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Antioxidant screening through DPPH-HPLC-PDA analysis on *Phlomis herba-venti L. subsp. Kopetdaghensis*

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Abstract : This study aimed to determine the antioxidant properties of three *phlomis herba-venti L. subsp. Kopetdaghensis* extracts from Lamiaceae family. The antioxidant activity of this plant was evaluated by DPPH test which was determined by high performance liquid chromatography. The solvents used in this test were methanol, dichloromethane and ethyl acetate. According to IC50 values the highest antioxidant activity belongs to dichloromethane extract which has decreased the sample peak area more than two other solvents.
Keywords: Antioxidant screening, DPPH-HPLC-PDA analysis, *phlomis herba-venti L. subsp. Kopetdaghensis.*

Introduction

Medicinal plants are promising sources of natural antioxidants. A significant number of different spices and aromatic herbs have been investigated for their antioxidant activity. In last forty years, synthetic and artificial antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tert-butylated hydroquinone (TBHQ), propyl gallate (PG) and octylgallate (OG) have been used extensively to inhibit oxidation in foods. However, in last years epidemiological studies have pointed to the possible health risks associated with consumption of synthetic antioxidants and strict regulations now govern their use in foods[1]. Nowadays, It is broadly accepted that certain classes of plant-based compounds such as dietary fiber, phenolic acids, flavonoids, vitamins, radical scavengers and neuropharmacological agents play preventive role against the incidence of some common diseases like cancer, cardiovascular and neurodegenerative disorders, inflammations as well as the ageing process[2,3]. Some, particularly those belonging to the Lamiaceae family, have been found to be very effective with regard to natural antioxidants. In various studies, rosemary, sage, oregano and thyme have shown strong antioxidant activity[4-8].

The plant chosen for the presented study belongs to one of the largest botanical family Lamiaceae. The family Lamiaceae consists of about 252 genera and more than 6700 species[9]. Some of Lamiaceae species are frequently used in cooking and are recognized as important preventive factor of many diseases[10-12]. Essential oils and extracts of these plants are known to possess antiseptic, anti-inflammatory and antimicrobial activities[13-14].

In Iran, the Lamiaceae family is represented by 46 genera, 406 species and 97 infraspecific taxa; of these, 165 taxa are endemic. This family in Iran is distributed in whole country but the species number

decreases from the centre towards the east, south-east and south. Species belonging to Lamiaceae contain flavonoids, phenolic acids, terpenes, saponins, polyphenols, tannins, iridoids, and quinones.

Phlomis is a large genus of the plant family Lamiaceae, having more than 100 species distributed throughout Euro-Asia and North Africa. The various species of this genus generally used as herbal tea against gastrointestinal troubles and to promote health by protecting liver, kidneys, bones and cardiovascular system[15]. Pharmacologically, some species are described to possess antidiabetic[16], antinociceptive, antiulcerogenic, anti-inflammatory, antiallergic[17], anticancer[18], antioxidant and antimicrobial properties[19]. Literature survey revealed that monoterpenes, sesquiterpenes, aliphatic compounds, fatty acids, flavonoids, iridoids and phenylethyl alcohol have been isolated from the relatives of this genus[20].

The purpose of presented study is to evaluate the antioxidant properties of three different extracts of *phlomisherba-venti L. subsp. Kopetdaghensi*s which is a local plant in North Khorasan province in Iran, by high performance liquid chromatography.

Plant material:

The Plant material was collected from a mountainouns area in North Khorasan Province called "Yamandagh". The plant was identified and confirmed by Natural Products & Medicinal Plants Research Centre, North Khorasan University of Medical Sciences (Iran) and Voucher specimen (No.NMP44/18-2) was deposited in herbarium of the Natural Products & Medicinal Plants Research Centre.

