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Effect of Extraction Methods on the Active Compounds and Antioxidant Properties of Ethanolic Extracts of *Echinacea purpurea* Flower

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Abstract

The extraction yields, active compounds and antioxidant properties of 50%-aqueous-ethanolic extracts of freeze-dried *Echinacea purpurea* flower with multi-steps and multi-batches extraction methods were assessed. In multi-steps extraction, the extraction yields of 1st, 2nd, and 3rd extracts were 21.52%, 9.33%, and 2.90%, and their total phenols contents were 182.08, 176.33, and 177.08 mg CAE/g, respectively, with cichoric acid (62.07 - 66.57 mg/g) being the main phenolic compound. No differences in the contents of individual and total caffeic acids derivates existed among 1st, 2nd, and 3rd extracts. The dodeca-2*E*, 4*E*, 8*Z*, 10(*E/Z*)-tetraenoic acid isobutylamide (alkamide 8/9) contents of 1st, 2nd, and 3rd extracts were 505.38, 598.61, and 585.99 μg/g, respectively. In multi-batches extraction, the extracted dry weight increased with increasing the sample batches, with the extraction yields and alkamide 8/9 contents of samples decreased from 19.93% to 12.98% and 534.36 to 269.76 μg/g, respectively. The total phenol (177.25 - 186.92 mg CAE/g), individual and total caffeic acid derivatives (85.99 - 95.06 mg/g) contents of extracts among different sample batches were not significantly different, with cichoric acid (63.66 - 70.31 mg/g) being the main phenolic compound. All the prepared extracts also exhibited potent antioxidant properties. Overall, the two-step sequential extraction is desirable for extracting bioactive compounds from freeze-dried *E. purpurea* flower.

Keywords

***Echinacea purpurea,* Extraction, Active Component, Antioxidant**

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1. Introduction

Echinacea purpurea L. is frequently used to alleviate colds, sore throats and other upper respiratory infections. Many types of *Echinacea* products (e.g., infusions, tinctures and capsules) are used to stimulate immune system. The immuno-stimulating properties of *Echinacea* products are mainly attributed to its bioactive phytochemicals including caffeic acid derivatives, alkamides, polysaccharides, and glycoproteins. Among these phytochemicals, caffeic acid derivatives, especially cichoric acid, possess many bioactive functions including anti-hyaluronidase activity, protection of collagen from free radical induced degradation, antiviral activity, inhibition of human immunodeficiency virus type 1 integrase and replication, promoting phagocyte activity *in vitro* and *in vivo*, a high free radical scavenging property and inhibition of human colon cancer cells growth [1]-[4]. Alkamides are reported to be a new class of cannabinomimetics modulating tumor necrosis factor α (TNF-α) gene expression via the cannabinoid type 2 (CB_2) receptor [5] [6]. They have exhibited a strong stimulating effect on phagocyte function and on lipoxygenase-inhibiting activity [1] [5]-[8]. Thus, cichoric acid and alkamides are commonly used as marker to determine the medicinal quality of *Echinacea* products [9].

Various extraction techniques have been developed to obtain phytochemicals from plant materials [10]. Many extraction methods, including solvent extraction (with or without mechanical agitation), and some novel techniques such as sonication-assisted and microwave-assisted extractions, are used to extract phytochemicals from various plant materials. Nevertheless, from commercial-scale production viewpoint, the selected extraction technique must be versatile, simple, low-cost and safe for both the operating personnel and the consumers [11]. In Taiwan, the classical solvent extraction coupled with higher temperature has been commercially used to extract bioactive compounds from various plant species for years.

E. purpurea has recently been grown in Taiwan for years. The total phenols and caffeic acid derivatives contents in different *E. purpurea* parts were in the descending order: flowers > leaves > stems > roots [12]. The optimal extraction conditions of *E. purpurea* flowers were 50% ethanol and 65˚C of extracting temperatures, and its ethanolic extract exhibited good antioxidant, antimutaginic, and anticancer activities in previous study [4] [13]. However, in solid-liquid extraction technique, a single extraction is generally not sufficient to remove all the phenolic compounds comparing to multiple extractions [14]. Therefore, the objectives of this study were to investigate the effects of multi-steps extraction and multi-batches extraction methods on the extraction yields, active compounds and antioxidant properties of the 50% ethanolic extracts of freeze-dried *E. purpurea* flower.

2. Material and Methods

2.1. Plant Materials and Chemicals

The flowers of *E. purpurea* (L.) Moench variety CLS-P2 harvested from six-month-old plants were donated by Echili Biotechnology (Dali, Taichung, Taiwan). The flowers were freeze-dried with vacuum on the same day as harvested. The dried flowers were ground in a mill, and screened through a 2.0 mm sieve. The particle size distribution of dried flower powders was > 1.680 mm (0.01%), 0.840 - 1.680 mm (1.74%), 0.149 - 0.840 mm (75.31%), 0.074 - 0.149 mm (13.74%), and < 0.074 mm (9.20%). The dried flower powders were sealed in a PET/Al/PE bag and then kept at −20˚C before use.

