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Antibacterial and antifungal activities of different polar extracts from *Anoectochilus roxburghii*

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Abstract: This study evaluates the antibacterial and antifungal activities of petroleum ether, acetic ether, n-butanol and aqueous extracts from *Anoectochilus roxburghii*. The *in vitro* antibacterial and antifungal effects against three bacterial strains (*Escherichia coli, Bacillus subtilis, Bacillus thuringiensis*) and three fungal species (*Exserohilum turcicum* (Pass.) Leonard et Suggs, *Botrytis cinerea Pers.*, *Fusahum graminearum Sehw.*) were assayed by the dilution and disc-diffusion methods. All of the polar extracts expressed dose-dependent antimicrobial activity against all tested microorganisms. The most active extract was aqueous extract, with a minimum inhibitory concentration below 0.625mg/ml in both bacteria and fungi. The results suggest that new chemical classes of natural antimicrobial substances (such as *A. roxiburghii* extracts) can be selectively exploited for the chemotherapy and control of infectious diseases.

Keywords: *Anoectochilus roxburghii*, antibacterial, antifungal, different polar extracts.

INTRODUCTION

Damage incurred by bacterial and fungal infection is a serious problem. For example, plant pathogenic fungi cause root rot in *Atractylodes macrocephala* Koidz. (Wu, 2005) and *A. lancea* (Zhou *et al*., 2014), which majorly reduces the yield and quality of the plants. Ginseng grown in Korea can be affected by diverse pathogenic fungi such as *Alternaria panax*, *Botrytis cinerea* and *Colletotrichum panacicola*, which cause many problematic diseases (Park *et al*., 2015). Following the massive use of antibiotics in recent decades, many bacteria and fungi have developed resistance to various antibiotics. Emerging antibiotic resistance seriously decreases the number of effective antimicrobial agents, and affects both agricultural production and public health. Therefore, finding new antibiotics with high efficacy and few adverse effects is strongly demanded. The antimicrobial properties of natural products are already being researched as alternative strategies to conventional antibiotics. Rashed *et al*. (2014) testified the antimicrobial effects of persimmon (*Diospyros virginiana* L.) extracts against eight bacterial strains and eight fungal species. Essential oil from *Genista quadriflora* Munby exhibits a marked antifungal activity against *Fusarium oxysporun* and significantly inhibits Gram-positive and Gram-negative bacteria (Kacem *et al*., 2016). Garlic, which is widely used in traditional medicine for its various therapeutic properties, also exhibits potent antimicrobial effects (Marchese *et al*., 2016). All of these natural products are potentially exploitable as new drugs against infectious diseases.

Anoectochilus roxburghii (Jin-Xian-Lian in Chinese, abbreviated as JXL) is a perennial herb of the Orchidaceae family that can treat diabetes, tumors, hyperliposis and hepatitis with few side effects. Because of its unique medicinal properties, JXL has been called ''the king of medicines'' in China (Liu *et al*., 2014). The herb is widely used in the health, food, cosmetic and flowers industries. Its reported chemical compounds include phenolic acids, polysaccharides, triterpenoids, and amino acids (Zheng *et al*., 2013; He *et al*., 2004). Previously, we confirmed the antioxidant activity of JXL in vivo, conferred by its phenolic compounds (Shao *et al*., 2014). However, the antibacterial or antifungal activities of JXL have been rarely reported. Therefore, the present study evaluates whether JXL extracts in different polar solvents inhibit diverse bacteria and fungi.

MATERIAL AND METHODS

Plant materials and preparation of polar extracts

Fresh JXL herbs were collected from the Baicaoyuan test base (latitude 30°15′, longitude 119°43′), and their identity was confirmed by Professor Runhuai Hu at Zhejiang A & F University. Samples were dried in a blast oven at 60°C until their moisture content stabilized at less than 5% and were processed in a high-speed rotary cutting mill. Finally, they were screened to separate the fraction containing particles with 40-mesh size.

The powder was heated and refluxed in 80% ethanol (at a solvent-to-material ratio of 40ml/g) for 30 minutes. The

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extract was collected and the residues were subjected to four further extractions. The combined extracts were concentrated to exclude their ethanol content, dissolved in water and sequentially extracted with petroleum ether, acetic ether and n-butanol. After recycling all solvents, the different polar extracts were dissolved in 4% dimethyl sulfoxide (DMSO) to 0.01g/ml.

