Antioxidant, Anti-inflammatory, and Analgesic Activities of Alcoholic Extracts of *Ephedra nebrodensis* From Eastern Algeria

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ABSTRACT

Objectives: *Ephedra nebrodensis* (Ephedraceae) presents a wide range of biological activities. It is used to treat respiratory problems and hepatic pathologies in traditional medicine. The aim of this study is to evaluate the antioxidant, *in vitro* and *in vivo* anti-inflammatory and analgesic properties of two hydro-alcoholic extracts of *E. nebrodensis* in mice.

Materials and Methods: The antioxidant capacity of hydro-methanolic (HM) and hydro-ethanolic (HE) extracts of *E. nebrodensis* was evaluated via assays of their superoxide radical scavenging capacity and ferrous ion chelating activity. The *in vitro* anti-inflammatory activity of the extracts (5, 10, and 20 mg/kg) was also determined using the bovine serum albumin denaturation test. Croton oil-induced ear edema was then employed to evaluate the *in vivo* anti-inflammatory effect of the extracts (200 and 400 mg/kg). Finally, the analgesic activity of the extracts (200 and 400 mg/kg) was determined by the acetic acid-induced torsion test.

Results: The hydro-alcoholic extracts of *E. nebrodensis* present significant antioxidant activity. The HE and HM could inhibit protein denaturation by 82.99±20.21% and 56.25±2.12%, respectively. The extracts (HM and HE) also show strong anti-inflammatory effects *in vivo* and could reduce ear edema by 70.37±2.00% and 72.22±1.94%, respectively. The HM extract (72.51±2.43%) demonstrates greater pain inhibitory effects than HE (70.76±2.58%).

Conclusion: The hydro-alcoholic extracts of *E. nebrodensis* produce antioxidant, anti-inflammatory, and analgesic effects. These results confirm the traditional use of the herb in the treatment of various diseases.

Key words *Ephedra nebrodensis*, anti-inflammatory activity, analgesic test, antioxidant capacity, hydro-alcoholic extracts

ÖZ


Gereç ve Yöntemler: *E. nebrodensis*‘in hidro-metanolik (HM) ve hidro-etanolik (HE) ekstrerinin antioksidan kapasitesi, süperoksid radikal süpürme kapasiteleri ve demirli iyon şelatlama aktivitelerinin belirlenmesi ile değerlendirildi. Eksrelerin (5, 10 ve 20 mg/kg) *in vitro* anti-inflamatuvar aktivitesi de sütür serum albüminin denatürasyon testi kullanılarak belirlendi. Daha sonra ekstrerinin (200 ve 400 mg/kg) *in vivo* anti-inflamatuvar etkisini değerlendirilmiş için kroton yağı ile indüklenen kulak ödemi modeli kullanıldı. Son olarak ekstrerinin analjezik aktivitesi (200 ve 400 mg/kg), asetik asit ile indüklenen kvarna testi ile belirlendi.

Bulgular: *E. nebrodensis*‘in hidro-alkollik ekstrerii, önemli antioksidan aktiviteye sahiptir. HE ve HM, protein denatürasyonunu sırasıyla %82.99±20, 21 ve %56.25±2.12 oranında inhibe edebilir. Eksreler (HM ve HE) ayrıca *in vivo* olarak güçlü anti-inflamatuvar etkiler gösterir ve kulak ödemi...
INTRODUCTION

Inflammation is a reaction of the immune system in response to external pathogens or injury to cells and tissues. The local coronary system, the immune system, inflammatory cells, mediators, and cytokines are implicated in this process. Macrophages play an important role in the production of numerous cytokines, reactive oxygen and nitrogen molecules, and growth factors and chemicals, such as lipopolysaccharides (LPS), which are organic mediators of inflammatory stimuli. Pain is a sign of tissue lesions due to mechanical, chemical, or physical stimulation. Pain perception is controlled by the neurosensory system and afferent nerve fibers in response to potential damage. Pain stimulates the production of substances called pain mediators, such as histamine, bradykinin, leukotriene, and prostaglandin; these substances activate pain receptors that channel the stimulus through to the brain via nerve points with numerous synapses through the spinal cord, bone marrow, and midbrain. Pain relief is achieved by a class of drugs known as analgesics. Despite their many benefits, however, analgesics also present a number of adverse side effects, including gastric ulcer.

