Comparison of activity and components of *Sophora flavescens* root and seed

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**Abstract.** Infectious diseases are amongst the leading causes of morbidity and mortality. The *Sophora flavescens* root (traditional Chinese medicine “kushen”) is recognized as a strong antibacterial and anti-inflammatory Chinese herb, and widely used for clinical application. However, the ever-increasing demand for natural antibacterial agent is fostering the insufficient supply of “kushen”. This study was conducted for the first time to explore the feasibility of *Sophora flavescens* seed as a supplement of *Sophora flavescens* root to alleviate the shortage of “kushen” resources. To accomplish this, the seed and root of *Sophora flavescens* were extracted by ultrasound-assisted extraction method. The antimicrobial activities of *Sophora flavescens* seed and root against *Escherichia coli*, *Bacillus subtilis*, *Micrococcus tetragenus* and *Proteus* species were compared by agar well diffusion method. And the phytochemical constituents from seed and root were analyzed by TLC, chromogenic reaction method, HPLC and GC/MS. The results revealed that different solvent fraction from *Sophora flavescens* root and seed exhibited different degrees of antibacterial activity. The chloroform fraction, ethyl acetate fraction and anhydrous ethanol fraction of *Sophora flavescens* root had obvious antibacterial activity. However, the best antibacterial activity of *Sophora flavescens* seed was achieved with the 80% ethanol extracts. Furthermore, the analysis of phytochemical compositions showed that the antibacterial-activity component profiles of *Sophora flavescens* seed were different from that of roots. This study clearly reveal that *Sophora flavescens* seed is unsuitable as a straight substitution of “kushen” due to the differences in their active component, but it has the potential to be used as a promising source of antibacterial agent, which could be utilized in clinical application and pharmaceutical industry, just as *Sophora flavescens* root.

1. Introduction

Nowadays infectious diseases are responsible of 30% global mortality. With the overprescription and misuse of traditional antibiotics to fight against pathogenic microorganisms, however, traditional antibiotics have made a bad situation worse for their potential to cause other health risks and emergence of antimicrobial resistance problems. In the previous studies, natural plant based antibacterial agents have proved to be reliable alternatives for traditional antibiotics [1, 2]. In China, many medicinal plants are traditionally employed in treatment of the most prevalent infections [3-5], such as *Sophora flavescens Ait. (S. flavescens)*.

*S. flavescens* belongs to Fabaceae family, and wildly grown in China. The root of the *S. flavescens*, well known as the traditional Chinese medicine “Kushen”, is considered as a powerful antibacterial and
anti-inflammatory herb which has been used for the treatment of viral and bacterial infection diseases such as viral hepatitis, chronic proctitis, bacillary dysentery, eczema, and psoriasis [6-13]. Moreover, alkaloids extracted from S. flavescens are also extensively used in agriculture as green antibacterial agents for the insecticidal effect [14, 15]. Since the root could not be applied in industry until grows up 3 years, the huge consumption of S. flavescens root makes its supply was not adequate to the demand.

S. flavescens seed can be harvested every year with yield of about 1500 kg per hectare, however, it is regarded as an undervalued byproduct which is not utilized in huge amounts by the agri-food industrial. In Chinese folk medicine, the seeds of S. flavescens are traditionally used for the cure of acute bacillary dysentery, constipation and parasite infections [16]. The studies of phytochemical profiles and the bioactivities demonstrated that the S. flavescens seed is also rich in terpenoids, alkaloids, saponins, and flavonoids [17]. So, it is necessary to exploit the possibility that S. flavescens seed is used as a promising resource to substitute, or partly substitute, S. flavescens root in pharmaceutical industry and agriculture in order to relieve the traditional Chinese medicine “kushen” resource deficiency. According to literature investigation, some phytochemical and pharmacological studies about S. flavescens seed have been performed, [18] and also, many researches about biological activity and chemical analysis of S. flavescens root have been conducted [19]. However, to our knowledge, few comparing studies of the biological activity and chemical constituents of S. flavescens root and seed were published.