Methanol, acetonitrile and phosphoric acid were purchased from Mallinckrodt Baker, Inc. (New Jersey, USA). Echinacoside and dodeca-2*E*, 4*E*, 8*Z*, 10(*E/Z*)-tetraenoic acid isobutylamide (alkamide 8/9) were purchased from ChromaDex Inc. (Santa Ana, CA, USA). Caftaric acid, chlorogenic acid, cichoric acid, caffeic acid, Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid, potassium ferricyanide, ferrous chloride, ferrozine, ascorbic acid, α-tocopherol, butylated hydroxyanisole (BHA), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anhydrous sodium carbonate was purchased from Shimakyu's Pure Chemicals (Osaka, Japan). Ferric chloride was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Sodium phosphate was purchased from Union Chemical Work Ltd. (Hsinchu, Taiwan). Ethanol (95%) was purchased from Taiwan Tobacco & Liquor Co. (Tainan, Taiwan).

2.2. Extraction of Active Compounds from *E. purpurea* Flowers

2.2.1. Multi-Steps Extraction Method

The extract was obtained according to the methods of Tsai *et al*. [13] with minor modifications. The freeze-dried

flower powders (15 g for each sample) were extracted with 150 ml of 50% aqueous ethanol in a shaking bath at 100 rpm for 30 min under 65˚C conditions, and then centrifuged at 3460 ×*g* for 10 min, filtered through Advantec No. 1 filter paper. The residue was re-extracted twice with two extra 150 ml portions of solvent as described above. The first, second and third extracts were designated as 1st, 2nd and 3rd, respectively. Each ethanolic extract was rotary evaporated at 40˚C and then freeze-dried with vacuum. The resultant dry extracts were stored at −20˚C before use. All experiments were done in triplicate.

2.2.2. Multi-Batches Extraction Method

The freeze-dried flower powders (15 g for each sample) were extracted with 150 ml of 50% aqueous ethanol in a shaking bath at 100 rpm for 30 min under 65˚C conditions, and then centrifuged at 3460 ×*g* for 10 min, filtered through Advantec No. 1 filter paper. The ethanolic extract was rotary evaporated at 40˚C and then freeze-dried with vacuum. The extract was designated as I. The filtrate from I was adjusted to 150 ml with 50% aqueous ethanol to extract another batch freeze-dried flower powders (15 g). The extract was designed as II. The filtrate from II was adjusted to 150 ml with 50% aqueous ethanol to extract another batch freeze-dried flower powders (15 g). The extract was designed as III. The filtrate from III was adjusted to 150 ml with 50% aqueous ethanol to extract another batch freeze-dried flower powders (15 g). The extract was designed as IV. Each ethanolic extract was rotary evaporated at 40˚C and then freeze-dried with vacuum. Dried extracts were stored at −20˚C before use. All experiments were done in triplicate.

2.3. Determination of Total Phenols

The content of total phenols in the extracts was determined using Folin-Ciocalteu's colorimetric method according to the method of Wu *et al.* [11] with minor modifications. The prepared extract (40 mg) was dissolved in 70% aqueous methanol (5.0 ml) using an ultrasonic bath with 40 kHz for 5 min, and then the volume was adjusted to 10 ml. A sample solution of 100 µl was mixed with 2.5 ml deionised water, followed by the addition of 0.1 ml (2 N) Folin-Ciocalteu's phenol reagent. The mixture was well stirred and allowed to stand for 6 min before 0.5 ml of a 20% sodium carbonate solution was added and then the volume was adjusted to 10 ml with deionised water. The color developed after 30 min at room temperature and the absorbance was measured at 760 nm using a UV-visible spectrophotometer, with chlorogenic acid used as a standard. The content of total phenols was calculated on the basis of the calibration curve of chlorogenic acid [the equation of standard curve: absorbance at 760 nm = 0.0005 $C_{\text{chlorogenic acid}}$ (µg/ml) + 0.0295, R^2 = 0.994]. Result was expressed as milligram of chlorogenic acid equivalents (CAE) per gram of dry extract. Each analysis was carried out in triplicate.

2.4. Determination of Caffeic Acid Derivatives

The content of caffeic acid derivatives in the prepared extract was determined according to the methods of Hu and Kitts [15] and Pellati *et al.* [3] with minor modifications. Briefly, the extract (40 mg) was dissolved in 70% ethanol (5.0 ml) using an ultrasonic bath with 40 kHz for 5 min, and the volume was adjusted to 10 ml, and then centrifuged at 1400 ×*g* for 5 min. The solution was filtered using a PVDF syringe filter (13 mm × 0.45 µm) prior to injection onto a HPLC. The HPLC system consisted of a Hitachi L-2130 pump, a Hitachi L-2400 UV detector, and a Luna 5u C18(2) 100A column (250 mm × 4.6 mm, 5 µm, Phenomenex Co., USA). The column temperature was maintained at 35˚C. The mobile phase was (A) acetonitrile/water containing 0.1% H_3PO_4 (10:90) and (B) acetonitrile/water containing 0.1% H_3PO_4 (25:75). A gradient elution profile was used with B increasing from 0% to 100% in 30 min and maintained at 100% for 10 min. Then a linear gradient of 100% B decreased to 0 B in 10 min. The flow rate was 1.5 ml/min, and the wavelength of the UV-visible detector was set at 330 nm. The sample injection volume was 10 µl. Contents of various caffeic acid derivatives were calculated on the basis of the calibration curve of each caftaric acid, chlorogenic acid, caffeic acid, echinacoside and cichoric acid. Each analysis was carried out in triplicate.

