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Preliminary Phytochemical Screening, Quantitative Estimation of Total Flavonoids, Total Phenols and Antioxidant Activity of *Ephedra alata* Decne.

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Abstract

This study designed to evaluate antioxidant activity, screen the existence of phytogenic chemical compounds and to determine the total flavonoid and phenol contents of the *Ephedra alata*. to prove its utilization in the Palestinian folk medicine for treatment of cancer. Total flavonoid contents of the plant were determined by using rutin reference standard method and total phenols determined by using Folin Ciocalteu method while antioxidant activity evaluated by using 2, 2-diphenyl-1-picryl-hydrazyl-hydrate assay.Phytochemical analyses indicated the presence of cardiac glycosides, reducing sugars, flavonoids, phenolic compounds and alkaloids. The total phenolic content in the ethanolic extract was 19.175 mg gallic acid while was the highest in the methanolic extract which was 47.62 mg gallic acid equivalent/g of extract powder. The total flavonoid content of the plant was 0.519 mg RU/g in the aqueous extract and 5.44 mg RU/g in the ethanolic extract while was the highest in the methanolic extract 54.66 mg RU/g. In the same time the *E. alata* methanolic extract showed that it has high antioxidant activity and powerful oxygen free radical scavenging abilities as well as the IC50 for the plant was almost equivalent to the Trolox standard antioxidant which justified its uses in the Palestinian traditional medicines and could represented as a good candidate for further biological and chemical analysis, and can be further subjected for isolation of the therapeutically active compounds with anticancer activity and also for further pharmacological evaluations.

Keywords*: Ephedra alata* Decne, Phytochemical screening, Total flavonoids, Total phenols, Antioxidant.

1. Introduction

The value of medicinal plants in drug discovery is known to us well and the human being used them for various purposes from the beginning of the human history [1]. Traditional folk remedies from plants have always guided scientists to search for new medications in order to maintain and promote healthy life for human and animals [2].

Ephedra alata Decne. (the Arabic name is Alanda, family Ephedraceae) is a perennial genus of non flowering seed herb belonging to the Gnetales plant, the closest living relative of the angiosperm [3].

The native land for this species is Iran, Algeria, Iraq, Chad, Egypt, Palestine, Lebanon, Jordan, Saudi Arabia, Morocco, Syrian Arab Republic, Libya, Mauritania, Mali, Somalia and Tunisia [4, 5]. This plant is light green densely branched dioecious small and perennial stiff shrub, about 50-100cm tall, the twigs appear leafless and the leaves reduced to small scales, cones sessile shaped, clustered in the axils or at branch tips (Fig.1). *E. alata* grows wildly on the gravely rocky, sandy and clay soil in arid environments often near shifting sand dunes [6-11].

The dried stems (green colored) are the used organs of the plant in traditional medicine, usually boiled about 30 minutes in water and administered orally as a hot tea and the usually daily herb dose is 1.5–9 g [12].

Ephedra alata stem is used in the folk medicine as decoction as a stimulant , a deobstruent, to treat kidney, bronchi, circular system, digestive system disorders and to relief asthma attack as well as used for treatment of cancer also the plant stems are chewed for treatment of bacterial and fungal infections [13-15]. It is a range plant with medicinal application used for treatment of cough as well as has decongestant effect [4]. The antimicrobial potential of some Ephedra species such as *E. altissima* Defs [16], *E. transitorai* [17], *E. nebrodensis* [18], *E. major* [19] and *E. breana* has been recognized [20].

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Figure 1. *Ephedra alata* plant

Additionally, the foliage of *E. alata* has acceptable aroma and used as food stuff during animal grazing in Saudi Arabia [7]. *Ephedra alata* contains flavonol glucosides, herbacetin 7-*O*-(6″-quinylglucoside), herbacetin 8-methyl ether 3-*O*-glucoside-7-*O*-rutinoside, viceninII, kaempferol 3-rhamnoside, lucenin III, herbacetin 7-glucoside and quercetin 3-rhamnoside [21].

In addition to the known *p*-coumaric acid, the furanofuran lignan (±)-syringaresinol and the digalloylglucose, nilocitin were obtained from the whole plant of *Ephedra alata*. Ephedrine, pseudoephedrine and a new alkaloid ephedralone was also have been isolated [9] Phyto- acids were identified in the organic extract like hexadecanoic acid, 2-Propenoic acid, benzoic acid,7, 2-propenoic acid, benzene-acetic acid, alpha.-hydrox benzene-dicarboxylic acid and benzene-propanoic acid [22]. According to the Global Species Program Red List Unit this plant had a risk to be distinct from the entire world, so its seeds have been collected and stored as part of the Millennium Seed Bank Project in the United Kingdom and the distribution intersects some protected areas [23].

