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Neuropharmacological effects of *Pistacia lentiscus* and its possible mechanisms of action

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Abstract

The objectives of the study were to investigate the analgesic, antianxiety and sedative effects of *Pistacia lentiscus* essential oil from fruits in mice. The antinociceptive effect was evaluated using three animal models such as writhing, hot plate, and formalin tests. While the sedative and antianxiety effects were evaluated using thiopental-induced sleeping time, elevated plus maze, hole-board, and open field assays. The essential oil possessed a strong antinociceptive effect at all doses. This effect was undone when animals were pretreated with naloxone and yohimbine in the neurogenic phase whereas atropine and glibenclamide were in the inflammation phase (formalin test). The administration of essential oil increased the latency of sleeping and extended time of sleeping in the thiopental test. On the other hand, essential oil possessed an anxiolytic effect. However, pretreatment with flumazenil reversed the effect indicating the possible implication of $GABA_A$ receptors.

Introduction

The major challenges of drugs acting on the central nervous system to treat neurological disorders are the adverse effects (in particular drug dependence) as well their ineffectiveness (Kesselheim et al., 2015). These increase the necessity to develop new bioactive molecules from plants.

The essential oils possess many neuropharmacological properties including anticonvulsant (Nobreg De almeida et al., 2011), antidepressant (Lopes et al., 2011), analgesic (Lenardao et al., 2016), anxiolytic (Mesfin et al., 2014) and sedative effects (Silva et al., 2013).

A species of the Anacardiaceae family, *Pistacia lentiscus* is currently considered an interesting plant to treat several diseases such as digestive (Bouyahya et al., 2017), cardiovascular, diabetes illnesses (Bouyahya et al., 2017), and analgesic effect (Benitez et al., 2012; Gras et al., 2019). The most remarkable biological effect is the anti-cancer effect due to its resin. Its essential oil has shown effects against oxidative stress and bacterial contamination (Maxia et al., 2011; Bozorgi et al., 2013; Giaginis and Theocharis, 2011; Catalani et al., 2017; Mharti et al., 2011). Different aromatic medicinal herbs are utilized in traditional pharmacopeia to treat pain and neurological illnesses such as *Mentha pulegium* (Rabiei et., 2016), *Piper nigrum* (Ghosh et al., 2021), and *Annona vepretorum* (Diniz et al., 2019).

To date, there are not enough studies that can justify the use of this oil for the therapy of the central nervous system disorders. In this report, the neuropharmacological potential of this oil in insomnia, nociception, and anxiety were studied. Their mechanisms of action were also studied.

Materials and Methods

Plant collection and identification

Fruits of *P. lentiscus* were harvested in the region of Ourika (31°23' latitude N/7°42' longitude W; 35 km from Marrakesh, Morocco). The identification of samples was done by one of the authors (AC) and stored as a voucher number (PL 19) at the plant herbarium of the laboratory, Department of Biology, Faculty of Sciences, Semlalia, Marrakesh, Morocco.

Essential oil isolation

The dry material of fruits (200 g) was used to obtain the essential oil of *P. lentiscus* by hydro-distillation technic, using clevenger apparatus type for 3 hours. The collected oil was dried with Na_2SO_4. Then kept in a refrigerator at 3°C.

Gas chromatography-mass spectrometry analysis

One microliter of the sample was injected into an Rtx-5 column (serial number: 952770) chromatography equipped with a flame ionization detector. The gas chromatography-mass spectrometry analysis was performed with a flame ionization detector and single quadrupole mass spectrometry using a Thermo Fisher Scientific ISQ 1712507 trace 1300. The mass spectrometer conditions were as follows: carrier gas 1 mL He/min, ion source and temperature detection were 200 and 250°C respectively. The column temperature was held at 35°C for 5 min, then raised to 250°C at 5°C/min. Finally, the operating electron impact mode was 70 Ev with a scan mass range m/z 40-450.

Animals

Male *Swiss* mice weighing 22-28 g were used to carry out pharmacological experiments. Animals were housed at 24 ± 1°C under (12 hours light/dark cycle). They were randomly housed in appropriate cages with free access to water and food *ad libitum*. One hour before all experiments, animals were acclimatized to the laboratory conditions.

Drugs and treatments

All animals were destined to different treatments using intraperitoneal route. Drugs used in the treatment of positive control groups were diazepam (2 mg/kg), indomethacin (20 mg/kg) or morphine (10 mg/kg) which were solubilized in saline solution. Vehicle (0.9% plus tween 80, 10 mL/kg) was used as negative control group. The essential oil at doses of 12.5, 25, and 50 mg/kg was used for the treatment of other groups in all experimental models.

