BIOTECHNOLOGY AND BIOENGINEERING
Chinese Journal of Chemical Engineering, **17**(3) 478—483 (2009)

Antioxidant and Antimicrobial Activities of Echinacea (*Echinacea purpurea* L.) Extracts Obtained by Classical and Ultrasound Extraction*

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Abstract Antioxidant and antimicrobial activities of *Echinacea purpurea* L. (*Asteraceae*) extracts obtained by classical and ultrasound solvent extraction were compared. The dry aerial part of plant was extracted by 70% ethanol at a solid-to-liquid ratio of 1∶10 (m/v) and 25°C. The extract obtained by classical solvent extraction contained 29% larger amounts of phenolic compounds and 20% higher content of flavonoids. 2,2-diphenyl-1-picril hydrazyl radical (DPPH) scavenging reached 93.6% and the values of EC50 were (34.16±0.65) $\mu g \cdot ml^{-1}$ and (65.48±1.12) $\mu g \cdot ml^{-1}$ for the extracts obtained by the classical and ultrasound extractions, respectively. The extracts, independent of the extraction technique applied, showed a considerable growth inhibition on *Candida albicans* and *Saccharomyces cerevisiae*, while no growth inhibition zones were observed for *Aspergillus niger*. The diameters of inhibition zone observed for all the microorganisms were larger for extracts obtained by classical extraction than those by ultrasound extraction.
Keywords antioxidant activity, antimicrobial activity, *Echinacea purpurea* L., total phenols, flavonoids, extraction

1 INTRODUCTION

Echinacea purpurea L., also known as purple coneflower, is an herbaceous perennial and a member of the *Asteraceae* family with a long, well-established tradition of medicinal use in North America, Europe [1] and Australia [2]. In modern cultures, *E. purpurea* is used for medicinal purposes, in treating acute upper respiratory infections, urinary tract infections, burns and disorders such as viral infections, cutaneous affections and chronic disease due to a deficiency of immunological responses [1, 3−5]. *E. purpurea* stimulates various immune cells including macrophages and natural killer cells and has anti-inflammatory effects [4].

The roots, the leaves or the whole plant may be also used in the dietary supplement preparation. The composition of the root extracts, compared to the upper plant extracts, is very different. Root parts have more volatile oils and pyrrolizidine alkaloids, such as tussilagine and isotussilagine, than the aerial parts. The main active compounds of the aerial parts are alkamides and polyacetylenes, caffeic and ferulic acid derivatives, polysaccharides (such as 4-O-methylglucuronylarabinoxylans, rhamnoarabinogalactans and acidic arabinogalactan) and glycoproteins [5, 6]. Of the caffeic acid derivatives, only cichoric acid, which is found to be the main phenolic compound in *E. purpurea*, shows immunostimulatory properties, promoting phagocyte activity *in vitro* and *in vivo*, antihyaluronidase activity and has a protective effect on the free-radical-induced degradation of collagen [7]. Cichoric acid shows also antiviral activity and has recently been found to inhibit HIV-1 integrase and replication [7]. The quantitative analysis indicates that alkamides and caffeic acids are present at significant concentrations in ethanol/water extracts of this plant stored for longer than one year [8]. This revelation is important because alkamides and caffeic acid derivatives have been identified as possible active constituents of *E. purpurea*.

Beside cichoric acid, typical constituents of *E. purpurea* extracts are echinacoside, chlorogenic acid, cynarine and caftaric acid. All of them are able to inhibit free radical production and lipid peroxidation, involved in the development of inflammation [1, 9]. Recent studies also suggest that melanin may contribute to the activity of *E. purpurea* extracts [10], while echinacoside and caffeic acid derivative do not possess immunostimulant activity, but have weak antibacterial and antiviral effects and are protectants against reactive oxygen species [11].

The antioxidant activity of *E. purpurea* extracts has been already shown [7, 12−15]. Generally, the tested *E. purpurea* extracts showed medium to low activity compared to the other investigated medicinal and aromatic plants [12]. The antioxidant activity could be ascribed to the polyphenolic components [16], such as flavonoids [15, 17], phenolic acids [18] or phenolic diterpenes [15]. Moreover, some studies demonstrated that the *E. purpurea* extracts protected immunosuppressed mice against systemic infections with *Listeria monocytogenes* and *Candida albicans* by stimulating macrophage and neutrophil function. The herb was non-toxic in mice, rats and humans even when administered intravenously at high doses [19].

