Original Article

Antifungal activity of *Momordica charantia* seed extracts toward the pathogenic fungus *Fusarium solani* L.

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ABSTRACT

Momordica charantia L., a vegetable crop with high nutritional value, has been used as an antimutagenic, antihelminthic, anticancer, antifertility, and antidiabetic agent in traditional folk medicine. In this study, the antifungal activity of *M. charantia* seed extract toward *Fusarium solani* L. was evaluated. Results showed that *M. charantia* seed extract effectively inhibited the mycelial growth of *F. solani*, with a 50% inhibitory rate (IC_{50}) value of 108.934 μg/mL. Further analysis with optical microscopy and fluorescence microscopy revealed that the seed extract led to deformation of cells with irregular budding, loss of integrity of cell wall, as well as disruption of the fungal cell membrane. In addition, genomic DNA was also severely affected, as small DNA fragments shorter than 50 bp appeared on agarose gel. These findings implied that *M. charantia* seed extract containing α-momorcharin, a typical ribosome-inactivating protein, could be an effective agent in the control of fungal pathogens, and such natural products would represent a sustainable alternative to the use of synthetic fungicides.

1. Introduction

Fungal diseases have a serious effect on the growth and yield of crops, therefore, conventional fungicides have been widely used. However, this has led to numerous environmental problems. To minimize the environmental contamination caused by conventional fungicides, many alternatives have been tried, including biological control, physical treatments, and use of low toxicity chemicals such as plant extracts or natural antifungal compounds [1]. In particular, the use of active plant extracts appears to be the best option, as they

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exert minimal environmental impact and pose little danger to consumers [2]. Extracts of many plants possess antimicrobial and insecticidal properties both under laboratory and field tests [3]. *Potentilla erecta* and *Salvia officinalis* extracts exhibited high antifungal activities toward *Phytophthora infestans* [4]. The antifungal activity of Moroccan medicinal plants has also been validated against *Geotrichum candidum* [5]. In order to search for the best plant-derived products with antifungal activities for microbial disease control in agriculture, more efforts need to be taken [6].

Plants have evolved various defense mechanisms to combat pathogens, such as the synthesis of plant pathogenesis-related (PR) proteins [7]. Antifungal proteins and peptides, found in different plant tissues, can effectively protect plants from fungal invasion. Based on biological activities and chemical structures, antifungal proteins are classified into several types: ribosome-inactivating proteins (RIPs), defensins, lectins, chitinases, chitin-binding proteins, thaumatin-like proteins, protease inhibitors, and lipid-transfer proteins [8]. In particular, RIPs have attracted much attention because of their broad antitumor, antifungal, and antibacterial activities [9]. RIPs can greatly improve disease resistance both in traditional crops and genetically modified crops. For example, transgenic tobacco transferred a barley RIP gene has a much higher level of resistance to fungal pathogens [10]; transgenic rice with an exogenous RIP gene has enhanced resistance to sheath blight [11].

Momordica charantia L. $(2x = 2n = 22)$, a pathogen-resistant species, is widely distributed in tropical and subtropical regions, including Asia, Africa, parts of the Amazon Basin, and South America [12]. Besides being a highly nutritious vegetable, *M. charantia* is also important in folk medicine, especially in China and India [13]. Medicinal properties such as antimicrobial, antitumor, immunotoxic, antiviral, antimutagenic, antifertility, and antidiabetic, have been clarified for *M. charantia* [13,14]. In our previous study, the recombinant alpha-momorcharin (α-MC), a typical RIP rich in *M. charantia* fruit and seed (about 10–20%; data not shown), showed significant antifungal activity toward *Fusarium solani* L. *in vitro*, with an IC$_{50}$ value of 6.23μM or 181.25 μg/mL [12]. It is not clear whether the *M. charantia* seed extract (MSE), which contains an abundant amount of soluble α-MC, protease inhibitors, and other active ingredients, possesses antifungal activity.

