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PHYTOCHEMICAL SCREENING, ANTIOXIDANT, ANTIBACTERIAL AND CYTOTOXIC ACTIVITIES OF DIFFERENT EXTRACTS OF *SELAGINELLA DOEDERLEINII*

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Abstract

Five fractions of ethanol extract, i.e. petroleum ether extraction (PEE), diethyl ether extraction (DEE), ethyl acetate extraction (EAE), methyl alcohol extraction (MAE) and water extraction (WE), were subjected to evaluate antitumor, antioxidant and antibacterial effects. Total phenols and biflavonoids content were followed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS) radical scavenging assay, ferric reducing power assay and chelation of ferrous ions assay for evaluating antioxidant potency. Among five fractions, EAE had efficiently high amount of total biflavonoids and MAE had higher phenols content. At 150 µg/ml of different fractions, EAE exhibited 85.77% DPPH radical scavenging activity, 90.39% ABTS radical scavenging activity, 82.51% chelation of ferrous ions effect and 1.227 reducing power. PEE showed more efficient zone of inhibition against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas*. In anticancer test, EAE showed marked cytotoxicity against A549 cell strain, 7721 cell strain, Hela cell line and Eca-109 cell line with IC_{50} value of 52.66, 66.20, 37.53 and 62.09 µg/ml, respectively than the other four fractions. Biflavonoids were found to play an important role for anticancer effect of *S. doederleinii* with R^2 values of 0.915, 0.834, 0.818, 0.718, respectively and their coefficient values were significantly higher than that of phenols. The extracts of *S. doederleinii* contain remedial potential and can be used as possible source for drug development by pharmaceutical industries.

Introduction

Selaginella doederleinii Hieron, used in traditional Chinese medicine, is a well-known herb belonging to the family *Selaginella Pteridophyta* growing mainly in Gui Zhou, Yun Nan, Si Chuan, Chong Qing and Guangxi of China at the low altitude (Dan *et al*. 2017; Wang *et al*.2014). It has been exhibited broad activities, including cytotoxic, antioxdant, antibactireal, leishmanicidal, antiplasmodial and inhibition of nuclear factor-kB activation activities (Wang *et al*. 2015). It has also been shown different types of compounds from *S. doederleinii*, including biflavonoid, alkaloid, xylogen, sterol and organic acid (Wang *et al*. 2015).

Anti-tumor effect is an important pharmacological activity of *S. doederleinii*. The herb was commonly adopted to treat some major cancers in clinic, including chorionic carcinoma, malignant hydatidiform mole, nasopharyngeal carcinoma, esophageal cancer, gastric cancer, liver cancer, lung cancer, cervical cancer etc (Wang *et al*. 2014). Acording to Kosuge *et al*. (1985), the ethanol extract in *S. doederleinii* could inhibit the growth of ehrlich ascites tumor of mice *in vivo* and *in vitro* experiments. Lee *et al.* (2005) considered that the methanol extracts from *S. doederleinii* showed strong inhibition against colon cancer cells, fine bronchoalveolar cancer and chronic myeloid leukemia *in vitro* experiments. Moreover, antitumor activity of the methanol extracts was far higher than those of ellipticine that was used as a positive reference.

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Meanwhile, modern medicine means that the formation of tumors had important relation with oxidative damage in human body. The reason was that reactive oxygen species (ROS) that was produced in body contributes to an array of normal physiology metabolism and clears excess free radicals at the same time under normal condition. But if the reaction mechanism was damaged, the excess free radicals will affect the human body, leading to the damage of tissues and cells. Therefore, when the free radicals were excessive or human antioxidants and repair function was damaged, it can cause oxidative stress damage, producing various tumor cells (Ayaz *et al*. 2014). It has always been a research hot spot to explore natural antioxidant of plants (Leiter 2014). There are reports that volatile oil, flavonoids and alkaloids can be used as potential antioxidant (Florence 1995).

Antitumor and antioxidant activities of volatile oil of *S. doederleinii* was evaluated from various habits (Wang *et al*. 2013). However, there is no other information on biological activity of other extract of *S. doederleinii*. The aim of this reasearch is to evaluate bioactive feature of different fractions of *S. doederleinii*, including phytochemical screening, antioxidant, antibacterial and cytotoxicity activities, which provide theoretical basis for further comprehensive research of *S. doederleinii*.

