*OnLine Journal of Biological Sciences*

Original Research Paper

# Antioxidant, Analgesic and Anti-inflammatory Effects of Ethanolic Extract of Moroccan *Lavandula stoechas* L.

**[1,2]Hind Ferehan, [2]Mehdi Ait Laaradia, [2]Sara Oufquir, [3]Rachida Aboufatima, [2]Soad Moubtakir, [2]Zahra Sokar, [2]Abderrahman Chait and [1]Abdellah Bagri**

*[1]Hassan First University of Settat, Faculty of Sciences and Techniques, Laboratory of Biochemistry and Neuroscience, Integrative and Computational Neuroscience Team, 26002, Settat, Morocco*
*[2]Cadi Ayad University, Faculty of Sciences Semlalia, Laboratory of Neurobiology, Pharmacology, Anthropology and Environment, 40000, Marrakech, Morocco*
*[3]Sultan Moulay Slimane University, Faculty of Sciences and Technology, Laboratory of Biological Engineering, 23000, Beni Mellal, Morocco*

*Article history*
Received: 06-08-2021
Revised: 06-10-2021
Accepted: 07-10-2021

Corresponding Author:
Hind Ferehan
Hassan First University of Settat, Faculty of Sciences and Techniques, Laboratory of Biochemistry and Neuroscience, Integrative and Computational Neuroscience Team, 26002, Settat, Morocco
E-mail: h.ferehan@uhp.ac.ma

**Abstract:** *Lavandula stoechas* L. is a widely used medicinal plant. In Moroccan traditional medicine, it is employed for the treatment of different illness and as an anxiolytic, analgesic or anti-inflammatory remedy. The purpose of this investigation was to assess the antioxidant, antinociceptive and anti-inflammatory effects of the ethanolic extract of Moroccan *Lavandula stoechas* L. in animal models. Therefore, *in vitro* anti-oxidant activity was appraised *via* two techniques, the 2,2-Diphenyl-1-Picryl-Hydrazyl (DPPH) radical scavenging method and reducing power approach. For the *in vivo* trials, the ethanolic extract of the herb was orally administered (0.5, 0.6 and 1 g/kg, p.o.). Furthermore, acetic acid induced-writhing, formalin induced-paw-licking, thermal hyperalgesia and tail-flick experiments were applied to examine the antalgic activity. The xylene induced ear-edema trial was achieved to estimate the anti-inflammatory activity. The results revealed an important anti-oxidant activity of our extract. Moreover, in the writhing-test, we observed that the concentrate inhibited abdominal contractions engendered by the acetic-acid in mice. In the hot-plate and the tail-flick tests, the extract caused a notable central analgesic reaction, the latency to the thermal stimuli intensified in a dose dependent way. Additionally, considering the formalin test, the pre-treatment with the extract reduced the licking-time in the first and late phase of the response. Lastly, the xylene induced ear-edema test demonstrated that the plant's extract remarkably restrained the edema formation occasioned by the xylene, which was confirmed by histological verification. This study allowed us to conclude that *Lavandula stoechas* is an ultimate medicinal plant that possesses antioxidant and anti-inflammatory activities along with the central and peripheral anti nociceptive properties.

**Keywords:** *Lavandula stoechas* L., Antioxidant Activity, Anti-Nociceptive Activity, Anti-Inflammatory Effect

## Introduction

As the other Lavenderis species, *Lavandula stoechas* is largely disseminated and exists in diverse regions in Morocco. This accessibility and its quite low price are a valuable benefit for common employment. *Lavandula stoechas* L., commonly known as *El-h'alhal* (Tahraoui *et al*., 2007), has been used for long time in Moroccan traditional medicine to make decoctions in case of rheumatism, cold and as digestive system benefices agent (El-Hilaly *et al*., 2003).

*Lavandula* genius is a major member of *Labiatae* (*Lamiaceae*) family. *Lavandula* spices are extensively spread in the Mediterranean area and are planted in France, Spain and Italy. Lavender has a considerable commercial value due to its extraordinary and agreeable fragrance. Both the plant materiel and its essential oil are largely claimed in perfumery, cosmetic and food

industries. The medicinal eminence of the plant is well described and the drugs composed from this plant are recorded in many Pharmacopeia (Gören *et al.*, 2002; Algieri *et al.*, 2016). For instance, Silexan, an oral lavender oil capsule preparation, has significant effect in the remedy of 'sub-syndromal' anxiety condition (Kasper *et al*., 2010). Indeed, traditional medicine practitioners in Morocco recommend this plant as antispasmodic and because of its anti nociceptive, calmative, aseptic and anti-microbial potencies. Lavender also has positive consequences on lesions, urinal-infections, cardiac problems and atopic dermatitis (Gülçin *et al*., 2004). It has been applied in epilepsy, depression, headaches, diabetes and as calmative therapy (Gilani *et al*., 2000; Gülçin *et al*., 2004).

Pain is one of the most predominant illness that restricts the productivity and reduces the quality of life. Although there is a plentitude of efficient and extensively utilized analgesics, there is some concern in regard to their safeness and side effects such as drowsiness, dizziness, upset stomach making their use problematic (Zhao *et al.*, 2003).

Drugs of natural origin are a prominent source for the medication of various ubiquitous ailments. The research and analysis of plants utilized as pain relievers and anti-inflammatory agents in popular ethno medicine is one among the most profitable and relevant approches in the search for current chemical substances with potential therapeutic uses (Algieri *et al.*, 2016).

Phytochemical investigations reported distinct secondary metabolites in *Lavandula* species (Shimizu *et al*., 1990; Gülçin *et al*., 2004) such as diterpenes (Politi *et al*., 2002), tri terpenes (Topcu *et al*., 2001), coumarins (Shimizu *et al*., 1990) and phenolic composites (Gabrieli and Kokkalou, 2003). Secondary metabolites are largely employed in food, pharmaceutical, chemical, cosmetics productions and agronomy (Ceylan *et al*., 2015).

In this study, anti-oxidant potentiality of *Lavandula stoechas* was assessed *via* DPPH technique and the reducing power assay. The total phenolic compounds, the flavonoids and tannins contents were also assessed. Additionally, we examined the anti-nociceptive property of the aerial parts' total extract in hot-plate, tail immersion, writhing and formalin trials. The anti-inflammatory effect was assessed using the xylene producing ear-edema test. Moreover, the possible mechanism implicated in the anti-nociceptive and anti-inflammatory properties was explored as well.

## Materials and Methods

### *Animals*

The experimentations were accomplished using *Swiss Albino* male and female mice (28 and 35 g) and *Sprague Dawley* male rats (180 and 220 g) raised in the central animal care facilities of Cadi Ayyad University, Marrakech, Morocco. Animals were housed under supervised temperature (22±2°C), humidity (60%) and 12h-light-dark cycle with an *ad libitum* access to food and water. All efforts were performed to reduce the animal suffering and to diminish the number of animals utilized. All the experimental protocols were accomplished in conformity with the European Council Directive (EEC, 1986/609) and judiciously confirmed by the Council Committee of research laboratories of the Faculty of Sciences, Cadi Ayyad University of Marrakech.

### *Plant Materials*

The *Lavandula stoechas'* aerial parts were collected from Demnat region, Morocco. Pr. Ahmed Ouhammou (Professor Responsible of the Regional Herbarium "MARK" at the Samlalia Science Faculty, Cadi Ayyad University, Marrakech) had identified and classified the plant material and a voucher specimen (Mark - 8259) was stored at the faculty herbarium.

### *Preparation of Ethanolic Extract*

The *Lavandula stoechas'* aerial parts were dried and triturated in order to get a powder. The dried powdered aerial parts (120 g) of the plant were extracted with ethanol (500 mL, 80% v/v) in a Soxhlet equipment for 16 h at 64°C. In addition, to get the total extract (34 g), the rotary device was utilized to eliminate the solvent at 45°C.

