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Enhanced antinociceptive activity of *Nigella sativa* oil after its combined treatment with honey in rats

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Abstract: Antinociceptive activity of honey and *Nigella sativa* (*N. sativa*) oil are well known. Therefore, aim of this study was to investigate the antinociceptive effect of *N. sativa* oil and its concurrent administration with honey in rats. The tested animals were randomized into 5 groups: Group (1) Normal saline (0.2ml, p.o.); Group (2) *N. sativa* oil (1gm/kg, p.o.); Group (3) honey (1gm/kg, p.o.); Group (4) *N. sativa* oil (1gm/kg, p.o.) + honey (1gm/kg, p.o.): Group (5) pethidine (20mg/kg, S.C.) as positive standard. The antinociceptive activity was tested using radiant heat and tail immersion tests. Antioxidant potential was determined by using *in-vitro* antioxidant assays. Our findings showed that *N. sativa* oil and honey have antinociceptive effect, the antinociceptive effect appeared after 30 and 60min of administration and declined after 120 and 180 min. Combined administration of *N. sativa* oil with Honey increased the antinociceptive effect by 20 to 30% in all models. In addition, the antinociceptive effect of the combination reduced the time for onset of action as well as prolonged its duration of action. In conclusion, combined treatment of *N. sativa* oil with honey increased its antinociceptive activity, showed faster onset of action and prolonged its duration, the fact that can be utilized in the management of painful conditions in humans.

Keywords: *Nigella sativa*, honey, analgesia, radiant heat, antioxidant.

INTRODUCTION

Pain is an unpleasant sensation, a protective mechanism of the body and is usually beneficial. However, occurrence of excruciating pain could be independent of any clear predisposing cause. Sometimes pain is a consequence of brain or nerve injury (Wilson, 2014). Although it is a protective response, pain is annoying sensation disturbing patient's life and sleep and most of therapeutic strategies for treatment of various diseases aim to overcome pain sensations by using painkillers or analgesics. Most of available analgesic drugs have a wide variety of side effects, the fact that necessitate research about natural analgesics either medicinal plants or natural products that can be used for relief or reduce pain sensation without unwanted effects.

Among various medicinal plants, *N. sativa*, (*Ranunculaceae*), is emerging as a miracle herb with a rich religious and historical background. Islamic medicine in prophetic terms, has been crafted from the central religious text and core source of all knowledge in Islam, the Qur'an, with natural remedies for most ailments and specific prayers that must be recited for any disease to be conquered (Monette, 2012). *N. sativa* is regarded one of the best healing medicine available due to its mention in one of the Prophetic hadith which states, "*N. sativa* is the remedy for all diseases except death" (Sahih Al-Bukhari, 1996). In the "Holy Bible" it has been mentioned as a curative black seed in the Book of Isaiah in the Old Testament (Isaiah 28:25-27 NKJV). It is described as the Melanthion by Hippocrates and Dioscorides and as the Gith by Pliny (Khan, 2018). Ibn Sina, a Persian physician, recommended its use to heal wounds, cure fever, skin diseases, treat fungal, helminth and parasitic infections, and worms, as well as against bites and stings of poisonous animals (Ijaz *et al*., 2017). It is used as traditional medicine to treat various diseases, as well as a spice for flavoring. For more than 2000 years, it has been used as an herbal medicine for treating respiratory, gastrointestinal, rheumatic and inflammatory disorders (Yarnell and Abascal, 2011). *N. sativa* seeds contain essential oils, fixed oils, saponins, alkaloids, flavonoids, and proteins (Al-Ghamdi, 2001). The medicinally important seeds exhibit many pharmacological activity including antibacterial, antiparasitic, antiviral, antifungal, antioxidant, anti-inflammatory activity and anti-stress activities. *N. sativa* extract has immunopotentiating, antioxidant, antihumoral and antidiabetic properties. Recently, around 67 constituents have been isolated from *N. sativa* oil, capable of eliciting beneficial pharmacological activity (Ahmad, 2013). Recent studies showed that *N. sativa* possesses potent analgesic and anti-inflammatory activity (Al-Ghamdi, 2001; Hajhashemietal *et al*., 2004).

