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Nelsonia Canescens Extract: Analgesic Properties in Male Rats

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A B S T R A C T

Plants used for many years for the treatment of diseases throughout the world can be the basis of the development of modern drugs. The benefits derived from plants have been linked to different functions including pain treatment. The extract of the aerial part of *Nelsonia canescens* was evaluated for its analgesic activity and its effect on spontaneous behavior in male rats subjected to a persistent painful stimulation (formalin test 2.5%, 50 µl) mimic a recurrent pain very common in clinic. The formalin-treated animals were divided into four groups and fed with almond oil (vehicle) or 12.5, 25 and 50 mg/kg of the extract for seven days. On the 8th day, a second formalin test was carried out (formalin test 1%, 50 µl) to mimic a recurrent pain condition and to quantify pain intensity. During both tests the behavior was monitored in all groups, analyzed and compared. The behavioral responses taken into account were licking, flexing and paw jerk as pain measures and rearing, self-grooming, locomotion and crouch time as measures of spontaneous behavior. The results showed that the extract, in its different concentrations, was able to decrease pain behavior with no significant changes in general activity. The most effective concentration was 25 mg/kg as it reduced pain behavior, jerking and flexing in particular, indicating an analgesic action. In conclusion, the extract of the aerial part of *Nelsonia canescens* has demonstrated analgesic activity in a model of recurrent pain. An estrogen-like action is proposed.

Keywords: Recurrent pain, behavior, analgesia, estrogens

A R T I C L E I N F O

CONTENTS

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1. Introduction

Medicinal plants still play an important role in the health of people living in rural societies [1], where herbs have been used to promote healing by traditional medical practitioners. In Africa the use of medicinal plants has been the unique health care for 4000 years, long before the advent of orthodox medicine [2]. Moreover, the World Health Organization (WHO) reports that even today about 80% of people in developing countries depend on phyto-therapy for their well-being [3]. In these countries, access to orthodox drugs is limited or even absent, so many communities still treat more common diseases with compounds extracted from plants found in the local environment.

Many analgesic drugs now in use were derived from molecules present in animals and plants. A good example is acetylsalicylic acid, which was derived from the bark of the willow tree. The use of the bark of this tree has persisted for many centuries in the traditional medicine of many geographical areas and is now one of the most commonly used analgesic, antipyretic and anti-inflammatory drugs. Other famous compounds extracted from plants include morphine and codeine, obtained from the opium poppy (*Papaver somniferum*)[4].

Nelsonia canescens (Lam.) Spreng, with the synonyms *Justicia brunelloides* (Lam.) [3] and *Justicia canescens* (Lam.), is a perennial herb of the family Acanthaceae and the subgroup Nelsoniaceae. It is distributed in the tropical regions of the world, especially in South and Southeast Asia, Africa, Brazil and Central America in moist deciduous forest but also in the plains. The use of this herb in traditional medicine has been reported in Nigeria [1], Burkina Faso [5], Cameroon [2] and India [6,7]. *Nelsonia canescens* (*N.C.*) has been used for a long time in diverse contexts, i.e. as an ornamental plant, antioxidant [5], antibacterial, anti-inflammatory, analgesic, purgative, anti-spasmodic[1], anti-ulcer [8], hepatoprotective [9], anti-cancer [10] and AChE inhibitor [11].

One of the first publications on the biological effects of *N.C.* was the paper by Owoyele *et al*. [12]. That group worked with the powder of *N.C.* leaves and tested its analgesic properties at various extract concentrations (50, 100, 200 mg/kg) against different types of inflammation and pain in rats. Their results showed that the oral administration of *N.C.* extract significantly reduced the paw edema in the carrageenan test and the time spent licking the formalin-injected paw in comparison to the control; moreover it inhibited granuloma formation by 5 to 18% depending on the dose and increased the latency time in the hot plate test. Another group [13] investigated the analgesic activity of the plant in albino rats but focused on the root extract. The doses administered to the rats were high, namely 270 mg/kg and 540 mg/kg. The formalin test and the tail flick test were performed after extract administration. The formalin test did not show any significant effect, as the extract did not change the paw licking response at either dose (the other pain responses were not determined). However, in the tail flick test the drug increased the pain threshold response in a dose-dependent manner.

