International Journal of Agricultural Technology 2022Vol. 18(2):489-502
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Potential use of extracts and active constituent from *Desmodium sequax* to control fungal plant diseases

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Do, T. H. T., Pham, T. H., Pham, G. V., Vo, K. A., Nguyen, T. T. T., Vu, D. H., Nguyen, X. C., Vu, V. H., Nghiem, D. T., Choi, G. J., Nguyen, N. H., Nguyen, H. T. Trinh, X. H. and Le Dang, Q. (2022). Potential use of extracts and active constituent from *Desmodium sequax* to control fungal plant diseases. International Journal of Agricultural Technology 18(2):489-502.

Abstract The methanol extract of the whole plant of *Desmodium sequax* was found to suppress efficiently fungal plant diseases. Especially, it remarkably controlled rice blast (RCB), tomato grey mold (TGM), and red pepper anthracnose (PAN) *in vivo*. Out of separated fractions, Hex-soluble fraction showed potent control values against RCB (93.75%), TGM (87.5%), wheat leaf rust (WLR) (80%) and PAN (95%) at 3000 μg/mL, respectively. Through bioassay-guided fractionation, compound **1** was isolated from the Hex-soluble fraction and this compound was identified as lupeol on the basis of NMR and ESI-MS data analysis. The *in vivo* and *in vitro* antifungal activity of **1** was evaluated against various fungal phytopathogens. Lupeol displayed a moderate inhibition against the mycelial growths of *Rhizoctonia solani*, *Colletotrichum orbiculare*, and *Magnaporthe oryzae in vitro*. Besides, *in vivo* antifungal efficacy of **1** against TGM and tomato late blight (TLB) over the concentration range of 125–500 μg/mL was described for the first time. The content of lupeol (2.94%) in Hex-soluble fraction was quantified by HPLC analysis. Our study demonstrated that *D. sequax* is a promising plant resource, contains lupeol as an antifungal constituent, and could be used to control fungal plant pathogens.

Keywords: *Desmodium sequax*, Antifungal activity, Lupeol, Triterpenoid, Plant diseases

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Introduction

Phytopathogenic fungi cause many serious crop damages and losses in yields. In past conventional agriculture, to protect the crops from fungal pathogens, the farmers have used chemical synthetic fungicides commonly. Nevertheless, the overuse of these synthetic fungicides results in environmental pollution, toxic effects on humans and non-target organisms, and the development of fungicide resistance. In current organic agriculture, to alternate toxic fungicides or reduce the use of synthetic fungicides and the resistance of fungal pathogens, biological methods have been employed (Han *et al*., 2018; Song *et al*., 2020). Among the biological methods used, botanical fungicides have been researched and applied in the control of fungal pathogens because they contain many different phytochemical constituents and display potential control efficacy against various plant diseases (Pham *et al*., 2020; Bae *et al*., 2021).

The plant *Desmodium sequax* Wall. is a shrub and belongs to the legume family (Fabaceae), which distributes mainly in India, the Himalayan mountains, Southeast China, Laos, Vietnam, and Malaysia. In Vietnam, the plant grows wildly at elevations from 200–1600 m, along rivers, forest edges, lawns, and open ground. This species is found to distribute in the Northern parts of Vietnam from Lai Chau, Lao Cai to Thanh Hoa provinces.

D. sequax is also a medicinal plant that has been widely used for many different medical purposes for a long time. According to folk knowledge, the whole plant has been used for the cure of conjunctivitis and burn wounds. In China, the plant is often used to treat placental failure, eye pain, internal injury and bleeding. The stems of *D. sequax* have been used to treat pulmonary tuberculosis in traditional Chinese medicine. The roots have the effect of preventing leprosy, reducing damage, antiseptic, antitussive, preventing asthma, and digestion. In India, the root is used to treat diarrhea, chronic fever, cough, asthma, snakebite and scorpion stings. The seeds are used for the treatment of internal injuries and bleeding (Ma *et al*., 2011).

A previous study has also shown that *D. sequax* displays strong antioxidant activity and the main active ingredient with antioxidant properties is chlorogenic acid (Tsai *et al*., 2011). Zeba and Asif (1998) reported that *D. sequax* possesses flavonoids and pterocarpanes such as karanjin, lanceolatin-B, pongapin, 5’-methoxypongapin, kanujin and glabra-II. However, up to now, the studies on antifungal activity and crop protection property of the extracts and constituents from *D. sequax* have been not yet published.

