Hydroxychavicol from *Piper betel* leave is an antifungal activity against plant pathogenic fungi

Narong Singburaudom

ABSTRACT

Ethanol crude extract of *Piper betel* Linn. (Piperaceae) leaves were tested under *in vitro* for antifungal activity against plant pathogenic fungi in economic crops. It exhibited 100 percent inhibition against all tested plant pathogenic fungi at a concentration of 1 % using the dilution method whereas inhibition was evident at 10% when tested by the diffusion method. The results suggested that crude extract exhibited fungistasis activity by inhibiting mycelial growth, and fungicidal activity by inhibiting spore germination. Fractionation of crude extract by column chromatography eluted with 51 solvent combination systems of increased polarity yielded 51 micro fractions which were tested for inhibition activity against plant pathogenic fungi *in vitro.* The micro fractions F4, F5, F7 and F8 showed antifungal activity and the TLC chromatogram indicated the common compounds were contained in these four fractions. Further re-fractionation of the most active fractions, F7 and F8 were eluted with mixture of petroleum ether and chloroform and tested for antifungal activity. The 21 submicro fractions of PE: $CHCl_3$ at ratio of 40:60 to 32:68 exhibited antifungal activity with the same results as in the former experiment. The TLC chromatograms indicated the presence of the like compounds and it is suggested that the active compounds might be contained in these sub micro fractions of PE: $CHCl_3$. The dosage response curve indicated a positive linear relationship between concentrations and the level of antifungal activity. NMR (Nuclear Magnetic Resonance) spectroscopy was conducted to elucidate the chemical structure of the active compound contained in the fractions F7 and F8 which was identified as hydroxychavicol. The study concluded that hydroxychavicol in the crude extract from *P. betel* leaves is an active constituent against plant pathogenic fungi.

MS History: 09.07.2015 (Received)-17.07.2015 (Revised)-18.09.2015 (Accepted)

Key words: *Piper betel*, active constituent fractionation, antifungal activity profile, hydroxychavicol

Citation: Narong Singburaudom. 2015. Hydroxychavicol from *Piper betel* leave is an antifungal activity against plant pathogenic fungi. *Journal of Biopesticides*, 8(2): 82-92.

INTRODUCTION

Piper betel L. (betel vine) (Piperaceae) is a plant with known ethnomedicinal properties which has been used in traditional herbal medicine in several countries (Kumar *et al.,* 2010, Amonkar *et al.,* 1986, Singh *et al.,* 2009, Norton, 1998, Prabhu *et al.,* 1995, Chang *et al.,* 2002, Sharma *et al.,* 2009 and Amonkar *et al.,* 1986). The leaves contain bitter compounds, phenol and terpene-like constituent, which indicates the quality of the leaves and exhibits various pharmacological activities. (Ernst *et al.,* 2002; Canto'n *et al.,* 2003; Nalina *et al.,* 2007; Avijit *et al.,* 2008; Murata *et al.,* 2009; Ali *et al.,* 2010; Al-Adhroey *et al.,* 2014). Additionally, allyl pyrocatecholmono acetate, eugenol, terpinen-4-ol and eugenol acetate alkaloid, tannins and steroid have been reported among others. Eugenol was identified as the antifungal principle in the oil (Craig *et al.,* 1996, Drugeon *et al.,* 1999, Jin *et al.,* 2003). The leaf extract fractions and purified compounds are found to play a role on different biological activities *in vitro* and *in vivo.* The active compounds isolated from this plant are hydroxychavicol, hydroxychavicol acetate, allypyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A and piperol B (Rooney, 1996; Singh *et al.,* 2009; Kumar *et*

al., 2010). The betel leaves extract shows antimicrobial activity against several pathogenic bacteria. The bioactive compound against bacteria is sterol and its mode of action is due to the disruption of the permeability barrier of the microbial membrane structure (Mula *et al.,* 2008). The chloroform extraction of *P. betel* is much more efficient than the methanol fraction against fungal activity because the presence of non-polar components in the fraction (Daniell *et al.,* 2009). Sharma (2009) evaluated purified hydroxychavicol from the leaves of *P. betel in vitro* against the oral cavity pathogen. It leads us to the fact that hydroxychavicol isolated from chloroform extraction shows inhibitory activity against all the tested oral cavity pathogens. The modes of action included preventing the formation of water-insoluble glucan, extending the postantibiotic effect, preventing the emergence of mutant of *S. mutans* and *Actinomyces viscosus,* inhibiting growth bio films and reducing the preformed bio film by bacteria. It also exhibited disruption of the permeability barrier of the microbial membrane structure, with efficacy against adherent cells of bacteria in water-insoluble glucan.

Ali *et al.* (2010) were the first to study the antifungal activity of hydroxychavicol isolated from the chloroform fraction of the aqueous leaf extract of *P. betel* L. The result indicated that hydroxychavicol exhibited significantly inhibitory effect on 124 strains of selected fungi. Hydroxychavicol inhibited the growth of biofilm and reduced the performance of biofilm generated by *C. albicans.* This indicated that the active compound from *P. betel* might be used as an antifungal compound for the treatment of fungal infection as well as a gargle mouthwash against oral cavity pathogens.

