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Preliminary Isolation and Antibacterial Activity of the Ethyl Acetate Extract of *Prinsepia utilis* Royle *in Vitro*

Zhonghui Pu[1], Zhongqiong Yin[2*], Renyong Jia[3], Xu Song[2], Jiao Xu[2], Xiongqing Wang[1], Xiwen Chen[1], Minghua Luo[1]

[1]College of Life Science and Technology, Mianyang Normal University, Mianyang, China
[2]College of Animal Medicine, Sichuan Agricultural University, Ya'an, China
[3]Key laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an, China
Email: [*]zhonghui.pu@163.com

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Abstract

Seven fractions were isolated from the ethyl acetate extract of *Prinsepia utilis* Royle by silica gel column chromatography. The antibacterial activities determined by disc diffusion method *in vitro*, indicated that the first and fourth fractions showed better antibacterial activity than the other fractions, while the sixth and seventh fractions did not showed any antibacterial activity. The diameters of the inhibition zone of first and fourth fractions were greater than 10 mm against three standard strains (*Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC44102 and *Salmonella sp.* CMCC(B)50041) at the concentration of 20 mg/ml. The first fraction was then repeatedly recrystallized in acetone to yield a white snowflake-like compound A, the inhibition zones of which were 14.03 mm, 11.54 mm and 12 mm, respectively. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were measured by broth dilution method at the concentrations ranging from 20 to 0.313 mg/mL. The MIC and MBC values of the first, fourth fractions and compound A were lower than that of oregano oil (positive control) against *S. aureus* ATCC25923.

Keywords

Antibacterial Activity, *Prinsepia utilis* Royle, MIC, MBC

[*]Corresponding author.

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1. Introduction

Herb is an important source of medicine treating human diseases in China and the use of herbal preparations and active ingredients from herb or natural product is becoming increasingly popular all over the world [1]. *Prinsepia utilis* Royle was cultivated commonly in Yunnan province of China [2], whose root, stem and leaf were used by indigenous people for curing various skin ailments and diseases [3] [4]. Used as foodstuff at one time due to its edible oil [5], the economic importance of *Prinsepia utilis* Royle is attracting the attention of researchers. Studies have shown that it has various functions such as maintaining skin humidity [6], enhancing poultry immunity, keeping the mature fruits freshness, antidotal [5], antibacterial [7] [8], antifungal and anti-inflammatory activity [9], etc. In addition, study of foreign scholars VK. Rai showed that more than 22 kinds of compounds in *Prinsepia utilis* Royle leaf by gas chromatography/spectrum, of which 13 species have been identified: Limonene, 1-8-cineole, o-cymene, bergamal, cis-linalooloxide, cis-sabinene hydrate, linalool, rans-terpineol, 2-undeca-none, isomenthol, α-terpineol, 2-dodecanol and tridecanone [10]. The objective of our study was to isolate active fraction from the ethyl acetate extracts of *Prinsepia utilis* Royle as well as determine its antibacterial activity *in vitro*.

2. Materials and Methods

2.1. Plant Materials

Prinsepia utilis Royle seeds obtained from Yunan province, China were identified and authenticated as *Prinsepia utilis* Royle seeds by the Kunming Institute of Botany, Chinese Academy of Sciences.

2.2. Extraction, Isolation and Purification of the Ethyl Acetate Extract

The dried seeds of *Prinsepia utilis Royle* was crushed and extracted with distilled water and then the aqueous extract was precipitated in ethanol. The supernatant was collected and condensed, subsequently extracted by petroleum ether, ethyl acetate and n-butanol respectively. The antibacterial activity *in vitro* of these three extracts was tested by disc diffusion method *in vitro*. As a result, the ethyl acetate extracts showed satisfactory antibacterial activity.

The ethyl acetate extract was purified by Silica gel column chromatography. The open glass column (45 mm by 600 mm) was packed with Silica gel G (100 - 200 mesh, Merck, Qingdao). The column, loaded with the ethyl acetate extract, was eluted with chloroform-methanol-acetone (8:7:0.5 [vol/vol/vol]) solvent system. Each fraction (50 mL) of the eluate was tracked by thin-layer chromatography (TLC) (silica gel GF_{254} [0.2 mm thick]; Merck). Seven fractions were collected, concentrated in the rotary evaporator and dried under vacuum, then used for assay of their antibacterial activity by disc diffusion method. The active fraction, which showed a high antibacterial activity, was further purified by repeatedly re-crystallized with acetone.

2.3. Microorganism

The bacterial strains used in this study were *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC44102 and *Salmonella sp.* CMCC(B)50041 were supplied from the Department of Food Engineering Sciences, Sichuan Agricultural University, Ya'an, China.

