A contribution to the therapeutic valorization of Moroccan Cistus Ladanifer L.

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Abstract. Cistus ladanifer L. is a medicinal shrub traditionally used against several diseases. The present study was performed for trying to enrich the potentialities of this plant by evaluating the possible analgaesic effect of aqueous extract of Cistus. ladanifer (AECL) and oral formulation prepared from AECL. The analgaesic effect was tested using the acetic acid induces writhing and formalin models. The results showed that both aqueous extract and herbal syrup induced a significant analgaesic activity in the two nociceptive tests. Moreover, the oral formulation produced the potent analgaesic effect. We conclude from the results of this work that the Moroccan C. ladanifer L orally administrated to mice possesses an efficient analgaesic effect, and may will contribute to developing a natural analgaesic drug.

1 Introduction

Cistus ladanifer L. and other species of Cistaceae have been used in many Mediterranean folk remedies. In traditional Moroccan medicine, this plant has been claimed to be anti-diarrhoeal, anti-acid and antispasmodic [1]. Moreover, it is used as aphrodisiacs [2], and for diuretic effect [3].

The investigations of the analgaesic activity of certain species of Cistus were previously carried out by several researchers, according to A.I. De Andres et al., (1999) [4], the aqueous extract of Cistus populifolius (L.) produced central antinociceptive effects, and M. Ark et al, (2004) [5], reported that the chloroform extract of Cistus laurifolius (L.) produced a central analgaesic activity. E. Yeşilada et al., (2007) [6], demonstrated that the flavonoids isolated from Cistus laurifolius are the effective principle responsible for the analgaesic effect. The analgesic activity of Cistus ladanifer, injected intraperitoneally into rats, was demonstrated by A. El Hamsas El Youbi, et al., (2016) [2], this study despite its importance due to the fact that it discovered a new potentiality of C. ladanifer, but still needs to be completed because, on the one hand, it was tested by a single test (hot plate) which studied the analgaesic response mediated only by the central system [7], while there is another way of the analgaesic response (not yet studied for this plant) mediated peripherally and which can be assessed by writhing test [8], and on the other hand to better understand the mechanism of action of C. ladanifer, there is another test, the formalin test which divides the analgaesic response into two phases, an acute peripheral, and a delayed second involves the production and release inflammatory mediators [9].

The aim of our study is, first of all, completing the studies previously carried out on the potentialities of C. ladanifer by testing the effect of AECL by other validated tests (formalin and whriting) and by another route of administration (oral), and secondly preparing a natural syrup as an alternative to analgaesic medication.

2 Materiel and methods

2.1 Collection and identification of plant material

C. ladanifer aerial parts were manually harvested with scissors during the month of July 2018 from its natural habitat, northern Morocco, and was identified by Professor Abdeslam Ennabili from sidi mohamed ben abdellah university, Morocco, and a voucher specimen ((2018-1 -CL), was prepared and deposited in the Herbarium of Natural Resources and Sustainable Development (NRSD). Faculty of Science, Ibn Tofail University, Kenitra, Morocco. We were not required to have any permission because this work does not concern protected or endangered species. Cistus ladanifer is a common plant growing near the sidewalk.

2.2 Extraction procedure

Aerial parts of C. ladanifer were dried in the shade at room temperature with occasional shifting, and

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powdered by using a mechanical grinder to achieve a fine particle size, and kept in the dark until future use.

Powder of C. ladanifer was subjected to decoction extraction as it described in folk Medicine [10]. To do this, 25 g of powder was extracted with 1 L of distilled water for 30 minutes. The obtained extract was filtered through a Whatman No. 1 filter paper and evaporated in a vacuum to give AECL.