Over the last few years, the use of medicinal plants as potential therapeutic agents for the treatment of pain and inflammation has generated great interest. Ephedra (Ephedraceae), a genus of non-flowering grained plants, includes approximately 67 species and is principally found in desert zones across Asia, Europe, North Africa, and North America. Over 145 organic molecules have been isolated from Ephedra, included alkaloids, polysaccharides, flavonoids, and tannins. Ephedra is known to show anti-asthmatic, anti-inflammatory, antiproliferative, hypoglycemic, antioxidant, and weight-reduction properties.

Research on Ephedra nebrodensis is scarce. Sureka et al. for example, showed that the aerial part of E. nebrodensis has cardio-protective effects. Short-term low-dose consumption of the hydro-ethanolic (HE) extract of Ephedra major has shown protective effects in cirrhotic patients. The ethanol:acetone extract of E. nebrodensis Tineo exhibits antihistaminic, adaptogenic, and antinociceptive activities. The data reported by Shah et al. suggest that the ethanol:acetone extract of E. nebrodensis has preventive effects against the cardio-toxicity induced by doxorubicin.

To date, however, no study describing the anti-inflammatory and antinociceptive effects of E. nebrodensis has yet been published. In this research, we studied the in vitro antioxidant and anti-inflammatory properties, the in vivo anti-inflammatory effect, and the analgesic activity of hydro-methanolic (HM) and HE extracts of the aerial parts of E. nebrodensis.

MATERIALS AND METHODS

Plant materials
The aerial parts of E. nebrodensis were collected in May 2017 from the mountains of Nafia, Commune de Hidoussa, Batna, Algeria. Species identification was conducted by Prof. Laouer Hocine (Laboratory of Natural Resources Valorization, Department of Biology and Vegetal Ecology, University of Setif 1, El Bez, 19000, Algeria). A plant specimen was deposited in the herbarium of the Laboratory of Botany of the Faculty of Natural and Life Sciences, University of Setif 1 (no: SNV004/20). The collected samples were dried in the shade in open air for 7 d.

Test animals
Two-month-old mice weighing 22-29 g were purchased from Institut Pasteur d’Algérie, Algiers. The animals were acclimatized in the pet shop at a temperature of 25-27 °C, relative humidity of 50–62%, and light/dark cycle of 12 h prior to the start of the experiments. All animal experimental protocols performed in this study were approved by the Ethics Committee of the Algerian Association of Sciences in Animal Experimentation (http://aasea.asso.dz/articles/) under Law No. 88-08/1988, which describes guidelines for veterinary medical activities and animal health protection (N° JORA: 004/1988).

Preparation of extracts
Approximately 100 g of the aerial part of E. nebrodensis in powder form was extracted with methanol (85%) and ethanol (70%). The samples were macerated for 72 h at room temperature prior to extraction. The sample/solvent mixtures were filtered, and the filtrates obtained were evaporated in an evaporator to eliminate the solvent. The residues were then dried in the oven to obtain crude HM and HE.

Determination of antioxidant capacity by alkaline dimethyl sulfoxide (DMSO) assay
Scanning capacity was established by the superoxide anion (produced in a non-enzymatic solution) assay with alkaline DMSO assay. Test mixtures consisting of 0.03 mL of NBT (1 mg/mL), 0.130 mL of alkaline DMSO (0.02 g of NaOH/100 mL of DMSO), and 0.04 mL of the crude extracts or standard were prepared and incubated for 5 min. The absorption of the solutions was then determined at 560 nm. The scavenging capacity of the samples was evaluated according to the following formula:

$$ [%\ inhibition] = \left( \frac{A_{ci} - A_{tci}}{A_{ci}} \right) \times 100 $$

where $A_{ci}$ is the absorbance of the sample and $A_{ci}$ is the absorbance of the control.