2.5. Determination of Alkamide 8/9

The content of alkamide 8/9 in the extracts was determined according to the methods of Perry *et al.* [16] and Bergeron *et al.* [17] with minor modifications. Briefly, each extract (40 mg) was dissolved in 70% ethanol (5.0 ml) using an ultrasonic bath with 40 kHz for 5 min, and the volume was adjusted to 10 ml, and then centrifuged

at 1400 ×*g* for 5 min. The solution was filtered using a PVDF syringe filter (13 mm × 0.45 μm) prior to injection onto a HPLC. The HPLC system consisted of a Hitachi L-2130 pump, a Hitachi L-2400 UV detector, and a LiChrospher® 100 RP-18e column (250 mm × 4.6 mm, 5 μm, Merck Co., Germany). The column temperature was maintained at 25˚C. The mobile phase was (A) acetonitrile and (B) water. A gradient elution profile was used with B decreasing from 60% to 20% in 15 min and maintained at 20% for 5 min. Then a linear gradient of 20% B increased to 60% B in 10 min. The flow rate was 1.5 ml/min, and the wavelength of the UV-visible detector was set at 254 nm. The sample injection volume was 20 μl. Contents of alkamide 8/9 were calculated on the basis of the purchased alkamide 8/9 standard. Each analysis was carried out in triplicate.

2.6. Antioxidant Properties

2.6.1. Scavenging Ability on DPPH Radicals

For DPPH radicals scavenging ability assay [18], each extract (30 - 150 μg/ml, 4 ml) in methanol was mixed with 1 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.5 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, the absorbance of the mixture was then measured at 517 nm against a blank. Ascorbic acid, BHA, and α-tocopherol were used for comparison. EC_{50} value (μg extract/ml) is the effective concentration at which DPPH radicals were scavenged by 50%.

2.6.2. Reducing Power

The reducing power was determined according to the method of Oyaizu [19]. Each extract (100 - 500 μg/ml, 2.5 ml) in methanol was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide, and the mixture was incubated at 50˚C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 2600 ×*g* for 10 min. The upper layer (3 ml) was mixed with 3 ml of deionized water and 0.6 ml of 0.1% ferric chloride, and the absorbance of the mixture was measured at 700 nm against a blank to determine the amount of ferric ferrocyanide (Prussian blue) formed. Ascorbic acid, BHA, and α-tocopherol were used for comparison. EC_{50} value (μg extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power.

2.6.3. Ferrous Ions Chelating Abilities

Chelating ability was determined according to the method of Dinis *et al*. [20]. Each extract (1 - 5 mg/ml, 1 ml) in methanol was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride. After 30 s of standing, 0.2 ml of 5 mM ferrozine was added. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. The EDTA, ascorbic acid, BHA, and α-tocopherol were used for comparison. EC_{50} value (mg extract/ml) is the effective concentration at which ferrous ions were chelated by 50%.

2.7. Statistical Analysis

All measurements were carried out in triplicate. All data were subjected to analysis of variance using the Statistical Analysis System software package (SAS Institute, Cary, NC, USA). When a significant difference was found among treatments, Duncan's multiple range tests were performed to determine the differences among the mean values at the level of $\alpha = 0.05$.

3. Results and Discussion

3.1. Effect of Multi-Steps Sequential Extraction Method on the Extraction Yields and Active Compounds

The extraction weights and yields of *E. purpurea* flower extracts obtained by a sequential extraction in three steps were shown in **Table 1**. The extract dry weight and extraction yield obtained from 15 g sample was 5.0629 g and 33.75%, respectively. The extraction yield was slightly lower than the value of 39.8% reported by Tsai *et al*. [13]. This result was not unexpected because only the petals were used in the study of Tsai *et al*. [13]. But in the present study both petals and receptacles were used for extraction. When the extraction steps were compared separately, the percentage of extract dry weight obtained from 1st-, 2nd- and 3rd-step extraction was 63.76%, 27.64%, and 8.60% of total extracted dry weight (summation of 1st, 2nd and 3rd extracts), respectively. How-

Table 1. The weight, extraction yields and active compounds contents of freeze-dried extracts from freeze-dried *Echinacea purpurea* flower with multi-steps extraction method.

Quality characteristics	Number of Extraction				Freezed-dried flower
	1st	2nd	3rd	1st + 2 nd + 3rd	
Weight (g)	3.2284 ± 0.2098 A[a]	1.4002 ± 0.0509 B	0.4343 ± 0.0197 C	5.0629 ± 0.1495	
Extraction yield[b] (%)	21.52 ± 1.40 A	9.33 ± 0.34 B	2.90 ± 0.13 C	33.75 ± 1.00	
Total phenols (mg CAE/g)	182.08 ± 3.74 A	176.33 ± 4.93 B	177.08 ± 4.27 B	180.06 ± 3.96	60.78 ± 1.32
Caffeic acid derivatives (mg/g)					
Caftaric acid	19.97 ± 1.40 A	19.89 ± 0.63 A	19.61 ± 1.43 A	19.92 ± 1.19	6.72 ± 0.11
Chlorogenic acid	2.98 ± 0.25 A	2.58 ± 0.21 AB	2.34 ± 0.16 B	2.81 ± 0.23	0.95 ± 0.01
Caffeic acid	0.40 ± 0.02 A	0.41 ± 0.04 A	0.38 ± 0.04 A	0.40 ± 0.03	0.14 ± 0.01
Echinacoside	1.09 ± 0.10 A	1.09 ± 0.13 A	1.09 ± 0.21 A	1.09 ± 0.12	0.37 ± 0.02
Cichoric acid	66.57 ± 3.53 A	66.54 ± 1.26 A	62.07 ± 2.14 A	66.18 ± 2.78	22.34 ± 0.20
Total caffeic acid derivatives	91.01 ± 5.29 A	90.51 ± 2.14 A	85.49 ± 3.90 A	90.40 ± 4.30	30.51 ± 0.32
Alkamide 8/9[c] (μg/g)	505.38 ± 28.52 B	598.61 ± 25.66 A	585.99 ± 36.46 A	538.08 ± 20.87	181.62 ± 15.28

