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In Vitro Antifungal Activity of *Boesenbergia rotundo* Linn. and *Syzygium aromaticum* L. Merr. & Perry. Extracts Against *Aspergillus flavus*†

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Abstract: *Aspergillus flavus* is a common human pathogen that releases mycotoxin into the host and is frequently treated with synthetic fungicides, but the fungicides have serious human health consequences. Natural products derived from higher plant species have long been investigated as a potential means of controlling pathogenic microorganisms. The indigenous vegetables *Boesenbergia rotunda* and *Syzygium aromaticum* are widely distributed in the tropical area. These plants have also been reported in traditional uses for the antimicrobial activity. The purpose of the study was to explore the antifungal susceptibility of dichloromethane and ethanol extracts of *B. rotunda* rhizomes and *S. aromaticum* flower buds by Soxhlet's apparatus against *A. flavus* using the poison food technique. The effective extract was also subjected to preliminary phytochemical screening tests. The experiment used a completely randomized design with triplications. *B. rotunda* ethanol extract demonstrated significantly higher potential antifungal activity. The values of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *B. rotunda* ethanol extract were 6.25 and 50 mg/ml, respectively, when tested using the macro-dilution method. According to phytochemical tests, the ethanol extract also contained alkaloids, flavonoids, cardiac glycosides, and saponins. The study suggests that a basic guideline for using this as an effective antifungal compound should be separated from the *B. rotunda* ethanol extract in the future for topical anti-pathogenic fungus.

Keywords: *Aspergillus flavus*; Antifungal activity; *Boesenbergia rotunda*; *Syzygium aromaticum*; MIC/MFC values

Introduction

Aspergillus flavus can grow rapidly in environments where the mycelia can use substrates provided by multiple carbon sources [1]. Besides that, the fungi produce aflatoxin, which has been related to class I liver cancer [2]. Synthesised chemical agents have long been used for the prevention of the fungus. However, the chemicals can accumulate and be harmful to the environment, humans, and animals.

Phytochemicals are also being developed to be a possible way to achieve outcome trends for antimicrobial agents [3-5]. Chemical groups from plants including alkaloids, flavonoids, tannins, and phenolics controlling microbial growth have been reported [6]. The indigenous vegetables *Boesenbergia rotunda* and *Syzygium aromaticum* are widely distributed in the tropical area. The plants belong to the Zingiberaceae and Myrtaceae

families, respectively. Various extracts and essential oils of these plants have also been reported in traditional uses for the antimicrobial activity against gram-positive and negative bacteria, filamentous fungi, and *Candida* species [7-8]. The chemical groups of the plants, including flavonoids, terpenes, terpenoids, aromatic compounds, and alkaloids, have been reported [9].

The purposes of the study were to investigate the antifungal activity of *B. rotunda* rhizomes and *S. aromaticum* flower buds obtained by dichloromethane and ethanol against *A. flavus* and to examine the phytochemical screening of the effective extract. The effective extract would then be isolated further to be used in the discovery of novel friendly topical agents.

Methods

Plant collection and authentication

Rhizomes of *B. rotunda* and flower buds of *S. aromaticum* were collected from the vegetable farm in Ongkharak province, Nakhon Nayok, Thailand. The plants were identified botanically in the Department of Botany, Faculty of Science, Chulalongkorn University.

Soxhlet's extraction

Ten kilogram of fresh rhizomes of *B. rotunda* and 500 g of flower buds of *S. aromaticum* were washed and dried at 60 °C until they reached a constant weight. After that, 200 g of each dried sample was powdered, packed in an extract bag, and subjected to Soxhlet's apparatus. Dichloromethane was firstly used to extract the samples, followed by ethanol. The crude extracts were filtered and then concentrated using a rotating vacuum evaporator until the crude extracts were constant weight. The crude extracts were stored in bottles covered with aluminium foil at 4 °C until they were studied.

Fungal strain

A flavus was provided by the Center of Excellence in Chemistry of Natural Products, Faculty of Science, Chulalongkorn University. The fungus was maintained on potato dextrose agar (PDA) at 28 °C in darkness.

Antifungal susceptibility

The antifungal activity was applied as a method of poisoning food techniques. Each extract was dissolved in 1% v/v DMSO and then 100 µl was added into PDA to give a final concentration of 1,000 mg/l. One hundred microliters of each extract was combined with melted potato dextrose agar (PDA) and poured into petri plates. A combination of PDA mixed with only 1% v/v DMSO or nystatin (0.05 mg/ml) was used as a negative and positive controls, respectively. Mycelia discs were plugged from the edges of the 5-day old culture with a cork borer (0.5 cm diameter). The plates were incubated at 28 °C in the dark, and susceptibility was determined by comparing the relative growth of fungus in each treatment [10]. The formula I= (C-T)/Cx100 was used to calculate the percentage of growth inhibition. Where I denoted percent inhibition, C denoted control colony diameter (cm), and T denoted treatment colony diameter (cm) [11]. The minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) in various concentrations of the effective extract were also determined using a macro dilution technique [12-13].

