Antifungal activity profile of *Piper longum* fruit extract against plant pathogenic fungi

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ABSTRACT

A series of five-step experiments was conducted to construct the antifungal activity profile of *P. longum* extracts against plant pathogenic fungi *in vitro.* The ethanol crude extract exhibited potent activity against all tested fungi using disc diffusion method. The crude extract was then partially fractionated in order by column chromatography using six organic solvents, and evaluated for their antifungal activity. The results revealed that the fractions eluted with diethyl ether (DE) and ethyl acetate (EtOAc) exhibited potent antifungal activity against tested plant pathogens including *Colletotrichum capsici*, *C. gloeosporioides* and *Fusarium oxysporum* f.sp. *cubense*. This crude extract was also further fractionated with fifty-one mixed solvents with a gradient system, which were also evaluated for their antifungal activity. The fractions obtained from eluting with the combination of DE: EtOAc and petroleum ether (PE): chloroform ($CHCl_3$) showed zone of inhibition towards tested plant pathogenic fungi ranged from 0.65 to 2.00 mm. The results suggested that the active compounds contained in DE and EtOAc fractions as well as micro-fractions from eluting with the mixture of PE: $CHCl_3$ (F5- F8) and DE: EtOAc (F22-F30). The TLC chromatograms and antifungal activity of the macro- and micro-fractions were analyzed for construction the antifungal activity profile of *P. longum*.

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INTRODUCTION

Plants belonging to genus *Piper* are well-known species in many countries. Seven hundred species have been reported in the world and five species have been used as folk medicines in Asia, namely, *P. betel*, *P. cubeba*, *P. retrofractum*, *P. longum*, and *P. nigrum* (Mohib and Siddiqui, 2007). In Thailand, there are some economically important species of *Piper* such as *P. betel, P. nigrum, P. longum* or *P. chaba*. Dee-plee is the local Thai name of two species of *P. longum* (syn. *Chavica roxburgbii*) and *P. chaba* Hunt (syn. *P. retrofractum*) that belong to the family Piperaceae which consists of various aromatic constituents, and they have been used for traditional medicine due to their antiflatulance, expectorant, antitussive, antifungal, sedative-hyponotic, appetizer, and counterirritant activities (Tewtrakul *et al.*, 2000). Several researchers have investigated the chemical analysis of *P. longum* and piperine piperonaline, piperrecosalidine, guineensine, methyl piperate, and fifiline were reported. Tewtrakul *et al.* (2000) described the essential oils composition of *P. chaba* (β-caryophyllene, ar-curcurmene, pentadecane, β-bisabolene, caryophyllene oxide, 8-heptadecene and heptadecane) and *P. longum* fruits (β-caryophyllene, ar-curcurmene, gormacrene D, 8-heptadecene and heptadecane). They contained a few monoterpene hydrocarbons, a moderate content of sesquiterpenes, and a high content of aliphatic hydrocarbons.

Mohib and Siddiqui (2007) evaluated the antimicrobial activity of the *Piper* species like *P. cubeba*, *P. chaba* (syn. *P. retrofractum* Vahl), *P. longum* and *P. nigrum*. The results indicated that crude extracts of these *Piper* species exhibited strong antibacterial and antifungal activities. Analysis of the bioactivity of the fractions concluded that the fractions containing piperine exhibited significant antibacterial activity.

Several *Piper* species have also been used in indigenous systems of medicine in several tropical and subtropical countries

(Parthasarathy *et al.*, 2006). There have been reports on the multidimensional effect of *Piper* on various systems in humans. Manisha *et al.* (2011) studied pharmacognostic, phytochemical, physiochemical, chromatographic, and antimicrobial activities of *P. nigrum* and *P. longum*, and the results indicated that aqueous and methanol crude extracts of *P. nigrum* and *P. longum* exhibited antimicrobial activity against human pathogens. They could isolate piperine from crude extracts of *P. nigrum* and *P. longum* with amounts of 1.20 and 1.58%, respectively. TLC analysis using a solvent system of toluene: ethyl acetate (8:2) and observed under UV spectrum revealed that the Rf value of piperine was 0.42. Phytochemical parameters of *P. longum* comprised alkaloids, tannin, phenols, coumarins, and essential oils. Ali *et al*. (2007) determined the antibacterial and antifungal activities of various solvent extracts of *P. longum* against pathogenic bacteria and fungi. The results showed that crude extract of *P. longum* exhibited moderate activity against bacteria but the effect was not prominent against fungi whereas ethyl acetate extract showed a relevant antimicrobial effect against the tested organisms.