Preparation of testing strains and suspensions

The antibacterial and antifungal activities of the extracts were separately tested on three bacteria (*Escherichia coli*, *Bacillus subtilis and*, *B. thuringiensis*) and three fungi (*Exserohilum turcicum* (Pass.) Leonard et Suggs, *Botrytis cinerea Pers.*, and *Fusahum graminearum Sehw.*). All strains were obtained from the Laboratory of Microbiology, Zhejiang A & F University.

Bacterial species were cultured on Mueller Hinton agar (MHA) at 37°C for 24h, and the fungal strains were maintained on potato dextrose agar (PDA) at 28°C for 3-5 d. Each microbial suspension was prepared from 2 or 3 colonies of a given strain collected from the plate and dissolved in 5ml pure water, then adjusted to approximately 10^5 CFU/ml.

Antibacterial activities

The antibacterial activities of the different polar extracts from JXL were evaluated by the disc-diffusion method [11]. Sterilized paper discs (6 mm) were impregnated with testing extracts (0.01g/ml), then placed onto Petri dishes pre-inoculated with 0.1ml of a bacterial stain suspension. Discs impregnated with 4% DMSO and pure water were used as negative controls. The dishes were incubated for 24h at 37°C. The antibacterial activities were assessed by measuring the diameters of the inhibition zone (mm) around the discs within the disc diameter. All tests were performed in triplicate.

Antifungal activities

The antifungal activities of the different polar extracts from JXL were evaluated by the disc-diffusion method as described above. After incubating the Petri dishes at 28-30°C for 48-72h, we measured the diameters of the inhibition zones (mm), including the disc diameters. All tests were performed in triplicate.

Minimum inhibitory concentration assay

The minimum inhibitory concentrations (MICs) of the JXL extracts were determined by the dilution method. The polar extracts were dissolved in 4% DMSO to different concentration gradients (100%, 50%, 25%, 12.5% and 6.25%). Sterilized paper discs (diameter 6mm) were impregnated with the above dilutions, and placed onto Petri dishes pre-inoculated with 0.1ml of an individual strain suspension. The MIC was defined as the lowest extract concentration that suppressed bacterial or fungal growth to non-visible levels. All tests were performed in triplicate.

STATISTICAL ANALYSIS

Results were expressed as their means ± SDs (standard deviations). The statistical significance was determined by one-way analysis of variance using SPSS 19.0 analysis software (SPSS Inc., IL, USA) followed by the least significant difference (LSD) test. The differences were considered significant at $p< 0.05$.

RESULTS

Antibacterial activities

The diameters of the inhibition zones (fig. 1) confirmed that all of the tested strains were sensitive to the four polar extracts. Intuitively, different polar extracts will exert various antibacterial effects. *E. coli* and *B. thuringiensis* were most inhibited by aqueous abstract, with inhibition zones extending to 10.8mm and 12.2mm respectively. However, *B. subtilis* was most susceptible to acetic ether extracts, with an inhibition zone reaching 9.8 mm. Similarly, among acetic ether, n-butanol, water, and petroleum ether extracts from *Sedum aizoon* L. in a previous study, acetic ether extract exhibited the highest antimicrobial activity against *B. subtilis* (Wang *et al*., 2013). In the present study, apart from its potency against *B. subtilis*, the inhibitory effects of acetic ether extract from JXL were close to those of petroleum ether extract. Moreover, *E. coli* and *B. subtilis* were the most sensitive and resistant bacterial species to n-butanol extract, respectively.

The MIC values confirm the different inhibitory effects of the same polar extract at different concentrations (see table 1). The MICs ranged from 0.625 to 1.25mg/ml. Meanwhile, the negative control (4% DMSO and pure water) exerted no antibacterial effect on any of the tested microorganisms. At 100% (10mg/ml), all of the extracts inhibited bacterial growth to below visible levels. The antibacterial activity weakened with decreasing concentration of the extracts. The MICs of the petroleum ether and acetic ether extracts reached 1.25mg/ml against all three strains, while those of n-butanol extract were 0.625mg/ml, 0.625mg/ml and 1.25mg/ml against *E. coli*, *B. thuringiensis* and *B. subtilis*, respectively.. The aqueous extract achieved the lowest MIC values (<0.625 mg/ml) against the tested bacteria. According to these results, all of the extracts from JXL inhibit microbial growth.