Materials and Methods

The herbs (collected from Gui Zhou Simianshan herbal market on 16/03/2016) were harvested in China, and identified as *S. doederleinii* (Wang *et al*. 2013) by vice-professor Zhang Yu-Jin from the Department of Pharmacy in Zunyi medical college. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzthia-zoline-6-sulphonate) (ABTS) and ferrozine, 3-(4,5- dimethyl-thiazolyl-2)- 2,5-diphenyltetrazolium bromide (MTT), butylated hydroxytoluene (BHT), vitamin C, quercetin, cisplatin and roxithromycin were purchased from Sigma Pure Chemical Industries (Berlin, Germany). All other reagents were used as analytical grade and purchased from Changzheng Chemical Company (Chengdu, China). Human lung carcinoma cell strain (A549), human hepatic carcinoma cell strain (7721), human Hela cell line and human Eca-109 cell line were purchased from Shanghai cell bank of Chinese Academy of Sciences. *Bacillus subtilis* (Ehrenberg) Cohn, *Staphylococcus aureus* Rosenbach, *Escherichia coli* T. Escherich and *Pseudomonas* were obtained from Chengdu Jinsheng Technology Co., Ltd. (Chengdu, China). One hundred g of dried *S. doederleinii* was powdered and extracted three times (each for 2 hrs) with 1.0 litre of 95% ethanol. The extracting solution was filtrated, mergered and centrifuged at 4000 rpm at 5°C. The supernatant was concentrated and dried under vacuum. Then 19.21 g extract was finally obtained. Then the extract was dissolved in warm water and used for liquid-liquid extraction with petroleum ether extraction (PEE), diethyl ether extraction (DEE), ethyl acetate extraction (EAE), methyl alcohol extraction (MAE) and water extraction (WE), successively. The five fractions were concentrated, dried and obtained 0.53, 0.78, 4.65, 3.79 and 8.21 g, respectively. All fractions were stored in refrigerator (4°C) for further use.

Phytochemical screening of all the five fractions was carried out to identify the phytochemical constituents (organic acid, phenols, alkaloids, anthocyanins, biflavonoids, chalcones, coumarins, anthraquinones, free steroids, free tetracyclic triterpenes and polysaccharide) using the standard procedures (Ayoola *et al*. 2008).

Standard procedure was followed for total phenols content using Folin-Ciocalteu's reagent and measured at 725 nm (Alhakmani *et al*. 2013). Gallic acid was used as standard for a calibration curve; the total phenol was expressed in gallic acid equivalents. Total biflavonoids were determined by measuring the sample absorbance at 415 nm (Kalita *et al*. 2013). Quercetin was used as standard for a calibration curve; total biflavonoids content was expressed in quercetin equivalents. Each of the above assays using five fractions of *S. doederleinii* extracts was performed in triplicate.

The determination method of ABTS radical scavenging ability was subtly improved following De *et al*. 2003. An amount of 0.007 mol/l ABTS solution was mixed well with 0.140 mol/l potassium persulfate in water solution and left standing overnight in the dark at room temperature to generate $ABTS^{+\cdot}$. The samples from five fractions were dissolved with methyl alcohol and was diluted to different concentrations (30, 60, 90, 120 and 150 µg/ml). 0.4 ml of each sample was mixed with 2.5 ml $ABTS^{+\cdot}$ solution, left standing for 10 min at room temperature and determined the absorbance of the sample at 734 nm in a UV spectrophotometer. The scavenging rate can be calculated according to the following formula: $I(\%) = [(A_b - A_s)/A_b] \times 100\%$. Where I is the inhibition percentage, A_b is the absorbance of the blank sample and A_s is the absorbance of the test sample. The regression equation can be done based on the radical scavenging ability to calculate IC_{50} value of radicals scavenging.

Determination of antioxidant activity was performed using the method of Chen *et al*. (1999). 0.5 ml of each sample with different concentrations from 5 fractions was mixed with 2 ml DPPH ethanol solution (0.2 mmol/l) separately. The mixture was shaken well, produced reaction and left standing at room temperature in the dark for 60 min. The absorbance was measured at 517 nm in the UV spectrophotometer. IC_{50} was calculated against sample of different concentrations.

