

The Inhibition of Human Pathogens: *Trichophyton rubrum* and *Trichoderma harzianum* by a Natural Product

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Abstract: Problem statement: A number of studies have recently conducted to identify novel and potent antifungal components from natural products. One of the reasons is to overcome the antifungal resistant developed against most of commercially available drugs. Studies confirmed that mung beans have increased phenolic compounds and enhanced defenses during germination. **Approach:** We hypothesized that antifungal activities might be found in sprouts of mung beans, or *Vigna radiate* (L.) R. Wilczek. The screening method was conducted using disc diffusion assay against 12 fungi. It was followed by the evaluation of the minimum inhibitory concentration and the minimum fungicidal concentration. **Results:** The screening results revealed a potential antifungal activity by mung bean sprout extract against 2 out of 12 fungi including remarkable antifungal activity against human fungal pathogens, *Trichophyton rubrum* and *Trichoderma harzianum*. The potential antifungal activity of mung bean sprout reflects effective quality/quantity of polyphenolic compounds present after bean germination. **Conclusion/Recommendations:** This unprecedented study showed that mung bean sprout extract is a potential source for novel antifungal compound (s) that is inexpensive and readily available at a large scale for pharmaceutical companies.

Key words: Antifungal, mung bean sprout, polyphenols

INTRODUCTION

In last few decades, the continuous escalation of resistant fungi against a wide range of antifungal drugs necessitates discovering novel unconventional sources of antifungal treatment. As a result of the indiscriminate use of antimicrobial drugs in the treatment of infectious diseases, microorganisms have developed resistance to many antimicrobes (Cowan, 1999). Therefore, there is a critical need to move fast and develop alternative antimicrobial drugs. One approach is to screen local medicinal plants which represent a rich source of novel antimicrobial agents. Medicinal plants are an integral component of research development in microbiology and pharmaceutical industry. Natural products from plants traditionally have provided the pharmaceutical industry with one of its most important sources of lead compounds and up to 40% of modern drugs are derived

from natural sources, using either the natural substance or a synthesized version (Gautam *et al.*, 2007).

Mung Bean Sprout (MBS), (*Vigna radiate* (L.) R. Wilczek), or previously known as *Phaseolus radiatus*, which is popular in Asian cuisine, is an excellent source of vitamins, minerals and protein with its essential amino acid profile comparable to that of soybean and kidney bean (Mubarak, 2005). The mung bean contains significant quantities of phenolic and polyphenolic compounds such as phenolic acids and flavonoids (Sosulski and Dabrowski, 1984). In the natural environment, seed sprouts survive during germination by enhancing their defensive responses through phenolics biosynthesis including modified vitamins, enzymes, and receptors (Randhir *et al.*, 2004). Among the enhanced defensive mechanisms during germination, the antimicrobial defenses might be highly involved. However, the antimicrobial defenses were not

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covered adequately in germinated sprouts in most of medicinal plants.

Plant phenolic metabolites are gaining interest due to their potential role in human disease prevention and treatment. The use of phytochemicals as natural antimicrobial agent commonly called 'biocides' are gaining popularity (Smid and Gorris, 1999). The main advantage of natural phenolic agents from plants is that they might contain a spectrum of phenolic antimicrobials, not only single antimicrobial substance, directed toward certain spectrum of microbes which potentially do not enhance the 'antibiotic resistance' phenomenon commonly seen with long-term use of synthetic antibiotics (Randhir *et al.*, 2004). Hence, some plant phenolics are being developed as potential antimicrobial agents and used in the defense against human pathogens (Nychas *et al.*, 2003; Tranter *et al.*, 1993).

Unfortunately, no previous studies scrutinized the possible enhancement of natural antimicrobial defenses during germination. Moreover, to the best of our knowledge, there was no previous comprehensive study conducted specifically to evaluate the antifungal activity of MBS against different notorious human fungal pathogens. We hypothesized that there is a good possibility to find potential antifungal activity in MBS basing on the enhanced antimicrobial defenses during germination. Accordingly, this study was designed to test the antifungal activity of MBS against 12 fungi including some of most notorious drug resistant bugs such as, *Trichoderma harzianum* and *Trichophyton rubrum*.

