Research Paper

Evaluation of DPPH Free Radical Scavenging Activity of Various Extracts of *Ligularia fischeri In Vitro*: A Case Study of Shaanxi Region

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A comparison study of the antioxidative activities was conducted *in vitro* on the different extracts from the different origins, harvest seasons, solvents and plant parts of *Ligularia fischeri* grown in Shaanxi. Different parts of *L. fischeri*, such as roots and rhizomes, stems, leaves, scapes and fruits, were extracted by an ultrasonic-assisted extraction method with water, 95% ethanol, n-butanol, ethyl acetate and chloroform, respectively. Diphenyl picryl hydrazyl method was used to evaluate the antioxidative activities of these extracts. The result of antioxidative study showed that *L. fischeri* picked in September showed higher activities than that those picked in October. The extracts of *L. fischeri* from different solvents presented different free radical scavenging activities: water>95% ethanol>n-butanol>ethyl acetate>chloroform. The different herbal parts also showed the different antioxidative activities. The present work will provide a reference for the further research and exploitation of *L. fischeri*.

Key words: *Ligularia fischeri*, extracts, antioxidative activities, DPPH method, radical scavenging activities

Ligularia fischeri (Ledebour) Turcz., a perennial herbaceous plant, belongs to the Compositae family. It is consumed as an important wild vegetable in Korea[1] and Jilin Province of China[2], and also the area of cultivation has increased gradually year by year. With the function of analgesia and cough expectorant, it has also been used for the treatment of traumatic injury, lumbocrural pain, whooping cough and other diseases[3]. The herbs, whose medicinal part is normally regarded as roots and rhizomes, are mainly distributed in Gansu, Shaanxi, Sichuan, Hubei, Henan, Anhui, Zhejiang and northeast of China. In Shaanxi province, it was harvested in the Qinling and Daba Mountain[4,5].

Free radicals, which are produced by the chemical reaction of organic compounds, could damage the body's tissues and cells, leading to human aging and causing a variety of diseases. Therefore, it is very important to find the antioxidants for scavenging these free radicals. Various *in vitro* and *in vivo* methods have been developed for the assessment of antioxidative activities.. From the standpoint of *in vitro*, several methods have been proposed for evaluating the antioxidative activity, such as 1,1-diphenyl-2-pierylhydrazyl (DPPH) method, 2,2'-azino-bis-(3-ethylbenzo thiazolin-6-sulfonic acid) diammonium salt (ABTS) method, ferric reducing antioxidant power (FRAP) method and so on[6-9]. DPPH method is one of the universal tools for estimating the antioxidative activities of the different products. DPPH radical, a very stable nitrogen-centered radical, can be used to determine the free radical scavenging ability, which is related to their antioxidative activities. The method is based on the spectrophotometric measurement of $DPPH^{\cdot}$ concentration changes resulting from the $DPPH^{\cdot}$ reaction with an antioxidant.

Up to now, some studies about the chemical composition and biological activities of *L. fischeri* have already been reported. Some studies of *L. fischeri* have shown that it contains sesquiterpene compounds in roots[10-12], volatile oils in rhizomes[13] and trace elements in leaves[14]. In addition, there are some reports showing that the tender stems of *L. fischeri* contain many

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organic nutrition compounds, such as protein, crude fiber, reducing sugar, vitamins, trace elements and so on[15,16]. In addition, these chemical constituents may be related to the biological activities of *L. fischeri,* including antioxidative activities.

Moreover, several experimental studies of *L. fischeri* have demonstrated some pharmacological effects. Researchers have found that the extracts of *L. fischeri* show significant antiinflammatory activity[17-19]. The ethanol extracts have protective effect on ethanol-induced gastric ulcer and acute alcoholism in mice[20,21]. Other studies have shown that it has strongly increasing humoral immunity function[22] and obviously expectorant effect in mice[23]. The extracts of *L. fischeri* were also reported to present the potent free radical scavenging activities by DPPH method[24].