The reducing power of the samples was measured using the method of Kim (Uchida *et al*. 1989) with minor modification. 1.2 ml of the sample with different concentrations were mixed with 1.0 ml phosphate buffer solution (PBS, 0.2 mol/l, pH 6.6) and 1.0 ml 1% potassium ferricyanide. The mixture was reacted for 20 min in 50°C and quickly chilled. Afterwards, the mixture was mixed well with 1.0 ml 10% trichloroacetic acid and 5 ml distilled water, and then centrifuged at 3000 r/min for 10 min. 2.5 ml of the supernate was mixed with 0.5 ml 0.1% ferric trichloride solution and 2 ml distilled water and left standing for 10 min. Absorbance (A) was measured at 700 nm in the UV spectrophotometer.

The chelation of ferrous ions is estimated using the method of Sharma (2006). 0.1 ml of each sample was added to a solution of 0.7 ml ferrous chloride (0.2 mmol/l). The mixture was diluted with 1.9 ml distilled water and reacted with 0.6 ml of ferrozine (5 mmol/l) at room temperature for 10 min. Absorbance was measured at 562 nm and then the IC_{50} of different samples was calculated.

In vitro cytotoxic activity was determined by the MTT assay against the A549 (lung), Eca-109 (esophageal), 7721 (liver), Hela (cervical) cancer cell lines (Wang *et al*. 2016). 20 µl of the samples with different concentrations (30, 60, 90, 120 and 150 µg/ml) of five fractions from *S. doederleinii* were mixed with each cell line, respectively. Absorbance of the mixture was measured at 570 nm in the enzyme-linked immunosorbent assay tester (DG5033A, Huadong electronics co., Nanjing, China). IC_{50} of each sample was calculated by plotting the inhibition percentage against different concentrations sample. Cisplatin (in the 5 - 30 µg/ml range) was used as a positive control.

The agar diffusion method was used for the antimicrobial evaluations. Wells of 6 mm diameter were poured into the sterile Mueller Hinton Agar with the test microorganisms and filled with 100 µl of five fractions (120 mg/ml). The plates were incubated for 18 hrs at 37°C. Antimicrobial activity was evaluated by measuring the inhibition zone in millimeter in diameter and recorded.

In order to verify the statistical significance, all analyses were carried out in triplicate and results were expressed as mean ± SD. In the assessment of correlation between total phenolics, total biflavonoids content, and the antitumor, antioxidant activity, respectively. Pearson correlation coefficients (R^2) were adopted to determine the differences. A value of $R^2 > 0.5$ was considered to be statistically significant correlationship.

Results and Discussion

Total biflavonoids content in fractions (Fig.1A) revealed that EAE fractions presented the highest content (31.98 ± 1.49 mg/g) followed by MAE fraction >DEE fraction >WE fraction >PEE fraction. Total phenolics content of the plant fractions is shown in Fig. 1B. Among five fractions, MAE had the highest phenolic content (28.31 ± 1.85 mg/g), followed by the fractions of EAE, PEE, WE and DEE.

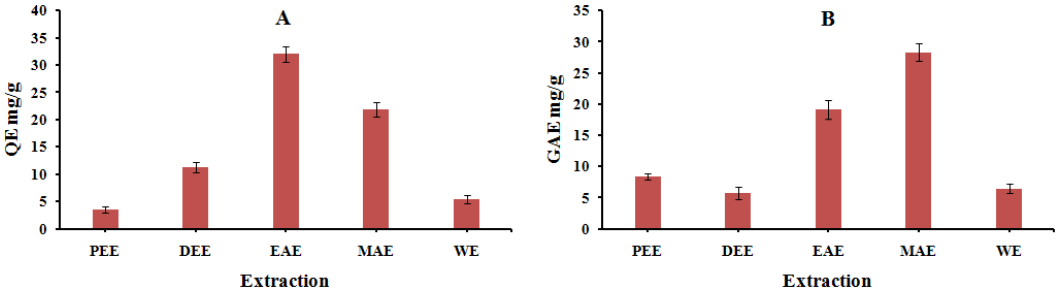

Fig. 1. Total biflavonoids (A) and total phenolics (B) contents of *S. doederleinii* fractions.