### *Acute Toxicity*

The toxicity examination was achieved out using male and female *Swiss Albino* mice. The animals were assigned at random to one control lot and seven treated lots, including 3 animals per sex. The treated groups got orally the plant extract at different doses: 100, 200, 500, 1000, 2000, 4000 and 5000 mg/kg. After the administration of the concentrate, the animals were observed for general behavioural changes or clinical signs and mortality. The observation was continuous for the first 4 h; then, for the next 24 h, they were observed every hour. Moreover, for the next 48 h the observation was one at 6 h intervals. The treated groups were kept under observation for one week for further changes.

### *Phytochemical Evaluation of the Ethanolic Extract of Lavandula Stoechas*

### *Evaluation of Total Phenolic (TPC), Flavonoids (TFC) and Condensed Tannin Compounds (TTC)*

The total phenolic composite of *Lavandula stoechas* (LS) ethanolic extract was appraised *via* the Folin-Ciocalteu technique **(**Singleton *et al*., 1999)**.** One hundred micro liter (µl) of the extract was diluted with 3.7 mL of distilled water and 200 µL of Folin-Ciocalteu reagent was included. After 3 min, 20% sodium carbonate (1 milliliter of $Na_2CO_3$) was added. The solution was agitated and incubated in dark for 45 min at 25°C. Then, the absorbance

was interpreted at 725 nm a UV-Vis spectro photometer (VR-2000, Spain). The total phenolic concentration of LS ethanolic extract is described in terms of milligram equivalent Gallic acid per gram of the dry matter of extract (mg EGA/g). All specimens were realized three times.

The flavonoids compound of LS extract was estimated based on the aluminum tri-chloride technique (Zhishen *et al*., 1999). Aliquots (200 µL) of diluted extract were added to 5% $NaNO_2$ solution (60 µL), followed by 10% AlCl3 (40 µL) and incubated for 6 min, followed by the addition of NaOH (400 µL, 1M). Then, distilled water (500 µL) was added. The absorbance of the solution was examined at 510 nm. The TFC in LS was mentioned as milligram catechin per gram of the extract (mg ECAT/g).

Tannins in LS extract were estimated as succeeding (Xu and Chang, 2007). Sample extract (300 µL) was added to a solution of vanillin (3 mL, 4% methanol) and HCl (1 mL). The absorbance was determined at 500 nm. The results were revealed as catechin per gram of the extract.

### *In vitro Anti-oxidant property*

### *DPPH (2,2-Diphényl 1-Picrylhydrazyle) Free Radical Scavenging Activity*

In order to evaluate the free radical scavenging property of LS ethanolic extract, we utilized the stable free radical 2,2-Diphenyl-1-Picryl Hydrazyl radical (DPPH) as described by Burits and Bucar (2000). Briefly, 50 µL of various extract concentrations were mixed with 2 milliliter of 60 mM methanol solution of DPPH. Furthermore, the samples' absorbance was noted at 517 nm after 20 min of incubation in the dark at room temperature. Blank solution was produced using the same amount of methanol and DPPH. As positive control, Quercetin and Butylated Hydroxytoluene (BHT) were utilized. To estimate the inhibition of the DPPH (%), the following equation was utilized (Badakhshan *et al*., 2012):

$$I\ \% = \left( \left( Ablank - A_{sample} \right) / A_{blank} \right) * 100$$

Where $A_{blank}$ is the absorbance of the control and $A_{sample}$ is the absorbance of the test compound. The sample concentration providing 50% Inhibition (IC50) was calculated from the graphic by plotting inhibition percentages against sample concentrations.

### *Reducing Power Assay*

As mentioned by Oyaizu (1986), the reducing power of the LS ethanolic extract was assessed. 1 milliliter of different sample concentrations was added to the phosphate buffer (2.5 mL, 200 mM, Ph 6.6) and potassium ferricyanide (2.5 mL, 1%). The resulted solution was incubated for 20 min at 50°C; after incubation period, 2.5 mL of 10% Tric-hloroacetic Acid (TCA) was mixed with the solution and then centrifuged at 3000 rpm for ten minutes. 2.5 mL of the upper layer solution was added to 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3$). The determination of coloration formed by the reduction of $Fe_3$+ at 700 nm was utilized to examine the sample concentration providing 0.5 of absorbance (IC50). Ascorbic acid, BHT and quercetin were employed as positive controls.

### *Assessment of Anti-Nociceptive Activity*

### *Hot Plate Test*

The heated area of a hot-plate analgesia meter (Ugo Basil, Italy Socrel DS-37 ®) was conserved at 55±0.2°C. Animals were located in an acrylic cylinder (20 cm in diameter) on the warmed area and the period (in seconds) after placing the animal on the hot plate and the manifestation of either the licking of the rear paw or the escape from the platform was monitored as response latency. A cut-off duration of twenty seconds was retained to prevent the paw surface injury (Shaheen *et al*., 2000).

Treated animals received orally the LS ethanolic extract, which was dissolved in distilled water (500, 600 and 1000 mg/kg). Control lot took distilled water orally at 0.1 mL/10 g. Morphine was administered at 10 mg/kg (intra-peritoneal).

### *Writhing-Test*

The anti nociceptive activity was investigated *via* the writhing test engendered, in mice, by acetic-acid at a concentration of 0.6% (0.1 mL/10 mg, (intra-peritoneal)). The various doses were given orally forty-five minutes earlier than the nociceptive agent. Five minutes after the acid injection, the number of writhes and stretching movements (contraction of the abdominal musculature and extension of hind limbs) was counted for a period of 30 min. Acetylsalicylic acid ASA (200 mg/kg body weight) was used as pharmacological reference (De Miranda *et al*., 2001; Aboufatima *et al*., 2021).

Anti-nociceptive activity was expressed as the reduction in the number of abdominal constrictions between the control animals and the mice pre-treated with compounds.

Inhibition percentage in the nociceptive response has been calculated; defined as follows:

$$I = \left[ \left( NC - NT \right) / NC \right] \times 100$$

Where:
*NC* : Number of contortions for the control group
*NT* : Number of contortions of a group under test

### *Formalin Test*

The formalin test was carried out as previously described by De Miranda *et al.* (2001) with slight modification, twenty microliters of a 2% (20 µL) formalin solution were subcutaneously injected into the right posterior paw of the mice. Immediately after the injection, the animal was placed in a transparent enclosure.

Throughout 5 min prior to this procedure, each mouse is allowed to adapt the testing box and left freely moving and exploring (habituation). For 30 min after injection, the time (in seconds) that the animal spent licking the injected paw was recorded.

There are two distinctive phases of intense licking performance that have been recognized. Early phase (the first five minutes) is primarily due to the C-fiber activation as a result of the peripheral stimulus caused by formalin (Tjølsen *et al*., 1992). A second burst of licking behavior occurs after 15 to 30 min and it is related to the inflammatory response elicited by formalin. This phase is termed inflammatory (Hunskaar and Hole, 1987; Milano *et al*., 2008).

The animals were pre-treated orally with the extract or injected with Acetylsalicylic Acid (ASA) (200 mg/kg b.w. i.p.) or morphine (10 mg/kg b.w. s.c.), before the formalin injection.

### *Tail-Immersion Nociceptive Test*

Rats in groups of six each were placed in individual restraining cages and the nociceptive reaction time in second was noted when the tail was immersed in a fixed-temperature water bath maintained at 55±1°C. The nociceptive end-point was characterized by a violent tail jerk. 15 sec was the time limit imposed for all animals that did not respond to the test (Sewell and Spence, 1976; Aboufatima *et al*., 2021).