Different antagonists were used to determine possible mechanisms of action involved in anxiolytic, sedative, and antinociceptive effects respectively such as flumazenil (2.5 mg/kg $GABA_A$ receptors antagonist). Atropine (5 mg/kg: muscarinic receptor antagonist), naloxone (2 mg/kg, selective opioid receptor antagonist), glibenclamide (8 mg/kg, ATP-sensitive K^+ channel inhibitor), nifedipine (10 mg/kg, L-type voltage-gated calcium channel inhibitor), granisetron (2 mg/kg, 5-HT_3 receptor antagonist) and yohimbine (3 mg/kg, α_2 receptor antagonist) were used as antagonists. The used drugs were purchased from Novartis Pharma, USA.

Experimental procedures

Toxicological profile

Four groups were created (5 in each) and randomly divided into control and experimental groups. They received an intraperitoneal single administration at 100, 500, 1000, and 1500 mg/kg. Mice were supervised for kinetic clinical signs of toxicity. During 14 days treatment, each animal was observed for the eventual clinical signs of toxicity such as lethargy, coma, piloerection, food and water consumption, hypersalivation, diarrhea, mood disorders, and mortality. The LD_{50}, LD_{90}, maximal tolerated dose, and lower lethal dose were estimated (Wu et al., 2018). On day 14 of post-treatment, all surviving animals were sacrificed by cervical dislocation to retrieve the blood samples to examine the possible modification of biochemical and hematological parameters. The liver, kidney, and spleen as concerned organs in acute toxicity were used to examine the possible microscopic alterations. Hematoxylin and eosin were used to color the microtome cuts (5 µm).

Antinociceptive effect

Abdominal constrictions induced by acetic acid

A method previously described was followed (Koster et al., 1959). Briefly, acetic acid 0.6% was injected intraperitoneally into mice (0.1 mL/10 g). Thirty minutes before injection, all groups (6 in each) were treated with vehicle, indomethacin (20 mg/kg) and essential oil of *P. lentiscus* (12.5, 25 and 50 mg/kg). Thereafter, separated cages were used to keep animals for counting the number of abdominal spasms during 30 min.

Hot plate assay

To measure the nociception reflexes in response to a thermal stimulus, the hot plate test was used. Animals put in the center of the hot plate were regulated at a temperature of 53 ± 0.2°C. The latency of jumping or hind paw licking was measured as reaction time over a limited period of 20 sec, at different intervals until 120 min post-treatments (Rios et al., 2013).

Formalin-induced paw licking

In this assay, experimental animals were divided into 7 groups (6 in each) as follows: negative control (vehicle NaCl 0.9% plus tween 80; 10 mL/kg intraperitoneal), positive control [morphine (10 mg/kg) and indomethacin (20 mg/kg), used as reference drugs], and essential oil at biological doses mentioned previously. A forma-

lin solution (2%) was administrated at 20 µL per right-hand paw for all groups. Then, the licking response was measured for 5 min (neurogenic phase) and 15 to 30 min after injection (inflammatory phase). To investigate the possible implication of opioid, ATP-sensitive K+ channels, muscarinic, adrenergic, serotonergic and voltage-gated calcium channel pathways, mice were pre-injected with naloxone, glibenclamide, atropine, yohimbine, granisetron, and nifedipine, respectively. Sixty minutes later, the animals were observed to estimate the spent licking or biting time of the treated paw (Rocha et al., 2011).

Sedative activity

Thiopental-induced sleeping time

Sodium thiopental was the drug used to determine the possible sedative effect of essential oil. The sleep behavior was provoked by intraperitoneal injection of thiopental at (60 mg/kg). Thirty minutes later, mice were submitted to all treatments (Badaoui et al., 2022). Experimental groups (n=6) were injected with essential oil at biological doses, vehicle and diazepam (2 mg/kg, used as reference drug). Different parameters were noted such as time of sleep state and latency. The cut-off time was taken as 120 min for all groups. To evaluate the possible implication of GABA receptors in the essential oil sedative activity, mice were pre-injected with flumazenil (2.5 mg/kg) 15 min before essential oil injection at a high dose or diazepam (2 mg/kg).

Anxiolytic activity

Elevated plus maze assay

The elevated plus maze test apparatus consists of 2 elevated close arms and 2 open arms above the floor surface (h=40 cm). Five groups (6 in each) had been injected with essential oil, diazepam (2 mg/kg; positive control) and vehicle. After 30 min, animals were positioned on the central intersection of the dispositive and both parameters had been recorded for 5 min: time spent by each animal and the number of entries in all arms (El Gabbas et al., 2018; Badaoui et al., 2022).