2.3 Preparation of Herbal Syrup

The formulation of herbal syrup (from AECL) and reference syrup (medication) were performed in three steps as described by JA. Avbunudiogba et al., (2013) [11]:

1- Preparation of simple syrup BP: we dissolved 667 g of sucrose in distilled water to obtain a final volume 1000 ml of concentrated simple syrup, which was filtered under sterile conditions and used as a vehicle.
2- Preparation of herbal syrup: to prepare the dose 500 mg/kg (analgaesic dose) of herbal syrup, 500 mg of AECL was dissolved in 10 ml of simple syrup BP.
3- For preparing medication (reference syrup) at dose 10 mg/kg, we dissolved 10 mg of Indomethacin in 10 ml of simple syrup BP.

2.4 Experimental animals

The experiment was conducted using healthy and sexually mature Swiss albino mice (average weight of 22 g). All rodents were maintained in environmentally controlled rooms at 24 ± 1 °C with relative humidity at 50±5% and 12/12 h light-dark cycle over the experiment duration. The animals were acclimatized to laboratory conditions for 1 week prior to the start of the experiment, and they were housed and maintained in clean plastic cages with proper supply of commercial rodents chow and tap water ad libitum. To preserve the hydration rate constant, Water was available at all times while were deprived of food for 12 h before doses administration. All animal work was carried out in general accordance with the general instructions for the use and care of

laboratory animals [12]. For statistical and ethical reasons, each group of animals used in this study consisted of 5 mice, providing enough data for statistical analysis but minimizing the number of animals used.

The different lots received orally either by distilled water, a reference Indomethacin dranaug, AECL at doses 300 and 500 mg/kg of body weight (BW)., simple syrup BP and herbal syrup.

2.5 Acute oval toxicity in mice

Procedure was performed to tested the acute oral toxicity according to the guidelines of OECD (2001) [13-14]. Twenty mice were randomly divided into five groups (n=5), the AECL (500, 1000, 2000 mg/kg, BW) was single treated by gavage to three groups of mice, and the mice in control group were received the same volume of water. Animals were nearly checked for mortality and changes in general behaviour every 30 min during the first six hours after dosing, then they were observed daily for possible behavior changes such as refusal of food or water, tremors, weakness, convulsions, sleep, salivation, impaired somato-motor activity and diarrhea for up to 14 days after treatment [15].

2.6 Evaluation of Anti-nociceptive activity

2.6.1 Acetic-acid-induced writhing

Peripheical analgaesic effect of AECL was assessed by writhing test [16], seven groups of mice (n = 5) were used in this study for testing samples. Group 1 received Indomethacin drug (10 mg/kg, BW), the second group was treated with distilled water (10 ml/kg, BW), for groups 3 and 4 they were received the AECL at doses 300 and 500 mg/kg, BW, respectively. Whereas group 5 was received 500 mg/kg, BW of herbal syrup and the sixth group served as a negative control of the herbal syrup was treated with simple syrup BP (10 ml/kg, BW) the last group received reference syrup (10 mg/kg, BW) Thirty minutes after the oral administration of test samples, the mice were intraperitoneally injected with 0.2 ml of acetic acid (0.6%). Five minutes later, the number of muscular contractions (writhing response) was counted during 30 min.The percentage of inhibition of the writhing response provoked by acetic acid was calculated by the following equation:

$$\% \text{ inhibition} = \frac{\text{average writhes (control)} - \text{average writhes (treated)}}{\text{average writhes (control)}} x\,100$$

2.6.2 Formalin Test

Twenty microliters of 1% formalin were administrated subcutaneously in the mouse's right hind [17]. Licking time of the injected paw (indicator of pain) was recorded for 5 min (phase 1, neurogenic) and from 15 to 30 min (phase 2, inflammatory), after the formalin injection [18]. One hour before formalin injection, the animals were given orally the following treatments:

- One group of positive control received Indomethacin drug (10 mg/kg, BW);
- First negative control group treated with distilled water (10 ml/kg, BW);
- An experimental group received 300 mg/kg, BW., of AECL;
- An experimental group treated with 500 mg/kg, BW., of AECL;
- An experimental group received herbal syrup (500 mg/kg, BW);
- second negative control group treated with simple syrup BP (10 ml/kg, BW), without any anti-healing molecules.
- Second positive control received reference syrup (10 mg/kg, BW).

