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EVALUATION OF THE ANTINOCICEPTIVE EFFECTS OF THE ESSENTIAL OIL FROM AERIAL PARTS OF *ANASTATICA HIEROCHUNTICA* IN EXPERIMENTAL MODELS

Esam Qnais*[1,3], Nabeel Modallal[1], Yousra Bseiso[1], Mohammad Wedyan[1] and Hakam Alkhateeb[2]

[1]*Department of Biology and Biotechnology, Faculty of Science, Hashemite University, Zarka*

[2]*Faculty of Medicine, Yarmouk University, Irbid*

[3]*Department of Medical Laboratories, Faculty of Health Sciences, American University of Madaba, Madaba, Jordan*

Esamqn@hu.edu.jo

Abstract

The aerial parts of the plant *Anastatica hierochuntica* are widely used in Jordan as well as several other countries in Middle East in treating painful complaints, gastrointestinal disorders, and diabetes. The antinociceptive potential of essential oil extracted from the aerial parts of *Anastatica hierochuntica* (EOAH) was examined using various experimental models. In our study EOAH was extracted using steam distillation method and was determined using gas chromatography coupled with mass spectrometry (GC-MS) analysis. Thirteen compounds were successfully identified from EOAH representing 80.3% of total oil. Our study showed that Eugenol (21.2%), 1.8- cineole (14.9%), α- phellandrene (11.5%), and β- caryophyllene (8.2%) were the major constituents of the oil. The median lethal dose (LD_{50}) of EOAH was estimated using the method of Lorke [24]. EOAH antinociceptive effect in rats was determined using chemical (acetic acid and formalin) as well as thermal (hot-plate) nociceptive tests at doses 10 , 31.6, 100, 316 and 1000 mg/kg (orally). At these test doses, EOAH significantly reduced the pain response in dose dependent manner ($p<0.05$) in all conducted experiments. The possible mechanism of antinociceptive action of EOAH was also examined. The partial blockage of the EOAH antinociceptive action by naloxone, suggests that its mode of action involved the participation of an opioid mechanism. These findings justify in part the traditional use of *Anastatica hierochuntica* in the treatment of various painful conditions.

Keywords: Antinociceptive, Opioid receptors, Essential oil, *Anastatica hierochuntica*, Formalin test, Gas chromatography.

Introduction

The discovery and development of new drugs from natural sources is a fundamental concern of ethnopharmacological researches. Large number of plant natural products are used in traditional folk medicine as well as in modern medicine [1]. For this reason, the preservation and clinical investigation of medicinal plant species are strongly required [2].

Essential oils are complex molecules composed mainly of monoterpenes and occur naturally in plants. They have been used in several industries around the world. Cosmetics have been one of the industrial uses of plant essential oils including manufacturing perfumes and beauty creams due to their pleasant scents. Beside industry, plant essential oils have been used in medicinal purposes such as treating inflammation and pain, as well as treatment of several human diseases [18].

In our study we used a plant commonly known as "Kaff Maryam" or "Rose of Jericho". Its scientific name is *Anastatica hierochuntica* L. (Brassicaceae) [3, 4]. It is a small gray plant, grows to a maximum height of 15 cm and produces small white flowers. It inhabits arid environments in the Middle East and parts of North Africa [5-7]. It curls inward under dry conditions and emerges from this dormant state when water is available.

When *A. hierochuntica* is used in traditional folk medicine, it is either consumed as tea beverage or it is powdered and mixed with honey. The plant is prescribed for treatment of arthritis, inflammation, pain, asthma, gastrointestinal disorders, headache, diabetes, heart diseases, management of female reproductive disorders and difficult labor [6-8].

Luteolin, quercetin, apigenin, isovitexin, kaempferol, eriodictyol, anastatin, glucosinolates, glucoiberin, glucocheirolin, terpenes, caffeoyl acids, dicaffeoylquinic acids, dihydroxybenzoic acid, silybins, isosilybins and hierochins are some examples on compounds have been isolated from *A. hierochuntica* [7 , 9-10]. Several phenolic compounds have also been identified from this plant species [11-12].