Due to there being few reports on the activity of secondary metabolites from *P. betel* against the phytopathogenic organisms, *P. betel* and related species are widely grown in the Asian region and plant diseases are a major problem in the production of economic crops and synthetic chemicals are still widely used in large quantities and at high prices with a consequential effect on the environment and the safety for human consumption of crop produce. The most important reason is that some diseases have no effective chemical control and the more effective chemicals are expensive. The objective of this investigation was to isolate and purify the active compound from *P. betel* and to test its efficacy to inhibit seven plant pathogenic fungi which are the causal agents of economic crop diseases. The inhibition potential of the extract will be used to develop natural fungicides for the control of economic crop diseases in the future.

MATERIALS AND METHODS

Plant materials

Fresh blue green leaves of commercial cultivar were purchased from the market and were used for plant material in this investigation. They were cut into small pieces and air dried at room temperature for 5-7 days. The small pieces of dry leaves were blended in a rotary blender (Model IKA A11 basic) to obtain fine powder and used for crude extraction.

Test plant pathogenic organisms

Seven plant pathogenic fungi: *Colletotrichum gloeosporioides, Rhizoctonia solani, Fusarium oxysporum* f. sp. *cubense, Sphaceloma ampelinum, Colletotrichum capsici, Alternaria brassicicola* and *Pyricularia oryzae* were tested for antifungal activity All tested fungi were obtained from the Mycology and Fungal Disease Branch of the Plant Pathology Department, Kasetsart University, Bangkok, Thailand. They were isolated from disease infected plants and tested for pathogenecity on their host plants cultured on Potato Dextrose Agar (PDA) slant and used for the experiments.

Crude extraction

Fifty g of fine powder of dry leaves were macerated in 500 ml of 95% ethanol for 10 days at room temperature. The macerated solution was filtered through cheesecloth and cotton wool to separate the plant debris, and the resulting solution was filtered through filter paper Whatmann grade 4. The maceration and extraction was carried out three times and the final filtered solution was

concentrated at reduced pressure, using a vacuum rotary evaporator, to yield crude extracts in the form of dark green sticky oily substance.

Fractionation of crude extract

Macro fraction extraction

Five g of ethanol crude extract was applied on a column chromatography over 20 g of silica gel 60 (0.2-0.5 mm, Merck) 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. They were then assayed against plant pathogenic fungi by diffusion method.

Micro fraction extraction

Further fractionation of the ethanol crude extract was performed also by column chromatography and eluted with 51 distinct solvent systems which were prepared by two combing between six organic solvents, PE, $CHCl_3$, DE, EA, AC and MeOH, wherein 51 microfractions of 600 mL were collected and concentrated at reduced pressure. The microfractions were assayed against plant pathogenic fungi, and subsequent re-fractionation was performed for the most active sub microfraction to purify them by eluting with the mixture of the most effective solvent combination systems.

Antimicrobial activity tests

Dilution method

Solutions of crude extracts were prepared in methanol at concentrations of 10%, 1%, 0.1%, 0.01% and 0.001%. One µL of each concentration was mixed into 9 µL of PDA to obtain cultures with concentrations of 1%, 0.1%, 0.01% and 0.001%, respectively and they were poured into petri dishes of 9-centimeter-diameter and 1.5 cm height. 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 transplanted from the culture and placed on the centre of the PDA containing crude extract in the Petri dish and incubated for 10 to 15 days at 25 °C. Diameters of each colony were measured and the percentage of mycelia inhibition was calculated (Singburaudom, 2015).

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 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 (10, 1, 0.1, 0.01 and 0.001%) 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 on 3 and 7 days after incubation (Singburaudom, 2015).

Antifungal activity of the fractions

The six macro fractions by the extraction solvents, PE, $CHCl_3$, DE, EA, AC and MeOH were prepared at four concentrations such as 10, 1, 0.1, and 0.001% and then tested for antifungal activity using the diffusion method. The experiment was carried out by completely randomized design with 2 replications. Fifty-one microfractions were prepared for one concentration of 10% in methanol and 20 µL of each microfractions was dipped onto the paper dishes and then placed on the PDA containing the spore suspension of the testing organisms. Also the selected active sub microfractions, $CHCl_3$: 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 sub-micro fractions that displayed antifungal activity were further purified by Preparative Thin Layer Chromatography (P-TLC). Briefly, the TLC plates were prepared

by coating Silica gel 60 (GF_{254} Merck), on 20 x 20-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 sub-micro fractions were isolated and purified with active constituents (Singburaudom, 2015).

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 MHz and 75.4 MHz respectively. The structure of the purified compound was elucidated by Professor Dr. AnakeKijjoa, Department of Chemistry, Instituto de Ciências Biomédicas Able Salazar, University of Porto, Portugal.