### *Anti-Inflammatory Activity*

### *Xylene Induced Mouse Ear Edema*

For inducing an acute inflammation, an application of 30µl of xylene was applied topically on the anterior and posterior surfaces on the right ear (Shang *et al*., 2011). Therefore, six groups of mice were tested: The control group (saline solution 0.9% i.p.), four treated groups with the LS extract (500, 600 and 1000 mg/kg p.o.) and a group of diclofenac (10 mg/kg i.p.). 30 min after the xylene usage, mice of the six groups of animals were given a euthanasia and 2 ear punches were collected and weighed. Comparing right and left ear weights, the edema's degree was estimated (Kebbou *et al*., 2019).

For the histology evaluation, 6 samples of inflamed ears from both the control and treated groups were taken and fixed in formaldehyde (10%). Fixed ear tissues have been embedded in parafin and sliced into 3-4 µm sections. Slices were mounted on glass slides, stained with Hematoxylin and Eosin (HE) for pathological studies (Sudoh *et al*., 2004).

### *Statistical Analysis*

The results were expressed as mean ± Standard Errors of the Mean (SEM). The comparison between the various groups was carried out with one-way Analysis (ANOVA) and the repeated measures ANOVA model monitored by Tukey's post hoc test. A value of $p<0.05$ was assumed to be statistically significant.

## Results

### *Acute Toxicity*

In this study, the oral administration of *Lavandula stoechas'* extract at the doses from 100 to 5000 mg/kg had no effect on animals' behavioral responses and body weight. Furthermore, no allergic manifestation nor mortality during the period of 48 h after administration were noticed or even after seven days observation.

Therefore, it can be indicated that ethanol extract has low toxicity profile and the LD50 is greater than 5 g/kg (b.w.).

### *Total Phenolic Compounds, Flavonoids and Tannins Content Determination*

The quantitative analysis of the total phenols, flavonoids and condensed tannins showed that the ethanolic extract contains total phenols, flavonoids and condensed tannins levels of 83.90 mg EGA/g dry extract, 72.80 mg ECAT/g and 37.90 mg ECAT/g dry extract respectively (Table 1).

### *In vitro Antioxidant Activity*

Antioxidant activity was evaluated using two complimentary *in vitro* antioxidant tests. Concentrations that led to 50% Inhibition (IC50) are presented in Table 2. The lower IC50 value reflects a higher protective action.

The results showed that the ethanolic extract of *Lavandula stoechas* revealed an important antioxidant activity, the lowest IC50 was obtained with reducing power assay (IC50 = 40.10±0.01) followed by DPPH (IC50 = 54.46±0.07). Comparing with Quercetin, BHT and Ascorbic acid, the IC50 values of the plant's extract were less effective than those of synthetic antioxidant agent.

### *Assessment of Pharmacological Activities*

### *Analgesic Tests*

### *Writhing Test*

The administration of the total extract at different doses caused a significant reduction in the number of writhing episodes induced by acetic acid compared to the control ($p<0.001$). The results for the second dose (600 mg/kg) and the third one (1000 mg/kg) were significant compared to the first dose (500 mg/kg) (Fig. 1). Moreover, it was stronger than that produced by ASA. On the other hand, no difference in the latency times was observed when the positive control mice were compared with those pretreated with the LS extract. The percentage of writhes inhibition was calculated as 59, 61% for ASA, 30, 36% for LS extract 500 mg/kg, 65, 46% for LS extract 600 mg/kg and 73, 54 % for LS extract 1000 mg/kg.

### *Formalin Test*

The results of formalin test have been represented in Fig. 2A. The ethanolic extract produced a significant ($p<0.001$) analgesia in the first and the second phases. Indeed, at doses of 500, 600 and 1000 mg/kg paw licking time in the early phase was reduced by 45.84, 75.37 and 80 %, respectively. In the late phase, pretreatment with different doses of the ethanol extract has significant ($p<0.001$) effect against the duration of licking activity compared to the negative control group.

Morphine (10 mg/kg b.w.) and the ASA (200 mg/kg b.w.) injections produced marked inhibition ($p<0.001$) of both the neurogenic pain phase and the inflammatory pain phase of the formalin test (Fig. 2A).

Using the drug naloxone reduced significantly both the analgesic effect of the morphine injection and the administration of the extract (EE.D3) (Fig. 2B).

### *Hot-Plate Test*

In the hot-plate test, there were significant differences in the anti-nociceptive effect of plant's extract at different doses. Administration of morphine at the dose of 10 mg/kg demonstrated a significant effect compared with the control group ($p<0,001$) (Fig. 3A).

Furthermore, these results also demonstrated that pretreatment with naloxone (a non-selective opioid receptor antagonist) significantly reversed the antinociceptive effect of morphine (10 mg/kg, i.p.) and ethanolic extract in the hot plate test (EE. D 3 = 1000 mg/kg) (Fig. 3B).

### *Tail-Immersion Nociceptive Test*

The results of tail immersion induced nociception method revealed that the ethanolic extract had substantially ($p<0.001$) augmented the reaction time in the thermal stimuli. The ethanolic extract of the plant showed an anti-nociceptive activity against the tail immersion induced nociception. The 1000 mg/kg of ethanolic extract manifested a significantly ($p<0.001$) increased reaction time in the thermal pain stimuli in 60 min and 120 min after the extract administration (Fig. 4). Treatment with morphine also showed significant ($p<0.001$) nociception inhibition activity within 30 min.

### *Effect of Ethanolic Extract of Lavandula Stoechas on Anti-Inflammatory Activity*

### *Xylene Induced Mouse Ear Edema*

The anti-inflammatory effect of various treatments of *Lavandula stoechas* ethanolic extract in xylene-induced ear edema is depicted in Fig. 5. The ethanolic extract significantly inhibited edema formation induced by the xylene at the doses of 500, 600 and 1000 mg/kg ($p<0.001$), compared to the vehicle control group. Moreover, ethanolic extract dose-dependently decreased xylene-induced edema by 39.66, 47.66 and 58.83% at doses of 500, 600 and 1000 mg/kg, respectively. The anti-inflammatory effect of ethanolic extract at the highest dose tested (1000 mg/kg) was comparable to the effect of diclofenac (10 mg/kg).

As indicated in Fig. 6, histologic assessment of tissue in the ear indicated that application of xylene produced a marked increase in epidermal thickness, edema and infiltration of Polymorph Nuclear Leukocytes (PMN). Oral administration of *Lavandula stoechas* extract (500, 600 and 1000 mg/kg) and diclofenac (10 mg/kg) significantly reduced the indicated changes.

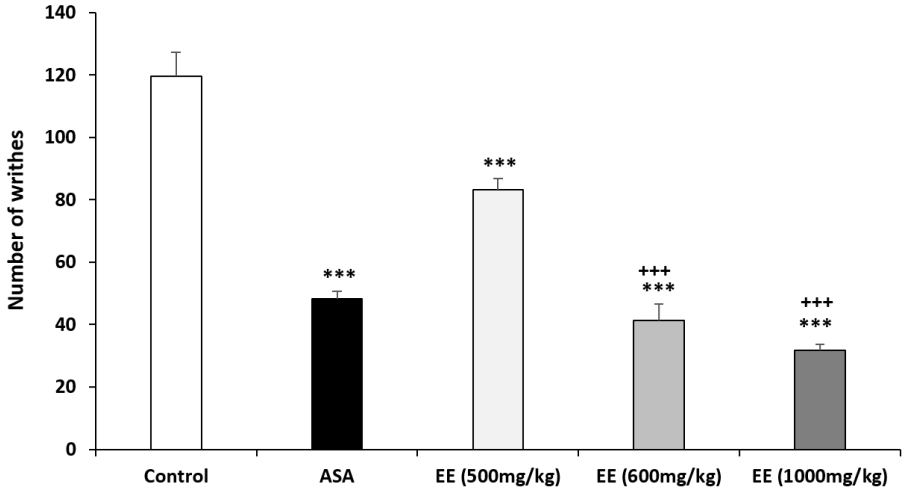



**Fig. 1:** Effect of *Lavandula stoechas* ethanolic extract on the acetic acid-induced writhing behavior in mice. Ethanolic extract (D1 = 500 mg/kg, D2 = 600 mg/Kg and D3 = 1000 mg/kg, p.o.) and acetylsalicylic acid (ASA = 200 mg/kg, i.p.) were administered. The results are illustrated as mean ± SEM, ***$p<0.001$ vs. control, $^{+++}$ $p<0.001$ Vs. EE. D 1