Several reports clearly showed that different kind of extracts from *A. hierochuntica* exhibited antibacterial activity [11,13], antioxidant activity [9-10, 13], hepato-protective activity [9, 14] , hypoglycemic activity [15] and anticancer activity [4, 16, 17].

Recent bibliographical survey showed that few phytochemical investigations have so far been conducted on *Anastatica hierochuntica*. Furthermore, the pharmacological potential of its essential oils still completely unrevealed.

Our work intended to investigate the chemical composition of essential oil extracted from *A. hierochuntica* (EOAH) and to examine its antinociceptive activity as well as to explore the possible antinociceptive mechanisms that lie behind the action of EOAH. Taking in concern the popular use of the plant in folk medicine as well as the shortage of pharmacological studies in literature on the antinociceptive activities of EOAH.

Materials and Methods

Plant material

Aerial parts of *Anastatica hierochuntica* were collected from Maan, Jordan, in April of 2015. The plant material was authenticated by botanist Yaser Ali, Associate Professor at Department of Biology. A voucher specimen was deposited at the Hashemite University herbarium, Zarka, Jordan, for future reference.

Determination of essential oil composition

The essential oil from the dried aerial parts of *Anastatica hierochuntica* was obtained by hydrodistillation for 8 h using a Clevenger-type apparatus according to Siani *et al*. [19]. The essential oil analysis of *Anastatica hierochuntica* was conducted by GC on a Trace GC ULTRA with FID detector gas chromatograph equipped with a column (30 m x 0.25 mm x 0.25 µm) type VB-5 (methylpolysiloxane with 5 % of phenyl) and split injection. Mass spectrometry (MS) analysis was performed on a Polaris Q MS mass spectrometer (with an ion-trap at 70 eV). Column temperatures were programmed from 40 °C for 2 min, raised to 180 °C at 4 °C.min^{-1}. The carrier gas was helium, with a constant flow rate of 1.4 mL/min. The identification of volatile constituents of the essential oil was made by automated comparison of their mass spectra with that of the NIST (National Institute of Standards and Technology) library [20].

Experimental animals

Non-fasting male Wistar rats weighing between 150 - 250 g were used in our experiments. Swiss albino mice weighing between 25 - 35 g were used for acute toxicity experiment. The animals were obtained from the Animal House, Department of Biology, Faculty of Sciences, Hashemite University. The animals were housed and kept in an Animal House provided with a 12 h light/dark cycle at 22 - 25 °C and free access to food and water *ad libitum*. All test solutions were administered in a volume of 10 ml/kg body weight. The animals were allowed to adapt to the laboratory for at least 2h before experimentation and were used only once. Animal care and handling

procedure were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research [21]. Experimental protocols and procedures were previously approved by Research Ethics Committee of Hashemite University.

Drugs and chemicals

All chemicals and drugs were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise stated. The chemicals and drugs were of analytical grade and dissolved in sterile saline. EOAH was prepared in 1 % v/v Tween 80 in sterile saline.

Acute toxicity test

Lethal dose (LD_{50}) of EOAH was estimated in mice according to the protocol described as by Lorke [24]. Animals were randomly assigned to different groups containing 5 mice in each group. EOAH (10, 100, 1000, 2000 and 3000 mg/kg) was administered orally to six groups of mice. The control group received 1 % v/v Tween 80 in sterile saline, (10 ml/kg). The mice were allowed food and water *ad libitum.* Signs of toxicity and mortality were recorded within 72 h.

Acetic acid-induced abdominal writhing test

This test was performed in rats according to the protocol described by Matheus *et al*. [23]. Briefly, after an intraperitoneal administration of a 2 % (v/v) acetic acid solution in a volume of 0.1 ml/10 g body weight. The nociceptive behavior was quantified by counting the number of writhes, a response consisting of contraction of an abdominal wall, pelvic rotation followed by hind limb extension, during continuous observation for 20 min beginning from 5 min after the acetic acid injection. Rats were orally pretreated 60 min before the administration of the nociceptive agent with EOAH (10 , 31.6, 100, 316 and 1000 mg/kg), or vehicle (1 % Tween 80 in normal saline). A positive control group was composed of animals pretreated with morphine (5 mg/kg, i.p.).

