Phytochemical Study, HPLC Chromatographic Analysis and Antioxidant Activity of *Ephedra alata* DC. Female Cones Extracts

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ABSTRACT:
The aim of this work is a phytochemical study, antioxidant activity and Chromatographic Analysis by HPLC of extracts of the female cones of *Ephedra alata* DC. Which grows in Oued Souf region (South East of Algeria). According to the results obtained from the estimation of polyphenols and flavonoids content, the methanolic extract has the best content of polyphenols (158.34±2.71mg GAE/g Extract), and the best values of flavonoids (88.50±1.12mg QE/g Extract). The chromatographic analysis by HPLC has identified eight phenolic compounds, which are Gallic Acid, Chlorogenic acid, Vanillic Acid, Vanillin, p-Coumaric acid, Rutin, Naringenin and Quercitin. The antioxidant activity was evaluated by three tests: DPPH• free-radical scavenging, Hemolysis and Reducing power, the results of test scavenging the free-radical DPPH• show the tannins extract had the best ~~scavenging~~ activity capacity than the other extracts (IC_{50}: 14.94±1.34µg/mL), But, in the hemolysis test, all the extracts were proximity except for the aqueous extract that was shown protected of the erythrocytes (50±0.5% of hemolysis percentage). Finally, in the reducing power assay, its results showed that the tannins extract has a best reducing power 27.16±0.25µg/mL in Abs_{700}= 0.5 compared to other extracts.

KEYWORDS: *Ephedra alata* DC., Phytochemical study, HPLC, Antioxidant activity.

INTRODUCTION:

The therapeutic value of plants is founded on the bioactive compounds that output a physiological action on the man's body[1], in recent years, there has been renewed interest in screening higher plants for novel biologically active compounds [2].

Secondary metabolites are copious and chemically diverse are of interest as sources of safer and effective substitutes[3]; and frequently used in the development of drugs[4].

In developing countries, the therapeutic use of medicinal plants is very present because of the absence of a medical system. The abundance of bioactive compounds gives the plant a remarkable pharmacological properties, which could justify its multiple therapeutic indications and used in traditional therapy[5].

The *Ephedra alata* is rich with bioactive compounds, including alkaloids, tannins, anthocyanins, flavonoids, cardenolides, terpenes, sterols and essential oils[6].

Ephedra was found among a series of medicinal plants at an archaeological site dating back to the Neanderthals (6.000 years ago). In ancient India, ephedra juice was called soma and it was consumed to ensure longevity[7], In Algeria, *E. alata* is used against influenza, whooping cough and general weakness in herbal tea and by inhalation as well as in the form of nasal drops against colds[8], and adverse effects clinically are tachycardia, hypertension, sweating, bronchodilation, restlessness and mydriasis can result. The use of *Ephedra* is also known to be associated with gastrointestinal and psychiatric manifestations[9], in traditional Chinese medicine, these effects may be the

reason of why the utilization of Ephedra is recommended only for intense situations and contraindicated for a long-range use[10].

The aim of this study is an estimate of the phytochemical properties, the antioxidant activity of flavonoids phase ethyl acetate, tannins and crude extracts of the female cones of *Ephedra alata* DC. collected from Oued Souf region Southeast of Algeria.

MATERIAL AND METHODS:

Plant material:

The female cones of *Ephedra alata* DC. have been collected during the flowering period in March 2018, from Adhel-Hassi Khalifa (El Oued, Southeast Algeria, 33° 40'42" N and 7°13'35" E). The female cones were drying at room temperature and protected from light and moisture.

Chemicals:

Ethanol (96%), Acetone, Ascorbic acid, Gallic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide ($K_4Fe(CN)_6$), Quercetin, Chlorogenic acid, p-Coumaric acid, Vanillic acid, Rutin, Vanillin and Naringenin were procured from Sigma-Aldrich (USA). Aluminum chloride ($AlCl_3$), ferric chloride ($FeCl_3$), sodium carbonate (Na_2CO_3), acetic acid and trichloroacetic acid were bought from Prolabo (USA). Methanol (99%), Ethyl acetate, Dichloromethane, Folin-Ciocalteu (FCR) reagent and Hydrogen peroxide H_2O_2 (30%) were obtained from the Biochem Chemopharma Co (France). All other reagents used in analytical grade and Acetonitrile of HPLC were purchased from Sigma Aldrich (USA).