Standards and reagents:

Methanol (CHROMASOLV, ≥99.9%, Sigma-Aldrich), CH2CL2(650463, CHROMASOLV, ≥99.9%, Sigma-Aldrich), ETOAC (439169, CHROMASOLV, ≥99.8% Sigma-Aldrich), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (257621, Sigma- Aldrich)

High Performance Liquid Chromatography condition:

High performance liquid chromatography (HPLC) was run on a LC-6AD pump (Shimadzu, Kyoto, Japan) connected to a SPD-M20A Diode Array detector (Shimadzu).The used column was analytical Shim-pack ODS-A, 4.6 × 250 mm, 5 µm (Shimadzu, Japan).

Preparation of plant extracts:

The aerial parts of the plant were dried under shade at room temperature and then cut into small pieces. About 100 g of sample was separately left in 1 Lit of three different solvents (methanol, dichloromethane and ethyl acetate) at 25°C. Each solvent was allowed to remain in contact with plant material for 2 days. Removal of solvents under vacuum at 40 ° using a rotary evaporator gave the crude extracts[21] and then weighed. The resulting extracts were stored in refrigerator at +4 ̊c for further analysis.

Evaluation of Antioxidant activity:

HPLC analysis for DPPH radical scavenging:

The extracts antioxidant activity were determined by DPPH-HPLC method[22]. Fresh DPPH (2.5mM) stock solution was prepared. Then, 100 µl of different concentration of methanolic, dichloromethane and ethyl acetate plant extracts were mixed with 100µl of DPPH. Mixtures were vortexed and kept in dark for 30 min at room temperature. 20 µl of these three samples were injected to HPLC. The blank was prepared by adding 100µL of DPPH to 100µL of methanolic solution. Analyses were carried out using a Reverse phase column and mobile phase was methanol/water (80:20, v/v) at a flow rate of 1 mL/min. The DPPH peaks were monitored at 517 nm. The difference between DPPH peak area in blank and samples was used for evaluating the percent radical scavenging activity of the sample by using equation (1):

$$\%Absorbance\ Inhibition = \frac{blank\ peak\ area - sample\ peak\ area}{blank\ peak\ area} * 100 \qquad \textit{Eq. 1}$$

The blank solution was ascorbic acid and methanol used as positive control. [23]AI was calculated as IC_{50} values which were calculated by using Graph Pad Prism software, version 5.0.1.

Results and Discussion

The antioxidant activity is generally due to presence of phenolic compounds in plant extracts[24]. The redox properties of phenolic compounds make them to behave as reducing agents, hydrogen donors and singlet oxygen quenchers[25]. The polarity of solvent has a significant role in phenolic compounds extraction and methanol is an efficient solvent in their extraction[26]. In the presented study three different solvents with different polarities were used. The highest yield of extraction goes to methanolic extracts (8.34%) among these three solvents due to its high polarity compared to dichloromethane(1.43%) and ethyl acetate(1.28%).

In last few years, HPLC-DPPH analysis developed for identification of antioxidant compounds. In antioxidant compounds, DPPH destroys the conjugated system. Consequently, the peaks of these compounds in HPLC chromatograms would be significantly reduced or disappeared. Figs 1,2 and 3 show chromatograms of the methanol, dichloromethane and ethyl acetate extracts with DPPH treatment monitored at 517 nm[27].