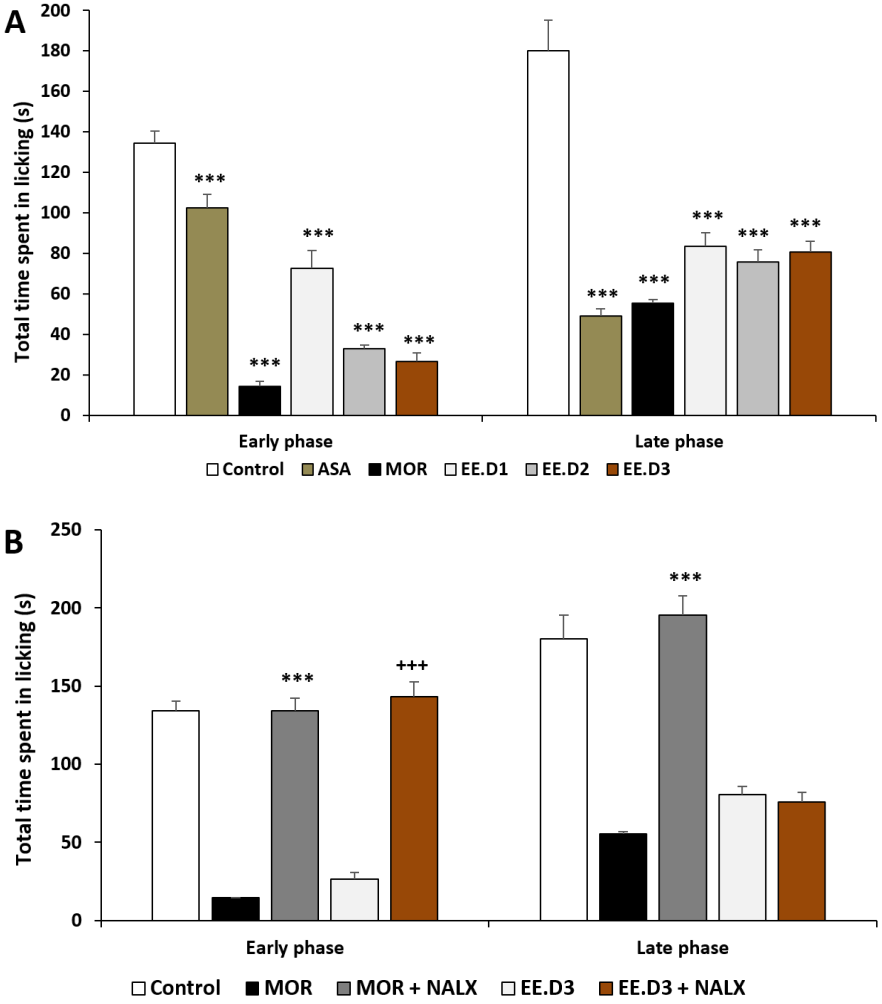
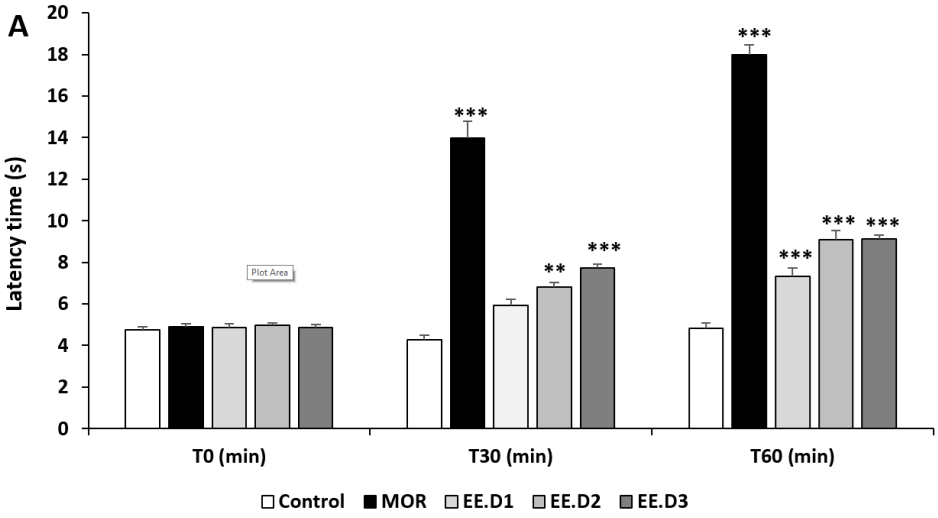



**Fig. 2:** The antinociceptive effect of *Lavandula stoechas* ethanolic extract, Acetyl Salicylic Acid (ASA), Morphine (MOR) (A) and Naloxone (NALX) reversible effect (B) on formalin-induced pain in mice. The values are represented as mean ± SEM. The results are reported as mean ± SEM, *******p<0.001 Vs. Control, $^{+++}$ p<0.001 Vs. EE. D 3



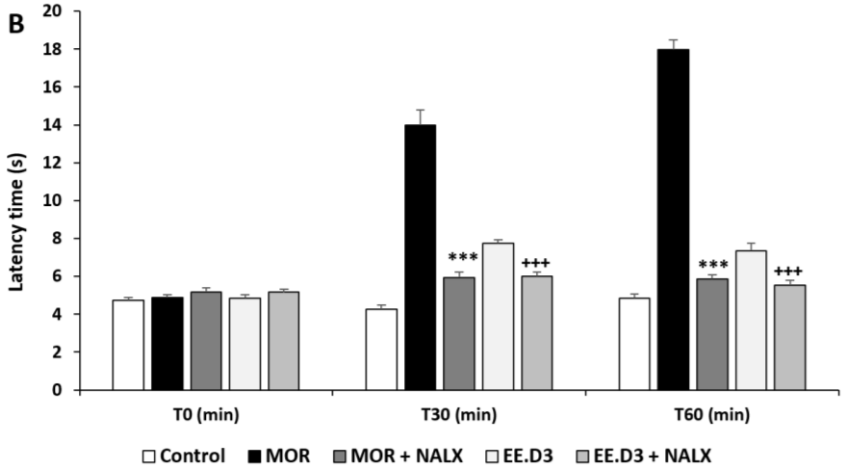



**Fig. 3:** The antinociceptive effect of *Lavandula stoechas* ethanolic extract, Morphine (MOR) **(A)** and reversal effect of naloxone (NALX) **(B)** on the hot plate test. Values are represented as the mean ± SEM. The results are reported as mean ± SEM, ***p<0.001 Vs. MOR; +++p<0.001 Vs. EED3