Free radical method 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) is an antioxidant assay based on electron transfer that gives a violet color ethanolic solution. This free radical, stable at ordinary temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. The use of the DPPH assay furnish rapid and easy way to estimate anti-oxidant activity by spectrophotometry, so it can be useful to assess various products at a time [24].

2. Materials and methods

2.1. Collection and preparing plant materials

Ephedra alata aerial parts were collected in July 2014 from the mountains of Jenin region of West Bank / Palestine. The plant was botanically identified by Dr. Nidal Jaradat from the Pharmacy Department at An-Najah National University. Voucher specimen was deposited in the Herbarium of the Pharmaceutical Chemistry and Technology Division (Laboratory of Pharmacognosy) and the *Ephedra alata* herbarium code is (Pharm-PCT-904).

The entire plant was washed and then dried in the shade at room temperature until all the plants parts became well dried. After drying, the plant materials were then powdered well by using grinder and placed into a well closed container.

2.2. Instrumentation

Shaker device (Memmert shaking incubator, Germany), rotary evaporator (Heidolph OB2000 Heidolph VV2000, Germany), spectrophotometer (Jenway 7135, England), freeze dryer (Mill rock technology, model BT85, Danfoss, China), grinder (Moulinex model, Uno, China), balance (Rad wag, AS 220/c/2, Poland), filter paper (Machrery-Nagel, MN 617 and Whatman no.1, USA).

2.3. Chemical Reagents

2.3.1. For antioxidant evaluation: Methanol was purchased from Lobachemie (India). N-hexane was obtained from Frutarum LTD (Israel). (DPPH) 2, 2-Diphenyl-1-picrylhydrazyl was ordered from Sigma-Aldrich (Germany). Trolox (6-hydroxy- 2, 5, 7, 8 –tetramethychroman-2 carboxylic acid) was purchased from Sigma-Aldrich (Denmark).

2.3.2. For phytochemical screening: Millon's reagent, NaOH and Benedict's reagent were obtained from Gadot (Israel). Ninhydrain solution, Molish's reagent, H_2SO_4 and iodine solution were purchased from Alfa Aesar (England). Chloroform was purchased from Sigma-Aldrich (Germany). HCl and magnesium ribbon were obtained from SDFCL (India). Acetic acid was ordered from Frutarom LTD (Israel) and $FeCl_3$ was purchased from (Riedeldehan, Germany).

2.3.3. For total phenolic content: Folin-Ciocalteu reagent was purchased from Sigma Aldrich, and for total flavonoid Rutin hydrate was purchased from MP-Biomedical (USA).

2.4. Preparation of plant extracts for phytochemical analysis

The phytochemical extraction was performed using organic solvent extraction as well as aqueous extraction. The organic extraction was performed by Soxhlet extraction method. This extraction was done by taking 20 gm of dried plant powder and was placed into a glass thimble then extracted with 250 ml of different solvents separately (ethanol, methanol, and acetone). The extraction processes carry on till the solvent in siphon tube of Soxhlet apparatus become colorless. After that the extract was heated on hot water bath at 35 ºC until all the solvent evaporated. The dried plant crude extract was kept in refrigerator at 2-8 ºC for their future use.

The aqueous extraction was done by taking five grams of the plant powder and mixed with 200 ml of distilled water in a beaker. The mixture was heated on a hot plate at 30º-40 ºC and mixed with continuous stirring for 20 minutes. The mixture was filtered using whatman filter paper filter and the filtrate was used for the further phytochemical analysis.

2.5. Preparation of plant extracts for antioxidant evaluation

About 10 g of the grounded plant were soaked in 1 Liter of methanol (99%) and put in a shaker device at 100 rounds per minute for 72 hours at room temperature and stored in refrigerator for 4 days. The extracts were then filtered using filter papers and concentrated under vacuum on a rotator evaporator. The crude extract was stored at 4 ºC and the antioxidant test was done directly with in five minutes.

2.6. Data analysis

The antioxidant activity was reported as percentage of inhibition. The inhibition of *E. alata* plant and Trolox standard at different concentration were plotted and tabulated and the IC50 for each of them was calculated using the BioDataFit fitting program in which the sigmoidal fitting model was the adapted model.