MATERIALS AND METHODS

Plant material and preparation of the extract: Fresh MSB, devoid of any preservative antimicrobials, was purchased from local markets in Selangor state in

Malaysia. The sprouts were left to dry in room temperature at dark area for approximately 7 days. After sprouts dryness, they were grounded to powder. The grounded powder was extracted (1/10) with 1:1 v/v 2.4 mol L⁻¹ HCl acidified aqueous methanol (Merck, Darmstadt, Germany) to extract all components of phenolic compounds, free and conjugated (Stratil *et al.*, 2006) and soaked for three days in dark at room temperature. The solvent was then removed by filtration and fresh solvent was then added to the plant material. The extraction was repeated twice and extracts were combined and evaporated to dryness under vacuum at 40°C. The pH of MBS extract ranged from 6.8-7.0 which is a neutral pH. The powder was then stored at -18°C in a desiccant until required.

Microorganisms and medium: A total of 12 fungi were used in this study (Table 1). All fungi were obtained from the microbiology laboratory of the institute of Bioscience, University Putra Malaysia. The fungi other than ATCC strains were identified depending on the macro and microscopic features (Ulloa and Hanlin, 2000). The fungi were maintained on sabouraud dextrose agar slants (Merck, Darmstadt, Germany) at 4°C until required.

Antimicrobial sensitivity tests:

Disc diffusion assay: The dried plant extracts were dissolved in the same solvent, methanol, to a final concentration of 200, 500 and 700 mg mL⁻¹ for antifungal tests and were sterilized by filtration through 0.45 µm Millipore filters (Nalgene, UK). The primary screening antifungal test was carried out by disc diffusion (Murray and Baron, 2007) using 100 µL of suspension containing 10⁵ spore mL⁻¹ of fungi, spread evenly on the surface of sabouraud dextrose agar plates.

Table 1: Antifungal activity of *Vigna mungo* methanol extract by disc diffusion method

Microorganisms		Inhibition zone in diameter (mm)				
		<i>B. oleracea</i> methanol extract (mg mL ⁻¹)			Negative control (MeOH*)	Positive control amphotericin B (10 µg disc ⁻¹)
Fungal species	Number of strains	200	500	700		
<i>Trichophyton rubrum</i>	1 (ATCC 11990)	-	-	12±1.3	- †	11±0.6
<i>Microsporum canis</i>	1 (ATCC 8137)	-	-	-	-	10±0.6
<i>Aspergillus niger</i>	1 isolate	-	-	-	-	18±1.0
<i>A. terreus</i>	1 (ATCC 20542)	-	-	-	-	10±0.8
<i>A. oryzae</i>	1 isolate	-	-	-	-	8±0.8
<i>Paecilomyces variatii</i>	1 isolate	-	-	-	-	18±1.3
<i>Phanerochaete crysosporium</i>	1 isolate	-	-	-	-	19±1.3
<i>Schizophyllum</i> sp.	1 isolate	-	-	-	-	20±1.6
<i>Granuloma</i> sp.	1 isolate	-	-	-	-	-
<i>Trichoderma</i> sp.	1 isolate	-	-	-	-	-
<i>Trichoderma harzianum</i>	1 (ATCC 20671)	-	-	15±0.6	-	10±0.8
<i>Trichoderma atroviride</i>	1 (ATCC 74058)	-	-	-	-	-

*: MeOH: methanol; †: (-) means no growth inhibition zone

Sterile Whatman No. 1 filter paper (MACHEREY-NAGEL, MN 615, Germany) was used to prepare 6 mm in diameter discs. These discs were processed, in triplicates, to contain 10 μL , i.e.; (2, 5 and 7 mg disc⁻¹) to concentrations of (200, 500 and 700 mg mL⁻¹), respectively, and were then impregnated in the inoculated agar. Negative controls were prepared, in triplicates per Petri dish, using the same solvents employed to dissolve the plant extracts. Amphotericin B “Sigma, Steinheim, Germany” (10 μg disc⁻¹) were used, in triplicates per Petri dish as positive control or reference standard drug to determine the sensitivity of each tested fungus towards the used extract in comparison with the positive control. The inoculated plates were incubated for 3-5 days at 30-35°C. Clear inhibition zones around discs indicated the presence of antifungal activity. For optimal fidelity of results, the disc diffusion assay was repeated three times. Therefore, the mean \pm SD was measured out of totally 9 inhibition zones, triplicate in each run.