Antimicrobial activity profile

The chemical profile of crude extract was determined by TLC using aluminium-backed, thin-layer chromatography plates (TLC silica gel 60 F_{254} 25 aluminium sheets 20x20 cm, MERCK). Fractions obtained from the column chromatography were chromatographed on TLC plate and the development of the chromatogram was performed by the mixture of solvent $CHCl_3$: PE: EA 10: 8: 2. The separated chemical components were visualized under visible and ultraviolet light at 254 and 360 nm using a UV lamp chamber (VILBER LOURMAT, IVAL). Quantitative measurements of antifungal activity of fractions tested by the diffusion method were used to construct the antimicrobial activity profile corresponding to the chemical profile of the fractions.

Correlation of concentration and antifungal activity of active fraction

The dosage response curve for active fraction of *P. betel* against selected plant pathogenic fungi *C. gloesporioides* and *S. ampelinum* was investigated. Active micro fraction F8 was prepared in methanol to obtain 20 concentrations which ranged from 0 to 10%. The interval between the lower and the higher level concentrations was decreased 10 times for each reduction. The antimicrobial activity test was conducted using the diffusion method. The experiment applied a completely randomized design with two replications. One Petri dish was used for each treatment. The diameter of the clear inhibition zone was measured 5 days after incubating. Statistical analysis was conducted to obtain the linear regression equation.

Statistical analysis

The experiments in this study were carried out by completely randomized design with and without replications. The data 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 (DMRT) and the linear relationship between concentration of hydroxychavicol and diameter of inhibiting clear zone was analyzed by linear regression analysis and the relationship was expressed by the linear equation; $Y= a+bX$

where X = concentration of crude extract,

Y = diameter of inhibiting clear zone,

b= slope of linear line and a= constant value.

RESULTS AND DISCUSSIONS

Acaricidal Antimicrobial activity

The antifungal activity test of ethanol crude extract against four plant pathogenic fungi exhibited 100 percent mycelial growth inhibition at a concentration of 1.0 percent by the dilution method. In concentration at and below 0.1 percent, the crude extract exhibited less than 50 percent mycelial growth inhibition against all tested fungi. The result of analysis of variance indicated a highly significant different of mean value of percent mycelial inhibition at 0.0005 level of probability (Table1). The result of experiment suggested that the effective concentration of crude extract to inhibit mycelial growth of these fungi might be between 0.1-2.0 percent. Because of the dilution method exhibited the effect on mycelial growth inhibition, it could be identified that crude extract of *P. betel* played on fungistatic activity against *C. gloeosporioides, C. capsici, F. oxysporum* f. sp. *Cubense* and *P. oryzae in vitro.* The diffusion method was conducted to evaluate

the fungicidal activity of crude extract using spore of fungi as the tester. The analysis of variance indicated significantly different mean of clear inhibiting zone of different concentrations at p=0.0005,0.013,0.04 and 0.097 for *C. capsici, C. gloeosporioides, S. ampelinum and F. oxysporum* f. sp. *cubense*, respectively (Table 2).The result indicated that a concentration of 10 percent exhibited the significantly different clear inhibiting zone against four tested fungi, whereas concentration of 1.0 percent exhibited weak or inexistent antifungal activity against *C. capsici, F. oxysporum* f.sp. *cubense* and *S. ampelinum* at concentration below 0.1 percent, crude extract exhibited none-inhibiting clear zone for all tested fungi (Table 2). The result suggested that the fungi, *C. capsici*, *F. oxysporum* f. sp. *cubense*, and *S. ampelinum* were more sensitive to crude extract than *C. gloeosporioides*.

Table 1. Inhibition activity of *P. betel* crude extract against four plant pathogenic fungi after culturing on medium containing at five concentrations by dilution method

Concentration (%)	Percent mycelial inhibition[a]			
	C. glo	*C. cap*	FOC	*P. oryzae*
1.0	100.0±0.00a	100.0±0.00a	100.0±0.00a	100.0±0.00a
0.1	24.5±2.40b	10.8±4.36b	21.1±3.75b	31.5±4.28b
0.01	2.9±1.39c	-0.07±10.08bc	9.5±1.21c	20.4±2.74c
0.001	-0.3±1.22c	-4.2±10.05c	4.3±1.82cd	14.2±2.50c
0.0	0.0±0.00c	0.0±0.00bc	0.0±0.00d	0.0±0.00d
Sig (p-value)	0.000	0.000	0.000	0.000

a = Mean in the same column followed by a different letter are significantly different by DMRT at p = 0.000
C.glo= C. gloeosporioides C. cap = C. capsici FOC= *F. oxysporum fspcubense*

Table 2. Inhibition activity of *P. betle*crude extract against three plant pathogenic fungi at five concentrations by diffusion method

Concentration (%)	Diameter of clear inhibition zone (cm)[a]			
	C. glo	*C. cap*	FOC	*Spha*
10.0	0.9±0.25a	1.5±0.10a	1.6±0.75a	1.3±0.20a
1.0	0.0±0.0b	0.6±0.10b	0.7±0.10ab	0.4±0.40b
0.1	0.0±0.00b	0.0±0.00c	0.0±0.00b	0.0±0.0b
0.0	0.0±0.00b	0.0±0.00c	0.0±0.00b	0.0±0.00b
Sig (p-value)	0.013	0.000	0.097	0.040

a = Mean in the same column followed by a different letter are significantly different by DMRTat p ranged between 0.000 – 0.097; *C. glo= C. gloeosporioides, C.cap= C.capsici; FOC=F. oxysporum fspcubense, Spha= S. ampelinum*

