The alkaloid Berberine isolated from *Coscinium fenestratum* is an inhibitor of phytopathogenic fungi

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ABSTRACT

Ethanol Crude extract of *Cosicinium fenestratum* stems (Gaertre) Coleb (Menispermaceae) exhibited inhibiting activity against seven plant pathogenic fungi and one antagonistic fungus when they were tested using the dilution method. Ethanolic crude extract of *C. fenestratum* stems was then fractionated by column chromatography using six organic solvents, and subsequent antimicrobial activity evaluation revealed that only the fraction eluted with methanol exhibited inhibitory activity against the tested phytopathogenic fungi. Further re-fractionation resulted in 60 subfractions eluted with 60 solvent combination systems of increased polarity, that were also evaluated for their antimicrobial effect, being the subfractions eluted with mixture of acetone: chloroform and chloroform: methanol identified as the most active against tested plant pathogenic fungi. The chromatographic profile of the active subfractions revealed a common metabolite, which was isolated by PTLC (Preparative Thin Layer Chromatography) and identified as berberine through NMR (Nuclear Magnetic Resonance) analysis. Berberine displayed relevant antimicrobial activity against several of the tested plant pathogenic fungi being identified as the main active constitutuent from the active subfractions.

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INTRODUCTION

Cosicinium fenestratum (Gaertre) Coleb. "HAM" locally known as Berberine vine and Weniwel., is widely known for its content biological activity effects. In addition to several relevant pharmacological effects both from aqueous and organic extracts of the plant stems (Singh *et al.,* 1990; Venukumar and Latha, 2004; Shirwaiker *et al.,* 2005; Puninha *et al.,* 2005; Rojsanga *et al.,* 2005), the plant also displayed remarkable antimicrobial activity (Kumar *et al.,* 2007). Several chemical studies on the bioactive metabolites content of the *C. fenestratum*, revealed that the plant is extremely rich in alkaloids such as palmatine, jatrorrhizine, calumbamine, berberine and isoquinoline (Narasimh and Nair, 2004; Siwon *et al.,* 1980; Molthotra *et al.,* 1989) and that its antimicrobial properties are mainly attributed to berberine (Nair *et al.,* 2005)

Several phytopathogenic fungi are causal agents of the destruction and a decrease on the quality of crops, leading to an important economical impact on agriculture. The use of synthetic antifungal agents, currently widespread in Asia, can be considered as extremely hazardous both to the environment as well as to human and animal health. With this work, we aimed to screen new natural sources that could lead the identification of new alternative antifungal agent that can effectively combat phythopathogenic fungi, with decreased cost and increased safety.

In this study we identified several subfractions obtains from *C. fenestratum* ethanolic extract, and subsequent chemical characterization of those subfractions led to the isolation and identification of purify compund as the main active constituent, responsible for the antifungal activity against several plant pathogenic fungi.

MATERIALS AND METHODS

Plant materials

Ham samples of the stem and stem pieces were collected from Phu Wau Wildlife Sanctuary, Nongkai province; Vanyai district, Mukdahan province and from the Medicinal Plant Garden, Chanthaburi province. Fresh samples were cut into small pieces and air dried at room temperature. The small pieces of dry samples were blended to obtain fine powder and used for extraction.

Crude Extraction

Fifty g of fine powder from stems of the different *C. fenestratum* samples were macerated in 500 mL of 95 % ethanol, and kept at room temperature for 10 days. Filtration through cheesecloth was performed to separate the plant debris (sediments), and the resulting solution was filtrated through filter paper Whatman grade 4. The extraction was carried out three times, and the final filtrated solution was concentrated at reduced pressure, using a vacuum rotary evaporator, to yield of a stick oily ethanolic crude extract.

Fractionation of crude extract

Five g of ethanolic crude extract was applied on a column chromatography over 20 g of Si gel (0.2-0.5 mm) and eluted with six solvents with increased polarity, namely petroleum ether (PE). chloroform ($CHCl_3$), diethyl ether (DE), ethyl acetate (EA), acetone (AC) and methanol (MeOH), wherein six fractions of 600 mL were collected with each solvent, and concentrated at reduced pressure. The preliminary fractions were then assayed against plant pathogenic fungi.