Microdilution assay: The Minimum Inhibitory Concentration (MIC) values were also studied for the fungi determined as sensitive to the extract by the disc diffusion assay. The microdilution method was used according to the methodology referred by (Zgoda and Porter, 2001) with some modifications. The inocula of the fungi were prepared from fresh fungal cultures 3-5 days according to the fungus type and standardized to 10⁵ spore mL⁻¹. The stock solution of extract at a concentration (600 mg mL⁻¹) was prepared in 10% dimethylsulfoxide (Merck, Darmstadt, Germany). The extract solution was diluted in sabouraud dextrose broth (Merck, Darmstadt, Germany). Two-fold serial dilutions of the stock extract solution were prepared in concentrations range from (600-75 mg mL⁻¹) in 1.5 mL test tubes (Eppendorf, Hamberg, Germany). One hundred, 100 μL well⁻¹ of each extract dilution and 100 μL well⁻¹ of each spore suspension were dispensed, in triplicates, into 96-well microtiter plate (Steriline, UK). On the other hand, triplicates of extract-free fungal spore suspensions were used as negative controls as well as triplicates of Amphotericin B at concentration range of (0.5-3 μg mL⁻¹) were used as standard antifungal positive controls. The final volume in each well was 200 μL . The plate was covered with a sterile plate sealer 80/140 mm (Greiner bio-one, Germany). The plates were incubated for 3-5 days at

30-35°C. The growth of fungi was determined by absorbance values at 530 nm using fully automated Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, Canada). The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

To confirm MIC and to establish Minimum Fungicidal Concentrations (MFC), 10 μL of each fungal culture medium with no visible growth were removed from each well and inoculated, in triplicates, on sabouraud dextrose agar plates. After incubation for 3-5 days at 30-35°C, the number of surviving organisms was determined. MFC was defined as the lowest extract concentration at which 99.9% of the microorganism was killed. The extract tested in this study was screened three times for each organism.

Statistical analysis: The disc diffusion assay was conducted in triplicates wells in three independent experiments. Therefore, the results were expressed as mean \pm SD. SPSS software version 12.0.0.2 was used.

RESULTS

Antimicrobial assays for fungi: Qualitative and quantitative results were obtained by both inhibition zone and zone diameter. The methanol crude extract of the MBS showed unprecedented antifungal action against two out of the 12 tested fungi; namely, *Trichophyton rubrum* and *Trichoderma harzianum* (Table 1). The clear inhibition zone on *T. rubrum* and *T. harzianum* using 700 mg mL⁻¹ MBS extract, 12 and 15 mm respectively, was greater than that of the standard drug, namely Amphotericin B, 11 and 10 mm respectively. Given that *T. rubrum* and *T. harzianum* are slow growing and were found usually resistant to most of antifungal drugs, the novel antifungal activity of MBS discovered in the current study might indicate a potent fact-acting antifungal substance. To confirm the antifungal activity of MBS extract, both MIC and MFC were measured. The MIC was 75 mg mL⁻¹ for both fungi, while MFC was 300 mg mL⁻¹ for *T. rubrum* and 150 mg mL⁻¹ for *T.harzianum* (Table 2). The growth inhibition was dose dependent and was reflected by the decrease in OD value (Fig. 1).

Table 2: The MIC and MFC values against the tested fungi by the microdilution method

Fungal species	Number of strains	MIC* (mg mL ⁻¹)	MFC [†] (mg mL ⁻¹)	MFC:MIC ratio	Antifungal mode	Positive control amphotericin B (μg mL ⁻¹)	
						MIC	MBC
<i>Trichophyton rubrum</i>	1 (ATCC 11990)	75	300	4:1	Fungistatic	2.0	3.0
<i>Trichoderma harzianum</i>	1 (ATCC strain 20671)	75	150	2:1	Fungicidal	2.5	2.9

