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In vitro Antioxidant, Anti-inflammatory (*in vitro* and *in vivo*) and analgesic activities of hydroalcoholic extracts of *Ephedra nebrodensis* from Eastern Algeria

M. Hamoudi[1*], D. Amroun[1], A. Baghiani[2], S. Khennouf[1], S. Dahamna[1]
1 Laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Natural and Life Sciences, University Ferhat Abbas Setif 1, Setif 19000, Algeria.
2 Laboratory of Applied Biochemistry, Faculty of Natural and Life sciences, University Ferhat Abbas Setif 1, Setif 19000, Algeria.

* Corresponding author
Hamoudi Meriem
meryoumamm2009@hotmail.fr
+213777150029
0000-0001-6659-3943
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Abstract
Objectives: *Ephedra nebrodensis* belonging to Ephedraceae family has a wide range of biological activities. It is used for the treatment of respiratory affections and hepatic pathologies in traditional medicine. The aim of this study was the evaluation of the antioxidant of two hydroalcoholic extracts of *Ephedra nebrodensis* and the anti-inflammatory activity *in vitro* and *in vivo*, and the analgesic property *in vivo* in mice.
Materials and Methods: The antioxidant capacity of extracts was evaluated by (Superoxide radical scavenging capacity and ferrous ion chelating activity). The *in vitro* anti-inflammatory activity of the hydro-methanolic (HM) and hydro-ethanolic (HE) extracts (5, 10 and 20 mg/kg) was determined by the BSA denaturation test. A model of croton oil induced ear edema was used to evaluate the *in vivo* anti-inflammatory effect of the extracts (200 and 400 mg/kg). The analgesic activity of the extracts (200 and 400 mg/kg) was determined by the acetic acid-induced torsion test.
Results: Results showed that hydroalcoholic extracts presented a significant antioxidant activity in the tests studied. HE and HM extracts have the potential to inhibit protein denaturation induced thermally with inhibition percentages of 82.99 ± 20.21 and 56.25 ± 2.12%, respectively. In addition, the extracts have a powerful anti-inflammatory effect *in vivo*. Indeed, they reduce ear edema with an inhibition percentage between 70.37 ± 2.00 and 72.22 ± 1.94%. The potent inhibitory effect of the abdominal contractions obtained with HM (72.51 ± 2.43) was greater than of HE (70.76 ± 2.58 %).
Conclusion: Based on the results obtained, it appears that the extracts (HM) and (HE) of *E. nebrodensis* produce antioxidant, anti-inflammatory and analgesic effects, which confirm its traditional use in the treatment of various diseases.
Key words *Ephedra nebrodensis,* anti-inflammatory activity, analgesic test, antioxidant capacity, hydro-alcoholic extracts.

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 all implicated in this process. In inflammatory tissue, macrophages play an important role in the production of many cytokines, reactive oxygen and nitrogen molecules, growth factors and chemicals, such as lipopolysaccharides, which are organic mediators of inflammatory stimuli [1]. On the other side, pain is a sign of tissue lesions due to mechanical, chemical or physical stimulation. The perception of pain is controlled by the neurosensory system and afferent nerve lanes, which are particularly responding to potential damage [2]. It also stimulates the liberation of some substances that are called pain mediators, such as histamine, bradykinin, leukotriene and prostaglandin [2]. All of these pain mediators stimulate the pain receptors that channel the stimulation through to the brain via nerve points that have many synapses through spinal cord, marrow advanced, and midbrain. For the treatment of this pain, there is a class of drugs known as analgesics. As we all know, this analgesics generally have side effects, especially gastric ulcer [3].
During the last few years, a great deal of interest has been given to medicinal plants as potential therapeutic agents in the treatment of pain and inflammation. Among them, the genus Ephedra (Ephedraceae) is the genus of unflowering grained plants [4], which includes about 67 species, principally in Asia's desert zones, Europe, North Africa and America [5]. The phytochemical research revealed that over one hundred forty-five molecules were singled and isolated for genus Ephedra, included alkaloids, polysaccharides, flavonoids and tannins. [6].The activities of Ephedra include anti-asthmatic [7], anti-inflammatory [8], anti-proliferative [9], hypoglycemic [10], antioxidant properties [11] and weight reduction [12]. The research conducted on *E. nebrodensis* is very few, among them the study of Sureka et *al*. [13] which showed that the aerial part of *E. nebrodensis*, is among the cardio-protective plants. Short term and low dose consumption of the hydro-ethanolic extract of *E. major*, it has a protective effect in cirrhotic patients [14].
They reported that the ethanol: acetone extract of *E. nebrodensis* Tineo has anti-histaminic, adaptogenic, anti-nociceptive activities [15]. The data reported by Shah et *al*. [16] suggested that the ethanol: acetone extract of *E. nebrodensis* has a preventive effect against the cardio-toxic effects induced by doxorubicin.
However, until now no study has proven its potential efficacy in treating inflammatory skin diseases and its anti-nociceptive effect. In this research we have studied the antioxidant and anti-inflammatory properties *in vitro*, the anti-inflammatory effect *in vivo* (using a croton oil caused skin inflammation model in mice) and the analgesic effect (induced by acetic acid in mice) of the HM and HE extracts of the aerial part of *E. nebrodensis*.