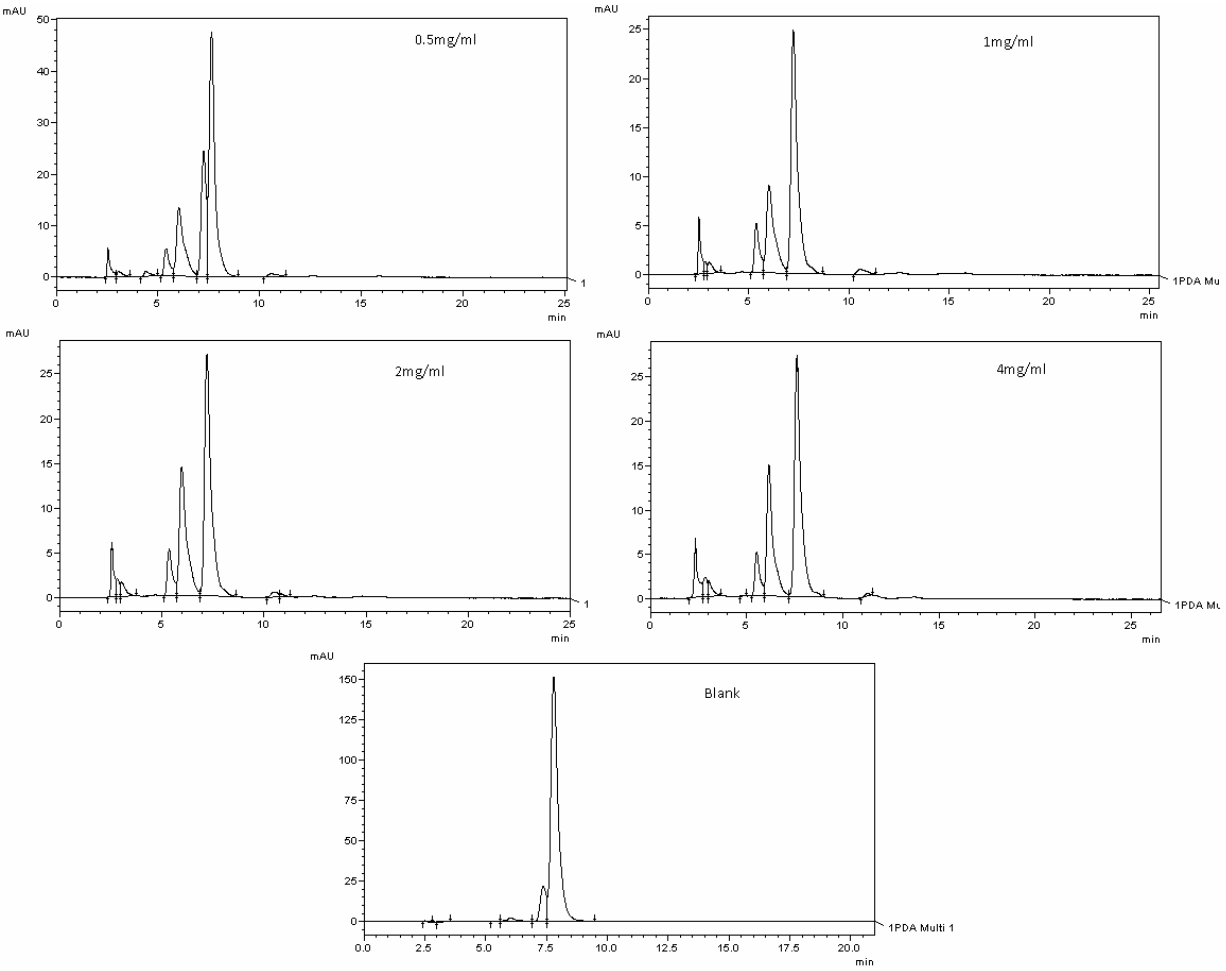

Fig.1:HPLC-DPPH chromatograms of methanolic extract withdiffrenet concentration