The aim of this study was to determine the antifungal activity of MSE toward *F. solani*, a filamentous fungus causing damping-off of many crops in fields and greenhouses. In addition, its antifungal mechanism was also explored. The results showed that MSE would be an effective sustainable alternative to synthetic fungicides in control of fungal pathogens.

2. Materials and methods

2.1. Materials

Seeds of *M. charantia* were obtained from a local shop (Wuhan, China). *F. solani* (AF93239) was provided by the China Center for Type Culture Collection (Wuhan University, Wuhan, China). Protein marker and DNA marker were from Takara (Dalian, China). Anti-α-MC polyclonal rabbit antibodies were prepared as described in our previous study. Propidium iodide (PI) was bought from Invitrogen (Paisley, UK). All other reagents were of analytical grade.

2.2. Preparation of seed extracts

Five grams of dried seed of *M. charantia* was homogenized with 10 mL protein extraction buffer (25mM Na$_3$PO$_4$, 250mM NaCl, 10mM EDTA, 10mM thiourea, 5mM dithiothreitol, 1mM phenylmethanesulfonyl fluoride, and 1.5% poly-vinylpyrrolidone, pH 7.4) at 4°C. The extract was first filtered through two layers of gauze, and then the filtrates were centrifuged at 10,000g for 30 minutes (4°C) to collect the supernatant. The concentration of total protein in the supernatant was measured with the Bradford method using bovine serum albumin as a standard [15]. Sterilized by passing through 0.22-μm-diameter Millipore Swinex filters, the MSE was adjusted to 1.0 mg/mL and kept at −20°C until further application.

2.3. Antifungal activity assay

Equal aliquots of *F. solani* fungal suspension were pipetted into each sterile tube containing 1 mL potato dextrose broth medium and cultured to logarithmic growth phase. Then, the *F. solani* suspensions were supplemented with different amounts of MSE (0–150 μg/mL) and cultured for another 24 hours. The optical density values of these fungal suspensions were measured using an Eppendorf Biophotometer Plus (Hamburg, Germany) at 595 nm. Accordingly, the growth inhibition percentage was calculated, which was defined as $100\times$ the ratio of the A_{595} of the control minus the corrected A_{595} of the sample over the corrected A_{595} of the control [9].

An assay of the antifungal activity of MSE toward *F. solani* was carried out in potato dextrose agar (PDA) medium containing filter-sterilized protein extraction buffer (control plate) or 1 mg/mL MSE (treated plate). Briefly, each PDA plate was placed with a 20-μL fungal suspension in the center, and further incubation was done at 28 ± 1°C. These fungal colonies were observed and photographed every 12 hours for 36 hours. Three independent replicates were performed for each sample.

2.4. Morphology observation of F. solani

Fungal hyphae treated with both protein extraction buffer (control sample) and MSE (treated sample) were mounted on glass slides for lactophenol blue staining. The slides were examined and photographed with a light microscope (Olympus BH-2, Tokyo, Japan) at 100× magnification. After staining with PI, the fungal cell viability was also assessed by fluorescence microscopy (×100). Each assay was repeated in triplicate.

2.5. Detection of genomic DNA fragments

Fungal hyphae treated with protein extraction buffer or the MSE were collected through centrifugation at 12,000g for 5 minutes at 4°C. The obtained cells were lysed and processed with Apoptotic DNA Ladder kit (Bioteke, Beijing, China)

according to the manufacturer's instructions. Then, the recovered DNA was dissolved in Tris–EDTA buffer (pH 8.0) and analyzed immediately on 3% agarose gel electrophoresis.

2.6.   Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of the seed extract

Proteins of MSE were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the molecular weights were estimated by comparison of electrophoresis mobility with those of the standard marker proteins (ranging from 4.4 to 94.0 kDa). In addition, the SDS-PAGE gel was electroblotted by semidry protein transfer to a nitrocellulose membrane (Whatman, Dassel, Germany). The nitrocellulose membrane was first saturated with 5% (w/v) defatted milk for 1 hour at 37°C, then incubated with a 1:2000 dilution

of anti-α-MC polyclonal rabbit antibodies specific for α-MC for 1 hour, and then incubated with 1:5000 dilution of alkaline phosphatase-conjugated goat antirabbit IgG secondary antibody (CWBIO, Beijing, China). Subsequently, the blotted protein was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Biosharp, Seattle, WA, USA) according to the manufacturer's instructions.

