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Determination of Antioxidant activity by High Performance Liquid Chromatography, Phenolic and Flavonoid contents of *Vincetoxicum nigrum*

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Abstract: The main purpose of this study is to evaluate the antioxidant properties, total phenolic content and total flavonoid content of *Vincetoxicum nigrum*. The determination has been done on three different extracts methanolic, dichloromethane and ethyl acetate. The methanolic fraction showed the highest antioxidant activity by DPPH-HPLC method, compared to dichloromethane and ethyl acetate. The total phenolic content was determined by Folin-Ciocalteu method and total flavonoid content was evaluated by equivalents of Quercetine. The methanolic extract has been found to be rich in flavonoids and phenolic compounds. To sum up, *Vincetoxicum nigrum* did not have a high antioxidant activity.
Keywords: *vincetoxicum nigrum*, antioxidant activity, High Performance Liquid Chromatography, total phenolic content, total flavonoid content.

Introduction

With the increasing aging of the world's population and people's lifestyle, the occurrence of oxidative stress in cells, and therefore, the production ofreactive species of oxygen (ROS) is also growing[1].

Oxidative stress, defined as ''an imbalance between oxidantsand antioxidants in favour of the oxidants, which is potentially causedamage''[2], is associated with higher risks of many disease including diabetes mellitus, hypertension, obesityand inflammation[3].

Reactive oxygen species (ROS) such as superoxide anions ($^{\cdot}OOH$), hydrogen peroxide (H_2O_2) and hydroxylradicals ($^{\cdot}OH$) induce Oxidative stress. ROS are generated as by-products of aerobic respiration and metabolism''[4], and modulated by antioxidant enzymes and non-enzymatic scavengers[5]. Natural antioxidants are generally obtained from plants and vegetables which are needed to counteractthe damage of ROS to cells.

Flavonoids are a large group of plant polyphenol secondary metabolites and can be found widely in the leaves, seeds, bark and plants flowers. It is already well recognised that flavonoids possess anti-tumoral, anti-ischemic, antiallergic,anti-inflammatory and anti-bacterial activities. Moreover,flavonoids show strong antioxidant capacities through scavenging oxygen free radicals, promote anti-oxidase or inhibit oxidative enzymes[6].

Phenolic compounds are secondary plant metabolites that play a key role in the sensory and nutritional quality of fruits, vegetables and other plants[7]. Antioxidant activities are known to increase proportionally to the polyphenol content, mainly due to their redox properties[8]. Among the diverse roles of polyphenols, they protect cell constituents against destructive oxidative damage, thus limiting the risk of various degenerative diseases associated with oxidative stress and thus tending to be potent free radical scavengers. Their ability to act as antioxidants is due to their chemical structure and ability to donate/accept electrons, thus delocalizing the unpaired electron within the aromatic structure[9].

The genus *Vincetoxicum* has traditionally been classified within the cosmopolitan family *Asclepiadaceae*. *Vincetoxicum nigrum* (*Cynanchumlouiseae)* is a species of plant that is native to Europe and is found primarily in Italy, France, Portugal, and Spain It is an invasive plant species in the northeastern United States, parts of the Midwest, southeastern Canada and California and is used as expectorant, diuretic and emetic agents in traditional medicine[10]. In Chinese medicine it was often used to treat rupture, internal fever, scrofula and scabies[11].

Cynanchumlouiseae is a perennial, herbaceous vine with oval shaped leaves that have pointed tips. The leaves are 3–4 inches (7.6–10.2 cm) long, and 2–3 inches (5.1–7.6 cm) wide, often occurring in pairs on the stem. The flowers have five petals that are star-shaped with white hairs. The flowers range in color from dark purple to black. The fruit of *Cynanchumlouiseae* is slender, tapered pods that range in color from green to light brown, which are tightly packed with seeds attached to puffy fibers[10].

Vincetoxicum genus produces phenanthroindolizidine alkaloids which have antibiotic activities towards insects[12].

There are no literature reports on antioxidant activity of *V. nigrum* and the main purpose of this study was to evaluate the antioxidant activity of *vincetoxicum nigrum* through DPPH-HPLC method and determination of total phenolic and flavonoid content of it.