Determination of iron-chelating activity
The ability of the extracts to chelate iron and inhibit the formation of the Fe$^{2+}$–ferrozine complex was tested. Briefly, 40
μL of ethylenediamine tetraacetic acid (EDTA) or the samples was added to 40 μL of FeCl₃ (0.2 mM) and 0.04 mL of methanol. Five minutes later, the complexation reaction was initiated via the addition of 0.08 mL of ferrozine (0.5 mM) and allowed to proceed for 10 min at ambient temperature. The absorption of the Fe²⁺-ferrozine complex produced was measured at 562 nm, and chelating ability was calculated in terms of inhibition percentage according to the following equation:

\[ \text{Fe}^{2+} \text{ chelating effect (\%)} = \left( \frac{A_{\text{TS}} - A_{\text{TS}}}{A_{\text{TS}}} \right) \times 100 \]

where \( A_{\text{TS}} \) is the absorbance of the test sample and \( A_{\text{C}} \) is the absorbance of the control sample.

**Determination of in vitro anti-inflammatory activity**

In vitro anti-inflammatory activity was evaluated according to the method of Karthik et al. with slight modifications. Briefly, 100 μL of different doses of the extracts or diclofenac was treated with 1 mL of 0.2% bovine serum albumin (BSA) solution prepared in Tris-HCl (pH: 6.6) and then kept in the oven for 15 min at 37°C. Thereafter, the solutions were placed in a water bath for 5 min at 72°C. The chilling turbidity of the solutions was determined at 660 nm by spectrophotometry. Blanks containing 1 mL of extract and 1 mL of Tris-HCl were prepared for each extract concentration.

**Determination of in vivo anti-inflammatory activity by the croton oil-induced ear edema method**

The anti-inflammatory properties of HM and HE from E. nebrodensis were tested via the croton oil-induced ear edema method according to Manga et al. The internal surface of the right ear of five groups of mice with a mean weight of 24.81±1.66 g was treated with 15 μL of acetone: water solution (1:1) containing 80 μg of croton oil as an irritant to induce skin inflammation. The same volume of solution without croton oil was applied to the left ear. The experimental mice were orally administered different concentrations of the extracts 1 h after croton oil application, the positive control group received 50 mg/kg indomethacin, and the negative control group received distilled water. Ear thickness was evaluated with a digital caliper 6 h after edema induction. The mice were randomized into six groups, each of which included six mice.

**Negative control group:** Received distilled water.

**Positive control group:** Received indomethacin (50 mg/kg).

**Groups A1 and A2:** Received 200 and 400 mg/kg HE, respectively.

**Groups B1 and B2:** Received 200 and 400 mg/kg HM, respectively.

The percentage of edema inhibition was defined in relation to the control group, which received the croton oil solution, according to the following formula:

\[ \text{Inhibition \%} = \left( \frac{D_{\text{Control}} - D_{\text{Treated}}}{D_{\text{Control}}} \right) \times 100 \]

where \( D_{\text{Control}} \) is the difference in edema thickness in the control group and \( D_{\text{Treated}} \) is the difference in edema thickness for the treated groups.

**Determination of in vivo analgesic activity by the acetic acid-induced writhing test**

Analgesic activity against acetic acid-induced pain was evaluated according to the method described by Koster et al. The negative control group was given distilled water orally, the treatment groups were given a single dose of 200 or 400 mg/kg HM or HE orally, and the positive control group was given 100 mg/kg aspirin. The mice were then injected with acetic acid (0.6%, 10 mL/kg) intra-peritoneally. The number of twitches exhibited by each mouse was counted at 5 min intervals for 30 min. The percentage of pain inhibition was determined using the following equation:

\[ \text{Inhibition \%} = 100 \times \left( \frac{C_{\text{nc}} - C_{\text{tr}}}{C_{\text{nc}}} \right) \]

where \( C_{\text{nc}} \) is the average number of twitches in the negative control group and \( C_{\text{tr}} \) is the average number of twitches in groups given various doses of HM or HE extracts or aspirin.

**Statistical analysis**

The results of the in vitro test were expressed as mean ± standard deviation, while the results of the in vivo experiments were expressed as mean ± standard error of the mean. Results were evaluated by One-Way analysis of variance and Dunnett’s test by using GraphPad Prism (version 5.01). \( P<0.05 \) was regarded as statistically significant.