Phytochemical profile of *S. doederleinii* is shown in Table 1. Organic acid, free steroids and free pentacyclic triterpenes were detected in PEE because of their small polarity. Coumarins and anthraquinones were detected in DEE, but they were not detected in other extracts obtained from *S. doederleinii*. Among five fractions, biflavonoids, anthocyanins and chalcones were only found in EAE obtained for its minor polarity. Phenolics and alkaloids were detected in MAE obtained for their major polarity. Polysaccharide was only identified in WE obtained while it was not found in other extraction. It was shown that as polarity of extracted solvent was different as a result of polarity difference of chemical compound extracted. This results suggested that the extraction method played an important role in the metabolite profile of the extracts. It was reported that ethanolic extracts obtained from *S. doederleinii* contained of biflavones, phenols, free steroids, triterpenes, anthraquinones and anthraquinones (Dan *et al*. 2017). However, according to the above research, anthocyanin and chalcones were firstly found in EAE fraction of ethanol extracts from *S. doederleinii*.

Compared with the antioxidant activity of five fractions, four antioxidant assays were investigated including DPPH radicals scavenging, ABTS radicals scavenging, chelation of ferrous ions and ferric reducing at different concentrations (30 - 150 μg/ml). As shown in Fig. 2, all the samples exhibited similar activities at high concentration. Among them, Fig. 2A revealed that the scavenging effects of three samples on ABTS radical activites decreased successively in the following order: EAE > MAE > EAE > WE > PEE. In addition, the inhibition percentage of five samples was obviously added with the improvement of each sample concentration. It was concluded that EAE exhibited better radical scavenging activity and the maximum ABTS inhibition percentage of EAE was 90.39 at 150 μg/ml.

The DPPH assay was also a common radical scavenging method of natural antioxidants. When considering the antioxidant effects of five samples, the results were evaluated (Fig. 2B). The results indicated that EAE has the strongest radical scavenging capacity with 85.77% at 150 μg/ml and MAE, EAE, WE, PEE follow suit. Moreover, the results of analysis were almost consistent with those of ABTS radical scavenging mentioned above (Fig. 2B). On the basis of the results of above

mentioned analysis, researchers thought the antioxidant capacity of EAE might contain many minor polarity compounds with phenolic hydroxyl groups (Birasuren *et al*. 2013).

Table 1. Phytochemical profile of the extracts obtained from *S. doederleinii*.

Class of metabolites	PEE	DEE	EAE	MAE	WE
Organic acid	+	-	-	-	-
Phenolics	-	-	-	+	-
Alkaloids	-	-	-	+	-
Coumarins	-	+	-	-	-
Polysaccharide	-	-	-	-	+
Biflavonoid	-	-	+	-	-
Anthocyanins	-	+	-	-	-
Chalcones	-	-	+	-	-
Free steroids	+	-	-	-	-
Free triterpenes	+	-	-	-	-
Anthraquinones	-	+	-	-	-