Antifungal activities

The antifungal susceptibilities of the fungal strains to different polar extracts are presented in fig. 2. The aqueous extract was potent against *F. graminearum*, *B. cinerea* and *E. turcicum*, with inhibition zones reaching 21.41mm, 22.89mm and 24.33mm, respectively. In addition, the sensitivities to each kind of extract varied among the fungi. Notably, a ranking of the sensitivities of the fungi to acetic ether extract were ordered as follows: *B.*

cinerea < *F. graminearum* < *E. turcicum*. In general, aqueous JXL extract achieved the strongest antifungal activity, followed by n-butanol and acetic ether extracts. The petroleum ether extract exerted a relatively weak effect against the fungal strains.

Table 1: Minimum inhibitory concentration of different extracts from *Anoectochilus roxburghii* against tested bacterial strains

		10mg·mL^{-1}	5mg·mL^{-1}	2.5mg·mL^{-1}	1.25mg·mL^{-1}	0.625mg·mL^{-1}
Escherichia coli	Aqueous	++	++	++	++	++
	n-butanol	++	++	++	++	++
	Acetic ether	++	++	++	++	+
	Petroleum ether	++	++	++	++	+
	Pre water control	-	-	-	-	-
	4% DMSO control	-	-	-	-	-
Bacillus thuringiensis	Aqueous	++	++	++	++	++
	n-butanol	++	++	++	++	++
	Acetic ether	++	++	++	++	+
	Petroleum ether	++	++	++	++	+
	Pre water control	-	-	-	-	-
	4% DMSO control	-	-	-	-	-
Bacillus subtilis	Aqueous	++	++	++	++	++
	n-butanol	++	++	++	++	+
	Acetic ether	++	++	++	++	+
	Petroleum ether	++	++	++	++	+
	Pre water control	-	-	-	-	-
	4% DMSO control	-	-	-	-	-

Table 2: Minimum inhibitory concentration of different extracts from *Anoectochilus roxburghii* against tested fungal strains

		10mg·mL^{-1}	5mg·mL^{-1}	2.5mg·mL^{-1}	1.25mg·mL^{-1}	0.625mg·mL^{-1}
FusaHum graminearum	Aqueous	++	++	++	++	+
	n-butanol	++	++	++	++	++
	Acetic ether	++	++	+	+	-
	Petroleum ether	++	++	+	+	-
	Pre water control	-	-	-	-	-
	4% DMSO control	-	-	-	-	-
Botrytis cinerea	Aqueous	++	++	++	++	++
	n-butanol	++	++	++	++	-
	Acetic ether	++	++	-	-	-
	Petroleum ether	++	+	-	-	-
	Pre water control	-	-	-	-	-
	4% DMSO control	-	-	-	-	-
Exserohilum turcicum	Aqueous	++	++	++	++	+
	n-butanol	++	++	++	-	-
	Acetic ether	++	++	++	++	-
	Petroleum ether	++	++	-	-	-
	Pre water control	-	-	-	-	-
	4% DMSO control	-	-	-	-	-

"++" means that the inhibition zone is greater than 9 mm; "+" means the inhibition zone 7-9 mm; "-" means no inhibition zone. Round filter paper piece diameter of 6 mm.