Phytochemical testing screening

The samples were examined against the powdered material using regular methodological approaches [14]. Alkaloids, anthraquinones, flavonoids, terpenoids, steroids, cardiac glycosides, saponins, tannins, and phlobatannins were all tested for in the phytochemical screening experiment.

Statistical analysis

The SPSS program for Windows version 22.0 was used to analyze the data. The Duncan's Multiple Range Test (DMRT) was used to compare the results, and significance was found at the $P<0.05$ level. Within a completely randomized design, the experiment was conducted as a generalized linear model with triplications.

Results and Discussion

Percentage yields

The percentage yields of the extracts are represented in Table 1. The ethanol extracts of each plant gave a much larger amount than the dichloromethane extracts. The extraction yields using different solvents were increased by the polarity of the solvent used in extraction (increasing in the polarity followed order: hexane<ethyl acetate<dichloro-methane<acetone<chloroform<ethanol<methanol) [15-16].

Table 1. Percentage yields

Plant	Solvent	% yield
B. rotundo	Dichloromethane	1.59
	Ethanol	4.49
S. aromaticum	Dichloromethane	2.08
	Ethanol	5.15

Antifungal activity

Table 2 shows the antifungal activity of *B. rotundo* and *S. aromaticum* extracts by dichloromethane and ethanol displyas.The dried rhizome extracts of *B. rotundo* from both organic solvents revealed higher antifungal activity than the extracts of *S. aromaticum* flower buds. The ethanol rhizome extract of *B. rotundo* had the highest percentage of mycelia growth inhibitory activity against *A. flavus* (50.93%) and closed to the positive control. So, the ethanol extract was chosen for MIC/MFC susceptibility and phytochemical screening.

Table 2. Antifungal activity of *B. rotundo* and *S. aromaticum* extracts against *A. flavus* at 1,000 mg/l.

Treatment	Extract	% Mycelia growth inhibition (%mean*±S.D.), *n*=3
B. rotundo	Dichloromethane	45.93±0.57[a]
	Ethanol	50.93±0.10[a]
S. aromaticum	Dichloromethane	25.19±0.14[b]
	Ethanol	27.41±0.12[b]
1% DMSO	Negative control	0.00[c]
Nystatin	Positive control (0.05 mg/ml)	49.25±0.23[a]

*Mean values with different superscript letters in each column are significantly different ($P<0.05$)

The MIC and MFC values of the ethanol extract of *B. rotunda* rhizomes on the fungus compared with the positive control of amphotericin B are shown in Table 3. The MIC/MFC values revealed 6.25/50 mg/ml that could be calculated to an MFC index of 8.00. The result was estimated at more than 4, suggesting that the extract was a fungistatic agent. A fungistatic agent is a chemical that inhibits the growth of fungi [17].

Table 3. The MIC/MFC values of ethanol extracts of *B. rotunda* against *A. flavus*

Treatment	MIC (mg/ml)	MFC (mg/ml)	MFC indice	Mode of Action
Ethanol extract	6.25	50.00	8.00	Fungistatic
Nystatin	2.15	3.50	1.63	Fungicidal

Phytochemical screening test

The result of the phytochemical screening test of ethanol rhizome extract of *B. rotunda* is shown in Table 4. In the ethanol extract, alkaloids, flavonoids, cardiac glycosides, and saponins were present.

Table 4. Phytochemical test results of ethanol extract.

Phytochemicals	Result*
Alkaloids	+
Anthaquinones	-
Flavonoids	+++
Terpenoids	-
Steroids	-
Cardiac glycoside	++
Saponins	+
Tannins	-
Phlobatannins	-

Note: *(-) = Negative test; (+) = Weak positive test; (++) = Positive test; (+++) = Test strongly positive

Conclusions

The ethanol rhizome extract of *B. rotunda* showed significantly potent antifungal activity against *A. flavus.* Alkaloids, flavonoids, cardiac glycosides, and saponins were discovered as phytochemicals. Furthermore, the ethanol rhizome extract of *B. rotunda* would isolate the anti-*A. flavus* compounds for a new generation of topical agents.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, P.C.; methodology, P.U. and P.C.; software, P.U. and P.C.; validation, P.C.; formal analysis, P.U. and P.C.; investigation, P.U. and P.C. resources, P.U. and P.C.; data curation, P.U. and P.C.; writing—original draft preparation, P.U.; writing—review and editing, P.C.; visualization, P.U. and P.C.; supervision, P.C.; project administration, P.C.; funding acquisition, P.U. and P.C. All authors have read and agreed to the published version of the manuscript.

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