In previous studies, *E. purpurea* was reported to increase chemotoxicity in neutrophils and bactericidal activity against *Staphylococcus* and to kill tumor cells (WEHI 164 cells) and cells infected either with the

Received 2008-06-25, accepted 2009-03-19.
* Supported by the Ministry of Science and Environmental Protection, Republic of Serbia (142073B).
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parasite *Leishmania enriettii* or with yeast *Candida albicans* [6]. Also, it was found that hexane extracts of echinacea variably inhibited growth of yeasts *Saccharomyces cerevisiae*, *Candida shehata*, *C. kefyr*, *C. albicans*, *C. steatulytica* and *C. tropicalis* under near UV irradiation (phototoxicity) and to a lower extent without irradiation (conventional antifungal activity) [20].

So far, the classical solvent extraction with [8, 7, 21] or without [22, 23] mechanical agitation, Soxhlet [21] and Goldfisch [24] extraction techniques have been applied for *Echinacea* sp. extraction. Recently, ultrasound extraction was used [5, 21, 25], but there is no comparative study of antioxidant and antimicrobial activities of *E. purpurea* extracts obtained by different extraction techniques.

The purposes of this study are to compare the efficiency of extractive substances, antioxidant and antimicrobial activities of extracts obtained by ultrasound and classical extraction techniques. Also, total phenolic compunds and flavonoids in *E. purpurea* aqueous ethanolic extracts obtained by two methods are compared. Antimicrobial and antiradical activity, antioxidant capacity, total phenolics and flavonoids of extracts are determined by *in vitro* assays.

2 EXPERIMENTAL

2.1 Materials

Ethanol was from Zorka-Pharma (Šabac, Serbia). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazil (DPPH), gallic acid and rutin were obtained from Sigma (St. Louis, MO). Sodium carbonate, potasium acetate and aluminium chloride were purchased from Merck-Alkaloid (Skopje, FYR Macedonia).

Dried aerial parts of *E. purpurea* L. were purchased from DOO. "Adonis" (Soko Banja, Serbia). Immediatly before being used, dry plant material was ground by an electrical mill with a fast-rotating knife (15000 r·min^{-1}; 1 min). The moisture content, determined by drying at 105°C to constant mass, was 11.2% and the yield of extractive substances, obtained by the Soxhlet extraction (9 h, 13 extraction cycles) with 70% aqueous ethanol as extracting solvent, was 15.1 g per 100 g of dry plant material, which was taken to represent the content of extractive substances present in the plant material.

2.2 Extraction of plant materials

2.2.1 *Classical extraction*

Ground plant material (10 g) and the predetermined volume of 70% aqueous ethanol were put in an Erlenmayer flasks (100 ml) at a ratio of plant material(g) and solvent(ml) of 1∶10. The extraction was performed at 25°C for 2.5, 5, 10, 20, 40, 60 and 90 min. The temperature was controlled and maintained at the desired level (±0.1°C). At the end of the extraction cycle the liquid extract was separated from the solid residue by vacuum filtration. The solid residue was washed twice with fresh solvent (20 ml each). The filtrates were collected and the solvent was evaporated in a rotary vacuum evaporator at 40°C.

2.2.2 *Ultrasound extraction*

The sonication was performed for 2.5, 5, 10, 20, 40 and 60 min with 70% aqueous ethanol, at a ratio of plant material (g) to solvent (ml) 1∶10 and 25°C using an ultrasonic cleaning bath (Sonic, Niš, Serbia; total nominal power: 150 W; operating at 40 kHz frequency and internal dimensions: 30 cm ×15 cm ×20 cm). The temperature was controlled and maintained at the desired level (±0.1°C) by water circulating from a thermostated water bath. Separation and further treatment of the filtrates were the same as described above.

