Turk J Pharm Sci 2021;18(5):574-580
DOI: 10.4274/tjps.galenos.2021.24571

ORIGINAL ARTICLE

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

Doğu Cezayir'den *Ephedra nebrodensis* Bitkisinin Alkol ile Hazırlanan Ekstrelerinin Antioksidan, Anti-inflamatuvar ve Analjezik Aktiviteleri

Meriem HAMOUDI[1]*, Djouher AMROUN[1], Abderrahmane BAGHIANI[2], Seddik KHENNOUF[1], Saliha DAHAMNA[1]

[1]Ferhat Abbas Setif 1 University Faculty of Natural and Life Sciences, Laboratory of Phytotherapy Applied to Chronic Diseases, Setif, Algeria
[2]Ferhat Abbas Setif 1 University Faculty of Natural and Life Sciences, Laboratory of Applied Biochemistry, Setif, Algeria

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

Amaç: *Ephedra nebrodensis* (Ephedraceae) bitkisinin çok çeşitli biyolojik aktivitesi bulunmaktadır. Geleneksel tıpta solunum problemlerini ve karaciğer patolojilerini tedavi etmek için kullanılır. Bu çalışmanın amacı, *in vitro* ve *in vivo* (farelerde) koşullarda *E. nebrodensis*'in iki hidro-alkollü ekstresinin antioksidan, anti-inflamatuvar ve analjezik özelliklerini değerlendirmektir.

Gereç ve Yöntemler: *E. nebrodensis*'in hidro-metanolik (HM) ve hidro-etanolik (HE) ekstrelerinin antioksidan kapasitesi, süperoksit radikal süpürme kapasiteleri ve demirli iyon şelatlama aktivitelerinin belirlenmesi ile değerlendirildi. Ekstrelerin (5, 10 ve 20 mg/kg) *in vitro* anti-inflamatuvar aktivitesi de sığır serum albümin denatürasyon testi kullanılarak belirlendi. Daha sonra ekstrelerin (200 ve 400 mg/kg) *in vivo* anti-inflamatuvar etkisini değerlendirmek için kroton yağı ile indüklenen kulak ödemi modeli kullanıldı. Son olarak ekstrelerin analjezik aktivitesi (200 ve 400 mg/kg), asetik asit ile indüklenen kıvranma testi ile belirlendi.

Bulgular: *E. nebrodensis*'in hidro-alkolik ekstreleri, önemli antioksidan aktiviteye sahiptir. HE ve HM, protein denatürasyonunu sırasıyla %82,99±%20,21 ve %56,25±%2,12 oranında inhibe edebilir. Ekstreler (HM ve HE) ayrıca *in vivo* olarak güçlü anti-inflamatuvar etkiler gösterir ve kulak ödemini

*Correspondence: meryoumamm2009@hotmail.fr, Phone: +213777150029, ORCID-ID: orcid.org/0000-0001-6659-3943
Received: 10.11.2020, Accepted: 03.02.2021

sırasıyla %70,37±%2,00 ve %72,22±%1,94 oranında azaltabilir. HM ekstresi (%72,51±%2,43) HE'den (%70,76±%2,58) daha yüksek analjezik aktiviteye sahiptir.

Sonuç: *E. nebrodensis*'in hidro-alkolik ekstreleri, antioksidan, anti-inflamatuvar ve analjezik etkiler üretir. Bu sonuçlar, bitkinin çeşitli hastalıkların tedavisinde geleneksel kullanımını doğrulamaktadır.

Anahtar kelimeler: *Ephedra nebrodensis*, anti-inflamatuvar aktivite, analjezik test, antioksidan kapasite, hidro-alkolik ekstreler

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.[1] 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 lanes in response to potential damage.[2] Pain stimulates the production of substances called pain mediators, such as histamine, bradykinin, leukotriene, and prostaglandin;[2] 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.[3]

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,[4] includes approximately 67 species and is principally found in desert zones across Asia, Europe, North Africa, and North America.[5] Over 145 organic molecules have been isolated from *Ephedra*, included alkaloids, polysaccharides, flavonoids, and tannins.[6] *Ephedra* is known to show anti-asthmatic,[7] anti-inflammatory,[8] antiproliferative,[9] hypoglycemic,[10] antioxidant,[11] and weight-reduction[12] properties.