The promotion of natural products for plant protection has placed more attention on natural products due to their higher safety profile than that of synthetic chemicals. Many efforts have discovered new antimicrobial compounds from various sources, especially microbes and plants. Systematic screening may result in the successful discovery of active compounds with the potential of product development. The objective of this study was to construct the TLC antifungal activity profile of *P. longum* fruits extracts by using five-step experiments.

MATERIALS AND METHODS

Plant material and extraction of secondary metabolites

Dried fruits of *P. longum* were purchased from a traditional medicine market in Thailand and were pulverized into fine powder using a grinding machine. Fifty grams of fine powder samples were macerated in 95% ethanol in the ratio plant sample: ethanol of 1:10 by w/v, for 7-10 days at 28^{o}. Filtration was performed through cheesecloth for separating plant debris (sediments), and then the organic layer was filtered through filtering paper Whatman No.4. The extraction was repeated for three times until the resultant extract from the filtrate solution was colorless and then evaporated under reduced pressure to get ethanol crude extract.

Fractionation of crude extract

Five grams of ethanol crude extract was subjected on a column chromatography 2.3 x 55 cm over 20g of silica gel 60 (0.2-0.5 mm) and eluted with six solvents including petroleum ether (PE), chloroform ($CHCl_3$), diethyl ether (DE), ethyl acetate (EtOAc), acetone (AC), and methanol (MeOH), wherein 500 ml fractions were collected and concentrated at a reduced pressure. These fractions were designed to macro-fractions, and they were evaluated for their antifungal activity against plant pathogenic fungi.

Further fractionation of the ethanol crude extract was also applied on a column chromatography as described previously by Kumla *et al*. (2014) and eluted with fifty-one distinct solvent systems, which are mixtures of increased polarity of PE, $CHCl_3$, DE, EtOAc, AC and MeOH (Table 1), wherein 200 ml sub-fractions were collected and was concentrated at a reduced pressure. The sub-fractions designed to micro-fractions and then assayed for their antifungal activity against plant pathogenic fungi.

Plant pathogens

Three plant pathogenic fungi namely *Colletotrichum capsici*, *C. gloeosporioides* and *Fusarium oxysporum* f.sp. *cubense* were used to evaluate antifungal activity of *P. longum* crude extract and fractions. All plant pathogens were obtained from the Mycology and Fungal Disease Branch of the Plant Pathology Department, Kasetsart University, Bangkok, Thailand.

Antimicrobial activity: Disc Diffusion method

Plant pathogenic fungi were cultured on separated potato dextrose agar (PDA) test tube for 2-3 weeks to obtain a large quantity of spores.

Table 1. Solvent combination systems.

Organic Solvent	Eluent
Petroleum Ether:Chloroform (PE:$CHCl_3$)	100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90
Chloroform:Diethyl Ether ($CHCl_3$:DE)	100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60 30:70, 20:80, 10:90
Diethyl Ether:Ethyl Acetate (DE:EtOAc)	100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90
Ethyl Acetate:Acetone (EtOAc:AC)	100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90
Acetone:Methanol (AC:MeOH)	100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90
MeOH	100

Nine mL of sterilized distilled water were mixed into a cultured test tube to prepare the spore suspension. The suspension was filtered using sterilized cheesecloth to obtain the spore suspension and adjust to 10^6conidia/ ml with water. One mL of spore suspension was mixed into 9 mL warm PDA and poured onto a sterile Petri dish. Four concentrations (100,000, 10,000, 1,000 and 100 ppm) of crude extract and fractions were prepared in methanol and 20 µL of each concentration was dropped on a sterile 0.5 cm diameter of filter paper and allowed to air dry before being placed onto the center of the PDA containing the spore suspension. Diameters of the zone of inhibition were measured at 7 days after incubation and levels of sensitivity were classified as follows: no sensitivity = 0 mm, low sensitivity = 0.1-0.5 mm, moderate sensitivity = 0.6-1.0 mm, high sensitivity = 1.1-1.5 mm, highly sensitivity = 1.6 mm (Hannarong, 2007; Singburaudom, 2015).