MATERIALS AND METHODS

Plant material

The aerial part of the *Ephedra nebrodensis* was collected in May 2017, in the mountains of Nafla (commune of Hidoussa), from Batna (Algeria). The identification was carried out by Prof. Laouer Hocine (Laboratory of Natural Resources Valorization, Department of Biology and Vegetal Ecology, University of University of Setif 1, El Bez, 19000, Algeria). A specimen of the plant was deposited in the herbarium of the Laboratory of Botany of the Faculty of Natural and Life Sciences, University of Setif 1, under the number SNV004/20. The aerial part was dried in the shadow and in the fresh air for seven days.

Test animals

Young mice of 2 months old and weighting 22–29 g were bought from 'Institut Pasteur d'Algérie', Algiers. They were acclimatized in a pet shop with the conditions of temperature between 25-27 °C, relative humidity between 50–62 % and Black-light cycle 12 hours before the start of experiments. The Committee of the "Algerian Association of Sciences in Animal

Experimentation" (http://aasea.asso.dz/ articles/) under law No. 88-08/1988, associated with veterinary medical activities and animal health protection (N° JORA: 004/1988) has approved the experimental protocols carried out on animals.

Preparation of extracts

Using the powder of the aerial part of *E. nebrodensis*, 100 g was extracted with methanol (MeOH 85%) and ethanol (EtOH 70%); this maceration lasted 72 hours at room temperature (to extract the maximum of the compounds). Then the mixtures were filtered and the filtrates obtained were evaporated an evaporator to eliminate the solvent and then dried in the oven to obtain two crude extracts, hydro-methanolic (HM) and hydro-ethanolic (HE) [17].

Antioxidant capacity

Alkaline DMSO assay

The scanning capacity was established by superoxide (produced in a non-enzymatic solution) by alkaline DMSO assay [18]. The sample test mixture consisted of 0.03 mL NBT (1 mg/ml), 0.130 mL of alkaline DMSO (0.02 g of NaOH/100 mL of DMSO) and 0.04 mL of extracts or standard. After a five-minute incubation period, absorptions have been determined at 560 nm. The scavenging capacity of sample was evaluated using the formula below:

$[\%\ \text{inhibition}] = [(A_{Ct} - A_{TS})/A_{Ct}] \times 100$.

Where, A_{TS}: the absorbance values of sample, A_{Ct}: the control absorbance values.

Iron ion chelation activity

The ability of extracts to present a chelating action is tested [19] from treatment of samples using Fe^{2+}, which inhibit the formation of the Fe^{2+}-ferrozine complex. Briefly, 40 µL of EDTA or the samples was added to 40 µL of $FeCl_2$ (0.2 mM) and 0.04 mL of methanol. Five minutes after, the reaction has begun with addition of 0.08 mL ferrozine (0.5 mM), allowing preparation of the mixture at ambient temperature for ten minutes. The absorption of produced Fe^{2+} -ferrozine complex was estimated at 562 nm and chelating ability in percent inhibition was given by the following equation:

Fe^{2+} chelating effect (%) $= [(A_C - A_{TS})/A_C] \times 100$.