Natural honey is a sweet food substance produced from nectar by honey bees. Its composition is influenced by

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various factors such as geographical location, botanical source of nectar, climatic and environmental conditions. It is mentioned in the "Holy Quran", "Then eat from all the fruits and follow the ways of your Lord laid down [for you]. There emerges from their (honey bees) bellies a drink, varying in colors, in which there is healing for people. "Indeed in that is a sign for a people who give thought" (Al-nahl, 16:69 Trans. Abdullah Yusuf Ali). Honey, contains at least 181 constituents, of which, monosaccharides and oligosaccharides are predominant (Bogdanov *et al*., 2008; Saranraj and Sivasakthi, 2018). Other bioactive constituents include organic acids, phenolic compounds, flavonoids, nitric oxide (NO) metabolites, Maillard reaction products, ascorbic acid, aromatics, vitamins, proteins, amino acids, and trace elements (Wang *et al*., 2011). Literature review indicates that antinociceptive and antimicrobial properties of some varieties of honey is attributed to presence of kynurenic acid (Beretta *et al*., 2007). Also various enzymes such as invertase, diastase, phosphatase, glucose oxidase, peroxidase and catalase are present in honey (Bogdanov *et al*., 2008). Recently, a renewed interest in natural honey research to investigate its beneficial effects on health, resulted in finding several medicinal effects, which include hypoglycemic, antioxidant, cardioprotective, antihypertensive, anti-inflammatory, antibacterial, anti-fungal, analgesic, and wound healing effects (Samarghandian *et al*., 2017; Rakha *et al*., 2008; Erejuwa *et al*., 2010; Erejuwa *et al*., 2011; Erejuwa *et al*., 2012; Tan *et al*., 2009; Feas *et al*., 2011; Zeina *et al*., 1996; Kassim *et al*., 2010; Gunduz *et al*., 2014; Biglari *et al*., 2013; Vogel *et al*., 2001). Hence, aim of this study was to determine the antinociceptive effect of acute treatment of *N. sativa* oil, honey and its combination with relatively large dose (1gm/kg, p.o) on the different models of nociception in rats, and to assess its *invitro* antioxidant potential.

MATERIALS AND METHODS

Animals

Thirty Wistar rats (male), 5-6 weeks old, weighing 150-180g, were procured from the animal house, College of Pharmacy, Najran University, Najran, Saudi Arabia. Rats were kept in properly ventilated cages (polycarbonate) on a 12-hour-light-dark cycle, animal food and water provided *ad libitum*. All experiments carried out during the light phase (9:00 Am to 2:00 Pm). Ethical approval vide number 05-01-16EC was obtained from the Research Ethics Committee, Najran University, Najran, Saudi Arabia.

Chemicals

N. sativa oil and honey were bought from a local supplier in Najran, Saudi Arabia, stored at room temperature and freshly used at the time of the experiment. Both the drugs were administered orally (p.o) using intragastric tube (gauge). Pethidine was obtained in the form of ampoule from Najran University Hospital and injected in dose 20 mg/kg subcutaneously.

Experimental Protocol

Animals were randomized into 5 groups containing 6 animals in each group. Group 1: Received 0.2ml normal saline orally (p.o.). Group 2: Received *N. sativa* oil (1 gm/kg) + 0.2ml distilled water orally (p.o.). Group 3: received Honey (1 gm/kg) + 0.2ml distilled water orally (p.o.). Group 4: mixture of *N. sativa* oil (1 gm/kg) + Honey (1gm/kg). Group 5: Pethidine 20mg/kg subcutaneously (S.C). Animals from all group were subjected to testing of analgesic activity at base time 0 min and at 30, 60, 120, 180 min after treatment.