Antifungal activity tests of the fractions

Six macro-fractions and fifty-one micro-fractions were prepared to a concentration of 100,000 ppm, and afterwards were tested for their antifungal activity using the disc diffusion method. Ten µL of each macro-and micro-fraction were seeded onto the sterile paper disc (0.5 cm) and then places on the PDA containing the spore suspension of the testing fungus. The procedure was the same as described in the disc diffusion method. Each treatment was performed in the triplicate with a complete randomized design.

RESULTS AND DISCUSSION

Antifungal activity of crude extract

The antifungal activity of *P. betle* ethanol crude extract is shown in Table 2. At 100,000 ppm, *P. betle* extract exhibited zone of inhibition ranging from 0.7 to 1.0 cm against all plant pathogenic fungi (Table 2). The fungus, *C. gloeosporioides*, exhibited the highest sensitivity to the crude extract of *P. longum*. The results of this experiment also suggested that the ethanol crude extract of *P. longum* might contain active compounds against tested plant pathogenic fungi.

Table 2. *In vitro* antifungal activity of the *P. longum* ethanol crude extract against three plant pathogenic fungi.

Concentration (ppm)	Diameter of zone of inhibition (cm)		
	C. capsici	*C. gloeosporioides*	*F. oxysporum*
100,000	1.0 ± 0.04	0.7 ± 0.05	0.7 ± 0.03
10,000	0.0	0.4 ± 0.04	0.0
1000	0.0	0.0	0.0
100	0.0	0.0	0.0

Non-sensitivity = 0 mm, Low-sensitivity = 0.1-0.5 mm, Moderate-sensitivity = 0.6-1.0 mm, High-sensitivity = 1.0-1.5 mm, Highly-sensitivity > 1.5 mm.

Antifungal activity of macro-fractions

Antifungal activity evaluation of the six macro-fraction obtained from PE, $CHCl_3$, DE, EtOAc, AC and MeOH fractionations revealed that the DE and EtOAc fractions displayed antifungal effects against tested plant pathogenic fungi, whereas the PE, $CHCl_3$, AC and MeOH fractions did not show zone of

inhibition against all tested plant pathogens (Table 3). The diameter of zone of inhibition ranged from 0.65 to 1.7 cm. The results suggested the existence of antifungal constituents were in both of DE and EtOAc fractions. The results also indicate that the antifungal constituents in *P. longum* crude extract could be extracted using DE and EtOAc or combinations of both solvents.

Table 3. Inhibition activity of six macro-fractions and crude extract of *P. longum* against plant pathogenic fungi at 100,000 ppm using the disc diffusion method.

Fraction	Diameter of zone of inhibition (cm)		
	C. capsici	*C. gloeosporioides*	*F. oxysporum* f.sp. *cubense*
PE	0.0	0.0	0.0
$CHCl_3$	0.0	0.0	0.0
DE	1.7 ± 0.06	0.76 ± 0.04	0.0
EtOAc	0.9 ± 0.05	0.65 ± 0.05	0.9 ± 0.04
AC	0.0	0.0	0.0
MeOH	0.0	0.0	0.0
CE	1.0 ± 0.04	0.7 ± 0.05	0.7 ± 0.03

Non-sensitivity = 0 mm, Low-sensitivity = 0.1-0.5 mm, Moderate-sensitivity = 0.6-1.0 mm, High-sensitivity = 1.0-1.5 mm, Highly-sensitivity > 1.5 mm.
PE: Petroleum Ether, $CHCl_3$: Chloroform, DE: Diethyl Ether, EtOAc: Ethyl Acetate, AC: Acetone, MeOH: Methanol, CE: Crude Extract