Where, A_{TS}: the absorbance values of the test sample, A_C: the control absorbance values.

Anti-inflammatory activity in vitro

In vitro anti-inflammatory capacity was evaluated by the method of Karthik et *al*. [20] with slight modifications. Briefly, 100 µL of different doses of extract or diclofenac was added to 1 mL of 0.2 % BSA solution prepared in Tris-Hcl (pH: 6.6), solutions are kept for 15 min at (37 °C) in the oven. After that, in a water bathroom for five minutes at (72 °C). Next chilling turbidity was determined at 660 nm by cuvette spectrophotometer. For each extract concentration. A blank was prepared in 1 mL extract and 1 ml Tris-Hcl.

Anti-inflammatory activity in vivo

Croton oil induced ear-edema

The anti-inflammatory capacity of HM and HE extracts from *E. nebrodensis* is tested by the model of ear-edema caused by topical application of croton oil according to Manga et *al*. [21]. In order to induce skin inflammation, five groups of mice with a mean weight of 24.815 ± 1.66 (g) were given 15 µL of acetone: water solution (1:1) containing 80 µg of croton oil as an irritant on the internal surface of the right ear. On the left ear the same volume was applied without the croton oil. The mice were treated orally by the extracts at the different concentrations, after one hour of application of the croton oil. The positive control group received 50 mg/kg indomethacin and the negative control group received distilled water. The ear's thickness was evaluated with a digital caliper after 6 hours of edema provocation [22]. The mice are randomized to six groups each consisting of six mice.

Negative group: Receives distilled water.

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

Groups A (A1, A2): received 200 mg/kg and 400 mg/kg of HE extract of *E. nebrodensis*, respectively.
Groups B (B1, B2): received 200 mg/kg and 400 mg/kg of HM extract from *E. nebrodensis*, respectively.
The percentage of edema inhibition is defined in relation to the control group (which receives the croton oil solution) according to the following formula:
Inhibition % = ($D_{Control}$ - $D_{Treated}$ / $D_{Control}$) x 100
Where $D_{Control}$: Difference in thickness for the control group. $D_{Treatment}$: difference in thickness for the treated group.
Analgesic activity in vivo
Acetic acid induced writhing test
The analgesic activity against acetic acid induced pain is evaluated by the approach described by Koster et *al*. [23]. A group was used as a control and was given distilled water orally and the other groups were given a single dose of (200 and 400 mg/kg) administered orally of extracts (MH) and (HE), or 100 mg/kg of aspirin as a positive control. A volume of 10 ml/kg of 0.6 % acetic acid then was injected intra-peritoneally. After a 5 minute latency period, the number of twists for each mouse was counted every 5 minutes for 30 minutes after injection of the acetic acid. The percentage of pain inhibition is determined with the following equation:
Inhibition % = 100 x (C_{nc}–C_{tr})/C_{nc}
Where: C_{nc} = average of twitching in group in negative lot, C_{tr} = average of twitching in groups given various doses of HM and HE extracts and aspirin.
Statistical analysis
The results of the *in vitro* test were expressed as mean ± standard deviation SD and the results of the *in vivo* experiments are given as mean ± standard error of mean (SEM). Results were evaluated by One Way ANOVA and Dunnet test and *P* value 0.05 is regarded as significant by graph pad prism (Version 5.01).

RESULTS

Antioxidant capacity

The capacity of the extracts to capture the superoxide anion radical was examined in our study. Based on results shown in Table 1, the better scavenging ability (IC_{50}) was registered for HE extract (1.84 ± 0.46 µg/ mL) which is more important ($p < 0.001$) than that of ascorbic acid (7.59 ± 1.16 µg/mL) and α-Tocopherol (31.52 ± 2.22 µg/mL). HM extract showed comparable effect (7.81 ± 0.28µg/mL) as ascorbic acid.