Research on *Ephedra nebrodensis* is scarce. Sureka et al.[13] 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.[14]

The ethanol:acetone extract of *E. nebrodensis* Tineo exhibits antihistaminic, adaptogenic, and antinociceptive activities.[15] The data reported by Shah et al.[16] 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 Nafla, 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.[17]

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.[18] 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:

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

where A_{TS} is the absorbance of the sample and A_{Ct} 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.[19] Briefly, 40

µL of ethylenediamine tetraacetic acid (EDTA) or the samples was added to 40 µL of $FeCl_2$ (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 Fe^{2+}-ferrozine complex produced was measured at 562 nm, and chelating ability was calculated in terms of inhibition percentage according to the following equation:

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

where A_{TS} is the absorbance of the test sample and A_C is the absorbance of the control sample.

Determination of in vitro anti-inflammatory activity

In vitro anti-inflammatory capacity was evaluated according to the method of Karthik et al.[20] 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.[21]

The internal surface of the right ear of five groups of mice with a mean weight of 24.815±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.[22]

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:

Inhibition % = $(D_{Control} - D_{Treated} / D_{Control}) \times 100$

where $D_{Control}$ is the difference in edema thickness in the control group and $D_{Treatment}$ 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.[23] 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:

Inhibition % = $100 \times (C_{nc} - C_{tr}) / C_{nc}$

where C_{nc} is the average number of twitches in the negative control group and C_{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 (IC_{50}) of HE (1.84±0.46 µg/mL) was significantly greater ($p<0.001$) than those of ascorbic acid (7.59±1.16 µg/mL) and α-tocopherol (31.52±2.22 µg/mL). HM showed effects (7.81±0.28 µg/mL) comparable with those of ascorbic acid.

All samples showed moderate chelation ability for Fe^{2+} (Table 1). Compared with other samples, HE appeared to be a more active chelator, and the IC_{50} of HE (168.12±1.13 µg/mL) was greater than that of HM (174.60±4.28 µg/mL). Neither extract appeared to be a more powerful Fe^{2+} 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

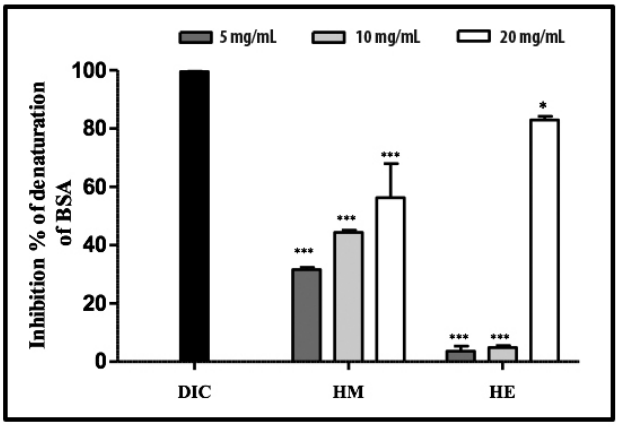

Figure 1. *In vitro* anti-inflammatory effect of hydro-alcoholic extracts of *Ephedra nebrodensis*. Data are presented as mean ± SD (n=3). *P<0.05 compared with the diclofenac group, ***P<0.001 compared with the diclofenac group.

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

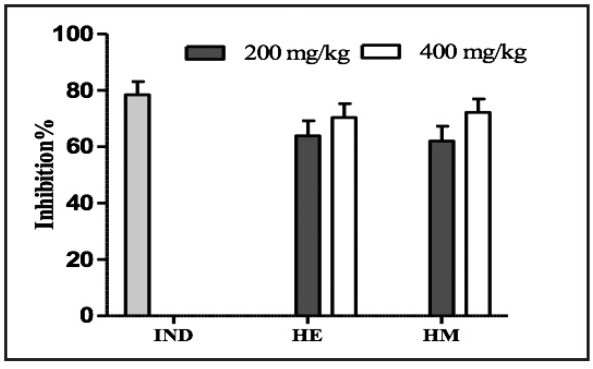

Figure 2. Effects of *Ephedra nebrodensis* on inflammation in Swiss albino mice (n=6; W: 24.815±1.66 g). Inflammation was induced by applying 15 µL of acetone:water solution (1:1, v:v) containing 80 µg of croton oil to the internal surface of the right ear. The same volume of solution without croton 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