Antifungal activity of micro-fractions

Fifty-one micro-fractions were tested for their antifungal activity against three plant pathogenic fungi using the disc diffusion method. The results revealed that the micro-fractions obtained from the mixtures of PE: $CHCl_3$ and DE: EtOAc eluting systems displayed relevant antifungal effects on all tested plant pathogens (Table 4). The micro-fractions obtained from the mixture of PE: $CHCl_3$ were F5, F6, F7 and F8 displayed antifungal activity that exhibited the zone of inhibition ranging from 0.7 to 2.0 cm while the micro-fractions obtained from the mixture of DE: EtOAc were micro-fractions F22, F23, F24, F25, F26, F27, F28, F29 and F30 that showed the zone of inhibition ranging from 0.55 to1.6 cm. The rest of the micro-fractions exhibited no activity. The results of this experiment suggested that there were at least two groups of active compounds in the crude extract of *P. longum* which were also contained in the micro-fractions obtained from eluting with PE: $CHCl_3$ and DE: EtOAc solvent systems.

Construction of activity profile of *P. longum* extracts

TLC chromatograms of the six macro-fractions suggested that there were at least one compound that appeared in $CHCl_3$ fraction whereas DE fraction exhibited four compounds, EtOAc exhibited two compounds, AC exhibited one compound, while MeOH exhibited no compounds (Figure 1). The results from antifungal activity evaluations of macro- and micro-fractions revealed that fractions obtained from DE, EtOAc, the combination of PE: $CHCl_3$ and DE: EtOAc eluting systems possess antifungal substances against tested plant pathogenic fungi. The results of antifungal activity and TLC chromatograms of six macro-fractions indicated that having main two groups of substances exhibited activity against the plant pathogenic fungi.

The first group consisted of four compounds contained in DE and EtOAc fractions. The second group consisted of at least two compounds contained in EtOAc fraction. The antifungal activity profile of *P. longum* crude extract was constructed using the combined results from antifungal activities, TLC chromatograms and Rf values is shown in figure 2. Several chemical constituents of *P. longum* have previously reported by Tewtrakul *et al.* (2000), but they have not been investigated for their antifungal activity, particularly against phyto pathogens. The results of this study indicate that *P. longum* ethanol crude extract at 10,000 ppm exhibited potent antifungal activity against tested phytopathogenic fungi, which are the causative agents of economic crop diseases in Thailand.

Table 4. Antifungal activity of fifty-one micro-fractions from *P. longum* crude extract against plant pathogenic fungi by disc diffusion method at 7 days.