2.7 Statistical analysis

All data (text, tables) are expressed as mean ± standard error of measurement (SEM) of 5 repetitions. Within group comparisons were performed by the analysis of variance using ANOVA test. Statistical significance between control and treated groups were determined by the student's t-test, and a p-value of 0.05 was used as the threshold for statistical significance.

3 Results

3.1 Acute toxicity study

The acute toxicity studies were executed following the OECD guidelines 420, in which the maximum threshold test dose used is 2000 mg / kg. No test substance- allied death was recorded in 2000 mg/kg [19]. Thus, testing at a dose greater than 2000 mg/kg may not be needed and the substance was considered nontoxic [20]. During the observation period (14 days), the AECL at a dose of 500, 1000 and 2000 mg/kg orally given to mice did not produce mortality in tested groups, and any behavioural changes or other signs of toxicity was detected, similar results were observed in the control group (Table.1).

Table 1. Evaluation of the AECL toxicity in mice.

Group	Dose (mg/kg)	Death/number of animals	Toxic symptoms
Control	0	0/5	None
AECL	500	0/5	None
	1000	0/5	None
	2000	0/5	None

None = no toxic symptoms during the 14-day period of observation after the oral administration.

3.2 Anti-nociceptive activity of AECL

*3.2.*1 Formalin-induced paw licking

AECL in both doses (300 and 500 mg /kg) caused reduction of the first phase of the licking response in the formalin test from 61,00 ± 1,15 to 26,67 ± 0,88 seconds (S) at 500 mg/kg dose, and increased the inhibition percentage of pain to 56,23%. Furthermore, the ACEL significantly ($p < 0.001$) reduced licking times of the second phase from 53,33 ± 2,03s to 20,67 ± 0,88s at 500 mg/kg dose and increased the inhibition percentage of pain to 61,05 % (Fig.1, Table.2).

Table 2. Effect of AECL on Formalin-induced paw licking in mice.

Group	Dose (mg/kg)	Inhibition (%)	
		First phase	Second phase
AECL	300	45,46 ± 2,83	46,72 ± 2,11
AECL	500	56,23 ± 1,92	61,05 ± 2,81
Medication	10	65,68 ± 1,17	71,03 ± 1,37

Values are expressed as mean ± SEM, n = 5.
P < 0.05 is considered significant in relation to the control

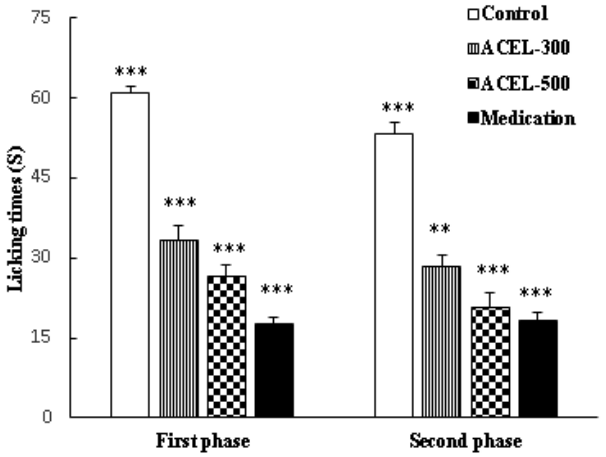

Fig. 1. Effect of the different doses of AECL and Medication on formalin-induced paw licking in mice.

Values are expressed as mean ± SEM, n = 5.
***P<0.001; **P < 0.001; compared to control group.

3.2.2 AECL reduced painful sensation in the Acetic-acid-induced writhing

AECL showed significant (P <0.001) and dose-dependent analgaesic activity. The extract caused inhibition of the number of writhes at 300 mg/kg (AECL-300) and 500 mg/kg (AECL-500) of the order of 34,01 % and 55,83%, respectively (Table 3).