Hole-board assay

The hole-board assay was performed to evaluate the potential antianxiety activity of essential oil. The apparatus was heightened 40 cm above the floor and contained five holes (Ø=3 cm). The recorded parameters were latency and the number of head dips. Five groups (n=6) were treated with essential oil, vehicle and diazepam 30 min before the test. After the treatment, all animals were surveyed for 5 min. To investigate the involvement of $GABA_A$ receptors in the anxiolytic-like effect of essential oil, another group was pre-injected with flumazenil (2.5 mg/kg) used as $GABA_A$ antagonist 15 min before essential oil at 50 mg/kg (high dose) administration or diazepam (2 mg/kg). Thirty minutes later, animals were observed for 5 min in the same conditions described previously (Costa et al., 2014).

Exploratory assessment

Locomotion and exploration activities were performed using open field assay. On the day of the test, each mouse was positioned in the center of the apparatus for 5 min. The measured parameters are the number of crossings, immobility time, and redress behavior (Brown et al, 1999).

Statistical analysis

All statistical differences were presented as mean ± SEM using ANOVA followed by Kruskal Wallis or Tukey's tests followed by post-hoc analysis.

Results

Chemical profile

P. lentiscus essential oil obtained from fruits produced a characteristic odor, a clear and yellowish color. The GC-MS analysis demonstrated that *P. lentiscus* essential oil possessed 31 volatile compounds, corresponding to 97.7% of biochemical compounds identified. Major compounds were terpineol (24.1%), α-myrcene (23.0%), and α-pinene (15.9%). Other compounds were found but at low concentrations (Data not shown).

Acute toxicity assessment

Estimation of LD_{50}: Four doses (100, 500, 1000, and 1500 mg/kg) were used. The last 3 groups received by intraperitoneal injection presented neurobehavioral signs of toxicity as motor coordination disorders, lethargy, agitation, piloerection, hypersalivation, abdominal constriction, and eyelid close. Concerning mortality, only high doses 1000 and 1500 mg/kg induced mortality (1-12 hours and 1-8 hours respectively). The mortal $dose_{50}$, mortal $doses_{90}$, maximal tolerant, and maximal lethal doses values were 547.2, 985, 100, and 1000 mg.kg respectively.

Biochemical analysis: The administration of *P. lentiscus* did not impair the concentration of biochemical markers (serum transaminases, urea, creatinine) at 100 and 500 mg/kg. In contrast, at a high dose (1000 mg/kg) increased the level of all investigated parameters, which suggests a possible alteration of hepatocytes or renal functions.

Histopathological examination: The excised organs (liver, spleen, and kidney) of groups treated with 100 or 500 mg/kg exhibited no histological damage. The essential oil at 1000 mg/kg provoked a minor injury of the liver, such as mild vascular congestion (data not shown).

Antinociceptive activity

Abdominal constriction

This test was utilized to investigate the antinociceptive effect of essential oil. The administration of essential oil at all doses reduced significantly the number of abdo-

minal constrictions compared to the control group (Figure 1). The observed effect was potentiated at 50 mg/kg of *P. lentiscus* and comparable to the morphine and indomethacin groups (positive control).

Hot plate test

In this assay, the latency of jumping or hind paw licking was calculated. The latency time was increased by administration of essential oil 25, 50, and 50 mg/kg 30 min later. However, only an elevated dose (50 mg/kg) of essential oil enhanced the latency time on the heightened plate after 60 min of treatment, indicating the antinociceptive action was probably mediated by a central mechanism. At 30, 60, and 90 min after treatment, morphine possessed a remarkable antinociceptive effect (Figure 1).

Formalin test

In the formalin nociception model, administration of essential oil at pharmacological doses reduced considerably the licking time in the right paw in comparison to the vehicle group in neurogenic and inflammatory phases (Figure 1). To assess the involvement of muscarinic, opioid, serotonergic adrenergic pathways, ATP-sensitive K+ and voltage-gated calcium channel mice were pretreated with different antagonists. The findings presented in Figure 1 show the effect of pretreatment of animals by yohimbine, granisetron, nifedipine, glibenclamide, atropine, and naloxone on the antinociceptive effect of essential oil at high doses. In the neurogenic phase, pretreatment with naloxone and yohimbine reversed the observed effect previously mentioned of essential oil suggesting the implication of central mechanisms of nociception. However, atropine and glibenclamide reversed significantly the analgesic effect of essential oil (50 mg/kg) only in the second phase of the test. In addition, pretreatment with nifedipine, and granisetron could not reverse significantly the antinociceptive effect of essential oil in both phases (Figure 1).

Neurobehavioral assessments

Sedative effect

The sedative effect of essential oil was examined by thiopental-induced sleep duration (Figure 2). The essential oil at 25 and 50 mg/kg reduced the latency time of sleeping compared to the negative group and extended sleeping time, indicating a possible sedative-like effect of essential oil. The same results were observed for diazepam. To determine the implication of $GABA_A$ receptor, mice were pretreated with flumazenil ($GABA_A$ receptor antagonist). The latency time and sleeping duration of essential oil and diazepam were statistically reversed, suggesting the involvement of $GABA_A$ receptor.