Further re-fractionation of the ethanolic crude extract was performed also by column chromatography and eluted with 60 distinct solvent systems, through mixtures of increased polarity of PE, $CHCl_3$, DE, EA, AC and MeOH, wherein 60 subfractions of 600 mL were collected and concentrated at reduced pressure. The subfractions were assayed against plant pathogenic fungi, and subsequent re-fractionation was performed for the most active subfractions to purity them.

Plant Pathogenic organisms preparation

Six plant pathogenic fungi such as *Colletotrichum capsici, Colletotrichum gloeosporioides, Fusarium oxysporum fsp. cubense, Rhizoctonia solani, Sclerotium roffsii, Lasiodiplodia therobromae* and one antagonistic fungi (*Trichoderma* sp.) were tested for antimicrobial activity. All tested fungi were obtained from the Mycology and Fungal Disease Branch of the Plant Pathology Department, Kasetsart University, Bangkok, Thailand. They were cultured to produce spores on PDA slant agar for 21 days. They were prepared for spore suspension in sterile distilled water and used for the experiments.

Antimicrobial activity tests

Dilution method

Solutions of crude extracts were prepared in methanol at concentrations of 100,000, 10,000, 1,000, 100, and 0 ppm. One µL of each concentration was mixed into 9 µL of PDA to obtain cultures with concentrations of 10,000, 1,000, 100, 10 and 0 ppm, respectively, and they were poured into 9-centimeter-diameter Petri dishes. Selected phytopathogenic fungi were cultured for 3-5 days on PDA to obtain young colony growth. A small dish of mycelia (0.5 cm diameter) was placed on the centre of the PDA containing crude extract in the Petri dish and incubated for 3, 5, 10 and 15 days at 25 °C. Diameters of each colony were measured and the percentage of mycelia inhibition was calculated (Hannarong, 2007; Hannarong *et al.*, 2013).

The dilution method in liquid medium was conducted by preparing crude extracts of five concentrations (100,000, 10,000, 10,000, 100 and 0 ppm) in MeOH. Five µL of each concentration, 44 µL of potato dextrose broth, and one ml of spore suspension were mixed together in 250 µL Erlenmeyer flask. Cultures

at concentrations of 10,000, 1,000, 100, 10 and 0 ppm were obtained and were incubated for 3-5 days with rotary shaking at 100 rpm. Colony plate counting and optical density were observed by spectrophotometry to calculate the spore density after culturing. This method was used for testing antimicrobial activity against the yeast-like form of the fungus, *Sphaceloma ampelinum*, the causal agent of grape scab.

Diffusion method

The diffusion method was conducted by culturing spore suspensions in PDA. Spores of test fungi were produced by culturing the fungi in PDA medium in a test tube for 2-3 weeks to obtain a large quantity of spores. Nine µL of sterilized distilled water was mixed into a test tube to make the spore suspension. The suspension was filtered to separate the mycelia using sterilized cheesecloth to obtain the spore suspension. One µL of spore suspension was mixed into nine µL of PDA and poured onto a Petri dish. Five concentrations (100000, 10000, 1000 100 and 0 ppm) of crude extract containing active fractions were prepared in methanol and were dipped on 0.5 cm diameter pieces of filter paper using 30 µL and allowed to air dry before placing on the surface of the culture medium containing the spore suspension. Diameters of the clear inhibition zone were measured at 3 and 7 days after incubation. (Hannarong, 2007; Hannarong *et al.,* 2013)

Antifungal activity of the fractions

The six preliminary fractions, namely by the extraction solvents, PE, $CHCl_3$, DE, EA, AC and MeOH were prepared for four concentrations; 100,000, 10,000, 1,000 and 0 ppm and then they were tested for antifungal activity using the diffusion method. The experiment was carried out by completely randomized design with 2 replications.

Sixty-one subfractions were prepared for one concentration of 100,000 ppm in methanol and 20 µL of each subfraction were dipped onto the paper dished and then places on the PDA containing the spore suspension of the testing organisms. Also the selected active subfraction, $CHCl_3$: MeOH at 90:10 and 80:20, were tested for antifungal activity against three plant pathogenic fungi by diffusion method. The procedure was the same as described in the diffusion method procedure.