[a]Each value is expressed as mean ± standard deviation (n = 3). Means with different capital letter within a row are significantly different ($P < 0.05$); [b]Extraction yield (%) = (dried extract weight/sample weight) × 100%; [c]Alkamide 8/9: dodeca-2*E*, 4*E*, 8*Z*, 10(*E*/*Z*)-tetraenoic acid isobutylamide.

ever, the summation of extracted dry weight of 1st- and 2nd-step extractions was over 90% of total extract dry weight obtained through three-step extractions.

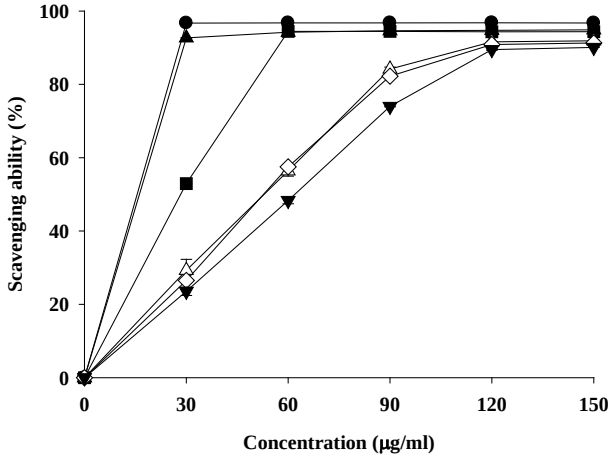
The contents of total phenols also differed significantly among the sequentially prepared 1st, 2nd and 3rd extracts, with the highest amount of total phenols were obtained from the 1st extract (**Table 1**). When the total extraction yields were taken into consideration, total phenols content of the two-steps and three-steps sequential extractions were 834.72 mg and 911.63 mg, respectively. The calculated total phenols content of freeze-dried *E. purpurea* flowers was 60.78 mg/g (**Table 1**).

As for the contents of caffeic acid derivatives in the aqueous ethanol extracts (**Table 1**), the cichoric acid (72.60% - 73.15%) represented the highest portion of phenolic substance, followed by caftaric acid (21.94% - 22.94%), chlorogenic acid (2.85% - 3.27%), echinacoside (1.20% - 1.28%), and caffeic acid (0.44% - 0.45%). These results are in agreement with the report of Tsai *et al*. [13]. No significant differences in the contents of caffeic acid derivatives (except chlorogenic acid) existed among the 1st, 2nd and 3rd extract. When the total extraction yields were taken into consideration, the cichoric acid, caftaric acid, chlorogenic acid, echinacoside and caffeic acid content obtained from 15 g sample were 335.06, 100.85, 14.23, 5.52 and 2.03 mg, respectively. The summation of cichoric acid, caftaric acid, chlorogenic acid, echinacoside and caffeic acid from 1st and 2nd extracts was 91.95%, 91.54%, 92.99%, 91.40%, and 91.89%, with the estimated contents (on freeze-dried flowers base) of 22.34, 6.72, 0.95, 0.37, and 0.14 mg/g, respectively (**Table 1**).

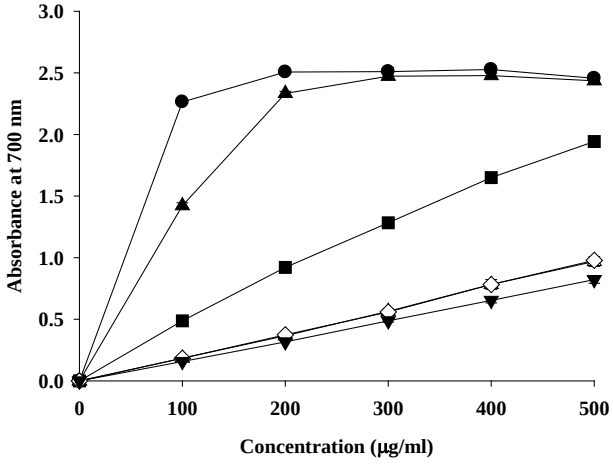
Many alkamides have been isolated and identified from the root, leaves and flowers of *E. purpurea*, with alkamide 8/9 being predominant [21]. Therefore, only alkamide 8/9 were measured in this study. As shown in **Table 1**, alkamide 8/9 were detectable in 1st, 2nd and 3rd extracts, and the contents of these compounds were progressively increased from 505.38 to 598.61 μg/g extract. When the total extraction yields were taken into consideration, the contents of alkamide 8/9 of the two-steps and three-steps sequential extractions were 2469.74 and 2724.24 μg, respectively. Nevertheless, the summation of alkamide 8/9 from 1st and 2nd extracts was 90.66%. The calculated alkamide 8/9 content of freeze-dried *E. purpurea* flowers was 181.62 μg/g (**Table 1**).