2.4. Determination of Antibacterial Activity

The disc diffusion method was used to evaluate the antibacterial activity [11] [12]. An inoculum of the test microbes were prepared for 24 h in Mueller Hinton Broth (MHB) cultures and inoculums were further diluted 1:10 in sterile MHB to obtain a final inoculum of 1×10^6 CFU/mL. 200 µl of the bacterial inoculum were spread evenly onto the surface of Mueller Hinton agar plates by a sterile L stick, before setting aside the seeded agar plate (9 cm in diameter) to solidify for 15 minutes. Sterile filterpaper disks with 6 mm diameter were used to screen the antimicrobial activity. Each sterile disk was impregnated with 10 µl of the fraction at the concentration of 20, 10, and 5 mg/ml respectively; Oregano oil (used as positive control) and Dimethylsulfoxide (used as negative control) were placed on the surface of the seeded plates. The plates were covered and incubated at 37˚C for 24 h, then diameter of inhibition zone was measured. Each test was performed in triplicate.

2.5. Determination of MICs and MBCs

The MICs and MBCs of active fractions were determined by the broth microdilution method, recommended by the Clinical and Laboratory Standards Institute/NCCLS [13]. The concentrations of active fractions ranging from 20 to 0.313 mg/mL and an inoculum of 1×10^6 CFU/mL were used. The MIC was taken as the lowest concentration of active fractions in the wells of the microtiter plate that showed no turbidity after 24 hours of incubation at 37˚C. The lowest concentration showing no visible growth on sterile agar plates by subculture was taken as MBC. Oregano oil was used as positive control and DMSO as negative control. All the experiments were performed in triplicate and the results were averaged.

2.6. Statistical Analysis

The results were expressed as the mean ± S.D. (Standard Deviation). The differences between the fractions were using one-way analysis of variance (ANOVA) by SPSS for Windows Programmer (SPSS 13.0; SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered significant statistically.

3. Results

3.1. Antibacterial Activity of Each Fraction Separated by Silica Gel Column Chromatography

Antibacterial activity each fraction obtained from the ethyl acetate extract of *Prinsepia utilis* Royle by silica gel column chromatography was shown in **Table 1**. Oregano oil (positive control) had been well known for its bacteriostatic properties [14]. We found that anterior fractions were active against all bacteria tested at concentration of 20 mg/mL, while the Fr.6 and Fr.7 showed no effect on all bacteria and produced no zone of inhibition.

The white snowflake-like compound A was isolated from the Fr.1 by re-crystallization with acetone. TLC indicated that Compound A has only one spot by petroleum ether, chloroform and ethanol (**Figure 1**). Fr.1, Fr.4 and compound A had a strong inhibitory effect, the inhibition zone diameter against standard *S. aureus* ATCC-25923 (14.16 mm, 15.26 mm, 14.03 mm) were greater than that of oregano oil (13.76 mm) in the same concentration, they had the same inhibition effect as oregano oil against standard *E. coli* ATCC44102 and *Salmonella* CMCC(B)50041.

3.2. The MIC and MBC Values

The MICs and MBCs of active fractions were shown in **Table 2**. Out of the three bacterial species tested, the range of MIC values of active fractions separated from the ethyl acetate extract of *Prinsepia utilis* Royle against

Table 1. The antibacterial activity of each fraction of acetic ether extract of *Prinsepia utilis* Royle separated by silica gel column chromatography.

Fractions	Zone of inhibition diameter (mean ± S.D., $n = 3$, mm)		
	Staphylococcus aureus ATCC25923	*Escherichia coli* ATCC44102	*Salmonella sp.* CMCC(B)50041
Fraction 1	14.16 ± 0.08	11.55 ± 0.05	12.04 ± 0.09
Fraction 2	12.59 ± 0.37	10.01 ± 0.02	10.41 ± 0.08
Fraction 3	12.93 ± 0.11	11.81 ± 0.62	10.28 ± 0.02
Fraction 4	15.26 ± 0.09	12.14 ± 0.03	11.00 ± 0.23
Fraction 5	9.99 ± 0.15	9.06 ± 0.10	8.740 ± 0.12
Fraction 6	—	—	—
Fraction 7	—	—	—
Compound A	14.03 ± 0.04	11.54 ± 0.04	12.00 ± 0.30
Oregano oil	13.76 ± 0.19	14.32 ± 0.06	12.32 ± 0.07
Dimethylsulfoxide	—	—	—

Note: "—"means no zone ofinhibition.