Elevated plus maze test

This test was used to determine the potential anti-anxiety effect of some drugs using different experimental models of neurological disorders especially anxiety (Figure 3). Administration of essential oil increased the number of entries and time of performance in the open arms. However, the same parameters were decreased significantly in the close arms when compared to a negative control. Similar outcomes were shown for mice treated with diazepam.

Hole-board test

To confirm the antianxiety effect found in the previous model, the hole-board test was carried out. The degree of anxiety was estimated by different parameters such as the number of head dips and latency. Administration of essential oil increased significantly the number of head dips and decreased latency. Diazepam exhibited the same results, indicating the involvement of the benzodiazepines pathway (Figure 3).

To validate this implication, flumazenil (2.5 mg/kg) was utilized as $GABA_A$ receptor antagonist. Mice treated by *P. lentiscus* only showed an important anxiolytic-like effect. Nevertheless, pretreatment with flumazenil was completely inverted to the anxiolytic effect shown previously.

Open field assay

This test is used widely to investigate the degree of anxiety, exploration, and locomotion. The number of crossings were reduced significantly when mice were treated with essential oil at all biological doses (54%, 63%, and 71% respectively) (Figure 4). These involved a possible dose-dependent antianxiety effect. However, the immobility time increased in comparison to the negative control. Diazepam exhibited the same results.

Discussion

The present study of *P. lentiscus* essential oil demonstrates that GC-MS analysis reveals two monoterpenes and one oxygenated monoterpene (α-myrcene, α-pinene, and terpineol. respectively) as major compounds. Concerning acute toxicity, the obtained results show no signs of toxicity and mortality for all biological doses. Nevertheless, biochemical parameters were slightly modified only at a high dose (1000 mg/kg). Furthermore, the removed organs show any histopathological alterations. On the other hand, it demonstrates that essential oil possesses remarkable antinociceptive, sedative, and antianxiety effects.

P. lentiscus is a plant rich in volatile compounds responsible for many biological and neuropharmacological effects (Milia et al., 2021). The results of the phytochemical analysis following previous reports (Bouyahya et al., 2019) on the same species from North West of Morocco, whose composition is almost similar (α-pinene 20.5%, myrcene 9.0%, and terpineol 5.8%) to

