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Biological Active Compounds of *Scleroderma Citrinum* That Inhibit Plant Pathogenic Fungi

Soytong, K. [*] [1], Sibounnavong, P.[2], Kanokmedhakul, K.[3] and Kanokmedhakul, S.[3]

[1]Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand, [2]Department of Plant Protection, Faculty of Agriculture, National University of Laos (NUOL), Vientiane, Lao, PDR, [2]Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand.

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Abstract The natural products were isolated from the fruiting bodies of *Scleroderma citrinum*. A new lanostane-type steroids were found namely 4,4'-Dimethoxymethyl vulpinate (DMV) and 4,4'-Dimethoxyvulpinic acid (DMVA). These compounds were tested for biological activities against plant pathogens *in vitro*. Results showed that 4,4'-Dimethoxyvulpinic acid had more active to inhibit the tested pathogens, *Phytophthora palmivora* and *Colletotrichum. gloeosporioides* than 4,4'-Dimethoxymethyl vulpinate at all tested concentrations. The effective dose (ED_{50}) of DMVA compound could significantly inhibit the mycelium growth of *P. palmivora* and *C. gloeosporioides* at the concentrations of 58 and 81 ug/ml, respectively. The ED_{50} of DMV compound for inhibition of such fungal mycelial growth was 2,114 and 5,231 ug/ml., respectively. The production of conidia of *C. gloeosporioides* was statistically significant inhibited by both tested compounds, with this the ED_{50} of DMA and DMVA compounds were 45 and 68 ug/ml., respectively.

Keywords: *Scleroderma citrinum*, Dimethoxymethyl vulpinate, Dimethoxyvulpinic acid

Introduction

In Thailand *Scleroderma citrinum* has been reported as ectomycorrhizal fungi in 1990 (Soytong, 1990). This species has a large fruiting body which belongs to Gasteromycetes, Basidiomycotina (Alexoporous and Mims, 1979). There are reports that the ectomycorrhizal fungi, *S. citrinum* are capable of forming ectomycorrhiza association with some pine, *Pinus abies* (Brunner *et al*., 1992), *P. patula* (Mohan *et al*., 1993), *P. menziesii*, *P. pinaster* (Parlade., 1996) and *Eucalyptus* spp. (Kumar *et al*., 1999) but no reports on the study of biological active compounds against pathogens. However, the presence of compounds with antimicrobial properties was studied in extracts of

[*] **Corresponding author:** Soytong, K.; **Email:** ajkasem@gmail.com.

Scleroderma flavidum. Extracts from *S. flavidum* grown in liquid culture media were processed to obtain 2 fractions, water and ethyl acetate soluble compounds. The fractions were tested for the presence of inhibitory constituents against *Fusarium roseum, Pythium* sp. and *Rhizoctonia solani* (Kasuya *et al*., 1996). Some species of Gasteromycestes, *Cyathus striatus* has been found to produce Cyathin e.g. cyathin A_3, Cyathin A_4, allocyathin A_4, Cyathin B_3, Cyathin B_4 and Cyathin C_5 and inhibited some species of bacteria and fungi (Bresinsky 1990). It has previously reported for chemical constituents and bioactivity of *S. citrinum* by Kanokmedhakul et al (2003) which reported that a new lanostane-type tripernoid, (20S,22S,23E)-22-0-acetyl-25-hydroxylanosta-8,23(E)-dien-3-one isolated as a new natural product for the first time, methyl 4,4'-dimethoxyvulpinate, together with the known compound 4,4'-dimethoxyvulpinic acid were isolated from *S. citrinum* and the 4,4'-dimethoxyvulpinic acid exhibited activity towards *Mycobacterium tuberculosis*. Our research finding was to investigate the biological active compounds of *S. citrinum* for inhibition of plant pathogens, *Phytophthora palmivora* causing stem canker and root rot of durian (*Durio zibithenus*) and *Colletotrichum gloeosporioides* causing anthracone of mango (*Mangifera indica*).