Accordingly, this research was conducted to characterize the chemical composition and antibacterial activity of S. flavescens seed, and compared with S. flavescens root extracts. The results of this research will help to demonstrate the possibility of extensive utilizing the S. flavescens seed in pharmaceutical industry, food industry and bio-pesticide industry.

2. Materials and methods

2.1. Chemicals
Methanol (Merck, Germany) was of HPLC grade and ultra-pure water was prepared using a Milli-Q purification system (Millipore, France). All other chemicals, such as ethanol, petroleum ether, chloroform, acetic ester and n-butanol were of analytical grade and used without further purification. Reagents for bacterial culture, beef extract-peptone agar medium were purchased from Sigma (Sigma Chemical Co., St. Louis, USA).

2.2. Plant material
Seeds and roots of S. flavescens were obtained from Qingdao Baicaoting Pharmacy (Qingdao, China) and authenticated by Dr. Wenying Zhao at Qingdao University of Science and Technology (Qingdao, China). The seeds and roots were grounded using a laboratory mill to obtain the seed and root powders (24 mesh).

2.3. Sample preparation
The dried seed powders (50 g) were immersed in 300 mL of petroleum ether and ultrasonicated three times with 40 kHz frequency, 100W power (JAC-4020, Kodo, Korea) for 20 min, then, the solvent was decanted and the marc was extracted in turn with chloroform, acetic ester, ethanol, and 80% ethanol under same condition, respectively. The resulting extracts were then filtered and concentrated to approximately 50 mL under reducing pressure. The extracts were dried in a 50°C water bath, and stored at -10°C until used.

The dried root powders (50 g) were extracted under same condition, the extracts of petroleum ether, chloroform, acetic ester, ethanol, and 80% ethanol were dried in a 50°C water bath, and stored at -10°C until used.

80% ethanol extracts (roots and seeds) were redissolved in hot water, and extracted in turn with petroleum ether, acetic ester and n-butanol, respectively. The resulting solutions were evaporated to dryness for the HPLC assay and colorimetric assay.
2.4. Antibacterial assay

The antibacterial activity of organic fractions of root and seed of *S. flavescens* were evaluated by agar well diffusion method and all four bacterial strains were obtained from Key Laboratory of Marine Drugs (China Ocean University), Ministry of Education. Agar well diffusion method was performed according to normalization method. One hundred microliters of inoculum, equivalent to 10^6 CFU/mL, was mixed with 20 mL of warm melted medium. The mixture was then poured into the plate with an 8 mm diameter metal cup. After solidifying of medium, the metal cups were removed and the well was added with 100 μL of each extracts (100 mg/mL), sterile water was taken as a negative control. The plate was incubated at 37°C for 24 h. The antimicrobial activity of each extract was determined by measuring the diameter of the zone of the inhibition in millimeters. Duplicates were maintained and the experiment was repeated thrice. The minimum inhibitory concentrations (MICs) of the Seeds of *S. flavescens* were determined via the dilution method.

2.5. TLC analysis

The extract (2 mg) of different samples was redissolved in methanol was used to develop a TLC method for identifying drug materials. Solutions were applied in the chromatographic plate, with a stationary phase of silica gel, using a capillary tube. The chromatographic chamber was prepared 30 min before performing analyses. The TLC sheet was developed with petroleum ether /AcOEt (7:3) as a developing solvent for petroleum ether extract and chloroform extract, with chloroform/methanol (9:1) for acetic ester extract and ethanol extract. When the solvent front reached the top of the TLC sheet, it was removed and air-dried. The plates were sprayed with 10% sulfate ethanol solution, and heated at 110°C for 5-10 min. Two elution systems were used for identifying 80% ethanol extract, firstly, toluene/acetone/methanol (16:6:1), then, toluene/acetic ester/methanol/water (7:14:7:3.5). The plate was migrated same distance in the two develop. After air drying, the developed plate was sprayed with 10% sulfate ethanol solution, and heated at 110°C for 5-10 min.