The percentage inhibitions of writhing was computed using Eq 2.

Percentage inhibitions of writhing = (N – Nt/ N) ×100 …… (2)

where *N* is the average number of stretching of control group and *N*t is the average number of stretching of test group.

Hot-plate test

The hot-plate test at 50 ± 1 °C was used as previously described by Qnais *et al* [22]. Latency to a discomfort reaction (licking paws) was determined in seconds before and 60 min after oral administration of vehicle (1 % v/v Tween 80 in sterile saline), EOAH (10, 31.6, 100, 316 and 1000 mg/kg) to 6 groups of male rats, (six animals per group). The 7^{th} group received morphine intraperitoneally (5 mg/kg; positive control). The largest doses were determined on the basis of LD_{50} experiments. The doses were calculated to be located at approximately 0.5 log units from each other on a log scale. The cut-off time was 60 s. The prolongation of the latency times was compared to the values of the control and used for statistical comparison. Baseline was considered as the mean of three readings of the reaction time obtained before administration of vehicle, EOAH or morphine and was defined as the normal reaction time of animals to this temperature. The increase over baseline (%) was calculated using Eq 1.

The increase over baseline (%) = (A-B/B) × 100…… (1)

where A is the mean of three readings of reaction time after treatment taken within 5 - 7 min; B is the mean of three readings of reaction time obtained before treatment. In this, and the following experiments, the concentration causing 50% of maximum effect (ED_{50}) was determined from the plot of individual experiments by the best visual fit.

Formalin test

Rats were divided into groups (six rats each) and were injected orally with either vehicle (1 % v/v Tween 80 in sterile saline) or EOAH (10, 31.6, 100 , 316 and 1000 mg/kg). A positive control group was composed of animals pre-treated with morphine (5 mg/kg, i.p.). Sixty min later, 50 µL of 5 % formalin was injected subcutaneously into the dorsal surface of the right hind paw of each rat using a microsyringe with a 27 gauge needle. Immediately after formalin injection, animals were placed individually in acrylic observation chambers (320 cm^2 × 40 cm). Mirrors were arranged at angles to allow clear observation of the paws of the animals. Licking of the injected paw was defined as the nociceptive response. The total time of the response was measured during the periods of 0 - 5 min (early phase) and 15 - 40 min (late phase). Inhibition of licking (%) was calculated using Eq 3.

The inhibition of licking (%) = (A-B/A) ×100 ……(3) where A is the time of licking before treatment; B is the time of licking after treatment.

Determination of possible mechanisms of antinociceptive action

To assess the possible involvement of opioid system in the *antinociceptive effect* of EOAH, the animals were pre-treated with antagonist naloxone (10 mg/kg). Naloxone

was administered i.p. 15 min before oral administration of vehicle (1 % v/v Tween 80 in sterile saline) or the ED_{50} of EOAH. Using the acetic acid-induced abdominal writhing test, hot-plate test, and the formalin test as described above, inhibition of the writhing response, latency times and licking was calculated after 60 min of vehicle (1 % v/v Tween 80 in sterile saline) or EOAH administration.

Statistical analysis

The data obtained are expressed as mean ± SEM, and were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test using GraphPad Prism 5.0 with significance set at $P < 0.05$.

Results

Essential oil

The chemical analysis of EOAH sample used in the study led to the identification of 13 compounds, representing 80.3% of total oil (Table 1). Table 1 show that Eugenol (21.2%), 1.8-cineole (14.9%), α-phellandrene (11.5%) and β-caryophyllene (8.2%) were the major constituents of EOAH.