Methods and Materials

Plant material:

The Plant material was collected in July 2014 from North Khorasan Province Mountains in Iran. Then, the plant was identified and confirmed by Natural Products & Medicinal Plants Research Centre, North Khorasan University of Medical Sciences (Iran) and Voucher specimen(No.NMP10/4-1) 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(, Folin-Ciocalteau reagent (F9252, Sigma-Aldrich(, Na2CO3 (451614, anhydrous powder, 99.999%, Sigma-Aldrich(, Gallic acid (91215, Fluka), aluminum chloride (563919, anhydrous powder, 99.999%, Sigma-Aldrich(, Quercetin (Q4951, ≥95%, Sigma-Aldrich), 2,2-diphenyl-1-picrylhydrazyl(DPPH) (257621, Sigma- Aldrich), ascorbic acid (A130000 , European Pharmacopoeia (EP) Reference Standard, Fluka).

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) and the column was analytical Shim-pack ODS-A, 4.6 × 250 mm, 5 μm (Shimadzu, Japan).

Preparation of plant extracts:

The aerial parts of theplant were dried under shade at room temperature and then cut into small pieces. About 100 g of sample was separately left in three different solvents(methanol, dichloromethane and ethyl

acetate) at 25°. Each solvent was allowed to remain in contact with plant material for 2 d, and replaced with fresh solvent four times. Removal of solvents under vacuum at 40 ° gave the crude extracts[13].

Total phenolic content Determination:

The total phenolic content was determined by Folin-Ciocalteu method. 100 µL of three extracts (1000 mg/L) were added to 100 µL of diluted Folin-Ciocalteu reagent (1/1). After 1 min, Sodium carbonate (Na_2CO_3) (2%, 2 ml) and 2.8ml of deionized water were added to each tube. Tubes were vortexed and incubated for 30 min at 25°. After that, the absorbance was read at 720 nm. Finally, the standard curve was prepared using 0.1 to 1 mg/ml solutions of Gallic acid in methanol. The analyses were done in triplicates. Total phenolic contents were expressed as Gallic acid equivalents (mg Gallic acid: (GA) per dry weight of extract)[14].

Total flavonoid content Determination:

For determination of total flavonoid contents, 1.5 ml of methanol with 0.5 ml of each extracts, 0.1 ml of aluminum chloride (10%), 0.1 ml of potassium acetate(1M) and 2.8 ml of distilled water were mixed. Then, tubes were incubated at 25° for 30 min and the absorbance of the mixtures were measured at 415 nm. All experiments were repeated three times and values were expressed in mean standard deviation in terms of totalflavonoid content (Quercetin equivalent: QE per dry weight of extract). The standard curve was sketched using 12.5 to 100 µg/ml of Quercetin in methanol[15].

Evaluation of Antioxidant activity:

HPLC analysis for DPPH radical scavenging:

The extracts antioxidant activity were determined by DPPH-HPLC method[16]. 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 20 min at 25°. 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):

$$\%Absorbance\ Inhibition = \frac{blank\ peak\ area - sample\ peak\ area}{blank\ peak\ area} \quad Eq.\ 1$$

The blank was methanol andascorbic acid was used as positive control. AI was calculated as IC_{50} values which were calculated by using Graph Pad Prism software, version 5.01[17].

Results and Discussion

The antioxidant activity is generally attributed to phenolic compounds in plant extracts[18]. The redox properties of phenolic compounds make them to behave as reducing agents, hydrogen donors and singlet oxygen quenchers[19]. Solvent polarity plays a remarkable role in phenolic compounds extraction and methanol is an efficient solvent in their extraction[20]. In this study three different solvents with different polarities were used. As shown in table 1 the yield of methanolic extracts was the highest(3.558%) among these three solvents due to its high plarity compared to dichloromethane(2.408%) and ethylacetate(1.935%).

