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A comparative study of echinacoside, oleuropein content and antioxidant properties of different solvent extracts from *Syringa pubescens* Turcz

Yanfang Wu[1], Qinyu Yang[2], Danjie Wang[1], Ruixue Deng[2], Miao Yu and Xinsheng Wang[2,1]
[1]School of Basic Medical Sciences, Henan University of Science and Technology, Luoyang, China
[2]Chemical Engineering and Pharmaceutical School, Henan University of Science and Technology, Luoyang, China

Abstract: *Syringa pubescens* Turcz is commonly used folk medicinal herb in west of Henan Province of China. In this work, water and various concentration of methanol, ethanol and acetone in water were used as solvent to extract echinacoside and oleuropein from *S. pubescens*. The antioxidant properties of different extracts were evaluated using various in vitro assays. The highest yields of echinacoside and oleuropein were obtained by using the 60% aqueous methanol and 80% aqueous ethanol, respectively. The extracts of water, aqueous ethanol or methanol showed strong antioxidant abilities. Furthermore, the high correlation between echinacoside content and antioxidant properties was found. The contribution of oleuropein content was not significant to antioxidant abilities. These findings indicate that *S. pubescens* can be used as a new natural antioxidant resource.

Keywords: *Syringa pubescens* Turcz, glycoside, antioxidant activity, effect of solvent, HPLC analysis.

INTRODUCTION

Syringa pubescens Turcz, as a member of the family of Oleaceae, is used in Chinese folk medicinein west of Henan Province of China to treat hepatitis and cirrhosis (Wang *et al.*, 2020). In previous studies revealed *S. pubescens* had the potential for prevention and treatment of CCl_4-induced liver damagein rat model with water extract. There was reduction in inflammation, decrease in alanine transaminase level and reduction in the degree. Hepatoprotective activities have been associated with plant extracts rich in antioxidants (Awaad *et al.*, 2006; Bo Huang *et al.*, 2010; Panahi Kokhdan *et al.*, 2017; Sha *et al.*, 2020). However, the antioxidant capacities of *S. pubescens* have not been studied *in vitro*.

The major bioactive compounds from *S. Pubescens* are secoiridoid glycosides (Wu *et al.*, 2003)and phenylethanoid glycosides (Deng *et al.*, 2010). The echinacoside and oleuropein were the dominant bioactive ingredients in these glycosides (Liu *et al.*, 2011). Echinacoside from *Cistanche tubulosa* (Morikawa *et al.*, 2019) and oleuropein from olive (Ranalli *et al.*, 2009) showed significant hepatoprotective and antioxidant activities against liver injury (Domitrovic *et al.*, 2012; Kim *et al.*, 2010). Solvent extraction is the most common technique employed to obtain natural compounds and antioxidants (Alcantara *et al.*, 2019). The mixtures of methanol, ethanol, acetone with water are widely used to extract glycosides and antioxidant compounds (Barreto *et al.*, 2008; B. Huang *et al.*, 2010). However, the extraction of glycosides from *S. Pubescens* has not been investigatedin great detail.

*Corresponding author: e-mail: wxs501@gmail.com

The objective of the present work was to investigate the extraction efficiency of different solvent and antioxidant capacities of extract from *S. pubscence*. The correlation between extract and antioxidant activities was analyzed. In addition, a High Performance Liquid Chromatograph (HPLC) method for simultaneous determination of echinacoside and oleuropein was developed.

MATERIALS AND METHODS

Materials and chemicals

S. pubescens was collected from Funiu Mountain of Henan Province, China. Plants were identified by Professor Yanfang Wu. The voucher specimens were deposited in the School of Basic Medical Sciences, Henan University of Science and Technology, Luoyang, China.

Echinacoside, oleuropein, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)were purchased from Shanghai Aladdin Bio-Chem Technology Co., (Shanghai, China). The HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (Shanghai, China) and the ultra-pure water was obtained from by Milli-Q system (Millipore, Bedford, MA, USA). All solvents used in the work were analytical grade.

Sample pretreatment

The samples collected were dried until constant weight in an oven (Yiheng Scientific Instrument Co., Shanghai, China) at 50^{o}C and pulverized, and then passed through a 60 mesh. Approximately 2g of samples were accurately weighed and were extracted with different solvent in an ultrasound bath (bath power 250W, 40kHz, Scientz, SB-5200DTD, Ningbo, China). After extraction, the solution

was centrifuged for 10 min at 10000 rpm to obtain the supernatant. The solution was filtered through a 0.45 μmsyringe membrane before HPLC analysis. The contents of echinacoside and oleuropein were measured using the corresponding calibration curves.