Materials and methods

Collection and identification of fungi

Fresh fruiting bodies of *S. citrinum* were collected from Nagatad Village, Arekadamnoy District, Sakornakon Province, Thailand which were naturally growing on the surface of plant debris in high organic soils at different growth stages during raining season. The specimens were brought to laboratory for identification and chemical elucidation. Specimens of fruiting bodies were deposited at Thai Mycological association Herbarium (TMAH) as a code No. TMAH-SC01, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

The tested pathogens were isolated from diseased-plant parts by tissue transplanting method. All isolates were then proved to be pathogenic to host plants on the basis of Koch's Postulation method. These are *Phytophthora palmivora* causing stem canker and root rot of durian (*Durio zibithenus* L.) and *Colletotrichum gloeosporioides* causing anthracnose of mango (*Mangifera indica* L.).

Chemical elucidation

The freshly fruiting bodies of *S. citrinum* were ground and extracted with MeOH at room temperature (27-30 C) for 2 days before filtration. The culture filtrates were combined together and the solvent was then removed *in vacuo* to obtain a red brown residue. The methanol crude extract was subjected to silica gel CC using hexane, EtOAC and MeOH as mobile phase with gradient elution. Fractions were collected and combined on the basis of TLC into different fractions and for further purification. DMV compound obtained from fractional recrystallization of F3 (hexane:EtoAc, 1:1) and F4 (hexane:EtoAc, 2:3) and DMVA compound obtained from fractional recrytallization of F4 (hexane :EtOAc, 2:3) and F5 (hexane:EtoAc, 1:2). CC and TLC were carried out on silica gel 60 (63-200 mesh) and silica gel 60 F_{254} precoated plates, respectively. NMR spectra were recorded in $CDCl_3$ on a Jeol JMN-4500 and a Bruker DRX400 spectometers, using residual $CHCl_3$ as an internal standard. IR spectra were carried out on a Bio-Rad FTS-7 spectrophotometer. EIMS and ESMS were measured on a Finningan Mat INCOS 50 and Bruker Esquie LC mass spectrometers. HR-FABMS was measured on a Finningan Mat 90 instrument.

Bioactivity test for inhibition of plant pathogenic fungi

The pure compounds were tested for inhibition of plant pathogenic fungi, *P. palmivora* (stem canker and root rot of durian) and *C. gloeosporioides* (anthracnose of mango). Potato dextrose agar were used as tested medium incorporating with different concentrations of each compound as follows:- 0, 10, 50, 100 and 500 ug/ml. The fungal growing on PDA for 7 d at room temperature (27-30 C) was cut at the edge of colony with sterilized cork borer (0.3 cm). The agar plug with fungal culture was then transferred onto PDA medium mixed with pure compound of *S. citrinum* in each concentration. The experiments were done using Completely Randomized Design with four repeated experiments. Data were collected as colony diameter or spore production of tested pathogens and computed analysis of variance. Treatment means were statistical compared by Duncan's New Multiple Range Test at P = 0.01. Per cent inhibition was computed and ED_{50} was analyzed by Probit analysis.

Results

The pathogenicity test was confirmed by culturing the isolates on potato dextrose agar for 7 days. Plants were inoculated by transferring the agar plugs

onto the leaf surfaces and incubated in a humid chamber for 48 h. After 5 days, the inoculated leaves were infected by the tested pathogens. The pathogens were reisolated and identified as *P. palmivora* and *C. gloeosporidiodes*, respectively. Results showed that *S. citrinum* strain Sc-1 produced some antifungal metabolites as the new lanostane-type steroids; 4,4'-dimethoxymethyl vulpinate (DMV) as seen in Fig.1 and 4',4'-dimethyoxyvulpinic acid (DMVA) as seen in Fig. 2. The antifungal activities of those pure compounds were tested for its ability to inhibit P. palmivora and *C. gloeosporidiodes* in vitro (Table 1, 2). It was showed that 4,4'-dimethoxymethyl vulpinate compound (DMV) significantly inhibited the mycelium growth of the tested plant pathogens, *P. palmivora* and *C. gloeosporioides* which the ED_{50} value of 2,114 and 5,231 ug/ml, respectively Moreover, 4',4'-dimethyoxyvulpinic acid compound (DMVA) inhibited the mycelium growth of *P. palmivora* which the ED_{50} value was 58 ug/ml. A result was also observed in 4',4'-dimethyoxyvulpinic acid (DMV) which inhibited the mycelium growth and conidia production of *P. palmivora* ; the ED_{50} values were 81 ug/ml ((Table 3). With this, the 4,4'-dimethoxymethyl vulpinate (DMV) also gave the highest inhibition for conidia production of *C. gloeosporioides* which the ED_{50} value was 45 ug/ml. (Table 4). It is suggested that these compounds may possible be developed to be a natural products for plant disease control.