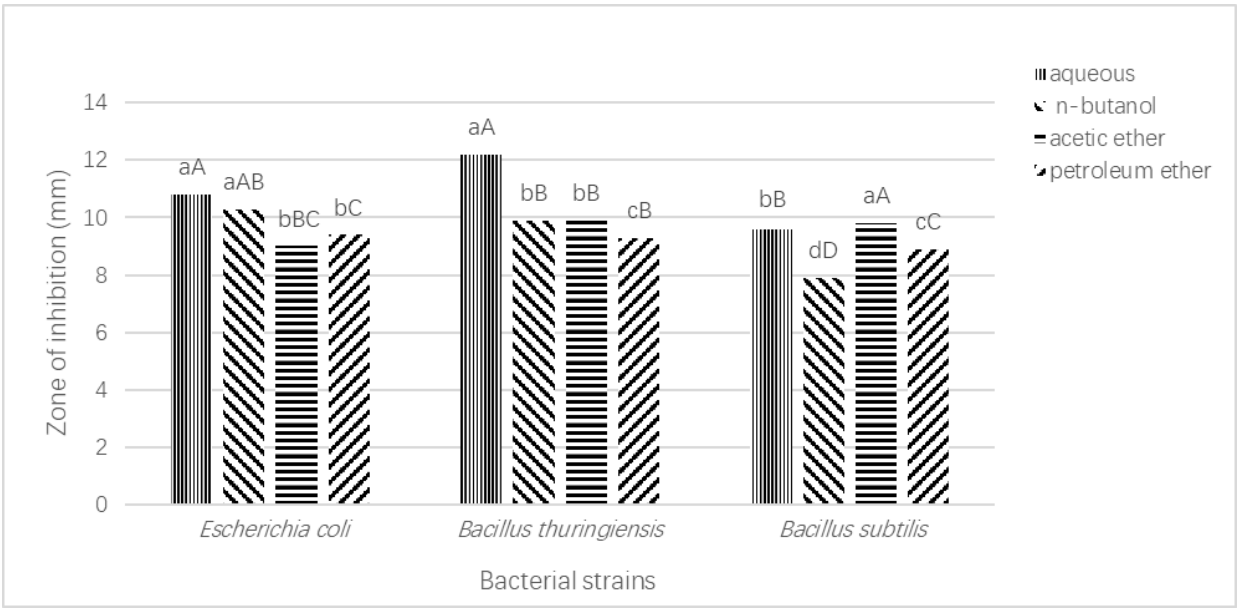

Fig. 1: Comparison of the inhibition zone of different extracts from *Anoectochilus roxburghii* against tested bacteria at 0.01 g/ml. Means with different small and capital letters respectively indicate that different polar extracts have significant differences in the inhibition zone of the same bacterial strains at $P< 0.05$ and $P< 0.01$.

Table 2 lists the MICs of the three fungi exposed to petroleum ether, acetic ether, n-butanol and aqueous extracts from JXL. The inhibitory effects increased with increasing concentration of the extracts. The MICs consolidated the above results; aqueous extract and petroleum ether extract presented the highest and lowest antifungal activities, respectively. Moreover, the petroleum ether extract more effectively inhibited *F. graminearum* (MIC = 1.25mg/ml) than *B. cinerea* (5mg/ml) and *E. turcicum* (also 5mg/ml). Even at the lowest concentration (0.625mg/ml), the aqueous extract displayed a strong antifungal property.

DISCUSSION

All of the JXL extracts inhibited the growth of all tested microorganisms. The aqueous extract most effectively inhibited *E. coli* and *B. thuringiensis* among the bacterial strains (MIC = 0.625mg/ml) and strongly inhibited the growth of all fungal strains (*F. graminearum*, *B. cinerea* and *E. turcicum*; all MIC< 0.625mg/ml). Aqueous extracts of *Syzygium buxifolium* leaves achieved the same results (Huang, 2005). However, in some studies, other polar extracts were more effective antimicrobial agents than aqueous extract. For instance, ethanol extract from *Tribulus terrestris* L. exerted a stronger antimicrobial effect than aqueous and chloroform extracts from the same plant. (AL-BAYATI & AL-MOLA, 2008) Methanol and chloroform extracts of sugar apples (*Annona squamosa* Linn.) also showed relatively higher activity than the aqueous extract (Kalidindi *et al*., 2015). These results indicate that different extracts contain diverse compounds or activate different modes of antimicrobial action.

