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Contents

Antifungal Compound Isolated from Leaf of *Cassia fistula* L. (Ngu Shwe Wah)

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Abstract

In the investigation of antifungal compound against rice plantpathogenic fungus *Magnaporthe grisea*, five kinds of plants grown in Pathein area were utilized. These plant parts such as leaves, barks and stems were extracted with ethanol, methanol, and acetone solvents and tested the antifungal activity on *M. grisea* by paper diffusion assay. In this study, methanol extract of leaf of *Cassia fistula* L. (Ngu Shwe Wah) showed highly activity on *Magnaporthe grisea* (24.1 mm inhibitory zone). Therefore, methanol extract of leaf *Cassia fistula* L. was selected for further investigations such as isolation, purification and biological properties. In the isolation and purification, silica gel column chromatography, C18 gel column chromatography, silica gel column rechromatography and Sephadex LH-20 gel chromatography were undertaken with using suitable ratios of organic solvents. MICs of this compound is 0.156 µg/ml on *M. grisea*, 0.3125 µg/ml on *Cladosporium* sp. and *Alternaria alterna*, and 0.625 µg/ml on *Aspergillus flavus*, *A. fumigatus*, *Curvularia oryzae*, and *Fusarium oxysporum*. This compound affects on the growth of *M. grisea* at a concentration of 0.156 µg/ml; any further increase in its concentration above 0.156 µg/ml resulted in a more suppressive effect on growth. At a concentration of 0.6.25 µg/ml, cell lysis was observed that indicating that this plant compound cut acts as a fungicidal agent.

Introduction

Rice is an Asian countries' most economically important product. However, decreasing yield continues to be a problem because of phytopathogenic fungus such as *Magnaporthe grisea* which cause a rice blast disease. The only form of control available at present is chemical treatment which resulted in accumulation of harmful chemicals in the environment. Alternative biological treatment is thus more environmental friendly and less dangerous to farmers and customers (Chutrakul, 2005). Fungal belonging to the genus *Magnaporthe* is either as plant pathogens, or saprophytic association with plant tissues. Rice blast disease, caused by

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Magnaporthe is also the most wide-spread and destructive fungal disease of rice (Oliver and Lionel, 2002). The discovery of antimicrobial agents from natural sources (plants) has been a goal of mankind since prehistoric times, and through previous efforts, plants have become a viable source of clinically useful antimicrobial agents (Richard, 1998). Plants have also afforded leads for synthetic modification and have served as tool for mechanistic studies (Colegate, and Molyneus, 1993).

The higher plants are logical; choices, chiefly because of their seemingly infinite variety of novel organic molecules, which are often referred to as secondary metabolites (Simon, and Alexander, 1991). *Cassia fistula* L. of the Caesalpiniaceae family belongs to a group of plants through Myanmar and are popular medicinal and ornamental plant.

Materials and Methods

Sample preparation

Five kinds of plants such as *Cassia fistula* L., *Oldenlandia diffusa* (Wild.) Roxb., *Apium graveolens* Linn., *Alostonia schlaris* R.Br., and *Mimosa pudica* Linn. were collected at Pathein Township. Initially the leaves of these plants were dried at 17°C and dried leaves (40 g) was extracted with organic solvents (each 100 ml, methanol, ethanol, and acetone) according to the method of Lammens, 1979 and Hostettmann, and Marston, 1994. The organic extracts were evaluated for their abilities to inhibit the *in vitro* growth of phytopathogenic fungi by paper disc diffusion assay method.

Investigation of antimicrobial activity

The sample (20µl) was put onto the paper disc. The assay medium (Glucose 1%, Polypepton 0.3%, KNO_3 0.1%, Agar 51.8%, Distilled water 100ml, pH 7.0) was used for the antimicrobial activity test. One percent (1.5 x 10^8/ml CFU) of test organism was added to assay medium, then poured into plates. The plates were incubated for 24-36 hours at 28 to 30°C. Clear zones (inhibitory zones) surrounding the agar well indicates the presence of bioactive metabolites which inhibit the growth of test organisms.