3.   Results

### 3.1.   Antifungal activity of MSE toward mycelial growth

MSE impaired both cell integrity and viability. When *F. solani* were treated with a low concentration of MSE, only faint growth inhibition occurred (Table 1, group 1). When MSE concentration was increased, the growth of *F. solani* was inhibited gradually, indicating that MSE inhibited the fungal growth in a dose-dependent manner. In addition, the inhibitor concentration leading to 50% inhibitory rate (IC_{50}) was calculated to be 108.934 μg/mL. Protein extraction buffer had no effect on *F. solani* growth, so the effect of fungal growth inhibition was attributed to MSE.

Compared with the negative control, in which *F. solani* hyphae grew normally and healthily (Figure 1A: a-1, a-2, a-3), the "treated plate" had almost no growth at all (Figure 1B: b-1, b-2, b-3). The diameter of the fungi plaque growing on the "control plate" had reached up to 3.8 ± 0.2 cm after a 36-hour culture (Figure 1A: a-3), but no change took place on the "treated plate." In addition to growth inhibition, the

Table 1 – Growth inhibition of *Fusarium solani* with different concentrations of MSE.

Groups	Concentrations of MSE (μg/mL)	Inhibition rate (%)
Control	0	0.00
1	25	10.22
2	50	33.44
3	75	39.744
4	100	45.856
5	125	57.216
6	150	63.136

MSE = *Momordica charantia* seed extract.
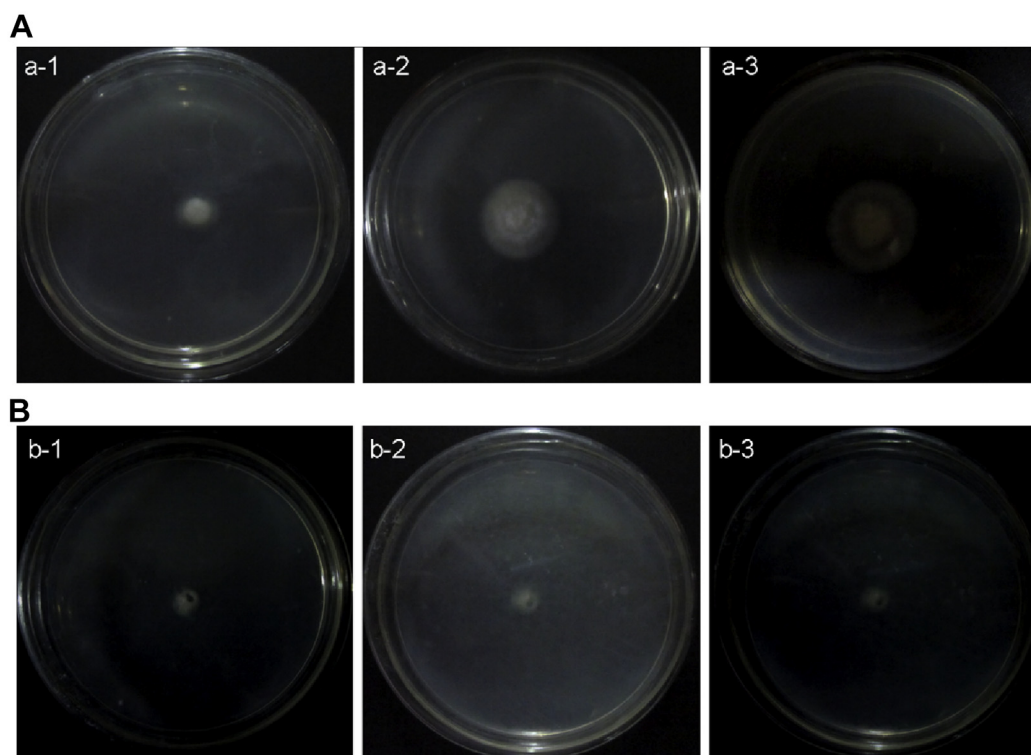
Figure 1 – Growth inhibition of *Fusarium solani* on PDA plates. (A) a-1 to a-3, *F. solani* was cultured on the "control plate" for 12 hours, 24 hours, and 36 hours, respectively. (B) b-1 to b-3) *F. solani* incubated on the "treated plate" coated with MSE for 12 hours, 24 hours, and 36 hours, respectively. MSE = *Momordica charantia* seed extract; PDA = potato dextrose agar.