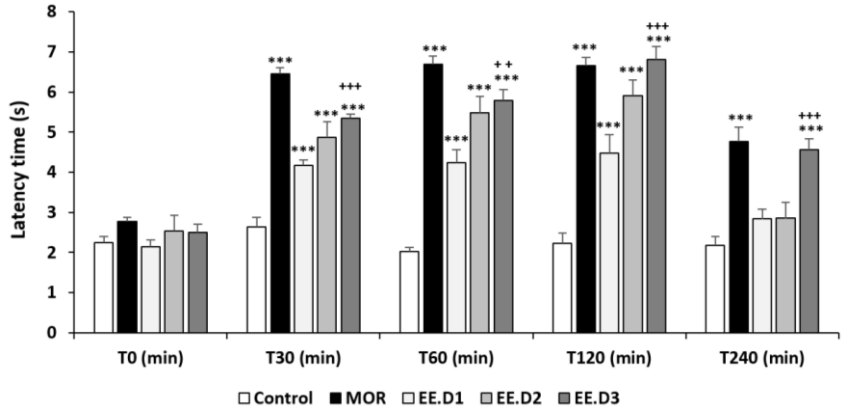



**Fig. 4:** Antinociceptive effect of ethanolic extract of *Lavandula stoechas*, Morphine (MOR) on the tail immersion test. Values are represented as the mean ± SEM. Results are presented as mean ± SEM, *******p<0.001 Vs. Control; $^{+++}$p<0.001, $^{++}$p<0.01 Vs. EE. D 1

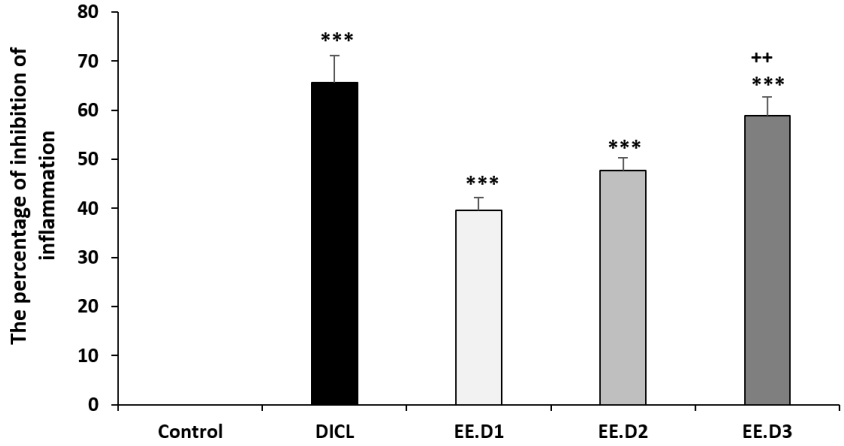



**Fig. 5:** Effect of ethanolic extract of *Lavandula stoechas* on the inflammation response in the xylene test. Diclofenac (DICL = 10 mg/kg i.p.), ethanolic extract were administered 45 min before xylene application (EE. D 1 = 500 mg/kg, EE. D 2= 600 mg/kg and EE. D 3 = 1000 mg/kg p.o.). The findings are reported as mean ± SEM, ***p<0.001 Vs. control

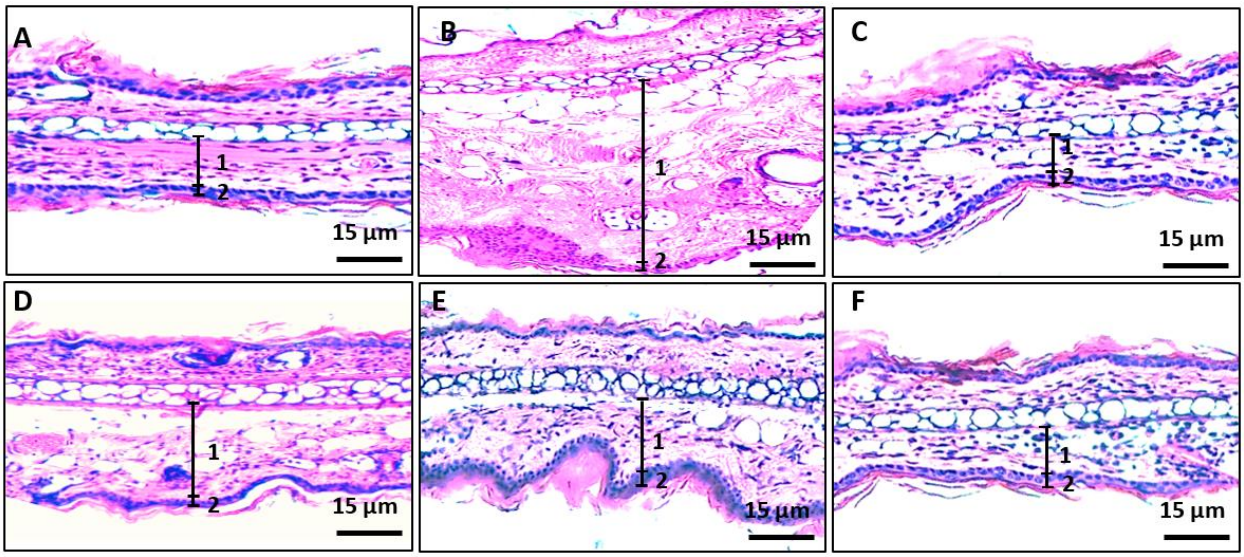



**Fig. 6:** Tissue sections indicating the swelling of the ear caused by xylene treatment. (A) The normal ear; (B) model control group induced by xylene; (C) the treatment with diclofenac (10 mg/kg); (D) an ethanolic extract treatment (500 mg/kg); (E) the treatment with ethanolic extract (600 mg/kg); and (F) treatment with Ethanolic Extract (1000 mg/kg). 1: Dermis thickness; 2: Epidermal thickness. (HE ×100). Scale - bar = 15 µm

**Table 1:** Total phenolics, flavonoids and tannins content of ethanol extract of the *Lavandula stoechas* (Mean ± SEM).mg equivalent gallic acid per g of extract (mg EGA/g); mg equivalent catechin per g of the extract (mg ECAT/g); the Dry Weight (DW)

| | Ethanol extract |
|---|---|
| Total phenolic contents (mg EGA/g DW) | 83.90±1.72 |
| Flavonoid contents (mg ECAT/g DW) | 72.80±3.91 |
| Tannin contents (mg ECAT/g DW) | 37.90±9.56 |

**Table 2:** IC50 (µg/ml) values of *Lavandula stoechas* ethanolic extract compared to synthetic antioxidants (Ascorbic acid, Quercetin and BHT). Results are presented as mean ± SEM

| | DPPH | Reducing power |
|---|---|---|
| BHT | 2.49±0.02 | 1.51±0.01 |
| Quercetin | 1.72±0.03 | 3.81±0.09 |
| Ascorbic acid | 1.14±0.02 | 4.33±0.01 |
| Ethanolic extract | 54.46±0.07 | 40.10±0.01 |

## Discussion

Nowadays, there are several pharmaceutical protocols to reduce pain and control inflammation. While most of these medications have been shown to be effective, their use may be accompanied by some alarming side effects (Lisa *et al*., 2020), hence limiting their long-term use. Therefore, the rising interest on alternative medicine could be justified. Within this frame of reference, our study focused on the anti-nociceptive, anti-inflammatory and antioxidant effects of the total ethanolic extract of *Lavandula stoechas*' aerial parts.

To begin with, the anti-nociceptive activity has been performed in a wide range of acute pain models in rodents, such as, the formalin, acetic acid-induced writhing, hot-plat and tail-immersion nociceptive tests. These are classical models of nociception used in the screening of potential anti-nociceptive drugs or plant extracts. They were chosen to study both peripherally and centrally mediated effects. In fact, acetic acid-induced abdominal writhes helped to elucidate peripheral activity, whereas both the tail flick and hot plate approaches investigated the central pain mechanism; however, the formalin test investigated the central and peripheral pain pathways (Paulino *et al*., 2003). For instance, the results represented above lead us to confirm that the *Lavandula stoechas* ethanolic extract exerted a significant effect against pain in the four current anti-nociceptive tests in mice and rats. Moreover, significant elevation of pain threshold demonstrated by the extract at different doses tested in these models indicates relevant peripheral and central mediated activities.

