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Research Article
Analgesic Effect of Melanin from (*Nigella sativa* L.) in the Hotplate Test in Mice (Possible Opioid Receptor Involvement)

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Abstract

Background: *Nigella sativa* L. seeds are famous seeds and have been regarded throughout the ages as one of the greatest healing seeds. They possess a vast range of pharmacological actions, among them analgesic effect was indicated. Recently, melanin has been extracted and shown to occur abundantly in the seed coats of *Nigella sativa* L. Melanin is a ubiquitous pigment of plants and animals. Studies indicated that it is associated with many protective effects but none of these studies addressed analgesic effects of melanin from *Nigella sativa* (NSM). **Objective:** This study has been designed to investigate the potential analgesic effects of NSM on hotplate animal models. **Methods:** Using the hotplate method with different NSM doses 10, 20 and 40 mg kg^{-1} intra-peritoneal injection (i.p.) in mice animal, reactions times (in seconds) were recorded and then the percent prolongations (% prolongation) and ED_{50} were calculated. The influence of naloxone 5 mg kg^{-1} i.p. against NSM 20 mg kg^{-1} i.p. was also tested. **Results:** The NSM at 20 and 40 mg kg^{-1} doses produced statistically significant analgesic effects compared with their control values at 15, 30, 45 and 60 min. A significant effect also produced following a treatment dose 20 mg kg^{-1} at 90 min. The analgesic effect of NSM was inhibited partially by naloxone (5 mg kg^{-1}) i.p. pretreatment. **Conclusion:** The obtained data direct the attention to the analgesic activity (central analgesia) of NSM. The effect is primarily assumed to be mediated partly through opioid receptors.

Key words: Analgesic, hotplate, *Nigella sativa*, melanin, naloxone

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pain is defined by the International Association for the Study of Pain IASP[1] as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage". It is an unpleasant sensation associated with many diseases (e.g., cancers, inflammatory diseases, diabetes ...etc.) and with deleterious conditions (e.g., physical trauma and noxious chemicals). This sensation arises from peripheral nociceptors on receiving inputs from different stimulus; neurons transmit these stimuli to the brain, where they are integrated in different brain centers. In fact, in most cases pain is a very important warning sign keeping the individual away from injuries and damaging stimulus; however, sometimes it gets very severe and persist even after withdrawal of stimuli. So, controlling pain is an important therapeutic goal. Opioids are the main drugs utilized as analgesics, other treatment options are also available depending on pain state such as NSAIDs, anticonvulsants and antidepressant drugs[2,3]. Although many analgesic drugs are currently available, still there is a need for investigating plant derived drugs[4].

Nigella sativa L. (family: Ranunculaceae) is an herbaceous plant growing in the Mediterranean countries and Western Asia. *Nigella sativa* seeds have been considered for many decades as one of the greatest healing seeds. A generally accepted belief in muslim countries is that black cumin is seeds with special healing powers and has long been used for support of the immune system and for protection against various diseases. Extensive study has been carried out highlighting the medicinal properties of the seeds as antioxidants, antimicrobial, anti-inflammatory, anti-diabetic, anti-ulcerogenic, anti-secretory, hepato-protective and even as anticancer[5-8].

Among these pharmacological actions associated with *Nigella sativa* analgesic activity was found to be associated with *Nigella sativa* seed's oil and different extracts and component using different animal models; formalin, acetic acid and hotplate etc. Some studies revealed that this effect could be mediated through opioids receptors and blocked by naloxone. In searching for compounds and chemical entities that are responsible from this effect studies indicated that thymoquinone, polyphenols form seeds and essential oil are all at least share part of analgesic effect[9-12].

Melanin is a pigment of plants and animals. It occurs both externally and internally in tissues and organs (e.g., in seed coats, hair, inner ear, substantia nigra and fertilized ova). Recently, it has been found to occur in the seed coats of *Nigella sativa* L. In humans, the absence of melanin is correlated with the onset of various diseases like albinism and parkinson's disease. It has been determined that melanins are built up of indolequinones at various degrees of combination and attachments to each other and other molecules. Melanin has been extracted from a few plants and recently it has been extracted from the seed coats of black cumin (*Nigella sativa* L.) (NS)[13].

The NSM has also been associated with many protective roles such as photo-protection[14], hepato-protection[15,16], nephron-protection[17] anti-inflammatory effect and antioxidant effect[18,19]. However, analgesic properties for *Nigella sativa* melanin (NSM) haven't been studied before.

According to the literature there is no information available on the potential NSM analgesic effect. The present study aimed to evaluate the possible NSM's analgesic activity and explaining the possible mechanisms underlying this effect using the hotplate animal model.

MATERIALS AND METHODS

Preparation of NSM: Extraction and characterization of NSM melanin from *Nigella sativa* L. have been carried as described before by El-Obeid *et al.*[5]. The seed coats of *Nigella sativa* L. were solubilized in an alkaline solution of NaOH (pH 12.5) for 1 day, which yielded a dark black solution. The solution was then centrifuged and filtered and melanin was precipitated from it at pH 2 using conc. HCl. This alkali–acid treatment was repeated 3 times to ensure a higher purity. The precipitate was thoroughly washed with distilled water, filtered out and dried at 60°C. The dry powder was stored and used later to prepare solutions at pH 7 for biological studies.