Isolation of the active constituents

The subfractions that displayed antifungal activity were further purified by PTLC. Briefly, the TLC plates were prepared by coating Silica gel 60(GF_{254} Merck), on 20x20 cm glass plates. Silica gel 60 was mixed with distilled water at the ratio of 40: 85 w/v. The slurry suspensions of silica 60 were coated on the surface of glass plates using a coating instrument. About 15 gm of silica 60 was applied to each plate. The plates were dried at 90 ºC for 2 hrs in a hot air oven. The selected subfractions were purified by TLC (Si Gel, butanol /glacial acetic acid / water 6: 3: 1) to yield of purified active constituents berberine.

Correlations

The dosage response curve for *C. fenestratum* active subfraction against plant pathogenic fungi; *Sphaceloma ampelinum*, the causal agent of grape scab disease was studied. The experiment was conducted with two replications using the diffusion method. The diameters of inhibiting clear zone were measured at 5 days after testing. The linear regression was analyzed to obtain a linear equation.

Structure Elucidation

H1 and 13C NMR spectra were recorded at ambient temperature on a Bruker AMC instrument (Bruker Bioscience Corporation, Billerica, MA, USA) operating at 300.13 and 75.4 MHz, respectively. The structure of the purified compound was elucidated by Professor Dr. Anake Kijjoa, Department of Chemitry, Instituto de Ciências Biomédicas Able Salazar, University of Porto, Portugal.

Antifungal Activity of Berberine

Purified berberine, isolated from the active subfractions of *C. fenestratum* ethanolic crude extract, and commercial berberine chloride were assayed for the antifungal activity against the selected plant pathogenic fungi. The antifungal activity was investigated through the diffusion method, previously described, at four different concentrations,

100000, 10000, 1000 and 0 ppm.

Statistical Analysis of the data

The experiments in this study were carried out by completely randomized design with and none replications. The datas were shown by mean values which were averaged by the number of replications. The difference of mean values were analyzed by Duncan Multiple Range Test and the linear relationship between concentration of berberine and diameter of inhibiting clear zone was analyzed by linear equation

y= a+bX where y= berberine concentration, X= diameter of inhibiting clear zone, b= slope of linear line and a= constant value.

RESULTS AND DISCUSSION

Antifungal activity of crude extract

The results showed that, at 10,000 ppm the ethanolic crude extract of the stems of *C. fenestratum* caused total inhibition of mycelial growth in all the selected phytopathogenic fungi species. Furthurmore, at the concentration of 1,000 ppm, the crude extract caused also a complete inhibition of *S. rolfsii*, as well as potent mycelial growth inhibitory activity (>50% inhibition mycelial growth) against *F. oxysporum* fsp. *cubense*, *R. solani*, and *Trichoderma* sp.. At concentration below 1,000 ppm the antifungal activity can be classified as weak or inexistent (Table 1).

The dilution method in liquid medium was conducted to evaluate the fungicidal activity of crude extract using the yeast-like form of the fungus *Sphaceloma ampelinum* as the tester. The results indicated that a concentration of 10,000 ppm exhibited the lowest optical density (0.07), number of spores per µL, (10.0), spore dry weight (0.18 gm) and diameter of inhibiting clear zone was the highest at 1.16 cm, respectively, (Table 2) which suggested that crude extract of *C. fenestratum* at this concentration was the most effective to exhibit fungicidal activity against *S. ampelinum*.

Antifungal activity of fractions of *C. fenestratum* ethanolic crude extract against plant pathogenic fungi.

Antifungal activity evaluation of the fractions obtained from the fractionation of crude extract revealed that only the fractions eluted with methanol exhibited antifungal activity against all tested fungi. The value of diameter inhibiting clear zone which was averaged from 5 tested fungi were 2.04 and 0.82 cm at concentration of 100,000 and 10,000 ppm, respectively. Most of the fungi were inhibited at concentration of 100,000 ppm, although, some of fungi, *S. ampelinum* and *R. solani* were inhibited at concentration 10,000 ppm. The result suggested the existence of antifungal constituents in methanolic fraction.

