosterol (Fig 1) through analysis of its ^{1}H- and ^{13}C-NMR spectra. The ^{1}H-NMR spectrum showed signals at δ 0.66-0.98 ppm, which were the signals of methyl protons of a steroid compound. The signals at 1.00-2.29 ppm were the signals of methylene and methine protons of a steroid compound. The multiplet signal at δ 3.50 ppm was the signal of the proton at C-3. The olefinic signal at δ 5.33 ppm (m) could be assigned as H-6, which was trisubstituted vinylic proton. The ^{13}C-NMR spectrum showed the signals of 3 quaternary, 9-methine, 11 methylene and 6 methyl carbons.

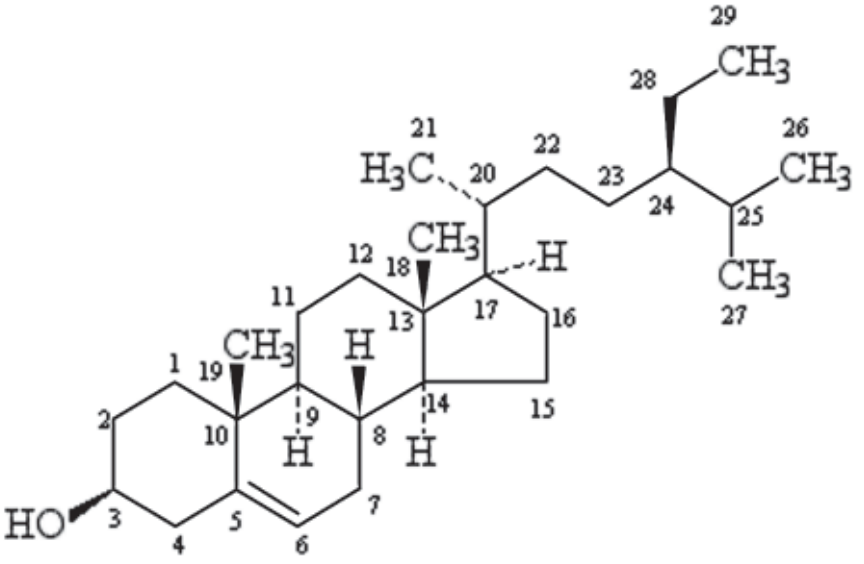

Fig 1- β-sitosterol.

DISCUSSION

Both the ^{1}H- and ^{13}C-NMR spectra were in accordance with those published previously for β-sitosterol (Wright *et al*, 1978; Kosin, 1996). Therefore, it could be concluded that the major compound of fraction V-3 was β-sitosterol.

A previous report by Ziegler *et al* (2002) has demonstrated that the *in vitro* antimalarial activity against *P. falciparum* 3D7 strain of lupeol; cholesterol reminiscent structure, appeared to be indirect, being due to stomatocytic transformation of the human erythrocyte and not to toxic effects via action on a drug target within the malaria parasite. Since the agents that inhibit malaria parasite growth through erythrocyte membrane modifications are unsuitable, this leads to the development of new antimalarials; fractions V-2 and V-4, still showed high level *in vitro* antimalarial activities against *P. faciparum* K1, and could be further purified. This investigation may lead to non-sterol candidates.

Conclusion

In order to study structure-activity relationships of 1-substituted-4-oxygenated-β-carbolines, the antimalarial activities and chemical constituents of *P. javanica* were reinvestigated. The result demonstrated that the hexane extract from *P. javanica* stembark showed the highest level of *in vitro* antimalarial activity against *P. falciparum* K1. Further fractionation provided fraction V, that still showed high level *in vitro* antimalarial activity against *P. falciparum* K1. Further isolation of fraction V provided six fractions. According to ^{1}H- and ^{13} C-NMR spectra, it could be concluded that the major compound in fraction V-3 was β-sitosterol. Unfortunately the antimalarial activity of β-sitosterol can not be determined because of its low solubility in DMSO. However, fractions V-2 and V-4 still showed high level *in vitro* antimalarial activity against *P. falciparum* K1. The further fractionation of these two active fractions could lead to promising antimalarials.

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