Antifungal activity profile of *P. betel* crude extract

Antifungal activity of the six macro fractions obtained from the crude extract fractionation were evaluated against three plant pathogenic fungi and it revealed that the fractions eluted with $CHCl_3$ and DE exhibited antifungal activity at concentrations of 100,000, 10,000 and 1,000 ppm, diameter of inhibiting clear zone were 2.82, 0.87 and 0.2 cm for $CHCl_3$ fraction and 1.24, 0.5 and 0.22 cm for DE fraction respectively when it was compared to antifungal activity of crude extract which exhibited inhibiting clear zone 1.4, and 0.23 respectively. The other macro fractions, PE, EA, AC and MeOH did not exhibit antifungal activity which indicated that the active antifungal constituent contained in these two macro fractions and the $CHCl_3$fraction was the most active fraction because it exhibited larger/ clearer inhibiting zone than DE fraction at concentration of 1.0 %. The result suggested that the $CHCl_3$ and DE were the most suitable solvents for isolation of active constituent in crude extract. Antifungal activity profile was conducted by testing fifty-one micro fractions for antifungal activity against fungi: *C. gloeosporioides* and *S. ampelinum* by diffusion method.

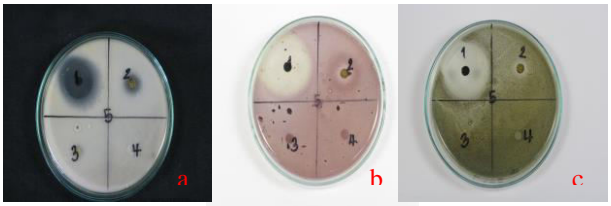


Fig 1. Clear inhibition zone of *P. betel* crude extract against (a) *C. gloeosporioides,* (b) *F. oxysporum* fsp. *oxysporum* and (c) *C. capsici.*

The experiment aimed at identifying the appropriate solvent combination system for isolating purified active constituent. The result suggested that the most active constituent which was indicated by diameter of inhibiting clear zone was contained in the solvent combination PE: $CHCl_3$ with a solvent ration range from 70:30 to 20:80 (F4, F5, F7 and F9) and this solvent combination was selected for re-fractionation to more purified active compound. All the active micro fractions were selected for re-fractionation by column chromatography to isolate sub micro fractions. The result of the antimicrobial activity test revealed that most of the active compounds were contained in the fractions of PE: $CHCl_3$ with a solvent ratio range from 40:60 to 32: 68. Sub micro fractions MF6 to MF14 exhibited a high degree of inhibiting activity against four tested plant pathogenic fungi (Table 3). The result of re-fractionation confirmed that the highest active fraction was from micro fraction F7 and F8. Furthermore, the results indicated that the active fraction of *P. betel* crude extract could be isolated by the solvent combination of petroleum ether (PE) and chloroform ($CHCl_3$). TLC chromatograms corresponded to the antimicrobial activity profile of 51 micro fractions which revealed that micro fraction F4-F9 exhibited the presence of active compounds.

Table 3. Antimicrobial activity profile of active submicro fraction of crude extract against four plant pathogenic fungi.

Submicro Fraction	Solvent combinations	Ratio of solvents	Diameter of clear zone (mm)			
			S. ampelinum	*C. gloeosporioides*	*F. oxysporum*	*R. solani*
MF_1	PE : $CHCl_3$	45: 55	0.6	0.9	1.0	0
MF_2	PE : $CHCl_3$	44 : 56	0	0	0	0
MF_3	PE : $CHCl_3$	43 : 57	0	0	0	0
MF_4	PE : $CHCl_3$	42 : 58	0	0	0	0
MF_5	PE : $CHCl_3$	41 : 59	0	0	0	0
MF_6	PE : $CHCl_3$	40 : 60	2.2	1.6	2.4	3.0
MF_7	PE : $CHCl_3$	39 : 61	2.5	2.4	2.8	3.5
MF_8	PE : $CHCl_3$	38 : 62	3.2	3.2	3.8	4.7
MF_9	PE : $CHCl_3$	37 : 63	3.0	3.1	3.8	4.9
MF_{10}	PE : $CHCl_3$	36 : 64	3.0	3.0	4.0	5.9
MF_{11}	PE : $CHCl_3$	35 : 65	3.4	3.5	3.5	4.0
MF_{12}	PE : $CHCl_3$	34 : 66	2.0	2.9	3.1	3.5
MF_{13}	PE : $CHCl_3$	33 : 67	1.5	1.5	2.6	3.0
MF_{14}	PE : $CHCl_3$	32 : 68	1.9	0	1.9	2.4
MF_{15}	PE : $CHCl_3$	31 : 69	0	0	0	0
MF_{16}	PE : $CHCl_3$	30 : 70	0	1.5	0	0
MF_{17}	PE : $CHCl_3$	29 : 71	0.6	0	0	0
MF_{18}	PE : $CHCl_3$	28 : 72	0.6	0	0	0
MF_{19}	PE : $CHCl_3$	27 : 73	0	0	0	0
MF_{20}	PE : $CHCl_3$	26 : 74	0	0	0	0
MF_{21}	PE : $CHCl_3$	25 : 75	0	0	0	0
Mean			1.17	1.12	1.38	1.66
Crude extract	EtOH	100	2.0	2.7	3.0	3.3

