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Antimicrobial efficacy of extracts and constituents fractionated from *Rheum tanguticum* Maxim. ex Balf. rhizomes against phytopathogenic fungi and bacteria

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ABSTRACT

The antimicrobial efficacies of extracts and constituents fractionated from *Rheum tanguticum* rhizomes were evaluated by using *in vitro* and *in vivo* bioassay against seven phytopathogenic fungi and ten pathogenic bacteria, respectively. Dichloromethane and ethyl acetate soluble extracts derived from *R. tanguticum* rhizomes effectively controlled plant diseases caused by five fungi *Magnaporthe oryzae*, *Blumeria graminis* f. sp. *hordei, Colletotrichum coccodes, Phytophthora infestans,* and *Puccinia recondita* in *in vivo* whole plant bioassay. In addition, these extracts showed strong inhibitory activity against the bacterial growth of *Acidovorax avenae* subsp. *cattlyae* (Aac), *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas arboricola* pv. *pruni* (Xap), and *Pseudomonas syringae* pv. *actinidiae* (Psa), with MIC values ranging from 125 to 250 μg/mL. The active extracts of *R. tanguticum* rhizomes were fractionated by using various and repeated chromatographic techniques to yield seven constituents that included three anthraquinones and four stilbenes. Notably, at concentrations ranging from 75 to 300 μg/mL, physcion (**DH03**) and chrysophanol (**DH04**) were significantly effective in suppressing the development of barley powdery mildew (BPM) caused by *B. graminis* f. sp. *hordei,* with control values from 80 to 96.7%. Moreover, stilbenes rhapontigenin (**DH02**) and desoxyrhapontigenin (**DH05**) had a broad spectrum and potent activity against phytopathogenic bacteria; **DH05** effectively inhibited the bacterial growth of Aac, *Burkholderia glumae* and Psa at MICs ranging from 38 to 150 μg/mL. In this paper, we report their antibacterial activity for the first time. The botanical materials containing anthraquinones and stilbenes were active against both phytopathogenic fungi and bacteria. These results suggested that *R. tanguticum* rhizomes could be used as a new source of antimicrobial substances for developing a botanical fungicide and bactericide to control plant diseases.

1. Introduction

Phytopathogenic fungi and bacteria cause substantial damage to many economically important crops. Synthetic chemicals have been used effectively to control the phytopathogenic microorganisms. However, due to the heavy and repeated use of synthetics, some fungicides and bactericides have become less effective when plant pathogens have developed resistance. Synthetic pesticides have also caused undesirable effects on non-target organisms, and environmental and human health concerns (Copping and Duke, 2007; Vidhyasekaran, 2004). As a result of increasing resistance to commercially synthetic pesticides, strategies to use less synthetic pesticides and more biocontrol agents have been considered in organic agricultural practices. In addition, research on alternative types with new modes of action to control plant pathogens is needed to avoid pathogenic resistance, to successively use with other pesticides in integrated pest management, and to reduce harmful effects on human and environment (Ben Ghnaya et al., 2016; Stević et al., 2014). Plant-derived chemicals appear to have a possible role in the development of botanical fungicides and bactericides, since they constitute a rich resource of bioactive compounds that

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show less environmental risk and mammalian toxicity (Copping and Duke, 2007; Kim et al., 2004; Vogt et al., 2013,b; Yoon et al., 2011a,b).

Rheum tanguticum Maxim. ex Balf. belongs to the family Polygonaceae, and is a one of the three genuine Rhubarbs that are commonly used in the folk medicines of China and Southeast Asia (Do, 2004; Hu et al., 2010; Hu et al., 2014). The plant is a high-altitude perennial herb that is sensitive to high temperature, and mainly found in the alpine regions of temperate and subtropical Asia, in particular Southwest and Northwest China (Chen et al., 2009; Yu et al., 2010). Stilbenes and anthraquinones are the main components occurring in the rhizomes of *R. tanguticum* (Jin and Tu, 2005; Jin et al., 2006, 2007; Ngoc et al., 2008), and show remarkable antioxidant, anti-inflammatory, anti-allergic, and cytotoxic properties (Jin et al., 2011; Ngoc et al., 2008). However, no report is available on the antimicrobial activity of *R. tanguticum* and its substances against plant pathogenic fungi and bacteria. In our screening program of antifungal and antibacterial compounds from Vietnamese plants, we have found that the ethyl acetate and dichloromethane soluble extracts from *R. tanguticum* rhizomes have strong *in vivo* antifungal activity against several fungal plant diseases, as well as strong *in vitro* antibacterial activity against phytopathogenic bacteria. Therefore, the aim of our research was to isolate and identify antimicrobial compounds, and evaluate the *in vivo* antifungal efficacy and *in vitro* antibacterial activity of the plant extracts and pure compounds derived from the rhizomes of *R. tanguticum* against various phytopathogens.

2. Materials and methods

2.1. Microbial strains and culture conditions

The plant extracts and compounds were tested against the seven phytopathogenic fungi, *Blumeria graminis* f. sp. *hordei*, *Botrytis cinerea, Colletotrichum coccodes, Magnaporthe oryzae, Phytophthora infestans, Puccinia recondita*, and *Rhizoctonia solani*, which were obtained from the Center for Eco-friendly New Materials, the Korea Research Institute of Chemical Technology. The fungal strains were maintained on potato dextrose agar (PDA) medium, and were sub-cultured in potato dextrose broth (PDB) with 1% inoculum at 25 °C for the period of 2–6 days, before use for *in vivo* bioassay.