Table 1. Inhibition activity of *C. fenestratum* crude extract against mycelial growth of plant pathogenic fungi at 5 concentrations by the dilution method at 3 days after culturing

Plant pathogen	% mycelial inhibition at concentration[1*]				
	10,000	1,000	100	10	0
C. capsici	100.0	39.5	23.2	-4.6	0.0
C.gloeosporioides	100.0	49.4	6.9	6.4	0.0
F.oxysporum fsp. *cubense*	100.0	63.5	-1.4	2.7	0.0
R. solani	100.0	64.1	-15.4	5.1	0.0
S. rolfsii	100.0	100.0	31.6	1.3	0.0
Lasiodiplodia sp.	100.0	0.0	0.0	0.0	0.0
Trichoderma sp.	100.0	71.2	18.2	-4.3	0.0
Mean	100.0	55.4	0.9	0.9	0.0

[1*] mean value averaged from 4 replications

Antimicrobial activity of sub fraction

Antifungal activity evaluation revealed that only the subfractions eluted with acetone: methanol and chloroform; methanol solvent systems, displayed relevant antifungal activity against *S. ampelinum*, *F. oxysporum* fsp. *cubense* and *C. Capsici*. The diameter of inhibiting clear zone of subfractions acetone: methanol (F41-F50) and chloroform: methanols (F51-F60) were ranged from 0.9-3.0 cm. While the subfractions eluted with the remaining solvent systems; PE: $CHCl_3$, $CHCl_3$: DE, DE: EA, and EA: AC exhibited minimal or no activity (Table 3).

The chromatograms of 60 subfractions indicated that one of common compound appeared in most of subfractions (F3-F60) but they were differed in degree of quantity which

Table 2. Inhibition activity of *C. fenestratum* crude extract against plant pathogenic fungi *S. ampelinum* at 5 concentrations by the dilution method

Concentration (ppm)	Optical density	No. of spore per mL (x 10^{11})	Spore dry weight (gm)	Diameter of inhibiting clear zone (cm) [1*]
10,000	0.07	10.0	0.18	1.16[a]
1,000	0.88	120.8	0.26	0.00[b]
100	0.98	210.2	0.38	0.00[b]
10	1.02	296.0	0.38	0.00[b]
0	1.14	496.0	0.41	0.00[b]

[1*] In a column means followed by a common letter are not significantly different by DMRT at 0.05 level of probability

Table 3. Antifungal activity profile of micro fractions from *C. fenestratum* crude extract against plant pathogenic fungi by the diffusion method

Fractions	**Solvent Combinations**	**Ratio of Solvent**	**Diameter of clear inhibition zone (cm)**		
			Spha	***FOC***	***Cap***
F40	EA : AC	10 : 90	0	0	0
F41	AC : MeOH	100 : 0	1.2	0	0
F42	AC : MeOH	90 : 10	1.0	0	0
F43	AC : MeOH	80 : 20	1.0	0	0
F44	AC : MeOH	70 : 30	2.5	2.2	1.7
F45	AC : MeOH	60 : 40	2.3	2.2	0
F46	AC : MeOH	50 : 50	1.8	1.9	1.7
F47	AC : MeOH	40 : 60	2.2	1.7	0
F48	AC : MeOH	30 : 70	2.3	1.6	2.1
F49	AC : MeOH	20 : 80	0.9	1.7	1.3
F50	AC : MeOH	10 : 90	2.6	2.0	2.1
F51	CHCl3:MeOH	100 : 0	3.0	2.3	1.3
F52	CHCl3:MeOH	90 : 10	2.0	2.3	1.6
F53	CHCl3:MeOH	80 : 20	1.0	1.7	1.3
F54	CHCl3:MeOH	70 : 30	1.3	1.8	1.4
F55	CHCl3:MeOH	60 : 40	1.6	2.4	1.7
F56	CHCl3:MeOH	50 : 50	-	-	-
F57	CHCl3:MeOH	40 : 60	-	-	-
F58	CHCl3:MeOH	30 : 70	-	-	-
F59	CHCl3:MeOH	20 : 80	-	-	-
F60	CHCl3:MeOH	10 : 90	-	-	-
	Crude		2.5	2.0	-
	MeOH		0.0	1.3	-
	Berberine		1.4	1.3	-