In this paper, we reported the *in vivo* antifungal activity of organic-soluble fractions of *D. sequax* against six fungal plant diseases and the identification of lupeol as a bioactive constituent from the plant. Besides, *in vitro* and *in vivo* antifungal activities and quantitative analysis of lupeol in the most active fraction were described.

Materials and methods

Plant materials and fractionation

The aerial parts of *D. sequax* were collected in May 2020. The sample of this species was identified by one of the authors (D. T. Nghiem). A voucher specimen (DS 29520) has been deposited in Institute for Tropical Technology. The aerial parts of *D. sequax* was dried and ground into fine powder. The dried powder was extracted with methanol (3 times) at room temperature for 2 weeks. The methanol extracts were pooled and concentrated by a rotary evaporator to remove methanol under reduced pressure at below 50 ℃. The combined methanol extract was suspended in water and successively partitioned with *n*-hexane (Hex), dichloromethane (DCM), butanol (BuOH) to give organic solvent-soluble fractions and an aqueous layer, respectively.

Isolation and identification of bioactive compound from the aerial parts of Desmodium sequax

Isolation of constituents from D. sequax: the Hex-soluble fraction of this plant displayed a remarkable *in vivo* antifungal activity; therefore it was subjected to separate by silica gel column chromatography [Merk, 60 Å (particle size: 40–63 μm)] eluting with the mixtures of Hex/EtOAc by increasing EtOAc at the step of 5% (v/v) to obtain 7 fractions. Fraction 3 eluted at Hex/EtOAc (9/1, v/v) was chromatographed on a silica gel column, eluting with the mixtures of Hex/Ac (20/1 and 95/5, v/v) to yield the main fraction, which showed antifungal effects. The main active fraction was purified by silica gel column and eluted with a gradient of Hex/EtOAc (from 90/1 to 20/1, v/v) to give compound **1**.

Identification of constituents from D. sequax: The structure of the isolated compound was determined based on nuclear magnetic resonance (NMR) spectra, combined with a comparison with previously published reports. Besides, compound **1** was identified by comparing it with an authentic compound on TLC.

NMR spectra of the isolated compound were recorded on a Bruker Avance 500 FT-NMR spectrometer. The ^{1}H and ^{13}C-NMR spectral data of compound **1** were presented in Table 1.

Quantitative analysis of lupeol in MeOH extract and Hexane-soluble fraction of Desmodium sequax

The content of lupeol in the extracts of *D. sequax* was determined by high-performance liquid chromatography (HPLC) on an Agilent 1260 Infinity system (Agilent Technologies, USA) using an Agilent Eclipse XDB-C18 column (4.6 × 250 mm; 5 μm) with a mobile phase of 50% MeOH and 50% Acetonitrile (0-23 min), injection volume of 20 μL, and a flow rate of 1 mL/min. The extract and fraction, and lupeol samples were dissolved in MeOH to the appropriate concentration (1.25-500 μg/mL for the pure compound, 30000 μg/mL for the extract and fraction), mixed with vortex, then sonicated for 15 min, and finally filtered by 0.45 μm syringe filters before injection.

In vivo evaluation of antifungal activity of extract, organic solvent-soluble fractions, and lupeol against various plant diseases

The *in vivo* bioassay experiment was performed as the previously reported protocols (Han *et al*., 2018; Pham *et al*., 2020; Bae *et al*., 2021). To examine the efficacy of the test materials (plant extract, fractions and compound **1**) against plant diseases, extract and organic-soluble fractions (1000 and 3000 μg/mL) and pure compound (125, 250 and 500 μg/mL) were dissolved in MeOH or DMSO to form the stock solutions. These stock solutions were diluted with water containing 0.025% Tween 20 to reach a concentration of 2% for MeOH and 5% for DMSO to spray onto plants. The solutions of DMSO (5%) and MeOH (2%) with 0.025% Tween 20 were used as negative controls, respectively. The tested plant diseases such as rice blast (RCB) caused by *M. oryzae*, tomato gray mold (TGM) caused by *B. cinerea*, tomato late blight (TLB) caused by *P. infestans*, wheat leaf rust (WLR) caused by *P. triticina*, barley powdery mildew (BPM) caused by *B. graminis* f. sp. *hordei*, and pepper anthracnose (PAN) caused by *C. coccodes* were employed in the *in vivo* bioassay. The test seedlings were inoculated with a spore suspension of each phytopathogenic fungus and incubated at 25 ℃ for 5 days. All experiments were conducted twice with three estimates for each treatment. The disease control efficacy was experessed as control percentage compared with the negative controls (Han *et al*., 2018; Pham *et al*., 2020). Chemical fungicides such as blasticidin-S for RCB, fludioxonil for TGM,

dimethomorph for TLB, flusilazole for WLR, benomyl for BPM, and dithianon for PAN were used as positive controls.