These results (Fig.1, Table 3) allow us to deduce that the ACEL administrated orally to mice at 300 and 500 mg/kg produced a significant ($P < 0.001$) and dose-dependent analgaesic effect in both acetic acid and formalin tests, and that the dose of 500 mg / kg of AECL is the most effective.

Table 3. Effect of AECL on acetic acid-induced writhing in mice

Group	Dose (mg/kg)	Number of writhes	Inhibition (%)
Control	-	62,67 ± 0,88	-
AECL	300	41,33 ± 0,88***	34,01 ± 1,77
AECL	500	27,67 ± 0,33***	55,83 ± 0,90
Medication	10	17,00 ± 1,00***	72,87 ± 1,56

Values are expressed as mean ± SEM, n = 5.
***P<0.001; compared to control group.

3.3 Analgaesic effect of herbal syrup

In order to enhance the use of this plant, we have chosen the most effective dose of our extract (500 mg/kg) for preparing it in form easy to administer (syrup) and we evaluated its analgaesic action in the formalin and acetic acid tests:

3.3.1 Acetic-acid Test

As noted in the results presented in the table 4, we noticed, on the one hand, the herbal syrup had a significant ($p < 0.001$) analgaesic effect, it reduced the

number of contortions to 24.33 compared to the simple syrup (62.33). On the other hand, the analgaesic effect shown by the herbal syrup is higher than that produced by AECL, the percentages of inhibition of constrictions were 60,98 %, and 55,83 % for herbal syrup and AECL-500, respectively. Moreover, the percentage of inhibition of the formulated form of AECL (herbal syrup) is almost similar (difference not significant) to that of reference syrup they are respectively 60,98% and 73,21%.

Table 4. Effect of herbal syrup on acetic acid-induced writhing in mice.

Group	Dose (mg/kg)	Number of writhes	Inhibition (%)
simple syrup BP	-	62,33 ± 0,67	-
Herbal syrup	500	24,33 ± 0,67***	60,98 ± 0,66
Reference syrup	10	16,67 ± 1,20***	73,21 ± 2,22

Values are represented as mean ± SEM, n = 5.
***P<0.001; compared to control group.

3.3.2 Formalin Test

According to the results of this test the herbal syrup at the doses of 500 mg/kg produced significant anti-nociception in the formalin test, with a maximum percentage of inhibition equal to 57,69 % at the earlier phase , and reduced the licking response from 60,67 ± 1,45s to 25,67 ± 0,67 s, followed by the late phase that increased the percentage of inhibition to 65,38 percent, and reduced the licking response to 19,67 ± 0,67s. While indomethacin syrup recorded as percent inhibition 60.48 and 72.98% in the first and second phase respectively (Table 5). In addition, there is no significant difference between the analgaesic effect expressed by herbal Syrup and that produced by the reference drug in the two phases of the formalin test (Fig. 2), therefore the analgaesic effect induced by the syrup is almost the same as that expressed by the medication.

Table 5. Effect of herbal syrup on Formalin Test in mice.

Group	Dose (mg/kg)	Inhibition (%)	
		First phase	Second phase
Herbal syrup	500	57,69 ± 0,67	65,38 ± 2,14
Reference syrup	10	60,48 ± 0,97	72,98 ± 2,31

Values are expressed as mean ± SEM, n = 5.
P < 0.05 is considered significant compared to the control