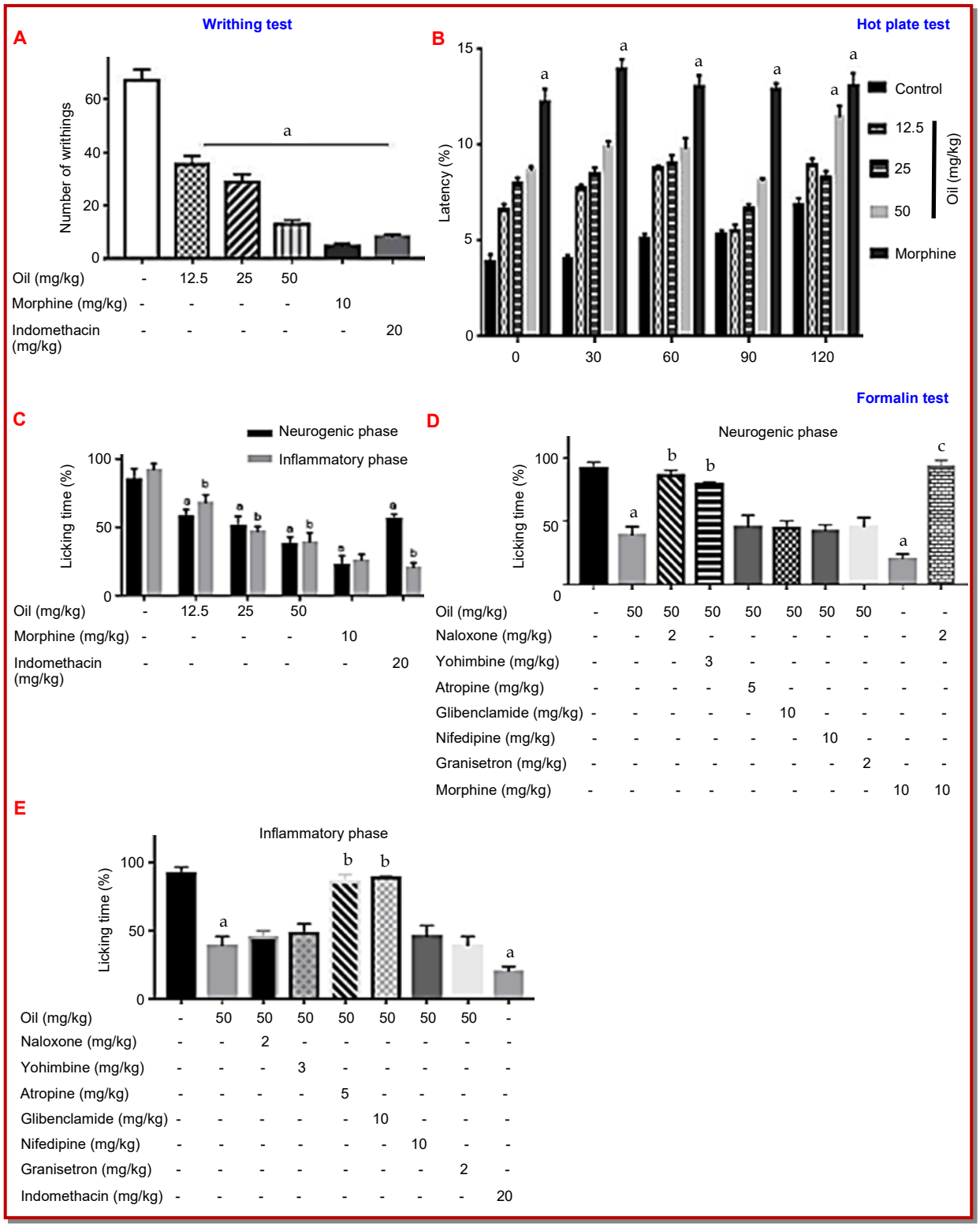

Figure 1: Antinociceptive (A-B) and analgesic (C-E) effects of *P. lentiscus* essential oil in writhing test, hot plate test and formalin test (neurogenic and inflammatory phases). Data are presented as mean ± SEM. 'a' indicates difference between negative control and treated groups, 'b' indicates differences between treated group and antagonists. 'c' indicates difference between indomethacin- and oil-treated group. Statistical analyses were done using ANOVA one-way followed by Tukey's post hoc test