Preparation of the crude extracts:

Ten grams of the dry plant were macerated with 150mL of methanol (99%) or ethanol (96%) or distilled water at normal temperature in dark for 24 hours. After filtration, the solvent was evaporated to dryness used the evaporator rotary at 50°C for obtaining the methanolic, ethanolic and aqueous crude extracts[11].

Extraction of Flavonoids phase Ethyl acetate:

Ten grams (10g) of dry matter were macerated with 150 mL of methanol at room temperature in dark for 24 hours. After filtration, the solvent was evaporated using the evaporator rotary at 50°C. Then, we added 150mL of warm distilled water and 150mL of Ethyl acetate and we put the new mixture in a separator funnel. After the separation, we got the Ethyl acetate phase for evaporated in an evaporator rotary at 50°C to get the Flavonoids extract (phase Ethyl acetate)[12].

Extraction of Tannins:

Thirty grams (30g) of dry matter were macerated with 60 mL of distilled water and 140mL of Acetone at dark and in room temperature for 72 hours. After filtration, the solvent was evaporated. Then, we added 150mL of Dichloromethane for separation of the organic and aqueous phases by separator funnel; the organic phases were further extracted with Ethyl acetate (150mL) and evaporate at 50°C[13].

Determination of phenolic contents in crude extracts:

The phenolic contents in crude extracts were estimated according to the Folin-Ciocalteu's reagent method citing in[14] with some modification; we mixed 0.2mL of the extract with 1mL of Folin-Ciocalteu reagent (10%), then we added 0.8 of sodium carbonate solution (7.5%). After stirring the test tubes we let them rest for 30 min, the absorbance was measured at 765nm by using the spectrophotometer UV. The calibration curve was prepared with gallic acid solutions in the concentration (0.02-0.12mg/mL). The total phenolic content was expressed as (mg of Gallic acid equivalents in a gram of extract).

Determination of flavonoids contents in crude extracts:

For the determination of flavonoids contents, we mixed 1mL of a crude extract with 1mL of $AlCl_3$ (2%). After 60 min at laboratory temperature, the absorbance measured at 430nm, by used the quercetin solution at different concentrations (0.03-0.1mg/mL) we prepared a calibration curve. The content of flavonoids in the extract was expressed in (mg of QE/g of extract)[15].

Chromatographic analysis by High-Performance Liquid Chromatography (HPLC):

HPLC is a technique used the identification, separation and quantification of the compounds of a mixture. The analyses can be separated based on hydrophobicity, size, charge and many other properties[16].

A High-Performance Liquid Chromatography (HPLC) system, type Shimadzu LC 20 AL equipped with the universal injector (Hamilton 25µL), an analytical column used was a Shim-pack VP-ODS C18 (4.6mm × 250mm,

5µm), type (Shimadzu). UV-VIS detector SPD 20A (Shimadzu) was used, According to[17], the principal work of HPLC was: 20µl of the methanolic extract solution was injected into the flow of mobile phase and we adjusted the high pressure that drives the mobile phase by using a pump. The separated compounds shall be determined using the column for 40-50 min with the mobile phase in the effluent of detecting λ = 268 nm and to the computer which records the results as chromatographic curves.

In this study, the quantification of separated peaks was performed by calibration with curves of standards.

Evaluation of Antioxidant activity:

DPPH• free-radical scavenging test:

According to the method citing in [18] with a small change, The scavenging was measured by mixing 1 mL of an extract with 1mL of DPPH• methanolic solution (0.1×10^{-3}M). After incubation for 15 min at laboratory temperature, the absorbance was measured at 517nm.

