Antinociceptive Activity and Acute Toxicity of Moroccan Black Propolis

M. Mountassir[1] , S. Chaib[1], Y. Selami[1], H. Khalki[1], A. Ouachrif [1], S. Moubtakir[1], R. Aboufatima[1] , A. Zyad [3], A. Benharref [2], A. Chait [1]

[1:] Laboratory of Neurobiology, Pharmacology and Behavior, Faculty of Sciences Semlalia, Marrakech, Morocco.
[2:] Laboratory of Chemical of Natural substances and Heterocycles. Faculty of sciences Semlalia, Marrakech, Morocco .
[3:] Laboratory of Biological Engineering. Natural Substances, Cellular and Molecular Immuno-pharmacology Group. Sultan Moulay Slimane University; Faculty of Science and Technology, Box: 523 Béni-Mellal 23000, Morocco.

***Abstract*--Propolis is a resinous natural hive product derived from plant exudates collected by honey bees. It has been used in folk medicine since ancient times. The chemical composition Depends qualitatively and quantitatively on with the geographical and botanical origins. In the present study, we attempted to verify the possible antinociceptive action of water extract obtained from Moroccan propolis (WEP). We used (acetic acid, formalin, and hot-plate tests) in order to characterize the analgesic effect. The extracts significantly, and in a dose-dependent manner, reduced the pain induced by intraperitoneal injection of acetic acid. The water extract of propolis (WEP) have also a significant effect in the hot plat test .The formalin test significantly reduced the painful stimulus in the early phase and the late phase of the test.**

These results suggest that the compounds present in the extract of propolis activated both central and peripheral mechanisms to elicit the analgesic effect.

Keywords: Moroccan propolis; antinociceptive action; analgesic.

I. INTRODUCTION

Propolis or bee glue is a complex resinous mixture of different plant exudates, which is gathered, modified and used by honeybees as a general purpose sealer and draught excluder in their hives. The uses of this propolis in the life of the colony are related to both mechanical and antibiotic properties [1] .

The first registers of the utilization of propolis were those involving its use in mummification of the ancient Egyptians. It was also used in the treatment of infections and swelling by the Assyrians. Later, the medicinal utility of propolis in both internal and external cicatrization was described by the early Greeks, especially Aristotle, Dioscorides and Hippocrates; the Romans, specifically Pliny and Galen, also described its medicinal uses [2,3] . Since then, there have been numerous references to its ethnopharmacological uses. The Incas employed it as an antipyretic [4] , and it was employed in the healing of wounds during the XIX century Anglo-Boer in South-Africa and the XX century Second World War in the ex-Soviet Union [5,2] . It had also been listed in the London pharmacopoeia of the XVII century [4].

Propolis has over 150 constituents, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids,and amino acids, but its composition varies qualitatively and quantitatively with the geographical and botanical origins [6,7] .

In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris depending on the place and time of collection [8,9].

The tentative of molecular analysis of Moroccan propolis extracts by HPLC-MS is reported for the first time. The probably composition is comparable to molecules and compounds found in samples from other regions [10]. The exact determination of this molecular composition requires further chromatographic and spectral studies. On the other hand, the interaction between these molecules ant the cell components remains to be established [11].

Due to propolis versatile biological and pharmacological effects, it has wide applications in medicine, cosmetics and food industry [12]. It has been suggested that the presence of a large number of flavonoids,aromatic acids and phenolics compounds are responsible for the most biological and pharmacological activities of propolis [13,14].

Several investigations on propolis have been done in Eastern Europe and South America, but there is no report about the water extract of Moroccan propolis. The aim of this study is to investigate antinociceptive activity and acute toxicity of water extract of Moroccan propolis (WEP).

II. MATERIALS AND METHODS

A. Propolis origin

Propolis sample has been kindly donated by Pr. A. CHAIT. From colonies of honeybees located in Oulad Ayyad near Beni-Mellal by using plastic nets in fall 2008.

B. *Animal models and habituation*

Male Sprague–Dawley rats and male mice, weighting 180–230 and 25–30 g, respectively, were used in this study. Animals were housed in groups of three rats or six mice per standard makrolon cage, on 12-h light/12-h dark cycle; and air temperature was maintained at 22±2 ◦C. They were offered food and water ad libitum. Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals [15]. All efforts were made to minimize the number of animals used and their suffering.

C. *Water extract of propolis (WEP) preparation*

Crude propolis was treated with ultrapure water and kept at 80°C 30 minutes. The insoluble portion was separated by filtration to obtain the water extract. Different concentrations of WEP were obtained by solubilisation of 1 ; 2,5 g and 5g in 100 ml of ultrapure water.