Experimental animals: Swiss albino mice 25 g b.wt. were housed in Plexiglas cages, in acclimatized colony rooms temperature of 22±1°C, 12 h/12 h light/dark cycle with free access to tap water and food. They were fed *ad libitum*. The handling of animals was done according to the ethical rules approved by King Saud University.

Hotplate procedures and recordings: Responsiveness to nociceptive stimulation was measured by placing mice on a hotplate (UGO Basile 7280) maintained at 57±0.5°C. The reaction time is defined as the interval (in seconds) from the time the mouse was placed on the hotplate until the moment the mouse licked its forelimbs or jumped off the hotplate. The timer was stopped by a foot-operated pedal and the rat was immediately removed from the hotplate, as previously described[20].

The baseline reaction time was obtained before treatment for all experimental animals, with maximum hotplate latency of 30 sec was adopted to prevent tissue damage to the mice's paws.

The mean reaction time each groups were obtained (control). The reaction time of each mouse were again evaluated at 15, 30, 45, 60 and 90 min after treatment with three NSM doses 10, 20 and 40 mg kg^{-1} through i.p. administration.. The mean values for each treatment group at each measurement were calculated. This final test mean was subsequently used to determine the percentage thermal pain protection by applying the following equation:

$$\text{Protection against thermal stimulus (\%)} = \frac{\text{Test mean-control mean}}{\text{Control mean}} \times 100$$

Drugs and treatment protocol: Drugs used here were NSM, aspirin, codeine and naloxone according to the following protocol:

- For evaluation of analgesic effect by hotplate and determination of ED_{50}, 4 groups (n = 6) were treated as the follows:
  - Group 1: Received NSM 10 mg kg^{-1}, i.p.
  - Group 2: Received NSM 20 mg kg^{-1}, i.p.
  - Group 3: Received NSM 40 mg kg^{-1}, i.p.
- For testing of opioid receptor mechanism of action, two groups (n = 6) were treated with as follows:
  - Group 1: 20 mg kg^{-1}, NSM
  - Group 2: Naloxone 5 mg kg^{-1}, i.p., then followed by NSM 20 mg kg^{-1}, i.p. after 5 min
- For testing the NSM synergistic effect with other analgesics, two groups (n = 6) were treated as following:
  - Melanin below ED_{50} (1 mg kg^{-1})+aspirin ED_{50} (93 mg kg^{-1})
  - NSM below ED_{50} (1 mg kg^{-1})+codeine ED_{50} (3 mg kg^{-1})

Statistical test: Data were presented as Mean±SEM. Statistical differences between the basal pretreatment times and those following the drugs were tested by one way ANOVA followed by Student's t-test. The differences were considered significant at $p<0.05$. Statistical analysis was performed using GraphPad instat version 3.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

RESULTS

Effects of NSM on hotplate test reaction time in mice: The effects of NSM on hotplate were presented in Table 1. At the dose 20 mg kg^{-1} NSM showed a significant analgesic effect ($p<0.001$) against thermally induced pain at both 30 and 90 min of the study period, at 45 and 60 min significances was ($p<0.01$) and the maximum protection was 110% at 30 min in a dose 20 mg kg^{-1} much more than other doses. The NSM at the dose of 40 mg kg^{-1} was showed significant analgesic activity at 15, 45 and 60 min ($p<0.05$). Administration of NSM at dose 10 mg kg^{-1} did not show any significant analgesic activity on hotplate test of analgesia.

Effect of naloxone on NSM analgesic effect: The effects of pre-treatment with naloxone (a non-selective opioid receptor antagonist) on the analgesic effect of NSM were presented in Table 2. Naloxone pretreatment was reduced the protection

Table 1: Effect of different NSM doses on hotplate latency in mice

		Reaction time (sec) percentage protection										
Groups	Doses (mg kg^{-1})	0 (min)	15 (min)	30 (min)	45 (min)	60 (min)	90 (min)	15 (min)	30 (min)	45 (min)	60 (min)	90 (min)
NSM	10	8.9±0.3	12.1±2.2	11.0±1.8	11.15±1.2	11.05±9.1	6.6±0.7	35.6	23.9	24.8	23.60	-25.74
NSM	20	7.6±0.8	10.5±1.5	16.2±1.3***	13.8±1.0**	11.3±0.7**	14.3±1.0***	36.9	110.9	80.4	8.40	86.90
NSM	40	6.6±0.3	9.1±0.9*	14.2±3.5	11.5±1.7*	12.5±1.8*	7.0±1.5	37.5	75.0	75.0	5.75	5.00

All values are presented as Mean±SEM (n = 6), *p-value<0.05, **p-value<0.01, ***p-value<0.001 and NSM: *Nigella sativa* melanin