2.3 Determination of free radical scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts [26]. Different concentrations of extracts (10, 20, 50, 100, 200, 500 and 1000 μg·ml^{-1}, in 70% ethanol) were added, at an equal volume (2.5 ml) to an ethanolic solution of DPPH (0.3 mmol·L^{-1}, 1 ml). After 30 min at room temperature, the absorbance of the plant extract with DPPH was measured at 517 nm on a spectrophotometer (VARIAN Cary-100) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH anti - radical scavenging capacity (\%)} = \left[1-\frac{(A_s - A_b)}{A_c}\right]\cdot 100$$

where A_s is the absorbance of the plant extract containing DPPH, A_b is the absorbance of ethanol (1.0 ml) plus plant extract solution (2.5 ml) and A_c is the absorbance of DPPH solution (1.0 ml) plus ethanol (2.5 ml). The EC_{50} values were calculated by sigmoid non-linear regression model using plots, where the abscissa and the ordinate represented the concentration of tested plant extracts the average percent of scavenging capacity from three replicates, respectively.

2.4 Determination of total phenols

Total phenols were determined by Folin Ciocalteu reagent using gallic acid as a standard [27]. The total phenols were expressed as mg gallic acid equivalents (GAE) per g dry extract [the equation of standard curve: absorbance at 765 nm = 12.722 $c_{\text{gallic acid}}$ (μg·ml^{-1})+0.0034, R^2 = 0.9994]. Since the assay measures all phenolics, the choice of gallic acid as a standard is based on the availability of a stable and pure substance. Each of plant extracts (0.2 ml, 20 μg·ml^{-1}) or gallic acid was mixed with Folin Ciocalteu reagent (1 ml) and aqueous Na_2CO_3 (0.8 ml, 7.5%). The mixtures were allowed to stand at room temperature for 30 min, and the absorbance of the reaction mixture was measured at 765 nm.

2.5 Determination of total flavonoids

The flavonoids content in extracts was determined spectrophotometrically using an aluminium chloride colorimetric method, based on the formation of a complex flavonoid-aluminium [28]. Each plant extract (0.5 ml, 20 $\mu g \cdot ml^{-1}$) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride ($AlCl_3$), 0.1 ml of 1 $mol \cdot L^{-1}$ potassium acetate (CH_3COOK) and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm against distilled water blank. Rutin was used as a standard to make the calibration curve and the data were expressed as mg rutin equivalents (RE) per g dry extract [the equation of standard curve: absorbance at 415 nm = 7.2328 c_{rutin} ($\mu g \cdot ml^{-1}$) – 0.2286, $R^2 = 0.9919$].

2.6 Antimicrobial activity

An agar well-diffusion method was employed for the determination of antimicrobial activities of extracts [29]. Seven microorganisms were selected to test the antimicrobial activity: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763 and *Aspergillus niger* ATCC 16404 (Oxoid, England).

For the yeast and mould, sabouraud dextrose agar (SDA) (Merck) was used; for cultures of bacteria, trypton soya agar (TSA) (Merck) was used, and plate count agar (Merck) was used for determination of the total number of microorganisms ($CFU \cdot ml^{-1}$). 0.1 ml of microorganism suspension, formed by 24 h culture on obliquely agar with 10 ml sterile 0.9% NaCl, was suspended into 10 ml of the nutritive medium (ca. 10^6 $CFU \cdot ml^{-1}$). Petri dish (86 mm internal diameter) was filled with this system. The wells (10 mm in diameter) were cut from the agar and 30 µl of extract solution (concentration 20 $mg \cdot ml^{-1}$ in methanol) was delivered into them. As a control, methanol (30 µl) was delivered into a well for each Petri dish. Erythromycin (997 $\mu g \cdot mg^{-1}$; [114-07-8]; Approx. 98%; H_2O content 4%; Sigma) and Tylosin Tartarat (950 $\mu g \cdot mg^{-1}$; [74610-55-2]; Sigma) were used as a positive control (concentration in methanol solution, 0.05 $mg \cdot ml^{-1}$). All dilutions were filtrated using a 0.45 µm membrane filter (Sartorius, Germany). After incubation at 37°C for 24 h, agar plates were examined for any zones of inhibition. Diameters of zones of inhibition (mm) were measured by Fisher Lilly Antibiotic Zone Reader (Fisher Scientific Co., USA) and each test was run in triplicate.

2.7 Statistical analysis

All of the measurements were carried out in triplicate and the results were expressed as mean ± standard deviation, except extract yields which were in duplicate. Comparison of means was analyzed by Student's t test and differences were considered significant when $p < 0.05$.