Aqueous extracts significantly inhibited the growth of the strains, suggesting that this extract contains chemical compounds that reinforce the antibacterial and antifungal activities of JXL. This herb is rich in phenolic compounds (Shao *et al*., 2014), which confer potential health benefits. For instance, many phenolic compounds are antioxidant, anti-allergic, anti-inflammatory, anticancer, anti-hypertensive and antimicrobial agents (Ke *et al*., 2012). One group of phenolic compounds, flavonoids with hydroxyl groups, exhibits effective antimicrobial activity against a wide array of microorganisms in vivo; for example *Scutellaria baicalensis* Georgi (Zeng *et al*., 2009) and *Sophora flavescens* (Zheng *et al*., 2008). These compounds probably interfere with the structural and functional properties of bacterial membranes, rendering them permeable to protons. Ultimately, proton permeability degrades the cellular integrity, and cytoplasmic contents leak out (Kirnpal-Kaur *et al*., 2011). The antimicrobial activity of flavonoids is mainly related to the number and positions of the hydroxyl groups on the aromatic ring (Yang *et al*., 2014), which greatly affect the polarity of the extracted substances. Thus, we can speculate that phenolic compounds are the active antimicrobial components in the aqueous extracts from JXL. Overall, the present study provides scientific evidence of the traditional medicinal uses of JXL. It also confirms the potential use of plants as bioactive substances in the chemotherapy and control of infectious diseases.

We investigated the antibacterial and antifungal activities of petroleum ether, acetic ether, n-butanol and aqueous extracts from JXL. All of the extracts demonstrated significant antimicrobial effects against all tested

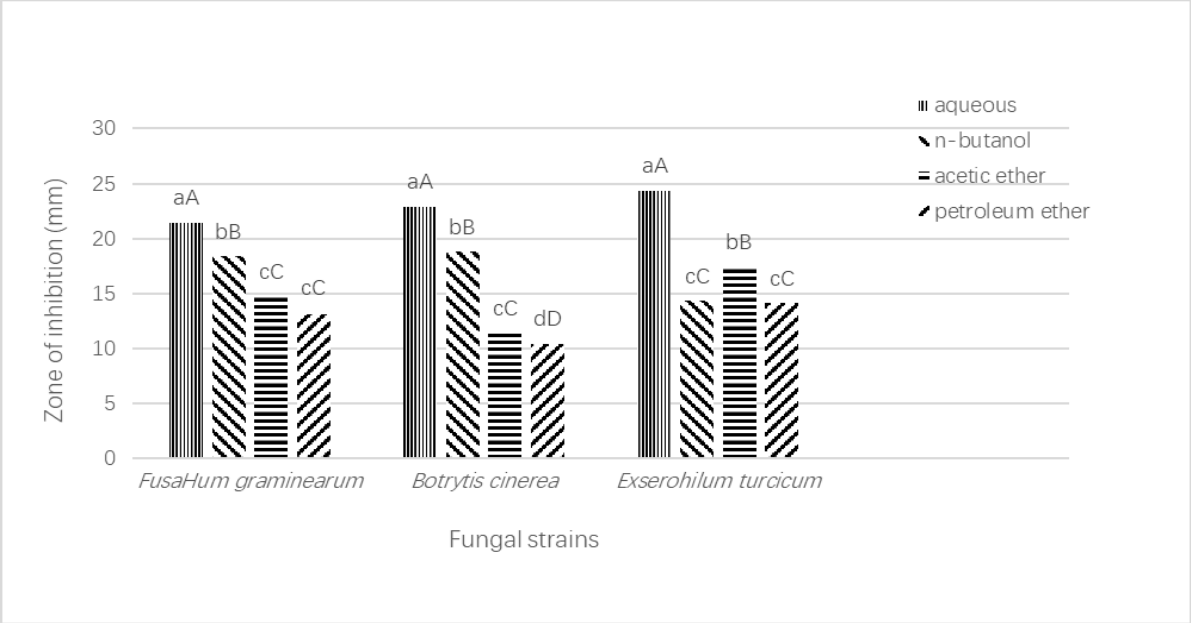

Fig. 2: Comparison of the inhibition zone of different extracts from *Anoectochilus roxburghii* against tested fungi at 0.01 g/ml. Means with different small and capital letters respectively indicate that different polar extracts have significant differences in the inhibition zone of the same fungal strains at P< 0.05 and P< 0.01.

microorganisms. The most effective extract against both bacteria and fungi was aqueous extract, with an MIC below 0.625mg/ml. We surmise that the aqueous extract contains the principal compounds of antimicrobial activity. The active compounds and the mechanisms underlying the observed antimicrobial activities of JXL extracts must be investigated in further studies.

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