Tableau1. Superoxide radical scavenging and metal chelating activities of *E. nebrodensis.*

Extracts/ standard	**$O^{\cdot -}$ 2 DMSO alkaline**		**Fe^{2+} ion chelating**	
	Inhibition % at 200 µg/mL	IC_{50} (µg / mL)[a]	Inhibition % at 200 µg/mL	IC_{50} (µg / mL)[a]
HM	94.86 ± 0.10[c]	7.81 ± 0.28[c]	54.55 ± 0.84[c]	174.60 ± 4.28[c]
HE	94.17 ± 0.01[c]	1.84 ± 0.46[c]	55.73 ± 0.63[c]	168.12 ± 1.13[c]
EDTA[B]	-	-	95.87 ± 0.06	8.80 ± 0.47
Ascorbic acid[B]	94.28 ± 1.12	7.59 ± 1.16	-	-
α-Tocopherol[B]	96.54 ± 0.10	31.52 ± 2.22	-	-

[a] IC_{50} values correspond to means ± SD of three simultaneous measures ([c] $p \leq 0.001$); [B] standards compounds. HE: hydro-ethanolic extract; HM: hydro-methanolic extract.

All samples showed a moderate chelation capacity of Fe^{2+} ions (Table 1). The HE extract was more active chelator and the descending order of IC_{50} was: HE extract (168.12 ± 1.13 µg/mL) > HM extract (174.60 ± 4.28 µg/mL). Neither extract appeared to be more powerful Fe^{2+} ion chelator than the EDTA positive standard (8.80 ± 0.47 µg/ mL) in this test system.

Anti-inflammatory activity in vitro

The anti-inflammatory *in vitro* effect of *E. nebrodensis* samples have been evaluated by denaturation of BSA (Bovine serum albumin) and the results are presented in (Figure 1). The results showed that the HE extract has the ability to stop denaturation of protein induced in a proportionally dependent dose, it gives a high inhibition level 82.99 % (20 mg/mL), followed by the HM extract with an inhibition percentage of 56.25 %. A dose of 5 mg/mL of diclofenac has an anti-inflammatory action with an inhibition of 99.82 %.

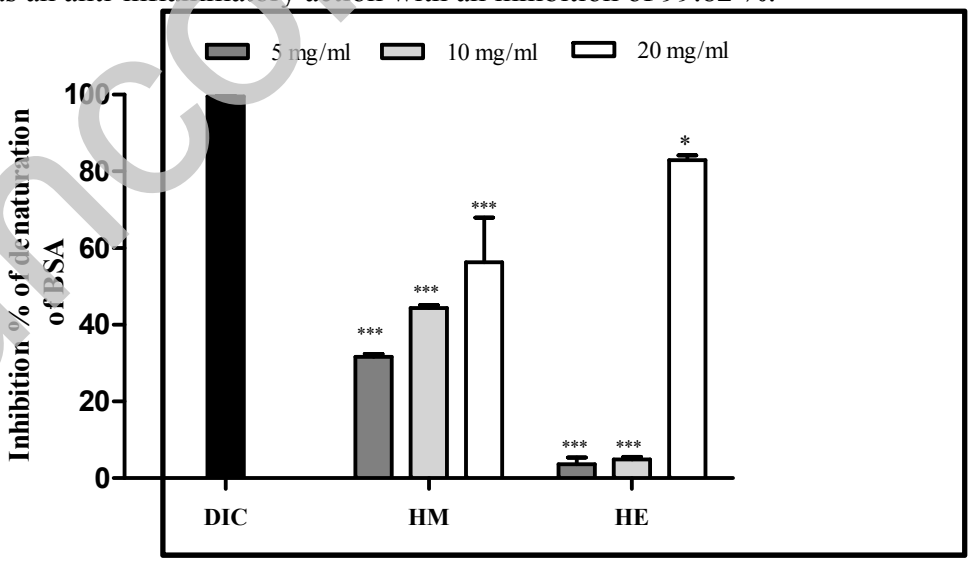

Figure 1. The *in vitro* anti-inflammatory effect of hydroalcoholic extracts of *E. nebrodensis.* Data are presented as the mean ± SD (n=3) (* $P < 0.05$ compared to diclofenac group, *** $P < 0.001$ compared to diclofenac group).
HE: hydro-ethanolic extract (70%), HM: hydro-methanolic extract (85%), DIC: diclofenac group 5g/kg, SD: Standard deviation.

