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Antithrombotic, Antihemolitic and Analgesic Activities of *Ephedra Alata Alanda Extracts*

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ABSTRACT

The aim of this study is part of a natural resource development and evaluation of biological activities *in vitro* and *in vivo* extracts of the medicinal plant *Ephedra alata alenda* (E.A.A.) aerial parts. In the present study, four extracts were prepared : hydromethanolic extract (Ep.MeOH/H_2O), methanolic extract (Ep.MeOH), ethyl acetate extract (Ep.Ac) and aqueous extract (Ep.Aq).The evaluation of the antioxidant power by the DPPH method revealed that all the extracts have a significant antioxidant potential, the highest of which is that of the crude extract Ep.MeOH (IC50 = 5,35 ± 0,63 µg/ml). Hydroxyl radical scavenging was also performed. The latter have a greater scavenger effect with respect to the OH radical, and whose lowest IC50 is that of the Ep.MeOH/H_2O (0.00138 mg/ml ± 0,00059). The bleaching of β-carotene followed for 24 hours is slowed down strongly in the presence of all the extracts and particularly Ep.MeOH/H_2O (85.84%). Analysis of the antithrombotic effect revealed that the fractions Ep.Aq, and Ep.MeOH / H_2O exhibited an increase in human clot lysis with a percentage of 21.19 ± 4.14 and 18.32 ± 0.02 relative to negative control (water) (5.6%). The anticoagulant activity is also evaluated vis-a-vis the endogenous and exogenous pathway using two tests, TP and TCA, this revealed that extracts of E.A.A. hase significant anticoagulant activity with respect to both coagulation pathways. The extracts also showed considerable anti-hemolytic activity expressed by a significant prolongation of the HT50 (hemolysis of 50% of the cells) from 73 mn for the AAPH control (2,2' azobis(2-amidinoprane) dichlorate) to (HT50=192 mn) for Ep.Aq. An *in vivo* approach is carried out on mice treated with 300 mg / ml and 150 mg / ml of the crude extract (Ep.MeOH) in order to estimate the relief of pain caused by acetic acid (0.9%). showed that the plant E.A.A. possesses a pharmacological power, reducing the number of contortions with a% inhibition of 74% for the dose 300 mg / kg and 60.09% for the dose of 150mg / kg, which supports its traditional use for the relief of various cramps abdominal.

Key words: *Ephedra Alata Alanda*, Antioxidant activity, Antihemolytic activity, Anticoagulant activity, Analgesic effect.

Introduction

The use of plants with medicinal purposes for the prevention and/or treatment of diseases is one of the most ancient forms of primary health care [1]. Plants produce several secondary metabolites that present many important biological activities. Anticoagulant and antioxidant activities could be

highlighted amongst these.

Although anticoagulant drugs are necessary for the treatment of arterial and venous thrombotic disorders and for the long-term prevention of recurrences but they have certain limitations related to its clinical application, such as bleeding complications, as side effects [2]. Thus, the search for new substances with anticoagulant and antithrombotic activity is relevant. Medicinal plants have historically been the primary source of anticoagulant and antithrombotic molecules.

The overproduction of free radicals or failure in endogenous antioxidant mechanisms can cause oxidative damage to biomolecules (lipids, proteins and DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, myocardial infarction, chronic inflammation, and other degenerative diseases in humans. Antioxidants are important substances that have the ability to protect the organism from the damage caused by the oxidative stress. Due to this ability, there is a special interest in the presence of natural antioxidants in medicinal plants that may help the organism to maintain the normal balance of ROS [3]. Plants are frequently reported as a good source of antioxidant components, such as phenolic compounds.

A traditional medicinal plant, *Ephedra alata alanda* (E.A.A.), has been selected, considering the chemical compounds, alkaloids, coumarins and flavonoids, which have been attributed to it [4].

The objectives of the current investigation is to quantify the phenolic and flavonoid contents in the areal part of this plant as potential source of natural antioxidants. An attempt made for confirmation of possible antithrombotic and antioxidant effects so that it may be useful in therapy thrombotic disorders.

Materiels and Methods

Plant material

The *Ephedra alata alenda* plant was harvested during the month of February 2019, in the wilaya of Oued Souf, Algeria.. The plant was botanically confirmed by Pr. Laouar, Farhat Abbes university, Setif1, Algeria.

Animal materiel

Male mice weighing 25-30 g each were purchased from the Pasteur Institute of Algeria. They were kept with free access to food and water and on a 12 h light/dark cycle. The experimental protocol was approved by the Ethics Committee of the University of Sétif 1. All procedures were performed in compliance with laws and institutional guidelines. The animals were housed for a period of at least seven days for acclimatization before the experiments.

Extraction

Aerial parts of E.A.A. were macerated three times with 80% MeOH and Methanol 100%. The

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macerates was then filtered and concentrated at 45 °C under reduced pressure using a rotary evaporator. The crude extract Ep.MeOH, was successively fractionated with different solvents of increasing polarity. Each fraction was evaporated to dryness under reduced pressure to afford hexane (Ep. HE), ethyl acetate (Ep.Ac) and the remaining aqueous (Ep.Aq) extracts.