Test organisms utilized in this study were phytopathogenic fungi, *Magnaporthe grisea*, *Cladosporium* sp., *Alternaria alterna*, *Aspergillus flavus*, *A. fumigatus*, *Curvularia oryzae*, and *Fusarium oxysporum*. Since it is intended to get the highly selective antifungal agent (Table 1 and 2),

based on antifungal activities, methanolic extract of *C. fistula* was selected to proceed the further isolation and purification.

Extraction, Isolation and Purification of antifungal agent

The methanolic extract of *C. fistula* leaves was concentrated *in vacuo* and the residue was dissolved in water (1:20, v/v) and was investigated the suitable pH for re-extraction with EtOAc. According to the results of pHs effects (Table 3), it was found that pH 4.0 was suitable for re-extraction with EtOAc. Based on the result, the methanolic extract (100ml) of *C. fistula* leaves was concentrated *in vacuo* (5 ml) and dissolved in water (1:20, v/v). This sample (105 ml) was adjusted at pH 4.0 and ethyl acetate (105 ml) was added for the re-extraction of antifungal agent. Ethyl acetate layer (105 ml) was concentrated *in vacuo*.

It is necessary to separate the bioactive compound by rechromatography, TLC was developed by the solvent such as hexane and hexane-acetone mixture. R_f values reported were acquired on Merck Kieselgel $60F_{254}$ pre-coated TLC plates, which were utilized for analytical preparative purpose. The obtained EtOAc extracted samples (20 µl) were applied on the TLC plate and allowed to dry. The TLC plates were developed in the solvent of hexane and hexane-acetone (9:1, 8:2, 7:3) mixture. Then, bioautography was done to check the antifungal activity of each. In this case also, the inhibitory zone was measured yielding an R_f value for the corresponding antifungal metabolites.

Based on the results of TLC, silica gel (Wako gel C-300, Japan) column chromatography was carried out with eluting solvent hexane and hexane-ethyl acetate mixture (9;1) and then tested the activity against *Magnaporthe grisea*. The column volume and flow rate were 5.25 cm x 20 cm and flow rate 2ml/min.

The active hexane-EtOAc fractions were combined and concentrated *in vacuo*. Silica gel column re-chromatography was then developed with dichloromethane-methanol (9:1) mixture. The column volume and flow rate were 5.25 cm x 20 cm and flow rate 1.5 ml/min.

Active fractions of silica gel coloum re-chromatography were combined and concentrated *in vacuo*. The residue was chromatographed (C 18 gel) by the eluting solvent acetonitrile-water (6:4) in the 4.5 x 18 cm column volume and 1.5 ml/min flow rate.

The active fractions from C 18 gel chromatography) were combined and concentrated. This residue was purified by Sephadex LH-20 with chloroform-methanol (7:3)(column volume 3.5 cm x 15 cm and flow rate 1.0 ml/min).

Determination of the minimun inhibitory concentration and Fungicidal effect on *Magnaporthe grisea* (Phay, 1999)

The minimum inhibitory concentrations (MICs) of compound were determined in GYA liquid medium (10 ml) inoculated with approximately 1.4 x 10^7 CFU of test organism *M. grisea* and were introduced with the treatment of compound (0.1562 µg/ml, 0.3125 µg/ml, 0.625 µg/ml, 1.25 µg/ml and 2.5 µg/ml) into 5 tubes. The control was also done. After incubation at 27°C for 24 hr, the MICs were determined by selecting the lowest concentration of compound which caused complete inhibition of fungicidal growth. Experiments were done in triplicate.

Test organism *M. grisea* (0.1 ml) was separately inoculated into 6 Conical flasks containing medium (100 ml, 1.0% glucose, 0.3% Yeast extract, 0.3% NZ amine type, pH 7.0) and incubated for 60 hours. At 24 hr incubation, it was introduced with the treatment of compound (0.3125 µg/ml 0.625 µg/ml 1.25 µg/ml, 2.5 µg/ml, and 5 µg/ml) into 5 flasks. PCV % was measured in 12 hr intervals. Control is one flask.