3.2. Effect of Multi-Steps Sequential Extraction Method on the Antioxidant Properties

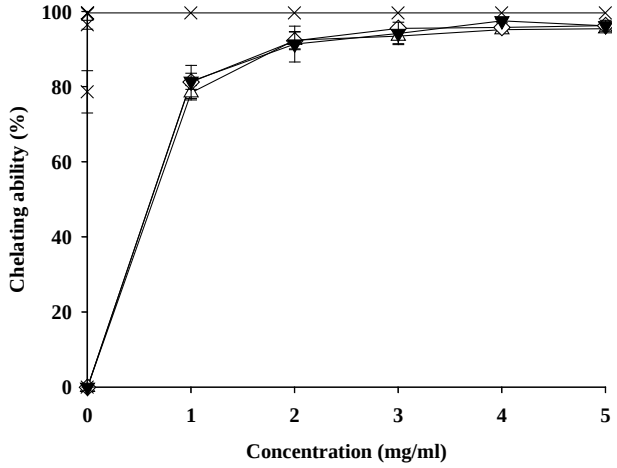
In this study, DPPH radical scavenging abilities of 1st, 2nd, and 3rd flower extracts plus three reference antioxidative compounds (ascorbic acid, BHA and α-tocopherol) presented in **Figure 1(a)** demonstrated dose-de-



(a)



(b)



(c)

Figure 1. Antioxidant properties of standards and extracts from freeze-dried *Echinacea purpurea* flower with different three time extraction. Each value is expressed as mean ± standard deviation (n = 3). Ascorbic acid (●), Butylated hydroxyanisole (▲), and *α*-Tocopherol (■) for scavenging ability on DPPH radicals and reducing power. Ethylenediaminetetraacetic acid (×) for chelating ability on ferrous ions. 1st extract (Δ), 2nd extract (◊), 3rd extract (▼). (a) Scavenging ability on DPPH radicals; (b) Reducing power; (c) Chelating ability on ferrous ions.

pendent responses. The DPPH radical scavenging abilities of 1st, 2nd, and 3rd extracts were lower than those of references. At 30 μg/ml dose, the scavenging abilities of tested samples on DPPH radicals were in the descending order of ascorbic acid (96.71%) > BHA (92.67%) > α-tocopherol (52.94%) > 1st extract (29.28%) ≈ 2nd extract (26.52%) > 3rd extract (23.55%). The estimated EC_{50} values of ascorbic acid, BHA, α-tocopherol, 1st extract, 2nd extract, and 3rd extract for DPPH radical scavenging ability were 15.51 μg/ml, 16.19 μg/ml, 28.34 μg/ml, 52.87 μg extract/ml (9.63 μg total phenols/ml), 52.39 μg extract/ml (9.24 μg total phenols/ml), and 61.45 μg extract/ml (10.88 μg total phenols/ml), respectively. The DPPH scavenging abilities of *E. purpurea* flower extract may be attributable to its high contents of caffeic acid derivatives, particularly the dominant presence of the cichoric acid (**Table 1**). The radical scavenging activity by the DPPH method depends on the number and substitution pattern of hydroxyl groups. Pellati *et al*. [3] reported that the cichoric acid had two adjacent hydroxyl groups on its phenolic rings, therefore it tended to have higher DPPH radical scavenging ability than other caffeic acid derivatives. The high DPPH scavenging ability of cichoric acid in *E. purpurea* leaf extract was also reported by Thygesen *et al*. [9].

The reducing powers of 1st, 2nd, and 3rd flower extracts in **Figure 1(b)** also exhibited dose-dependent responses. At 100 μg/ml dose, the reducing powers of samples were in the descending order of ascorbic acid (2.264 AU) > BHA (1.422 AU) > α-tocopherol (0.487 AU) > 1st extract (0.184 AU) ≈ 2nd extract (0.183 AU) > 3rd extract (0.157 AU). The estimated EC_{50} values of 1st extract, 2nd extract, and 3rd extract for reducing power were 264.98 μg extract/ml (48.25 μg total phenols/ml), 268.74 μg extract/ml (47.39 μg total phenols/ml), and 309.05 μg extract/ml (54.73 μg total phenols/ml), respectively. These values were significantly greater than the EC_{50} values of 22.12 μg/ml, 35.13 μg/ml, 107.67 μg/ml obtained from ascorbic acid, BHA, α-tocopherol, respectively.

Ferrous ions are the most effective pro-oxidants in the food system; therefore it is frequently used as an antioxidant assessment index [22] [23]. The ferrous ions chelating ability of flower extracts and reference compounds were illustrated in **Figure 1(c)**. The ferrous ions chelating ability of ascorbic acid, BHA and α-tocopherol were not detectable. The flower extracts exerted > 80% chelating effect at 1 mg/ml dosew when compared with the 10 μg/ml of EDTA. The EC_{50} values of 1st extract, 2nd extract, and 3rd extract for ferrous ions chelating ability were 637.99 μg extract/ml (116.17 μg total phenols/ml), 616.74 μg extract/ml (108.75 μg total phenols/ml), and 614.04 μg extract/ml (108.73 μg total phenols/ml), respectively. The ferrous ions chelating ability of *E. purpurea* flower extracts may be attributed to the adjacent hydroxyl and carbonyl groups in its flavanol structure, which could chelate ferrous ion to form a complex [24].

