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Original Article

Effect of Solvent Type on Rosmarinic Acid, Total Phenol, Flavonoids, and Antioxidant Activity of *Nepeta asterotricha* Rech. f: An Endemic Plant from Iran

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Abstract

The current study aims to investigate the effect of solvent variety on some phytochemical factors of *Nepeta asterotricha* Rech. f.: as an endemic plant from Iran. It is suspected that quantity and quality of chemical compounds would be affected by solvent type. For this purpose, aqueous, hydro-alcohol (50/50 ethanol/water), and methanol were used as solvents. The studied factors were total phenol, flavonoid, antioxidant activity, and rosmarinic acid that were measured by Folin-Ciocalteu, colorimetric, FRAP, and HPLC methods, respectively. The outcomes showed hydro-alcohol extraction could significantly isolate phenol compounds (156.84 mg GAE/g DW) with considerable antioxidant properties (318.55 mg AA/g DW). However, methanol was more effective to extract total flavonoid content (101.34 mg RE/g DW) and rosmarinic acid (140.39% DW). In addition, more study should be done to investigate the importance of each compounds for both medicinal and industrial uses.

Keywords: Folin-Ciocalteu, Colorimetric, FRAP, HPLC, Endemic.

Introduction

Nepeta L. is demonstrated by 79 species in the Iranian flora, 42 of which are endemic such as *Nepeta asterotricha* Rech. f. [1,2]. The species has been used as febrifuge, anti-septic, antitussive, diaphoretic, sedative, anti-asthmatic, antipyretics, against snakes and scorpion bites, feline and canine attractants [3,4]. Other therapeutic effects are antioxidant [5,6], cytotoxic [7], Antifungal [8], and anti-inflammatory [9].

Medicinal plants are valuable for their therapeutic effects that have chemical components serving as natural products, or secondary metabolites such as phenol, flavonoids, organic acids, terpenes, terpenoids, among others. In other words, these compounds can be used for human disease treatment [10-18]. Characterization and isolation of compounds would be influenced by the extraction methods and condition. In addition, variation in composition and pharmacologic properties are related to technical practices among different laboratories briefly named as post-harvest techniques such as solvent type, sample preparation, and extraction method [19-22].

The present study aims to investigate total phenolic compounds, antioxidant activity, and variation the content of rosmarinic acid from *N. asterotricha* Rech. f. in different solvent. For this purpose, ethanol, methanol, and aqueous were used to extract aerial part of the genus. It is noteworthy that the current research consists of two main objective, which the first one is influence of solvent on the studied phytochemical parameters in *N. asterotricha* Rech. f. The other one is evaluation of the effect of solvents on antioxidant activity and the amount of rosmarinic acid.

Method and Material

Plant Material

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The plant materials were prepared at full flowering stage in Deh-Bala, Yazd, Iran. The sample were identified in Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. The Herbarium code of *N. asterotricha* Rech. f. is MPH-2569.

Plant Extraction

In order to formulate different extract, several solvents were prepared for maceration method. The studied solvents were methanol (MeOH), water (aqueous), and hydro-alcohol (50% water: 50% EtOH). Each solvent was separately added to dried material of the aerial part of *N. asterotricha* with 20 times ratio. Then, the new mixtures were shaken for 24 hours and were separately filtered. Each sample was extracted two times. Finally, the solvents were completely removed and three types of extraction were kept in shade for further investigation.

Total Phenol Content

Folin–Cicalteu reagent was used to determine the concentration of total phenolic compounds [22]. Briefly, 5 µl of each extract (2 mg/ml), 195.5 µl of distilled water, and 12.4 µl of Folin–Cicalteu was mixed to microplate wells. After 3 minutes, 37.15 µl of 7% (w/v) sodium carbonate was added, too. The newly obtained solution was shaken for 120 minutes and absorbance was measured at 765 nm. Finally, based on standard Gallic acid (GA) curve, total phenol compounds were calculated and expressed as mg of Gallic acid equivalents (GAE) per g of dry weight (DW).