Acute toxicity

It was not possible to obtain the LD_{50} of EOAH, because administering mice with increasing doses of EOAH up to 3000 mg/kg (orally), showed no lethal effects. The 3000 mg/kg dose of EOAH was not lethal indicated the low toxicity profile of EOAH. Diarrhea, motor impairment, ataxia, hyperactivity, alterations on respiratory frequency, piloerection or other signs of toxicity, were not noticed in both the control or experimental animals at all doses. Furthermore, no gastric ulcerogenic effect was observed in experimental or control animals.

Effect of EOAH on latency time

The pretreatment of animals with EOAH (10, 31.6, 100, 361 and 1000 mg/kg, orally) significantly increased latency (time of response) to thermal stimulation in a dose-dependent manner compared with control rats (vehicle only) with ED_{50} value of 92.7±3.7 mg/kg (Fig. 1). The percentage of increase in baseline produced by the standard drug morphine (5 mg/kg, i.p.) was 91.2±5.7%.

Effect of EOAH on acetic acid-induced abdominal writhing

As shown in Fig. 2, the oral pretreatment with EOAH (10, 31.6, 100, 361 and 1000 mg/kg, orally) evoked a dose-dependent ($P < 0.05$) inhibition of acetic acid-induced abdominal writhes in mice when compared to control group (vehicle only), with an ED_{50} value of 74.1±4.6mg/kg. The standard drug reference morphine (5 mg/kg, i.p.) resulted in a significant ($P < 0.05$, Fig. 2) reduction (89.2± 5.8%) of the control writhes.

Effect of EOAH on formalin-induced nociception

As demonstrated in Fig. 3A and B, EOAH (10, 31.6, 100, 361 and 1000 mg/kg, orally) significantly inhibited both the early (neurogenic, 0–5 min) and late (inflammatory, 15–30 min) phases of formalin-induced licking compared with control rats. The calculated ED_{50} value for the first and second phases were: 88.1 ± 5.1 and 74.3 ± 4.2 mg/kg, respectively. The inhibitions observed were 83.2±3.2 % and 81.4±4.6 % at a dose of 1000 mg/kg, for the first and second phases, respectively (Fig. 3A and B). The positive control drug, morphine (5 mg/kg i.p), significantly attenuated the neurogenic and inflammatory pain (both phases). The percentage inhibitions of licking were 86.3 ± 6.6 and 84.2 ±4.1%, for the first and second phases, respectively.

Possible mechanisms of antinociceptive action of *anastatica hierochuntica*

Pretreatment of animals with antagonists naloxone partially reversed the antinociceptive action of EOAH (Table 2).

Pain management is probably one of the most important aspect and still the most difficult in medical practice. Many plant material work as analgesics agents have been developed. Up till now, there is no known studies on the antinociceptive activity of *Anastatica hierochuntica* essential oil.

Our study demonstrated that the EOAH elicited effective antinociceptive activity in rats using hot plate test, formalin test and acetic acid-induced abdominal writhing. Naloxone, the non-selective opioid receptor antagonist, was used in an attempt to focus some light into the mechanisms involved in EOAH antinociceptive properties.

The hot-plate test is a good method to evaluate the centrally acting analgesics material. The paws of rats are very sensitive to heat at high temperatures, in this method the behavioral responses as jumping, pulling out of the paws and pasting of the paw, can be measured in terms of their reaction times [25]. Our experiments showed that EOAH extract has a significant dose-dependent increase in time of response (latency) to thermal stimulation compared to control rats treated with vehicle. The same test also showed increase in latency baseline when morphine was used in 5 mg/kg dose (i.p.). This result suggested that EOAH inhibits centrally located pain receptors. EOAH also decreased writhing number in acetic acid-induced abdominal

constriction which was used as a standard test for measuring analgesia induced by opioids receptors [26].

The acetic acid-induced abdominal writhing test depends on the abdominal writhing as an obvious response to the intense pain induced by irritant principles via nociceptors. It is characterized by stretching, tension to one side, extension of hind legs, contraction of the abdomen so that the abdomen of animal touches the floor, rotating of trunk (twist). Inhibition of the writhing number by analgesics is easily quantifiable [27]. In our study EOAH at test doses 10, 31.6, 100, 316 and 1000 mg/kg, (orally) caused a significant dose-dependent decrease in the number of acetic acid-induced writhes in rat in comparison to control. This observation indicated that EOAH possesses peripherally-mediated antinociceptive property.