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.[24] Phytochemicals are ubiquitous compounds found in herbs that provide a wide range of benefits, such as anticancer, antibacterial, anti-inflammatory, antidiabetic, and antioxidant activities.[25] 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.[11] 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 superoxide radical scavenging activity of our extracts may be related to their contents of flavonoids and polyphenols, which are major contributors to the antioxidant potential of the aerial parts of *E. nebrodensis*. Good correlations between the antioxidant effect and polyphenol and flavonoid contents of various plant extracts have been well established in the literature.[11,26,27]

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

Table 1. Superoxide radical scavenging and metal-chelating activities of *Ephedra nebrodensis*

Extracts/standard	O^{-2} DMSO alkaline		Fe^{2+} ion chelating ability	
	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 the mean ± standard deviation of three simultaneous measures, [b]Standard compounds, [c]p≤0.001. HE: Hydro-ethanolic extract, HM: Hydro-methanolic extract, DMSO: Dimethyl sulfoxide, EDTA: Ethylenediamine tetraacetic acid

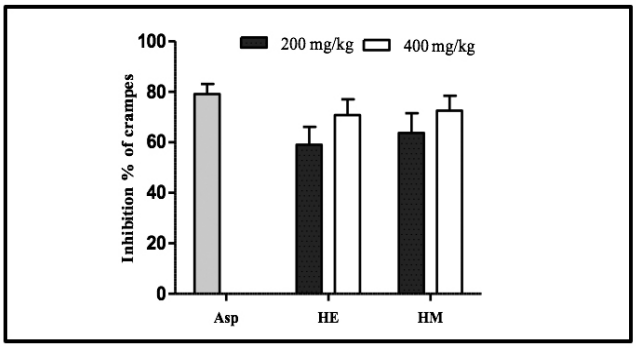

Figure 3. Effects of hydro-alcoholic extracts of *Ephedra nebrodensis* on peripheral nociception in Swiss albino mice (n=6; W: 27±5 g). Peripheral antinociceptive activity was determined by the acetic acid-induced writhing test. The results are expressed as mean ± SEM. P>0.05 compared with 100 mg/kg aspirin

HE: Hydro-ethanolic extract (70%), HM: Hydro-methanolic extract (85%), Asp: Aspirin 100 mg/kg, SEM: Standard error of the mean

relationship between phenolic level and Fe^{2+}-chelating activity.[28] This result suggests that the iron-chelating effect of the extracts may be attributed to the existence of other antioxidants that able to chelate metal ions, such as phosphoric acid, carnosine, acid citric, amino acids, protein, and ascorbic acid.[29]

Protein denaturation refers to changes in the structure of proteins as a result of altered hydrogen, hydrophobic, electrostatic, and disulfide bonds. The majority of proteins loses their biological activities following denaturation and generates auto-antigens, leading to a series of autoimmune dysfunctions, such as inflammatory and rheumatoid disorders. Thus, drugs that could inhibit the denaturation of proteins are regarded as essential anti-inflammatory agents.[30] The *in vitro* anti-inflammatory effect of the hydro-alcoholic extracts observed in this work indicates that the extracts could preserve the three-dimensional profile of proteins that control the production of auto-antigens. This finding may be explained by the presence of phytochemicals, such as flavonoids and phenols, in *Ephedra*.

Croton oil is often used as an inflammatory agent.[31] The oil activates phospholipase A2, which induces the secretion of arachidonic acid from the cell membrane. This compound is then metabolized to prostaglandins and leukotrienes.[32] Dermal exposure to croton oil can cause reactive oxygen species production and an inflammatory skin response resembling that occurring during contact dermatitis.[33,34]

Analogues of ephedrine, which consist mainly of ephedrine, ephedrroxane, and pseudoephedrine, have potent *in vivo* anti-inflammatory capacity, which has been attributed to the blockage of prostaglandin E2 biosynthesis.[35] Kim et al.[36] found that ephedranin A and ephedranin B in *Ephedra* root extracts have anti-inflammatory effects. These substances can impede the transcription of IL-1β and TNF-α and block the inflammation induced by LPS. These substances could also inhibit the translocation of NF-κB and phosphorylation of p38 kinase in mitogen-activated protein.

