

Original Article

MICROWAVE-ASSISTED EXTRACTION OF BIO-ACTIVE COMPOUNDS (PHENOLICS AND ALKAMIDES) FROM *ECHINACEA PURPUREA*

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Received: 26 Jun 2014 Revised and Accepted: 18 Aug 2014

ABSTRACT

**Objective:** *Echinacea purpurea* is used widely for its unspecific enhancement of the immune system. It contains multiple bioactive substances, including, phenolics (caffeic acid derivatives), flavonoids, anthocyanins and alkamides. In this currently investigation, efforts are focused on the evaluation of extraction techniques viz., conventional extraction (CE) and microwave-assisted extraction (MAE) for the regeneration of the active constituents of *Echinacea purpurea*.

**Methods:** The aerial parts have been separately extracted with 80% methanol by two methods; CE and MAE. Identification of the isolated compounds was carried out by spectroscopic analysis viz., UV, TLC, <sup>1</sup>H-NMR and MS. TLC scanner system and RP-HPLC with UV detection have been employed for the quantitative determination of caffeic acid derivatives and that of alkamides content in both extracts.

**Results:** Chicoric and caftaric acids have been isolated and identified. The results of the TLC scanner system have revealed that the concentration is about 1.19 ± 0.02 and 1.35 ± 0.03 % (w/w) for caftaric and chicoric acid, respectively of the dry plant material extracted with CE, while the concentration is 1.81 ± 0.04 and 6.19 ± 0.26 % (w/w) for the two acids, respectively of the dry plant material extracted by MAE. The results of the HPLC have revealed that the concentration of alkamides is 0.25 and 0.33 % (w/w) of the dry plant material obtained with CE and MAE, respectively.

**Conclusion:** Our study proved that MAE is a more effective technique compared to the CE. Extraction time was reduced, with the use of less solvent and the yield amount of the extracted active compounds was increased.

**Keywords:** *Echinacea purpurea*, Chicoric acid, Alkamides, HPLC, Microwave-assisted extraction.

INTRODUCTION

The use of plant remedies, known to possess natural antioxidants, immunomodulatory and other activities has increased in the last decade in human medicine, as it is perceived as a natural approach to treat several diseases [1]. One of the most popular medicinal plants is *Echinacea* species. *Echinacea* has received a global attention, because of its increasing medicinal and economic value, it is an excellent example of a plant that contains bioactive phytochemicals including phenolics, flavonoids and anthocyanins. It is widely used in Europe, North America and Australia for the treatment of cold, flu and upper respiratory tract infections [2, 3]. *Echinacea* species such as *E. purpurea*, *E. angustifolia* and *E. pallida* are members of the *Asteraceae* family [4]. The extract of these plants has significant immunomodulatory, antibacterial, antiviral, antifungal properties [5, 6] and antioxidant activities [7].

Two classes of compounds in these species are believed to be responsible for its activity; caffeic acid derivatives (CADS); chicoric, caftaric, chlorogenic and caffeic acid, and alkamides [8]. Chicoric acid is of special interest, due to its rarity in the plant kingdom and its biological multifunctional effects, it has shown phagocytic, antihyaluronidase, antiviral, antioxidant activity, and inhibits HIV-1 integrase and replication [9]. *Echinacea* species produce dodeca-2E, 4E, 8Z, 10E (10Z)-tetraenoic acid isobutylamide, as well as, a number of other alkamides [10]. *Echinacea purpurea* plant contains at least nine alkamides in the roots [11].

Extraction is one of the critical steps in achieving complete recovery of targeted compounds. The drawback of many extraction methods is that, time consuming, requires relatively large quantities of solvents and samples are extracted at high temperatures and for prolonged periods of time, which may lead to reduced phenolics concentration [12]. Recently, innovative extraction techniques are applied. Microwave-assisted extraction (MAE) which represents a reliable alternative to traditional extraction techniques has been used to result in a yield increase in shorter time at the same temperature using less solvent [13]. MAE has found its application

for fast extraction of plant polyphenols [14], however, there has been no report about its use for extracting *Echinacea* polyphenolic compounds. The main objective of this study is designed to demonstrate the utility of MAE for *Echinacea purpurea*, so as to evaluate the influence of MAE on the extraction yield of phenolic acids and alkamides with the comparison to conventional extraction. MAE is fully validated and its practical applicability is proved by the analysis of *Echinacea purpurea*. Furthermore, MAE conditions can be optimized in order to determine the extraction parameters for optimal and significant extraction of natural compounds. Total caffeic acid derivatives and alkamide contents were quantified during this study.