The percentage of inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

In the DPPH• free radical trapping test, ascorbic acid and BHT were used as standard. IC_{50} values are deduced from the regression equation corresponding to its calibration curve is expressed in µg/mL, IC_{50} is defined as the concentration necessary to inhibit 50% of DPPH• free radical[17].

Hemolysis test:

This test is used to determine the protected of the plant extracts of the erythrocyte blood cells from damage or disruption of the cell membrane after exposing them to oxidative stress and free radicals by measuring the percentage of exploded erythrocytes[19].

According to[20], a volume of 40µl of erythrocytes of human was added to 2mL of extract and conserved for 5 min at 37°C. Then, we added 40µl of H_2O_2 (30×10^{-3} M), 40µl of $FeCl_3$ (80×10^{-3} M) and 40µl of Ascorbic acid solution (50×10^{-3} M) respectively.

After 1 hour of incubation at 37°C, the mixture was centrifuged with 700 Tour/min for 10 min, at λ=540nm the absorbance of the supernatant was read.

The percentage of hemolysis was determined using the following formula:

$$\text{Hemolysis } \% = (A_{control} / A_{sample}) \times 100$$

Reducing power test:

The presence of antioxidants results in the reduction of the ferri-cyanide complex (Fe^{3+}) to the ferro-cyanide form (Fe^{2+})[21].

A volume 0.5mL of different concentration of extract was added to 1.25mL of a phosphate buffer (0.2 M, pH 6.6) mixed with 1.25mL of potassium ferro-cyanide (1%). In a hot bath at 50°C for 20 min, The mixture was incubated, and by adding 1.25mL of trichloroacetic acid (10%) we stopped the reaction, the tubes were centrifuged at 3000rpm for 10 min. After that we mixed 1.25mL of the supernatant with 0.25mL of $FeCl_3$ (0.1%) and 1.25mL of distilled water, the absorbance was read at 700nm[22].

Statistical analysis:

The results obtained are expressed as an average ± SEM. Data analysis was performed by applying the ANOVA One Way test, using Excel software (Version 2010) which we used to carry out the tests as well as the curves.

RESULTS AND DISCUSSION:

Determination of total polyphenols and flavonoids:

In (Fig. 1) observed the methanolic extract had the best content of polyphenols (158.34±2.71mg GAE/g Extract), and the best values of flavonoids (88.50±1.12mg QE/g Extract). Statistically; the difference between the content of polyphenols and flavonoids in extracts is very highly significant ($p < 0.001$).

Fig. 1: Polyphenols (mg GAE/g Extract) and Flavonoids contents (mg QE/g Extract) in *Ephedra alata* crude extracts

The content of polyphenols and flavonoids in the ethanolic extract is linked to the high solubility of phenols in polar solvents[23].

The variations of the content of polyphenols and flavonoids is due to several factors: the drying and extraction conditions method, time, temperature, particle size, solvent, number of extraction steps[24].

Chromatographic analysis by HPLC:

The results of the separation of methanol extract from *Ephedra elated* by HPLC are illustrated in the chromatogram (Fig. 2).

Fig. 2: Profile of HPLC chromatogram of methanolic extract of *Ephedra alata*

The HPLC has identified eight phenolic compounds out of 63 peaks in the methanolic crude extract of the *Ephedra alata* are gallic acid, chlorogenic acid, vanillic acid, vanillin, p-coumaric acid, rutin, naringenin and quercetin. The retention time of these compounds and their concentration are recorded in (Table 1).

Table 1: Retention time and the concentration of phenolic compounds identified in *Ephedra alata* methanolic extract

Compounds	Retention Time (min)	Concentration (µg/mg extract)
Gallic Acid	5.151	0.289
Chlorogenic acid	13.371	0.926
Vanillic Acid	15.306	0.392
Vanillin	21.286	0.276
p-Coumaric acid	23.869	0.278
Rutin	28.59	0.637
Naringenin	34.961	3.507
Quercetin	45.096	0.166

Many studies have looked at the identification of phenolic compounds in the extract of *Ephedra alata* by HPLC analysis[6]. These studies are in agreement with our results, despite the geographic differences of the studied sites, we find that the plant *Ephedra alata* has the same phenolic profiles. This shows that there is no significant qualitative difference. We can explain some our results in the following: gallic acid has helps the plants to adapt to the climatic conditions, the vanillin does accelerate the maturity of fruits, and it has the role of antibiotic stress and ρ-coumaric acid is a good stimulated for antioxidant activity[20].