**RESULTS**

**Antioxidant capacity**

The capacity of the extracts to capture superoxide anion radicals was examined in our study. According to the results shown in Table 1, the scavenging ability (IC₅₀) of HE (1.84±0.46 μg/mL) was significantly greater (\( p<0.001 \)) than those of ascorbic acid (7.59±1.6 μg/mL) and α-tocopherol (31.52±2.2 μg/mL). HM showed effects (7.81±0.28 μg/mL) comparable with those of ascorbic acid.

All samples showed moderate chelation ability for Fe²⁺ (Table 1). Compared with other samples, HE appeared to be a more active chelator, and the IC₅₀ of HE (168.12±113 μg/mL) was greater than that of HM (174.60±4.28 μg/mL). Neither extract appeared to be a more powerful Fe²⁺ chelator than the EDTA positive standard (8.80±0.47 μg/mL) in the present test system.

**In vitro anti-inflammatory activity**

The in vitro anti-inflammatory effect of the E. nebrodensis extracts was evaluated via the BSA denaturation method, and the results are presented in Figure 1. HE could inhibit BSA denaturation in a dose-dependent manner and demonstrated a maximum inhibition rate of 82.99% at a dose of 20 mg/mL. HM inhibited protein denaturation by 56.25%. Diclofenac at a dose of 5 mg/mL inhibited inflammation by 99.82%.

**Anti-inflammatory effects against croton oil-induced ear edema**

The anti-inflammatory effects of HE and HM on ear edema induced by croton oil are presented in Figure 2. Edema was inhibited by the extracts in a dose-dependent manner, and the highest dose of the extracts demonstrated the most significant activity. HM and HE reduced ear edema with maximum
inhibition percentages of 72.22% and 70.37%, respectively, at a dose of 400 mg/kg. This effect is statistically similar to that of indomethacin (78.49%).

**Antinociceptive effects against acetic acid-induced pain**

The results presented in Figure 3 show that administration of 200 and 400 mg/kg *E. nebrodensis* extracts exerts a protective effect against pain caused by acetic acid. HM and HE demonstrated good analgesic activity with inhibition rates of 63.74% and 59.06%, respectively, when administered at a dose of 200 mg/kg. Higher doses of HM and HE (400 mg/kg) resulted in higher pain inhibition rates of 72.51% and 70.76%, respectively. These effects are similar to that of aspirin at 100 mg/kg (79.14%). No significant difference between the effects of the extracts at different concentrations and the standard (aspirin) was observed.

**DISCUSSION**

Herbal medicines are widely acknowledged to represent a beneficial approach for the treatment of many types of human diseases. Numerous sources have documented the ethno-pharmacological use of herbs by many populations since ancient times. Phytochemicals are ubiquitous compounds found in herbs that provide a wide range of benefits, such as anticancer, antibacterial, anti-inflammatory, antidiabetic, and antioxidant activities.

In this study, we report the antioxidant, anti-inflammatory (*in vitro* and *in vivo*), and analgesic effects of *E. nebrodensis* extracts on a mouse model. HE demonstrated stronger *in vitro* antioxidant activity compared with HM. Hamoudi et al. used 2,2-diphenyl-1-picrylhydrazyl and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assays to show that an ethyl acetate fraction of *E. nebrodensis* exhibits potent antioxidant properties.

The results obtained in this work indicate that HE and HM are strongly able to chelate Fe$^{2+}$. Previous research found no

Figure 1. *In vitro* anti-inflammatory effect of hydro-alcoholic extracts of *Ephedra nebrodensis*. Data are presented as mean ± SD (n=3). *P<0.05 compared with the diclofenac group. ***P<0.001 compared with the diclofenac group.