MATERIALS AND METHODS

General

Thin layer chromatography (TLC) was carried out on pre-coated silica gel F254 plates (Merck, Darmstadt, Germany) developed with methylene chloride: methanol (8:2) and ethyl acetate: formic acid: acetic acid: H<sub>2</sub>O (30: 1.5: 1.5: 7). Spots were detected using Neu's reagent (1% 2-aminoethyl diphenylborinate in methanol) for caffeic acids, and *p*-anisaldehyde reagent (glacial acetic acid, sulfuric acid, and *p*-anisaldehyde 10:5:0.5) for alkamides (Aldrich). Column chromatography (CC) was performed using sephadex LH-20 (Pharmacia, Merck, Darmstadt, Germany). NMR was recorded on a Delta 2 spectrometer operating at 500 MHz in DMSO. Chicoric, caftaric, chlorogenic acids and dodecanoic acid (2-hydroxy-1,1-Bis-hydroxymethyl-ethyl) amide were purchased from Aldrich sigma. All solvents used were HPLC grade, double distilled water was used.

Plant material

*Echinacea purpurea* was collected from the Botanical Garden of Sekam Co. in Belbais during April 2010. The plant was identified by Prof. Dr. Kamal M. Zayed, taxonomist, Botany Dept., Faculty of Science, Cairo University, Egypt. Aerial parts of the plant were air dried at room temperature and ground into fine powder. A voucher specimen has been deposited at the herbarium of the NRC.

## Extraction

The air dried and ground aerial parts of *E. purpurea* were exhaustively extracted with two methods; Conventional extraction: 1 kg was percolated with 80% aqueous methanol (5Lx 3) at room temperature for 3 days.

Microwave-assisted extraction: 1 kg was extracted with the solvent in a 2L glass vessel using a monomode focused microwave apparatus with a closed-vessel system (Mars, CEM). The instrument was able to provide continuous non-pulse microwave heating focusing on a single cavity where the sample was placed. During the extraction, magnetic stirring was applied to homogenize the sample. The experimental conditions were set as follows: extraction solvent 80% aqueous methanol, extraction temperature at 60°C and extraction time for 20 min, with a radiation output of 800 W [15]. After the extraction time had elapsed, the vessel was allowed to cool at room temperature, and the extract was filtered.

## Fractionation and isolation of the main phenolic compounds

A weighed amount of the extract residue (MAE) was dissolved in aqueous methanol, then passed through a column of fuller's earth (30x10 cm) and then through a charcoal column. Then, the residue obtained after evaporating the solvent was washed with methanol, and the filtrate was concentrated under reduced pressure. The residue was subjected to CC on sephadex eluted with 100% methanol with gradient increase of distilled water (10% at each time) till 60% water concentration. 24 fractions (100 ml each) were collected, and monitored by TLC using silica gel plates. The spots were visualized under UV before and after spraying. Similar fractions were pooled together into 6 subfractions. Subfraction 2, eluted with 90% methanol, was rechromatographed on sephadex column (70 x 5), eluted with methanol: water 1:1. The main fraction upon standing overnight in 80% methanol yielded white homogenous residue, which was further purified to give chicoric acid (**1**) (70 mg). Subfraction 4, eluted with 70 % methanol, was subjected to a CC on sephadex eluted with chloroform and gradient increase in methanol concentration till 100%. Similar subfractions were pooled together. The main subfraction was further subjected to CC on sephadex to give caftaric acid (**2**) (40 mg) which was purified by CC.

## TLC scanner: densitometric analysis

The CAMAG HPTLC system (Muttensz, Switzerland) consisted of TLC scanner 3 connected to a computer running WinCATS version 1.2.3; and Linomat V sample applicator connected to a nitrogen tank.

Samples of both extracts (CE, MAE) and standard solutions were prepared by dissolving 2 mg in 1 ml HPLC grade methanol. 25 µl sample solutions and 10 µl standard solutions were applied in the form of 10 mm bands with CAMAG microlitre syringe on precoated silica gel glass plate 60 F<sub>254</sub> (20x10 cm with 0.2 mm thickness; Merck, Germany). The TLC procedure was optimized with a view to quantify the alcoholic extract of *Echinacea purpurea*. Initially two phenolic standards were used (chicoric and caftaric acids).