2.6. HPLC analysis

Analyses were carried out on DIONEX U3000 RP HPLC instrument (DIONEX, California, USA), equipped with Variable wavelength UV-Vis detector, using reverse phase C18 (Agilent) analytical column (250 mm × 4.6 mm i.d., 5 μm particle size), and the column temperature was maintained at 30°C. The mobile phase A was water, while mobile phase B was methanol. Gradient elution was applied according to the following scheme: 0-20 min, 70-50% A; 20-50 min, 50% A; 50-60 min, 50-70% A, and the flow rate of 0.8 mL/min. The injection volume was 20 μL. Detection wavelength was set at 254 nm.

2.7. Phytochemical screening

The 80% ethanol extracts of *S. flavescens* seed and root, which fractionated with petroleum ether, acetyl acetate and n-bathanol, were subjected to the colorimetric test. Around 10 mg of each sample was dissolved in 5 mL of methanol, and afterwards, 1 mL of an aliquot of the extract was added into an evaporating dish. Then, in the same dish were added 1 mL of chromogenic agent. The tiles were visualized under natural daylight.

2.8. GC/MS analysis

Chemometric profiling of 80% ethanol extracts of *S. flavescens* seed was done by GC/MS analysis. The standard operating conditions were followed, and the analysis was carried out on an Agilent 7890b-7000e Triple Quadrupole GC/MS system (Agilent Technologies Inc., California, USA). The gas chromatograph was linked to a mass spectrometer instrument employing the following conditions: Agilent HP-5 MS column (30 m × 0.25 mm × 0.25 μm), electron impact (EI+) mode at 70 eV, helium (99.999%) used as carrier gas at a constant flow of 1 mL/min, injection volume of 1.0 μL, split ratio of 20:1, injector temperature of 260°C, and transfer line temperature of 280°C. The oven temperature
was programmed from 60°C (isothermal for 1 min), with a gradual increase in steps of 20°C/min to 260°C, and held for 20 min. Mass spectra were taken at 70 eV, and full mass scan range from 15 m/z to 600 m/z. The reference mass spectrum was obtained from the MS- NIST database.

3. Results and discussion

3.1. Antibacterial activity of the extracts of S. flavescens
The antibacterial properties in the various extracts derived from S. flavescens were assayed against Escherichia coli, Bacillus subtilis, Micrococcus tetragenus and Proteus species. All four bacterial strains are commonly parasitic in the intestines of humans and often cause diarrhea and other digestive problems which are consistent with the therapeutic uses of S. flavescens. Table 1 presents diameters of inhibition zones exerted by each extract towards four bacteria.

Table 1. Antibacterial activity of different solvent extracts from S. flavescens root and seed (punch diameter 8 mm).

| Bacterial strain          | Petroleum ether extract | Chloroform extract | Ethyl acetate extract | Anhydrous ethanol extract | 80% ethanol extract |
|---------------------------|-------------------------|--------------------|-----------------------|--------------------------|---------------------|
|                           | root        | seed    | root        | seed    | root        | seed    | root        | seed    | root        | seed    |
| Bacillus subtilis         | 8           | 8       | 20          | 8       | 22          | 8       | 16          | 17      | 9           | 21      |
| Proteus species           | 8           | 8       | 17          | 8       | 19          | 8       | 16          | 16      | 9           | 20      |
| Micrococcus tetragenus    | 8           | 8       | 19          | 8       | 23          | 8       | 18          | 18      | 9           | 23      |
| Escherichia coli          | 8           | 8       | 18          | 8       | 20          | 8       | 15          | 17      | 9           | 20      |