Results

Investigation of antimicrobial activity

In the study of antimicrobial agent isolated from the leaves of five kinds of plants, it was found that although all extract of *Cassia fistula* showed antifungal activity, methanolic extract *Cassia fistula* L. showed highly antifungal activity against *Magnaporthe grisea*, *Cladosporium* sp., *Alternaria alterna*, *Aspergillus flavus*, *A. fumigatus*, *Curvularia oryzae*, and *Fusarium oxysporum* (Table 1). Other plant extracts did not show the activity.

Table 1. Antifungal activity of extracts of organic solvents from *Cassia fistula* L

Extract	*M. grisea*	*Clado.*	*A. alterna*	*A. flavus*	*A. fumi*	*C. oryzae*	*F oxys*
MeOH	24.1 mm	16.2 mm	18.8 mm	18.6 mm	18.7 mm	17.4 mm	20.4 mm
EtOH	13.2 mm	-	-	13.6 mm	13.4 mm	-	16.5 mm
Acetone	20.6 mm	16.0 mm	17.6 mm	18.4 mm	16.8 mm	16.6 mm	20.8 mm

M. grisea = *Magnaporthe grisea* *Clado* = *Cladosporium*

A. alterna = *Alternaria alterna*

A. flavus = *Aspergillus flavus* *A. fumigatus* = *Aspergillus fumigatus*

C. oryzae = *Curvularia oryzae* *F. oxysporium* = *Fusarium oxysporium*

Table 2. The effects of pHs for re-extraction of antifungal agent

pHs	Inhibitory zone (mm)
3	20.2
4	21.8
5	19.6
6	18.4

Extraction, Isolation and Purification of antifungal agent

According to the R_f value of paper chromatography bioassay, it was considered that the antifungal agent could be re-extracted by ethyl acetate solvent at pH 4.0.

Re-extraction was undertaken by EtOAc at pH 4.0. The purification was developed with silica gel column chromatography (hexane-acetone), silica gel column chromatography (dichloromethane-methanol), C18 gel

column chromatography (acetonitrile-water) and Sephadex LH-20 (chloroform-methanol) to afford pure compound (27.8 mg).

Determination of the minimun inhibitory concentration and Fungicidal effect on *M. grisea* (Phay, 1999)

It was observed that MIC of compound is 0.156 μg/ml on *M. grisea*. This compound affects on the growth of *M. grisea* at a concentration of 0.156 μg/ml; any further increase in its concentration above 0.156 μg/ml resulted in a more suppressive effect on growth. At a concentration of 0.625 μg/ml, cell lysis was observed that indicating that compound acts as a fungicidal compound.

Conclusion

In the studies on the antifungal agent for the control the growth of *Magnaporthe grisea* , antifungal compound was isolated from the leaves of five kinds of plants using three solvents. Among them, the methanolic extract *Cassia fistula* Linn. showed highly antifungal activity. The methanolic extract was purified by silica gel column chromatography (hexane-acetone), silica gel column chromatography (dichloromethane-methanol), C18 gel column chromatography (acetonitrile-water) and Sephadex LH-20 (chloroform-methanol) to afford pure compound (27.8 mg) from 40 g of dried leaves. This antifungal compound potently inhibited the growth of *Magnaporthe grisea* with MIC of 0.625 μg/ml, comparable to that of griseofulvin.

YMICs of compound is 0.156 μg/ml on *M. grisea*. This compound affects on the growth of *M. grisea* at a concentration of 0.156 μg/ml; any further increase in its concentration above 0.156 μg/ml resulted in a more suppressive effect on growth means at a concentration of 0.625 μg/ml, cell lysis was observed that indicating that compound acts as a fungicidal compound.

From the traditional medicine knowledge, it was possible to select a plant species used to control the growth of fungi. The pharmacological value of this plant was demonstrated and was able to purify and isolate and antifungal agent, which represents a new molecular weight. Therefore, it is necessary to elucidate the compound structure.

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