The mobile phase ethyl acetate: acetic acid: formic acid: H<sub>2</sub>O (30: 1.5: 1.5: 7) gave good resolution for the sample. The bands of chicoric and caftaric acids from sample solution were confirmed by comparing the R<sub>f</sub> and spectra of the bands with that of standards. The peak purity of phenolic acids was assessed by comparing the spectra at three different levels, i. e., peak start, peak apex and peak end positions of the bands [16-18].

## HPLC analysis

High performance liquid chromatography method was used for quantification of the alkamide with an Agilent Technologies (Waldbronn, Germany) modular model 1200 series instrument, equipped with photodiode array detector and the chromatographic data analyses were done using Chemstation soft-ware. Gradient elution method with acetonitrile: H<sub>2</sub>O (40: 60 v/v to 80/20 v/v) in 20 min was used for the chromatographic analyses. At 25°C, the separation was conducted with a reversed phase column (Eclipse) DB C18. Flow rate was fixed at 1 ml/ min, injection volume 50 µl.

Detection was set at UV 210 nm and 254 nm [19]. A weighed amount of the methanol residues of CE and MAE were separately dissolved in chloroform, and hexane was added. The supernatant, containing the crude alkamide (Fig. 1), was concentrated and subjected to HPLC and spectrophotometric determination [20].

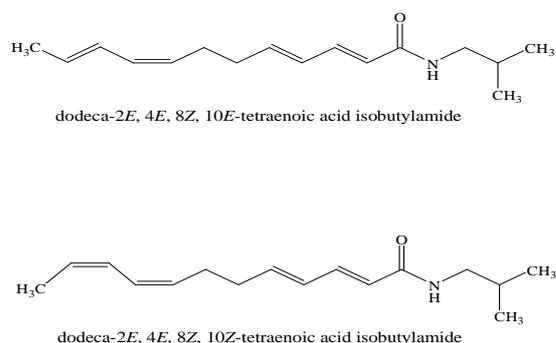


Fig. 1: Alkamides of aerial parts of *Echinacea purpurea*

## RESULTS AND DISCUSSION

### Isolated compounds

By correlating with spectral data (TLC, UV, MS, <sup>1</sup>H-NMR) of literature values, the tartaric esters of caffeic acid; Chicoric and caftaric acids were identified (Fig. 2). R<sub>f</sub> (TLC) = 0.89 and 0.57 respectively, with fluorescent spot appearance (UV) before and after spraying with spray reagent. UV λ max in MeOH: 270, 350. The mass spectrum of compound **1** showed fragment ions m/z at 473 for (M-H<sup>-</sup>), 311 for (M-H-caffeoyl), and 293 for (M-H-caffeoyl-H<sub>2</sub>O) which was found to be in agreement with that reported in literature for chicoric acid, for caftaric acid, it showed 311 for (M-H) as parent ion, 149 for (tartaric acid-H) and 179 for (caffeic acid-H) [21].

The <sup>1</sup>H-NMR spectrum of the isolated compounds showed a well defined typical pattern of trans-caffeate moiety and a singlet at 5.45 ppm in good accordance with chicoric acid, and well defined data of caftaric acid as compared to literature [22].