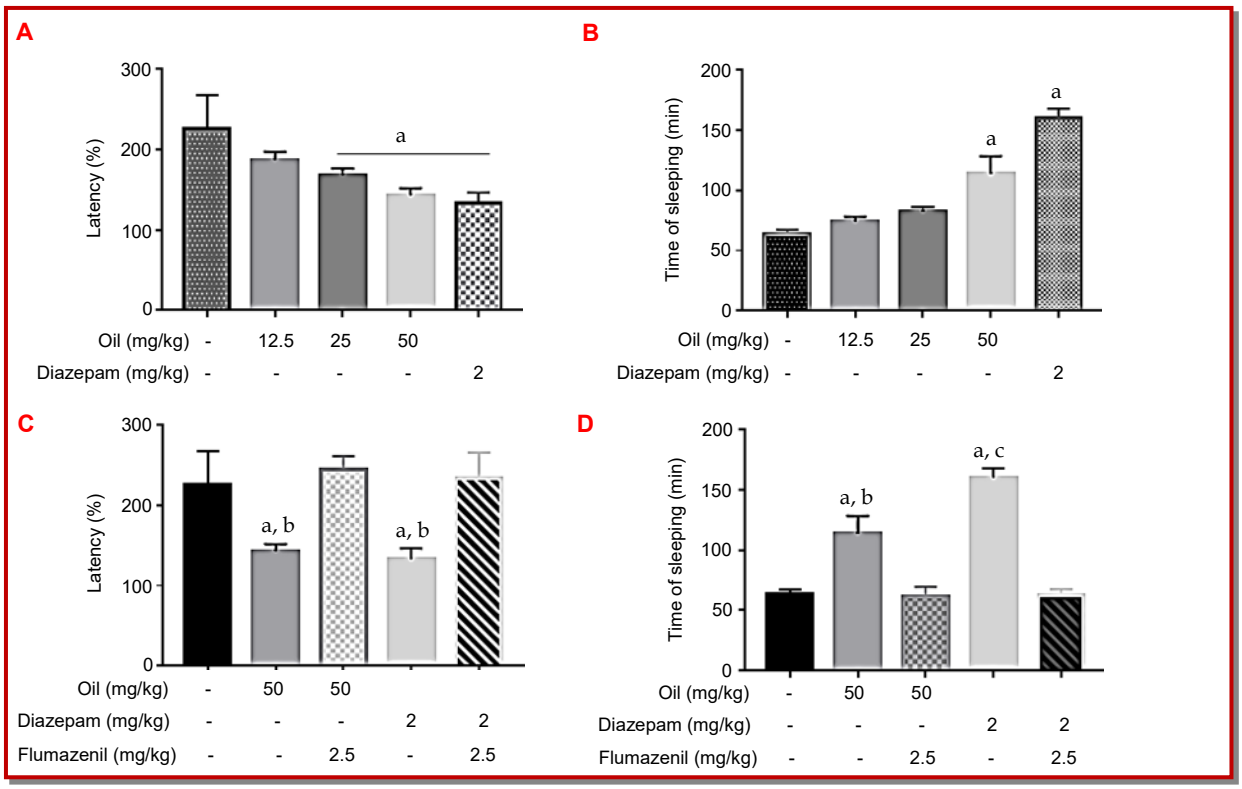

Figure 2: Effect of essential oil of *P. lentiscus* in the thiopental-induced sleeping time test and latency (n=6/group). Data are expressed as mean ± SEM, where 'a', 'b' and 'c' indicate $p<0.05$, significant difference from the treated group according to ANOVA one-way followed by the Kruskal Wallis test

that identified in the present samples from South Morocco. Another study report that *P. lentiscus* essential oil from Tunisia possesses several monoterpenes and sesquiterpenes compounds (Ben khedir et al., 2016). The variation in chemical composition could be due to the growth environment, harvesting season, altitude, and climatic conditions.

The analgesic activity of *P. lentiscus* essential oil was examined using three animal models. The abdominal constrictions are a classic model to determine the antinociceptive potential of some drugs. However, the injection of acetic acid solution provokes an increase in peritoneal fluid of histamine, prostaglandin, substance P, and serotonin (Gawade, 2012). First, *P. lentiscus* decreased significantly the numbers of cramps. These results are similar to those exhibited by reference drugs indomethacin and morphine. The present findings corroborate previous reports elsewhere in another species belonging to Anacardiaceae family (*P. atlantica*). In a study, *P. atlantica* extract produces a significant analgesic effect in different animal models (Nadri et al., 2018). *P. atlantica* possesses a significant anti-inflammatory activity (Hajjaj et al., 2018).

The hot plate model is one of the most important central models to evaluate the analgesic effect (Jeong and Holden, 2008). The high temperature causes two behaviors (jumping and licking). In this assay, intraperitoneal administration of *P. lentiscus* increased licking time up to 60 min post-treatment, this indicates a possible central analgesic effect. For this purpose, another test was used for this end, injection of formalin solution in the right paw. In this test, animal responses to moderate pain caused by a wounded tissue may be confirmed with the ability to produce two responses neurogenic and inflammatory (Hunskaar et al., 1987). The finding demonstrates that *P. lentiscus* produces a remarkable antinociceptive effect by decreasing time licking in both phases. In general, drugs that can reduce nociception response via a central process such as morphine could exhibit their effect by binding to opioid receptors. Therefore, *P. lentiscus* could be acting similarly to morphine. The hot plate confirmed this hypothesis.

In the aim of investigating the possible mechanism implied in the antinociceptive effect of *P. lentiscus*, mice were pretreated with antagonists (naloxone, atropine, yohimbine, nifedipine, granisetron, and glibenclamide). In the neurogenic phase, the analgesic response was inverted by pretreatment with yohimbine and naloxone suggesting the implication of adrenergic and opioid receptors. Nevertheless, in the inflammatory phase, the analgesic activity was reversed only by pretreatment with atropine and glibenclamide indicating the possible involvement of muscarinic and ATP-sensitive K^+ channel pathways.