Discussion

The nature of *S. citrinum* habitats usually growing in association with the forest trees as ectomycorrhizal fungus (Putra *et al*., 1999). S. citrtinum was also reported to be capable of forming ectomycorrhiza with some pine, eucalypt and fagaceous tree species and able to withstand high concentration of heavy metals such as Al, Fe, Cu and Zn and might have potential for revegetation schemes in metal-contaminated soils (Tam, 1995), but the mode of actions to inhibit plant pathogenic fungi of this growing fungus has never known. Our research findings have been proved that *S. citrinum* not only associated with some forest trees but also could produced some active chemical compounds to inhibit plant pathogenic fungi as well such as *C. gloeosporioides* causing anthracnose disease and *P. palmivora* causing root rot disease.

S. citrinum strain Sc-1 produced the new lanostane-type steroids; 4,4'-dimethoxymethyl vulpinate (DMV) and 4',4'-dimethyoxyvulpinic acid (DMVA) as antifungal metabolites to control *P. palmivora* (stem canker and root rot of durian) and *C. gloeosporioides* (anthracnose of mango). With this, Gong *et al*. (1999) reported that field and nursery trials were established in Guangdong Province, China, to determine the efficacy of antagonism of

ectomycorrhizal fungal isolate of *Scleroderma polyrhizum* to the bacterial wilt pathogen, *Pseudomonas solanacearum* (*Ralstonia solanacearum*) on *Eucalyptus* sp. The results showed that these ectomycorrhizal fungi were effective in controlling the occurrence and development of bacterial wilt. The disease rates of mycorrhizal seedlings were reduced by 40.00-72.78 % in the nursery and by 20.0-38.9% in the field, compared with those in un-inoculated seedlings. The height and basal diameter growth of trees in the [field] trials were enhanced by 11.67 to 59.7%, respectively, in inoculated plants. But it was not proved that which kind of fungal metabolites could active control the tested pathogen.

Our study finding the antifungal activities of those pure compounds were tested for its ability to inhibit *P. palmivora* and *C. gloeosporidiodes* in vitro which 4,4'-dimethoxymethyl vulpinate compound (DMV) significantly inhibited the mycelium growth and conidia production of the tested pathogens.

These research findings may possible to serve a control mechanism of S. citrinum for promote plant growth as its ectomycorhizal fungi with healthy plant. It is possible to release some antifungal substances to protect plants from some plant pathogens as well. As Parlade *et al*. (1996) reported that the container-grown *P. menziesii* and *P. pinaster* seedlings were inoculated with water suspensions of spores of ectomycorrhizal fungus commonly found in northeastern Spain. *P. pinaster* seedlings were inoculated with basidiospores of *S. citrinum.* The spore concentration were 103-107 spores/seedling for *S. citrinum* colonized more short roots in a larger proportion of plants at 107 spores/seedling than at any other rate. *S. citrinum* colonized a high percentage of short roots on all inoculated plants when applied at 105 spores/seedling.

It is suggested that these compounds may further investigate to be a natural products for plant disease control.