Determination of total phenolics contents

Total phenolic content was determined with the Folin–Ciocalteu's reagent (FCR) according to the published method [5]. Such method consists of the phosphotungstic (WO4-2)- phosphomolybdic (MoO4-2) acid (Folin-ciocalteu's reagent) reduction by the phenolic hydroxyl groups, resulting in the formation of a blue product in alkaline solution. Briefly, 200 µl of appropriate dilution of each extracts were added to 1 ml of 1:10 diluted Folin-ciocalteu's reagent. After 4 mn, the reaction mixture was neutralized with 800 µl of saturated sodium carbonate (75 g/l). Subsequently, the shaken mixture was allowed to stand for 2 h at room temperature, and then measured at 765 nm. Gallic acid (20-140 mg/l) was used for the standard calibration curve. The results were expressed as µg gallic acid equivalent (GAE)/mg of each extracts.

Determination of flavonoids contents

The aluminium chloride ($AlCl_3$) method [6] was used to determine the flavonoids contents of the E.A.A. extracts, employing the reaction of complex formation between flavonoids and $AlCl_3$. Briefly, 1 ml of extracts was added to equal volume of a solution of $AlCl_3$ (2%). The mixture was vigorously shaken, and absorbance was read at 430 nm after incubation in dark at room temperature for 10 mn. Quercetin (1-40 mg/l) was used as standard for calibration curve. Flavonoids contents were expressed as µg quercetin equivalent (QE)/mg of each extracts.

Evaluation of antioxidant activity

Effect on clotting time

The anticoagulant propensity of compounds were assessed by adopting the procedure of Koffuor and Amoateng [7]. A solution of 74 µl of each compound (0.5 mg/ml), positive control (citrate) and negative control (water) were distributed in respective test tubes and 370 µl of fresh blood of healthy volunteer (The volunteers were duly informed about the research, and their willingness to participate in the research was documented by the signing of written informed consent) was immediately added to the respective test tubes. The time required for complete blood clotting was recorded using a stopwatch.

Thrombolytic activity

A solution of examined compounds (0.1 mg/ml) was prepared. Venous blood was drawn from the healthy volunteer. Blood was distributed (370 µl/ tube) to each previously weighed sterile eppendorf tubes and incubated at 37°C for 45 mn to form the clot. Then, serum was totally removed after clot formation without disturbing the clot and each tube having clot was again

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weighed to find out the clot weight. In each eppendorf tube containing pre-weighed clot, a volume of 70 µl of test solution was added. 70 µl of distilled water was separately added to the control tube considered as a negative control. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. Fluid released was removed after incubation and tubes were again weighed to observe the difference in the weight of blood clot [8].

% Lysis = Weight of released clot/Weight of clot X 100.

Where weight of released clot = Weight of clot before lysis – Weight of clot after lysis

Effect on *in vitro* prothrombin time and activated partial thromboplastin time

Trisodium citrate was added in all centrifuge tubes. 3ml of blood sample from human volunteers (n=3) was added to the centrifuge tubes and subjected to centrifugation at 3,000 rpm for 5 min. Plasma was separated with the help of micropipettes and saved in appendorf tubes. 250 µl of each, extracts (0.1mg) were mixed with 250 µl of plasma in an eppendorf tube. For evaluation of prothrombin time, samples were incubated at 37°C for 5 mn then 200 ul of prothrombin time reagent was added to 100 µl of test plasma and clotting time was measured as prothrombin time.
For evaluation of activated partial thromboplastin time, 100 µl of activated partial thromboplastin reagent was added to the 100 ul of test plasma (platelet poor plasma + extract) and the mixture was incubated for 1 min after which 100 µl calcium chloride (25 mmol) was added and incubated for 15 sec and clotting time was recorded as activated partial thromboplastin time [9].

Anti-hemolytic assay

The inhibition of mice erythrocytes hemolysis by each extracts was assessed according to the method described by Guemmaz et *al.* [10] with slight modifications. Mice erythrocytes were isolated by centrifugation at 3000 rpm for 10 mn and washed three times with phosphate buffer (10mM, pH 7.4) until the supernatant became colourless. The erythrocytes were then diluted with phosphate buffer to give 2% (v/v) suspension. Briefly, 80 µl of 2% erythrocytes suspension was added to 20 µl of each extracts (0.1 mg/ml), the mixed were treated then by 136 µl of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH, 300 mM). We have controlled the whole blood hemolysis with a 96-well microplate reader device. The kinetics of erythrocytes resistance to hemolysis was determined at 37°C by continuous monitoring by measuring the rate of decrease at 630 nm. Results were expressed as the time corresponding to 50% of maximal hemolysis (half-hemolysis time, HT50 in mn). Vit C was used as standard.