morphology of the *F. solani* filament fungi had also been seriously affected by MSE (Figure 1B: b-3), which showed that MSE was highly active toward *F. solani*.

3.2. Mechanism of antifungal activity

The "control sample" had homogeneous cell structure and normal mycelial apex (Figure 2A: Con-1, Con-2, Con-3, Con-4). However, the "treated sample" showed irregular shapes and heterogeneous inner structure (Figure 2B: Tre-1 to Tre-8); and the fungal tube elongation seemed to be substantially affected by MSE, as extensive septum formation was observed (Figure 2B: Tre-1 to Tre-8). Loss of symmetry, distorted apex, small swellings along the mycelia and mycelial apex, and abnormal mycelia with bursting cells were also observed in the "treated sample" (Figure 2B: Tre-1 to Tre-8).

PI, membrane-impermeant and generally excluded from viable cells, is commonly used to distinguish dead cells from live cells. Hyphae treated with MSE were more sensitive to PI stain than the control hyphae (Figure 3). Different from the "control sample" (Figure 3: Con-1-2), a large increase in the number of fluorescent cells was observed in MSE-treated fungal cells (Figure 3: Tre-1-2 and Tre-2-2). Moreover, the "treated sample" had an enlarged and deformed nucleus, as well as masses of densely stained granular material localized in cytoplasm (Figure 3: Tre-1-2 and Tre-2-2). MSE could induce cell membrane permeabilization, destruction of cell inner structures, and correspondingly apoptosis-mediated cell death.

PI staining and subsequent fluorescence microscopy observation showed that the nucleus of *F. solani* had been markedly affected by MSE. Therefore, a detailed analysis needs to be carried out to characterize whether *F. solani* genomic DNA was also affected, for example, the DNA ladder phenomenon. The genomic DNA of both the treated sample and control sample was isolated by the Apoptotic DNA Ladder kit. In addition, the right amount of RNase A was added into the incubation buffer prior to lysis extraction and subsequent extraction process. Results from 3% agarose gel showed that a major DNA band bigger than 4000 bp appeared on the top of the gel (Figure 4). Moreover, extra fragments shorter than 50 bp existed in the "Treated" lane (Figure 4), which might be caused by MSE. As MSE-treated *F. solani* (with damaged genomic DNA) grew unhealthily and irregularly, we speculated that MSE affected the integrity of fungi DNA, which correspondingly restrained the fungi growth. Although *F. solani* hyphae treated with MSE exhibited the characteristics of apoptosis, no typical DNA ladder was observed. Triplicate assays were carried out, and the results remained the same.