The aim of the present study was to provide further information (lower doses, different behaviors) on this plant's analgesic effects in a model of recurrent pain. In particular, we investigated the analgesic properties in a long-lasting model able to mimic the clinical use of analgesics: pain followed by treatment. Treatment strongly indicates an analgesic action exerted by the plant extract.

2. Materials and method

Preparation of the plant extract

The plant (*Nelsonia canescens*) used for this study was harvested from the Tanke area of the Ilorin metropolis in Nigeria. It was previously [12] identified at the Forestry Research Institute of Nigeria. The shade-dried leaves were reduced to a powdery form and 180 g of the powdered sample were exhaustively extracted with 3.75 L of ethanol (analytical grade) for 3 days by maceration. The solvent was removed at 45°C using a vacuum evaporator to give a dark solid extract weighing 6.45 g. The extract was stored in a refrigerator at 4°C and dilutions of the extract were made in sweet almond oil for pharmacological studies

Subjects

The experiments were carried out on 32 male Wistar rats (Harlan-Nossan, Milan, Italy) which weighed 224-248 g upon arrival. They were kept in the animal house for adaptation, in large cages with 6 rats each. Cages were kept at room temperature (21±1 C°), relative humidity (60±10 %) and on a 12/12 h light/dark inverted cycle (light off at 7 a.m.). They received food and water ad libitum.

The experimental procedures were pre-approved by the local and national ethics committees. In all experiments, attention was paid to the regulations for handling laboratory animals of the European Communities Council Directive (86/609/EEC) and the ethical guidelines for investigation of experimental pain in conscious animals issued by the ad hoc Committee of the International Association for the Study of Pain [14]. Particular efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental procedure (Fig. 1)

On day 1 of the experiment (one week after arrival), each rat was subjected to the first formalin test. The test was carried out during the dark phase, the active period of rodents between 09:30 a.m. and 12:30 a.m., in a dedicated room under red light and white background noise. After the first test, the animals were housed two per cage (separated with a transparent Plexiglas wall with holes to allow social

interaction) in plastic-bottomed cages with sawdust bedding. The animals were then randomly divided into the following treatment groups:

- OIL (N=8), rats treated *per os* with almond oil (vehicle)
- NC 50 (N=8), rats treated *per os* with *N.C.* (50 mg/kg in almond oil)
- NC 25 (N=8) rats treated *per os* with *N.C.* (25 mg/kg in almond oil)
- NC 12.5 (N=8) rats treated *per os* with *N.C.* (12.5 mg/kg in almond oil)

On day 8 the second formalin test was performed.

Treatment

From day 1 to day 7, the animals were treated by gavage once a day (at about 4 pm each day) with vehicle or extracts. The *N.C.* extract was dissolved in almond oil to obtain 12.5 mg/ml, 25 mg/ml and 50 mg/ml solutions. The doses were selected on the basis of the previous experiment by Owoyele *et al*. [8]. Each animal was weighed immediately before treatment and administered 1 ml/kg of the solution according to its body weight.

Formalin injection

All animals were subjected to the formalin test on day 1 (FT1) involving a subcutaneous injection of dilute formalin (2.5% in 0.9% NaCl, freshly prepared) in the dorsal right hind paw (50 l). After 7 days of treatment (day 8) they were re-subjected to the formalin test (FT2) consisting of a subcutaneous injection of dilute formalin (1% in 0.9% NaCl, freshly prepared) in the same paw (50 µl).