Ion chelating assay

Ferrous iron-chelating activity of each extracts was measured by inhibition of the formation of Fe^{2+} -ferrozine complex after treatment of test extract with Fe^{2+}, following the method of Zerargui et *al.* [11]. The reaction mixture contained 250 µl of each extracts, 50 µl $FeCl_2$ (0.6 mM in water) and 450 µl methanol. The control contained all the reaction reagents except the extract. The mixture was shaken and allowed to react at room temperature for 5 mn. An aliquot of 50 µl of ferrozine (5 mM in methanol) were then added; the mixture shaken again, followed by further reaction at room temperature for 10 mn to complex the residual Fe^{2+} ion. The absorbance of the Fe^{2+} -ferrozine complex was measured at 562 nm against a blank contained all the reaction reagents except

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ferrozine. Lower absorbance indicates a higher chelating power. Ethylene diamine tetraacetic acid (EDTA) was used as reference chelator. The chelating activity was calculated as percentage:

$$\text{Chelating activity \%} = Ac\text{-}Ae\ /Ac \times 100$$

Where Ac: control absorbance and Ae: absorbance in the presence of each extracts.
EC50, effective concentration which produces 50% Fe^{2+} ions chelating, was calculated.

Reducing power assay

The reducing power of extracts was determined according to the method of Bencheikh *et al.* (12]. An aliquot of each extracts (125 µl) was mixed with 125 µl of sodium phosphate buffer (0.2 M, pH 6.6) and 125 µl of 1% K_3Fe (CN)6 followed by incubation at 50°C for 20 mn. After adding 125 µl of 10% trichloroacetic acid (TCA), the mixture was centrifuged at 3750 g for 10 mn. The supernatant solution (100 µl) was mixed with 100 µl of distilled H_2O and 20 µl of 1% ferric chloride to react for 10 mn. Subsequently, the absorbance was measured at 700 nm. The results were expressed as EC 50 which means effective concentration at which the absorbance is 0.5. Vit C was used as standard.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extracts was estimated by the method of Guemmaz et *al.* (13]. The reaction mixture contained 1 ml of $FeSO_4$ (1.5 mM), 0.7 ml of H_2O_2 (6 mM), 0.3 ml of sodium salicylate (20 mM) and varied concentration of the different extracts. These mixtures were incubated for 1 hour at 37 °C. The absorbance of the hydroxylated salicylate complex was mesured at 562 nm. 2, 6 di-tert-butyl-4-methyl phenol (BHT) was used as a positive control. The percentage of hydroxyl radicals scavenging activity was calculated by the following equation:

$$I\ \% = [1-(A1-A2) / A0] \times 100$$

A0 is absorbance of the control without extract
A1 is absorbance in the presence of the extract
A2 is absorbance without sodium salicylate Hydrogen

β- carotene bleaching assay

Antioxidant capacity is determined by measuring the inhibition of each extracts and the conjugated diene hydroperoxides arising from linoleic acid oxidation [14]. A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1ml of chloroform, and then 25 µl linoleic acid and 200 mg Tween 40 were added in flask. Chloroform was evaporated using a rotavapor. A volume of 100 ml distilled water saturated with oxygen (30 mn, 100 ml/mn) was added with vigorous shaking to form emulsion. Aliquot 350 µl of each extracts, prepared in methanol and/or distilled water at concentration of 2 mg/ml, were added to 2500 µl aliquot of reaction mixture, and the emulsion system was incubated up to 48h in dark at room temperature. Control samples (BHT, H_2O and methanol) received only the emulsion, while blank consisted only of corresponding sample or control. After this incubation period, absorbance of the mixtures was measured at 490 nm after 0h, 1h, 2h, 4h and 24h of incubation. The rate of bleaching of β-carotene was calculated as antioxidant activity and calculated using the equation:

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Antioxidant activity%= Ae Ac X 100

Where Ac: absorbance at t = 0, and Ae: absorbance at t = x (1h, 2h, 4h and 24h).

DPPH scavenging assay

The DPPH assay measures hydrogen atom (or one electron) donating activity and hence provides a measure of free-radical scavenging antioxidant activity. The DPPH is a purplecolored stable free radical; it becomes reduced to the yellow-colored, diphenyl picryl- hydrazine. According to Guemmaz. et *al.* [13] method with slight modification, 50 µl of various dilutions of each extracts or standards were mixed with 1250 µl of a 0.004% methanol solution of DPPH. After an incubation period of 30 mn in dark at room temperature, the absorbance of the samples was read at 517 nm. Butylated hydroxytoluene (BHT) and quercetin were used as standards. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The ability to scavenge the DPPH radical was calculated by using the following equation:

Scavenging effect = Ac-Ae Ac x 100
Where Ac: control absorbance and Ae: absorbance in the presence of each extracts.
Effective concentration (EC50) values of the extract, concentration of extract necessary to decrease the initial concentration of DPPH by 50%, were calculated.