In vitro evaluation of antifungal activity of lupeol against phytopathogenic fungi

By using the poisoned food technique, compound **1** (lupeol) was tested at concentrations of 100 and 250 µg/mL by dissolving in DMSO to form the stock solutions. The solutions were diluted with melt PDA containing 0.025% Tween 20 to reach a concentration of 2% DMSO and poured into Petri dishes (4 cm diameter). The phytopathogenic fungal strains such as *Rhizoctonia solani* (RS), *Fusarium oxysporum* (FO), *Colletotrichum orbiculare* (CO), *Magnaporthe oryzae* K (MO-K), and *Magnaporthe oryzae* BV (MO-BV) were used in the mycelial growth inhibition tests. Each Petri dish was inoculated with a mycelial plug in the center and incubated at 20–25 ℃ for 2–7 days. DMSO (2%) was used as a negative control. Score 250EC (a commercial fungicide containing 250 g/L of difenoconazole, Syngenta Vietnam Co. Ltd.) was used as a positive control in the test against *M. oryzae* and *C. orbiculare* at 250 µg/mL. Each treatment contained two replicates and was conducted twice (Tan *et al*., 2021).

Results

In vivo antifungal activity of the extract and organic solvent-soluble fractions

In vivo bioassay: The results of the *in vivo* antifungal activity of the extract, organic solvent-soluble fractions are shown in Table 1. At a concentration of 3000 µg/mL, the MeOH extract showed remarkable efficacy in controlling of RCB (62.5 %), TGM (60 %), WLR (66.7 %) and PAN (95%). Hex and DCM-soluble fractions suppressed the development of RCB, WLR and PAN with control efficacies ranging from 53.3 to 95%. Hex-soluble fraction was active against TGM with suppressions of 62.5 (at 1000 µg/mL) and 87.5% (at 3000 µg/mL). At 3000 µg/mL, EtOAc-soluble fraction inhibited the development of RCB (56.3 %), WLR (53.3 %) and PAN (92.5%). The BuOH-soluble fraction was virtually inactive against RCB, TGM, TLB, and BPM. The aqueous layer also did not affect the development of all test plant diseases.

Table 1. *In vivo* antifungal efficacy of methanol extract and organic solvent-soluble fractions from *Desmodium sequax* against six fungal plant diseases

Extract	Conc. (μg/mL)	Control efficacy (%)					
		RCB[a]	TGM	TLB	WLR	BPM	PAN
MeOH	1000	43.8[b]±6.3d	50±0d	6.3±6.3c	26.7±6.7e	0±0	80±10ab
	3000	62.5±0c	60±10c	37.5±0b	66.7±0b	33.3±0	95±0a
Hex	1000	75±0b	62.5±0b	6.3± 6.3c	53.3 ± 0c	0±0	75±15abc
	3000	93.8±6.3a	87.5±0a	18.8±6.3c	80 ± 0a	0±0	95±0a
DCM	1000	50±0d	0±0	0±0	33.3 ± 0d	0±0	65±5b
	3000	87.5±0a	20±0	68.8±6.3a	80 ± 0a	0±0	87.5±7.5a
EA	1000	0±0b	0±0	0±0	3.3±3.3f	0±0	40±20bc
	3000	56.3±6.3d	0±0	0±0	53.3 ± 0c	0±0	92.5±2.5a
BuOH	1000	0±0	0±0	0±0	0±0	0±0	0±0
	3000	0±0	0±0	0±0	33.3±0d	0±0	50±0c
W	1000	25±0e	0±0	0±0	0±0	0±0	0±0
	3000	25±0e	0±0	0±0	0±0	0±0	0±0

[a]RCB: rice blast; TGM: tomato grey mould; TLB: tomato late blight; WLR: wheat leaf rust; BPM: barley powdery mildew; PAN: red pepper anthracnose. The seedlings were inoculated with spores or a mycelial suspension of the test fungi 1 day after the chemical solution was sprayed to run-off on the leaves. MeOH: methanol extract; Hex: *n*-hexane-soluble fraction; DCM: dichloromethane-soluble fraction; EA: ethyl acetate-soluble fraction; BuOH: butanol-soluble fraction; W: aqueous layer. Disease ratings were measured 3-7 days after inoculation. [b]Each value represents the mean (standard derivation) of two runs with three replicates each. The means followed by the same letter in each column were not significant different ($P<0.05$) according to Duncan's multiple range test.