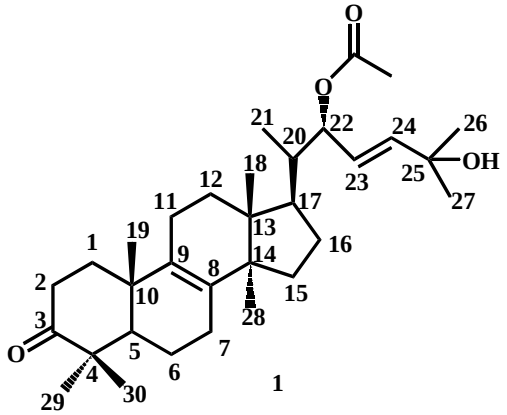

Fig. 1. Structure of 4,4′-dimethoxyvulpinic acid

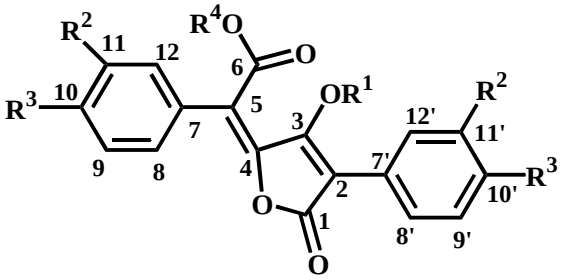

Fig. 2. Structure of 4,4′-dimethoxymethyl vulpinate

Table 1. The effect of *4,4'-dimethoxymethyl vulpinate and 4',4' dimethyoxyvulpinic acid* from *Scleroderma citrinum* to inhibit the colony growth (cm) of plant pathogenic fungi

Concentrations	*P. palmivora*		*C. gloeosporioides*	
(μg/ml)	DMV[1]	DMVA[2]	DMV	DMVA
0	5.00 a[3]	5.00 a	4.80 a	4.47 a
10	4.05 b	3.83 b	4.15 b	3.05 b
50	3.71 c	2.23 c	4.07 b	2.50 c
100	3.40 d	1.96 cd	3.40 c	2.72 bc
500	3.05 e	1.26 d	2.97 c	0.95 d
C.V.(%)	2.25	13.72	5.81	8.73

[1]*4,4'-dimethoxymethyl vulpinate,* [2] *4',4'-dimethyoxyvulpinic acid*

[3]Average of four replications. Mean follows by a common letter in each column are not significant different at DMRT at P=0.01.

Table 2. The effect of *4,4'-dimethoxymethyl vulpinate and 4',4'-dimethyoxyvulpinic acid* from *Scleroderma citrinum* to inhibit conidia production of *Colletotrichum gloeosporioides*

Concentrations (μg/ml)	Number of conidia ($x10^6.ml^{-1}$)	
	DMV[1]	DMVA[2]
0	16.25 a[3]	16.00 a
10	12.81 b	14.00 a
50	10.37 b	13.50 a
100	3.50 c	7.37 ab
500	1.18 c	1.25 b
C.V.(%)	26.38	37.96

[1]*4,4'-dimethoxymethyl vulpinate,* [2] *4',4'-dimethyoxyvulpinic acid*

[3]Average of four replications. Mean follows by a common letter in each column are not significantly differenve by DMRT at P=0.01.

Table 3. The effect of *4,4'-dimethoxymethyl vulpinate and 4',4'-dimethyoxyvulpinic acid* from *Scleroderma citrinum* to inhibit the mycelia growth of plant pathogenic fungi

Concentrations (μg/ml)	Inhibition of *P. palmivora* (%)		Inhibition of *C. gloeosporioides* (%)	
	DMV[1]	DMVA[2]	DMV	DMVA
10	19.00	23.40	13.00	31.76
50	25.80	55.40	14.60	44.07
100	32.00	60.80	28.00	39.14
500	39.00	74.80	36.00	78.74
average	28.95	53.60	22.90	48.42
ED_{50}(μg/ml)	2,11	58	5,23	81

[1]*4,4'-dimethoxymethyl vulpinate*, [2] *4',4'-dimethyoxyvulpinic acid*

Table 4. The effect of *4,4'-dimethoxymethyl vulpinate and 4',4'-dimethyoxyvulpinic acid* from *Scleroderma citrinum* to inhibit the conidia production of *Colletotrichum gloeosporioides*

Concentrations (μg/ml)	Inhibition of conidial production (%)	
	DMV[1]	DMVA[2]
10	21.16	12.50
50	36.18	15.62
100	78.46	53.93
500	92.73	92.18
average	57.13	43.57
ED_{50}(μg/ml)	45.00	68.00

[1]*4,4'-dimethoxymethyl vulpinate*, [2] *4',4'-dimethyoxyvulpinic acid*

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