Analgesic activity by acetic acid induced writhing

In mice Writhing study was performed by following the method of Adeyemi et *al.* [15] to investigate the analgesic effect of extracts. In this protocol, female mice were divided into four groups of five animals each (n=5). Group 1 (Positive control): treated orally with aspirin, used as a standard drug (100 mg/Kg). Group 2: (Negative control), treated orally with distilled water. Groups 3–4: treated orally with Ep.MeOH from stems (150 and 300 mg/kg in 0.5 ml H_2O, respectively). After 60 mn, writhes was induced in mice through intra-peritoneal injection with 0.6% (v/v) acetic acid. After 5 mn of injection, the number of abdominal contractions was counted over a period of 30 mn. The percentage inhibition of writhing reflex was calculated using the formula:

Inhibition (%) = 100 x (Cn–Ct)/Cn,

Where Cn = mean of contractions' count in animals in the negative control, and Ct = Mean of contractions' count in animals treated with different concentrations of extracts or aspirin.

Statistical Analysis

The results were expressed as mean ± SD. Allcomparisons were performed by the analysis of variance (ANOVA) followed by Tukey's test. All calculations were performed using Graph Pad software version 5.0. $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ were considered as indicative of significance, as compared to control groups.

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Results and discussion

Extraction yield

According to our results, the highest extraction yield ((14.34%) was obtained with Ep.MeOH followed by Ep.MeOH/H_2O (4.6%). The variation can be explained by the difference in solubility of the different compounds in the sample. These results show that the water showed a better yield compared to organic solvents methanol. Fractionation of 9 g of Ep.MeOH by solvents of increasing polarity gave the ethyl acetate extract (Ep.Ac) 8.6%, and the aqueous extract (Ep.Aq) 56%. According to the results obtained, a large part of the constituents of the crude extracts remain in the aqueous fraction.

Determination of total polyphenol and flavonoids contents

Our results (table 1) revealed that Ep.Ac extract had the highest total phenolic (TPC) and flavonoid (TFC) content. On the other hand, E.A.A. aqueous extract EP.Aq had the lowest phenolic. The difference can be explained by the different polarities of the solvents, which selectively extracted targetable phenol and flavonoid compounds from the materials. Furthermore, the extract had higher phenolic and flavonoid content when compared to E. A.A. collected from Tatouine Sahara (Tunisia) which had a TFC of 2.8 ± 0.0 mg Q/gE and a TPC of 53.3±0.1mg GA/gE [4]. This difference may be due to various factors such as the harvesting location and date, soil properties, rainfall, plant storage and extraction methods [16].

Table 1. Total phenolic and flavonoids content of E.A.A. extracts.

The values represent the means of three measurements ± SD.

Extracts	**Total phenolics (mg EAG/g E)**	**Flavonoids (mg EQ/g E)**
EP.MeOH/H_2O	61.42±0.003	1.65±0.003
EP.MeOH	48.64±0.006	3.25±0.001
EP.AC	116.73±0.009	5.87±0.008
EP.Aq	31.52±0.002	1.75±0.004

Anticoagulant Activity

Antithrombotic drugs are pivotal in the prevention and/or treatment of thrombotic disorders. Secondary metabolites from vegetal origin are a potential source of anti-coagulant drugs [17]. Although there is no study in literature about the possible anticoagulant activity of this plant. Therefore, with the aim of therapeutic purposes, the *in vitro* anticoagulant action of the extract of E.A.A was investigated in the present study.

Effect of extracts on *in vitro* prothrombin (PT) and activated partial thromboplastin time (aPTT)

The anticoagulant activity of the crude extract and the different fractions of E.A.A. was evaluated by the prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays, using

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normal citrated human plasma.

As indicated in table 2, the results obtained in the TP test, the Ep.MeOH extract and the Ep.Aq fraction exert considerable anticoagulant activity and consequently a total inhibition of coagulation, the Ep.Ac exhibit a coagulation time of 90 mn. With regard to the results of the TCA test, this results in an inhibition of total coagulation on the part of the extracts, Ep.MeOH and Ep.Ac. The aPTT time for the Ep.Aq extract is 20 mn. The pro-longation of aPTT and PT indicates the inhibition of the intrinsic and extrinsic pathway of coagulation [18].

Table 2 .Effect of different extracts on *in vitro* prothrombin, activated partial thromboplastin time and clot lysis of human blood. Results are proposed in Mean ± SD (n=3).

Extracts	**TP (mn)**	**aPTT(mn)**	**% of clot lysis**
EP.MeOH/H_2O			13.45 ± 1.85
EP.MeOH	no coagulation	no coagulation	6.12 ± 0.05
EP.Ac	90 ± 03	no coagulation	21.19 ± 4.14
EP.Aq	no coagulation	20 ± 1.5	18.32 ± 0.02
Citrate			37.35 ± 0.03
H_2O			5.72 ± 0.09

***In vitro* thrombolytic activity**

As can be observed in table 2 , that the Ep. Ac extract showed an increase in human clot lysis compared to the negative control (water) ($p < 0.001$) followed by the Ep.Aq extract, the Ep.MeOH/H_2O and Ep.MeOH.