It can be seen that S. flavescens root and seed extracts prepared by different solvents exhibited various degrees of antibacterial activity. All of the tested strains were inhibited by the chloroform fraction (RCE), EtOAc fraction (RAE) and EtOH fraction (REE) from S. flavescens root. Specifically, the tested strains were more sensitive to the RAE and RCE, while petroleum ether fraction (RPE) and 80% EtOH fraction (80REE) from root shows no, or almost no, inhibition on all the tested strains. The main ingredients, which exhibited excellent antibacterial property, in S. flavescens roots are quinolizidine alkaloids according to Zhao [20], and it is well known that these alkaloids are easily soluble in moderately polar solvents, such as chloroform, acetyl acetate and alcohol, but insoluble in water. RCE, RAE, and REE exert higher antibacterial activity, due to quinolizidine alkaloids high solubility in those solvents, and this result verified the previous reports on the alkaloid activities from S. flavescens roots [21, 22]. Extracts from seed have showed different inhibition on the four tested strain compared with extracts from root. The strongest antibacterial activity was observed in 80% ethanol fraction (80SEE), a higher polar solvent extraction section, while the petroleum ether fraction (SPE), chloroform fraction (SCE), and EtOAc fraction (SAE) showed no inhibition on the four tested strains. These findings are in good agreement with existing report of Weng et al. [23] for the fraction with antibacterial activity was extracted with 80% ethanol from S. flavescens seeds. Furthermore, antimicrobial activities of S. flavescens seed extracts were to be referred to the presence of alkaloids in their study. This is not surprising because through previous works, a basic phytochemical screening revealed the presence of alkaloids in these seeds, and other phytochemicals such as flavonoids, saponins and glycosides [24]. The difference observed in the results of antibacterial activity between the root and seed extracts may be attributed to the main antibacterial constituents from S. flavescens seeds different from roots, and higher polar solvent, such as ethanol and aqueous ethanol could be preferable solvent systems for the efficient extraction of these activity compounds.

To observe which concentrations of 80% ethanol extracts were still able sufficiently to inhibit the bacteria, a serial of concentrations were reexamined. The data presented in Table 2, indicate that 80%
ethanol extracts reached the best activity at the highest applied concentration of 100 mg/mL, and no differences in activity against the bacteria tested regardless of the type of the bacteria. However, there were significant differences at the lowest concentrations of inhibitory activity, 80% ethanol extracts showed more effective against *Escherichia coli* at concentration of 5 mg/mL while *Bacillus subtilis* and *Micrococcus tetragenus* were inhibited at 10 mg/mL, and *Proteus* species only at higher concentration of 15 mg/mL. The 80% ethanol extract was found active against both gram-positive and gram-negative bacterial species, and this result is in agreement with recently report[23] (Weng et al. 2017). Although, in their study, only *Escherichia coli* and *Staphylococcus aureus* were selected for evaluation of extracts from *S. flavescens* seeds. But it is noteworthy that a MIC of 3.125 mg/mL against *Escherichia coli* confirmed our study. Numerous studies have demonstrated antibacterial properties of *S. flavescens* roots, the root extracts were therefore not investigated further.

**Table 2.** Inhibition Zone in diameter of 80% ethanol extracts of *S. flavescens* seed with different concentrations (punch diameter 8 mm).

| Bacterial strain | The concentration of the extract (mg/ml) |
|------------------|----------------------------------------|
|                  | 100 | 90  | 60  | 40  | 15  | 10  | 5  |
| Gram-positive bacteria |     |     |     |     |     |     |    |
| *Bacillus subtilis* | 24  | 22  | 17  | 16  | 10  | 9   | 8  |
| *Micrococcus tetragenus* | 23  | 20  | 19  | 17  | 14  | 9   | 8  |
| Gram-negative bacteria |     |     |     |     |     |     |    |
| *Escherichia coli* | 22  | 21  | 20  | 19  | 12  | 10  | 9  |
| *Proteus species* | 22  | 16  | 14  | 14  | 9   | 8   | 8  |

3.2. **TLC of the extracts of *S. flavescens***

To verify the chemical composition of the *S. flavescens* root and seed extracts and to determine if the differences of antibacterial activity were qualitative or only quantitative, an analytical TLC was performed of all extracts (Fig 1 and Fig. 2).