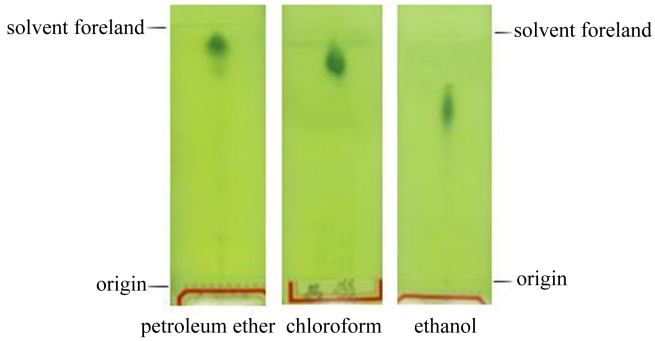

Figure 1. The TLC of compound A.

Table 2. Determination of MICs and MBCs of active fractions against *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC44102 and *Salmonella sp.* CMCC(B)50041.

Samples		*Staphylococcus aureus* ATCC25923	*Escherichia coli* ATCC44102	*Salmonella sp.* CMCC(B)50041
Fr.1	MIC	0.625	2.5	5
	MBC	2.5	5	10
Fr.2	MIC	1.25	5	5
	MBC	10	10	10
Fr.3	MIC	2.5	2.5	5
	MBC	5	10	10
Fr.4	MIC	0.625	1.25	5
	MBC	1.25	10	10
Fr.5	MIC	10	20	20
	MBC	20	20	>20
Compound A	MIC	1.25	2.5	20
	MBC	1.25	5	>20
Oregano oil	MIC	5	1.25	2.5
	MBC	5	2.5	5

S. aureus ATCC25923 (0.625 mg/mL to 10 mg/mL) were lower compared with Gram-negative bacteria *E. coli* ATCC44102 and *Salmonella* CMCC(B)50041 (2.5 mg/mL to 20 mg/mL). The MICs of Fr.1 and Fr.4 against *S. aureus*ATCC25923 were the lowest, 0.625 mg/mL. The MIC of Oregano oil had better antibacterial effects on *E. coli* ATCC44102 and *Salmonella* CMCC(B)50041 (1.25 mg/mL, 2.5 mg/mL) than *S. aureus* ATCC25923 (5 mg/mL).

Based on the results obtained from the MICs, the MBCs were higher than the MIC values of active fractions against the bacteria. The MBC values of Fr.1 and Fr.4 against *S. aureus* ATCC25923 (2.5 mg/mL, 1.25 mg/mL) were 4 times and 2 times greater than MIC values (0.625 mg/mL, 0.625 mg/mL), respectively. The MICs and MBCs of the compound A were the same against *S. aureus* ATCC25923 (1.25 mg/mL), while the MBC value was 2 times greater than its MIC values for *E. coli* ATCC44102, greater than 20 mg/mL for *Salmonella* CMCC(B)50041.

4. Discussion

The results showed that the ethyl acetate extract of *Prinsepia utilis* Royle by silica gel column chromatography was active against all bacteria investigated, as supported by other researchers who reported that *Prinsepia utilis* Royle was found to be active against Gram-positive bacteria and Gram-negative bacteria [15]. We found that the antibacterial activity of active fractions separated from the ethyl acetate extract of *Prinsepia utilis* Royle against *S. aureus* ATCC25923 were better compared with *E. coli* ATCC44102 and *Salmonella* CMCC(B)50041. The

results indicated that the Gram-negative bacteria were more susceptible to the effect of the *Prinsepia utilis* Royle compared to its Gram-positive counterpart. Oregano oil was developed a new type of plant-type antibiotics in recent years [14], the inhibitory effect of which was a well-recognized. In our study, oregano oil had better antibacterial effects on *E. coli* and *Salmonella* than *S. aureuss*, which was unanimous in the reports that oregano oil had a better inhibitory effect for Gram-negative bacteria, especially *E. coli* and *Salmonella* [16]. Compared to oregano oil, separated fractions had better activity against *S. aureus* ATCC25923 than *E. coli* ATCC44102 and *Salmonella* CMCC(B)50041. Meanwhile, based on the results obtained from the MICs and MBCs, these results further indicated *Prinsepia utilis* Royle had broad application prospects in antibacterial plant agents. However, which particular active ingredient was poorly understood, there is a need for further to study and research.

The MICs and MBCs of the compound A against *S. aureus* ATCC25923 than *E. coli* ATCC44102 and *Salmonella* CMCC(B)50041 were 1.25, 2.5, 20 mg/mL and 1.25, 5, more than 20 mg/mL, respectively, the results indicated that A had antimicrobial potency, may represent a new type of bacteriostatic agent. Therefore, further studies should be done for making the constituents of these fractions clear, and structural identification would be taken on compound A.

5. Conclusion

In conclusion, *Prinsepia utilis* Royle had broad application prospects in plant-derived antibacterial agents. Our survey showed that an active fraction (compound A) with remarkable *in vitro* antibacterial activity has been isolated from the *Prinsepia utilis* Royle ethyl acetate extract.

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