3.3. Effect of Multi-Batches Extraction Method on the Extraction Yields and Active Compounds

The extraction dry weight of extract was increased significantly with increasing the number of extraction batches (**Table 2**), but with a concomitant decline in extraction yield. The contents of total phenols in I, II, III, and IV batch extracts varied between 177.25 and 186.92 mg CAE/g extract (**Table 2**). When the total extraction yields were taken into consideration, total phenols content of I, II, III, and IV extracts obtained from 15, 30, 45, and 60 g sample were 558.70, 924.94, 1278.63, and 1404.63 mg, respectively. The obtained total phenols content (mg CAE/g freeze-dried flowers) in the tested I, II, III, and IV batches was 37.25, 30.83, 28.41, and 23.41, respectively (**Table 3**).

As shown in **Table 2**, the contents of chlorogenic acid, caffeic acid, echinacoside, cichoric acid, and total caffeic acid derivatives (except caftaric acid) were not significantly different among the tested extracts. The cichoric acid (73.89% - 74.47%) was the major phenolic substance, followed by caftaric acid (20.50% - 21.24%), chlorogenic acid (3.07% - 3.22%), echinacoside (1.29% - 1.36%), and caffeic acid (0.45% - 0.50%). When the total extraction yields were taken into consideration, the calculated individual and total caffeic acid derivatives contents (mg/g freeze-dried flowers) of freeze-dried *E. purpurea* flowers was I > II > III > IV (**Table 3**).

The contents of alkamide 8/9 in I, II, III, and IV batch extracts was decreased with increasing the number of batch, ranging from 534.36 to 269.76 μg/g extract (**Table 2**). When the total extraction yields were taken into consideration in this study, the contents of alkamide 8/9 in I, II, III, and IV extract samples were 1597.95, 2396.03, 3115.30, and 2101.21 μg, respectively. The estimated alkamide 8/9 content (μg/g freeze-dried flowers) of freeze-dried *E. purpurea* flowers was I > II > III > IV (**Table 3**).

Table 2. The weight, extraction yields and active compounds contents of freeze-dried extracts from freeze-dried *Echinacea purpurea* flower with multi-batches extraction method.

Quality characteristics	Multi-batches extraction			
	I	II	III	IV
Weight (g)	2.9904 ± 0.0807 D[a]	5.2183 ± 0.0971 C	6.8405 ± 0.0297 B	7.7892 ± 0.1327 A
Extraction yield[b] (%)	19.93 ± 0.53 A	17.39 ± 0.33 B	15.20 ± 0.07 C	12.98 ± 0.22 D
Total phenols (mg CAE/g)	186.83 ± 5.03 A	177.25 ± 2.05 A	186.92 ± 8.91 A	180.33 ± 2.53 A
Caffeic acid derivatives (mg/g)				
Caftaric acid	20.08 ± 0.20 A	19.19 ± 0.70 AB	18.47 ± 0.32 AB	18.03 ± 1.43 B
Chlorogenic acid	2.92 ± 0.17 A	2.82 ± 0.19 A	2.90 ± 0.08 A	2.71 ± 0.36 A
Caffeic acid	0.48 ± 0.09 A	0.41 ± 0.01 A	0.44 ± 0.04 A	0.42 ± 0.04 A
Echinacoside	1.27 ± 0.25 A	1.17 ± 0.06 A	1.19 ± 0.17 A	1.17 ± 0.12 A
Cichoric acid	70.31 ± 3.54 A	66.76 ± 3.03 A	67.09 ± 0.92 A	63.66 ± 5.32 A
Total caffeic acid derivatives	95.06 ± 4.09 A	90.35 ± 3.73 A	90.09 ± 1.02 A	85.99 ± 7.18 A
Alkamide 8/9[c] (μg/g)	534.36 ± 40.94 A	459.16 ± 8.57 B	455.42 ± 33.26 B	269.76 ± 8.81 C

[a]Each value is expressed as mean ± standard deviation (n = 3). Means with different capital letter within a row are significantly different ($P < 0.05$); [b]Extraction yield (%) = (dried extract weight/sample weight) × 100%; [c]Alkamide 8/9: dodeca-2*E*, 4*E*, 8*Z*, 10(*E*/*Z*)-tetraenoic acid isobutylamide.

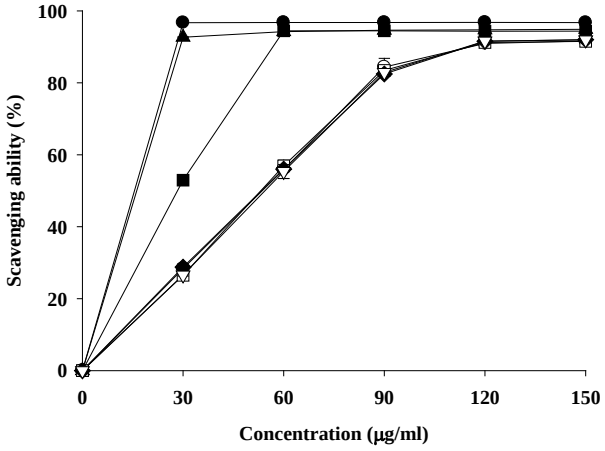
Table 3. Active compounds contents of freeze-dried *Echinacea purpurea* flower with multi-batches extraction method.