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.[37,38]

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-lipooxygenase, complement and elastase functions.[39,40] 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, lipooxygenases, 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.

ACKNOWLEDGMENTS

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (MESRS, DGRSDT). We

would like to thank Pr. Laouer Hocine (Laboratory of Natural Resources Valorization, Department of Biology and Vegetal Ecology, University of Ferhat Abbas Setif 1, El Bez, 19000, Algeria) for identifying the plant materials used in this study.

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

1. Somsil P, Ruangrungsi N, Limpanasitikul W, Itthipanichpong C. *In vivo* and *in vitro* anti-infammatory activity of Harrisonia perforata root extract. Pharmacogn J. 2012;4:38-44.
2. Ghosh, AK, Banerjee M, Mandal TK, Mishra A, Bhowmik M.K. A study on analgesic efficacy and adverse effects of aloe vera in Wistar rats. Pharmacologyonline. 2011;1:1098-1108.
3. Nalamachu S. An overview of pain management: the clinical efficacy and value of treatment. Am J Manag Care. 2013;19(Suppl 14):s261-s266.
4. D'Auria M, Emanuele L, Racioppi R. FT-ICR-MS analysis of lignin. Nat Prod Res. 2012;26:1368-1374.
5. Xie M, Yang Y, Wang B, Wang C. Interdisciplinary investigation on ancient *Ephedra* twigs from Gumugou Cemetery (3800 B.P.) in Xinjiang region, northwest China. Microsc Res Tech. 2013;76:663-672.
6. Zhang BM, Wang ZB, Xin P, Wang QH, Bu H, Kuang HX. Phytochemistry and pharmacology of genus *Ephedra*. Chin J Nat Med. 2018;16:811-828.
7. ILiu YG, Luo JB. [Effects of among compositions of Herba Ephedrae decoction on genic xpression of 5-lipoxygenase activating protein, IL-4 and leukotriene C4 in asthmatic mice]. Zhongguo Zhong Yao Za Zhi. 2007;32:246-249.
8. Aoki K, Yamakuni T, Yoshida M, Ohizumi Y. Ephedorae herba decreases lipopolysaccharide-induced cyclooxgenase-2 protein expression and NF-kappaB-dependent transcription in C6 rat glioma cells. J Pharmacol Sci. 2005;98:327-330.
9. Danciu C, Muntean D, Alexa E, Farcas C, Oprean C, Zupko I, Bor A, Minda D, Proks M, Buda V, Hancianu M, Cioanca O, Soica C, Popescu S, Dehelean CA. Phytochemical characterization and evaluation of the antimicrobial, antiproliferative and pro-apoptotic potential of ephedra alata decne. Hydroalcoholic extract against the MCF-7 breast cancer cell line. Molecules. 2018;24:13.
10. Ben Lamine J, Boujbiha MA, Dahane S, Cherifa AB, Khlifi A, Chahdoura H, Yakoubi MT, Ferchichi S, El Ayeb N, Achour L. α-Amylase and α-glucosidase inhibitor effects and pancreatic response to diabetes mellitus on Wistar rats of *Ephedra* alata areal part decoction with immunohistochemical analyses. Environ Sci Pollut Res Int. 2019;26:9739-9754.
11. Hamoudi M, Amroun D, Khennouf S, Dahamna S. Antioxidant evaluation and polyphenol contents of hydro ethanolic extract's fractions from *Ephedra nebrodensis*. Journal of Drug Delivery and Therapeutics. 2020;10:314-319.
12. Lim J, Lee H, Ahn J, Kim J, Jang J, Park Y, Jeong B, Yang H, Shin SS, Yoon M. The polyherbal drug GGEx18 from Laminaria japonica, Rheum palmatum, and *Ephedra sinica* inhibits hepatic steatosis and fibroinflammtion in high-fat diet-induced obese mice. J Ethnopharmacol. 2018;225:31-41.
13. Sureka M, Sumathi R, Kanagavalli U. A Comprehensive Review on Cardiotoxic Drugs and Cardioprotective Medicinal Plants. IJPRR. 2016;5:21-34.