Overall, researches on chemical constituents and biological activities of *L. fischeri* have a certain foundation. It has been reported that the leaf extracts of *L. fischeri* from methanol and *n*-butanol have effective antioxidative activities *in vitro*, which were evaluated by DPPH method. The extract solvent included methanol, dichloromethane, *n*-butanol and water[24]. However, the antioxidative activities of other herbal parts or extraction parts of *L. fischeri* have not been reported yet. Therefore, the antioxidative activities of different herbal parts (such as roots and rhizomes, stems, leaves, scapes and fruits) of *L. fischeri* extracts from different solvents (e.g. water, 95% ethanol, n-butanol, chloroform, and ethyl acetate) were determined in this work. Moreover, it is employed to compare the antioxidative activities of the different extracts among the different solvents, origins, parts, as well as harvest seasons. Thus, the present research is necessary for screening the potential medicinal parts, and also contribute to the in depth research and exploitation of natural antioxidant from *L. fischeri*.

MATERIALS AND METHODS

L. fischeri was collected from Hu County and Mei County (Shaanxi, China). The herbs were authenticated by Xiezhimin from Xi'an Food and Drug Inspection Institute and Shijuan from Xi'an Jiaotong University based on their morphology. The samples of *L. fischeri* (Table 1) included the roots and rhizomes from Mei County in September; the roots and rhizomes, stems, leaves, scapes, fruits from Hu County in September and October. After washing and drying under the natural condition, the herbs then dried for 48 h by 101-2AB type electrothermal blowing at 50° (Tianjin Teste Instrument Co., Ltd.) and then pulverized respectively by FZ102 type micro mill plants (Tianjin Teste Instrument Co., Ltd.). DPPH was purchased from Shanghai Sonny Biological Technology Co., Ltd. All the reagents used were of analytical grade.

All samples of the dried *L. fischeri* herbal parts were ground to powder, and then 1.0 g of each sample was weighed and extracted for 60 min by ultrasonication (KQ3200DB, Kunshan Ultrasonic Instrument Co., Ltd., China) with 50 ml of water, 95% ethanol, *n*-butanol, ethyl acetate and chloroform, respectively. Shaken well, filtered and got the subsequent filtrate. The concentration of extracts was equal to 20 mg/ml. Then the above solution was diluted in methanol at the following concentrations as the samples: 2, 4, 6, 8 and 10 mg/ml.

The appropriate amount of ascorbic acid as a positive control was accurately weighed and then prepared as the stock solution with a concentration of 25 μg/ml. Different volumes of 1, 2, 4, 6, 8 and 10 ml from the stock solution were transferred into different volumetric flask with 10 ml methanol. The concentrations of ascorbic acid were equal to 2.5, 5.0, 10.0, 15.0, 20.0, 25.0 μg/ml, respectively. The samples and the stock standard solution were stored at 4° before analysis.

Scavenging activities on DPPH radical:

The radical scavenging activities of the extracts were measured via the reference method[25]. A two milliliter of the sample solution in methanol was mixed with two

TABLE 1: DESCRIPTION OF THE SAMPLE OF *L. FISCHERI* FROM SHAANXI PROVINCE OF CHINA

Sample number	*L. fischeri* parts	Origin
1	roots and rhizomes	Mei County in September
2	roots and rhizomes	Hu County in September
3	leaves	Hu County in September
4	fruits	Hu County in September
5	stems	Hu County in September
6	scapes	Hu County in September
7	roots and rhizomes	Hu County in October

milliliter of 0.04 mg/ml DPPH solution in methanol. After reaction for 60 min at room temperature in dark conditions, the absorbance values of the sample were measured by a UV probe-2450 spectrophotometer (Shimadzu, Kyoto, Japan) at 515 nm (λ_{max}) and calculated as a percentage of radical scavenging activities (%RSA). The assays were carried out in five copies. The %RSA values in the examined system, associated with the amount of remaining DPPH•, was the measure of antioxidative activities of samples as follows: $\%RSA = [1-(A_i-A_j)/A_o] \times 100\%$. Where, the A_i are the absorbance of 2 ml of sample solution mixed with 2 ml of 0.04 mg/ml DPPH solution; the A_o are the absorbance of 2 ml of MeOH mixed with 2 ml of 0.04 mg/ml DPPH solution; the A_j are the absorbance of 2 ml of MeOH mixed with 2 ml of sample solution.