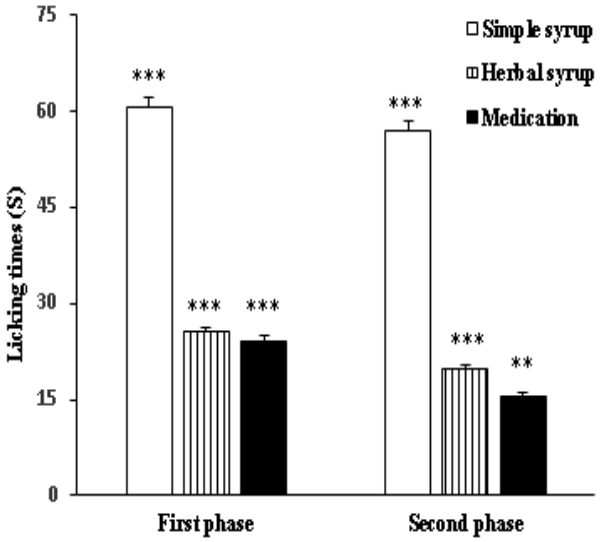

Fig. 2. Effect of the herbal syrup on formalin-induced paw licking in mice. Values are expressed as mean ± SEM, n = 5. ***P<0.001; **P < 0.001; compared to control group.

4 Discussion

In the first part of this work we examined the toxicity of AECL orally treated in mice, we noticed that the animals received up to 2000 mg / kg produced no deaths or signs of adverse effects. These findings indicate that the LD50 value is higher than 2000 mg / kg. Moreover, as per the Classification of Globally System Harmonized (GSH) for Chemical Mixtures and Substances adopted by the OECD, we can classify the ACEL as a class 5 substance, and therefore considered it as non-toxic drug [14]. These results corroborate the non-toxicity of Cistus ladanifer, reported in previous acute toxicity studies, such as the study conducted by M. Aziz et al., 2011[21]. in which they found that oral administration of Cistus ladanifer decoction at doses up to 5000 mg / kg did not induce death or adverse effects in mice. In addition, M. El Kabbaoui and his team concluded that the infusion of Cistus ladanifer at doses below 2000 mg / kg did not show mortality or toxicological effect in mice [22].

The anti-nociceptive activity was carried out in mice using two validated pain models: acetic acid test induced writhing reflux following an injection of acetic acid, useful to evaluate visceral pain [23], and the formalin test caused paw licking after injection of formalin to assess spontaneous pain [24]. The results of the writhing test show that at all doses studied, AECL and herbal syrup of C. ladanifer significantly (P<0.001) reduced writhing induced by acetic acid which suggests that a part of its antinoceceptive effects may be peripherally mediated [8]. Moreover, AECL (in both doses 300 and 500 mg /kg) and herbal syrup caused inhibition of all phases of formalin-induced pain, which allowed us to suggest that its analgaesic effects may mediate both centrally and peripherally [16-17]. Furthermore, the analgaesic effect shown by the herbal syrup is higher like the effect of the non-steroidal anti-inflammatory agent (indomethacin).

This effect may be due to the fact that the plant contains flavonoids [25-29], because the effect analgaesic of flavonoids has been proven by several studies [30-32], this allows us to suggest that the analgaesic effect induced by ACEL and herbal syrup is due to the presence of flavonoid compounds.

Finally, we formulated a syrup based on the aqueous extract at its effective analgaesic dose (500 mg / kg), in order to assess its possible analgaesic effect, the results of this part showed a slight increase (statistically not significant) of the anti-nociceptive effect of AECL-Syrup compared to EECL-500, in both the analgaesic models studied, this slight increase may be due to the anti-nociceptive potentiality of the sucorse demonstrated by several previous works [33-34].

5 Conclusion

In summary, the results of this study showed the analgaesic effect of the AECL and the herbal syrup of the aerial parts of C. ladanifer administrated orally and evaluated by both acetic acid and formalin tests.

Considering also that AECL shows no signs of short-term toxicity, our work will contribute to developing a natural analgaesic drug (appropriate for patients and easy to administer) without preservatives or other chemical additives (which may be harmful to health).

However, additional chronic toxicity studies and phytochemical analyses must be carried out in order to exclude any long-term undesirable effect and to identify exactly the chemical family responsible for this analgaesic activity.

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