EOAH also reduced the licking time in response to pain induced by formalin during the first and second phases of the formalin test. Injection of formalin induces a biphasic pain response; the first phase (short phase) is thought to result from direct activation of C-fiber afferent nociceptors, After a short period, the acute phase is followed by a continuous prolonged response (Phase II) which believe to reflect the combined effects of afferent input and central sensitization in the dorsal horn. A central sensitization of dorsal horn neurons occurs during the inflammatory pain, a phase in which amines (such as histamine and serotonin), prostaglandins, bradykinin and cytokines (such as TNF-a, IL1b, IL-8) play the major role [28-29]. In the present study, the highest concentrations used of EOAH significantly reduced the measured pain behavioral parameter in both phase 1 and phase 2 to a degree that was almost similar to that caused by morphine. Inhibiting both phases of pain response relative to controls, suggested that EOAH has both central and peripheral antinociceptive effects. Moreover, the inhibitory effect of EOAH on nociceptive response in the late phase of formalin test, which is an inflammatory reaction in the peripheral tissue, suggested that part of EOAH antinociceptive effect could be due to its anti-inflammatory effect by inhibiting the synthesis or production of inflammatory cytokines and mediators such as histamine, prostaglandins, kinins and cytokines.

The mechanisms by which EOAH produced antinociception in the three models of nociception studied are not completely understood. However, our results showed that EOAH acts partially by interaction with opioid receptor, since the antagonist naloxone partially attenuated EOAH antinociception when assessed using hot-plate test, abdominal writhing test and formalin-induced pain test. Additional study is needed to understand the precise receptors participating in the analgesic effect of EOAH.

The antinociceptive effect of EOAH may be related to the single or synergic action of a number of constituents present in the oil, such as eugenol, α-phellendrene, 1,8-cineole, and β- caryophyllene. For example, previous study showed that eugenol, the main chemical constituent of EOAH (21.2%), possess antiinflammatory, antioxidant, anaesthetic and muscle relaxant properties [30]. Another study showed that eugenol has antinociceptive effects through different mechanisms that may involve both central and peripheral pathway. One of these mechanisms is via blockade of calcium channels and vanilloid receptor modulation [31]. An additional study by Dal Bó et al. [32] proved that eugenol promotes significant antinociception by the inhibition of glutamatergic neurotransmission and cytokine signaling. A study by Santos and Rao showed that 1,8-cineole (14.9 %) inhibited the chemical nociception induced by intraplantar formalin with no participation of opioid system [33].

Our work suggests the possible involvement of α-phellendrene (11.5%) in EOAH antinociceptive effect. The compound has been shown to reduce the licking time induced by formalin test with participation of the glutamatergic, adrenergic and opioid systems [36].

The presence of β- caryophyllene (8.2%) may also play a possible role in EOAH antinociceptive effect. This naturally occurring monoterpenoid was able to reduce the edema formation induced by carrageenan, histamine, bradikinin, PGE2 and platelet activating factor [34]. Furthermore, β- caryophyllene is a known CB-2-agonist. CB-2 is a cannabinoid receptor. CB-2-selective agonists are promising candidates for pain treatment [35].

In conclusion, the present findings indicate, for the first time, that EOAH has analgesic effect arising from both CNS and peripheral actions and was related to its ability to activate opioid receptors.

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Declaration of interest

All authors declare that there are no conflicts of interest.

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Table 1: Chemical composition of the essential oil of aerial parts of *Anastatica hierochuntica.*

Constituents	**Percentage (%)**
Eugenol	21.1
1.8- cineole	14.9
α- phellandrene	11.5
β-Caryophyllene	8.5
Copaene	4.3
Cuminyl acetate	4.1
Selinene	3.9
Humulene	3.1
Muurolene	2.5
Amorfene	2.4
Eremoligenol	1.7
Germacrene	1.3
α- agarofuran	1.2
Total identified	**80.3**

Table 2: Influence of naloxone on EOAH antinociceptive activity in rats assessed using the hot-plate test, abdominal writhing test and formalin-induced pain test. Control indicates vehicle administration, and theone asterisk (*) denotes significance levels when compared with the control group. Two asterisks (**) denote significance levels when compared with the EOAH treated group (P>0.01). Each valuerepresents mean ±SEM (n = 6 per group).

