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Extraction Separation and Antifungal Activities of *Cynanchum komarovii* Al. Iljinski

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Abstract

This study researched the antifungal activity of *Cynanchum komarovii* Al. Iljinski extract prepared using ethanol solvent. The result showed that ethanol extract (50 mg·mL^{-1}) had inhibitory effect against *Fusarium semitectum* Berk. & Rav., *Rhizoctonia solani* Kühn, *Setosphaeria turcica*, *Botrytis cinerea* and *Valsa mali*, the inhibition ratio ranged from 44.0 to 61.2%. The ethanol extract was extracted successively with petroleum ether, ethyl acetate and n-butyl alcohol, the n-butyl alcohol extract (50 mg·mL^{-1}) exhibited significant antifungal activity, the inhibition ratio ranged from 76.4 to 100.0%, particularly, the inhibition ratio was 100.0% against *F. semitectum.* Compound ③ was isolated from n-butyl alcohol extract by column chromatography and characterized as 7-demethoxylophorine by comparing spectral data and physical properties. 7-Demethoxylophorine had significant inhibition against *F. semitectum,* with EC_{50} values of 4.6637 μg·mL^{-1}, and that could be used as a new bio-fungicide against *F. semitectum* for further study.

Keywords

***Cynanchum komarovii*, *Fusarium semitectum*, Antifungal activity, Column chromatography.**

1. INTRODUCTION

Cynanchum komarovii Al. Iljinski is one of the indicators of serious sandy desertification of the land, is a kind of poisonous psammophytes belonging to the family of Asclepiadaceae, widely distributed in the northwest part of China. *C. komarovii* is a perennial herb, it not only has insecticidal, antibacterial and antiviral activities, is used as a Chinese herbal medicine to cure fever, diarrhea, pain, inflammation, tumor, cough, asthma and cholecystitis [1-4]. Several alkaloids were isolated from the *C. komarovii*, such as 2,3-dimethoxy-6-(3-oxo-butyl)-7,9,10,11,11a,12-hexahydrobenzo[*f*]pyrrolo[*1,2-b*]isoquinoline, 7-demethoxytylophorine and 7-demethoxytylophorine N-oxide had antiviral activities against tobacco mosaic virus [1]. The antifungal proteins CkTLP, CkChn134 and CkPGIP1 were isolated from *C. komarovii*, which are good candidate proteins or genes for contributing to the development of disease-resistant crops [5-6]. The gross alkaloids from *C. komarovii* have insecticidal activity against *Spodoptera litura*, the LC_{50} value was 2669.88 mg·L^{-1} [7]. Two alkaloids 6-hydroxyl-2,3-dimethoxy phenanthroindolizidine and 7-demethoxytylophorine were isolated from the *C. komarovii*, which had insecticidal, antifeedant and growth inhibitory effects against *Plutella xylostella* [8]. Five C_{21} steroidal glycosides isolated from *C. komarovii* showed potent inhibitory activities against human leukemia cell lines [9-10]. Tylophora alkaloids, isolated from *C. komarovii*, offer

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a new skeleton for the development of anticoronavirus drug candidate, such as NK007(S,R), a tylophorine malate, displays high antiviral activity against SARSCoV-2 [11].

According to the above reports, the *C. komarovii* has insecticidal and antibacterial activities. The objective of this study was to identify the antifungal activity of *C. komarovii* and to track and isolate the active compounds, which laid a theoretical foundation for the research and development of botanical fungicides.

2. EXPERIMENTAL

Plant Materials and Extraction. The whole plants of *C. komarovii* were collected from Yulin in Shaanxi, China, in June 2021. The botanical identification of the collected materials was done by senior experimentalist Gang Li, College of Life and Science, Yulin University. A voucher specimen has been deposited in the herbarium of the College of Life and Science, Yulin University. Plant materials were dried in the shade at room temperature for 15 days, then pulverized in a mill and stored in a sterile air-tight container. For preparation of ethanol extraction, 5 kg sample of *C. komarovii* was added to 50 L industrial alcohol and the mixture was stirred by a magnetic stirrer during 3 days. Then, the extract was filtered from filter paper. The extract was concentrated under reduced pressure by a vacuum rotary evaporator (SIBATA SRE-M3, Japan) to yield an ethanol extract of *C. komarovii*. The ethanol extract was suspended in water and extracted successively with petroleum ether, ethyl acetate and n-butyl alcohol, respectively.