3 RESULTS AND DISCUSSION

3.1 Antioxidant capacity

The percentage of DPPH radical-scavenging activity was plotted against the plant extract concentration (Fig. 1) to determine the concentration of extract necessary to decrease DPPH radical concentration by 50% (so called EC_{50}). The EC_{50} value was used to measure the antioxidant activity of extracts: the lower EC_{50}, the higher the value of the antioxidant activity.

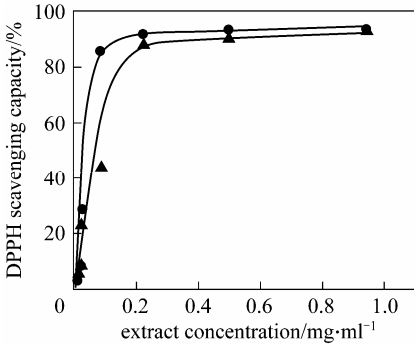

Figure 1 Antioxidant activity for *E. purpurea* extracts obtained by classical and ultrasound extraction
● classical extraction; ▲ ultrasound extraction

As can be seen in Table 1, the extract obtained by the classical extraction shows higher antioxidant activity, where the differences observed are statistically significant with 95% confidence interval. This extract also contains larger amount of total phenolic compounds per gram of either dry extract or dry plant material. The total flavonoides content of the extract

Table 1 Total antioxidant capacity, amount of phenolic and flavonoids compounds of *E. purpurea* extracts (25°C, 40 min)

Extraction technique	EC_{50}/ $mg \cdot ml^{-1}$	Total phenolics		Total flavonoids	
		(GAE/dry extract)/ $mg \cdot g^{-1}$	(GAE/dry plant material)/ $mg \cdot g^{-1}$	(RE/dry extract)/ $mg \cdot g^{-1}$	(RE/dry plant material)/ $mg \cdot g^{-1}$
classical	65.48±1.12	60.2±0.1	3.91±0.01	32.3±0.2	2.09±0.02
ultrasound	34.16±0.65	46.8±0.3	3.73±0.02	27.0±0.4	2.15±0.03

Note: Data were expressed as the mean of three replicates ± standard deviation.

obtained by classical extraction is higher if calculated per gram of the extract but it is lower if calculated per gram of the dry plant material. Thus, ultrasonic extraction gave higher yield but lower purity of total flavonoides. The total amount of these compounds is accepted as an indication of antioxidant potential because they act in plants as antioxidants, antimicrobials and photoreceptors [30].

The extract obtained by classical solvent extraction contained 29% larger amount of phenolic compounds and 20% higher content of flavonoids. The differences observed were statistically significant with 95% confidence interval. We believed that the observed reduction of phenolic compounds and flavonoids in the extract obtained by ultrasound extraction was the result of their degradation by interaction with highly reactive hydroxyl radicals formed during sonication. Sonication of water results in the formation of hydroxyl radicals, which can combine to form hydrogen peroxide that may or may not be beneficial to the extraction process itself [31]. Organic compounds in aqueous solution exposed to an ultrasonic irradiation behave differently according to their physical and chemical properties [32]. In the cases of some compounds with antioxidant activity, aqueous solvents appeared to be unsuitable for ultrasonic extractions due to the formation of free radicals from the insonation of the solvent [31].

3.2 Antimicrobial activity

The antimicrobial activity of *E. purpurea* extracts obtained by different extraction techniques were tested against the seven microorganisms mentioned above by the agar well-diffusion method.

As shown in Table 2, independent of the extraction technique, the ethanolic *E. purpurea* extracts show activity against almost all of the tested microorganisms, exception is only mould *A. niger*. The control treatment (methanol) has no inhibitory effect on any of the test microorganisms. Sensitivities of *S. aures*, *C. albicans*, and *S. cerevisiae* are higher for both echinacea extracts than the case of tested antibiotic (Erytromycin and Tylosin tartarat).

The diameters of inhibition zone observed for all microorganisms were larger for extracts obtained by classical extraction than those by ultrasound extraction. The differences observed were statistically significant (with 95% confidence interval) in the case of *E. coli*, *B. subtilis*, *C. albicans* and *S. cerevisiae*. No growth inhibition zones were observed for *A. niger* for both tested extracts.