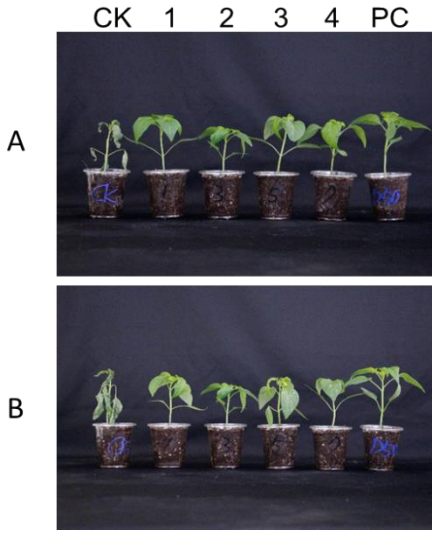

Figure 1. The *in vivo* antifungal activity of methanol extract and and organic solvent-soluble fractions from *Desmodium sequax* against anthracnose caused by *Collectotrichum coccodes* in red pepper plants. **A**: 1st experiment, **B**: 2nd experiment, **CK**: Negative control, **1**: MeOH extract; **2**: Hex-soluble fraction; **3**: DCM-soluble fraction; **4**: EA-soluble fraction. **PC**: positive control (dithanol 50 μg/mL)

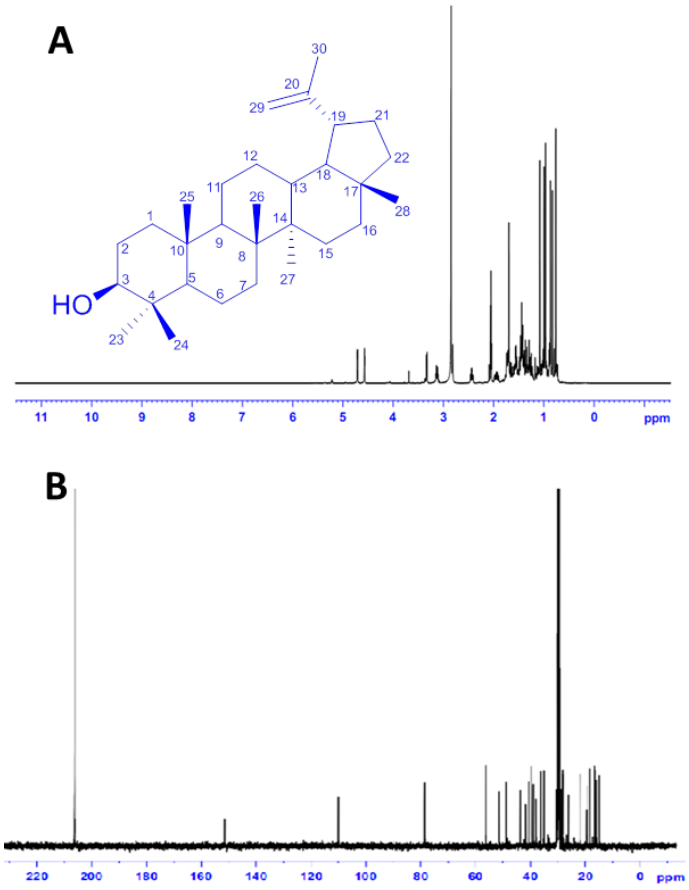

Figure 2. Chemical structure and NMR spectra of compound **1**. **A**: ^{1}H-NMR spectrum, **B**: ^{13}C-NMR spectrum. The ^{1}H and ^{13}C-NMR spectra of compound **1** were recorded in acetone-d_6 at 500 MHz and 125 MHz, respectively

Identification, quantification and in vitro antifungal activity evaluation of isolated compound from Desmodium sequax

Through antifungal bioassay-guided fractionation, compound **1** was isolated as a white powder. The ^{1}H-NMR spectrum (500 MHz, acetone-d_6) and HSQC spectrum of **1** showed 7 singlet signals of methyl groups at position δ_H ranging from 0.745 to 1.694 ppm. In the low-field region, there are proton signals of exo-olefin groups at δ_H 4.708 (m, H-29a) and 4.567 (m, H-29b). A double doublet of an oxygenate carbon at δ_H 3.13 (dd, J=11.0, 4.5 Hz, H-3α) is assigned for a proton at the C_3-OH in the structure of **1**.