D. *Acute toxicity*

The toxicity study was carried out using male and female Swiss mice weighing 25–30 g each. The animals were randomly distributed into one control group and three treated groups, containing five animals each. They were maintained on animal cages, provided with water ad libitum. The animals were observed continuously for the first 4 h and then each hour for the next 24 h and at 6 hourly intervals for the following 48 h after administering of the extract, to observe any death or changes in general behaviour and other physiological activities [16, 17].

E. *Antinociceptive tests*

- *Writhing test*

The antinociceptive effect was evaluated in mice by the writhing test induced by acetic acid 0.6% (0.1 ml/10 g; i.p.).Each dose of the extract was administered 30 min before the acetic acid injection. Five minutes after the administration of the acid, the number of writhes and stretching movements (contraction of the abdominal musculature and extension of hind limbs) was counted over a 5 min for a period of 30 min. The strength of the elicited analgesic effect was compared to that of an effective dose of acetylsalicylic acid (ASA, 200 mg/kg, i.p.) [18].

- *Formalin test*

The method used in the present study was similar to that described previously by De Miranda et al. (2001) [18] with slight modifications. It consists briefly of injecting subcutaneously 20µl of 20% formalin into the right posterior paw of mice 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). The formalininduced licking of the paw was considered as indicative of the nociceptive behaviour. Using a chronometer; the total time spent in licking and biting the injected paw is recorded, quantifying the nociceptive behaviour. However, as the formalin test in rodent consists of two successive phases [19]. The initial nociceptive response normally peaked 5 min after formalin injection (early phase) and 15–30 after formalin injection (late phase), representing the tonic and inflammatory pain responses, respectively. The animals were pre-treated intraperitoneally with the extract, or with morphine (10 mg/kg) or ASA, 30 min beforehand.

- *Hot plate test*

The heated surface of a hot plate analgesia meter (Ugo Basil, Italy; Socrel DS-37) was maintained at 55±0.2 ◦C. Each animal was placed into a glass cylinder (diameter 20 cm) on the heated surface of the plate. Treated rats received orally three doses of aqueous extract (1%, 2,5% and 5% b.w.). Control group received orally water at 10 ml/kg. Morphine was administered intraperitoneally at 10 mg/kg (b.w.). The latency to nociceptive response was recorded before treatment and at 30 min after intraperitoneal administration of morphine and at 60 min after oral treatment with aqueous extract. Licking of paws and jumping were the parameters evaluated as the thermal reactions.

- *Statistical analysis*

The results were presented as means±S.E.M. and the comparisons between the experimental groups were made using Student's t-test (ANOVA two way) . p values less than 0.05, 0.01 or 0.001 were considered as indicative of significance. The inhibition percents were calculated by the following formula: inhibition percent = (1−Vt/Vc)×100, where Vt and Vc represent the number of writhes or the licking paw time of the treated and control groups, respectively.

III. RESULTS

A. *Acute toxicity*

In this study there were no death and toxicological changes in clinical and behavioral signs in test animals group up to 48 h. The water extract of propolis is not toxic.

B. *Anti- nociceptive studies*

The water of propolis at dose of 5% showed anti-nociceptive in all the tree different models of nociception used to investigate the anti-nociceptive effects of the extract in this study .

In the wrinthig test (Table 1), the extract decreased the number of acetic acid-induced abdominal constrictions in mice. The effect was dose-dependent and the values were found to be significant at doses level tested ($\rho<0,05$) at dose 2,5% and ($\rho<0,01$) at 5% of extract .The maximum percentage inhibition of constrictions of 49% was observed at 5% for the extract.

In the hot plate test (Table 2), treatment of rats with the WEP significantly increased the time spent on the hot plate ($\rho<0,05$) at both of doses 2,5% and 5%.

In the formalin test (Table3), the effect was not dose-dependent. There was a significant (ρ<0, 05) reduction in responses to nociception during both phases I and II for the extract at 5%.

TABLE 1: Effect of water exract of propolis (WEP) on the acetic acid-induced writhing behavior in mice

Treatment	**Dose**	**Number of writhings**	**%Inhibition**
Control WEP	_ 1%	92,2±3,31 74±0,85	_
WEP	2,5%	67,4± 0,77 *	27%
WEP	5%	47,4±18,6**	49%
ASA	200 mg/kg	11,4±1,17	88%

Each value is represented in mean±S.E.M. in seconds, n = 6. *p < 0.05; **p < 0.01; ***p < 0.001 significantly different from control group.