The following ten phytopathogenic bacterial strains were used for the antibacterial bioassay: *Acidovorax avenae* subsp. *cattlyae* (bacterial blight of konjac), *Agrobacterium tumefaciens* (crown gall disease), *Burkholderia glumae* (bacterial grain rot of rice), *Clavibacter michiganensis* subsp. *michiganensis* (bacterial wilt and canker of tomato), *Pectobacterium carotovora* subsp. *carotovora* and *Pectobacterium chrysanthemi* (bacterial spot rot), *Pseudomonas syringae* pv. *lachrymans* and *Pseudomonas syringae pv. actinidiae* KW11 (cucumber angular leaf spot), *Xanthomonas arboricola* pv. pruni (pepper bacterial spot), and *R. solanacearum* (tomato bacterial wilt). All bacterial strains were grown on nutrient agar (NA) (Becton, Dickinson and Co., Sparks, MD, USA) and nutrient broth (NB), except for *R. solanacearum*, which was grown in tryptic soy agar (TSA) (Becton, Dickinson and Co.) and tryptic soy broth (TSB) (Becton, Dickinson and Co.). *X. arboricola* was cultured aerobically at 25 °C for 18–36 h, and the remaining strains were cultured aerobically at 30 °C for 18–36 h. Before antibacterial activity test, the bacteria were aerobically cultured in NB or TSB for 24 h, and then suspended in sterile saline at a density equivalent to that of the 0.5 McFarland standard. Bacterial suspensions with a concentration of 10^5 cfu/mL were used for *in vitro* antibacterial activity test.

2.2. Plant materials

R. tanguticum Maxim. rhizomes were purchased from Ninh Hiep traditional medicine market in Vietnam in August 2014, and were identified by Mr Nghiem Duc Trong, Hanoi University of Pharmacy. An authenticated voucher specimen (Acc. No. 2014 DH-RT) (Fig. 1) was deposited at the Laboratory of Bioactive Compound Technology, Institute of Chemistry. The rhizomes were dried in the dark, and ground into powder by using a blending machine.

Fig. 1. Image of *Rheum tanguticum* rhizomes.

2.3. Isolation of constituents from R. tanguticum rhizomes

R. tanguticum rhizomes (3 kg) were ground and extracted with methanol (4.5 L, 3 times). The solvent was evaporated under reduced pressure using a rotary evaporator at a temperature below 40 °C to yield 882 g methanol extract. The methanol extract was suspended in 4 L of distilled water, and partitioned with dichloromethane (DCM) (4 L; 3 times). The aqueous layer was acidified with HCl 9N to yield a solution containing 0.5% of HCl. The resulting aqueous layer was partitioned three times with ethyl acetate (EtOAc). Each organic layer was pooled, washed with distillated water, and evaporated under reduced pressure, to yield DCM soluble extract (269.65 g) and EtOAc soluble extract (201.4 g) (Fig. 2).

MeOH extract and DCM and EtOAc soluble extracts were tested for their antimicrobial activity against phytopathogenic fungi and bacteria in *in vivo* and *in vitro* bioassay. DCM and EtOAc soluble extracts displayed significant activity against phytopathogenic fungi and similar activity spectrum pattern to MeOH extract. These extracts were used as a raw material to isolate constituents with antimicrobial activity.

A portion of DCM soluble extract (20 g) was mixed with 40 g of silica gel 60 Å (40–63 μm), and applied on the top of a silica gel column [400 g silica gel 60 Å (40–63 μm), 4.5 × 70 cm]. The column was eluted with *n*-hexane (H):EtOAc solvent stepwise gradient (100:0, 99:1, 98:2, 96:4, 92:8, 85:15, 70:30; 30:70 and 0:100; 4 L each), and then with the mixtures of EtOAc:MeOH (9:1 and 5:5, 4 L each). The collection fraction volume was 400 mL, and the fractions with patterns on TLC chromatogram were pooled. At the end, the column was washed with MeOH. The fractionation of DCM extract yielded 12 sub-fractions F1–F12. Fraction 5 (2.7169 g) was subjected to a silica gel column [190 g silica gel 60 Å (40–63 μm), 3.5 × 60 cm] eluting with H:DCM (100:0, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50 and 40:60; 800 mL each and fraction volume of 100 mL) to yield two pure compounds **DH04** (426.3 mg) and **DH03** (284.6 mg). Fraction F6 (947.7 mg) was fractionated by silica gel column chromatography [50 g silica gel 60 Å (40–63 μm), 2.5 × 50 cm] eluting with H:EtOAc (85:15) to give **DH01** (357.1 mg). Fraction F8 (1,353.2 mg) was chromatographed on a silica gel column [78 g silica gel 60 Å (40–63 μm), 2.5 × 50 cm] with EtOAc:MeOH (70:30, v/v) as eluting solvent, and the resulting fractions were crystallized to afford **DH02** (942.7 mg). Fraction F7 (953.2 mg) was chromatographed on a silica gel column [50 g silica gel 60 Å (40–63 μm), 2.5 × 50 cm] with mixtures of DCM:MeOH (99:1, 98:2, 96:4 and 90:10) as eluting solvents, and the resulting fractions were crystallized in MeOH to yield **DH05** (7.6 mg) (Fig. 2).

Fractionation of a 20 g sample of EtOAc soluble extract was performed by silica gel column chromatography [450 g silica gel 60 Å (40–63 μm), 4.5 × 70 cm], eluting with a gradient elution of DCM:MeOH from 100% DCM to 30:70 (v/v), to give 9 fractions from E1 to E9. Fraction E6 (1.5 g) was chromatographed on a silica gel column [120 g silica gel 60 Å (40–63 μm), 3.5 × 60 cm] and eluted with