The ^{13}C-NMR and DEPT spectra of **1** showed a signal pattern of 30 carbons, in which the signals of seven -CH_3 groups, eleven -CH_2 groups, six -CH groups and six quaternary carbons were observed. The C-3 hydroxyl group appears at δ_C 78.57 ppm, while the olefinic carbons occur at δ_C 151.50 and 109.95. Combining data from ^{1}H-NMR, ^{13}C-NMR spectra of **1** and comparing with spectral data in the previous report of Pham *et al.* (2017) led to the confirmation of **1** as lupeol (Table 2).

Table 2. Comparison of ^{1}H and ^{13}C-NMR spectra of compound **1** and lupeol (Pham *et al.* 2017)

No	Compound 1 (acetone-d_6)		Lupeol (chloroform-*d*) (Pham *et al.* 2017)	
	δ_c (ppm)	δ_H (ppm)	δ_c (ppm)	δ_H (ppm)
1	39.60		38.74	
2	28.56		27.44	
3	78.57	3.13 (dd; *J*=4.5; 11.0 Hz)	79.02	3.19 (dd; *J*=4.5; 11.0 Hz)
4	39.57		38.87	
5	56.29	0.761 (d; *J*=11.0 Hz)	55.34	0.68 (d; *J*=10.0 Hz)
6	21.66		18.34	
7	35.14		34.32	
8	41.65		40.86	
9	51.33		50.48	
10	37.93		37.20	
11	20.9		20.95	
12	28.27		25.19	
13	39.0		38.09	
14	43.56		42.86	
15	29.34		27.47	
16	36.27		35.61	
17	16.07		43.01	
18	49.05	0.965 (1H; t)	48.34	
19	48.80	2.932 (1H; dt; *J*=4.5;11.0 Hz)	48.00	2.37 (1H; dt; *J*=6.0; 11.0 Hz)
20	150.8		150.96	
21	30.51	1.958 (2H; m)	29.88	1.93 (2H; m)
22	40.62		40.02	
23	30.26	0.993 (s)	28.00	0.97 (s)
24	16.61	1.079 (s)	15.36	1.03 (s)
25	19.09	0.827 (s)	16.12	0.83 (s)
26	18.31	0.761 (s)	15.99	0.76 (s)
27	16.45	0.950 (s)	14.56	0.95 (s)
28	19.51	0.745 (s)	18.09	0.79 (s)
29	109.95	4.567 (d. *J*= 1.5 Hz) and 4.708 (m)	109.31	4.69 (d.; *J*= 2.5 Hz) and 4.57 (m)
30	26.03	1.694 (s)	19.32	1.68 (s)

Based on HPLC analysis, specifically, the correlation of peak area and concentration of standard substances, a standard calibration curve of lupeol in extracts with R^2 = 0.9966 was determined (Figure 4). HPLC analysis showed that lupeol occurred on the HPLC chromatogram of the MeOH extract. It was noteworthy that the Hex-soluble fraction contains a high content of lupeol up to 2.94% (Figures 3 and 4).