Statistical analysis:

All the results were expressed as the mean±standard deviation (SD). In order to determine the measurements reproducibility, the antioxidative activity assay of each sample was measured for three times. Statistical analysis was performed by one-way ANOVA and Student's t-test. All analyses were conducted at the 95% confident level, with solvents, origins, parts, and harvest seasons as class variables in the experiment model. P-value less than 0.05 was assumed as the statistically significant difference between the experimental points.

RESULTS AND DISCUSSION

Many preliminary experiments have been conducted in order to select the optimal experimental conditions. The maximum absorption wavelength of 515 nm was determined by scanning the wavelength in the range between 400 nm and 760 nm for DPPH solution and DPPH mixed with the sample solution. In this case, the decrease in the DPPH absorbance was registered in continuous manner to the reaction steady state (about 60 min). Therefore the reaction time was chosen as 60 min. The DPPH solution in methanol was stable within 5 h through the whole process of the stability test study.

The following equation of calibration curve was used to calculate the concentration of DPPH• in the reaction system: y=0.0272x+0.0098. A standard curve was prepared using the different concentrations of DPPH•. The DPPH• scavenging capacities were calculated from the obtained calibration curve determined by linear regression (R^2=0.9993).

The %RSA of ascorbic acid is showed in Table 2. The ascorbic acid presents the potent free radical scavenging activities, which is associated with strong antioxidative activities.

The roots and rhizomes samples of *L. fischeri* in September were collected from Mei County and Hu County. As shown in fig. 1, the %RSA of extracts from Mei County were higher than that of Hu County (P<0.05), indicating that the *in vitro* antioxidative activities of Mei County were more effective, and all extracts of *L. fischeri* from Mei County in different solvents had the same results. The extracts of *L. fischeri* from Mei County in different solvents had high antioxidative activities, which should be the preferred option to research and develop.

L. fischeri was collected from Hu County in September and in October. It could be seen from the fig. 2 that the %RSA of roots and rhizomes extracts from different harvest seasons (in September and in October) had no significant difference in water and 95% ethanol

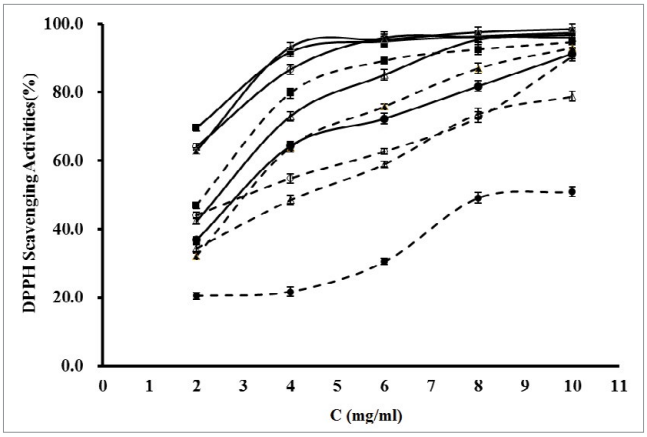

Fig. 1: %RSA of *L. fischeri* samples of different concentrations extracts in different solvents.

Mei County (solid line) and Hu County (dotted line). The extracted samples in different solvents collected from Mei County: water (—■—), 95% ethanol (—▲—), *n*-butanol (—○—), ethyl acetate (—△—), chloroform (—●—); and from Hu County: water (– ■ –), 95% ethanol (– ★ –), *n*-butanol (– ○ –), ethyl acetate (– △ –), chloroform (– ● –). Data are expressed as mean±SD of five independent experiments for each antioxidant. Means marked with different letters are significantly different (P< 0.05).

TABLE 2: FREE RADICAL SCAVENGING ACTIVITIES OF THE ASCORBIC ACID

Number	1	2	3	4	5	6
Concentration (μg/ml)	2.5	5.0	10.0	15.0	20.0	25.0
DPPH scavenging rate (%)	26.3	70.1	82.5	87.6	96.0	100.0

(P>0.05). While in the rest of the solvents, chloroform, ethyl acetate, *n*-butanol, the mean values of the DPPH scavenging activities of *L. fischeri* harvested in September were higher than that of in October (P<0.05). This shows that the *in vitro* antioxidative activity of *L. fischeri* was more effective in September than that of in October. These results may be related to climate. In addition, the %RSA of leaves extracts from all solvent had the greater significantly difference (P<0.05) than other herbal parts extracts, indicating that the antioxidative activities of *L. fischeri* leaves were affected by the harvest seasons.