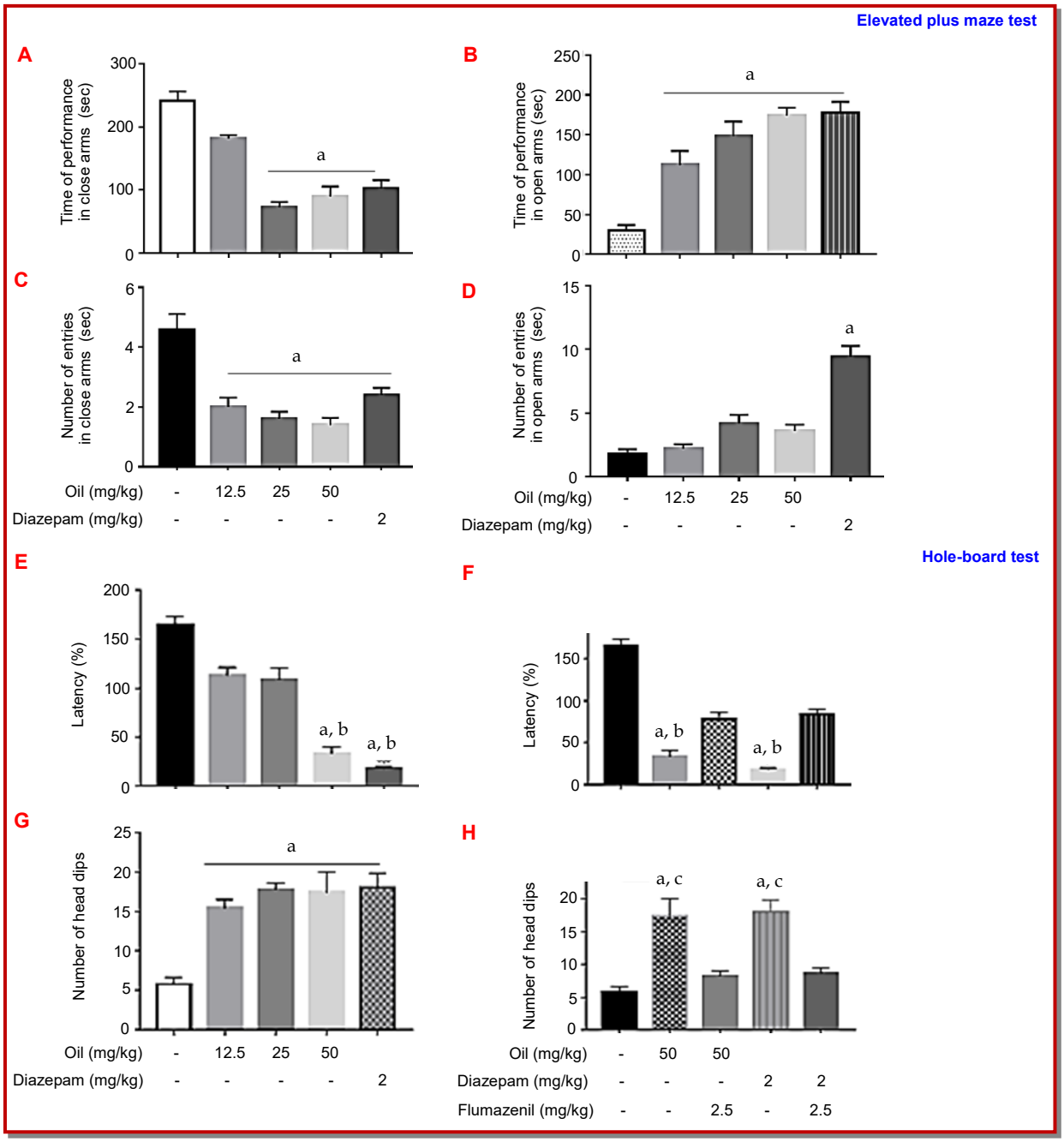

Figure 3: Effect of *P. lentiscus* essential oil in elevated plus maze test (A-D) and hole-board test (E-H) in mice (n=6 in each group). The results are expressed as mean ± SEM, "a" indicate significant difference between control group and treated groups. "b" and "c" indicate significant difference between treated group (50 mg/kg), diazepam and antagonists. Statistical analyses were done according to ANOVA one way followed by Kruskal Wallis/Tukey's test

Sleeping disease constitutes another factor of central nervous system illness that affect several people worldwide. Thiopental-induced sleeping behavior is one of the major animal models to investigate the possible sedative action of biomolecules. The administration of 25 and 50 mg/kg decreased the latency time and extended the sleeping time. These results following a previous investigation reported (Milanos et al., 2017).

The degree of anxiety was evaluated using different animal models. The elevated plus maze test is one of the currently utilized assays to investigate a potential anxiolytic effect of new drugs. Anxiolytic molecules can increase the number of entries and time of permanence in the open arms. In this end, *P. lentiscus* at biological doses decrease the time of performance, number of entries in the close arms and increases the time of