**Figure 1.** Thin layer chromatography (TLC) of different solvent extracts from *Sophora flavescens* root and seeds after spraying with alcoholic solution of sulfuric acid. (1)*Sophora flavescens* root (petroleum ether); (2)*Sophora flavescens* seed(petroleum ether); (3)*Sophora flavescens* root (chloroform); (4)*Sophora flavescens* seed (chloroform); (5)*Sophora flavescens* root (Ethyl acetate);
(6) Sophora flavescens seed (Ethyl acetate); (7) Sophora flavescens root (Anhydrous ethanol); (8) Sophora flavescens seed (Anhydrous ethanol)

As can be seen, similar compositions were obtained for petroleum ether extracts of root and seed, but there were different compositions between root and seed extracts, for the same solvent, of chloroform, ethyl acetate, ethanol and 80% ethanol. This means the differences at antibacterial activity were mainly qualitative. Furthermore, more difference observed for ethanol and 80% ethanol samples. The spot of ethanol sample of S. flavescens seed did not move from the application point (Fig 1), and further analysis of the 80% ethanol extracts with a method developed for polar compounds analysis (see Fig 2) shows the spots from S. flavescens root extract at Rf > 0.5, however, the main spots from S. flavescens seed extract at Rf < 0.5. It can be deduced from the TLC that compounds from these seed extracts were mainly polar, and the antibacterial activity of 80% ethanol extract being markedly different from the root extracts maybe due to these polar compounds.

Figure 2. Thin layer chromatography (TLC) of different solvent extracts from Sophora flavescens root and seeds after spraying with alcoholic solution of sulfuric acid. (1) Sophora flavescens root (chloroform); (2) Sophora flavescens root (80% ethanol); (3) Sophora flavescens seed (80% ethanol).

3.3. Phytochemical tests of the extracts of S. flavescens

The composition and antibacterial activity of the extracts prepared by 80% ethanol varied much between the S. flavescens root and seed, so the chemical nature of 80% ethanol extracts were further analysis with phytochemical tests. The tests were performed according to normalization method described by Cao [25] and Lay [26], and the results were present in Table 3. The major natural chemical groups such as terpenoids, alkaloids, saponins, flavonoids, phenols were identified in the extracts of S. flavescens roots and seeds. Among the phytochemicals, phenols, flavonoids, and alkaloids have been proven to be of great important secondary metabolites of plants because they serve as defence mechanism against predation by many micro-organisms and insects [27, 28]. The presence of these phytochemical in the both extracts could possibly play a significant role in the antibacterial activity observed in this present study, and the differences of antibacterial activity between root and seed extracts maybe mainly due to the different content and composition of phytochemical constituents.
3.4. **HPLC profiles of the extracts of S. flavescens**

The composition of the extracts prepared by 80% ethanol of the *S. flavescens* root and seed (with same concentration) were also studied by HPLC. As it can be observed, there were differences on fingerprint characteristic from 15 to 40 min of retention time, special on relative abundance of peaks (Fig. 3).

![HPLC profiles](image-url)
Many peaks, but smaller peak areas, were found in extracts of *S. flavescens* root, whereas there were one main big peak at retention times of 27 min, and three other, smaller peaks in extracts of petroleum ether and ethyl acetate fraction from seed. The result of antibacterial activity indicated the ethyl acetate fraction of 80% ethanol extract of seed showed much higher activity as compared to the other fractions (Fig 4), and n-BuOH fraction, which only a minor peak was observed at 27 min, shows no antibacterial activity. This may be an indication of a positive effect of peaks at 15 to 40 min on the antibacterial activity. However, these components of the extract are not identified in view of the fact that some standards could not be available. Therefore, it is worth further works on the characterization, antibacterial activity, and structure-function relationship of compounds in *S. flavescens* seed.