Microorganisms and Medium. The strains of *Fusarium semitectum* Berk. & Rav., *Rhizoctonia solani* Kühn, *Setosphaeria turcica*, *Botrytis cinerea* and *Valsa mali* were provided by the the Plant Protection Laboratory, College of Life Sciences, Yulin university. The strains were cultured on PDA (potato 200 g, sugar 20 g, and agar 17 g in 1 L of water) medium at 25 °C for 3-5 d.

Antifungal Activity Assay. The effect of different solvent extracts against *F. semitectum*, *R. solani*, *S. turcica*, *B. cinerea* and *V. mali* were measured according to the method described by Wang *et al.* [12-13]. The extract of ethanol, petroleum ether, ethyl acetate, n-butyl alcohol and water was dissolved in ethanol, petroleum ether, ethyl acetate, n-butyl alcohol and water, respectively. The concentrations of ethanol, petroleum ether, ethyl acetate, n-butyl alcohol and water never exceeded 3% of the testing solution. This concentration of solvent above did not affect the different stages of *F. semitectum*, *R. solani*, *S. turcica*, *B. cinerea* and *V. mali* development. Controls always contained the same solvent concentration as the test samples. Solutions of extracts were diluted and added to PDA when the PDA had cooled to approximately 50ºC. Agar punches (5 mm diameter) were removed from the edge of a 5-day-old colony of strains above cultured on PDA and placed face up in the center of the Petri dish (9 cm diameter) containing different extracts (50 $mg \cdot mL^{-1}$). Blank and solvent controls were prepared in parallel. The experiment was repeated three times, and each treatment was with three replicate plates. We calculated the inhibition rate according to the method described by Wang *et al.* [12-13].

Isolation and Antifungal Activity Tracing of N-butanol Extract. The N-butanol extract was subjected to and eluted with $CHCl_3/CH_3OH$ (50:1, 40:1, 30:1, 20:1, 10:1, 1:1, 8:1, 5:1, 1:10, 1:20, 1:30, 1:40, 1:50) to give six fractions. The antifungal activity was determined, showed that the second fraction had significant antifungal effect. The second fraction was repeatedly subjected to column chromatography over silica gel to afford compounds ①, ②, ③ and ④. The effect of different compounds against *F. semitectum* were measured according to the method described by 2.3. Agar punches were removed from the edge of a 5-day-old colony of strains above cultured on PDA and placed face up in the center of the Petri dish containing compounds ①, ②, ③ and ④ (5, 10, 20, 40, 80 and 160 $\mu g \cdot mL^{-1}$), respectively.

Spectroscopic Determination of Compound ③. Nuclear magnetic resonance (NMR) spectra were acquired on Bruker Avance NEO 600 (Germany) spectrometers with CD_3OD as

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solvent. High resolution mass spectrum (HRMS) were obtained on a Aglient 7250 (America) & JEOL-JMS-T100LP AccuTOF (Japan) instrument. Liquid chromatography-mass spectrometry (LCMS) were measured on a SHIMADZU-LC20A & LCMS-8050 (Japan) instrument. The structure of compound ③ was determined based on NMR spectroscopy, HRMS and LCMS elemental analysis.

3. RESULTS AND DISCUSSION

Extraction Yields. The results showed that the total extraction rate of *C. komarovii* using industrial alcohol was 20.3%, which obtained ethanol extract 1015.2 g (Fig. 1A). The ethanol extract was suspended in water and extracted successively with petroleum ether, ethyl acetate and n-butyl alcohol, to give petroleum ether 320.7 g, ethyl acetate 23.8 g, n-butyl alcohol 217.1 g and water 286.7 g, respectively (Fig. 1B).