Apparatus and chromatographic conditions

The HPLC system consisted of an Agilent 1100 Series supplemented with a diode array detector (Agilent Technologies, Santa Clara, CA, USA) and a reverse-phase Zorbax Eclipse Plus C18 column (250mm×4.6mm, 5μm) (Agilent Technologies, Santa Clara, CA, USA) at a column temperature of 25°C. The mobile phase consisted of acetonitrile (A) and 0.5% acetic acid (B) using a gradient elution of 5% A at 0-10min, 5%-20% A at 10-20 min, 20%-25% A at 20-25min. The flow rate was 1mL/min and injection volume was 10μL.

DPPH radical scavenging assay

The DPPH test of the different extracts was evaluated according to the methods described by our previous report (Wu *et al.*, 2017). Extract solution at various concentrations (1mL) and 0.2 mM DPPH solution (1mL) were mixed. And then the mixture was shaken vigorously and kept for 45 min to reach a steady state at room temperature. The absorbance was measured at 517 nm. Radical scavenging capacity was calculated using the following equation:

Scavenging rate = $[(A_s\text{-}A_i)/A_s]\times100$

Where $A_s$is the absorbance of DPPH alone and A_i is the absorbance of DPPH in the presence of various extracts Ascorbic acid was used as a reference. The antioxidant ability of the sample was expressed as IC_{50}.

ABTS radical scavenging assay

The capacity to scavenge the ABTS radical cation was measured according to Wu *et al.* (2017). The solution of ABTS radical cation ($ABTS^+$) was obtained by the reaction of 2.45mM potassium persulfate and 7mM ABTS and kept for 16h at room temperature in the dark and then the mixture was diluted with ethanol to the absorbance of 0.70±0.02 at 734 nm. The $ABTS^+$ solution (3.6 mL) and extract solution at various concentrations (0.4mL) were mixed and allowed to be kept for 30 min. The absorbance was measured at 734 nm. Radical scavenging activity was calculated as the following percentage: $[(A_s-A_i/A_s)]\times100$ (A_s=absorbance of pure $ABTS^+$, A_i=absorbance of $ABTS^+$ in the presence of various extracts). Ascorbic acid was used as a reference.

Scavenging assay of •OH radical

The ability to scavenge the hydroxyl radical was measured using commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the generation of hydroxyl radical results from the combination of Fe^{2+} and H_2O_2 *in vitro*. In this assay method, the reaction system was mixed with the Griess' reagent. The absorbance of mixed solution was measured at 550 nm against a blank. Ascorbic acid was used as a reference. Hydroxyl radical scavenging activity was calculated as the following percentage: $[(A_0-A_i/A_0)]\times100$ (A_0=absorbance without sample, A_i=absorbance in the presence of various extracts).

STATISTICAL ANALYSES

All experiments were performed in triplicate, and results were expressed as means ± standard derivations (SD). Statistical analysis was carried out with one-way analysis of variance using SPSS software (ver. 18.0IBM, Armonk, NY, USA) and p value< 0.05 was regarded as significant. The Pearson correlation coefficients were calculated in order to identify the correlation between yields and antioxidant abilities.

RESULTS

HPLC method validation

The validation was assessed in terms of linearity and precision. The calibration curves were obtained by plotting the peak areas and corresponding concentrations. As shown in table 1, the correlation coefficients were above 0.999 within tested ranges. The RSD values of intra- and inter-day precision were less than 3%. These results showed the method developed was precise and accurate. The representative HPLC chromatograms of the standard substances and sample were presented in fig. 1.

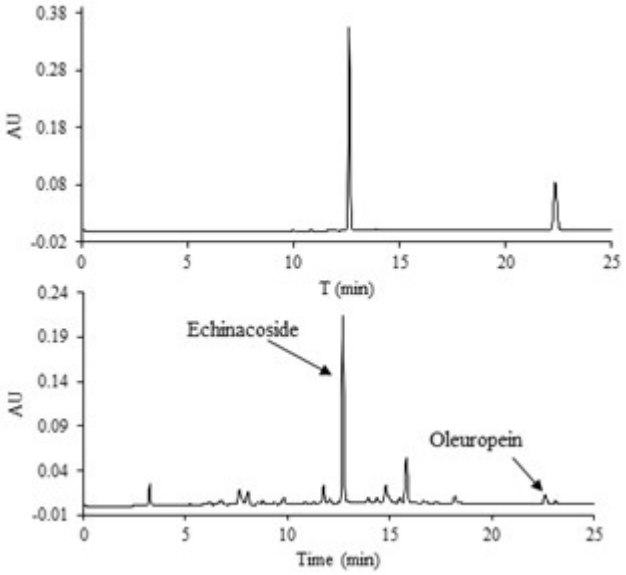

Fig. 1: Representative HPLC chromatograms of the standard substances (A) and sample (B).