Behavioral Apparatus

The open field apparatus was used to monitor pain-induced and spontaneous behaviors; it consists of a square transparent Plexiglas cage (50x50 cm) with 30 cm high walls situated on a platform 50 cm above the floor. The floor of the chamber was painted black and divided into 25 equal squares (16 external and 9 internal) by intersecting white lines. The orientation and position of the open field and other objects in the room were not changed during the experiment. A video-camera was used to record the tests carried out in this apparatus. The recordings were later analyzed with Observer behavioral software (Noldus Information Technology, Amsterdam, the Netherlands) by a researcher who was blind to the treatments.

Formalin-induced responses

The following formalin-induced and spontaneous behavioral responses were considered:

a) Formalin-induced responses: licking duration (time spent licking the injected foot), flexing duration (time spent with the leg held off the floor, flexed close to the body) and paw jerk frequency (number of phasic flexions of the leg)

b)Spontaneous behaviors: rearing frequency (number of times the animal stood on its rear limbs), self-grooming duration (time spent washing or scratching the face or body), locomotion duration (time spent sniffing and looking around the environment), sit alert duration (time spent motionless in an alert position), crouch duration (time spent motionless in a sleeping-like position); locomotion was also evaluated by counting the internal crossings (number of times the animal crossed the internal squares) and external crossings (number of times the animal crossed the external squares).

Statistical analysis

Data are reported as mean ± SEM in tables and figures. To determine pain intensity and to verify the behavioral effects of treatment, all behaviors were recorded for 60 min and analyzed in 12 periods of 5 min.

To determine differences among the treated groups, repeated-measures ANOVA was carried out with the factors Group (4 levels: OIL, NC 12.5, NC 25, NC 50) and Time (12 levels: 12 5-min intervals) on the pain-induced behaviors recorded during the formalin test.

To determine the effect of treatment on the repetition of the formalin test, two-way ANOVA was carried out with the factors Group (4 levels: OIL, NC 12.5, NC 25, NC 50) and Test (2 levels: formalin test 1 and formalin test 2). Details are given in the Results section.

The Fisher Least Square Deviation (LSD) test was used as post-hoc analysis. $P<0.05$ was considered significant.

3. Results and Discussion

The formalin concentrations used for treatment induced pain responses lasting for about 1 hr. No signs of pain were observed during the inter-test period. No animal showed any sign of behavioral impairment. Similarly all rats showed a physiological weight increase (data not shown) without significant differences among groups.

Formalin tests

Formalin test 1

During the first formalin test carried out before the start of treatment, all animals showed comparable frequencies and/or durations of the recorded behaviors (data not shown). The subjects were then randomly assigned to one of the experimental groups.

Formalin test 2: differences among groups

All pain responses showed the classic biphasic time-course [15] in all groups, with an initial burst of licking of the injected paw (first phase) followed by a pain-free period (interphase) and a recovery of pain responses between 25 and 45 minutes after formalin injection. One-way repeated ANOVA with the factors Group (4 levels: OIL, NC 12.5, NC 25, NC 50) and Time (12 levels, repeated) was carried out on the pain-induced responses (Fig. 2).

Licking: There were no significant differences in licking duration among groups (Fig. 2).

Flexing: As shown in Figure 2, there was a clear effect of treatment on flexing duration as seen by the significance of the factor Group ($F(3,26)=3,98$ $p<0.01$) and Group x Time interaction ($F(11,286)=1.62$ $p<0.01$). This was due to the reduced flexing duration in 25 mg/kg-treated animals with respect to oil-treated ones at different time periods (see Fig. 2, $p<0.04$ for all). The same tendency was observed during the end of the second phase (see Fig. 2) in the group treated with the highest dose.

Paw jerk: As reported in Figure 2, ANOVA revealed a significant effect of the factor Group ($F(11,286)=2.1797$ $p=0.01$) due to a clear reduction of paw jerk frequency in the group treated with 25 mg/kg with respect OIL ($p<0.01$).

Similar non-significant reductions were also present with the other concentrations.