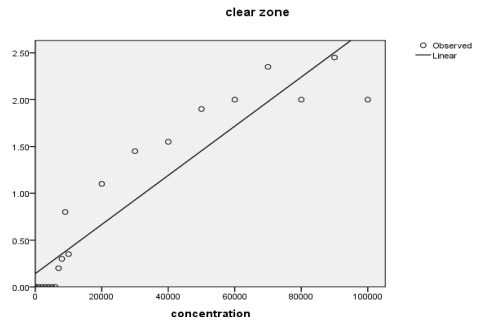
PE: Petroleum Ether $CHCl_3$: Chloroform

This antifungal activity profile will serves as guided information for fractionation and isolation of purified active compound.

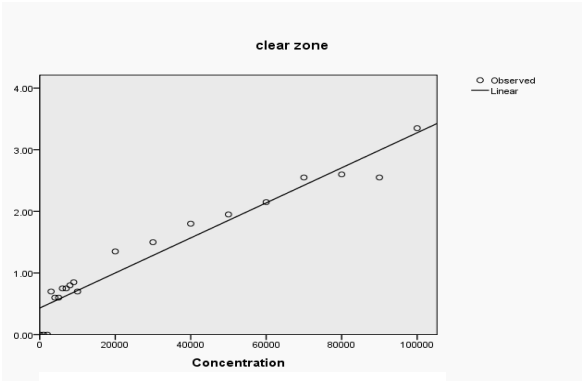
Relationship between concentration and antifungal activity

The relationship between concentrations and antifungal activity of crude extract against plant pathogenic fungi was investigated using the active microfraction PE: $CHCl_3$ (40:60) by the diffusion method. The results indicated the existance of a linear relationship between concentrations and the clear inhibition zone also which suggested that as the concentration of crude extract increased, the diameter of the

inhibiting clear zone was increased. The relationships were expressed by the linear equation: $Y_{C.glo}$= 0.142+0.000026X for *C. gloeosporioides* and Y_{spha} = 0.431 + 0.000028X for *S. ampelinum*. The linear regression analysis indicated the regression line slope of both pathogens were significantly different at $p \leq 0.000$. The r^2values also exhibited high, 0.933 and 0.868 for *S. ampelinum* and *C. gloeosporioides* respectively, which indicated a close relationship existing between the concentrations of crude extract and diameter of clear inhibition zone. The result of regression analysis is shown in Figure 2.



A. *Colletotrichum gloeosporioides*
$Y = 0.142 + 2.623e^{-5}X$
$R^2 = 0.868$
Sig (p) = 0.000
F = 118.07***
t = 10.866***



B. *Sphacelomaampelinum*
$Y = 0.431 + 2.844e^{-5}X$
$R^2 = 0.933$
Sig (p) = 0.000
F = 252.61***
t = 15.894***

Fig 2. Dosage response curse of active fraction of *P. betel* crude extract against plant pathogenic fungi.

Identification of active compound

The results of column chromatography indicated that fractions of petroleum ether and chloroform at ratios of 40:60 (F7) and 30:70 (F8) exhibited significant antimicrobial activity against test fungi. The compounds in the submicro fractions exhibited effective activity to inhibit the fungus at concentrations of 10%. Submicro fractions of PE: $CHCl_3$ from 35:65 to 38:62 exhibited the highest antimicrobial activity which was indicated by a bigger and clearer inhibition zone. (Table 3) The TLC chromatography results of these sub-micro fractions were observed under UV light at a wavelength of 254 nm and only one compound was present in all the submicro fractions. The active compound in each sub-micro fraction was located at the same position on each TLC plate which revealed that these sub-micro fractions contained the same active compound that exhibited antimicrobial activity to inhibit *C. gloeosporioides* and *S. ampelinum*. The 1H and 13C NMR spectra analysis and comparison with data from literature revealed that the crystalline compound isolated from the active sub-microfraction was elucidated to hydroxychavicol (Figure 3).

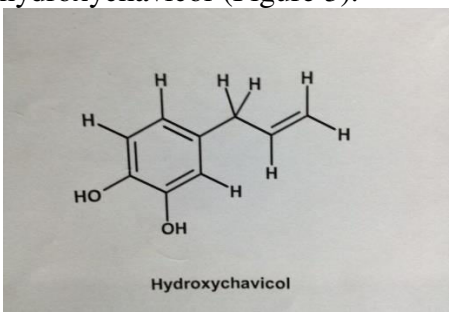


Fig 3.Structure of Hydroxychavicol.