A prolonged aPTT and PT result may indicate the inhibition of the intrinsic and extrinsic pathway of coagulation. Based on this results, it is possible to conclude that E.A.A. contains the main compounds responsible for the anticoagulant action observed in the different such as phenolic and flavonoid. Studies have shown these compounds have inhibitory effects on platelet function, thus having beneficial effects on heart disease and atherosclerosis [19]. It has been reported that coumarins and ephedrine have antithrombotic and thrombolytic activity [8]. Several articles showed their presence in the aerial parts of E.A.A. [20, 21]. Extracts rich in phenolics are increasingly of interest to the pharmaceutical and food industry [22].

Anti-hemolytic assay

Oxidation of erythrocytes serves as a good model for the oxidative damage of biological membrane of their ease of isolation, their simplicity, the richness of their polyunsaturated fatty acid membranes and the high oxygen and hemoglobin cell concentration [23]. In this study, lipid oxidation of mice erythrocyte by AAPH (300 mM) induced peroxyl radicals (ROO•) leading to erythrocyte hemolysis. This experiment was aimed to assess whether different extracts from Ephedra alata plant prevented oxidative damages to erythrocyte membrane or not.

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The results presented in figure 1 showed that Ep.MeOH/H_2O, Ep.MeOH, Ep.Ac, Ep.Aq and Vit C had a very large effect ($p < 0.001$) antihemolytic activity. Ep.Aq revealed an extension of HT 50 of 76.92 ± 3.59 mn from control at 188.15 ± 1.91 mn, but the other extracts Ep.MeOH/H_2O, Ep.MeOH, Ep.Ac,did not reach the HT50 even after 195 mn (the time of 100% Hemolysis reached by AAPH).

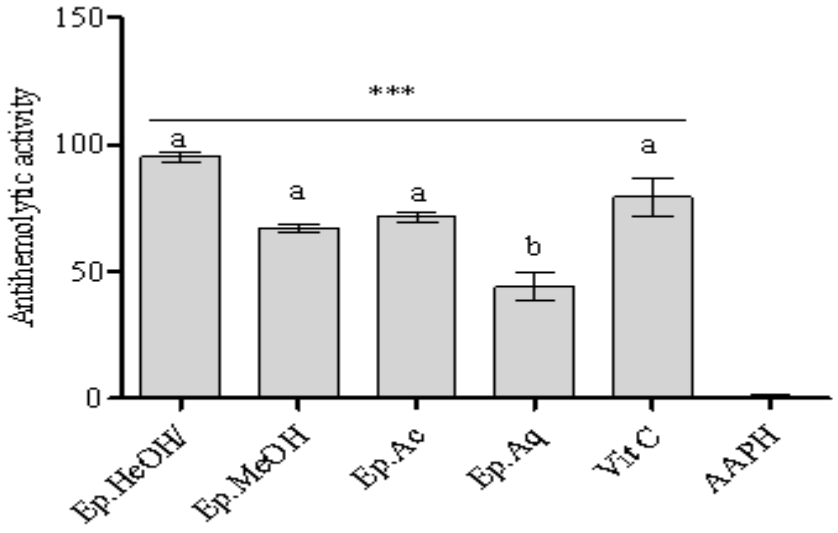

Figure 1. antihemolytic activity of Ep.MeOH/, Ep.MeOH, Ep.Ac, Ep.Aq, Vit C and control (AAPH). Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other.

Biological membranes can be affected by many natural products present in medicinal plants. Various authors mentioned that phenolic compounds and flavonoids had beneficial effect on the erythrocyte membrane stability [24]. The correlation analysis indicated that there is a significant positive correlation between total phenolics content and antihemolytic activity. Our findings are in agreement with studies showing that polyphenols protect erythrocytes from oxidative stress or increase their resistance to oxidative damage [25].

Ion chelating assay

Ion chelating agents also may inhibit the Fenton reaction and lipid peroxidation [26]. Thus, an antioxidant's ability to chelate metals is an important antioxidant property to measure. The strongest chelating activity was showed by Ep.MeOH/H_2O (EC50 = 0,60 ± 0,07 mg/ml), Ep.Aq (EC50 = 0,80 ± 0,10 mg/ml) and Ep.MeOH (EC50 = 1,53 ± 0,04 mg/ml) and Ep.Ac exhibited the lowest activity (figure 2).

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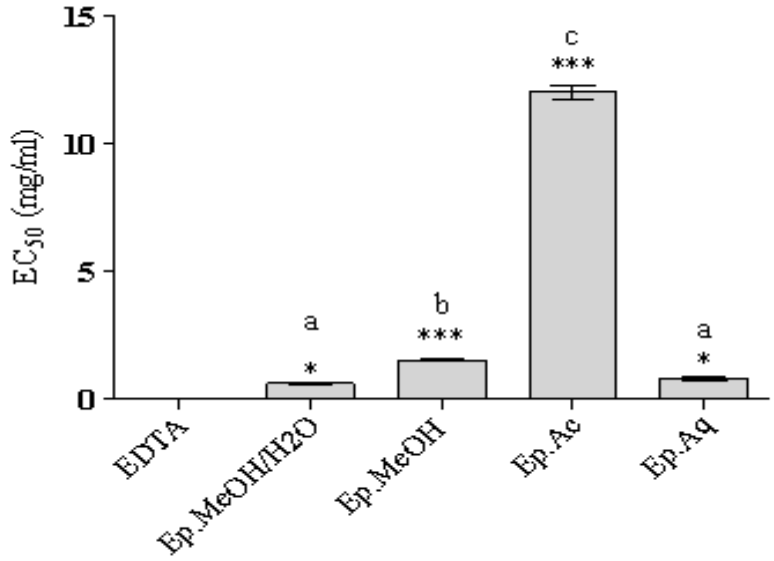