Effect of solvent on the contents echinacoside and oleuropein

Solvent extraction is the most widely used method for extraction of desired compounds from plant material. Extraction efficiency is influenced by the chemical nature

Table 1: Calibration curves and precision of the assay of echinacoside and oleuropein

Analyte	Calibration curve	r^2	Test range (mg/mL)	Intra-day RSD (%)	Inter-day RSD (%)
Echinacoside	$y=10^7x$-2882	0.9997	0.02-0.4	0.61	0.60
Oleuropein	$y=3\times10^6x$+13392	0.9999	0.03-0.6	0.64	0.63

Table 2: The contents of echinacoside and oleuropein from *S. pubescens* using different solvent

Solvent system	Echinacoside (mg/g)	Oleuropein (mg/g)
Water	20.93±1.50^{f}	4.88±0.36^{b}
Pure methanol	19.46±0.96^{e}	7.79±0.92^{fg}
80 % aqueous methanol	23.84±1.21^{gh}	9.77±0.56^{h}
60 % aqueous methanol	27.08±0.36^{j}	8.23±0.48^{g}
40 % aqueous methanol	24.56±1.02^{hi}	7.29±0.23^{f}
Pure ethanol	11.60±0.36^{d}	6.20±0.15^{de}
80 % aqueous ethanol	18.27±0.68^{e}	11.81±0.25^{i}
60 % aqueous ethanol	25.77±0.33^{ij}	5.27±0.22^{bc}
40 % aqueous ethanol	22.79±0.95^{g}	5.07±0.15^{bc}
Pure acetone	2.68±0.09^{a}	3.56±0.09^{a}
80 % aqueous acetone	5.62±0.10^{b}	5.66±0.08^{cd}
60 % aqueous acetone	8.79±0.26^{c}	6.56±0.12^{e}
40 % aqueous acetone	11.36±0.38^{d}	8.21±0.28^{g}

Note: Different letters in superscript ($^{a\text{-}j}$) indicate significant difference from one another ($p < 0.05$).

of desired compounds, the extraction technique applied, the extraction solvent applied, and the presence of interfering chemicals (Do *et al.*, 2014). The solvent is the most important parameter to extract objective compounds. In the present study, the extraction was performed using water and different concentrations of aqueous methanol, ethanol and acetone (40%, 60%, 80% and 100%). The contents of echinacoside and oleuropein were measured using calibration curve. The results were presented in table 2.

Effect of solvent on the antioxidant activities

The stable free radicals DPPH and ABTS were widely used to evaluate the antioxidant capacities of plant extract (Bendif *et al.*, 2018; Costa *et al.*, 2012; Fan *et al.*, 2020). In this work, the IC_{50}values of different extracts were calculated using the graph by plotting inhibition and listed in table 3. The hydroxyl radical reactive oxygen species generated during the metabolism of organisms. The excessive production of hydroxyl radical can result in cell damage *in vivo* (Ambigaipalan *et al.*, 2016).Therefore, it is vital to remove the excessive hydroxyl radical of organisms (Pisoschi & Pop, 2015). table 3 indicated that different solvent extracts exhibited different antioxidant properties.

Correlation analysis

Pearson correlation coefficients among echinacoside content, oleuropein content, and antioxidant properties are listed table 4. Negative correlations imply that a higher compound content leads to lower IC_{50} values and higher antioxidant activity.

DISCUSSION

It is well known that the solvent type and its polarity can influence the extraction efficiency of target compounds. It can be found from table 2 that the methanol, ethanol, and water extracts show higher echinacoside content than acetone extracts. The results also revealed that the mixture of water and ethanol or methanol shows higher extraction efficiency to echinacoside compared to a single solvent such as water, ethanol and methanol. This is due to the interactions between the polar sites (hydrogen bonds) and the solvent. The employment of 60% aqueous methanol resulted in the highest extraction efficiency of echinacoside (27.08mg/g), followed by 60% aqueous ethanol (25.77 mg/g). These results indicated that the extraction efficiency of echinacoside increased with increasing the methanol or ethanol concentration. However, a further increase in methanol or ethanol concentration above 60% decreased the extraction yield. This may be attributable to the higher solubility of echinacoside in 60% of methanol or ethanol concentration than in other solvents (table 2). The data obtained from this work are in line with the extraction yields of other medicinal plant materials (Martins *et al.*, 2016; Wölkart *et al.*, 2004).