*: Minimum inhibitory concentration; [†]: Minimum fungicidal concentration

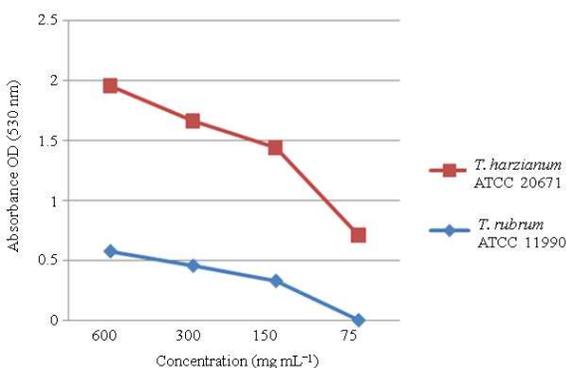


Fig. 1: The growth inhibition of the sensitive fungi as detected by OD value in the microdilution assay

Mode of antimicrobial action: The nature of the antifungal effect of the extract in regard to inhibition/killing of tested fungi is important. The MFC: MIC for fungi is used to specify the nature of the antimicrobial effect against any given pathogen (Barry *et al.*, 1999). When the MFC:MIC ratio of a pathogen is between 1:1-2:1, the chemical substance is considered as fungicidal against that pathogen (Barry *et al.*, 1999). On the other hand, if ratio was >2:1, the mode of antimicrobial action is more likely to be fungistatic. Therefore, MFC: MIC ratio was calculated for each fungal pathogen. It was found that the extract exerted fungicidal effect against *T. harzianum* and fungistatic against *T. rubrum* (Table 2).

DISCUSSION

The wide spread of drug resistant microbe raises the need for new, cheap, effective, and safe drugs. One of the best candidates to address this need appears to be the natural sources. Nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds. Therefore, they are most often obtained through initial ethanol or methanol extraction (Cowan, 1999; Lin *et al.*, 1999; Eloff, 1998; Ahmad *et al.*, 1998). Therefore, we used methanol as a solvent to extract almost all of the proposed antimicrobial agents in order to prepare the basis for monitoring different antifungal agents as a prelude for the future separation of single antifungal compound (s).

Seed sprouts have long been used in the diet as health food and recent research shows that in addition to being a good source of basic nutrients, they also have important phytochemicals with disease preventive and health promoting properties

(Kurtzweil, 1999). Moreover, germinating seeds, or sprouts, are believed to have stronger defenses and metabolic pathways than the parent seeds (Mwikya *et al.*, 2001; Fernandez-Orozco *et al.*, 2008) This study assumed that one of the enhanced defense mechanisms and modified phytochemical activities in sprouts might be the synthesis of competent antimicrobial phytochemicals that might share their antimicrobial effect with human and animal pathogens.

The findings of the current study backed up strongly such assumption. The current study unveiled a novel, powerful and broad spectrum antifungal activity of MBS against two of human pathogenic fungi. On the other hand, methanol extract of Mung Beans (not MBS) was subjected to the same experiment done for MBS. Unlike MBS extract, Mung bean extract showed no remarkable antifungal activity against all tested fungi (data not shown). This provided stronger evidence that MBS chemical structure is much different from that of Mung beans and the germination of Mung beans into MBS resulted to synthesis and/or modification of the phytochemical structure leading to formation of new antimicrobial active compounds. Previous studies had isolated a combination of antimicrobial protein from mung bean which appeared to be active against a range of bacteria and fungi (Wang *et al.*, 2004a; 2004b; 2005; 2006; Ye and Ng, 2005; Lin *et al.*, 2007). However, few or no studies had focused on the phytochemical profile of the mung bean or mung bean sprout, such as the current methanol extract of MBS, as antifungal agents. A study found that sprouting improved the antioxidant activity due to the higher demand for oxygen during early stages of the germination; therefore, phenolics might be protecting the cells from potential oxidation-induced deterioration (Lambert, 2008). Another study confirmed the presence of six flavonoids, i.e., robinin, rutin, kaempferol, quercetin, isoquercitrin, and kaempferol-7-O-rhamnoside, and found that the content of these flavonoids increased during the germination of mung beans (Sawa *et al.*, 1999). Therefore, the increased antioxidant activity found in MBS together with the potent type of flavonoids might explain the significant antifungal activity against the tested fungi.