Indeed, the acetic acid test is widely used for the evaluation of peripheral anti-nociceptive activity. Also known as the abdominal constriction response, it is highly sensitive and capable of detecting the anti-nociceptive effects of compounds (Bentley *et al*., 1981; Collier *et al*., 1968; Oluyomi *et al*., 1992). In this pharmacological test, because the intraperitoneal injection of acetic acid immediately activates somatic and visceral nociceptors innervating the peritoneum, inducing inflammation in the sub diaphragmatic visceral organs as well as in the subcutaneous muscle walls, the observed anti-nociceptive effect of acetic acid conditioning could be more than just

visceral (Kurihara *et al*., 2003). Indeed, when administered orally, the ethanolic extract of *L. stoechas* caused a strong analgesic effect decreasing the number of acetic acid-induced abdominal twinges compared to the control group. This finding suggests that the extract of plant may be well absorbed from the gastrointestinal tract and induces peripheral analgesic activity. This is supported by the finding that the twitch-lowering drug demonstrates analgesia via inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition, as reported by Loganayaki *et al*. (2012) and Alam *et al*. (2009).

The tail-immersion and hot plate nociceptive tests are pain tests in which central analgesics apply their anti-nociceptive activities via supra-spinal and spinal receptors (Sewell and Spence, 1976; Abbot and Melzack, 1982). Moreover, morphine, a centrally acting analgesic drug, produced an inhibitory effect on the nociceptive response in these tests in this study, the oral pretreatment with the *Lavandula stoechas* total extract showed a significant activity as well. Furthermore, the application of the opioid antagonist (naloxone) relatively reversed the analgesic effect of morphine and extract in the hot plate test. It could then be proposed that the ethanolic extract includes compounds that can act on the central nervous system and are therefore related to the opioid system.

Formalin test is a frequently used and well established model to study pain mechanisms and to evaluate the analgesic action of various endogenous compounds (Dubuisson and Dennis, 1977; Abbott *et al*., 1999; Oluyomi *et al*., 1992; Murray *et al*., 1988; Zhao *et al*., 2003). The intra-plantar formalin injection generates a reproducible syndrome of nociceptive behavior, which appears in two distinct phases. The first phase starts at the time of the injection and lasts about 10 min. However, the second phase starts at 10 min after the injection and lasts for about 50 min. Indeed, it is considered that the first phase results from a direct activation of nociceptive afferent fibers. Yet, the second phase is thought to be mediated by activation of central sensitized neurons due to peripheral inflammation and continued activity of primary afferents (Hunskaar and Hol, 1987; Zhao *et al*., 2003; Puig and Sorkin, 1996; Dubner and Ren, 1999; Milano *et al*., 2008). The application of formalin to a hind paw was shown by Dickenson and Sullivan (1987) to stimulate primary afferent C-fibers in a biphasic manner and to follow a time course similar to that observed in behavioral studies (Dickenson and Sullivan, 1987).

In this way, it has been shown that formalin intra-plantarly injected in animal's hind paw increased significantly in the early phase the release of aspartate, taurine, glutamate, citrulline, glycine, serine, glutamine, PGE2 and asparagine; while in the second phase, it augmented the liberation of the citrulline, PGE2, glutamate and aspartate. In similar studies conducted on thymoquinone (Abdel-fattah *et al*., 2000), supraspinal opioid systems, particularly the M1 and K opioid receptor subtypes, have been shown to be involved in the early phase. Whereas the antinociception induced by thymoquinone in related late phase is due to the inhibition of inflammatory mediators (Houghton *et al*., 1995).

The *Lavandula stoechas* ethanolic extract showed nociceptive activity by inhibiting both phases of the formalin response. Indeed, the manifestation of the late phase is due to inflammation causing a release of serotonin, prostaglandins, histamine and bradykinin, which at least to some extent, can lead to sensitization of central nociceptive neurons (Verma *et al*., 2005). It then implies that the anti-nociceptive activity of the extract possibly resulted from its central and peripheral action, which might also suggest an anti-inflammatory action.

Since prostaglandins are important mediators of pain and lavender extract, due to the rich composition in phenolic flavonoids and flavonols compounds (Ezzoubi *et al*., 2014) can inhibit the production of thromboxane and prostaglandins (Picq *et al*., 1991), mediators involved in the maintenance and initiation of the pain and inflammation cascade. Therefore, *Lavandula stoechas* ethanolic extract may induce its analgesic and anti-inflammatory effects in this way.

To screen for the effects of anti-inflammatory drugs, xylene-induced acute inflammation in the ear of mice was used (Kou *et al*., 2005). Indeed, our study examined the impacts of *Lavandula stoechas* on acute inflammation induced by the xylene model. The application of xylene caused a significant increase in the weight of the mouse ear due to the inflammatory reaction. These ear weight increases were used as pertinent markers of anti-inflammatory effects (Ojewole, 2005). For example, pretreatment with the plant extract inhibited the increase in ear weight in a dose-dependent manner. Furthermore, the histopathological manifestations such as inflammatory cell infiltration, vasodilatation and edematous skin changes were decreased on a dose-dependent basis. This inhibition could be considered as a direct evidence that the extract of *Lavandula stoechas* has positive effects on decreasing the acute inflammatory reaction in comparison with those of diclofenac.

The anti-inflammatory action of this extract could be attributed to its chemical composition. Indeed, several major constituents of *Lavandula sp.* can explain their positive effects, including polyphenols (Somani *et al*., 2015). In fact, flavonoids are polyphenols that show antioxidant properties that can contribute to the anti-inflammatory effects exerted by this extract in this experimental model of inflammation (Comalada *et al*., 2005; 2006).

## Conclusion

Significant analgesic and anti-inflammatory properties of the total extract of the aerial parts of the plant were found in these models, which suggests a reasonable basis for the popular and traditional uses of this plant for certain painful and inflammatory conditions.

Many mechanisms are involved in the anti-inflammatory and analgesic actions of the drugs and should be investigated for these fractions in the future. In addition, further biological and phytochemical assays are proposed to determine the chemical active constituents involved in these activities.

## Acknowledgment

We are grateful to Prof. A. Ouhammou for his help in identifying plant material and we thank Mr. A. Regragui for the provision of animals.

## Data Availability

All of the data used to substantiate the findings are included in the article.

## Author's Contributions

**Hind Ferehan, Mehdi Ait Laaradia, Sara Oufquir and Rachida Aboufatima:** Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Drafting the manuscript, Revising the manuscript critically for important intellectual content, Final approval of the version submitted and any revised version.

**Soad Moubtakir:** Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Final approval of the version submitted and any revised version.

**Zahra Sokar:** Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Drafting the manuscript, Final approval of the version submitted and any revised version.