L. fischeri collected from Hu County in September (fig. 3), when extracted in water or ethanol, the %RSA of leaves or fruits was higher than that of other herbal parts (P>0.05). And the highest %RSA of roots and rhizomes could be obtained by extracting in chloroform or ethyl acetate. The %RSA of water extracts was higher that of ethyl acetate extracts. It could be ascribed that the stronger polarity solvent used to be extracted, the higher clearance rate of the extracts would be. Shown as the fig. 3, no matter what solvent used to be extracted, the %RSA of extracts from roots and rhizomes, leaves, fruits were higher than that of stems and scapes (P<0.05). Therefore, based on the fig. 3 a-c, the antioxidative activities of different parts *in vitro* were as follows: leaves≈fruits>roots and rhizomes>stems>scapes. Medicinal part of *L. fischeri* is normally regarded as the roots and rhizomes, while its leaves and fruits have a better effect in the antioxidative activities than that of other portions. It might provide valuable information for screening the new antioxidant effective parts of *L. fischeri*.

The radical scavenging activities of different concentrations of *L. fischeri* extracts in different solvents from three different parts (roots and rhizomes, leaves, fruits) were compared (fig. 4). It could be known from fig. 4 that the %RSA of *L. fischeri* extracts from water and ethanol were higher than that of chloroform, ethyl acetate and *n*-butanol with no significant difference (P>0.05). In a word, the %RSA of different solvents extracts was as following: water≈95% ethanol>*n*-butanol>ethyl acetate>chloroform (fig. 4).

Previous research already reported that the leaves extracts of *L. fischeri* from methanol and *n*-butanol shown potent free radical scavenging activities with DPPH method, which is associated with antioxidative activities. However, the antioxidative activities of other herbal parts or extraction parts of *L. fischeri* have not been reported. In this study, we used five kinds of solvents to extract the various parts of *L. fischeri* from two origins in different harvest seasons. %RSA was compared in five different concentrations. The results showed that *L. fischeri* picked in September had higher activities than that of in October. The activities of *L. fischeri* picked from Mei County were more effective than that of from Hu County. The antioxidative activities effective parts of the plant were leaves, fruits, roots and rhizomes. The better solvents for extracting *L. fischeri* were water and 95% ethanol for antioxidative activity research. In other words, the stronger polarity solvent used for extraction, higher the antioxidative activities of extracts would be. These results suggested that the antioxidative ingredient in the plant of *L. fischeri* might be some polarized compounds, which needed to be clarified and characterized by further relative research.