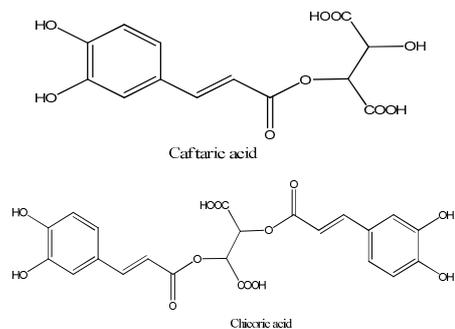


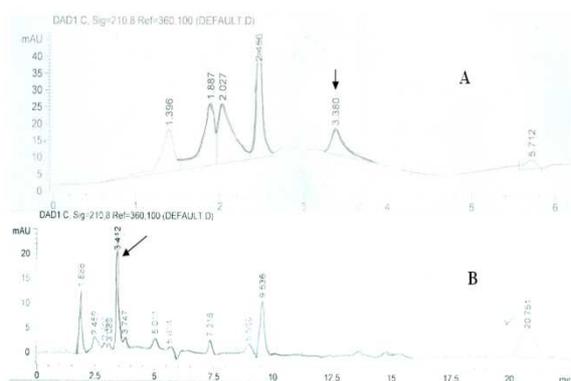
Fig. 2: Caffeic acid derivatives

### Densitometric analysis

The phenolic acids were detected by UV scanning at 350 nm and sprayed with Neu's reagent to compare the band colour with that of the standard. Quantitative analysis was done to compare the yield of the caffeic acid derivatives in both extracts (CE and MAE). The percentage of chicoric and caftaric acids were calculated with the use of two calibration curves obtained from their correspondent standard solutions [23]. Calculation results showed higher concentrations of the acids obtained by MAE. In this case, the total amount for caftaric acid was 1.19 and 1.81% (w/w) in the dried aerial parts of *Echinacea purpurea*, obtained by CE and MAE, respectively. The total amount for chicoric acid was 1.35 and 6.19 (w/w) obtained by CE and MAE, respectively.

### HPLC analysis

Quantification was done via a calibration with standard (external standard method) [24]. A stock solution (1 mg/ml) of standard alkamide derivative (dodecanoic acid (2-hydroxy-1,1-Bis-hydroxymethyl-ethyl) amide in methanol) was prepared. Standard calibration curve was prepared using serial amounts of standard (20- 50 µg) from stock solution. Typical calibration curve was prepared by plotting peak area (y) against injected amount (x, µg). The calibration curve had correlation coefficient,  $R^2 = 0.960$ . The content of alkamides was 0.25 and 0.33 % (w/w) of the dry plant weight (expressed as dodecanoic acid (2-hydroxy-1,1-Bis-hydroxymethyl-ethyl) amide obtained with CE and MAE respectively (Fig. 3).



**Fig. 3: HPLC chromatograms of alkamide (authentic), A and *Echinacea purpurea* extract obtained by MAE, B, detection 210 nm: retention time 3.380.**

### DISCUSSION

The analysis of phenolic compounds in plants attracts considerable attention. CE as well as MAE of phenolic substances from aromatic plants using different solvents have been studied [25]. In recent years, MAE has found its application for fast extraction of plant polyphenols, it is proved that MAE is a more effective technique compared to CE, consequently, considered as a potential alternative to traditional solid-liquid extraction for the isolation of metabolites such as phenolic compounds from plants [26]. MAE is a process that uses microwave energy to heat solvent rapidly and effectively, it is fast, reliable with better efficiency of extraction.

This may be attributed to the better absorption of microwave energy, which enables homogenous heating of solvent and plant matrix, thus increasing temperature inside the plant cells which contains significant amount of water that strongly absorbs microwave energy, resulting in internal superheating, causing disruption of cell walls and releasing compounds into the surrounding solvent, facilitating extraction process. Further-more, the migration of dissolved ions, which increases solvent penetration into the matrix and subsequently, increases extraction yield [27].

*Echinacea purpurea* is one of the few plants that stimulate the non-specific immune system [28]. TLC scanner system and HPLC with UV detection were employed for the quantitative analysis of the caffeic acid derivatives as well as that of alkamides of *Echinacea purpurea* aerial parts extracted with MAE and CE. Results showed that MAE was more efficient than CE, all studied compounds were present in higher concentrations using MAE compared to CE. It showed that each phenolic acid as well as alkamides determined for MAE with 80% methanol was two to four times, that determined for CE with the same solvent. The ratio of the investigated compounds, extracted with CE to MAE was 1: 1.5 for caftaric acid, 1: 4.5 for chicoric acid and 1: 1.3 for alkamides. Since, the phenolic content in *Echinacea purpurea* is one of the important markers for controlling the quality of drugs containing it, we suggest that extraction of the active compounds of this important plant could be enhanced via this

technique of extraction. It is obviously clear that the development of a MAE method for fast and economical application in enriching the concentration of total phenolics may enhance the market potential for medicinal plants.

### CONFLICT OF INTERESTS

Declared None

### ACKNOWLEDGEMENT

The authors are grateful to project "Economically and Environmentally Friendly Innovated Techniques for Extraction and Isolation of Medicinal and Aromatic Plants (MPA) of Egypt: A Technology Transfer Proposal" (Phase I); Project ID 973, for making the extraction techniques available. This Project is funded by Science and Technology Development Fund (STDF).

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