2.7. Anti oxidant activity

2.7.1. Trolox standard and plant working solutions

A stock solution of a concentration of 1mg/ml in methanol was firstly prepared for the plant extract and trolox. The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/ml) were prepared by serial dilution with methanol from the stock solution.

2.7.2. Spectrophotometric measurements

DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above prepared working concentration in a ration of 1:1:1 respectively. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only. The solutions were incubated in dark for 30 minute at room temperature before the absorbance readings were recorded at 517nm.

2.7.3. Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of the plants and the trolox standard were calculated using the following formula:

Percentage of inhibition of DPPH activity (%) = (A-B)/A ×100%

Where: A = optical density of the blank,

B = optical density of the sample.

The antioxidant half maximal inhibitory concentration (IC50) for the plant samples and the standard were calculated using BioDataFit edition 1.02 (data fit for biologist).

2.8. Phytochemical qualitative analysis

The plant aqueous, ethanolic, acetone and methanolic extracts were screened for the presence of the phytochemical classes by using the standard following methods.

2.8.1. Tests for proteins

Millon's test: 2 ml of Millon's reagent mixed with the entire plant crude extract, appeared white precipitate, which upon gentle heating turned into red color which indicated the presence of protein in the plant.

Ninhydrin test: Boil 2 ml of 0.2% Ninhydrin solution with the entire plant Crude extract, appeared violet color indicate the presence of proteins and amino acids.

2.8.2. Tests for carbohydrates

Fehling's solutions test: Boil a mixture of Fehling solutions A and B with equal volumes were added to crude plant extract. A red color precipitate indicated the presence of reducing sugars.

Benedict's reagent test: Boil 2 ml of Benedict's reagent with a crude extract, a reddish brown color indicated the presence of the carbohydrates.

Molisch's solution test: Shake 2 ml of Molisch's solution with crude plant extract then add 2 ml of H_2SO_4 concentrated and poured carefully along the side of the test tube. a violet ring appeared at the inter phase of the test tube indicated the presence of carbohydrate.
Iodine test: 2 ml of iodine solution mixed with crude plant extract. Purple or dark blue colors prove the presence of the carbohydrate.

2.8.3. Test for phenols and tannins
Two milliliter of 2% solution of $FeCl_3$ mixed with crude extract. Black or blue-green color indicated the presence of tannins and phenols.

2.8.4. Tests for flavonoids
Shinoda test: pieces of magnesium ribbon and HCl concentrated were mixed with crude plant extract after few minutes pink colored scarlet appeared that indicated the presence of flavonoids.
Alkaline reagent test: 2 ml of 2% NaOH solution was mixed with plant crude extract, intensive yellow color was formed, which turned into colorless when added 2 drops of diluted acid to solution, this result indicated the presence of flavonoids.

2.8.5. Test for saponins
Five milliliter of distilled water was added to crude plant extract in a test tube and it was shaken vigorously. The foam formation indicated the presence of saponins.

2.8.6. Tests for glycosides
Liebermann's test: 2 ml of acetic acid and 2 ml of chloroform mixed with entire plant crude extract. The mixture was then cooled and added H_2SO_4 concentrated, green color indicated the entity of aglycone steroidal part of glycosides.
Salkowski's test: H_2SO_4 concentrated (about 2 ml) was added to the entire plant crude extract. A reddish brown color produced indicated the entity of steroidal aglycone part of the glycoside.

2.8.7. Keller-kilani test
A mixture of Acetic acid glacial (2 ml) with 2 drops of 2% $FeCl_3$ solution was added to the plant extract and H_2SO_4 concentrated. A brown ring produced between the layers which indicated the entity of cardiac steroidal glycosides.

2.8.8. Test for steroid
Two milliliter of chloroform and concentrated H_2SO_4 were mixed with the entire plant crude extract. In the lower chloroform layer produced red color that indicated the presence of steroids.
Another test was performed by mixing 2 ml of each of acetic acid with H_2SO_4 concentrated and crude extract with 2 ml of chloroform. Green color indicated the entity of steroids.

2.8.9. Test for terpenoids
Two milliliter of chloroform was mixed with the plant extract and evaporated on the water path then boiled with 2 ml of H_2SO_4 concentrated. A grey color produced indicated the entity of terpenoids.

2.9. Determination of total phenol content in the different plant extracts
Total phenolic content (TPC) in the plant methanolic and ethanolic extracts was determined using spectrophotometric method [25] with some modifications. 1 mg/ml aqueous solutions for both methanolic and ethanolic extracts were prepared in the analysis. The reaction mixture was prepared by mixing 0.5 ml of plant extract solution, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO3 aqueous solution.