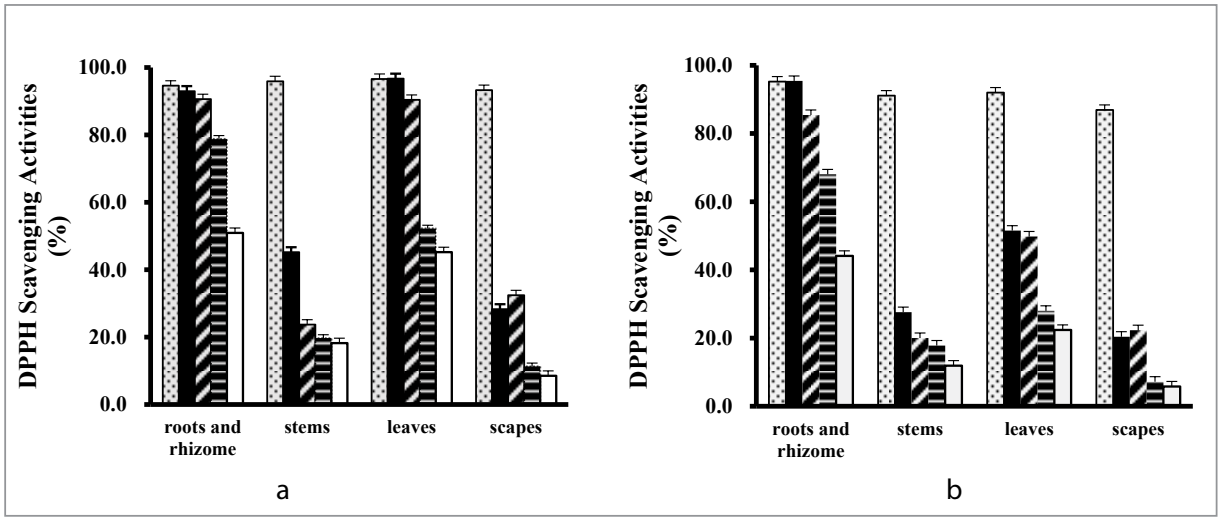

Fig. 2: %RSA of *L. fischeri* samples of the different herbal parts in different solvents harvested in September and October.

The samples harvested in September (a) and October (b) extract in different solvent are expressed as the following: water (▨), 95% ethanol (■), *n*-butanol (▨), ethyl acetate (▤), chloroform (□). Results were expressed by mean±SD (n=5).

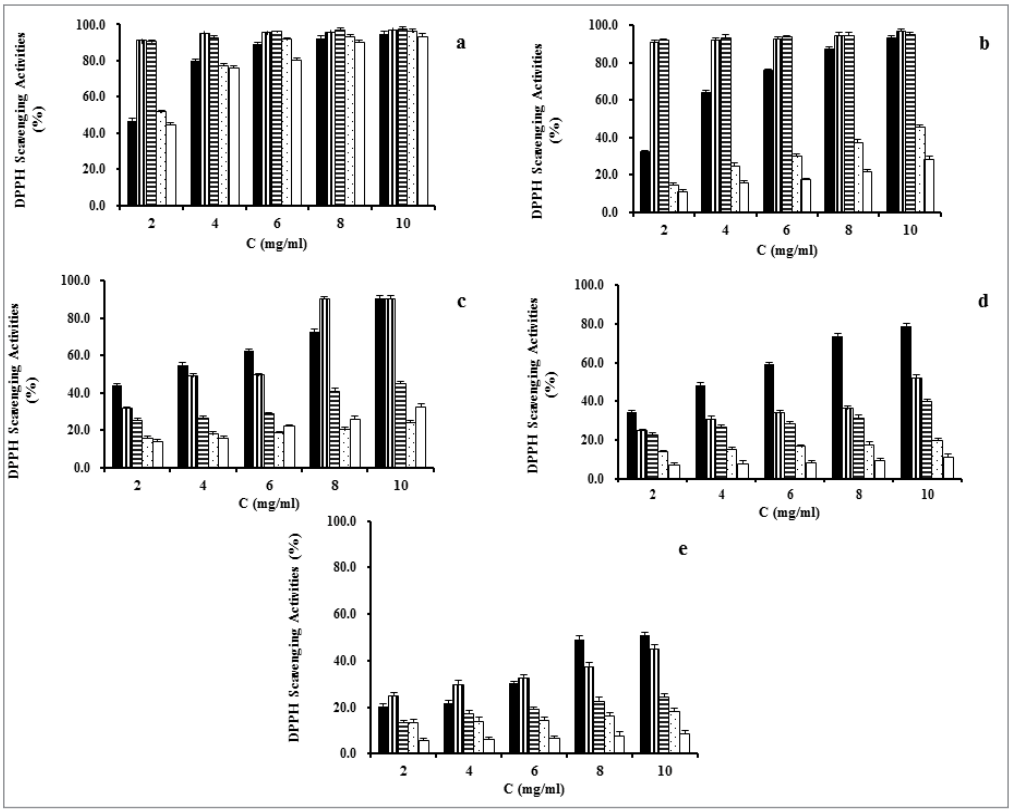

Fig. 3: %RSA of *L. fischeri* samples of different concentrations extracts from different parts in different solvents.
The samples of different herbal parts extract in different solvent (water (a), 95% ethanol (b), *n*-butanol (c), ethyl acetate (d), chloroform (e)) are expressed as the following: roots and rhizomes (■), leaves (▥), fruits (▤), stems (▨), scapes (□). Results are expressed as mean±SD (*n*=5).