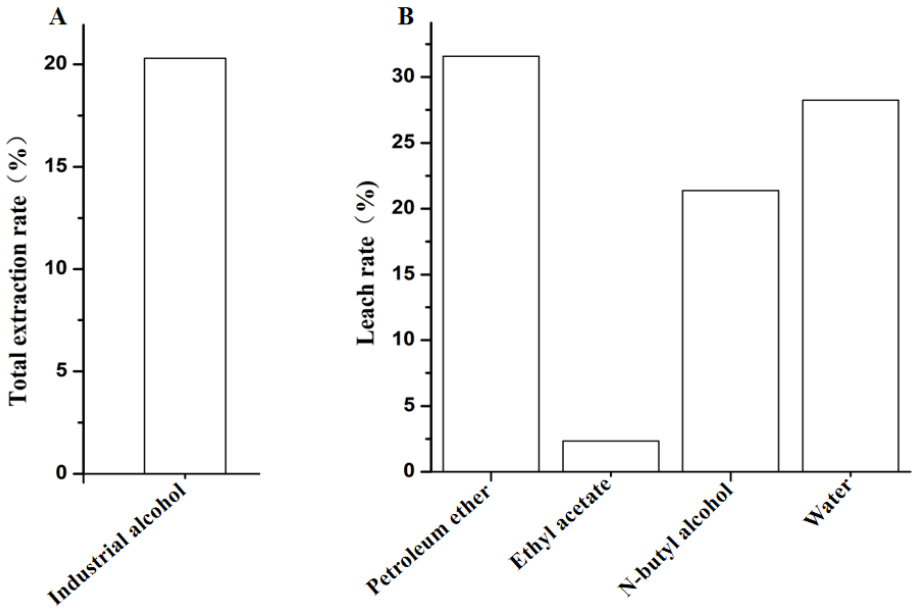

Figure 1. The extraction rate of *C. komarovii*. A. The total extraction rate of *C. komarovii* using industrial alcohol. B. The ethanol extract was extracted with petroleum ether, ethyl acetate and n-butyl alcohol and water, respectively.

Antifungal Activity of Different Extracts. The strains of *F. semitectum, R. solani*, *S. turcica*, *B. cinerea* and *V. mali* were tested against five different extracts (50 mg·mL^{-1}) from *C. komarovii* (Fig. 2). The result showed that ethanol extract, which is the total extract, had inhibitory effect against five strains, the inhibition ratio ranged from 44.0 to 61.2%. Petroleum ether extract had inhibitory effect against five strains, the inhibition ratio ranged from 46.5 to 60.5%. Ethyl acetate extract had inhibitory effect against five strains, the inhibition ratio ranged from 52.8 to 72.4%. However, among all the tested extracts, n-butyl alcohol extract exhibited significant antifungal activity, the inhibition ratio ranged from 76.4 to 100.0%, particularly, the inhibition ratio was 100.0% against *F. semitectum.* Whereas, the weakest activity shown by the water extract, the inhibition ratio ranged from 22.8 to 34.9%. Studies have shown that the alkaloids were main active ingredient of *C. komarovii*, the alkaloids have insecticidal, antibacterial and antiviral activities [14-17]. The result showed that among all the tested extracts, nbutyl alcohol extract exhibited significant antifungal activity, therefore, we speculate that alkaloids are mainly concentrated in the n-butanol extract.

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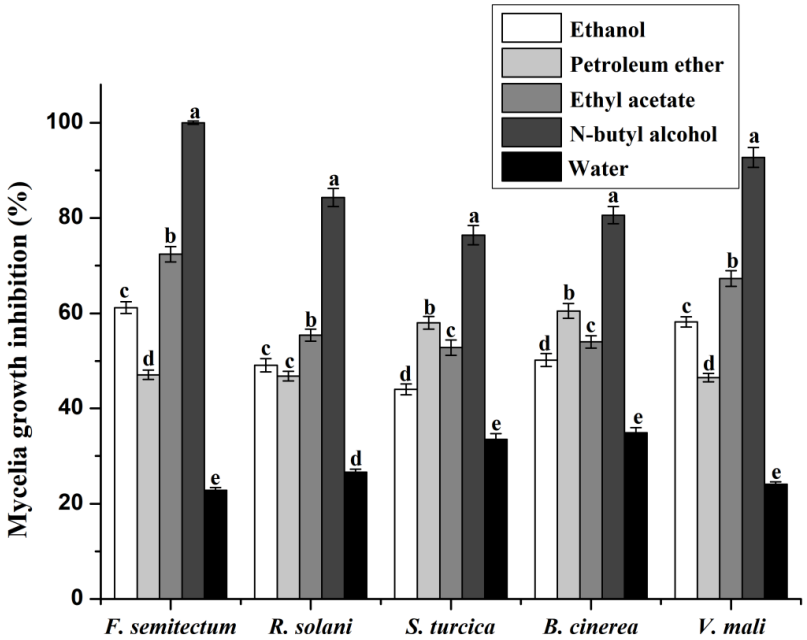