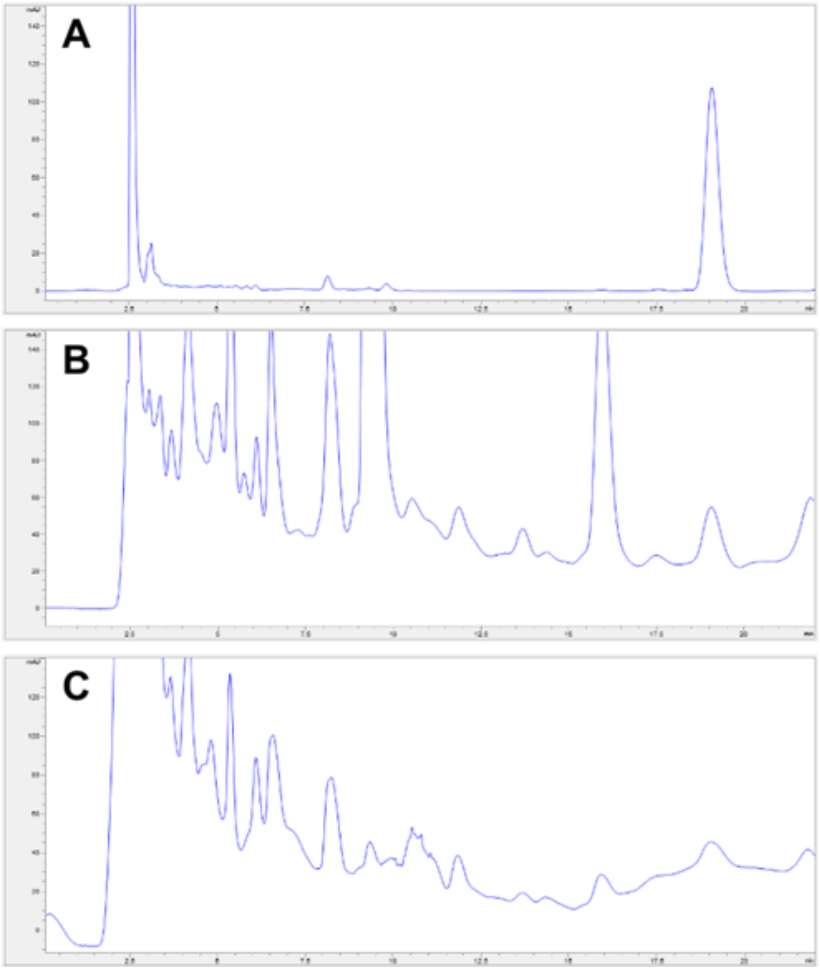

Figure 3. HPLC chromatogram of lupeol (**A**), Hex-soluble fraction from *Desmodium sequax* (**B**) and MeOH extract of *Desmodium sequax* (**C**). HPLC analysis was run on an Agilent 1260 Infinity system (Agilent Technologies, USA) using an Agilent Eclipse XDB-C18 column (4.6 × 250 mm; 5 µm) with a mobile phase of 50% MeOH and 50% acetonitrile

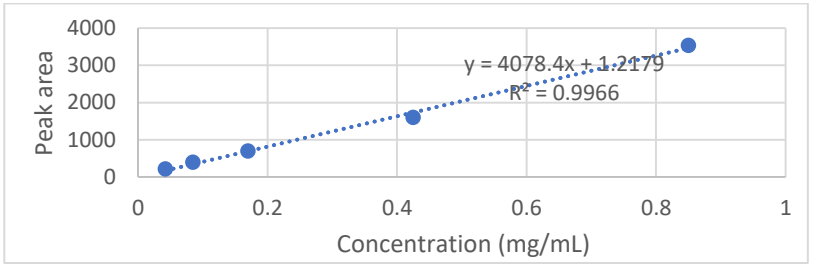

Figure 4. Lupeol standard calibration curve. HPLC analysis was run on an Agilent 1260 Infinity system (Agilent Technologies, USA) using an Agilent Eclipse XDB-C18 column (4.6 × 250 mm; 5 µm) with a mobile phase of 50% MeOH and 50% acetonitrile

In vitro and in vivo antifungal activity of lupeol against various fungal plant pathogens

Compound **1** was isolated from the Hex-soluble fraction by column chromatography. As for *in vitro* antifungal activity of compound **1**, the isolated material was tested at concentrations of 100 and 250 µg/mL based on the poisoned food technique. The bioassay results showed inhibitory efficacies from 17% to 39% against all tested fungal strains. Lupeol possessed moderate inhibition against RS, CO and MO strains (Table 3 and Figure 5); however, it exhibited a relatively weak inhibition for FO (17.03 ± 3.28% at 250 µg/mL).

The *in vivo* bioassay results showed that lupeol significantly suppressed the disease development of TCM (50 %) and TLB (48 %) at 500 µg/mL. The other test plant diseases were not affected by lupeol at concentrations from 125 to 500 µg/mL (Table 4).

Table 3. *In vitro* inhibition of compound **1** against the mycelial growths of five phytopathogenic fungi

Fungi	Concentration (ppm)	Control value (%)
RS	100	36.37 ± 6.41ab
	250	38.50 ± 1.01a
FO	100	11.63 ± 1.57cd
	250	17.03 ± 3.28c
CO	100	30.45 ± 6.23abc
	250	36.59 ± 3.02ab
MO-K	100	29.67 ± 7.27abcd
	250	32.23 ± 7.83abc
MO-BV	100	28.32 ± 3.52b
	250	35.26 ± 4.26ab

RS: *Rhizoctonia solani*; **FO**: *Fusarium oxysporum*; **CO**: *Colletotrichum orbiculare*; **MO-K**: *Magnaporthe oryzae* K; **MO-BV**: *Magnaporthe oryzae* BV. The means followed by the same letter in each column were not significant different ($P < 0.05$) according to Duncan's multiple range test.