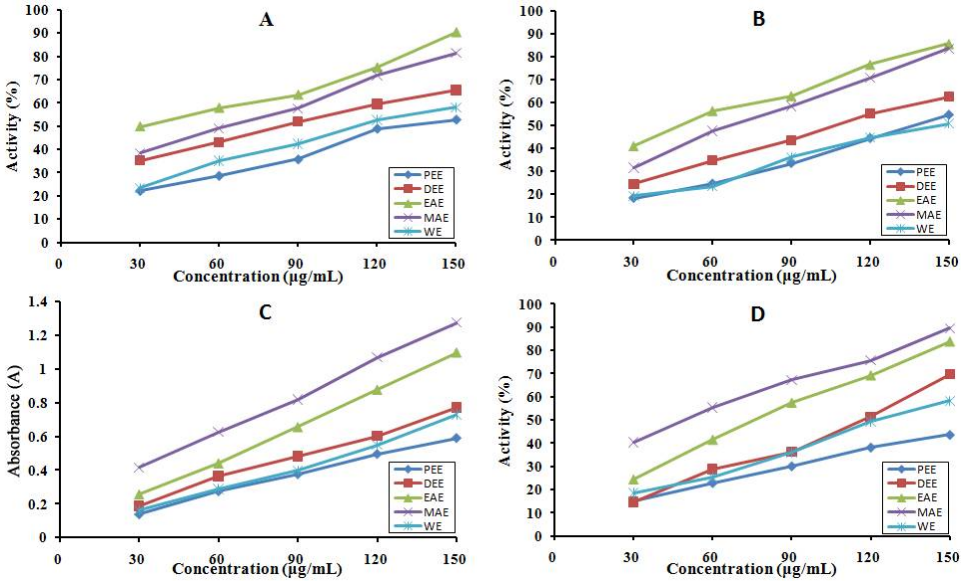

Fig. 2. Comparison of antioxidant of different fractions from *S. doederleinii*. A: ABTS radical scavenging ability. B: DPPH radical scavenging ability. C: Reducing power. D: Ferric chelation power.

Reducing power test was usually evaluated the antioxidant ability of natural products by the principle of Fe^{2+} reduction. Fig. 2C implied that all the samples exhibited similar activities at high concentration. When the concentration of five samples at different concentrations was constantly increased, the reducing power significantly increased. Among five fractions, the antioxidative activity of MAE has the strongest reducing power.

According to Sharma (2006), chelation power of ferrous principle was described as follows: Iron ions were known to promote the conversion of less reactive species such as hydroxyl, peroxyl/alkoxyl radicals and the release of iron ion can accelerate oxidative damage. Fig. 2D indicated that ferrous chelation capacity increased significantly. The antioxidative activity of MAE has the highest ferrous chelation capacity at different concentrations with 82.51% at 150 μg/ml. The antioxidant capacity of the MAE might contain due to mainly many phenolic compounds (Birasuren *et al*. 2013).

The correlation coefficient (R^2) was assessed between four antioxidant activities, namely ABTS, DPPH, reducing capacity and chelation power of ferrous and phenolic content or biflavonoid content, respectively. The results illustrated that biflavonoids of five fractions showed better antioxidant activity to four antioxidant methods with high correlation coefficient values (R^2 = 0.883, 0.878, 0.834, 0.814, respectively), and phenols also had potent antioxidant effects with correlation coefficient values (R^2 = 0.797, 0.665, 0.790, 0.721, respectively). Biflavonoids and phenolics tended to be significant activity for exhibiting antioxidant activity.

Among various fractions against A-549 cell line, 7721 cell line, Hela cell line and Eca-109 cell line, EAE has the strongest cytotoxicity (52.66 ± 2.72, 66.20 ± 3.04, 37.53 ± 1.91, 62.09 ± 2.41 μg/ml, respectively), while WE has the weakest cytotoxicity (253.73 ± 7.56, 346.94 ± 10.15, 311.69 ± 9.17, 297.15 ± 10.26 μg/ml, respectively) in Table 2. But the anticaner activity of five fractions was less potent than the positive controls, namely cisplatin (20.89 ± 1.44, 6.27 ± 1.53, 16.36 ± 1.88, 29.08 ± 3.29 μg/ml, respectively). The above results showed that biflavonoids of five fractions exhibited better anticancer activity against four cell lines with high correlation coefficient values (R^2 = 0.915, 0.834, 0.818, 0.718, respectively), and phenols had potential antitumor effects with general correlation coefficient values (R^2 = 0.792, 0.752, 0.654, 0.649, respectively). Biflavonoids tended to have obvious influence for exhibiting anticancer activity than phenols.

Table 2. IC_{50} values (mg/ml) of different extracts obtained from five fractions for anticancer test.

Treatment	A 549 cell line IC_{50} (μg/ml)	7721 cell line IC_{50} (μg/ml)	Hela cell line IC_{50} (μg/ml)	Eca-109 cell line IC_{50} (μg/ml)
PEE	214.29 ± 8.02	255.65 ± 9.36	186.27 ± 7.76	173.76 ± 8.10
DEE	174.12 ± 4.13	190.35 ± 6.21	155.40 ± 5.49	106.44 ± 4.59
EAE	52.66 ± 2.72	66.20 ± 3.04	37.53 ± 1.91	62.09 ± 2.41
MAE	90.51 ± 2.99	81.74 ± 4.67	79.02 ± 3.58	86.58 ± 3.69
WE	253.73 ± 7.56	346.94 ± 10.15	311.69 ± 9.17	297.15 ± 10.26
Cis	20.89 ± 1.44	6.27 ± 1.53	16.36 ± 1.88	29.08 ± 3.29