Treatment	**Increase in baseline (hotplate test, %)**	**Inhibition of licking (%) (Phase I)**	**Inhibition of licking (%) (Phase II)**	**Inhibition of abdominal writhing (%)**
Control	2.1±0.3	2.6±1.6	1.4±0.5	2.8±0.4
EOAH	48.2±1.3*	51.1±2.6*	51.9±5.1*	52.1±4.1*
Morphine	87±2.5*	89±3.6*	89±6.1*	85±2.7*
Naloxone	3.2±1.4	2.5±1.5	3.4±0.9	3.8±1.3
Naloxone + EOAH	19.5±2.8**	22.3±4.2**	21.3±4.1**	18.3±2.2**
Morphine +Naloxone	4.1±1.6	3.2±1.4	4.7±0.9	3.4±1.6

Doses of EOAH used were those used to give ID_{50}.

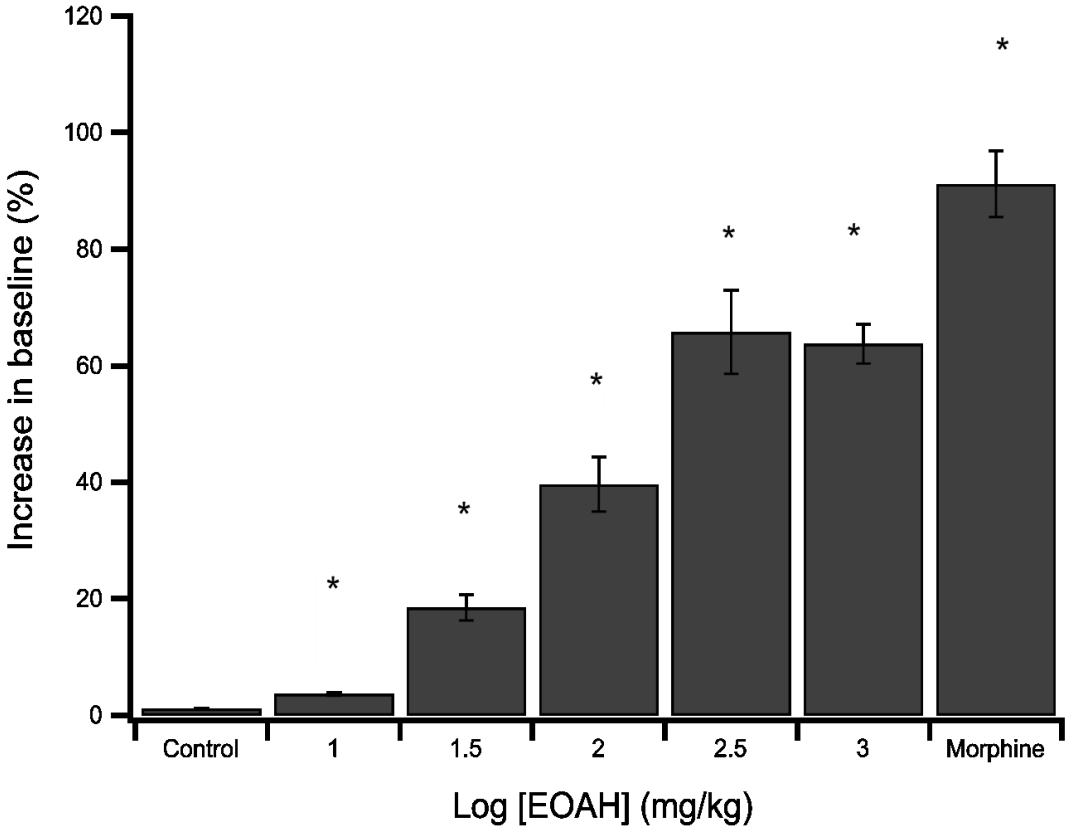

Figure 1. Antinociceptive activity of essential oil of *Anastatica hierochuntica* (EOAH) in hot plate reaction time. Bar represents mean±SEM (n = 6). Control values indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups. (p>0.05).