**Figure 3.** HPLC chromatograms of different solvent fraction of extracts from *S. flavescens* root and seeds at 254 nm. (A) extracts of petroleum ether fraction from seed; (B) extracts of petroleum ether fraction from root; (C) extracts of ethyl acetate fraction from seed; (D) extracts of ethyl acetate fraction from root; (E) extracts of n-BuOH fraction from seed; (F) extracts of n-BuOH fraction from root.

**Figure 4.** Antibacterial activity of the extracts from *Sophora flavescens* seeds and root. 1) petroleum ether layer, 2) ethyl acetate layer and 3) n-butanol layer of alcohol extract from *Sophora flavescens* seeds (ultrasonic extraction); 4) Petroleum ether layer, 5) ethyl acetate layer and 6) n-butanol layer of alcohol extract from *Sophora flavescens* seeds (pressure extraction).
3.5. GC–MS of the extracts of *S. flavescens* seed

Now a day, the GC–MS is the most ideal technique for qualitative and quantitative analysis of volatile and semi volatile bioactive compounds [29]. There are seldom volatile and semi volatile compounds in the extracts of *S. flavescens* root, therefore, the extracts obtained by extraction with 80% ethanol from *S. flavescens* seed was analyzed with GC–MS in the present study. The result clearly shows that the extracts of *S. flavescens* seed consisted of complex mixtures of various types of organic compounds (Table 4), which included alcohols, aldehydes, esters, fatty acids and their derivatives. In the investigated extracts, (Z,Z)-9,12-Octadecadienoic acid (linoleic acid), an essential fatty acid, was found in quite high amount about 32.76%, but the value was lower than in previous reports [30]. In their study, (Z,Z)-9,12-Octadecadienoic acid constitutes up to 46.77% of the total fatty acid content. Other main fatty acids, such as hexadecanoic acid (3.30%) and 10-methyl-heptadecanoic acid (0.92%) were also identified in the extracts of *S. flavescens* seed (Table 5), and the results reported by Weng [23] were slightly lower than in our study. This difference in the results might be due to cultivar, geographical or ecological variations, and also attributed to the solvent of extraction, nature of processing method and micro change in lab environment. (Z,Z)-9,12-octadecadienoic acid and hexadecanoic acid are pharmaceutical fatty acids known for their antioxidant, anti-inflammatory, hypocholesterolemic activity, and effective against other heart related problems[31-37] (Patra et al., 2015; Mohamad et al., 2018; El-Shouny et al., 2018; Kata et al., 2018; Zhang et al., 2012; Xie et al., 2012; Wang et al., 1994). Thus, the presence of these compounds in *S. flavescens* seeds demonstrates their potential use in the pharmaceutical and food industries.

4. Conclusion

In the present study, the antibacterial activity and chemical components of *S. flavescens* seed was compared with those of *S. flavescens* root to explore its potential as novel raw material to substitute “kushen” for clinical application. The results show that *S. flavescens* seed, as a by-product of traditional Chinese medicine “kushen”, possesses comparable antibacterial activity, and can be employed for agriculture and pharmaceutical industry. However, the TLC and HPLC analysis indicated the antibacterial activity fraction and component profiles of *S. flavescens* seed different from root, and *S. flavescens* seed is not suitable to be a straight substitution to relieve the traditional Chinese medicine “kushen” resource deficiency. Furthermore, it was observed that the more powerful antibacterial effect of *S. flavescens* seed is attributed to 80% ethanol fraction, and further studies are need to focus on isolation and characterization of these biologically components for the potential clinical application value of *S. flavescens* seeds.

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