Total Flavonoid Content

According to colorimetric method, total flavonoid content was measured. In detail, 20 µl of each extract (10 mg/ml) was mixed with 80 µl of distilled water and 6 µl of $NaNO_2$ (15%) and allowed to stand for 6 minutes. Then, 6 µl of 10% $AlCl_3$ solution was added. After 6 minutes, 80 µl of 4% NaOH and 8 µl distilled water was added to the solution. The obtained mixture was shaken for an hour and absorbance was measured at 510 nm. The total flavonoid content of the different extracts was expressed as mg of rutin equivalents per g of dry weight of plant material (mg RE/g DW).

Antioxidant Activity

The ferric reducing antioxidant power (FRAP) was carried out using standard method. In order to prepare FRAP reagent, 300 mmol sodium acetate buffer (pH 3.6), 20 mmol iron (III) chloride solution, and 10 mmol TPTZ solution in 40 mmol HCl in a volume ratio of 10:1:1 were mixed. It is worth to note that prepared FRAP have to use fresh that is stable for 30 minutes. After that, 200 µL of FRAP was added to 20 µL of each extract (0.5 mg/mL). Then it was incubated at 37 °C for 30 min and the absorbance was determined at 593 nm. Different concentrations of iron (II) sulfate solution (0.0625-1 mmol) were prepared to do standard solutions for the calibration curve. It should be mentioned that ascorbic acid was used as control sample and the results were expressed in mg ascorbic acid per g of dry weight extract.

Rosmarinic Acid in the Plant Extracts

Isolation of rosmarinic acid was performed using HPLC. A waters liquid chromatography apparatus consisted of a Separations module: waters 2695 (USA) and Dual absorbance Detector waters 2487 (USA) were used for the HPLC analysis. Injection was auto sampler injector equipped with a 100 µl loop. Data acquisition and integration was performed with Millennium32 software. The chromatographic assay was performed on a 25 cm×4.6 mm with pre-column, Eurospher 100-5 C_{18} analytical column provided by KNAUER (Berline, Germany) reversed phase matrix (5 µm) (Waters) and elution was carried out in a gradient system with acetonitrile as the organic phase (solvent A) and distilled water (solvent B) with the flow-rate of 1 mL min^{-1}. Peaks were monitored at 330 nm wavelength. Injection volume was 20 µL and the temperature was maintained at 25°C. Calibration graphs (figure 1) were plotted subsequently for linear regression analysis of the peak area with concentration 6, 10, 20, 50, 100, and 200 mg L^{-1}.

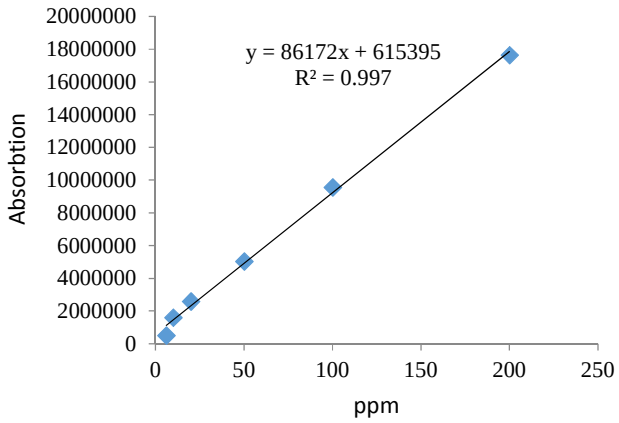

Fig. 1 Calibration curve

Statistical Analysis

In order to analyze the data, all of them were subjected to SAS version 9.4. Probability levels of 1% and 5% ($P < 0.01$ or 0.05) were used to test the significance among the treatments. All analyses were conducted in three independent replicates. The mean values and standard deviations for all studied factors were calculated and the obtained outcomes were expressed as mean ± SD. Least significant difference (LSD) was used to split the means of main effect, when an F-test showed statistical significance.