### 3.3. Characterization of antifungal protein in MSE

The sodium phosphate buffer (pH 7.0) was used to isolate MSE. In total, 5 g of dried seed yielded 8.53 mL mixtures, and the protein concentration was considered to be 191.5722 mg/mL. Through characterization on 12% SDS-PAGE, proteins in the mixture covered a broad range of 10.0−97.2 kDa, and the major portion concentrated between 18.0 and 45.2 kDa (Figure 5A). A main protein band appeared around 29−31.5 kDa, similar to the previously isolated α-MC, a highly active antifungal protein. Western blotting results showed that the special protein spot was really α-MC (Figure 5B). As a seed storage protein, α-MC may contribute significantly to the antifungal activity of seed extract. However, other antifungal proteins and peptides found in the MSE need to be further identified.
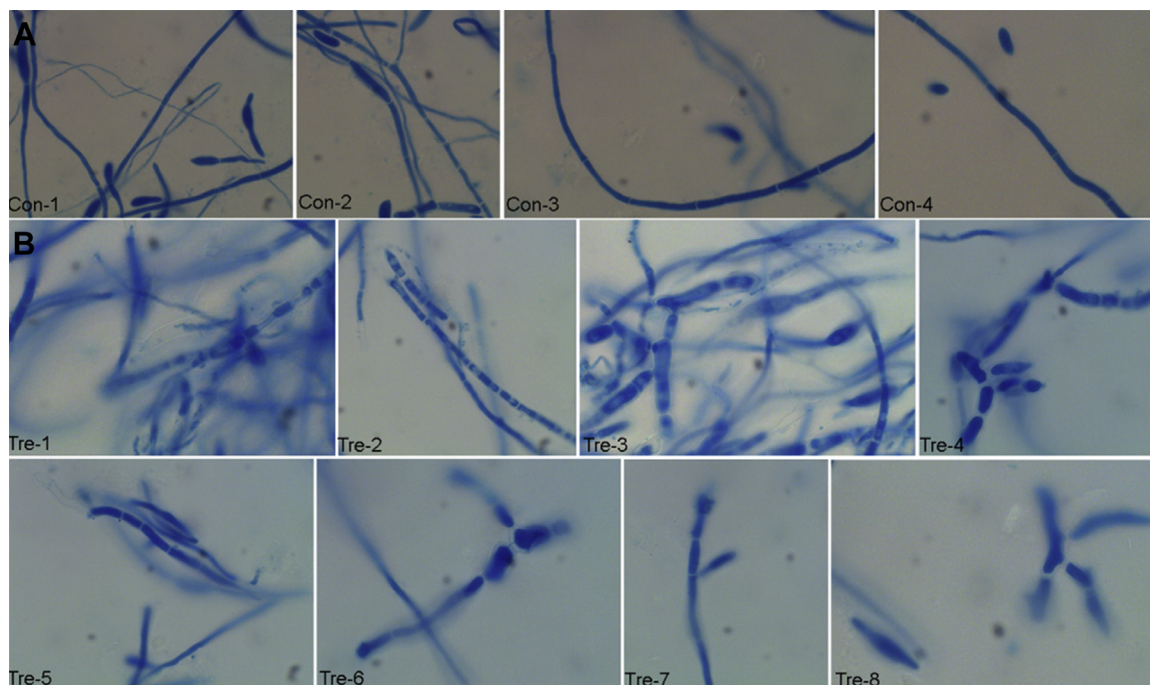