Effect of extracts on ear edema induced by croton oil

The anti-inflammatory action of HE and HM extracts of *E. nebrodensis* caused by the applying of croton oil on the ear is presented by (Figure 2). This study shows that the edema was inhibited in a dose-dependent manner; the highest dose of extracts gives the significant activity. The HM and HE extracts reduced ear edema with the highest inhibition percentage of (72.22 % and 70.37 %, respectively) at 400 mg/kg. This effect was statistically similar to that induced by indomethacin 78.49 % as shown in (Figure 2).

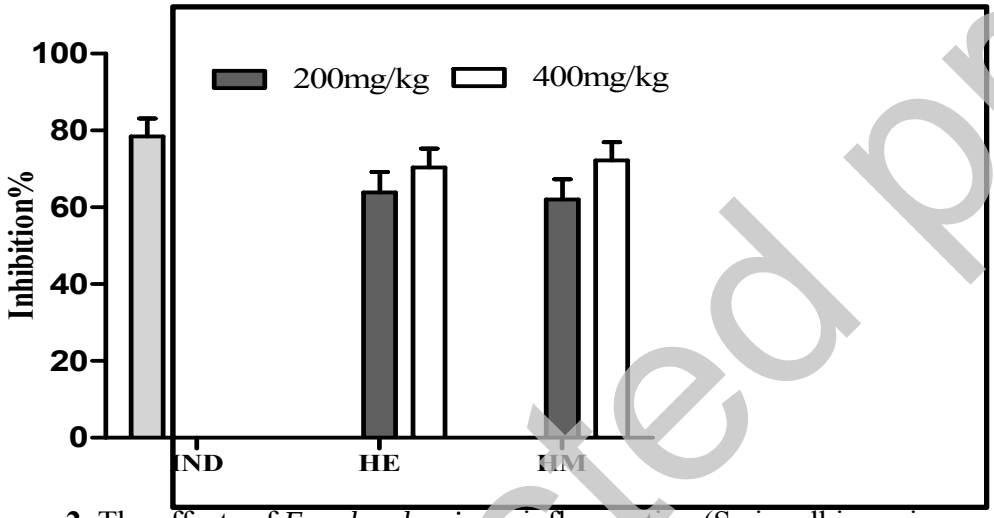

Figure 2. The effects of *E. nebrodensis* on inflammation (Swiss albino mice; n=6; W: 24.815 ± 1.66 g). The inflammation was induced by croton oil (15 µL, 80 µg in acetone: water V: V) on the internal surface of the right ear. On left ear the same volume was applied without the croton oil. After one hour of application, the mice were administered orally by the extracts. The ear's thickness was measured after 6 hours. The results are expressed as the mean ± SEM, ($P > 0.05$ compared to 50 mg/kg indomethacin).
HE: hydro-ethanolic extract (70%), HM: hydro-methanolic extract (85%), IND: indomethacin (50 mg/kg), SEM: Standard error mean.

Analgesic effect of extracts induced by acetic acid

The results presented in Figure 3 showed that the administration of 200 and 400 mg/kg of *E. nebrodensis* extracts exerted a protective effect against the pain caused by acetic acid. The extracts show an important analgesic activity with an inhibition percentage of: 63.74 % and 59.06 % for the extracts HM and HE respectively at 200 mg/kg. Thus the potent inhibitory effect of abdominal contractions is reported with the HM and HE extracts at the higher dose (400 mg/kg). In the following order: 72.51 % > 70.76 %, respectively. These effects are similar to that of aspirin at 100 mg/kg (79.14 %). There was no significant difference between these extracts at different concentrations and the standard used (aspirin) as shown in the Figure 3.