Figure 2. The EC50 values of ferrous iron chelating activity of Ep.MeOH/H_2O, Ep.MeOH, Ep.Ac, Ep.Aq and EDTA. Values were expressed as mean ± SD (n = 3). Bars with superscript with different letters in histogram were significantly ($p < 0.05$) different from each other.

Noting that Ep.Aq contained the lowest amount of both total phenolics and flavonoids; however, it exhibited the highest chelating activity. This result can be explained by the work of Sahreen *et al*. [27] showing that plant fractions induce chelating activity directly proportional to the polarity of their solvents. According to the literature, studies undertaken in recent years have demonstrated that the polyphenols and flavonoids can be excellent chelators for iron and copper [28]. However, through the correlation analysis, the chelating activity of Ep.MeOH/H_2O, Ep.MeOH, Ep.Ac, Ep.Aq did not point to any correlation with phenolic compounds content and flavonoids content. The lack of correlation indicated that phenolic compounds and flavonoids played a weak role on the ferrous chelating activity. Our results are consistent with those found by Esmaeili *et al.* [29] who reported that there is no correlation between phenolic compounds and flavonoids contents of the extract from the in vitro grown Trifolium pretense and ion chelating activity. Several studies show that only phenolic compounds with a certain structure and functional groups can act as transition metals chelators and show a chelating activity [28]. From our findings, we can suggest that the chelating activity of different extracts of E.A.A. did not return only to the amount of polyphenols but also to their structure and to the possible synergism with other compounds.

Reducing power assay

The presence of the antioxidants in the samples leads to Fe3+/ferricyanide complex reduced to the Fe2+ form, and Fe2+ can be monitored through the measurement of the formation of Perl's Prussian blue at 700 nm [30].

The E.A.A. extracts showed reducing power in a concentration-dependent manner. The Ep.Ac

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exhibited the highest reducing power (EC_{50} = 0,038,47±0,001mg/ml) which was significantly higher than that of Vit C (EC_{50} = 0.064± 0.0015 mg/ml), followed by Ep.Aq (EC 50 = 0.044±0.009 mg/ml) (figure 3).

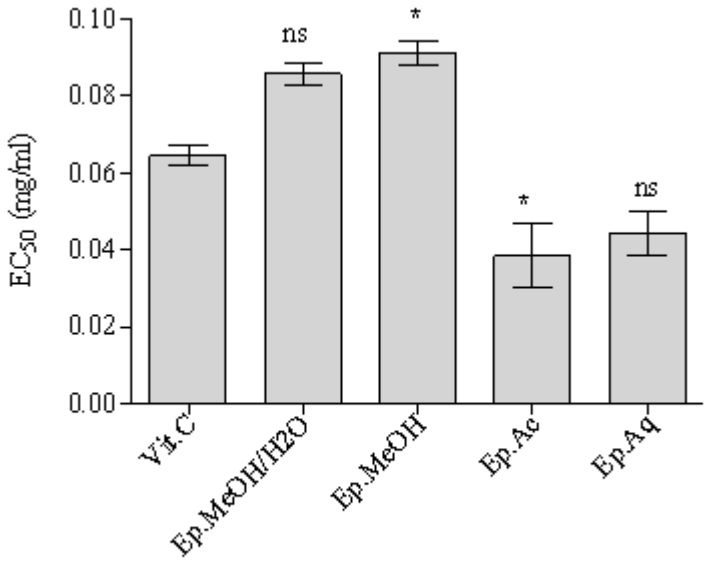

Figure 3. The EC50 values of reducing power of Ep.MeOH/H2O, Ep.MeOH, Ep.Ac, Ep.Aq and Vit C. Values were expressed as mean ± SD (n = 3). ns: p> 0,05, *: $p \leq 0,05$

Thus, it is suggested that Ep.Ac and Ep.Aq have a remarkable potency to donate electron to reactive free radicals, converting them into more stable nonreactive species and terminating the free radical chain reaction. The fact that Ep.Ac exhibited the strongest scavenger activity could be explained by its richness in phenolic compounds (116.73±0.009 mg EAG/g E) compared to the other extracts. Many reports have revealed that the electron-donating ability of polyphenols reflects the reducing power of these biomolecules and is also associated with their antioxidant activity. Polyphenols can break the free radical chain by donating electrons and, for this reason; the transition metal reducing power of polyphenols is correlated with their antioxidant activity [28].