Antioxidant activity:

DPPH• free-radical scavenging test:

The results in (Fig. 3) show that the IC_{50} values of tannins, flavonoids and methanol extracts have the best values of scavenging of DPPH• free-radical were 14.94±1.34 µg/mL, 26.55±1.51 µg/mL and 50.66±0.64 µg/mL, respectively.

Statistically, the difference between the IC_{50} values according to the extracts was very highly significant ($p<0.001$).

Fig. 3: Value of IC_{50} (µg/mL) of DPPH free radical of extracts of *Ephedra alata* and standards

Our results of DPPH• radical, tannins extract has a very high antiradical power compared to the other extracts, because there is a direct correlation between antioxidant activity and the capacity of the compounds in extracts[25]. The tannins reduce and decolorize DPPH due to their ability to donate hydrogen to lipid free radicals produced during peroxidation. More stable tannic radicals are then formed, which has the effect of stopping the chain reaction of the auto-oxidation of lipids[26]. The extracts reduce and discolor the DPPH• radical due to their ability to yield hydrogen to the free radicals produced during peroxidation[27].

Hemolysis assay:

In the hemolysis test (Fig. 4), all extracts were proximity except for the aqueous extract was the low protected of the erythrocytes.

Fig. 4: Percentage of Hemolysis (with C: 1 mg/mL) of extracts of *Ephedra alata* and Ascorbic Acid.

Statistically, the difference between the extracts of the plant was very highly significant ($p<0.001$) in the hemolysis test.

The hydrogen peroxide destroys the cell membrane and consequently the release of hemoglobin from cells[20]. According to [28] H_2O_2 cause degradation of hemoglobin in erythrocytes thus formed Fe^{2+} ions generated by the reaction of the OH hydroxyl radical.

The antihemolytic activity of plant extracts may be due to inhibition of the radical by the bioactive compounds in the extract which releases electrons to H_2O_2 thus neutralizing a water molecule.

Fig. 5: The concentration (µg/mL) values (at Abs_{700}: 0.5) in educing power test of extracts of *Ephedra alata* and Ascorbic Acid

Reducing power assay:

Our results of the reducing power test (Fig. 5), show that the tannins extract has the best reducing power of all the other extracts, but the ascorbic acid registered the best values in this test.

Statistically, the difference between the extracts of *Ephedra alata* was very highly significant ($p<0.001$) in the reducing power test.

Antioxidants are therefore considered to be reducers and activators of oxidants[29]. The solvent and the quality of extraction also directly influence the antioxidant activity[15]. The reducing capacity of a compound can serve as a significant indicator of its potential antioxidant activity[27,30].

CONCLUSIONS:

The phytochemical study of the female cones *Ephedra alata* was high contents of polyphenols and flavonoids in the methanol extract.

The result of the separation of methanol extract by HPLC has identified eight phenolic compounds out of 63 peaks are gallic acid, chlorogenic acid, vanillic acid, vanillin, p-coumaric acid, rutin, naringenin and quercetin.

The antioxidant activity of *Ephedra alata* extracts showed that have the best values of scavenging and reducing power.

ACKNOWLEDGEMENT:

The authors are grateful to Dr. Rebiai A. Vtrs Laboratory, El Oued University, Algeria. for Chromatographic analysis by HPLC.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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Received on 19.01.2021 Modified on 18.03.2021
Accepted on 20.04.2021

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Asian Journal of Research in Chemistry. 2021; 14(4):259-264.
DOI: 10.52711/0974-4150.2021.00044