Comparison between formalin tests 1 (FT1) and 2 (FT2)

To evaluate the effect of treatment on pain-induced responses in the different *N.C.*-treated groups and in the OIL group, we calculated the percentage of variation of each pain response at FT2 with respect to FT1 (Fig. 3). One-way ANOVA applied to these data for each pain-induced response with the factor Group (4 levels: OIL, NC 12.5, NC 25, NC 50) revealed no significant differences for all three formalin-induced behaviors.

The OIL group had pain responses, which were about the same in the two formalin tests, with a tendency to an increase of all three pain-related behaviors notwithstanding the lower concentration of formalin used. In contrast, in the *N.C.*-treated animals, all pain responses showed a strong tendency to lower levels in the second test with respect to the first one, suggesting the lack of long-term sensitization in these groups unlike the OIL one.

Spontaneous behaviors

To verify that *N.C.* administration did not interfere with the normal behavior of the animals, we compared the spontaneous behaviors observed during the first formalin test with those of the second one (Fig. 4 and 5). Two-way ANOVA with the factors Group (4 levels: OIL, NC 12.5, NC 25, NC 50) and Test (2 levels: FT1 and FT2) was applied to each spontaneous behavior (see Fig. 4 for locomotion, grooming and rearing). No significant differences were found among groups for all the behaviors.

Crossings

To determine if the treatment influenced the locomotor activity of the animals, we measured the floor line crossings in both formalin tests by counting the internal crossings, i.e. the number of lines crossed in the internal part of the behavioral apparatus (9 central squares), and the external crossings, i.e. the number of lines crossed in the external part of the behavioral apparatus (16 peripheral squares). Two-way ANOVA with the factors Group (4 levels: OIL, NC 12.5, NC 25, NC 50) and Test (2 levels: FT1 and FT2) was applied.

As for internal crossings, there was no effect of the factor Group (Fig. 5A). For the factor Test, no significant differences were found between FT1 and FT2, even though an increase in internal crossings in FT2 could be detected in the groups administered the 25 and 50 mg/kg doses. As for external crossings (Fig. 5B), there were no significant differences among groups or between tests. In particular, in FT1 the scores were homogeneous for all the groups and in FT2 only a slight increase was observed in the group administered 50 mg/kg compared with the OIL group. Repetition of the formalin test (FT1 vs FT2) did not produce any significant effect.

Discussion

This study provides new information on the analgesic effect of administration of a *Nelsonia canescens* extract in a model of persistent, inflammatory, repeated pain in male rats.

Nelsonia canescens is a perennial herb of the family Acanthaceae used in traditional medicine. Various parts of the plant are used for different purposes. The whole plant is used to shorten labor during delivery and for other gynecological uses [2]. The aerial part (stems and leaves) is employed for the treatment of diarrhea, fever, smallpox, vomiting, and hypertension and also for the treatment of skin infection, pain, inflammation and ulcer. It is usually boiled and the decoction is drunk twice a day for the treatment of ailments. The root is used to treat nausea and inflammation [5]. Several recent studies on the healing properties of the plant were carried out to justify its use in traditional medicine. Thus, the focus of the present study was on inflammatory pain.

Our results showed that *N. canescens* induced significant changes in pain-related behaviors, an index of analgesia. All concentrations used produced a general decrease of pain behaviors during the second formalin test, although the 25 mg/kg dose caused a remarkable reduction in the duration of the tonic flexing of the paw and in the paw jerk frequency, particularly in the second phase of the formalin test. These changes in flexing and paw jerk indicate that, at the concentration used and with this experimental procedure, only the motor responses involved in the more spinally mediated actions (flexing and jerking) are affected by treatment. Thus we can hypothesize that the analgesic agent in the extract acts on receptors present in the spinal cord or in a higher center involved in spinal cord modulation like the periaqueductal gray (PAG) area.

It has been documented that paw jerk reduction may be mediated by the descending endogenous analgesia system due to the action on opioid receptors [16]. For this reason, even though the active compounds in *N. canescens* were not identified, the substances might have an opioid-like activity or, as suggested later, an estrogenic effect able to modulate the opiate system at the hypothalamic level. Dasgupta *et al.* [9] showed that the extract contains alkaloids, flavonoids and saponins [9,12] which have the ability to inhibit nociception.