DPPH radical scavenging activity

According to DPPH radical scavenging activity results (figure 4), the Ep.MeOH/H_2O presented a low scavenging activity with a high IC_{50} of 170,23±6,62µg/ml (***p< 0.001) and the crud extract Ep.MeOH had the best free radical scavenging activity with an IC_{50} of 5,35 ± 0,63 µg/ml while the IC_{50} of the BHT used as a standard was found to be 32,63 ± 0,64 µg/ml.

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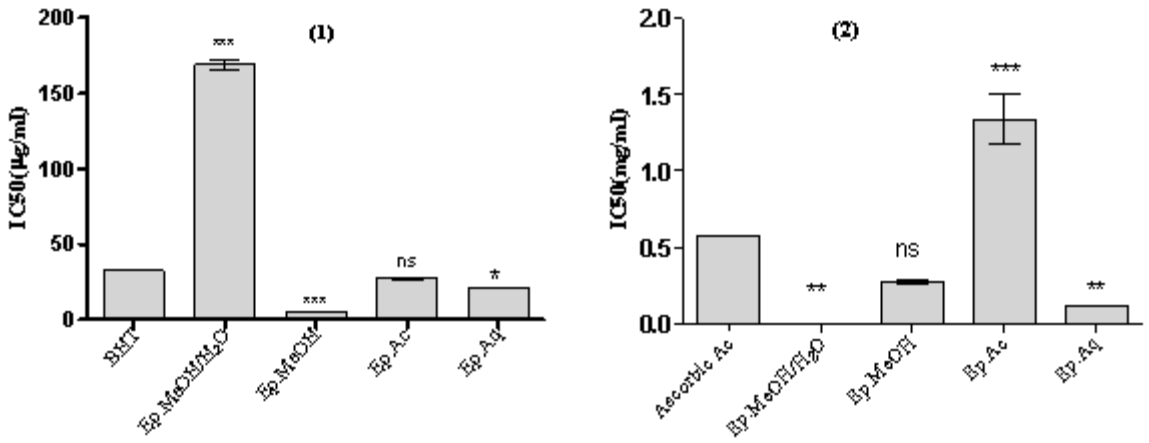

Figure 4. (1) DPPH radical scavenging activity of E.A.A. extracts and standards. Data are presented as IC_{50} values. (2) The IC50 values of hydroxyl radicals scavenging of E.A.A. extracts and Ascorbis acid. Values were expressed as mean ± SD (n = 3). (***: $p \leq 0.001$, * *$p \leq 0.01$, * $p \leq 0.05$, ns: non significant).

Antioxidant activity of methanol, ethyl acetate and aqueous extracts, demonstrating positive results which correlated with its higher content of phenolic compounds. The authors attributed the free radical scavenging activity to the presence of flavonoids [31].

Hydroxyl radical scavenging activity assay

Hydroxyl radicals are ROS im-plicated in cell damage. The hydroxyl radical is the most reactive of the radicals, making it extremely harmful. Its main source of production in vivo is due to the reaction of transition metals with the superoxide ion by the Fenton reaction. In view of this, the radical scavenging activity of all extracts from E.A.A. was evaluated. According to the results obtained (figures 4), all the extracts showed antioxidant activity against the hydroxyl radical, the most active extract is that of Ep. MeOH/H_2O (IC50 =0.00138 µg/ml ± 0,00059) followed by Ep.Aq extract (0.115 mg /ml ± 0.029), Ep.MeOH (0.272 mg /ml ± 0.032). These extracts have a very high activity compared to ascorbic acid ($p<0.001$), with the exception of the extract, Ep.Ac (1,007 mg/ml ±0.042) which is a little less than the standard. Therefore Ep .A.A. can be considered very good hydroxyl radical scavengers.

According to the DPPH and Hydroxyl radicals scavenging activity results, the E. A.A. extract had higher antioxidant potentials which could be explained by its higher phenolic and flavonoid content. These results are better than those reported by Zatout et *al.* [32].

β -Carotene Bleaching Assay

Lipid peroxidation is an oxidative process that causes the disruption of essential macromolecules like DNA, proteins, phospholipids, among others. It is associated to chronic diseases, like asthma, hepatitis and cardiovascular illnesses [33]. As can be seen in figure 5, all the extracts were capable

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of inhibiting the bleaching of β-carotene by scavenging linoleate derived free radicals. The inhibition extent of lipid oxidation by *E.alata* extracts when compared to BHT, which had 97.4±1,57% at the same concentration (2 mg/ml) showed a marked activity effects. The high inhibition ratios by the Ep.MeOH/H_2O were showed for 85.84 % followed by Ep.Aq (79.93%), Ep.Ac (79.37%) and Ep.MeOH (76.13%).