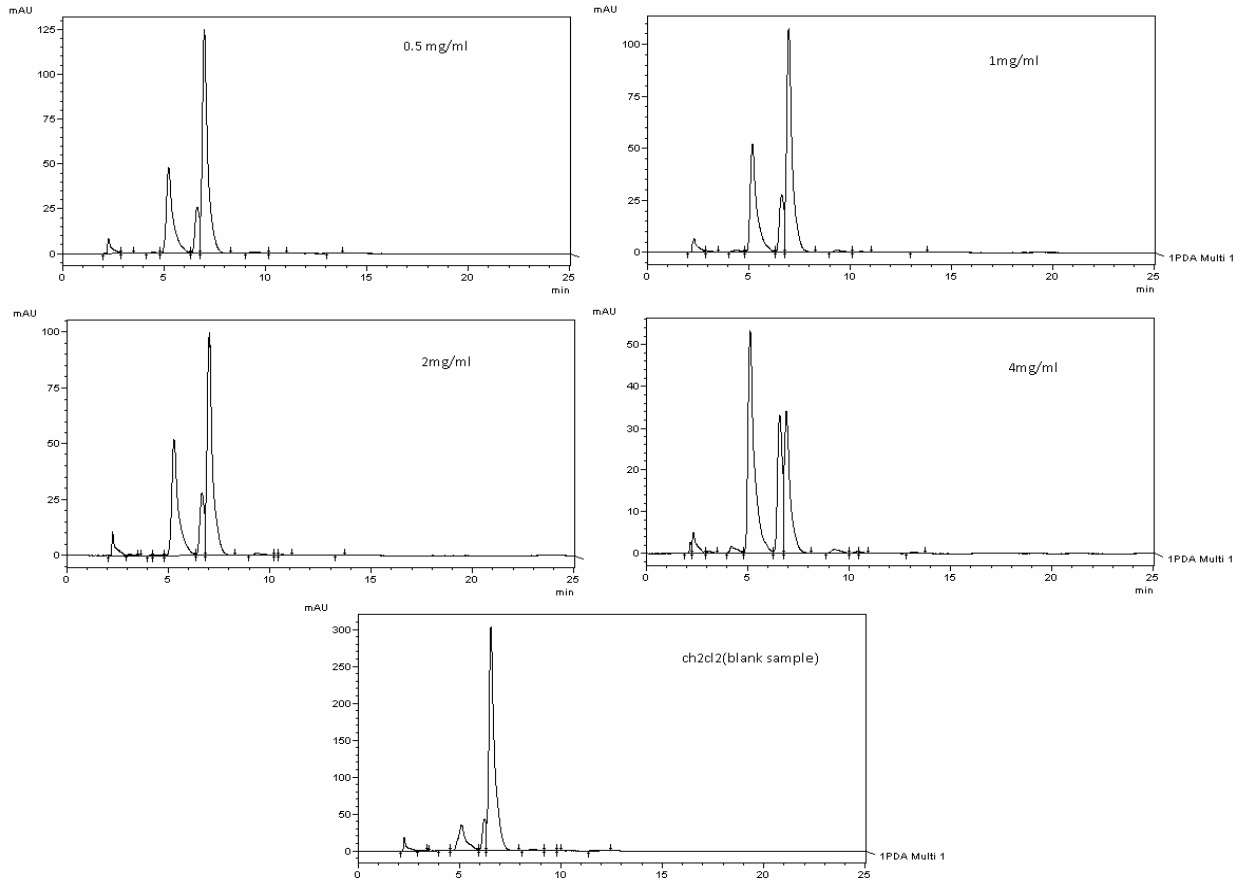

Fig.2:DPPH-HPLC chromatograms for dichloromethane extract with different concentration