In addition to the clear analgesic effect demonstrated by the decreased flexing and paw jerk, there was no effect on licking behavior. Licking is a complex supraspinal reflex certainly induced by peripheral inputs. With the quite low *N.C.* concentrations used in the present study, licking of the injected paw was not affected by the treatment, whereas in other studies using higher doses the *N. canescens* extracts decreased the time spent licking [12]. Different results were obtained in a previous experiment by Owoyele *et al*. [12] carried out with the powder of *N. canescens* leaves to test its analgesic properties at various extract concentrations (50, 100, 200 mg/kg) against different types of inflammation and pain in rats. The study used the hot plate and formalin-induced paw-licking model. In the hot plate test the animals were administered 50-200 mg/kg of the extract and placed on the hot plate maintained at 55^{o}C at 30, 60 and 90 min after extract administration. The rats were also tested after injection of 100 µl of 3% formalin in the

left hind paw 1 hr after extract administration and the licking duration was observed only in the first 5 min and from 20-30 min after injection. The results showed that with a shorter time after extract administration (unlike the 24 hrs before as in the present study) and with a stronger painful stimulation (3 vs 1% formalin), the plant extract significantly reduced the time spent licking during the formalin test in comparison to the control. On the other hand, the study by Mohaddesi *et al*. [13] on the root extract of this plant involved very high doses (270 mg/kg and 540 mg/kg). The root extract was administered 1 hr before formalin injection and paw licking alone was measured as an index of pain in the first 10 min and 20-30 min after formalin injection. Moreover the tail flick test was used as phasic pain. The results showed that the root extract had anti-nociceptive effects in the tail flick test but not on the formalin-induced licking response.

In the present study, 25 mg/kg of *N. canescens* extract was the optimal dose while the higher concentration (50 mg/kg) had a weaker effect. Considering these results together with the small but consistent increase in locomotor activity in the latter group, we can hypothesize an *estrogen-like* effect of the treatment. Indeed, since licking was the only pain response not significantly decreased, we suggest that while the analgesic activity is present at the spinal cord level the *N.C.* active molecules work at the supra-spinal cord level by activating the arousal system (as occurs in females with estrogens) and thus increase the attention level towards the stimulus. It should also be underlined that the second formalin test was carried out with a much lower formalin concentration than the first (1% vs 2.5%). Hence, the same behavioral response would correspond to an increase while with the highest *N.C.* concentration (50 mg/kg) the licking levels were at about the control levels! This was not present in the flexing or paws jerk response. Interestingly, these data can be compared with those from a previous experiment by our group [17] in which icv administration of estradiol in male rats increased their licking behavior without affecting (or decreasing) flexing and jerking responses. Also in this case there was a clear pro-nociceptive action of estrogen in the licking response and an analgesic effect in the other more spinally-mediated measures. Finally, this hypothesis agrees with the analgesic effect found when a high concentration of the substance is used in a model of phasic pain. In that case, treatment probably acts as an analgesic, as do estrogens at high levels [18], which is indirectly confirmed by the various uses of *N. canescens* for reproductive situations in traditional medicine.

Another important aspect is the interesting action on the injection point in the hind paw. As previously reported by Owoyele *et al.* [12], the external 'normal' condition of the injected paw observed before the second formalin test in the treated animals, showed that the extract also played a role in the periphery. The lack of edema in the rats receiving *N. canescens* demonstrates that the extract has anti-inflammatory potential.

Spontaneous behaviors were also measured to verify the homogeneity of the groups before the treatment, to monitor the anxiety of the rats and to determine if the treatment has a negative influence on the activity and rest of the animals. However, no effect on the locomotor or rest activity was found. Moreover, the count of line crossings demonstrated that the treatment with *N. canescens* did not decrease locomotion or increase anxiety, thus demonstrating the absence of toxicity. The involvement of toxicity can also be ruled out because a previous study by Dasgupta *et al.* [9] reported no toxic effects of the plant even though they used much higher doses than those used in the present study.