Generally, the activities of crude extract from *P. betel* leaves are related to phenolic constituents such as hydroxychavicol (HC), hydroxylchavicol acetate, allypyrocatechol (APC), *piper betel* methyl, *piper betel* piperol A and piperol B. The activities of these compounds are attributed to several types of antibiological activity of human diseases (Kumar *et al.,* 2010). *P. betel* leaf extract contains essential oil, and its phenolic constituents (APC and HC) have been demonstrated to have antimicrobial properties against a number of oral bacteria and pathogens such as *Vibrio cholerae*ogawa, *Diplococcus pneumoniae* and *Klebsiella*

aerogenes (Shitut *et al.,* 1999; Ramji *et al.,* 2002; Rozak *et al.,* 2003; Glupta *et al.,* 2009). Hydroxychavicol has been reported as the major active compound which was isolated from the aqueous extract of *P. betel* leaf. It is a phenolic component which exhibits various pharmacological activities (Chang *et al.,* 2002, 2007). There have been a few reports on the antibacterial and antifungal activities of hydroxychavicol (Ramji *et al.,* 2002; Sharma *et al.,* 2009) Recently, Ali *et al.* (2010) reported on the *in vitro* antifungal activity of hydroxychavicol isolated from *P. betel* involving a total of 124 fungal strains including *Aspergillus* spp. such as *A. flavus, A fimigatus, A. niger* and *A. parasiticus*. However, there are still few reports on the antifungal activity of plant pathogenic fungi and no reports on the anti-activity of plant pathogenic organisms, particularly plant pathogenic fungi which are the causal organisms of economic crop diseases.

Prior to the commencement of this study, we conducted a preliminary screening of 100 kinds of Thai medicinal plants for their antifungal activity against major plant pathogenic fungi and selected for study antimicrobial activity profile and isolation active compounds. *Piper betel* L. is a herbal medicinal plant which exhibited a high potential because it has been used for oral hygiene since ancient times. It has a high potential for developing a natural fungicide to combat plant diseases because the crude extraction from the *Piper betel* leaves produced more than 20 percent of crude extract from the ethanol extraction of dried leaves (data not shown). In this present study, ethanol crude extract exhibited antimycelial growth at a concentration of 1% against tested fungi (*Colletotrichum gloeosporioides,* F. *oxysporum* f.sp. *cubense* and *Sphaceloma ampelinum*) the causal organisms of mango anthracnose, banana wilt and scab of grape respectively (Table 1). The fungicidal activity of crude extract was evaluated by diffusion method (Table 2) and the results indicated that the active compound contained in crude extract of *P. betel* posseses fungicidal activity to kill or inhibit the spores of fungi.

The results of the investigation on antifungal activity suggested that the active compound in crude extract of *Piper betel* demonstrated fungistasis and fungicidal effects against plant pathogenic fungi. The same result was reported by Ali (2010) who said that hydroxychavicol which was extracted from *P. betel* leaves exhibited fungicidal activity with all 124 stains of tested fungi. Isolation of hydroxychavicol has been reported by Sharma *et al.* (2008). Hydroxychavicol extraction has been reported previously. Freshly procured leaves of *P. betel* (1 kg) were extracted in boiling water (3 liters) with stirring for 4 h. The resulting extract was filtered through muslin cloth, centrifuged and concentrated to one-sixth of the original volume under reduced pressure at 50 ± 5 °C on a film evaporator. The concentrate extract was then extracted with chloroform in a separating funnel. The chloroform fraction was concentrated under reduced pressure to yield a residue (5.06 g) containing 80% hydroxychavicol as monitored by High Pressure Liquid Chromatography (HPLC) and TLC. The $CHCl_3$ crude extract was separated using a silica gel column with 1.0% methanol in chloroform (v/v) as the eluting agent. Fractions of 100 ml each were collected and subjected to TLC in $CHCl_3$: MeOH (19:1). Fractions containing hydroxychavicol were crystallized from the benzene-petroleum ether as a collected solid.

In this study the search for an active compound against plant pathogenic fungi was conducted using silica gel column chromatography. Ethanol crude extract was isolated using six solvents (petroleum ether, chloroform, diethyl ether, ethyl acetate, acetone and methanol). Solvent combination systems were made using two solvents to obtain 51 solvent combinations (fractions) which had different properties with their increasing polarity from low to high polarity. All fractions were collected and were tested for antifungal activity by the diffusion method. The results demonstrated that we were successful in isolating and selecting the active compound for use in studying its

chemical structure. The results of NMR demonstrated that the active compound is hydroxychavicol and this is a significant finding on the antimicrobial activity of hydroxychavicol against plant pathogenic fungi. Quantification of the antimicrobial activity of the active compound can be determined by a linear response as reported by Sharma *et al.* (2008). Quantification of hydroxychavicol exhibited a linear response in the concentration range 17.5 to 35 µg/mL, and the calibration curve was prepared using the multipoint calibration curve method. A working solution was injected with different concentrations. An excellent calibration curve was obtained for hydroxychavicol (r^2= 0.9986) determined on the basis of six levels of concentration.