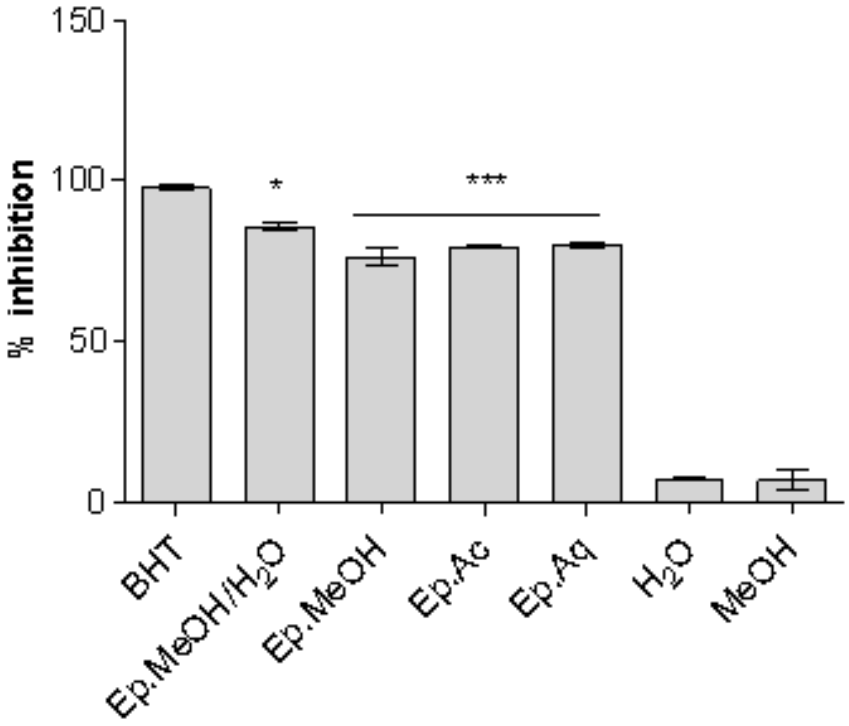

Figure 5. Relative antioxidant activities of E.A.A. extracts and BHT in β-carotene/ linoleic acid system after 24h. Values were means ± SD of triplicate (***: $p \leq 0.001$, * $p \leq 0.05$).

Indeed, Frankel and Meyer [34] have suggested that the polarity of an extract is important in water: oil emulsions, apolar extract exhibit most important antioxidant properties as they are concentrated within lipid-water interface, thus helping to prevent radical formation and lipid oxidation of β-carotene. While polar extract are diluted in the aqueous phase and are thus less effective in protecting lipids.

Evaluation of Analgesic Activities of the Extract

In this test, E.A.A. extract (Ep.MeOH) at two test doses employed (150 et 300 mg/kg)) showed peripheral analgesic activities by reducing the number of writhing with the respective values of 74 % and 60.09 % as compared to the negative control (figure 6). These findings confirmed that the peripheral analgesic activity of the extract increased from the lower dose (150 mg/kg) to the higher dose (300 mg/kg) in dose dependent manner.

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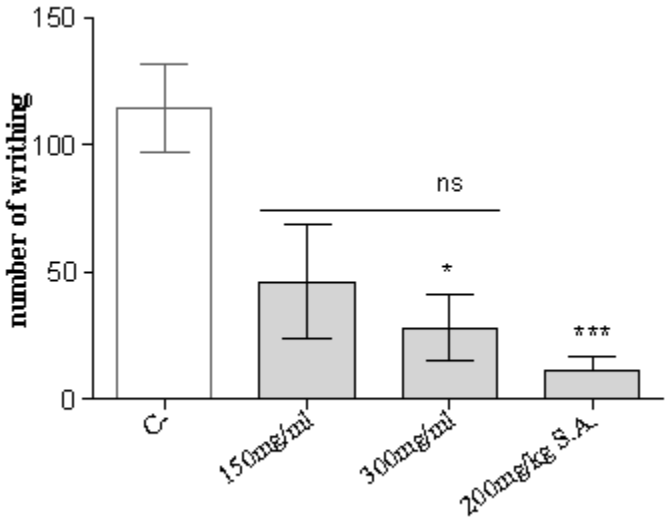

Figure 6. Analgesic activity showing the writhing response in 30 minutes after acetic-acid injection (0,6%) in the presence of two doses of E.A.A. in experimental animals. S.A = Salicylic acid, C^- = distilled water. Values were expressed as mean ± SEM (n = 5) $p < 0.05$.

The increase in analgesic activity with increasing doses of the extract might be attributed with an increase in concentration of phyto constituents that possess analgesic activity with the maximum dose. The possible mechanism by which the extract produced peripheral analgesia in this model might be associated with inhibiting the synthesis and release of various endogenous inflammatory mediators and suppression of sensitivity of peripheral nociceptors in the peritoneal free nerve endings for chemical-induced pain. These proposed mechanisms are in line with the principles that stated, any agent that decreases the number of writhing will demonstrate analgesia by inhibiting the synthesis and release of PGs, and by inhibiting the peripheral pain transmission [35].

Conclusion

The plant extracts have shown an important antioxidant effect as well as an interesting anticoagulant and analgesic activity. It is suggested by our results that E.A.A. extracts can be adopted as an effective and safe antioxidant source. Further determination of compounds from this plant and the study of other biological effects may provide more information on their medicinal value.

Conflict of Interest

No conflict of interest was declaredby the authors.

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