Table 1. Extraction Yield, Total Phenolic and Flavonoid Content and Antioxidant Activity of *Vincetoxicum nigrum*

Extracts	Extraction yield (%)	Total phenolic (Gallic acid equivalents mg/g of dry extract)	Total flavonoid (Quercetin equivalents mg/g of dry extract)	IC_{50} via HPLC-DPPH (mg/mL)
Dichloromethane	6.875±0.013	2.408	0.89	4.2
Ethyl acetate	5.7±0.0049	1.935	0.751	15.47
Methanol	10.15±0.01	3.558	1.247	1.44
Ascorbic acid	---	---	----	0.00097

The total phenolic content of these three extracts were determined by Gallic acid(mg) per dried extracts (gr). The standard curve was prepared and the equation 2 obtained:

$$y = 0.005x + 0.001 \quad (R^2=0.995) \text{Eq(2)}$$

Methanolic extract had the highest phenolic content which showed that methanol can be an efficient solvent for extracting phenols in comparison with ethyl acetate and dichloromethane.

In the same way, the total flavonoid content of methanolic fraction was higher than the two other fractions. The total flavonoid content was determined by Querctin(mg) per dried extract (gr). The standard curve for flavonoid was prepared and its equation is equation 3:

$$y = 0.008x + 0.066 \quad (R^2=0.956) \quad \text{Eq(3)}$$

Antioxidant activity of phenol and flavonoid compounds is because of their chemical structures, which make them reducing agents[21].

In last few years, HPLC-DPPH analysis developed for identification of antioxidant compounds. In antioxidant compounds, the conjugated system would be destroyed by DPPH. As a matter of fact,the peaks of these compounds in HPLC chromatograms would be significantly reduced or disappeared. Figs 1,2 and 3 show the methanol, dichloromethane and ethyl acetate extracts chromatograms with DPPH treatment monitored at 517 nm[22].