The samples were thereafter incubated in a thermostat at 45 ºC for 45 min. The absorbance was determined using spectrophotometer at wave length = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of gallic acid equivalent expressed in terms of (mg of GA/g of extract).

2.10. Determination of flavonoid concentrations in the different plant extract
The total flavonoid content (TFC) was determined from the calibration curve of Rutin and expressed as milligram of Rutin Equivalent per gram of extract (mg RU/g extract) .Total flavonoid content was determined according to the procedure of Chang *et al.*(2002) [26], validated by Nugroho (2011) [27] with some modifications using rutin as reference standard. Rutin (100 mg) was dissolved in 10 ml distilled water and diluted to 100 ml. Subsequently, the stock solution was diluted to provide a series of concentrations (5, 10, 20, 40, 100 µg/ml). from each solution (0.5 ml) was mixed with 3 ml methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml potassium acetate 1M and 5 ml distilled water, and then incubated at room temperature for 30 minutes.

This procedure is repeated for aqueous, methanolic and ethanolic *E. alata* extracts. Furthermore, absorbance was measured at 415 nm wavelength, and distilled water with methanol, 10% $AlCl_3$ and potassium acetate was used as a blank. Total flavonoid in extracts was expressed in terms of Rutin equivalents (mg of RU/g of *Ephedra alata* extract).

3. Results and Discussion

Selfish genes in human beings and other living organisms fighting all the time for existence and surviving from all diseases to maintain the health for human genus and other living organisms.

Discovering and screening for potential anticancer and antioxidant agents from natural plant products still in the recent time the main scope for many of the pharmaceutical and medical scientists. In the entire world, tremendous resources are being invested in diagnosis, prevention and treatment of cancer.

3.1. Phytochemical screening

Phytochemical screening tests for different extracts of *Ephedra alata* showed the active phytochemical classes as cardiac glycosides, alkaloids, reducing sugars, phenols and flavonoids as presented in table 1.

Table1: Phytochemical screening tests for the aqueous, methanolic , acetone and ethanolic *Ephedra alata* extracts.

Phytochemical compounds	Aqueous extract	Methanol extract	Acetone extract	Ethanol extract
Cardiac glycosides	+	+	-	+
Saponin glycoside	-	-	-	-
Alkaloids	-	+	+	+
Amino acids	-	-	-	-
Starch	-	-	-	-
Reducing sugars	+	+	+	+
Phenols	-	+	-	+
Volatile oil	-	-	-	-
Tannin	-	-	-	-
Steroids	-	-	-	-
Flavonoid	+	+	-	+

3.2. Total flavonoid content

The total flavonoid content for different extracts of *E. alata* plant presented in table 2 as well as absorbance of standard compound (Rutin) was shown in this table at different concentrations.

Table 2: Absorbance of standard compound (Rutin) at $\lambda_{max = 415nm}$

Rutin concentration(µg/ml)	Absorbance (mean value) at $\lambda_{max =415}$
0.3	0.143
0.6	0.197
1.2	0.213
2.4	0.344
6	0.563

Standard curve of Rutin indicated the equation of y = 0.072x + 0.1409 and $\boldsymbol{R^2}$ = 0.9844 clarified in Fig.2.

3.3. Total phenolic content

Absorbance of standard compound (Gallic acid) *at* $\lambda_{max =765nm}$ in *Ephedra alata* presented in table3 and Fig.3.

Table 3: Absorbance of standard compound (Gallic acid)

Gallic acid concentration (mg/l)	Absorbance (mean value) at $\lambda_{max =765nm}$
0.0312	0.241
0.0468	0.348
0.0625	0.393
0.125	0.922
0.25	2.021