In this study, we used the dosage response curve to identify the relationship between antifungal activity and concentrations of active compound (fractions containing hydroxychavicol). We quantified the antimicrobial activity of hydroxychavicol which exhibited a linear response in the concentration range from 0.1% to 10%. The results revealed that it exhibited a linear relationship when the concentrations were tested for anti-activity with two fungi; the linear equations are shown in Figures 2 for the *C. gloeosporioides* and *S. ampelinum*, respectively. These equations are useful for the quantification of antimicrobial activity of a natural product composed of hydroxychavicol as the active ingredient.

The results presented in this study are the first information of purified hydroxychavicol for antifungal activity against plant pathogenic fungi. Hydroxychavicol is one of the major constituents of *P. betel* leaves. It exhibited fungistasis and fungicidal effects to inhibit plant pathogenic fungi. It exhibited antifungal activity to a broad range of plant pathogenic fungi. Further studies are exploring the possibility of developing a natural fungicide for plant disease control.

ACKNOWLEDGEMENT

Kasetsart University Research Development Institute (KURDI), Kasetsart University, Bangkok, Thailand; Institute de CienciasBiomedicas de Abel Salazar, University of Porto, Portugal.

REFERENCES

Ali, I., G. F. Khan, K. A. Suri, B.D. Gupta, N.K. Satti, P. Dutt, F. Afrin, G.A. Qazi and I.A. Khan. 2010. *In vitro* antifungal activity of hydroxychavicol isolated from *Piper betel* L. *Annals of Clinical Microbiology and Antimicrobials,* **9**:7.

Amonkar, A. J., M. Nagabhuchan, A.V. D' Souza and S.V. Bhide. 1986. Hydroxychavicol: A New phenolic antimutagen from betel leaf. Fd. *Chemical Toxicology*, **24**:1321-1324.

Al-Adhroey, A.H., Z.M. Nor, H.M. Al-Mekhlafi, A. A. Amran and R. Mahmud. 2010. Antimalarial activity of methanolic leaf extract of *Piper betle* L. *Molecules,* **16**(1): 107-18.

Avijit, S., S. Rupashree, S. Piu, G. Sudipto, M. Goutam, and C. Mitali. 2008. An ethanolic extract of leaves of *Piper betle* (Paan) Linn mediates its antileishmanial activity via apoptosis. *Parasitology Research,***102**:1249-1255.

Bhattacharya S., D. Banergee ,A.K. Bauri, S. Chattopadhyay, and S.K. Bandyopadhyay. 2007. Healing properties of the *Piper betle* phenol, allylpyrocatechol against indomethacin-induced stomach ulceration and mechanism of action. *Word Journal of Gastroenterology,* **21**:3705-3713.

Canto, N. E., Pema, J. A. N., Viudes, G., Quindo, S., Gobernado, M. and A. Espinel- Ingroff. 2003. Minimum fungicidal concentrations of amphotericin B for bloodstream *Candida* species. *Diagnostic Microbiology and Infectious Disease,***45**: 203–206.

Chang, C., B.J. Uang, H.L. Wu, J.J. Lee and J.H. Jeng. 2002. Inducing the cell cycle arrest and apoptosis of oral KB carcinoma cells by hydroxychavicol: Roles of glutathione and reactive oxygen species. *Brazilian Journal of Pharmacology*, **135**:619-630.

Chang, C., B.J. Uang, C.Y Tsai, H.L. Wu, B.R. Lin, C.S. Lee, Y.J. Chen, C.H.

Change, Y.L. Tsai, C.J. Kao and J.H. Jeng. 2007. Hydroxychavicol, a novel betel leaf component, inhibits platelet aggregation by suppression of cyclo oxygenenase, thromboxane production and calcium mobilization. *Brazilian Journal of Pharmacology*, **152**:73-82.

Craig, W.A. and Gudmundsson, S. 1996. Postantibiotic effect. P. 296-329. In V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams and Wilkins Co., Baltimore, MD.

Daniell, H., N.D. Singh, H. Mason and S.J. Streatfield, 2009.Plant-made vaccine antigen and biopharmaceuticals. *Trends Plant Sciences*, **14**: 669-679.

Drugeon, H.B., Juvin, M.E. and Bryskier, A. 1999. Relative potential for selection of fluoroquinolone- resistant *Streptococus pneumoniae* strains by levofloxacin: comparison with ciprofloxacin, sparfloxacin and ofloxacin. *Journal of antimicrobial Chemotherapy.* **43**: 55-59.

Ernst,E. J., Ellen, E., Roling, C., Rosemarie, P., Douglas, J. K. and Klepser, M. E. 2002.*In vitro* activity of micafungin (FK-463) against *Candida* spp. microdilution, time-kill, and postantifungal-effect studies.*Antimicrobial Agents and Chemotherapy*,**46**: 3846–3853.

Ghosh, K. and Bhattacharya. T. 2005. Chemical constituents of *Piper betle* Linn. (Piperaceae) roots. *Molecules*, **10**:798-802.

Gülçín, Í. 2005.The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. *International Journal of Food and Science Nutritions,* **56**:491-499.