Figure 2 − Morphology observations of *Fusarium solani* hyphae under light microscope (×100). (A) Hyphae treated just with the protein extraction buffer. (B) The mycelia incubated with *Momordica charantia* seed extract (MSE).
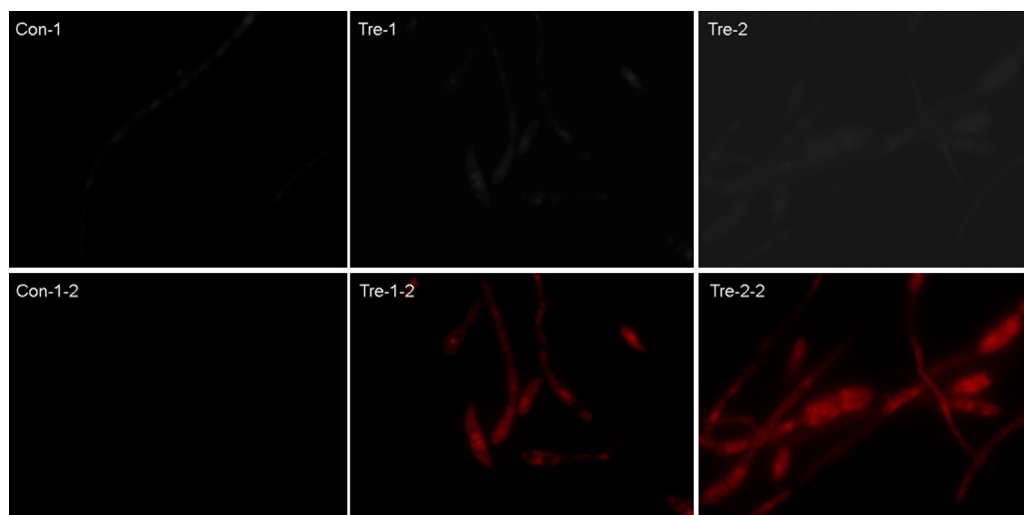
Figure 3 – Microscopic observation of hyphae stained with propidium iodide (PI). "-1" and "-2" = the hyphae stained with PI was observed under the light microscope (×100); "-1-2" and "-2-2" = the PI stained hyphae was observed under the fluorescence microscopy (×100); "Con-" = the hyphae treated with protein extraction buffer, the healthy hyphae; "Tre-" = the seed extract treated hyphae, the unhealthy hyphae.
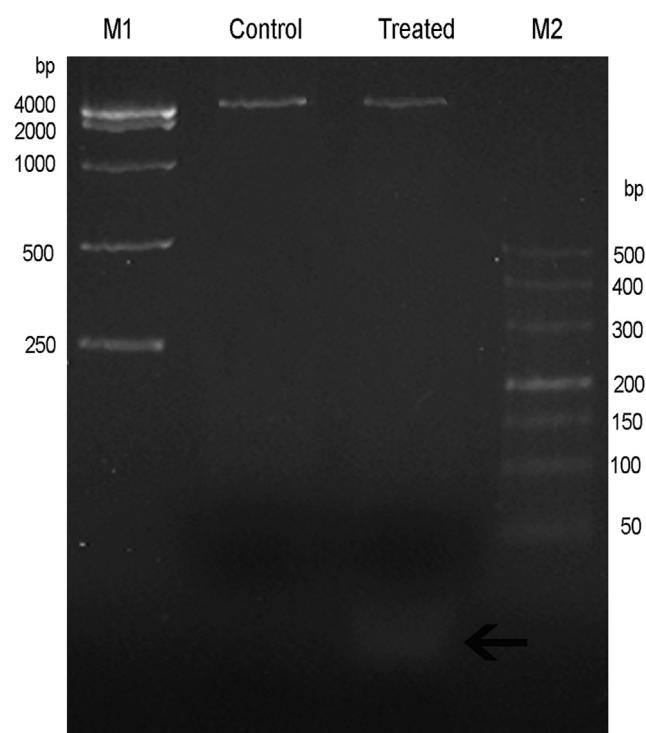
Figure 4 – Characterization of *Fusarium solani* genomic DNA on 3% agarose gel. Control = genomic DNA from the protein extraction buffer treated hyphae; M1 and M2 = two different DNA markers; MSE = *Momordica charantia* seed extract; Treated = genomic DNA of MSE-treated hyphae, the black arrow indicating the short fragments.