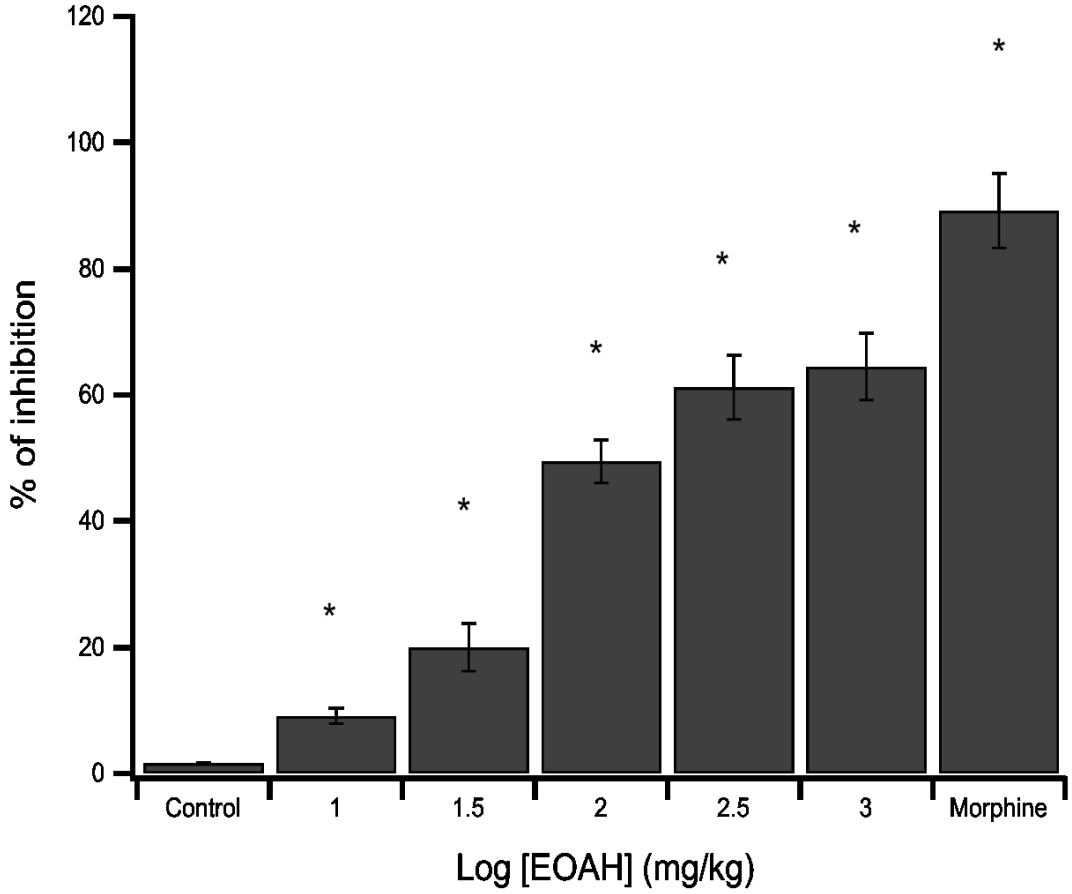

Figure 2. Antinociceptive activity of essential oil of *Anastatica hierochuntica* (EOAH) in acetic acid induced writhing test. Bar represents mean±SEM. (*n* = 6). Control values indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups ($p>0.05$).