HE: Hydro-ethanolic extract (70%), HM: Hydro-methanolic extract (85%), DIC: Diclofenac group 5 g/kg, SD: Standard deviation, BSA: Bovine serum albumin

Figure 2. Effects of *Ephedra nebrodensis* on inflammation in Swiss albino mice (n=6; W: 24.815±1.66 g). Inflammation was induced by applying 15 μL of acetone-water solution (1:1, v:v) containing 80 μg of croton oil to the internal surface of the right ear. The same volume of solution without croton oil was applied to the left ear. After 1 h, the mice were orally administered the extracts. Ear thickness was measured after 6 h. The results are expressed as mean ± SEM. *P<0.05 compared with 50 mg/kg indomethacin

HE: Hydro-ethanolic extract (70%), HM: Hydro-methanolic extract (85%), IND: Indomethacin (50 mg/kg), SEM: Standard error of the mean

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<tr>
<th>Extracts/standard</th>
<th>O$_2^\cdot$ DMSO alkaline</th>
<th>Fe$^{2+}$ ion chelating ability</th>
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<tr>
<td></td>
<td>Inhibition % at 200 μg/mL</td>
<td>IC$_{50}$ (μg/mL)$^*$</td>
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<tr>
<td>HM</td>
<td>94.86±0.10$^c$</td>
<td>7.81±0.28$^c$</td>
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<tr>
<td>HE</td>
<td>94.17±0.01$^c$</td>
<td>1.84±0.46$^c$</td>
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<td>EDTA$^b$</td>
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<td>Ascorbic acid$^b$</td>
<td>94.28±1.12</td>
<td>7.59±1.36</td>
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<td>α-tocopherol$^b$</td>
<td>96.54±0.10$^c$</td>
<td>31.52±2.22</td>
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$^*$IC$_{50}$ values correspond to the mean ± standard deviation of three simultaneous measures. $^*$Standard compounds, $^p<0.001$. HE: Hydro-ethanolic extract, HM: Hydro-methanolic extract, DMSO: Dimethyl sulfoxide, EDTA: Ethylenediamine tetraacetic acid
Acute inflammation reactions are manifested by the formation of swelling and infiltration of leukocytes into the inflamed tissue. The chemicals released by resident cells promote the alteration of vascular permeability and, in turn, the formation of edema. Sequential processes and activities between the endothelium and inflammatory tissue cells (principally neutrophils) then lead to the development of inflammatory cells at the tissue.27,38

We noted that the *E. nebrodensis* extracts and indomethacin, which was used as a positive control, similarly inhibited the formation of atrial edema. Anti-inflammatory effects may be achieved through various mechanisms of action, including inhibition of histamine release, 5-lipoxygenase, complement and elastase functions.29,40 Many studies have attributed the anti-inflammatory properties of phenolic compounds to their antioxidant activity.41,42

In acetic acid assay, it is apparent that abdominal muscle pain is not a specific pattern and involuntary abdominal muscle pain may be due to its similarities to some of the known visceral pain patterns.43 Activation of prostaglandins, histamines, serotonin, lipoxygenases, cyclooxygenases, and endogenous cytokines (e.g., IL-8 and IL-1β) in peripheral tissues is activated by acetic acid injection into the abdominal cavity of mice.44 These substances penetrate the dorsal horn of the central nervous system and stimulate primary nociceptors, resulting in enzyme pain and torsion disorder.45 In our experiment, the hydro-alcoholic extracts significantly (p<0.001) and dose-dependently reduced the number of acetic acid-facilitated abdominal contractions or torsions manifested by the mice. This finding clearly indicates that the anti-nociceptive effect produced by the extracts prevents the endogenous synthesis of inflammatory substances or directly inhibits their receptors.46

The peripheral analgesic capacity of the different extracts studied in this work may be due to the phenolic compounds and alkaloids produced by the plant. These compounds are known to be responsible for the analgesic properties of other medicinal plants, such as *Jasminum amplexicaule* and *Elephantopus tomentosus*.47,48

CONCLUSION

This study reports for the first time the antioxidant and anti-inflammatory activities of hydro-alcoholic extracts (i.e., HE and HM) of *E. nebrodensis*. The extracts exhibited excellent *in vitro* antioxidant activity, as determined by superoxide radical scavenging and metal chelation assays. The extracts also demonstrated significant *in vitro* anti-inflammatory effects, with inhibition rates of 56-82%.

The plant extracts studied in this work demonstrated important *in vivo* anti-inflammatory effects, as well as moderate analgesic activity, in mice. Further phytochemical characterization of the active compounds responsible for these biological activities is necessary to improve the understanding on the mechanism through which *E. nebrodensis* reduces inflammation and pain.

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Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

REFERENCES