3.3 Kinetics of extraction

The changes of the extract yield from the aerial parts of *E. purpurea* L. during the classical and ultrasound extraction are shown in Fig. 2. Independent of the extraction method, the extraction occurs in two main stages: first, dissolution of material near the surface characterized by a rapid increase in the extractive substance yield in the beginning of the process (washing or fast extraction), and second, diffusion of the solute from the porous plant residue into the solution (slow extraction). The optimum time for both extraction techniques was approximately 40 min, ensuring nearly the maximum oil yield.

As can be seen in Fig. 2, the main benefits of ultrasound included the increase of extractive substance yield and faster extraction. In recovering the extractive substances from *E. purpurea* L. the ultrasound extraction was more efficient than the classical solvent extraction, but less efficient than the Soxhlet extraction. The total extract content obtained by ultrasound extraction after 40 min was 22.8% higher than that obtained by the classical extraction. At the same time, it was 52.8% of the yield obtained by the Soxhlet extraction, which was 15.1 $g\cdot(100\ g)^{-1}$. However, a shortcoming of the Soxhlet extraction is higher operating temperature and in this case more than tenfold longer extraction time.

Table 2 Antimicrobial activity of *E. purpurea* extracts (40 min, 25ºC) **and antibiotic sensitivity of microorganisms** (zone size, mm)

Test microorganisms	Extracts (20 $mg\cdot ml^{-1}$)		Antibiotics (0.05 $mg\cdot ml^{-1}$)	
	Ultrasound	Classical	Erytromycin	Tylosin tartarat
Escherichia coli ATCC 25922	10.5±0.3	11.2±0.2	21.2±0.1	18.4±0.0
Pseudomonas aeruginosa ATCC 9027	11.3±0.3 a	11.9±0.4 a	25.2±0.9	17.6±0.1
Bacillus subtilis ATCC 6633	10.5±0.1	10.9±0.1	19.1±0.1	17.3±0.7
Staphylococcus aureus ATCC 6538	11.0±0.1 b	11.2±0.1 b	23.6±0.0	18.5±0.6
Candida albicans ATCC 10231	18.2±0.3	21.0±0.1	23.0±0.0	16.2±0.3
Saccharomyces cerevisiae ATCC 9763	21.2±0.1	25.7±0.1	naa	naa
Aspergillus niger ATCC 16404	naa	naa	20.5±0.7	18.1±0.1

Note: Control treatment (methanol) had no inhibitory effect on any of the test microorganisms. Differences between values for parameters designated with the same letters (a and b) were not statistically significant with 95% confidence interval (Student's *t* test). "naa" means no antimicrobial activity.

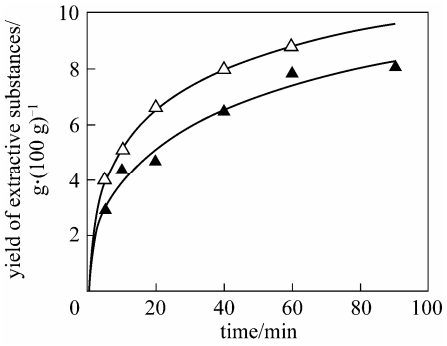

Figure 2 Variation of the extractive substance yield (based on dry plant meterial) **from the *E. purpurea* L.**
△ ultrasound extraction; ▲classical extraction

4 CONCLUSIONS

The present study suggests that 70% aqueous ethanolic extracts of *E. purpurea* is a potential source of active natural and non-toxic substances, which have functions as antioxidants, antimicrobials and antibiotics. The extract obtained by the classical extraction from aerial parts of *E. purpurea* L. contained larger amount of bioactive compound (total phenols and favonoid). Also, it showed stronger antioxidant and antimicrobial activities than the extract obtained by ultrasound extraction. Independent of the extraction technique, the ethanolic *E. purpurea* extracts showed antimicrobial activity against all tested microorganisms except in the case of *A. niger*. Ultrasound had a positive effect on the extractive substance yield from *E. purpurea* L., but negative effects on the content of total phenolic compounds and flavonoids. Some bioactive compounds were probably degradated by interaction with highly reactive hydroxyl radicals formed during sonication.

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