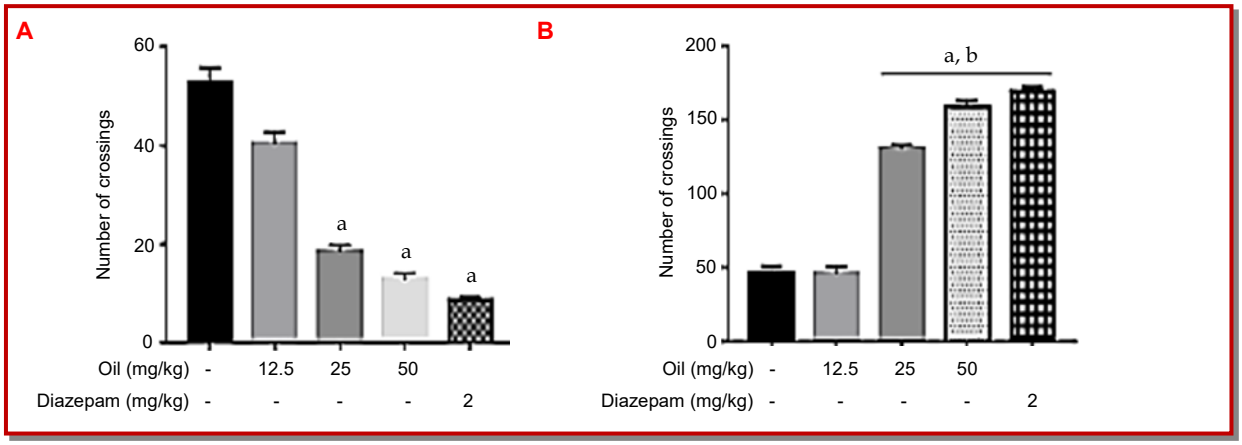

Figure 4: Effect of the essential oil of *P. lentiscus* in the open field test (n=6 per group). Data are expressed as mean ± SEM, where 'a' and 'b' p<0,05 indicate difference between control group and essential oil (12.5 mg/kg) respectively. Statistical analysis were done using ANOVA one-way followed by post-hoc Tukey's test

performance, frequency in the open arms significantly in comparison to the negative control suggesting a remarkable antianxiety effect of *P. lentiscus* essential oil from fruits. The observed anxiolytic effect was confirmed in hole-board test, in which *P. lentiscus* increased significantly the number of head dips and reduced the latency time compared to the control group. These findings are comparable to the diazepam group. This pharmacological effect was inversed when animals were pretreated with flumazenil indicating that *P. lentiscus* acts by $GABA_A$ receptors pathway. These outcomes are in agreement with a previous study (Souza et al., 2015). On the other hand, exploratory and locomotor activities were performed using open field assay. Outcomes demonstrate that the administration of *P. lentiscus* increases the time of immobility and decreases the number of crossings.

In pain control systems, the mechanisms of spinal and/or supraspinal pathway integration include the liberation of several neuromediators especially acetylcholine, noradrenaline, endogenous opioid, and serotonin (Fiorino and Garcia-Guzman, 2012). As a result, the implication of these modulatory systems in *P. lentiscus* nociception was studied. The antinociception of *P. lentiscus* essential oil was completely inverted when mice were pretreated with yohimbine (α_2-adrenergic receptors) in the first phase of the formalin assay. The spinal noradrenergic and descending noradrenergic pain pathways that originate in the supraspinal region have been explained by noradrenergic control systems (Pertovaara, 2006). Thus, it is suggested that the analgesic action of essential oil could be primarily mediated by increased concentration of noradrenaline and by adrenoceptor stimulation. Among the systems involved in pain control is the opioid system as descending inhibitory pathways. To this end, naloxone was used to antagonize the opioid receptors. The antinociceptive activity of opioids can be mediated by several receptor subtypes such as mu, kappa, and delta (Al-hasani and Bruchas, 2011). It was demonstrated that the analgesic effect of this oil may be mediated by spinal/supraspinal -organized. In this study, analgesic effect obtained was inverted when animals were pretreated with glibenclamide in the second phase. These out-comes suggested the involvement of ATP-K^+ sensitive channels (Boakye-Gyasi et al., 2017). In addition, the implication of the muscarinic pathway in the analgesic effect of *P. lentiscus* essential oil was also investigated using atropine. The obtained finding demonstrates that cholinergic receptors are involved in the inhibition of pain only in the second phase of the formalin test. Various monoterpene compounds are known for their antinociceptive effects like α-pinene, myrcene, and terpineol, which are identified as major compounds in *P. lentiscus* essential oil (De Lavor et al., 2018).

Essential oil of *P. lentiscus* possesses a remarkable anxiolytic and sedative effect, but pretreatment with a competitive $GABA_A$ receptor antagonist reversed the antianxiety effect. Monoterpenes and sesquiterpenes may be implicated in these effects acting by GABA transmission and chloride-channel complex (De Sousa et al., 2015; Milanos et al., 2017). In addition, terpenoid compounds can cross the blood-brain barrier easily and act on the central nervous system due to their hydrophobic characteristics (De Oliveira Junior et al., 2018) which could explain the anxiolytic effect.

Conclusion

P. lentiscus essential oil possesses a remarkable antinociceptive activity. This effect could be mediated by opioid, adrenergic systems, cholinergic receptor, and ATP-sensitive channels. It also provides beneficial effects on sedation and anxiety. This effect may be correlated to interaction with the GABAergic pathway.

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Ethical Issue

All experiments were performed in accordance with the European Community Guidelines (EEC directive 86/609/EEC, November 24, 1986). All efforts were made to keep animals suffering to a minimum as possible and to reduce the number of animals used in all experiments. The acute toxicity assessment followed the standard procedure described in OECD guidelines 425, published in 2008.

Conflict of Interest

Authors declare no conflict of interest

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