Figure 2. The effect of different extracts (50 mg·mL^{-1}) on the mycelial growth of *F. semitectum*, *R. solani*, *S. turcica*, *B. cinerea* and *V. mali.* Data represent the mean ± standard deviation (SD) of three independent experiments. In each row, different lower case letters indicate a significant difference ($P < 0.05$).

Antifungal Activity of Different Fractions from N-butyl Alcohol Extract. The strains of *F. semitectum, R. solani*, *S. turcica*, *B. cinerea* and *V. mali* were tested against six different fractions of n-butyl alcohol extract (100 μg·mL^{-1}) from *C. komarovii* (Table 1). From the table 1, we knew that the weakest activity showed by the first fraction of n-butyl alcohol extract against five strains, the inhibition ratio ranged from 6.4 to 10.1%. Nevertheless, among all the measured fractions of n-butyl alcohol extract, the second fractions demonstrated significant antifungal activity, the inhibition ratio ranged from 83.6 to 100.0%, particularly, the inhibition ratio was 100.0% against *F. semitectum.* The third and the fourth fractions of n-butyl alcohol extract had analogouslyve inhibitory effect against five strains, the inhibition ratio ranged from 54.7 to 75.9%. The fifth fraction of n-butyl alcohol extract had inhibitory effect against five strains, the inhibition ratio ranged from 16.9 to 29.3%. The sixth fraction of n-butyl alcohol extract had inhibitory effect against five strains, the inhibition ratio ranged from 50.7 to 62.3%. The solubility of alkaloids are related to the existence state of nitrogen atom, polarity size, number of functional groups and solvent, and most alkaloids dissolve easily in n-butanol solvents [18-19]. The results showed that alkaloids with significant antifungal activity were mainly concentrated in the second fraction.

Table 1. The effect of different fractions from n-butyl alcohol extract on the mycelial growth of *F. semitectum*, *R. solani*, *S. turcica*, *B. cinerea* and *V. mali*

Fractions (100 μg·mL^{-1})	***F. semitectum***	***R. solani***	***S. turcica***	***B. cinerea***	***V. mali***
	Inhibition(%), mean ± SD				
First	10.1 ± 0.11	6.5± 0.07	6.4 ± 0.05	8.2 ± 0.07	9.3 ± 0.08
Second	100.0 ± 0.11	86.5 ± 2.01	83.6 ± 1.98	92.4 ± 1.77	94.1 ± 1.64
Third	71.7 ± 1.07	61.4 ± 1.35	54.7 ± 1.14	63.5 ± 1.44	68.2 ± 1.59
Fourth	75.9 ± 1.25	59.3 ± 1.10	65.3 ± 1.27	61.8 ± 1.54	67.3 ± 1.19
Fifth	29.3 ± 0.41	25.4 ± 0.52	18.9 ± 0.12	16.9 ± 0.63	28.1 ± 0.47
Sixth	62.3 ± 1.29	50.7 ± 0.94	54.6 ± 1.05	58.1 ± 1.04	60.1 ± 1.17

Data represents the mean value of triplication.

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Antifungal Activity of Different Compounds from the Second Fractions. The strain of *F. semitectum* was tested against four different compounds of the second fractions of n-butyl alcohol extract from *C. komarovii* (Table 2). The result showed that the weakest activity exhibited by compounds ① and ④ against *F. semitectum*, with EC_{50} values of 120.0802 μg·mL^{-1} and 100.4090 μg·mL^{-1}. The compound ② had inhibitory effect against *F. semitectum*, with EC_{50} values of 67.2009 μg·mL^{-1}. However, the compound ③ had significant inhibition of *F.* semitectum, with EC_{50} values of 4.6637 μg·mL-1. The main compounds of total alkaloids from the C. komarovii were 7-demethoxylophorine, (13aR, 14R)-14-hydroxy-7-demethoxylophorine, (13aR, 14R)-14-hydroxy-7-demethoxylophorine N-oxide, desoxytylophorinin N-oxide, N-oxide-7-demethoxylophorine, tylophorinine, tylocrebrine, 6-hydroxy-2, 3-dimethoxy-fizoindolicidine and so on, and most of these alkaloids belong to pheniоindolicidine [1, 20]. The above alkaloids have certain physiological activity, and 7-demethoxylophorine is the main one with significant antifungal activity, which has been widely reported [21-23].