Jin, Y., Yip, H. K., Samaranayake, Y. H., Yau, J. Y. and Samaranayake, L. P. 2003. Biofilm-forming ability of *Candida albicans*is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. *Journal of Clinical Microbiology,* **41**: 2961-2967.

Katsura, H., Tsukiyama, R. Suzuki, A. and Kobayashi, M. 2001. In vitro antimicrobial activity of bakuchiol against oral microorganisma. *Antimicrobial Agents Chemotherapy*, **45**:3009-3013.

Koga, T., Hamada, S., Murakawa,S. and Endo,A. 1982. Effect of a glucosyl-transferase inhibitor on glucan synthesis and cellular adherence of *Streptococcus mutans.* Infect. *Immunology*, **38**: 882-886.

Kolen brander, P.E., Andersen, R.N. and Moore, L.V. 1990. Intra generic co-aggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. *Applied Environment Microbiology,* **56**: 3890-3894.

Kolenbrander, P.E. 2000. Oral microbial communities: biofilms, interactions, and genetic systems. *Annual Review of Microbiology*, **54**:413-437.

Kumar, N., Misra, P., Dube, A., Bhattacharya, S., Dikshit, M. and Ranade, S. 2010.*Piper betel* Linn. A maligned Pan-Asiatic plant with an array of pharmacological activities and prospects for drug discovery. *Current Sciences,* **99**: 922-932.

Lei D., Chan, C., Wang, Y., Wang, T., Lin, B., Huang, C., Lee, J., Chen, H., Jeng, J. and Chang, M. 2003.Antioxidative and anti platelet effects of aqueous inflorescence *Piper betel* extract. *Journal of Agricultural and Food Chemistry,* **51**: 2083-2008.

Manoharan, S. and Kaur, J. 2013. Anticancer, antiviral, antidiabetic, antifungal and phytochemical constituents of medicinal plants. *American Journal of Pharm Tech Research*, **3**(4): 149-169.

Mula, S., Banerjee, D., Patro, B.S., Bhattacharya, S., Barik, A., Bandopadahyay, S.K. and Chattopadhyay, S. 2008. Inhibitory property of the *Piper betel* phenolics against photosensitization-induced biological damages. *Bio organization of Medicinal Chemistry*, **16:** 2932-2938.

Murata, K., Nakao, K., Hirata, N., Namba, K., Nomi, T., Kitamura, Y., Moriyama, K., Shintani, T., Iinuma, M. and Matsuda, H. 2009.Hydroxychavicol: a potent xanthine oxidase inhibitor obtained from the leaves

of betel, *Piper betel*. *Journal of Natural Medicine,* **63**:355-359.

Nalina, T. and Rahim, Z.H.A. 2007.The crude aqueous extract of *Piper betle* L. and its antibacterial effect towards *Streptococcus mutant. American Journal of Biotechnology and Biochemistry*, **3**:10-15.

Norton S. A. 1998. Betel: Consumption and consequences. *Journal of American Academical Dermatology*, **38**:81-88.

Prabhu M.S. Patel, K., Saraawathi, G. andSrinivasan,K.1995. Effect of orally administered betel leaf (*Piper betel* leaf Linn.) on digestive enzymes of pancreas and intestinal mucosa and on bile production in rats. *Indian Journal of Experimental Biology,* **33**:752-756.

Ramji, N., Ramji, N., Iyer, R. and Chandrasekaran, S. 2002. Phenolic antibacterials from *Piper betel* in the prevention of halitosis. *Journal of Ethnopharmacology,* **83**: 149-152.

Rathee J., Patro, B., Mula, S., Gamre, S. and Chattopadhyay, S. 2006.Antioxidant activity of *Piper betle* leaf extract and its constituents. *Journal of Agricultural and Food Chemistry,* **54**:9046-9054.

Sharma, S., Khan, I. A., Ali, I., Ali, F., Kumar, M., Kumar, A., Johri, R. K., Abdullah, S. T., Bani, S., Pandey, A., Suri, K. A., Gupta, B. D., Satti, N. K., Dutt, P. and Qazi, G. N. 2009. Evaluation of the antimicrobial, antioxidant, and anti-inflammatory activities of hydroxychavicol for its potential use as an oral care agent. *Antimicrobial Agents and Chemotherapy,* **53**: 216–222.

Sigh R. and Rao, H. 2008. In vitro antioxidant activity of *Piper nigrum* Linn. *Phcog. Mag*. **4**:115-120.

Sigh, M., Shakya, S., Soni, V.K., Dangi, A., Kumar, N. and Bhattacharya, S. M. 2009. The n-hexane and chloroform fractions of *Piper betel* L. trigger different arms of immune responses in BALB/c mice and exhibitanti filarial activity against human lymphatic filarid *Brugiamalayi*. *International Journal of Immunopharmacology,* **9**:716-728.

Trakranrungsie. N., Chatchawanchonteera, A. and Khunkitti, W. 2006. Anti dermatophytic Activity of *Piper betle* Cream. *Thailand Journal of Pharmacology,* **28**:16-20.

Narong Singburaudom
Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
Office phone number+6625791026;
Fax number +6629428044;
Email: agrnrs@ku.ac.th