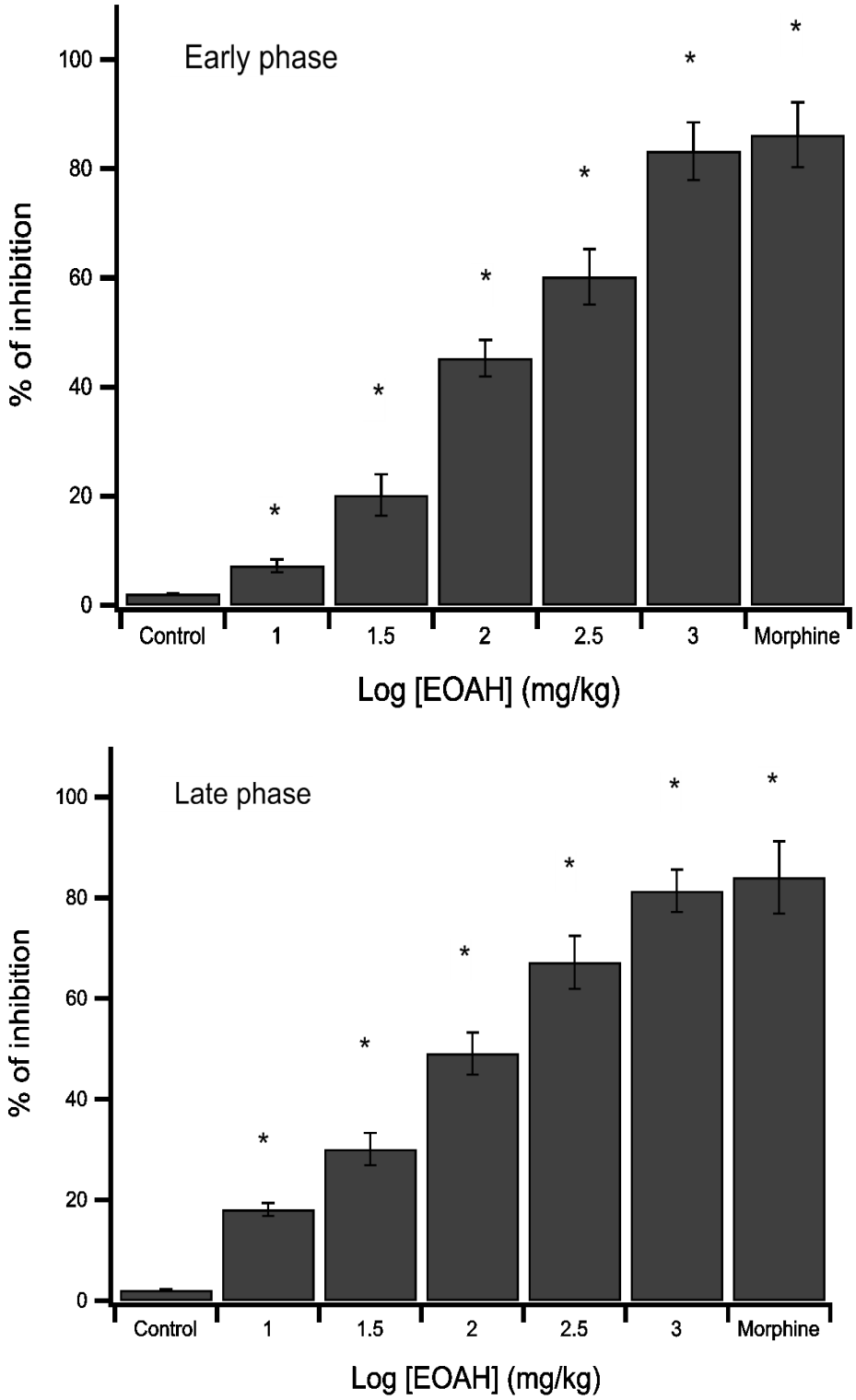

Figure 3 Antinociceptive activity of essential oil of *Anastatica hierochuntica* (EOAH) and morphine (5mg/kg) in formalin-induced licking test. Bar represents mean±SEM. (*n* = 6). Control values indicate vehicleadministration, and the asterisks denote significance levels when compared with the control group (*P* > 0.05).