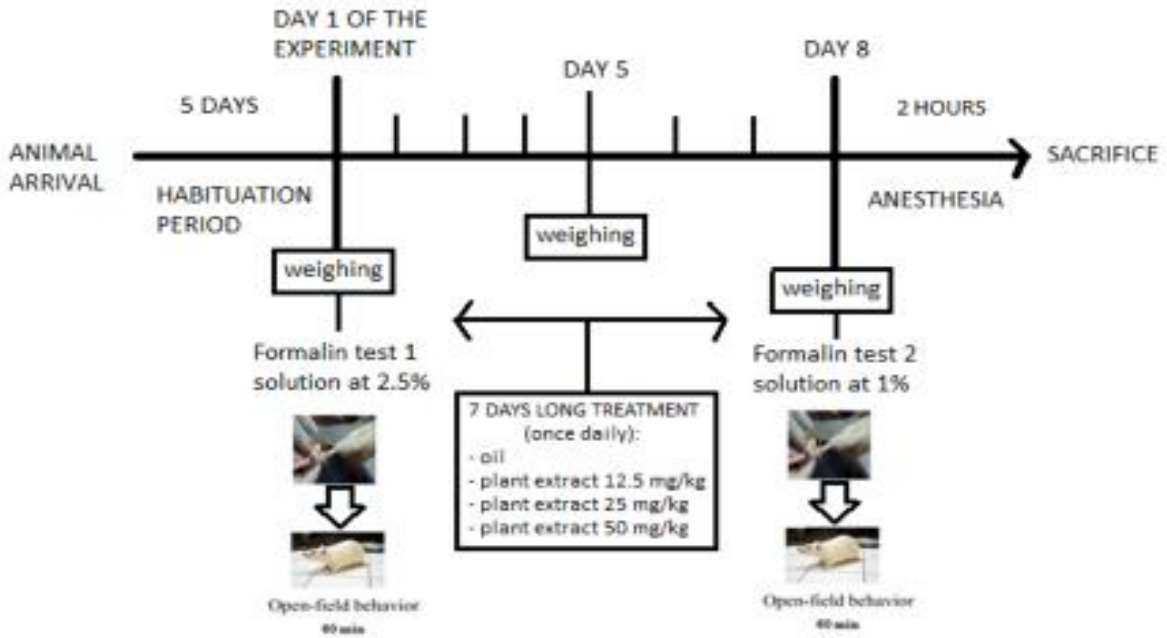

Figure 1: Schematic representation of experimental design.