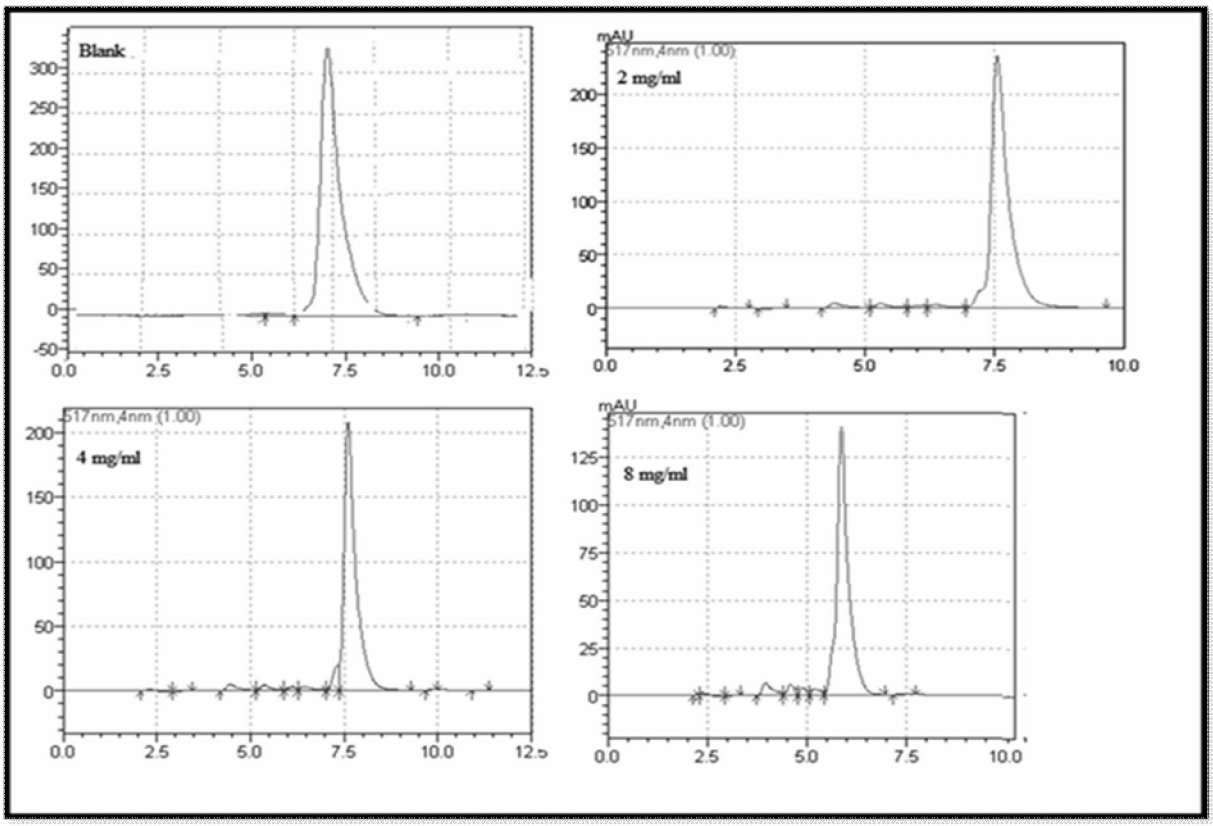

Fig.1:HPLC-DPPH chromatograms of methanolic extract in different concentration

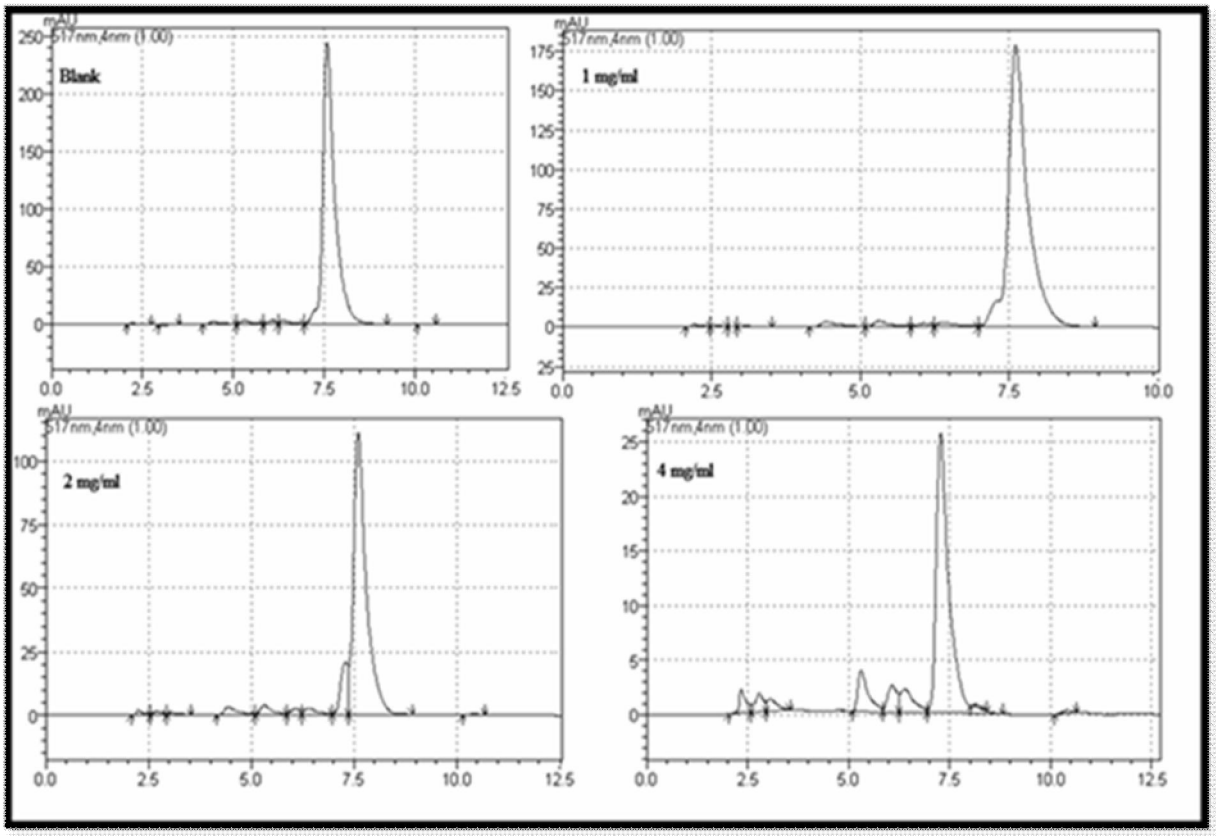

Fig.2:DPPH-HPLC chromatograms for dichloromethane extract in different concentrations