PEE fraction showed significant antibacterial activities against all bacterial strains tested, which demonstrated 11 - 13 mm zone of inhibition against Gram positive and Gram negative bacterial strains among five fractions, followed by WE fraction (10 - 12 mm, Table 3). In addition, antimicrobial activities of five fractions were obviously less than that of roxithromycin (17 - 33 mm). It was worth mentioning that five fractions of *S. doederleinii* had similar antimicrobial activities on *E. coli* and *Pseudomonas.* Five fractions had potent antibacterial activities against Gram positive and Gram negative bacterial strains, specially PEE fraction.

As shown in Fig. 2 and Table 2, EAE and MAE of ethonal extracts from *S. doederleinii* had relatively stronger anti-oxidative and anticancer capacity, while DEE, WE and PEE had relatively weaker anti-oxidative and anticancer capacity. This activity of EAE and MAE had been likely attributed to their phenolics and biflavonoids which are secondary metabolites present in various

plant parts. These compounds have properties to neutralize the active moieties during the metabolism such as free radicals and may prevent pathological conditions involving free radicals including cancer, cardiovascular and neurodegenerative anomalies (Chen *et al*. 1999). Both phenolics and biflavonoids produce their antioxidant activity by acting as hydrogen or electron donor to free radicals, by reacting with radicals to form less reactive compounds or by chelating transition metals which may act as prooxidants and can lead to degeneration of body systems (Renukadevi *et al*. 2011). The difference of phenolics or biflavonoids in various fractions utilized in the study may be due to the nature of polarity of different solvents and the deviation of phenolics or biflavonoids solubilized from the solvent, which may result in the difference of anti-oxidative and cytotoxicity activity tested in five fractions from *S. doederleinii.*

Table 3. Antibacterial activities of different fraction of ethanol extracts of *S. doederleinii* (IZ, mm).

Fractions	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas*
PEE	12.2 ± 0.5	13.1 ± 0.4	13.5 ± 0.6	11.7 ± 0.5
DEE	8.5 ± 0.1	8.2 ± 0.2	9.3 ± 0.4	8.2 ± 0.2
EAE	9.1 ± 0.2	7.3 ± 0.3	8.3 ± 0.1	7.6 ± 0.1
MAE	7.6 ± 0.2	8.1 ± 0.1	11.6 ± 0.3	9.4 ± 0.3
WE	10.4 ± 0.3	12.2 ± 0.3	11.2 ± 0.4	10.3 ± 0.2
Roxithromycin	26.3 ± 0.3	33.1 ± 0.2	17.9 ± 0.6	28.5 ± 1.1

As shown in Table 3, PEE of ethonal extracts had the highest antibacterial effects among five fractions. This might be related to rich terpene components, which had significant antibacterial activity. For instance, 6-oxo-genipin obtained from the aerial parts of *Canthium multiflorum* demonstrated significant inhibitory activity against five microbial strains tested, specially the pathogen of *Staphylococcus aureus* with minimum inhibitory concentrations (MIC) of 0.01 µg/ml (Simeon *et al*. 2013). Among five pseudoguaianolide sesquiterpenes, neoambrosin showed the highest antibacterial activity with MIC values of 150 and 90 mg/l against two plant pathogenic bacteria, namely *Agrobacterium tumefaciens* and *Erwinia carotovora*, respectively (Mohamed *et al*. 2014).

Present investigation concludes that fractions from *S. doederleinii* had better anti-oxidation and antitumor activity, particularly EAE. A very good correlation was found between biflavonoid of five fractions and antioxidant or anticaner capacity, which was implied that biflavonoids played a critical role for anticancer action of *S. doederleinii*. Among five fractions, PEE showed more efficient inhibition zone against four bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas*, which indicated that *S. doederleinii* can be used as potential antibacterial agent. *S. doederleinii* is extensively growing in South China and is of very high medicinal value. However, the research of *S. doederleinii* is currently very limited, specially there was no systematic pharmacological studies, resulting in lack of theoretical supports to its clinical application. The study in this paper is of certain reference values for further development about utilization of *S. doederleinii* resources and its application in food and medical domains.

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