PE- Petroleum Ether, CHCl3- Chloroform, DE-Diethyl Ether, EA-Ethyl Acetate, AC-Acetone, MeOH-Methanol, *Sphaceloma ampelinum* (Spha),*Fusarium oxysporum* fsp. *cubense* (FOC), *Colletotrichum capsici* (Cap)

F_1-F_{40} = exhibited negative antimicrobial activity and it is not necessary shown in table

was indicated by the size of spots which exhibited on TLC plates. Subfractions F1-F2 exhibited none compound, F3-F34 one compound at a low quantity, F35-F45 one compound at moderate quantity and F46- F60 exhibited two compounds which one compound was larger quantity than the second one.

The TLC Chromatograms were corresponded to the antimicrobial activity profile of 60 subfractions which revealed that subfractions 44-60 exhibited high degree of antifungal activity and large quantity of active compound. It suggested that the antifungal constituents were contained in those subfractions. This antifungal activity profile will be guided information for fractionation.

Antifungal activity of selected subfractions

The results indicated that at the highest concentration tested the selected subfractions could effectively inhibit the growth of *S. ampelinum*, *F. oxysporum* fsp. *cubense* and *C. capsici*. *S. ampelinum* and *F. oxysporum* fsp *cubense* exhibited a clear inhibition zone at a concentration of 10,000 ppm (Table 4) which confirmed that the active compound of *C. fenestratum* was contained in the fraction of $CHCl_3$:MeOH or in a fraction combining with MeOH. Column chromatography was conducted to obtain the subsub fraction. The results indicated that all the subsub fractions of $CHCl_3$: MeOH exhibited a clear inhibition zone. Fractions of $CHCl_3$: MeOH at 90:10 and 85:15 showed the highest inhibiting activity with clear inhibition zones having diameters of 3.1 and 3.0 cm, respectively. The results strongly suggested that the main active constituents responsible for the antifungal activity of *C. fenestratum* ethanolic crude extract, could be contained in the referred subfractions. It also confirmed that chloroform alone could not extract the active fraction.

Table 4. Inhibitory activity of active sub fractions against plant pathogenic fungi at four concentrations by the diffusion method.

Fraction of CHCl3:MeOH	Plant Pathogenic Fungi	Concentration (ppm)			
		100,000	10,000	1,000	0
90:10	*Sphaceloma ampelinum*	2.6	1.6	0.6	0.0
	Fusarium oxysporum fsp. *cubense*	2.9	1.7	0.0	0.0
	Colletotrichum capsici	1.0	0.0	0.0	0.0
80:20	*Sphaceloma ampelinum*	2.0	0.6	0.0	0.0
	Fusarium oxysporum fsp. *cubense*	2.6	2.0	0.0	0.0
	Colletotrichum capsici	0.9	0.7	0.0	0.0
MeOH Crude extract	*Sphaceloma ampelinum*	2.5	1.2	0.0	0.0
	Fusarium oxysporum fsp. *cubense*	2.5	1.6	0.0	0.0
	Colletotrichum capsici	2.7	0.0	0.0	0.0

Relationship between concentration and antimicrobial activity of active fraction

The relationship between concentrations and antifungal activity against the fungus *S. ampelinum* was investigated using the active subfraction CHCl3: MeOH (90:10) by the diffusion method. The results indicated the existing of a linear relationship between concentrations and the clear inhibition zone which suggested that as the concentration of active subfraction increased, the diameter of the inhibiting clear zone was increased (Figure 1). The relationship was expressed by the linear equation Y (conc.) = (-40060.02+53496.67X (diameter) as shown in Figure 1 and the value of r^2 was 0.82.