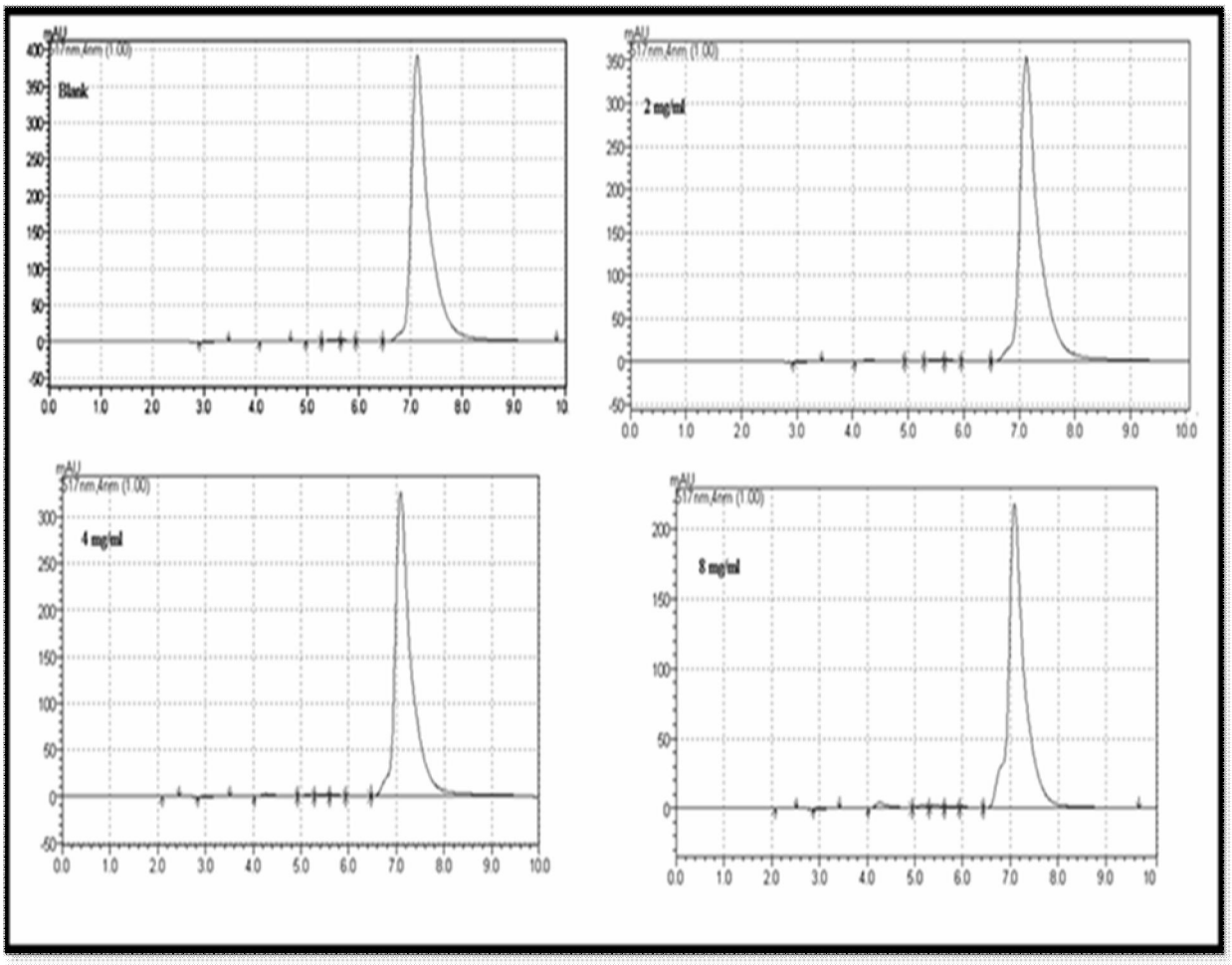

Fig.3:HPLC-DPPH chromatograms of ethyl acetate extract in different concentration

As shown in figs 1,2and 3 with increasing in sample concentration the peak areas remarkably decreased after spiking the DPPH solution. The sample peak areas are shown in Table 2.