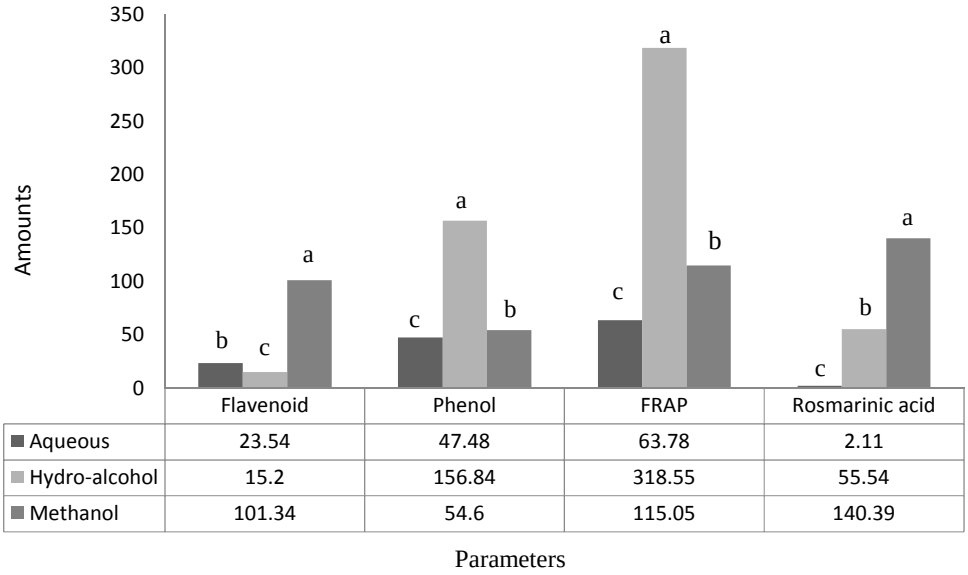

Fig. 2 The effect of solvent type on the studied parameters

Results and Discussion

In order to extract phytochemical compounds, solvent type is one of the most important factor [23]. For this purpose, various solvents (water and organic solvent) with different polarities were used to comprise the solvent effect. The TPC, TFC, antioxidant activity, and rosmarinic acid of the studied solvent were in the range of 54.6-156.84 mg GAE/g DW, 15.2-101.34 mg RE/g DW, 63.78-318.55 mg AA/g DW, and 2.11-140.39% DW, respectively. There is noteworthy that high level of TPC in extract does not cause a high amount of TFC. The present results showed hydro-alcohol extract could isolate more phenol compounds, significantly. Use of ethanol as a polar organic solvent could cause this result and it is in accordance with other research that revealed combination of aqueous organic solvent is more useful than absolute organic solvent to extract TPC [24]. However, some researchers believed that the amount of TPC could be increased using ethanol as an absolute organic solvent [25,26]. Comparing the effect of the studied solvents showed highly significant difference for the total flavonoids. The present outcome showed methanol was the best solvent to extract the total flavonoids content and it was similar to previous study [27].

The antioxidant properties of hydro-alcohol extraction was the best one. This activity is associated with the high level of phenol compounds, which is in accordance with previous studies [18,19,28,29]. On the other hand, the present results like other study emphasized aqueous-organic solvents verified higher free radical scavenging activity than their absolute organic preparations [24]. Despite of these outcomes, some research showed the radical scavengers were more soluble in absolute organic solvent than aqueous one [30]. Based on these variations, it is speculated the type of solvent, polarities, and the existence of polyphenolic compounds have effect on the antioxidant activities.

One of the most important phenolic acids is rosmarinic acid that is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid derived from hydroxycinnamic acid [31]. This compound showed several biological properties such as antioxidant and anti-mutagen [32], hepatoprotective [33], anti-acetyl cholinesterase properties [34], neuroprotective [35], antiviral, antibacterial, and anti-inflammatory [36,37], so investigation on this compound is valuable. Use of methanol as a solvent can obtained the highest amount of rosmarinic acid. In addition, the current research like other researches showed the phytochemical compositions have been significantly affected by solvent [20,23].

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