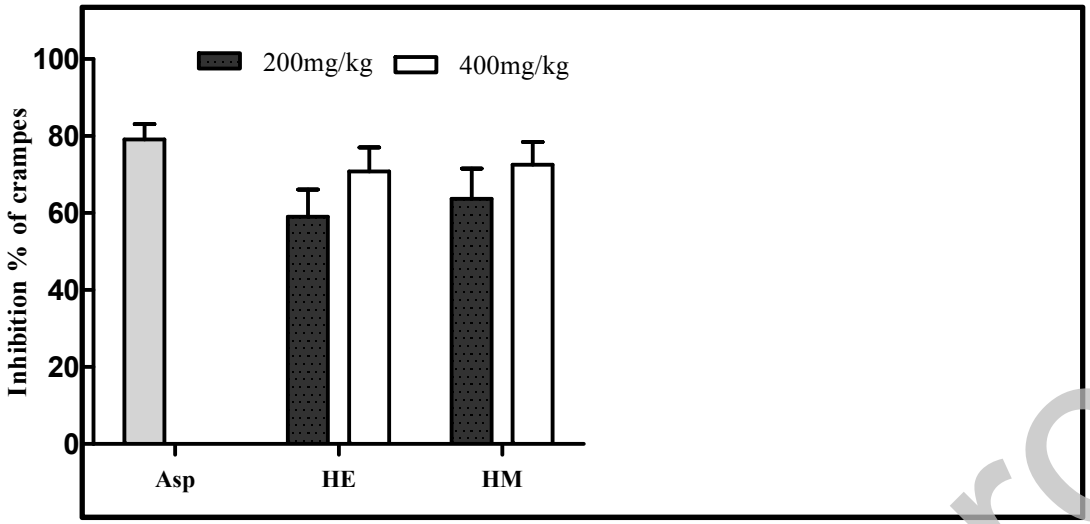

Figure 3. The effects of hydroalcoholic extracts of *E. nebrodensis* on peripheral nociception (Swiss albino mice, n=6; W: 27±5 g). The peripheral anti-nociceptive activity was determined by the acetic acid-induced writhing test. The results are expressed as the mean ± SEM. $p > 0.05$ compared to 100 mg/kg aspirin.
HE: hydro-ethanolic extract (70%), HM: hydro-methanolic extract (85%), Asp: Aspirin 100 mg/kg, SEM: Standard error mean.

DISCUSSION

It is usually recognized that herbal medicines represent potentially beneficial approaches for treatment of many types of human diseases. Numerous sources of supporting documents provide documentary records of the ethno-pharmacological use of herbs from ancient times by many populations [24]. Phytochemicals are omnipresent compounds in herbs, known to providing a wide range benefits to health like anticancer, antibacterial, anti-inflammatory, anti-diabetic and antioxidant effects [25]. In this study we report experimental data concerning antioxidant, anti-inflammatory (*in vitro* and *in vivo*) and analgesic effect of *E. nebrodensis* extracts in mice models.

In the *in vitro* antioxidant activity, the HE extract had a stronger antioxidant power using superoxide radical scavenging activity in comparison to HM extract. The study of Hamoudi et *al*. [11] signalized that ethyl acetate fraction (EF) from *E. nebrodensis* exhibit potent antioxidant using DPPH and ABTS assays.

The obtained results for superoxide radical scavenging activity may be related to the content of flavonoids and polyphenols, which are the major contribution to the antioxidant potential of the aerial part extracts of *E. nebrodensis*. In fact, the literature has demonstrated that a good correlation has been established for antioxidant effect and the content of polyphenols and flavonoids [11; 26; 27].

The presented results indicate that HE and HM extracts had a strong affinity to chelate Fe^{2+} utilizing ferrozine test. A previous research found that no relationship between phenolic level and ferrous ion chelating activity [28]. This suggests that the effect is caused by the existence of other antioxidants able to chelate metal ions, such as phosphoric acid, carnosine, acid citric, amino acids, protein and ascorbic acid [29].