Micro-Fraction	Solvent Combination	Ratio	Diameter of zone of inhibition (cm)		
			Colletotrichum capsici	*Colletotrichum gloeosporioides*	*Fusarium oxysporum* f.sp. *cubense*
F_1	PE : $CHCl_3$	100 : 0	0	0	0
F_2	PE : $CHCl_3$	90 : 10	0	0	0
F_3	PE : $CHCl_3$	80 : 20	0	0	0
F_4	PE : $CHCl_3$	70 : 30	0	0	0
F_5	PE : $CHCl_3$	60 : 40	0.7 ± 0.03	0.8 ± 0.05	0.7 ± 0.02
F_6	PE : $CHCl_3$	50 : 50	1.4 ± 0.05	1.4 ± 0.02	2.0 ± 0.05
F_7	PE : $CHCl_3$	40 : 60	1.3 ± 0.05	1.4 ± 0.04	1.2 ± 0.03
F_8	PE : $CHCl_3$	30 : 70	1.0 ± 0.04	1.0 ± 0.04	1.0 ± 0.02
F_9	PE : $CHCl_3$	20 : 80	0	0	0
F_{10}	PE : $CHCl_3$	10 : 90	0	0	0
F_{11}	$CHCl_3$: DE	100 : 0	0	0	0
F_{12}	$CHCl_3$: DE	90 : 10	0	0	0
F_{13}	$CHCl_3$: DE	80 : 20	0	0	0
F_{14}	$CHCl_3$: DE	70 : 30	0	0	0
F_{15}	$CHCl_3$: DE	60 : 40	0	0	0
F_{16}	$CHCl_3$: DE	50 : 50	0	0	0
F_{17}	$CHCl_3$: DE	40 : 60	0	0	0
F_{18}	$CHCl_3$: DE	30 : 70	0	0	0
F_{19}	$CHCl_3$: DE	20 : 80	0	0	0
F_{20}	$CHCl_3$: DE	10 : 90	0	0	0
F_{21}	DE : EtOAc	100 : 0	1.0 ± 0.06	0.55 ± 0.03	0
F_{22}	DE : EtOAc	90 : 10	1.1 ± 0.02	1.1 ± 0.04	1.1 ± 0.02
F_{23}	DE : EtOAc	80 : 20	1.3 ± 0.04	0.8 ± 0.02	1.0 ± 0.02
F_{24}	DE : EtOAc	70 : 30	1.3 ± 0.03	1.3 ± 0.02	1.5 ± 0.05
F_{25}	DE : EtOAc	60 : 40	1.6 ± 0.03	1.6 ± 0.05	1.2 ± 0.02
F_{26}	DE : EtOAc	50 : 50	1.5 ± 0.05	1.5 ± 0.03	1.5 ± 0.04
F_{27}	DE : EtOAc	40 : 60	1.4 ± 0.04	1.4 ± 0.04	1.4 ± 0.05
F_{28}	DE : EtOAc	30 : 70	0	1.0 ± 0.04	0
F_{29}	DE : EtOAc	20 : 80	0	1.2 ± 0.05	0
F_{30}	DE : EtOAc	10 : 90	0	0.9 ± 0.03	0
F_{31}	EtOAc : AC	100 : 0	0.8 ± 0.03	0.5 ± 0.06	0.8 ± 0.02
F_{32}	EtOAc : AC	90 : 10	0	0	0
F_{33}	EtOAc : AC	80 : 20	0	0	0
F_{34}	EtOAc : AC	70 : 30	0	0	0
F_{35}	EtOAc : AC	60 : 40	0	0	0
F_{36}	EtOAc : AC	50 : 50	0	0	0
F_{37}	EtOAc : AC	40 : 60	0	0	0
F_{38}	EtOAc : AC	30 : 70	0	0	0
F_{39}	EtOAc : AC	20 : 80	0	0	0
F_{40}	EtOAc : AC	10 : 90	0	0	0
F_{41}	AC : MeOH	100 : 0	0	0	0
F_{42}	AC : MeOH	90 : 10	0	0	0
F_{43}	AC : MeOH	80 : 20	0	0	0
F_{44}	AC : MeOH	70 : 30	0	0	0
F_{45}	AC : MeOH	60 : 40	0	0	0
F_{46}	AC : MeOH	50 : 50	0	0	0
F_{47}	AC : MeOH	40 : 60	0	0	0
F_{48}	AC : MeOH	30 : 70	0	0	0
F_{49}	AC : MeOH	20 : 80	0	0	0
F_{50}	AC : MeOH	10 : 90	0	0	0
F_{51}	MeOH	100 : 0	0	0	0

PE: Petroleum Ether $CHCl_3$: Chloroform DE: Diethyl Ether EtOAc: Ethyl Acetate
AC: Acetone MeOH: Methanol

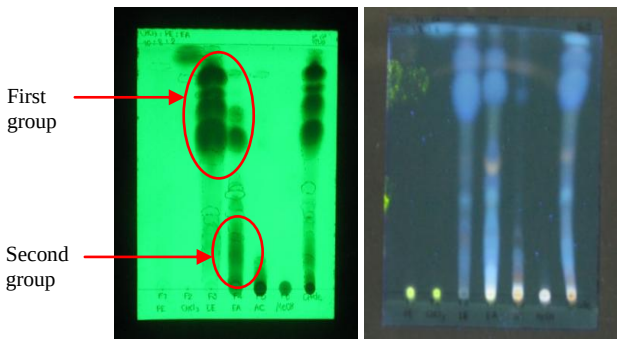

Fig.1. TLC chromatograms of six maco-fractions and crude extract of *P. longum* under UV at 254 nm (left) and infrared at 365 nm (right). Left to Right; Lane 1= Petroleum Ether (PE), 2 = Chloroform ($CHCl_3$), 3 = Diethyl Ether (DE), 4 = Ethyl Acetate (EA), 5 = Acetone (AC), 6 = Methanol (MeOH), 7 = Crude Extract (CE)