Table 2: Effect of naloxone on NSM effect

		Reaction time (sec)			
Groups	Doses (mg kg^{-1})	0 (min)	30 (min)	Protection (%)	Effectiveness of naloxone (%)
NSM	20	6.30±5.0	12.0±1.1	91.2	
NSM+naloxone	20	6.25±0.32	8.0±1.1	21.8	69.4**

All values are presented as Mean±SEM (n = 5), **p-value<0.01 and NSM: *Nigella sativa* melanin

Table 3: Additive effect of NSM on other analgesics

Groups	Doses (mg kg^{-1})	Reaction time (sec) 0 (min)	30 (min)	Protection (%)
NSM	1*[1]	6.87±0.2	8.4±0.4	11.6
Aspirin	93*[2]		11.0±1.6	71.9
Codeine	3*[3]		11.5±2.3	59.7
NSM+asprin	1+93		10.0±1.0	53.8
NSM+codeine	1+3		9.8±1.7	45.7

All values are presented as Mean±SEM (n = 5), NSM: *Nigella sativa* melanin, *[1]: Below NSM ED_{50}, *[2]: Aspirin ED_{50} and *[3]: Codeine ED_{50}

of NSM 20 mg kg^{-1} on hotplate from 91.2-21.8% and the percentage of naloxone effectiveness was 69.4% ($p<0.01$).

Synergistic effect of NSM with other anlgesics: The NSM in combination with other analgesics, aspirin and codeine did not show any significant synergistic effect. Table 3 presented the use of NSM in a dose below its ED_{50} (1 mg kg^{-1}) with ED_{50} of either aspirin or codeine did not show synergism and even the analgesic effects of aspirin and codeine have been reduced.

DISCUSSION

Hotplate test is one of the most common and well-known animal model for testing analgesic activity, which is based on induction of pain through high intensity thermal stimulus. It is considered specific for centrally mediated nociception and which involved opioids receptors[21,22]. A number of studies previously reported the analgesic effect *Nigella sativa* oil and its components thymoquinone, essential oil and polyphenols in different animal models[9-12]. Some of these studies have proposed that opioid receptors system is at least involved in the analgesic effect [9].

Melanin has been extracted from a few plants and recently it has been extracted from the seed coats of black cumin (*Nigella sativa* L.) (NS)[13]. Generally, it has been found to possess many activities, such as photo-protective, hepato-protective, nephron-protective, anti-inflammatory effect and antioxidant effect[14-19]. Regarding NSM, El-Obeid *et al.*[13] reported its ability to activate toll like receptors type 4 to stimulate the release of IL-8 from PBMCs and other cell lines. Also it has been reported its ability to protect against alcohol, aspirin, indomethacin and stress-induced ulcers in presence or absence of commensal gastric bacteria[23]. However, analgesic properties for *Nigella sativa* melanin (NSM) haven't been studied before.

In this study, NSM at the dose of 20 and 40 mg kg^{-1} by intraperitoneal injection produced statistically significant prolongation of the hotplate latency with time is produced up to 1 h at time intervals 30, 45 and 60 and 90 min as compared with control. However, no analgesic effect was observed with dose 10 mg kg^{-1}, which indicated that the analgesic effect was produced in a dose dependent manner. The NSM failed to produce synergistic effects with other analgesic drugs, e.g., aspirin and codeine (Table 3) and there is even a slight reduction in their analgesic activity suggesting that some competitive interaction is exist, due to targeting the same receptors (Table 1).

To evaluate the nature of the possible analgesic mechanisms, it has been investigated that the effect of naloxone (a non-selective opioid receptor antagonist) on the analgesic activity of NSM. The data obtained showed that the analgesic effect produced by NSM is naloxone-sensitive. Since naloxone in a dose 5 mg kg^{-1}, i.p. antagonized the analgesic of NSM significantly (about 72%, p-value<0.01) (Table 2), which a confirmed the involvement of opioid receptor in NSM analgesic action. This results in agreement to that already reported by Abdel-Fattah *et al.*[9] for *Nigella sativa* oil and thymoquinone. However, the results contradicated the findings reported by Ghannadi *et al.*[12], which stated that the opioid receptors are not involved in analgesic effects of a polyphenol-rich extract, fractionated from *Nigella sativa* seeds. This is may be due to difference in tests utilized, in this case it has been used hotplate test, which is considered specific for centrally mediated nociception and which, involved opioids receptors[21,22].

This study indicates for the first time, that the NSM extracted from *Nigella sativa* seed coats produces an analgesic effect on nociceptive responses caused by thermal nociceptive stimuli in mice (Received patent No. 3295, Sudan). Some studies reported the analgesic effect of *Nigella sativa* and its active constituents, such as thymoquinone, but none of these studies certainly studied NSM as analgesic. Thus, NSM could be considered as one of the main participants to the known *Nigella sativa's* analgesic action. The study also supports the view that the opioid system is involved in the analgesic effect of melanin due to the blocking of analgesic effect of NSM significantly with naloxone.

CONCLUSION

The NSM has analgesic effect possibly mediated through opioid receptors. Moreover, the known *N. sativa* analgesic effect is in part, due to its active ingredient NSM. More studies are needed to fully characterize this effect.

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