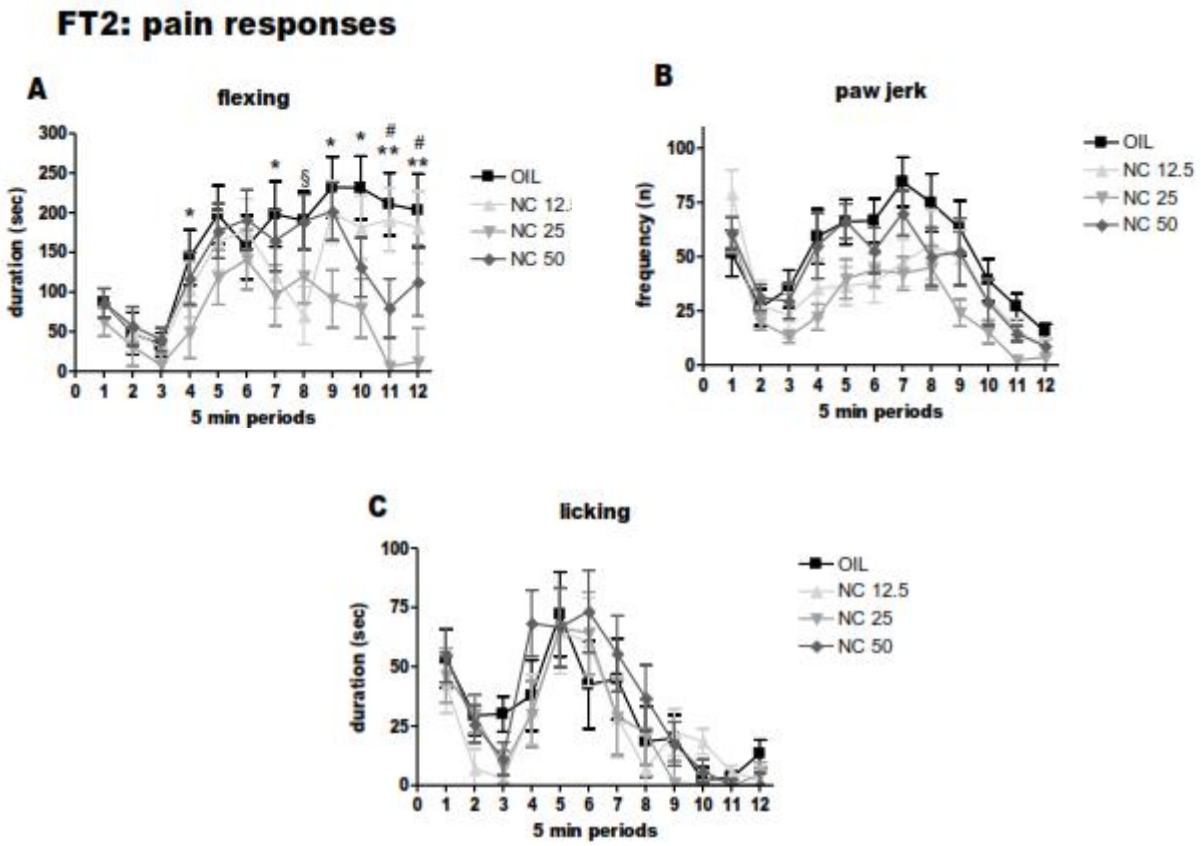

Figure 2: Time-course of flexing (A), paw jerk (B) and licking (C) during the 60 minute formalin test divided into 12 5 – minute interval. * Significant differences between oil- treated and 25 mg/kg groups (p=0.05).

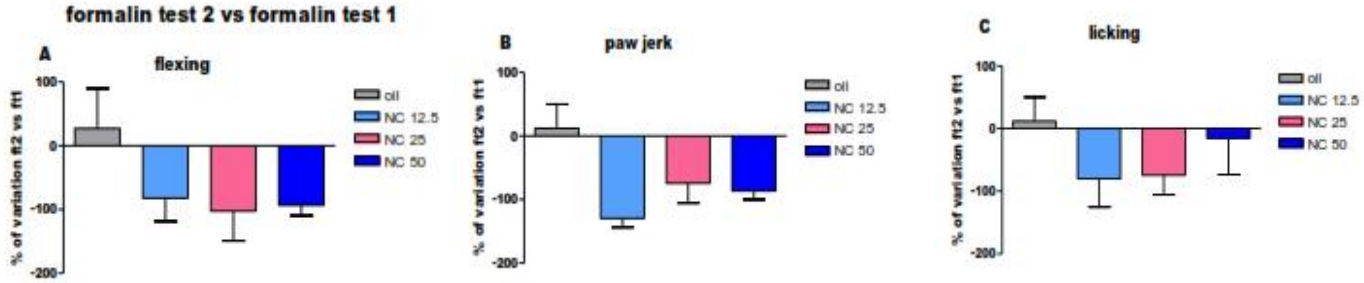

Figure 3: Percentage of variation of flexing (A), paw jerk (B) and licking (C) in FT1 vs. FT2 in animals belonging to all the experimental groups: oil-treated and *N. canescens*- treated animals at the three doses: 12.5, 25 and 50 mg/kg. Bar represent mean±SEM.