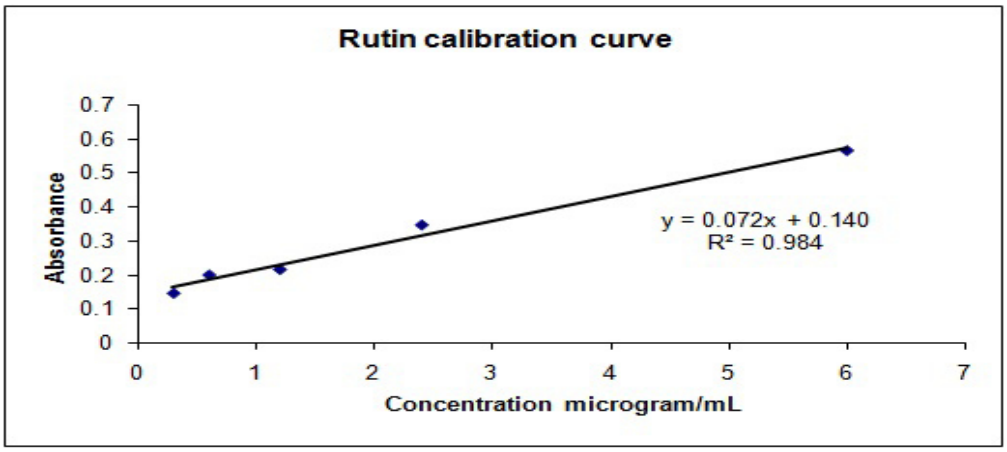

Figure 2. Standard calibration curve of Rutin

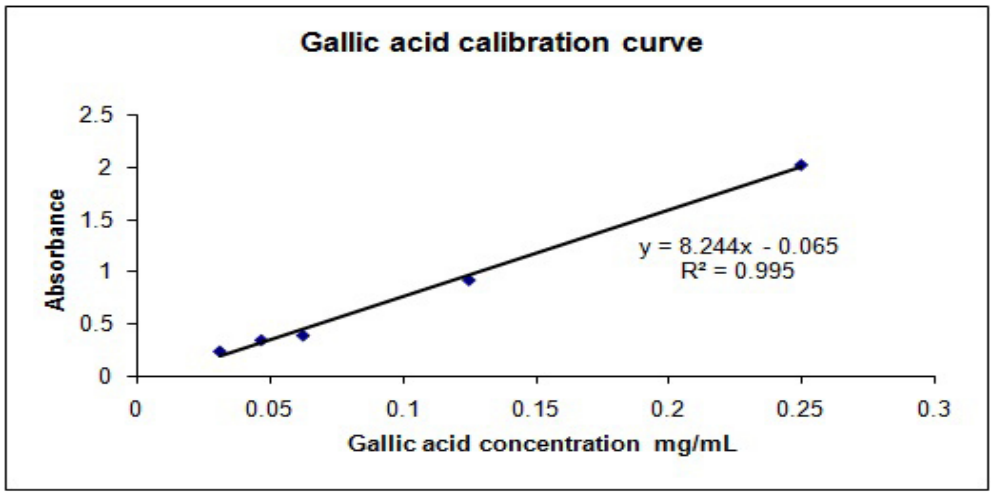

Figure 3. Standard calibration curve of Gallic acid

Natural phytophenols considered antioxidant compounds which have ability to damage free radical in the organisms related to their bioactivity to inhibit lipoxygenase, scavenge free radicals and to chelate metals [28]. Estimation of total phenol in the extracts was determined by using Folin Ciocalteu method and the standard compound (Gallic acid).

The total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: Y = 8.2442X + 0.065, $\boldsymbol{R^2}$=0.9959, Where

Y- Absorbance at 760 nm

X- Total phenol in the extracts.

Table 4 presented the contents of total phenols that were measured by Folin Ciocalteu reagent in terms of gallic acid equivalent and total flavonoid content expressed as mg/g Rutin equivalent.

Table 4: Total phenolic and flavonoid content in different *E. alata* extracts

E. alata extract	Total phenolic content (mg GA/g) ±SD	Total flavonoid content (mg RU/g) ±SD
Aqueous extract	---	0.519 ±0.09
Methanol extract	47.62 ±0.94	54.66 ±0.12
Ethanol extract	19.175 ±0.625	5.44 ±0.625

The total phenolic content of the aqueous plant extract was absented and in the ethanolic extract was 19.175 mg gallic acid while was the highest in the methanolic extract which was 47.62 mg gallic acid equivalent/g of extract powder. The total flavonoid content of the plant was 0.519 mg RU/g in the aqueous extract and 5.44 mg RU/g in the ethanolic extract while was the highest in the methanolic extract 54.66 mg RU/g (Fig. 4).