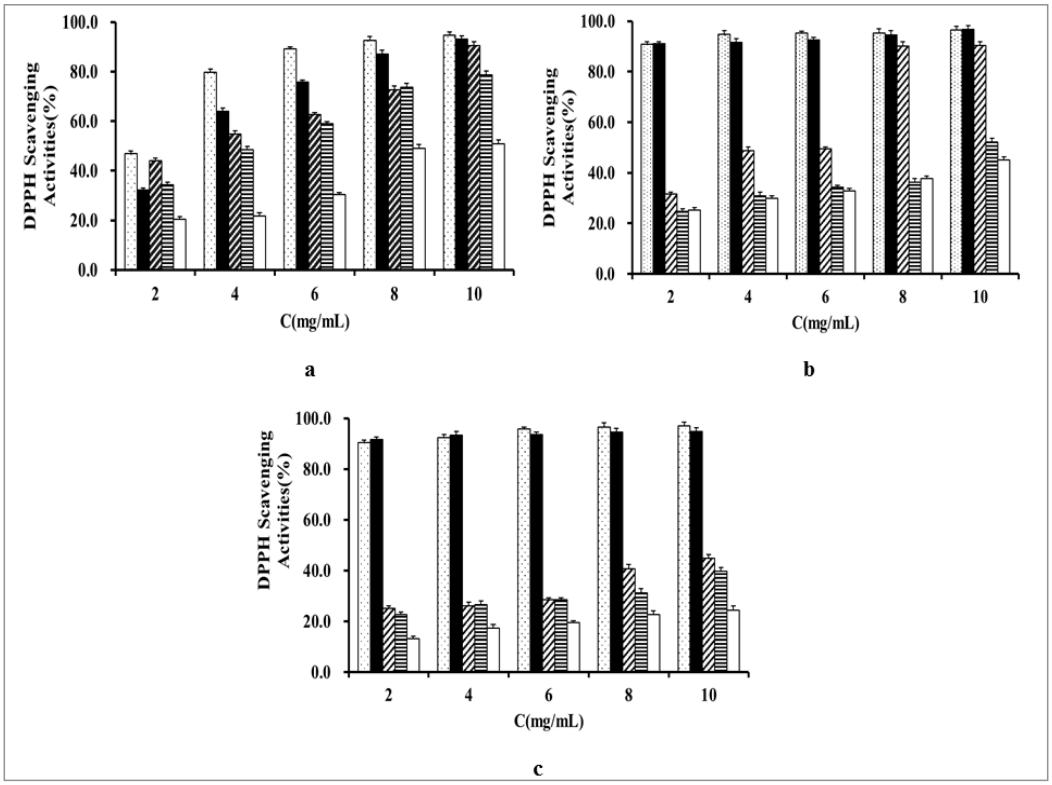

Fig. 4: %RSA of *L. fischeri* samples in different solvents from three herbal parts.
***L. fischeri* samples of three herbal parts, roots and rhizomes (a), leaves (b), fruits (c) in different solvent are expressed as the following: water (▨), 95% ethanol (■), *n*-butanol (▨), ethyl acetate (▤), chloroform (□). Results were expressed by mean±SD (*n*=5).**

Since the hydroxyl group was a positive functional group in a structure, it was speculated that the active compound might also contain these groups in their skeletons. Therefore, these polar properties should be considered during the possible further isolation process. In addition, the antioxidative activity compounds with polar properties in *L. fischeri*, which can be served as effective natural antioxidants, need further isolation and structure identification.

In this work, different parts of *L. fischeri,* including roots and rhizomes, stems, leaves, scapes and fruits, were extracted in water, 95% ethanol, *n*-butanol, ethyl acetate and chloroform respectively, which were carried out by an ultrasonic-assisted extraction method. The extracts from different origins, harvest seasons, solvents and the herbal parts showed different antioxidative activities, which is measured by the DPPH method. Leaves and fruits of *L. fischeri* could be antioxidant effective parts of the herbs. And the stronger polarity solvent used for extraction, the higher antioxidative activities of *L. fischeri* extracts would be. This present work will contribute to the in depth research and exploitation of natural antioxidant from *L. fischeri.*

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Conflicts of interest:

There are no conflicts of interest.

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