Table 2. The effect of four different compounds of the second fractions of n-butyl alcohol extract on the mycelial growth of *F. semitectum*

Compounds	Concentrations (μg·mL^{-1})	Inhibition(%), mean ± SD	Regression equation (Y =)	EC_{50} (μg·mL^{-1}) (95% confidence	r
①	10	8.02 ± 0.03	Y=1.2610X+2.3778	120.0802 (91.1130-158.2567)	0.9966
	20	15.80 ± 0.17			
	40	32.07 ± 0.94			
	80	44.34 ± 1.06			
	160	52.67 ± 1.23			
②	10	11.05 ± 0.06	Y=1.4641X+2.3246	67.2009 (63.4541-71.1691)	0.9990
	20	22.14 ± 0.31			
	40	39.26 ± 1.11			
	80	53.08 ± 1.39			
	160	71.37 ± 1.48			
③	5	57.78 ± 1.35	Y=4.2448+1.1625x	4.6637 (2.6785-8.3144)	0.9947
	10	63.89 ± 1.41			
	20	70.00 ± 1.67			
	40	87.22 ± 1.58			
	80	100.0 ± 1.74			
④	10	8.02 ± 0.07	Y=1.4216X+2.1542	100.4090 (0.0104-51.5739)	0.9993
	20	15.80 ± 0.24			
	40	29.31 ± 1.06			
	80	45.07 ± 1.35			
	160	61.64 ± 1.47			

Data represents the mean value of triplication. The EC_{50} was assessed based on log-transformation analysis.

Structural Identification of Compound ③. The compound ③ was characterized as 7-demethoxylophorine by comparing spectral data and physical properties with that of references [24-27]. The compound ③ molecular formula and molecular weight were $C_{23}H_{25}NO_3$ and 363.45, respectively. And the structural formula of the compound ③ is shown in Fig. 3. The results showed that 7-demethoxylophorine (50 μg·mL^{-1}) had a significant inhibitory effect against *Cladosporium cucumerium*, *Cercospora arachidicola*, *Alternaria solani* and *Physalospora*

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piricola, the inhibition ratio ranged from 70 to 100% [21]. 7-Demethoxylophorine had a significant inhibitory effect against *Penicillium digitatum*, the minimum inhibitory concentration was 1.5625 μg·mL^{-1}, and the minimum fungicidal concentration was 12.5 μg·mL^{-1}. However, further study revealed that 7-demethoxylophorine could damage cell membrane and mitochondrial membrane structure of *P. digitatum* [22-23]. Certainly, 7-demethoxylophorine also has antibacterial, antiviral and anticancer activities [28-31].

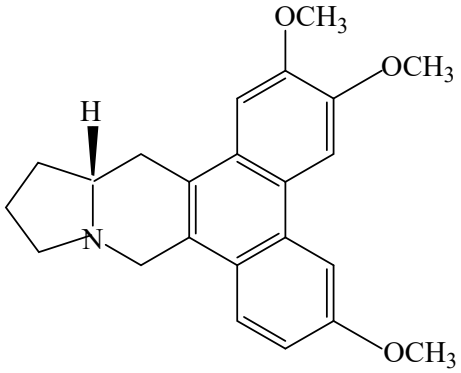

Figure 3. The structural formula of the compound ③

4. CONCLUSIONS

In this study, a antifungal active substance was successfully isolated from *C. komarovii*, which had significant inhibition against phytopathogen, particularly *Fusarium semitectum,* with EC_{50} values of 4.6637 μg·mL^{-1} and characterized as 7-demethoxylophorine. 7-Demethoxylophorine can be developed as a new bio-fungicide against *F. semitectum* for further study.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants performed by any of the authors.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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