TABLE 2: Effect of water extract of propolis (WEP) on the nociceptive in hot plate test in rat

Treatment	Dose	Hindpaw lick latency (s)	
		0	1h
Control WEP	 1%	6,6±0,15 6,78±0,47	5,7±0,19 6,94±0,34
WEP	2,5%(	6,8±0,34	9,72±0,29*
WEP	5%	8,42±0,45	11,94±0,61*
Morphine	10 mg/Kg	6,2±0,8	15,5±0,6***

Each value is represented in mean±S.E.M. in seconds, n = 6. *p < 0.05; **p < 0.01; ***p < 0.001 significantly different from control group.

TABLE 3: Effect of water extract of propolis (WEP) on formalin-induced pain in mice

Treatment	Dose	Total time spent in licking (s)			
		0–5 min	Inhibition (%)	15–30 min	Inhibition (%)
Control (NaCl 9‰)	_	80.5 ±8,3217	_	167.6 ± 18,45	_
WEP	1%	84 ± 20,02	_	131.5 ± 34,66	22%
WEP	2.5%	53.3 ± 5,86	34%	119.16 ± 13,76	29%
WEP	5%	53.16 ±7,75*	35%	59 ± 26,38*	71%
ASA	200 mg /kg	91.5	_	150.16	11%

Each value is represented in mean±S.E.M. in seconds, n = 6. *p < 0.05; **p < 0.01; ***p < 0.001 significantly different from control group.

IV. DISCUSSION

This work is the first undertaken study that reveals the analgesic effect of black Moroccan propolis using hot plate test, writhing test and formalin test.

Acetic acid induced writhing response and hot plate method are the two most used methods for screening peripheral and central analgesic activity.

The acetic acid induced writhing method is widely used for the evaluation of peripheral antinociceptive activity [20]. It is also called as the abdominal constrictions response; it is very sensitive and able to detect antinociceptive effects of compounds and dose levels that may appear inactive in other methods like tail- flick test [21].

This test is useful for the evaluation of mild analgesic non steroidal. Among the several models of visceral pain, the writhing test has been mostly used as standard screening method specially when evaluating for antispasmodic properties.

Acetic acid causes an increase in peritoneal fluid levels of prostaglanding (PGE2) and (PGE2 α), involving in part peritoneal receptors and inflammatory pain by inducing capillary permeability. Therfore, the analgesic and anti-inflammatory actions of propolis extracts seems to be mediated by inhibition of lipoxygenase and/or cyclo-oxygenase activity or by release of cytokines such as TNF-α, interleukin-1β and interleukin; by resident peritoneal macrophages and mast cells, as shown by Reibero et al. (2000) [22], or both mechanisms.

We observed that water extracted black Moroccan propolis caused a marked analgesic effect using the hot plate test . To our knowledge ,it's the first study that showed that propolis can be have an effect in hot plate test. . Previously, Paulino et al.(2003) [23] reported that Bulgarian propolis was ineffective when assessed in the hot plate .The same result for Brazilian propolis [24] .it could be suggested that the water extract of proplois contains products that may exert analgesic effect through activation of central mechanisms it may contain more hydrophilic constituents than other propolis, knowing that the region of Béni-méllal contains plants originate from other areas. These constituents may have synergic effects, which leads propolis to have such different pharmacological activities.

Indeed, the hot plate test is commonly used to assess opiodergic analgesic mechanisms [25] and narcotic analgesia [26].

In the formalin test there is a distinctive biphasic nociceptive response termed early and late phases.Drugs that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs inhibit the late phases [27,28]. The early phase is probably a direct result of stimulation of nociceptors in the paw and reflects centrally mediated pain while the late phase is due to inflammation with a release of serotonin, histamine, bradykinin and prostaglandins [29]. But also could be due, to a lesser degree, to fthe activation of central nociceptive neurons [29, 30].

It has been demonstrated that the nociception produced by formalin (first phase), is quite resistant to the great majority of non-steroidal anti-inflammatory drugs, while it is sensitive to dipyrone,opioid drugs such as morphine and drugs that antagonize substance P or glutamate receptors[27,31].

Supression of both phases of pain as observed with the exract (5%) in this study also lends strongs credence to the presence of both central and peripheral effects.This speculation of dual activity is further buttressed by the significant activity observed on both the acetic acid abdominal constriction and hot plate tests.

In conclusion, our results show that the water extract of propolis possess a central and peripheral antinociceptive activity. They support the traditional use of WEP in some painful conditions . Seasonal and geographic differences may affect composition of propolis, further investigations are needed to clarify influence of these differences on the analgesic effect propolis.

As a next step, studies in our laboratory are currently under way to isolate and characterize the active principles of the water extract of black Moroccan propolis..

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