14. Hassanzadeh M, Dianat M, Torabizadeh P, Badavi M. Protective effect of hydroalcoholic extract of *ephedra* major on an experimental model of bile duct ligation in rats. Int. J. LifeSc. Bt & Pharm Res. 2014;3:44-50.
15. Ballero M, Foddis C, Sanna C, Scartezzini P, Poli F, Petitto V, Serafini M, Stanzione A, Bianco A, Serilli AM, Spina L, Longoni R, Kasture S. Pharmacological activities on *Ephedra nebrodensis* Tineo. Nat Prod Res. 2010;24:1115-1124.
16. Shah S, Mohan MM, Kasture S, Sanna C, Maxia A. Protective Effect of *Ephedra nebrodensis* on Doxorubicin-Induced Cardiotoxicity in Rats. Iranian Journal of Pharmacology & Therapeutics. 2009;8:61-66.
17. Annapandian VM, Rajagopal SS. Phytochemical evaluation and *in vitro* antioxidant activity of various solvent extracts of *Leucas aspera* (willd.) link leaves. Free Radic Antioxid. 2017;7:166-171.
18. Kunchandy E, Rao MNA. Oxygen radical scavenging activity of curcumin. Int J Pharm. 1990;58:237-240.
19. Decker EA, Welch B. Role of ferritin as a lipid oxidation catalyst in muscle food. J Agric Food Chem. 1990;38:674-677.
20. Karthik K, Bharath R, Kumar P, Priya VR, Kumar SK, Rathore RSB. Evaluation of anti-inflammatory activity of *canthium parviflorum* by *in-vitro* method. IJRPB. 2013;1:729-731.
21. Manga HM, Brkic D, Marie DE, Quetin-Leclercq J. *In vivo* anti-inflammatory activity of *Alchornea cordifolia* (Schumach. & Thonn.) Müll. Arg. (Euphorbiaceae). J Ethnopharmacol. 2004;92:209-214.
22. Delaporte RH, Sarragiotto MH, Takemura OS, Sánchez GM, Filho BP, Nakamura CV. Evaluation of the antioedematogenic, free radical scavenging and antimicrobial activities of aerial parts of Tillandsiastreptocarpa Baker-Bromeliaceae. J Ethnopharmacol. 2004;95:229-233.
23. Koster R, Anderson M, De Beer EJ. Acetic acid for analgesic screening. Federal Proceeding. 1959;8:412-416.
24. Zengin G, Mahomoodally F, Picot-Allain C, Diuzheva A, Jekő J, Cziáky Z, Cvetanović, A Aktumsek, A, Zeković Z, Rengasamy KRR. Metabolomic profile of *Salvia viridis* L. root extracts using HPLC-MS/MS technique and their pharmacological properties: a comparative study. Ind Crops Prod. 2019;131:266-280.
25. Guldiken B, Ozkan G, Catalkaya G, Ceylan FD, Ekin Yalcinkaya I, Capanoglu E. Phytochemicals of herbs and spices: Health versus toxicological effects. Food Chem Toxicol. 2018;119:37-49.
26. Bouaziz A, Djidel S, Bentaher A, Khennouf S. Polyphenolic content, antioxidant and anti-inflammatory activities of melon (cucumis melo L. var. inodorus) seeds. J Drug Deliv Ther. 2020;10:22-26.
27. Mamache W, Amira S, Ben Souici C, Laouer H, Benchikh F. *In vitro* antioxidant, anticholinesterases, anti-α-amylase, and anti-α-glucosidase effects of Algerian *Salvia aegyptiaca* and *Salvia verbenaca*. J Food Biochem. 2020;00:e13472.
28. Belkhiri F, Baghiani A, Zerroug MM, Arrar L. Investigation of antihemolytic, xanthine oxidase inhibition, antioxidant and antimicrobial properties of *Salvia verbenaca* L. aerial part extracts. Afr J Tradit Complement Altern Med. 2017;14:273-281.
29. Lee J, Renita M, Fioritto RJ, St Martin SK, Schwartz SJ, Vodovotz Y. Isoflavone characterization and antioxidant activity of ohio soybeans. J Agric Food Chem. 2004;52:2647-2651.

30. Mouffouk C, Hambaba L, Haba H, Mouffouk S, Bensouici C, Hachemi M, Khadraoui H. Acute toxicity and *in vivo* anti-inflammatory effects and *in vitro* antioxidant and anti-arthritic potential of *Scabiosa Stellata*. Orient Pharm Exp Med. 2018;18:335-348.