Quality characteristics	Multi-batch extraction			
	I	II	III	IV
Total phenols (mg CAE/g flower)	37.25 ± 0.25 A[a]	30.83 ± 0.57 B	28.41 ± 1.16 C	23.41 ± 0.59 D
Caffeic acid derivatives (mg/g flower)				
Caftaric acid	4.00 ± 0.07 A	3.34 ± 0.09 B	2.81 ± 0.05 C	2.34 ± 0.20 D
Chlorogenic acid	0.58 ± 0.04 A	0.49 ± 0.03 B	0.44 ± 0.01 B	0.35 ± 0.05 C
Caffeic acid	0.10 ± 0.02 A	0.07 ± < 0.01 B	0.07 ± 0.01 B	0.05 ± 0.01 B
Echinacoside	0.25 ± 0.04 A	0.20 ± 0.01 B	0.18 ± 0.03 B	0.15 ± 0.02 B
Cichoric acid	14.02 ± 0.33 A	11.61 ± 0.31 B	10.20 ± 0.16 C	8.26 ± 0.69 D
Total caffeic acid derivatives	18.95 ± 0.32 A	15.72 ± 0.38 B	13.69 ± 0.20 C	11.16 ± 0.95 D
Alkamide 8/9[b] (μg/g flower)	106.53 ± 8.19 A	79.87 ± 2.33 B	69.23 ± 5.19 C	35.02 ± 1.12 D

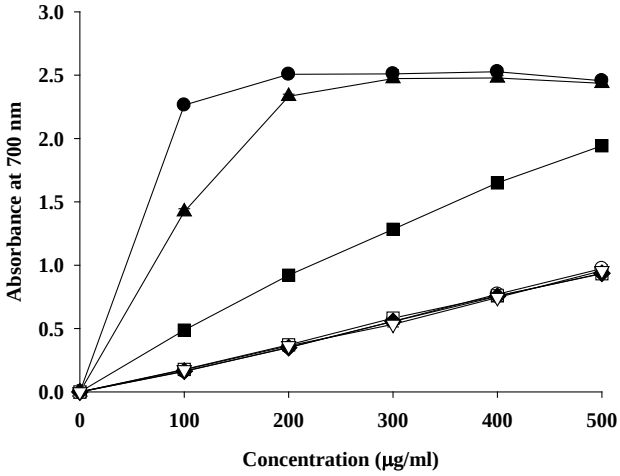
[a]Each value is expressed as mean ± standard deviation (n = 3). Means with different capital letter within a row are significantly different ($P < 0.05$); [b]Alkamide 8/9: dodeca-2*E*, 4*E*, 8*Z*, 10(*E*/*Z*)-tetraenoic acid isobutylamide.

3.4. Effect of Multi-Batches Extraction Method on the Antioxidant Properties

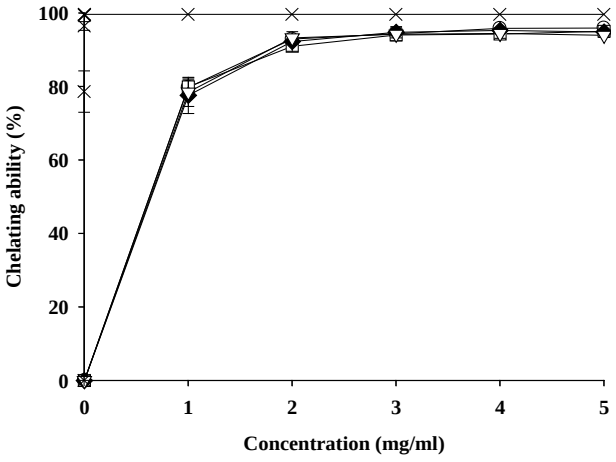
DPPH radical scavenging abilities of I, II, III, and IV flower extracts plus three reference chemicals (ascorbic acid, BHA and α-tocopherol) presented in **Figure 2(a)** also demonstrated dose-dependent responses. The DPPH radical scavenging abilities of I, II, III, and IV flower extracts were lower than those of reference chemicals. At 30 μg/ml dose, the scavenging abilities of tested samples on DPPH radicals are in the descending order of ascorbic acid (96.71%) > BHA (92.67%) > α-tocopherol (52.94%) > I extract (28.36%) ≈ II extract (26.49%) ≈ III extract (28.77%) ≈ IV extract (26.56%). The calculated EC_{50} values of I, II, III, and IV extracts in DPPH radical scavenging ability were 53.64 μg extract/ml (10.02 μg total phenols/ml), 53.43 μg extract/ml (9.47 μg total phe-



(a)



(b)



(c)

Figure 2. Antioxidant properties of standards and extracts from freeze-dried *Echinacea purpurea* flower with different batches extraction. Each value is expressed as mean ± standard deviation (n = 3). Ascorbic acid (●), Butylated hydroxyanisole (▲), and *α*-Tocopherol (■) for scavenging ability on DPPH radicals and reducing power. Ethylenediaminetetraacetic acid (×) for chelating ability on ferrous ions. I (○), II (□), III (♦), IV (▽). (a) Scavenging ability on DPPH radicals; (b) Reducing power; (c) Chelating ability on ferrous ions.

nols/ml), 53.30 μg extract/ml (9.96 μg total phenols/ml), and 54.66 μg extract/ml (9.86 μg total phenols/ml), respectively.

The reducing powers of I, II, III, and IV extracts in **Figure 2(b)** also exhibited dose-dependent responses. The calculated EC_{50} values (μg extract/ml) for I, II, III, and IV extracts in reducing power were 274.34 μg extract/ml (51.25 μg total phenols/ml), 267.99 μg extract/ml (47.50 μg total phenols/ml), 273.82 μg extract/ml (51.18 μg total phenols/ml), and 283.68 μg extract/ml (51.16 μg total phenols/ml), respectively.