Denaturation of proteins is a pathway where proteins shed their structures as a result of altered hydrogen, hydrophobic, electrostatic and disulfide bonds. The majority of proteins losing their biological activities following denaturation and cause generation of auto-antigens, leading to a series of autoimmune dysfunctions, such as inflammatory and rheumatoid Disorder. Thus, drugs which inhibit denaturation of protein are regarded as essential anti-inflammatory agents [30]. The finding of the anti-inflammatory (*in vitro*) effect showed that activity of the hydroalcoholic extracts to preserve the three dimensional profile of the proteins that control

production of auto-antigens. This is perhaps explained by the presence of the main phytochemicals present in Ephedra, such as flavonoids and phenols.
On the other hand, croton oil is known for its irritating properties and can cause inflammation, so it is used as an inflammatory agent [31]. This oil has the mechanism to activate phospholipase A2, which then secretes arachidonic acid from the cell membrane. This compound is then metabolized to prostaglandins and leukotrienes [32]. Thus, dermal exposure to croton oil can cause the production of ROS and a high inflammatory skin response resembling to that occurring irritation dermatitis [33; 34].
In the genus of Ephedra, It has been reported that analogues of ephedrine, which consists mainly of ephedrine, ephedrroxane and pseudoephedrine, have potent anti-inflammatory capacity *in vivo*. This anti-inflammatory activity was probably due to blockage of biosynthesis of prostaglandin E2 [35]. The results of Iksoo and his collaborators in ephedra root extracts found that (ephedranin A and ephedranin B) had anti-inflammatory effect. They had the potential to inhibit transcription of (IL-1β and TNF-α) than to block the inflammation induced by LPS (lipopolysaccharide). They inhibited the translocation of NF-κB and phosphorylation of the p38 kinase of the mitogen-activated protein [36].
The acute inflammation reaction is recognized by the formation of swelling and infiltration of leukocytes into the inflamed tissue. Firstly, the chemicals released by the resident cells promote the alteration of vascular permeability and consequently the formation of edema. In parallel, the sequential processes and actions between endothelium and inflammatory tissue cells (principally neutrophils) lead to the development of these inflammatory cells at the level of their tissue lesions [37; 38].
We only noted that the administration of *E. nebrodensis* extracts also indomethacin, used as a positive compound, inhibited formation of atrial edema. In addition, the anti-inflammatory capacity appears through many mechanisms of action, including inhibition of histamine release, 5-lipooxygenase, complement and elastase functions [39; 40]. Many studies have attributed the anti-inflammatory activity of phenolic constituents to their antioxidant activity [41; 42].
In acetic acid assay, whereas abdominal muscle pain is not a specific pattern and unintentional abdominal muscle pain may be due to its similarities to some of the known visceral pain patterns [43]. Activation of prostaglandins, histamines, serotonin, lipooxygenases, cyclooxygenases and endogenous cytokines (IL-8 and IL-1β), in peripheral tissue was activated by acetic acid injection into abdominal cavity of mice [44], which often penetrates the dorsal horn of the CNS (central nervous system) and stimulates primary nociceptors, resulting in enzyme pain and torsion disorder [45]. In our experimental observation, hydroalcoholic extracts significantly ($P < 0.001$) reduced the number of acetic acid-facilitated abdominal contractions or torsions in the dose-dependent effect. This finding clearly indicates that the anti-nociception produced by the extracts prevented the endogenous synthesis of the infamous media, or directly inhibited the receptors [46].
The peripheral analgesic capacity of different extracts studied would probably be due to the presence of phenolic compounds and alkaloids present in this plant. These compounds are known to have analgesic properties in other medicinal plants such as *Jasminum amplexicaule* and *Elephantopus tomentosus* [47; 48].

CONCLUSION

This study reports for the first the antioxidant and the inflammatory activities of hydroalcoholic extracts (HE and HM) of *E. nebrodensis.* These extracts exhibited *in vitro* antioxidant activity marked by superoxide radical scavenging and metal chelation methods. Also, the extracts have significant anti-inflammatory effect *in vitro*, with an inhibition percentage between 56% and 82%.

The plant extracts have shown an important anti-inflammatory effect *in vivo* as well as an interesting analgesic activity in mice. To better understand the mechanism by which *E. nebrodensis* reduces inflammation and pain, it is necessary to carry out a phytochemical characterization of the active compounds responsible for these biological activities.

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COMPLIANCE WITH ETHICAL STANDARDS

Experimental assays in mice were approved by the Committee of the 'Association Algerienne des Sciences en Experimentation Animale' (http://aasea.asso.dz/ articles/) under law No. 88-08/1988, associated with veterinary medical activities and animal health protection (N° JORA: 004/1988).

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