31. Lan M, Wan P, Wang ZY, Huang XL. [GC-MS analysis of chemical components in seeds oil from Croton tiglium]. Zhong Yao Cai. 2012;35:1105-1108.

32. Shah B, Seth A, dan Maheshwari K. A review on medicinal plants as a resource of antiinflammatory agents. Res J Med Plant. 2011;5:101-115.

33. Pinto NDCC, Machado DC, da Silva JM, Conegundes JLM, Gualberto ACM, Gameiro J, Chedier LM, Castañon MCMN, Scio E. Pereskia aculeata Miller leaves present *in vivo* topical anti-inflammatory activity in models of acute and chronic dermatitis. Journal of Ethnopharmacology. 2015;173:330-337.

34. Siddiqui F, Naqvi S, Abidi L, Faizi S, Lubna, Avesi L, Mirza T, Farooq AD. Opuntia dillenii cladode: opuntiol and opuntioside attenuated cytokines and eicosanoids mediated inflammation. J Ethnopharmacol. 2016;182:221-234.

35. Kasahara Y, Hikino H, Tsurufuji S, Watanabe M, Ohuchi K. Antiinflammatory actions of ephedrines in acute inflammations. Planta Med. 1985;325-331.

36. Kim IS, Park YJ, Yoon SJ, Lee HB. Ephedrannin A and B from roots of *Ephedra sinica* inhibit lipopolysaccharide-induced inflammatory mediators by suppressing nuclear factor-κB activation in RAW 264.7 macrophages. Int Immunopharmacol. 2010;10:1616-1625.

37. Tamura EK, Jimenez RS, Waismam K, Gobbo-Neto L, Lopes NP, Malpezzi-Marinho EA, Marinho EA, Farsky SH. Inhibitory effects of Solidago chilensis Meyen hydroalcoholic extract on acute inflammation. J Ethnopharmacol. 2009;122:478-485.

38. Vestweber D. How leukocytes cross the vascular endothelium. Nat Rev Immunol. 2015;15:692-704.

39. Díaz AM, Abad MJ, Fernández L, Recuero C, Villaescusa L, Silván AM, Bermejo P. *In vitro* anti-inflammatory activity of iridoids and triterpenoid compounds isolated from *Phillyrea latifolia* L. Biol Pharm Bull. 2000;23:1307-1313.

40. Ryu SY, Oak MH, Yoon SK, Cho DI, Yoo GS, Kim TS, Kim KM. Anti-allergic and anti-inflammatory triterpenes from the herb of *Prunella vulgaris*. Planta Med. 2000;66:358-360.

41. Middleton E Jr, Kandaswami C. Effects of flavonoids on immune and inflammatory cell functions. Biochem Pharmacol. 1992;43:1167-1179.

42. Kassim M, Achoui M, Mansor M, Yusoff KM. The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin E(2) in inflammatory tissues. Fitoterapia. 2010;81:1196-1201.

43. Hajhashemi V, Sajjadi SE, Heshmati M. Anti-inflammatory and analgesic properties of Heracleum persicum essential oil and hydroalcoholic extract in animal models. J Ethnopharmacol. 2009;124:475-480.

44. Lu TC, Ko YZ, Huang HW, Hung YC, Lin YC, Peng WH. Analgesic and anti-inflammatory activities of aqueous extract from Glycine tomentella root in mice. J Ethnopharmacol. 2007;113:142-148.

45. Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. Pharmacol Rev. 2001;53:597-652.

46. Franzotti EM, Santos CV, Rodrigues HM, Mourão RH, Andrade MR, Antoniolli AR. Anti-inflammatory, analgesic activity and acute toxicity of *Sida cordifolia* L. (Malva-branca). J Ethnopharmacol. 2000;72:273-277.

47. Jia Q, Su W, Peng W, Li P, Wang Y. Anti-diarrhoea and analgesic activities of the methanol extract and its fractions of *Jasminum amplexicaule* Buch.-Ham. (Oleaceae). J Ethnopharmacol. 2008;119:299-304.

48. Yam MF, Ang LF, Ameer OZ, Salman IM, Aziz HA, Asmawi MZ. Anti-inflammatory and analgesic effects of Elephantopus tomentosus ethanolic extract. J Acupunct Meridian Stud. 2009;2:280-287.