The ferrous ions chelating ability of I, II, III, and IV extracts and reference compounds were illustrated in **Figure 2(c)**. The flower extracts demonstrated 70% - 80% chelating effect at 1 mg/ml dose comparing to 10 μg/ml of EDTA (**Figure 2(c)**). The estimated EC_{50} values of I, II, III, and IV extracts in ferrous ions chelating ability were 626.89 μg extract/ml (117.12 μg total phenols/ml), 625.69 μg extract/ml (110.90 μg total phenols/ml), 646.03 μg extract/ml (120.76 μg total phenols/ml), and 638.27 μg extract/ml (115.10 μg total phenols/ml), respectively.

3.5. Comparisons of Quality Characteristics of Freeze-Dried Flowers among Extraction Methods

In multi-steps sequential extraction, the total extract weight of two- and three-steps extraction obtained from 15 g freeze-dried *E. purpurea* flowers were 4.6286 and 5.0629 g, respectively (**Table 1**). When the results are recalculated and expressed on 60 g freeze-dried flowers base, the estimated total extract weights for two- and three-steps sequential extraction is around 18.5144 and 20.2516 g, respectively (**Table 4**). In multi-batches extraction method, the extract weight obtained from IV batches of extraction was only 7.7892 g (**Table 2**).

The contents of total phenols of two- and three-steps sequential extraction obtained from 15 g freeze-dried flowers were 834.72 and 911.63 mg, respectively (**Table 1**). The total phenols content of two- and three-steps sequential extraction obtained from 60 g freeze-dried flowers were 3338.88 and 3646.52 mg, respectively (**Table 4**). In multi-batches extraction, total phenols of IV sample (60 g) only was 1404.26 mg, which is about 42.1% and 38.5% of two- and three-steps sequential extraction obtained from 60 g freezed-dried flowers, respectively. The total caffeic acid derivatives content of two- and three-steps sequential extraction obtained from 15 g freezed-dried flowers were 420.55 and 457.68 mg, respectively (**Table 1**). The total caffeic acid derivatives content of two- and three-steps sequential extraction obtained from 60 g freezed-dried flowers were 1686.20 and 1830.72 mg, respectively (**Table 4**). In multi-batches extraction, the total caffeic acid derivatives content of IV sample (60 g) only was 669.79 mg, which was about 39.8% and 36.6% of two and three sequential extractions obtained from 60 g freezed-dried flowers, respectively.

The alkamide 8/9 content of two- and three-steps sequential extraction obtained from 15 g freezed-dried flowers was 2469.74 and 2724.24 μg, respectively (**Table 1**). The alkamide content of two- and three-steps sequential extraction obtained from 60 g freezed-dried flowers was 9878.96 and 10896.96 μg, respectively (**Table 4**). In multi-batches extraction, the alkamide 8/9 of IV sample (60 g) only was 2101.21 μg, which was about 21.3% and 19.3% of two- and three-steps sequential extraction obtained from 60 g freeze-dried flowers, respectively.

When the amounts of recovered ethanol were taken into consideration, the consumption of 50% aqueous ethanol in two- and three-steps sequential extraction method for 15 g freeze-dried flowers were 98.1 and 124.7 ml, respectively. The total consumption of 50% aqueous ethanol of two- and three-steps sequential extraction for 60 g freezed-dried flowers was 392.4 and 498.8 ml, respectively (**Table 4**). On the other hand, the consumption of 50% aqueous ethanol for IV batch extraction sample was 217.9 ml (**Table 4**).

Table 4. Comparison of total extract weight, active compounds, and ethanol consumption of freeze-dried *E. purpurea* flower with multi-steps and multi-batches extraction methods.[a]

Extraction method[b]	Total extract weight (g)	Total phenols (mg CAE)	Total caffeic acid derivatives (mg)	Alkamide 8/9[c] (μg)	50% Ethanol consumption (ml)
Two-steps sequential extraction	18.5144	3338.88	1682.20	9878.96	392.4
Three-steps sequential extraction	20.2516	3646.52	1830.72	10896.96	498.8
Multi-batches extraction IV	7.7892	1404.62	669.79	2101.21	217.9

[a]The quality characteristics result calculated on the basis of 60 g freezed-dried flowers. [b]Multi-steps extraction 1 and 2 indicated that 2 and 3 sequential extraction, respectively, in multi-steps extraction method. Multi-batches extraction IV: same as **Table 2**. [c]Alkamide 8/9: dodeca-2*E*, 4*E*, 8*Z*, 10(*E*/*Z*)-tetraenoic acid isobutylamide.

4. Conclusion

The weight and yield of extract were decreased significantly different with increased number of extraction in multi-steps extraction. The increased extract weight was not proportioned to the sample batches in multi-batch extraction. The total phenols, individual and total caffeic acid derivatives content of extracts were not significantly different obtained from multi-steps or multi-batches extractions. The alkamide 8/9 contents of extracts were 2nd ≈ 3rd > 1st in multi-steps extraction, and I > II ≈ III > IV in multi-batches extraction. When the total extraction yields were taken into consideration, the results showed that 1st > 2nd > 3rd in multi-steps extraction, and I > II > III > IV in multi-batches extraction. Thus, the optimal extraction method is recommended to be two-steps sequential extraction, when the extract weight, active compounds content, and ethanol consumption were taken into consideration.

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