In terms of oleuropein, extraction yields ranged from 3.56 mg/g for acetone extract to 11.81 mg/g for 80% aqueous ethanol extract. The extraction yield decreased in the following order: 80% ethanol >80% methanol >60% methanol ≥40% acetone ≥pure methanol ≥40% ethanol >60% acetone >pure ethanol >80% acetone >60%

Table 3: Antioxidant properties of the different solvent extract

Sample	IC_{50} values		
	DPPH (mg/mL)	ABTS (mg/mL)	·OH (mg/mL)
Water	0.55±0.06	0.67±0.08	1.83±0.16
Pure methanol	0.65±0.04	0.73±0.09	1.43±0.13
80 % aqueous methanol	0.57±0.09	0.67±0.11	1.89±0.08
60 % aqueous methanol	0.39±0.06	0.42±0.08	1.23±0.12
40 % aqueous methanol	0.56±0.03	0.66±0.08	1.75±0.10
Pure ethanol	2.37±0.10	2.51±0.13	5.02±0.18
80 % aqueous ethanol	1.56±0.21	1.71±0.18	2.63±0.16
60 % aqueous ethanol	1.13±0.13	1.39±0.08	2.33±0.11
40 % aqueous ethanol	1.23±0.16	1.50±0.12	2.56±0.03
Pure acetone	4.71±0.18	4.94±0.26	8.92±0.33
80 % aqueous acetone	3.81±0.21	3.87±0.17	6.92±0.13
60 % aqueous acetone	3.11±0.11	3.26±0.13	5.71±0.08
40 % aqueous acetone	2.76±0.23	3.00±0.17	4.52±0.22
Ascorbic acid	0.02±0.005	0.06±0.003	0.12±0.006

Table 4: Correlation among echinacoside, oleuropein, DPPH, ABTS and ·OH

	Echinacoside	Oleuropein	DPPH[a]	ABTS[b]	·OH[c]
Echinacoside	1	0.331	-0.954	-0.946	-0.947
Oleuropein		1	-0.399	-0.411	-0.467
DPPH			1	0.999	0.989
ABTS				1	0.985
·OH					1

DPPH[a] : DPPH radical assay, ABTS[b] : ABTS radical assay, ·OH[c]: Hydroxyl radical assay.

methanol ≥40% methanol >water >pure acetone. These results further confirm that solvents play a vital role in the extraction of oleuropein from *S. pubescens*. The combination of water and organic solvent such as methanol, ethanol, and acetone is the optimum solvent for extraction of oleuropein. Similar results were reported in previous studies, which indicated that the mixture of water and organic solvent gave high oleuropein content (Lama-Muñoz *et al.*, 2019; Malik & Bradford, 2008; Yateem *et al.*, 2014).This can be explained by the fact the combined use of water and organic solvent may enhance the solubility and mass transfer of target compounds.

In DPPH scavenging assay, the methanol and water extracts exhibited higher radical scavenging activity than those of ethanol and acetone. This may be due to the fact that methanol and water favor the solubility and mass transfer of polar compounds including echinacoside, oleuropein and other phenolic chemicals, which has strong antioxidant potency to scavenging radicals (Ulewicz-Magulska & Wesolowski, 2019). Moreover, water-soluble polysaccharide is extracted by water extraction and shows antioxidant properties (Ji *et al.*, 2020). These results further confirm that extraction solvents significantly influence antioxidant activities. However, the antioxidant capacities of different solvent extracts are weaker than that of ascorbic acid. Similar scavenging capacity patterns were found in the ABTS assay. It could be found that the 60% methanol extract showed the strongest scavenging hydroxyl radical, and the pure acetone exhibited the lowest antioxidant ability. Compared with the scavenging DPPH and ABTS free radicals effect, the scavenging ·OH ability was weaker.

As seen from table 4, the antioxidant properties presented significant correlation with echinacoside content (-0.954, -0.946, -0.947) and low correlation with oleuropein content (-0.399, -0.411, -0.467). The results indicated significant contribution of echinacoside to these antioxidant capacities. Similar to our findings, Xiong *et al.* (Xiong *et al.*, 1996) reported that echinacoside from *Cistanche desericola* exhibited strong free radical scavenging abilities. On the other hand, the oleuropein showed synergistic effects to antioxidant capacities. In addition, these results coincide very well with previous studies where hydroxyl radical was found to show similar results to DPPH and ABTS due to high correlation (Bhardwaj *et al.*, 2020).

CONCLUSION

The investigation is the first report of comparative analysis of different extraction solvent for echinacoside content, oleuropein content, and antioxidant properties

analysis from *S. pubescens*. The 60% aqueous methanol gave higher echinacoside yield compared with other solvent. In terms of oleuropein extraction, 80% aqueous ethanol was the optimum extracting solvent. Furthermore, the extracts of water, aqueous ethanol or methanol exhibited remarkable antioxidant capacities. Correlation analysis indicated that the echinacosied content attribute to high antioxidant ability. The results obtained from this study further confirmed that the *S. pubescens* possessed potential hepatoprotective activities. In addition, a method for simultaneous determination of echinacoside and oleuropein was proposed.

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