Unfortunately, the antifungal potential of MBS extracts against pathogenic fungi has long been underestimated and not fully documented. The two previous reports found studying the antimicrobial activity of MBS were conducted on one bacteria only, *Helicobacter pylori*, that causes gastroduodenal disease (Randhir *et al.*, 2004; Mitchell and Megraud, 2002). Surprisingly, no other studies were conducted on other bacteria or fungi. Therefore, we believe that the findings of the current study might be a breakthrough in

the field of microbiology, antimicrobials, and natural products. MBS crude extract yielded a very promising antifungal activity against 2 opportunistic and strictly pathogenic fungi. The most interesting point, MBS exerted fungicidal rather than fungistatic effect on one of newly known human pathogen, i.e., *T. harzianum* and fungistatic effect on one of commonly drug resistant pathogen, i.e., *T. rubrum*.

Although *T. harzianum* is a rare opportunistic pathogen, it had been detected as a disseminated fungal infection in the postmortem examination of a renal transplant recipient. The case illustrates the widening spectrum of opportunistic *Trichoderma* spp. in immunocompromised patients (Guarro *et al.*, 1999). Recently, it was isolated from blood serum, skin lesions, sputum, and throat of a pediatric patients with neutropenia (Kantarciolu *et al.*, 2009) On the other hand, *T. rubrum* is the most common dermatophyte species and the most frequent cause of fungal skin infections in humans worldwide. It's a major concern because feet and nail infections caused by this organism is extremely difficult to cure (Yang *et al.*, 2007). In addition, it is resistant to the most of the commercially available antifungal agents that had been recorded (Mukherjee *et al.*, 2003). The effective antifungal activities obtained by using MBS extract against *T. harzianum* and *T. rubrum* were remarkable. The reason behind, the antifungal activity of MBS was compared with one of the most powerful antifungal drugs, namely amphotericin B. The inhibition zones of MBS for *T. rubrum*, 12 mm, and *T. harzianum*, 15 mm, were higher than that of amphotericin B, 11 and 10 mm respectively. However, the mechanism of action is not yet explained which needs, in the future, to separate and test the single antifungal compounds present in MBS.

Since, MBS extract exerted powerful antifungal effect against these two fungi, the antifungal effect of MBS seems to bypass the most common pathways of antifungal resistance. Therefore, this gave a clue on the high possibility for the presence of novel antifungal compound (s) in MBS extract that might not share the same mode of action with the above common antifungal drugs or at least have some modified interactions of the same pathway.

There are many possibilities for the effective antifungal activities gained in the results of the current study. It is known that flavonoids are substances which are synthesized by plants in response to microbial infection (Dixon *et al.*, 1983), it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls

(Tsuchiya *et al.*, 1996) and may be with fungal cell wall too. Modes of action reported for flavonoids are: lysis and leakage of intracellular constituents, perturbation of cell homeostasis, inhibition of enzymes, electron transport, oxidative phosphorylation, interaction with macromolecules and effects on macromolecular biosynthetic processes (Lambert, 2008).

Finally, the potent and novel antifungal activity of this study open the gate for further future studies on more fungi and trying to isolate and identify the potent component (s) of MBS. Furthermore, finding new antifungal drug against *T. rubrum* and *T. harzianum* may solve the problem of many patients and doctors around the world who suffer from the rapid resistant development of these two human pathogens against most of commercially available antifungal agents.

CONCLUSION

Taken together, it was concluded that the MBS extract using acid methanol solvent has novel and potent antifungal effect against the tested fungi while the mung bean extract did not show similar potent antifungal activity. These findings support the hypothesis of the enhanced/modified phytochemicals synthesis and defense mechanisms in sprouting seeds. Most interestingly, for the first time, MBS extract showed potent antifungal activity against *T. rubrum* and *T. harzianum*. The antifungal activity of MBS against those two fungi points out to the possibility of the presence of novel antifungal component. Further investigations on the active antifungal component (s) in the MBS methanol crude extract are necessarily required to provide the pharmaceutical companies with cheap, effective, and most likely novel single antifungal agent (s).

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