## Ethics

This article is an original work done by the authors. The corresponding author asserts that the other authors have read and approved the manuscript and no ethical issues could be involved

## References

Abbot, F. V., & Melzack, (1982). Brainstem lesions dissociate neural mechanisms of morphine analgesia in different kinds of pain. Brain Res., 251, 149-155. doi.org/10.1016/0006-8993(82)91282-3

Abbott, F. V., Najafee, R., & Franklin, K. B. (1999). Improving the efficiency of the formalin test. PAIN®, 83(3), 561-569. doi.org/10.1016/S0304-3959(99)00168-2

Abdel-Fattah, A. F. M., Matsumoto, K., & Watanabe, H. (2000). Antinociceptive effects of Nigella sativa oil and its major component, thymoquinone, in mice. European journal of pharmacology, 400(1), 89-97. doi.org/10.1016/S0014-2999(00)00340-X

Aboufatima, R., Farouk, L., Ait Laaradia, M., Oufquir, S., Laadraoui, J., El Alami, A., El Gabbas, Z., Bezza, K., Marhoume, F. Z., Sokar Z., & Chait, A., (2021). The Antinociceptive effects of central and peripheral administration of Peganum harmala L. extracts in animal models. OnLine Journal of Biological Sciences, 21 (2), 267.279. doi.org/10.1016/S0014-2999(00)00340-X

Alam, M.A., Subhan, N., Abdul Awal. M., Alam, M.S., Sarder, M., Nahar, L., & Sarker, S.D., 2009. Anti-nociceptive and anti-inflammatory properties of Ruellia tuberosa. Pharm. Biol., 47, 209-214. doi.org/10.1080/13880200802434575

Algieri, F., Rodriguez-Nogales, A., Vezza, T., Garrido-Mesa, J., Garrido-Mesa, N., Utrilla, M. P., ... & Galvez, J. (2016). Anti-inflammatory activity of hydroalcoholic extracts of Lavandula dentata L. and Lavandula stoechas L. Journal of ethnopharmacology, 190, 142-158. doi.org/10.1016/j.jep.2016.05.063

Badakhshan, M.P., L.J. Subramanion, Y.L. Lachimanan, C. Yeng. and S. Sreenivasan, 2012. Antioxidant activity of methanol extracts of different parts of Lantana camara. Asian. Pac. J. Trop. Biomed., 2: 960-965. https://doi.org/10.1016/S2221-1691(13)60007-6

Bentley, G. A., Newton, S. H., & Starr, J. (1981). Evidence for an action of morphine and the enkephalins on sensory nerve endings in the mouse peritoneum. British Journal of Pharmacology, 73(2), 325-332. doi.org/10.1111/j.1476-5381.1981.tb10425.x

Burits, M., & Bucar, F. (2000). Antioxidant activity of Nigella sativa essential oil. Phytotherapy research, 14(5), 323-328. doi.org/10.1002/1099-1573(200008)14:5<323::AID-PTR621>3.0.CO;2-Q

Ceylan, Y., Usta, K., Usta, A., Maltas, E., & Yildiz, S. (2015). Evaluation of antioxidant activity, phytochemicals and ESR analysis of Lavandula stoechas. Acta Phys Pol A, 128, 483-487. http://przyrbwn.icm.edu.pl/APP/PDF/128/a128z2bp148.pdf

Collier, H. O. J., Dinneen, L. C., Johnson, C. A., & Schneider, C. (1968). The abdominal constriction response and its suppression by analgesic drugs in the mouse. British journal of pharmacology and chemotherapy, 32(2), 295-310. doi.org/10.1111/j.1476-5381.1968.tb00973.x

Comalada, M., Ballester, I., Bailon, E., Sierra, S., Xaus, J., Galvez, J., ... & Zarzuelo, A. (2006). Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure–activity relationship. Biochemical pharmacology, 72(8), 1010-1021. doi.org/10.1016/j.bcp.2006.07.016

Comalada, M., Camuesco, D., Sierra, S., Ballester, I., Xaus, J., Gálvez, J., & Zarzuelo, A. (2005). In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-κB pathway. European journal of immunology, 35(2), 584-592. doi.org/10.1002/eji.200425778

De Miranda, F. G. G., Vilar, J. C., Alves, I. A. N., de Holanda Cavalcanti, S. C., & Antoniolli, Â. R. (2001). Antinociceptive and antiedematogenic properties and acute toxicity of Tabebuia avellanedae Lor. ex Griseb. inner bark aqueous extract. BMC pharmacology, 1(1), 1-5. doi.org/10.1186/1471-2210-1-6

Dubner, R., & Ren, K. (1999). Endogenous mechanisms of sensory modulation. Pain, 82, S45-S53. doi.org/10.1016/S0304-3959(99)00137-2

Dubuisson, D., & Dennis, S. G. (1977). The formalin test: A quantitative study of the analgesic effects of morphine, meperidine and brain stem stimulation in rats and cats. Pain, 4, 161-174. doi.org/10.1016/0304-3959(77)90130-0

El-Hilaly, J., Hmammouchi, M., & Lyoussi, B. (2003). Ethnobotanical studies and economic evaluation of medicinal plants in Taounate province (Northern Morocco). Journal of Ethnopharmacology, 86(2-3), 149-158. doi.org/10.1016/S0378-8741(03)00012-6

Ezzoubi, Y., Bousta, D., Lachkar, M., & Farah, A. (2014). Antioxidant and anti-inflammatory properties of ethanolic extract of Lavandula stoechas L. from Taounate region in Morocco. International Journal of Phytopharmacology, 5(1), 21-26. file:///C:/Users/WindowS%2010/Downloads/articleyassine%20(4).pdf

Gabrieli, C., & Kokkalou, E. (2003). A new acetylated glucoside of luteolin and two flavone glucosides from Lavandula stoechas ssp. stoechas. Die Pharmazie, 58(6), 426-427. https://europepmc.org/article/med/12857010

Gilani, A. H., Aziz, N., Khan, M. A., Shaheen, F., Jabeen, Q., Siddiqui, B. S., & Herzig, J. W. (2000). Ethnopharmacological evaluation of the anticonvulsant, sedative and antispasmodic activities of Lavandula stoechas L. Journal of Ethnopharmacology, 71(1-2), 161-167. doi.org/10.1016/S0378-8741(99)00198-1

Gören, A. C., Topçu, G., Bilsel, G., Bilsel, M., Aydoğmusç, Z., & Pezzuto, J. M. (2002). The chemical constituents and biological activity of essential oil of Lavandula stoechas ssp. stoechas. Zeitschrift für Naturforschung C, 57(9-10), 797-800. doi.org/10.1515/znc-2002-9-1007

Gülçin, İ., Şat, İ. G., Beydemir, Ş., Elmastaş, M., & Küfrevioğlu, Ö. İ. (2004). Comparison of antioxidant activity of clove (Eugenia caryophylata Thunb) buds and lavender (Lavandula stoechas L.). Food chemistry, 87(3), 393-400. doi.org/10.1016/j.foodchem.2003.12.008

Dickenson, A. H., & Sullivan, A. F., (1987). Subcutaneous formalin-induced activity of dorsal horn neurons in the rat: Differential response to an intrathecal opiate administered pre or post formalin. Pain, 30: 349-360. doi.org/10.1016/0304-3959(87)90023-6

Houghton, P. J., Zarka, R., de las Heras, B., & Hoult, J. B. S. (1995). Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation. *Planta* Med., 61, 33-36. doi.org/10.1055/s-2006-957994

Hunskaar, S., & Hole, K. (1987). The formalin test in mice: Dissociation between inflammatory and non-inflammatory pain. Pain, 30(1), 103-114. doi.org/10.1016/0304-3959(87)90088-1

Kasper, S., Gastpar, M., Müller, W. E., Volz, H. P., Möller, H. J., Dienel, A., & Schläfke, S. (2010). Silexan, an orally administered Lavandula oil preparation, is effective in the treatment of 'subsyndromal'anxiety disorder: A randomized, double-blind, placebo controlled trial. International clinical psychopharmacology, 25(5), 277-287. https://journals.lww.com/intclinpsychopharm/Abstract/2010/09000/Silexan,_an_orally_administered_Lavandula_oil.5.aspx

Kebbou A., Ait Laaradia, M., Oufquir, S., Aarab, A., El Gabbas, Z., Rais, H., Zyad, A. and Chait, A. Antioxidant Activity, 2019. Anti-inflammatory and analgesic effects of C*aralluma europaea* (eddaghmouss) in mice. OnLine Journal of Biological Sciences, 19 (4): 272.285. doi.org/10.3844/ojbsci.2019.272.285