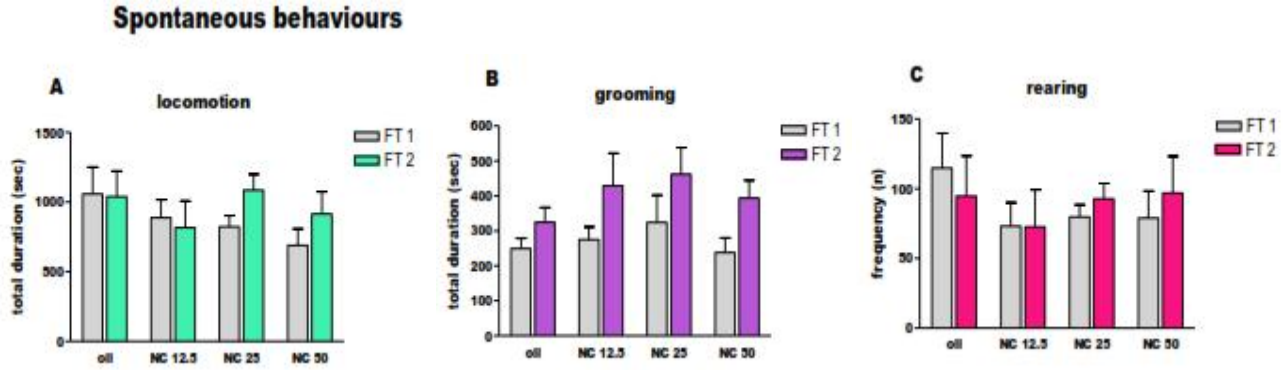

Figure 4: Activity behavior: total duration of locomotion (A) and grooming (B) and total frequency of rearing (C) recorded during the 60- minute formalin test (1 and 2) in animals belonging to all experimental groups: oil-treated and *N. canescens*-treated animals at the three doses: 12.5, 25 and 50 mg/kg. Bar represent mean±SEM.

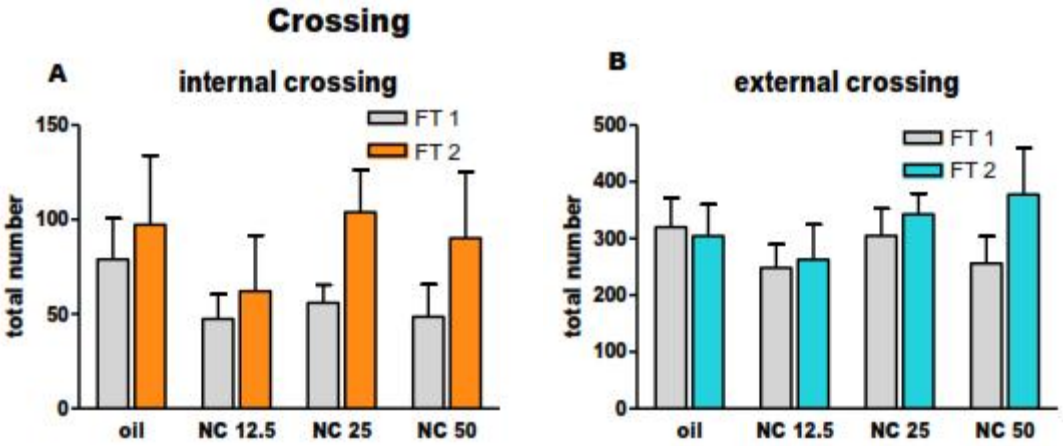

Figure 5: Total number of internal crossing (A) and total number of external crossing (B) recorded during the 60- minute formalin test (1 and 2) in animals belonging to all experimental groups: oil-treated and *N. canescens*- treated animals at the three doses: 12.5, 25 and 50 mg/kg. Bar represent mean±SEM.

4. Conclusion

In conclusion, *Nelsonia canescens* can be considered a plant with analgesic activity at the doses used in the present study. It appears to act especially at the spinal cord level with a possible estrogen-like effect. The focus of further studies could be on how to isolate and characterize the active molecules and the receptors involved.

5. Acknowledgements

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