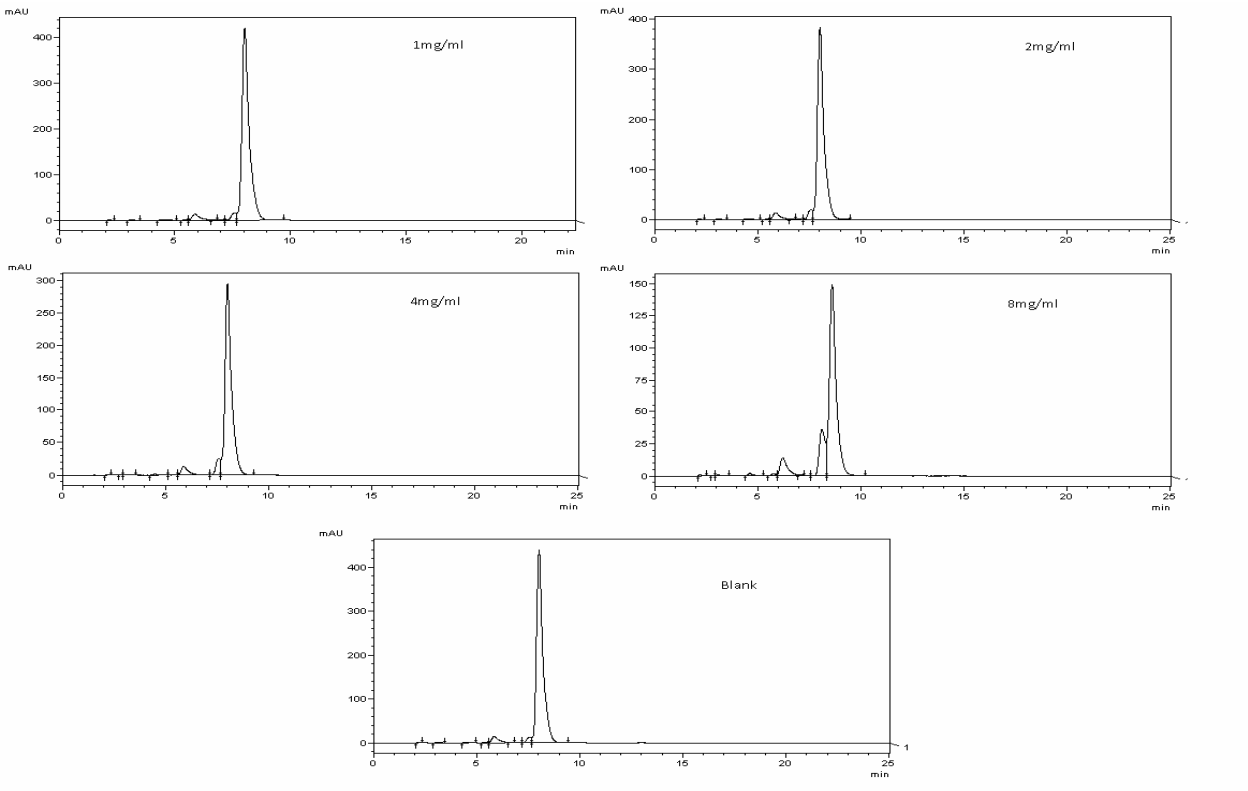

Fig.3: HPLC-DPPH chromatograms of ethyl acetate extract with different concentration

As shown in figures 1,2 and 3 as the sample concentration increased, after spiking the DPPH solution the peak areas decreased remarkably. The sample peak areas are shown in Table 1.

Table 1: Sample Peak Areas for Different Solvents with Different Concentrations

Solvent	Blank	0.25mg/ml	0.5mg/ml	1mg/ ml	2mg/ ml	4mg/ ml	8mg/ ml
Methanol	3179487	1622037	952139	565525	617499	636146	-------
Dichloro methane	5827421	----------	2346050	2033929	1841936	662109	-------
Ethyl acetate	9397782	---------	---------	---------	8382904	6479100	3380389

The maximum (4mg/ml) and minimum concentration (0.25mg/ml) of methanolic extract has inhibited 79.99% and 48.98% of DPPH compared to Vitamin C which inhibited 89.66% of DPPH. For dichloromethane extract, the %DPPH inhibition of the maximum concentration is 88.63% which is lower than that of methanol and higher than the ethyl acetate maximum concentration (8mg/ml) with 64.02% DPPH Inhibition. The obtained results were reported as IC50, which is defined as the amount of antioxidant required to inhibit 50% of DPPH free radicals and in this method, it is well-known that the lower IC50 has the higher antiradical activity. IC50 values of methanolic, dichloromethane and ethyl acetate extracts are 0.407,0.292 and 5.867, respectively. According to IC50 values, dichloromethane extract has the highest antioxidant activity which has extracted the non-polar compounds which may shown the antioxidant properties.

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