Kou, J., Ni, Y., Li, N., Wang, J., Liu, L., & Jiang, Z. H. (2005). Analgesic and anti-inflammatory activities of total extract and individual fractions of Chinese medicinal ants Polyrhachis lamellidens. Biological and Pharmaceutical Bulletin, 28(1), 176-180. doi.org/10.1248/bpb.28.176

Kurihara, T., Nonaka, T., & Tanabe, T. (2003). Acetic acid conditioning stimulus induces long-lasting antinociception of somatic inflammatory pain. Pharmacology Biochemistry and Behavior, 74(4), 841-849. doi.org/10.1016/s0091-3057(03)00014-5

Lisa, S. R., Islam, M. K., & Qais, N. (2020). Plants and Plant Constituents with Analgesic and Anti-inflammatory Activities: A Systematic Review. Dhaka University Journal of Pharmaceutical Sciences, 19(2), 207-224. doi.org/10.3329/dujps.v19i2.50638

Loganayaki, N., Siddhuraju, P., & Manian, S. (2012). Antioxidant, anti-inflammatory and anti-nociceptive effects of Ammannia baccifera L.(Lythracceae), a folklore medicinal plant. Journal of Ethnopharmacology, 140(2), 230-233. doi.org/10.1016/j.jep.2012.01.001

Milano, J., Oliveira, S. M., Rossato, M. F., Sauzem, P. D., Machado, P., Beck, P., ... & Bonacorso, H. G. (2008). Antinociceptive effect of novel trihalomethyl-substituted pyrazoline methyl esters in formalin and hot-plate tests in mice. European journal of pharmacology, 581(1-2), 86-96. doi.org/10.1016/j.ejphar.2007.11.042

Murray, C. W., Porreca, F., & Cowan, A. (1988). Methodological refinements to the mouse paw formalin test: An animal model of tonic pain. Journal of pharmacological methods, 20(2), 175-186. doi.org/10.1016/0160-5402(88)90078-2

Oluyomi, A. O., Hart, S. L., & Smith, T. W. (1992). Differential antinociceptive effects of morphine and methylmorphine in the formalin test. Pain, 49(3), 415-418. doi.org/10.1016/0160-5402(88)90078-2

Oyaizu, M. (1986). Studies on products of browning reaction antioxidative activities of products of browning reaction prepared from glucosamine. The Japanese journal of nutrition and dietetics, 44(6), 307-315. doi.org/10.5264/eiyogakuzashi.44.307

Ojewole, J. A. (2005). Antinociceptive, anti-inflammatory and antidiabetic effects of Bryophyllum pinnatum (Crassulaceae) leaf aqueous extract. J. Ethnopharmacol., 99, 13-19. doi.org/10.1016/j.jep.2005.01.025

Paulino, N., Dantas, A. P., Bankova, V., Longhi, D. T., Scremin, A., de Castro, S. L., & Calixto, J. B. (2003). Bulgarian propolis induces analgesic and anti-inflammatory effects in mice and inhibits in vitro contraction of airway smooth muscle. Journal of Pharmacological Sciences, 93(3), 307-313. doi.org/10.1254/jphs.93.307

Picq, M., Cheav, S. L., & Prigent, A. F. (1991). Effect of two flavonoid compounds on central nervous system. Analgesic activity. Life sciences, 49(26), 1979-1988. doi.org/10.1016/0024-3205(91)90640-W

Politi, M., De Tommasi, N., Pescitelli, G., Di Bari, L., Morelli, I., & Braca, A. (2002). Structure and Absolute Configuration of New Diterpenes from Lavandula m ultifida. Journal of natural products, 65(11), 1742-1745. doi.org/10.1021/np020260p

Puig, S., & Sorkin, L. S. (1996). Formalin-evoked activity in identified primary afferent fibers: Systemic lidocaine suppresses phase-2 activity. Pain, 64(2), 345-355. doi.org/10.1016/0304-3959(95)00121-2

Sewell, R. D. E., & Spencer, P. S. J. (1976). Antinociceptive activity of narcotic agonist and partial agonist analgesics and other agents in the tail-immersion test in mice and rats. Neuropharmacology, 15(11), 683-688. doi.org/10.1016/0028-3908 (76)90037-X

Shaheen, H. M., Ali, B. H., Alqarawi, A. A., & Bashir, A. K. (2000). Effect of Psidium guajava leaves on some aspects of the central nervous system in mice. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 14(2), 107-111. doi.org/10.1002/(sici)1099-1573(200003)14:2<107::aid-ptr602>3.0.co;2-z.

Shang, X., Wang, J., Li, M., Miao, X., Pan, H., Yang, Y., & Wang, Y. (2011). Antinociceptive and anti-inflammatory activities of Phlomis umbrosa Turcz extract. Fitoterapia, 82(4), 716-721. doi.org/10.1016/j.fitote.2011.03.001.

Shimizu, M., Shogawa, H., Matsuzawa, T., Yonezawa, S., Hayashi, T., Arisawa, M., Suzuki, S., Yoshizaki, M., Morita, N., Ferro, E., Basualdo, I. and Berzanga, L.H., 1990. Anti-inflammatory constituents of topically applied crude drugs. IV. Constituents and anti-inflammatory effect of Paraguayan crude drug "Alhucema" (*Lavandula latifolia* Vill.). Chemical, Pharmaceulical Bulletin, 38(8): 2283-2284.

Singleton, V.L., R. Orthofer and R.M. Lamuela Raventós, 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Meth. Enzymol*., 299: 152-178

Somani, S. J., Modi, K. P., Majumdar, A. S., & Sadarani, B. N. (2015). Phytochemicals and their potential usefulness in inflammatory bowel disease. Phytotherapy research, 29(3), 339-350. doi.org/10.1002/ptr.5271

Sudoh, Y., Desai, S. P., Haderer, A. E., Sudoh, S., Gerner, P., Anthony, D. C., ... & Wang, G. K. (2004). Neurologic and histopathologic evaluation after high-volume intrathecal amitriptyline. Regional Anesthesia & Pain Medicine, 29(5), 434-440. doi.org/10.1016/j.rapm.2004.06.008

Tahraoui, A., El-Hilaly, J., Israili, Z. H., & Lyoussi, B. (2007). Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). Journal of ethnopharmacology, 110(1), 105-117. doi.org/10.1016/j.jep.2006.09.011

Tjølsen, A., Berge, O. G., Hunskaar, S., Rosland, J. H., & Hole, K. (1992). The formalin test: An evaluation of the method. Pain, 51(1), 5-17. doi.org/10.1016/0304-3959 (92)90003-T

Topcu, G. Ü. L. A. Ç. T. I., Ayral, M. N., Aydin, A., Gören, A. C., Chai, H. B., & Pezzuto, J. M. (2001). Triterpenoids of the roots of Lavandula stoechas ssp. stoechas. Die Pharmazie, 56(11), 892-895. https://europepmc.org/article/med/11817178

Verma, P. R., Joharapurkar, A. A., Chatpalliwar, V. A., & Asnani, A. J. (2005). Antinociceptive activity of alcoholic extract of Hemidesmus indicus R. Br. in mice. Journal of Ethnopharmacology, 102(2), 298-301. doi.org/10.1016/j.jep.2005.05.039

Xu, B. J., & Chang, S. K. C. (2007). A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. Journal of food science, 72(2), S159-S166. doi.org/10.1111/j.1750-3841.2006.00260.x

Zhao, C. S., Tao, Y. X., Tall, J. M., Donovan, D. M., Meyer, R. A., & Raja, S. N. (2003). Role of μ-opioid receptors in formalin-induced pain behavior in mice. Experimental neurology, 184(2), 839-845. doi.org/10.1016/S0014-4886 (03)00346-7

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food chemistry, 64(4), 555-559. doi.org/10.1016/S0308-8146(98)00102-2