Table 4. *In vitro* antifungal efficacy of compound **1** derived from *Desmodium sequax* against six fungal plant diseases

Conc. (µg/ml)	RCB[a]	TGM	TLB	WLR	BPM	PAN
500	0	50[b] ±0a	48.3 ±1.7a	0	0	0
250	0	50 ±0a	35.4 ±2.1b	0	0	0
125	0	50 ±0a	3.3 ±3.3c	0	0	0

[a]RCB: rice blast; TGM: tomato grey mould; TLB: tomato late blight; WLR: wheat leaf rust; BPM: barley powdery mildew; PAN: red pepper anthracnose. The seedlings were inoculated with spores or a mycelial suspension of the test fungi 1 day after the chemical solution was sprayed to run-off on the leaves. Disease ratings were measured 3-7 days after inoculation. [b]Each value represents the mean (standard derivation) of two runs with three replicates each. The means followed by the same letter in each column were not significantly different ($P < 0.05$) according to Duncan's multiple range test.

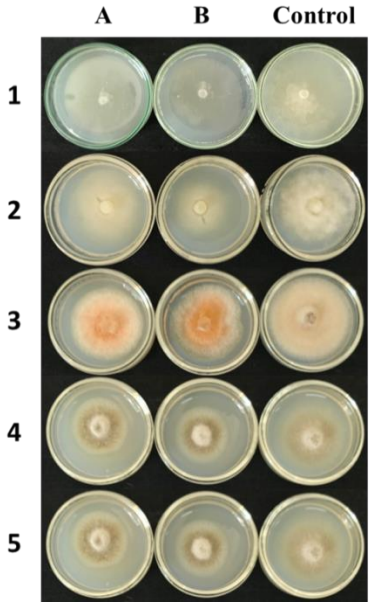

Figure 5. *In vitro* inhibition of compound **1** against the mycelial growths of five phytopathogenic fungi. Lane **A**: 250 µg/mL; Lane **B**: 100 µg/mL; Control: untreated dish. **1**: *Rhizoctonia solani*; **2**: *Fusarium oxysporum*; **3**: *Colletotrichum orbiculare*; **4**: *Magnaporthe oryzae* K; **5**: *Magnaporthe oryzae* BV

Discussion

Ongoing study of antifungal plants in Vietnam, the methanol extract of legume plant *D. sequax* exerted the considerable *in vivo* control efficacy against RCB, WLR and PAN at 3000 µg/mL. The Hex and DCM-soluble fractions derived from the methanol extract of *D. sequax* also strongly reduced the development of RCB, WLR and PAN at the same dose. Especially, these test

materials highly suppressed the development of anthracnose on red paper seedlings with control values in the range of 87.5% to 95% at a concentration of 3000 µg/mL (Figure 2). Even though, at 1000 µg/mL, methanol extract and Hex-soluble fraction still suppressed PAN with control values of 80 and 75 %, respectively. The *in vivo* antifungal properties of various plants against fungal plant diseases were described previously. Bae *et al*. (2021) and Han *et al*. (2018) reported the *in vivo* antifungal activity of *Platycladus orientalis* and *Curcuma zedoaria* under greenhouse conditions. At 3000 µg/mL, methanol extract of *C. zedoaria* suppressed WLR (90% of inhibition); however, it caused a few inhibition for TGM and TLB, and did not affect the development of BPM *in vivo* (Han *et al*., 2018). The methanol extract (at 3000 µg/mL) and EtOAc fraction (at 2000 µg/mL) of *P. orientalis* were found to be active against RCB (90 and 75%) and WLR (90 and 67%), respectively. However, the extract and fraction displayed little to no antifungal activity against TGM, TLB and BPM and PAN (Bae *et al*., 2021). Compared with the methanol extract of *D. sequax*, those from *P. orientalis* and *C. zedoaria* exhibited superior control efficacy against WLR. In contrast, all extracts and fractions from *P. orientalis* and *C. zedoaria* have weak to no inhibition against TGM, TLB and PAN (Han *et al*., 2018; Bae *et al*., 2021). The hexane fraction of *C. zedoaria* strongly inhibited WLR and its active constituents have been identified. The phytochemical study of the two plants showed that they contain antifungal terpenoids; *C. zedoaria* contains sesquiterpene lactone and curcumin as antifungal constituents; and *P. orientalis* has antifungal diterpenes such as labdanes and isopimaranes (Han *et al*., 2018; Bae *et al*., 2021). Similarly, Hex-soluble fraction of *D. sequax* exhibited not only inhibition against WLR but also an excellent control efficacy against RCB, TGM, and PAN. This *in vivo* antifungal activity was strong and wider-spectrum compared to those of *P. orientalis* and *C. zedoaria* previously reported. By antifungal bioassay-guided isolation, the main active compound of the Hex-soluble fraction of *D. sequax* was identified to be lupeol, a pentacyclic triterpenoid that occurs in a variety of plant species (Figure 2A).