4. Discussion

Plant disease caused by pathogenic fungi severely affects crop growth and reduces crop yields worldwide, especially in developing countries. Besides yield losses and food decay, fungi also pose a serious risk for consumers because of dangerous secondary metabolites [16]. Therefore, the control of pathogenic fungi is urgent and important. For a long time, synthetic fungicides have been used widely to fight against pathogenic fungi, which further led to the selective proliferation of fungicide-resistant isolates. Therefore, the search for a natural substitute with antifungal activity has been encouraged.

Crude plant tissue extracts, generally a mixture of active and nonactive compounds, provide many advantages as antimicrobial agents [17,18]. The first is their natural origin, which seems much safer for consumers and the environment; second, they are at low risk for resistance development of pathogenic fungi; third, they represent a rich source of potential bioactive compounds [18]. As various modes of action exist in tissue extract, it is difficult for pathogens to develop resistance to such a mixture of components. Therefore, the development of management strategies to replace or supplement synthetic fungicides with natural bioactive products is desirable. In particular, the antifungal proteins collected from the Cucurbitaceae family, pathogen-resistant species in the natural environment, are most promising because of their specific pathogen-related activities [19].

To date, many types of antimicrobial components have been isolated and characterized, including proteins, peptides, phenolics, and essential oils [20]. In particular, RIPs, a type of N-glycosidases cleaving the N-glycosidic bond of adenine-4324 in the GAGA hairpin of the sarcin/rincin loop of the 28S rRNA, play an important role in plant–pathogen interaction [21]. For example, *Zea mays* b-32 efficiently inhibits the growth of *Fusarium verticillioides* [22]. Many mechanisms have been speculated upon, such as N-glycosidase activity, DNase-like activity, and oxidative stress-inducing stimulus [23].

Active proteins in the MSE mixture may permeate cellular membrane, lead to the instability of the membrane, and accordingly affect macromolecular synthesis. At the same
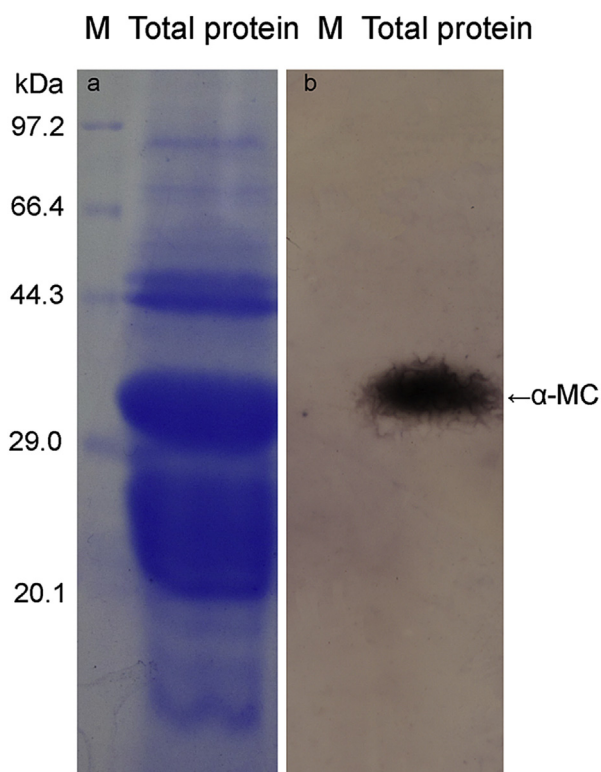
Figure 5 — SDS-PAGE analysis (a) and Western blotting test of MSE with alpha-momorcharia antibody (b).

time, functions of organelles can also be affected. For example, protein synthesis on ribosomes may be arrested by RIPs present in the seed extract, as fungal ribosomes are more sensitive to RIP than that of mammalian and plant [24]. In this assay, genomic DNA degradation of the MSE-treated *F. solani* hyphae was observed. As no smear bands appeared on the gel (effect of DNase), the short DNA fragments might be generated by *N*-glycosidase activity and DNase-like activity of α-MC. Compared with the IC_{50} value of recombinant α-MC toward *F. solani* (181.25 μg/mL), the MSE had an even smaller IC_{50} value (108.934 μg/mL), inferring that MSE could be more effective as potential fungicide in the control of fungal pathogens. Other antifungal proteins present in MSE might collaborate with RIP α-MC and enhance the overall antifungal activity.

Fungicides have been considered to act directly against fungi, as well as induce a defense response in plant. The same mode of action has also been presumed for plant extracts against fungal pathogens [6,17,25—28]. Genes encoding PR proteins, such as RIP, could be activated during the pathogen attack process, which are induced through the action of signaling compounds such as salicylic acid, jasmonic acid, or ethylene [6]. The plant extracts may also act in synergy with elicitors or other defense proteins, which can have an additive effect when used together.

Plant extracts have been used for the treatment of plant diseases caused by pathogenic microbes since the 1990s. MSE has proved to be a highly active antifungal agent in this study, which implies that MSE may be effective in the control of fungal pathogens, and such natural products would

represent a sustainable alternative to the use of synthetic fungicides. We also believe that extracts of plants belonging to the Cucurbitaceae family, including *M. charantia*, may be considered as natural and nontoxic biofungicides against pathogens.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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