Table 2: Sample Peak Areas for Different Solvents in Different Concentrations

Solvent	Blank	0.5mg/ ml	1mg/ ml	2mg/ ml	4mg/ ml	8mg/ ml
Methanol	5621009	4397055	4097637	2370394	560084	551107
Dichloro methane	6334935	6302178	6258054	5609521	3600628	1152595
Ethyl acetate	9229653	9012546	8758435	8355044	7697582	5086724

Fig 4 shows that as the concentration of extract increased the %DPPH inhibition increased. The maximum concentration of methanolic exract(8mg/ml) was able to inhibit 90.19% of DPPH compared to Vitamin C which inhibited 89.66% of DPPH. For dichloromethane the %DPPH inhibition of the maximum concentration is 81.8% which is lower than that of methanol and higher than ethyl acetate with 44.18% DPPH Inhibition. Fig 4 shows %DPPH inhibition with concentration. Results were reported as IC_{50}, 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 IC_{50} has the higher antiradical activity. IC_{50} values of extracts were shown in Table 1.

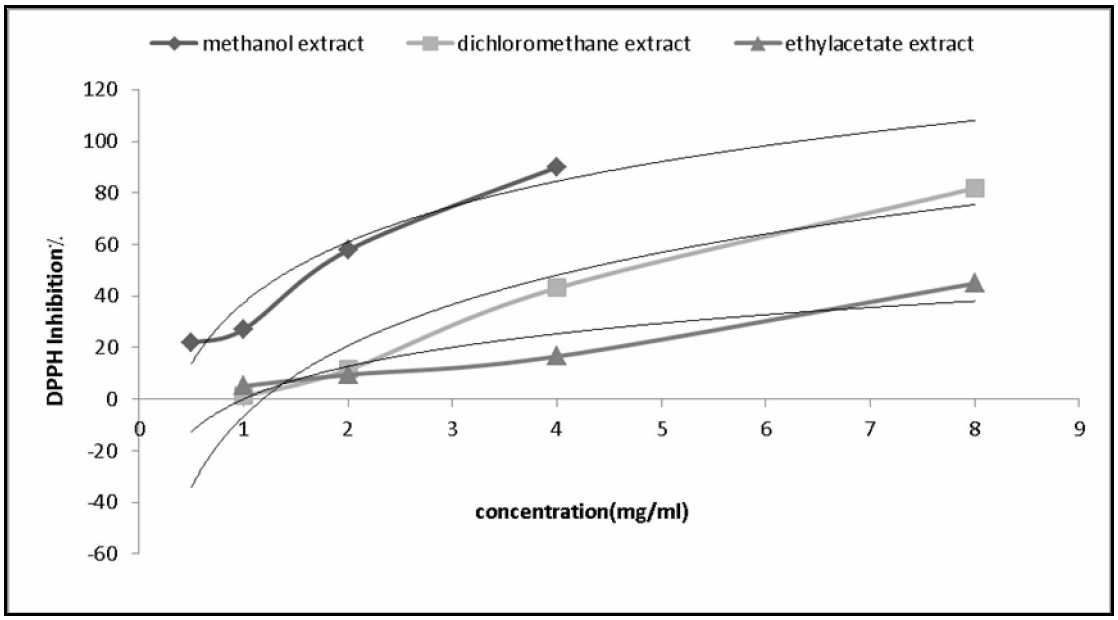

Fig.4:%DPPH inhibition in different concentrations

In this study, Ascorbic acid had the highest radical scavenging activities than *Vincetoxicum nigrum* extracts. Among these extracts, methanolic extract showed the highest antioxidant activity. Moreover, this extract had the highest amount of phenolic and flavonoid content (IC_{50}=1.44 mg/ml) in comparison with ethyl acetate and dichloromethane extracts. The difference between the total phenolic and flavonoid contents of three extracts leads to different antioxidant activity[23]. As we mentioned before this research was the only one which investigated the antioxidant activity of this genus and *vincetoxicum nigrum* did not show remarkable antioxidant activity and was not rich in phenols and flavonoids.

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