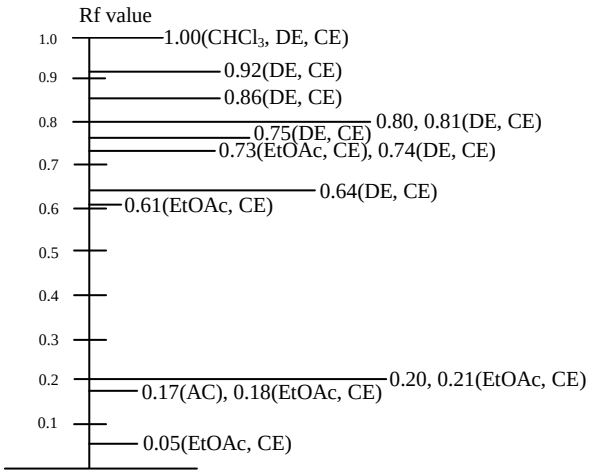

Fig. 2. Antifungal activity profile showed Rf values of macro-fractions of *P. longum* crude extract against plant pathogenic fungi.
$CHCl_3$ = Chloroform, DE = Diethyl Ether, CE = crude extract, EtOAc = Ethyl Acetate, AC = acetone

The results of the fractionation of crude extract suggested that the solvent fractions or macro-fractions, DE and EtOAc exhibited promising antifungal activities. The current study is corresponding with several reports which reported that DE and EtOAc fractions exhibited antifungal and antibacterial activities (Ali *et al.*, 2007; Mohib and Siddiqui, 2007; Aneja *et al.*, 2010; Dahiya *et al.*, 2011; Singh *et al.*, 2011; Sindhu *et al.*, 2013; Singh and Nagendra, 2013). Thus, the results of this current study have confirmed that DE and EtOAc were superior solvents for extracting the active constituents from *P. longum* fruits.

Bioactivity guided fractionation-based phytochemical analysis of *P. longum* fruits extract has reported by fractionation using different organic solvents as well as screened for their bioactivity against *Mycobacterium* by Singh *et al.*, (2011). The results indicated that EtOAc fraction was active against this bacterium and piperine, a well-known alkaloid that was isolated from this fraction displayed key active compound. The authors concluded that piperine is the main active ingredient, which means the fraction-contained piperine has significant activity against bacteria. However, there are still no reports on testing its antimicrobial activity on plant pathogenic fungi. Many researchers have discovered new antifungal compounds from various kinds of sources, particularly from plants. Systematic screening may result in the successful discovery of active compounds with the potential for commercial product development. In the current study, we propose the antifungal activity profile of *P. longum* fruit extracts as the model of bioactivity-guided fractionation for searching the active compounds against plant pathogens. This is the first report on the proposal of five steps in the search for antifungal compounds against plant pathogens from medicinal plants *in vitro*. The first step is antifungal activity evaluation of ethanol crude extract as well as the potential for product development. The most important criterion used for screening potentially crude extract is the effective dosage to inhibit plant pathogens—the concentration should be lower than 10,000 ppm. The results from preliminary screening in this study indicate that ethanol crude extract of *P. longum* fruit exhibited potent mycelial growth inhibition against tested plant pathogenic fungi at 1,000 and 10,000 ppm *in vitro*.

Thus, it is promising for the second step involving the fractionation of ethanol crude extract to separate the fractions containing the active compounds. This step is carrying out by column chromatography and elute with six organic solvents (PE, $CHCl_3$, DE, EtOAc, AC, and MeOH) as well as their combinations to obtain the six macro-fractions and fifty-one micro-fractions to further evaluate for their antifungal activity using disc diffusion method. However, the disc diffusion method is not superior when tested on non-spore forming or sterile mycelial fungi such as *Sclerotium rolfsii*, *Rhizoctonia solani*, and some of the Oomycetes fungi and also not suitable for biological activity evaluations of water-insoluble fractions because the diffusion of the compounds contained in the fractions was limited in a medium. The third step comprised the application of each fraction on TLC to examine the presence of compounds under UV and infrared spectra. The fourth step is the

isolation of the active compounds by chromatography techniques. The fifth step is the construction of the antifungal activity profile of the crude and solvent extracts, which the results on antifungal activities against plant pathogenic fungi and the chromatogram profiles of macro-fraction are pool and analyze to construct the antifungal activity profile.

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