Some plant triterpenoid aglycones and saponins were reported to possess antifungal activity against phytopathogenic fungi. Saponins alliospirosides A and B isolated from shallot (*Allium cepa*) were found to inhibit the mycelial growth of *C. gloeosporioides* at 100 ppm *in vitro* and alliospiroside A suppressed strawberry anthracnose at concentrations ranging from 50–500 ppm *in vivo* (Teshima *et al.*, 2013). The bulbs of *Allium cepa* also contain ceposides A, B, and C that inhibited the mycelial growth of *Botrytis cinerea* and *Trichoderma atroviride* at concentrations of 10, 50 and 200 ppm (Lanzotti *et al*., 2012a). A study by Lanzotti *et al*. (2012b) also reported the *in vitro* antifungal

activity of seven saponins from *Allium sativum* against *B. cinerea* and *T. atroviride.*

In previous studies, lupeol was found in *Lepisanthes rubiginosa* and the root bark of *Euclea natalensis* (Lall *et al*., 2013; Pham *et al*., 2017). Lupeol is known to have anti-inflammatory and anti-cancer properties, arthritis, diabetes, heart disease, kidney and liver toxicity. Of these effects, the anti-inflammatory and anti-cancer effects are the most notable. Similar to our results, Muhammad *et al.* (2013) showed that at a concentration of 2.5–10 mg/mL lupeol inhibited *Fusarium solani* with zones of inhibitions from 20–28 mm. In addition, at a concentration range of 0.01–0.1 mg/mL, lupeol was effective against *Cladosporium cladosporioides* and *Phytophthora* sp. with inhibition zones from 10–12 mm *in vitro* (Lall *et al*., 2013). The previous results of Lall *et al*. (2013) and Muhammad *et al.* (2013) indirectly supported our finding of antifungal activity of lupeol against phytopathogenic fungi. However, in our study, lupeol was proved to possess *in vivo* antifungal potential of lupeol against TGM and TLB and *in vitro* inhibition of *R. solani*, *F. oxysporum*, *C. orbiculare*, and *M. oryzae* strains for the first time. This evidence directly confirmed the suppression of TGM by Hex-soluble fraction of *D. sequax* is due to the presence of lupeol (in high content) and other active constituents therefrom. In the future, the researches of the mode of action of *D. sequax*'s extract, fractions and lupeol against various phytopathogenic fungi and relevant formulations are necessary to improve the effectiveness of these botanical materials for controlling these plant diseases.

This study demonstrates for the first time the *in vitro* and *in vivo* antifungal activity of *D. sequax* and the identification of a main active constituent, lupeol, therefrom. The MeOH extract, Hex and DCM-soluble fractions remarkably suppressed the development of RCB, TGM, TLB and PAN. Lupeol was proved to be an active constituent in the Hex-soluble fraction with a content of 2.94% based on quantitative analysis in HPLC. The substance exhibited a moderate inhibition for the mycelial growth of *R. solani*, *C. orbiculare* and *M. oryzae in vitro*. Besides, it significantly suppressed the development of TGM and TLB *in vivo*. Our results confirmed the antifungal property of *D. sequax* and its active constituent and also suggested that MeOH extract and Hex and DCM-soluble fractions of *D. sequax* could be used to develop a novel botanical fungicide for controlling the fungal plant diseases.

Acknowledgements

The author would like to Thanh Huong Nguyen and Thoa Thi Vu for technical assistance. This research was supported by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant no. 106.03-2019.17.

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(Received: 13 October 2021, accepted: 20 February 2022)