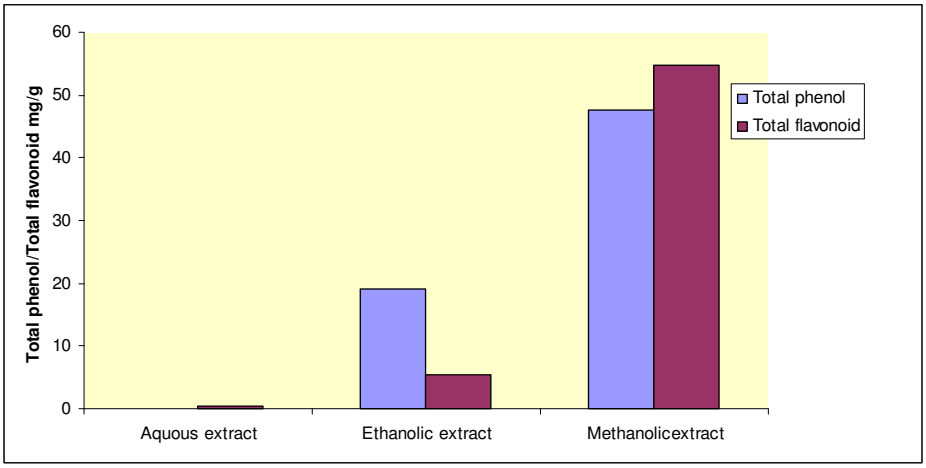

Figure 4. Total phenolic and total flavonoid contents of different *Ephedra alata* extract

3.4. Antioxidant activity using Trolox as standard equivalent

The free radical scavenging activity of the methanolic extract of *Ephedra alata* has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100 µg/ml. The zero inhibition was considered for the solution which contained only DPPH without any plant extract. The results are showed in (Table 5), and the table readings are explained in Fig. 5.

Table 5: Percentage inhibition activity for Trolox and *Ephedra alata*

Concentration µg/ml	% inhibition by Trolox ±SD	% inhibition by *Ephedra alata* ±SD
1	38.65 ±1.08	29.14 ±1.11
2	47.55 ±1.34	29.38 ±1.72
3	52.09 ±1.05	32.09 ±1.28
5	64.19 ±1.32	32.09 ±0.96
7	64.19 ±1.83	33.33 ±1.21
10	69.38 ±1.33	38.76 ±1.53
20	76.29 ±2.12	43.46 ±1.65
30	81.23 ±1.43	46.17 ±1.77
40	92.1 ±1.65	71.36 ±1.21
50	96.05 ±1.40	73.83 ±1.43
80	98.02 ±1.87	73.83 ±1.11
100	98.32 ±1.58	75.02 ±1.67

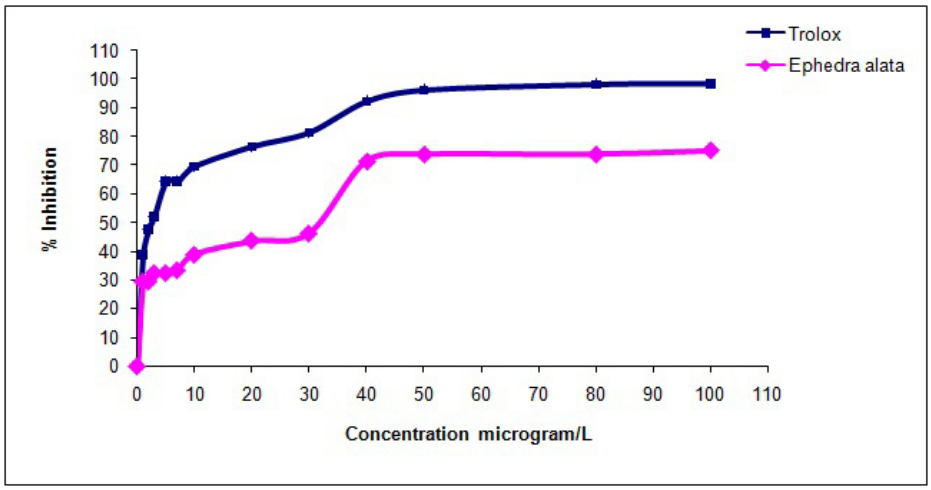

Figure 5. Inhibition activity of Trolox standard and *Ephedra alata* extract.

From the Fig.5 the calculated half maximal inhibitory concentration (IC_{50}) was 16.03 µg/ml for *Ephedra alata* and 3.6 µg/ml for Trolox standard.

Conclusion

The phytochemical screening showed that the *Ephedra alata* plant extract contain a mixture of phytochemicals as cardiac glycosides, reducing sugars, flavonoids, phenolic compounds and alkaloids. The quantitative total flavonoids and total phenol screening indicated that the methanolic plant extract has the highest contents of flavonoids and phenols and the DPPH assay showed that the plant has potent antioxidant activity which can be an excellent choice for biological and chemical analysis, and can be further subjected for the isolation of the therapeutically active compounds with anticancer potency.

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