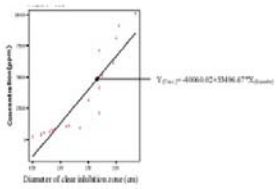

Fig. 1. Dosage response curves of active fractions of *Coscinium fenestratum* against the fungus *Sphaceloma ampelinum.*

NMR spectral analysis and comparison with data from literature revealed that the crystalline compound isolated from the active subfraction eluted with $CHCl_3$: MeOH (90:10) is berberine. Further comparison with a commercial sample of berberine was performed by TLC, confirming the identity Rf value of the isolated active compound (Rf = 0.69) and commercial berberine (Rf= 0.65).

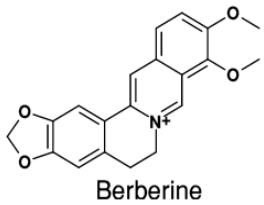

Fig. 2. Structure of berberine (1) *(Wei et al.,* 2012*)*

Coscinium fenestratum is wildly used in folk and Ayurvedic systems of medicine. Both stems and roots of *C. fenestratum* have been reported by their richness in active secondary metabolites, mainly alkaloids (Anjaliet *et al.*, 2013). During our preliminary screening on the antifungal properties of plants collected from Thai flora, we have identified *C. fenestratum* as a potential, causing the inhibition on the growth and spore multiplication of the phytopathogenic fungi *S. ampelinum*, the grape scab disease causal agent (Hannarong *et al.*, 2013).

Bioassay-guided fractionation of the ethanolic crude extract of the stems of *C. fenestratum* collected from different locations, revealed that the subfractions eluted with solvent systems containing methanol, displayed relevant antifungal activity. Further purification of those subfractions led to the isolation and identification of the alkaloid berberine. The purified berberine was assessed for its antifungal activity displaying relevant inhibitory effect on the mycelial growth of *S. ampelinum* and *F. oxysporum* fsp. *cubense*. Additionally, the resultd from the antifungal activity against the selected plant pathogenic fungi were compared with commercial berberine, displaying similar growth inhibitory activities.

Berberine has been widely reported from several plants such as Berberis (e.g. *Berberis aquifolium, Berberis vulgaris and Berberine aristata*), *Hydrastis canadensis, Phellodendron amurense and Coptis chinensis* amongs others. The isoquinoline alkaloid has been previously reported from marketable sample of *C. fenestratum* inThailand, and identified as a major constituent of the plant, reaching 9.87 to 16.38% of dry weight of samples and 11.84 to 18.45 % of dry weight crude extracts (Rojsanga *et al.*, 2005), indicating that the selected natural source is a great reservoir of this antifungal agent. Furthermore, in a study that aimed to quantify the content of berberine in methanolic extracts was high (0.98 to 0.99 % w/w), which is in agreement with our results, suggesting the extraction with polar solvents could successfully lead to an efficient berberine extraction.

In the present study we reported the antifungal activity of berberine, apparently the main constituent from the stems of *C. fenestratum*, in agreement with Tran *et al.* (2012). Despite the reports on the synergistic antifungal activity of berberine in combination with the imidazole antifungal agents, miconazole and fluconazole, against the human pathogenic fungus *Candida albicans*, this is the first report in the antifungal activity against plant pathogenic fungi. Since the inhibitory effect on the mycelial growth, of both crude extract and berberine was comparable, it is suggested that the alkaloid is the main active compound, responsible for the antifungal properties of the plant. However, there are several reports on the phytochemical characterization of *C. fenestratum* extracts, dealing with the isolation of new secondary metabolites such as deoxypalmitine (Talat *et al.*, 2012), amongs others (Malhotra *et al.*,1989; Pinho *et al.*, 1992; Siwon *et al.*, 1980), indicating that this source has great potential as a source of bioactive compounds, namely with antifungal activity.

Preliminary antifungal screening of plants

from Thai flora led us to identify "HAM" (*C. fenestratum*) as a promising source of antifungal agent. Bioassay-guided fractionation and antifungal activity evaluation revealed that several subfractions displays relevant inhibitory activity on the mycelial growth of phytopathogenic fungi. Subsequent purification of those subfractions led to the isolation of the alkaloid berberine, which also proved to be extremely active against the tested plant pathogenic fungi. These results, in conjugation with the reports on literature indicating that *C. fenestratum* is a rich source of berberine, and that could be effectively and easily extracted with methanol, in a cheap and fast way.

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