Radiant heat analgesia

The test was performed based on the previous method (Vogel *et al*., 2001). The animal is restrained in a small cage with the tail kept outside through an opening in the rear wall. The proximal third of the tail was exposed to source of light and the reaction time was determined. Endpoint was recorded when rat tried to pull its tail away and turned the head. Basal reading at time 0 was recoded before administration of test or standard drug. The animals were submitted to the same testing procedure at time 30, 60, 120 and eventually 180 min.

Tail immersion test

The test was performed based on the previous method (Vogel *et al*., 2001). Tip of the tail 5cm from distal end was immersed in hot water (55°C). The cut off time was 15 seconds to avoid harm to animal tail. Basal reading at time 0 was recoded before administration of test or standard drug. The animals were submitted to the same testing procedure at time 30, 60, 120 and 180min and reaction time recorded.

In-vitro antioxidant assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging activity of test drugs and their combination was measured as per the method described by Blois (1958). 0.2mM DPPH solution (100µl) was added to different concentrations of test drugs (20 to 200 µg/ml). Absorbance was measured at 517nm after 30 min. Standard curve was plotted using different concentrations of Ascorbic acid. The scavenging activity was calculated using the following equation:

% DPPH scavenging activity = (A0-A1/ A0) × 100
A0: absorbance of control

A1: absorbance of sample

Reducing power

Reducing power activity was estimated based on the previously mentioned method (Yildirim *et al*., 2001). Varying concentrations of test drugs and their combination (50 to 450µg/ml); were added to a test tube

previously containing a mixture of 1ml of 1% potassium ferriccyanide solution and 1ml of 200mM sodium phosphate buffer (pH 6.6) and incubated for 20 min at 50^0C. The above mixture was subjected to centrifugation for 10 minutes (3000 rpm) after adding 10% trichloro acetic acid (1ml). Supernatant was collected and added to a tube containing 1% ferric chloride (0.5ml) and distilled water (2ml) and incubated (10 min) and absorbance measured (700nm). Greater absorbance denotes higher reductive power of the test compounds (Jamuna *et al*., 2012).

Hydroxyl radical scavenging activity

The deoxyribose method was used to determine the $^{\bullet}$OH scavenging activity (Halliwell *et al*., 1988). The reaction mixture contained 10 mM 2-deoxyribose (0.15ml), 0.2M Na_3PO_4 buffer (0.45ml), 10 mM H_2O_2 (0.15 ml), 10mM FeSo4-EDTA (0.15ml), distilled water (0.525 ml), test drug (0.075 ml) and incubated for 4h at 37^0C. After incubation, 2.8% trichloroacetic acid (0.75ml) and 1.0% thiobarbituric acid (0.75ml) was added, followed by boiling for 10 min, cooled, absorbance was measured at 520 nm. Mannitol (0.5-4.5mg/ml) was used as standard for comparison and standard curve was plotted.

% ·OH radical scavenging activity = (A0-A1/ A0) × 100.

A0: absorbance of control

A1: absorbance of sample

STATISTICAL ANALYSIS

SPSS software (version 18.0) was used for statistical analysis. Differences between means were determined by One-way analysis of variance (ANOVA) followed by Dunnet's post hoc test and values of $p<0.05$ were statistically measured as significant.

RESULTS

Effect of N. sativa oil, Honey and its combination on Radiant heat test

Treatment of the tested animals with NS oil and honey (1 gm/kg, p.o) each, in a separate treatment, lead to significant ($p<0.05$) increase in the antinociceptive latency of the radiant heat test. The effect started 60 min after treatment (fig. 1C) and started to decrease 120 and 180 min after treatment (fig. 1D and 1E). Combined treatment of *N. sativa* oil with honey significantly ($p< 0.01$) increased the antinociceptive effect through increasing the latency of radiant heat test compared with control and compared with the single *N. sativa* oil group. The antinoceptive effect of the combination also appeared earlier, after 30 min of treatment (fig. 1B) and prolonged for 180 min (fig. 1E) of treatment. The time profile of the tested drugs is shown in fig. 1F.

Effect on N. sativa oil, honey and its combination on tail immersion test

Studying the antinociceptive activity using tail immersion test of thermal pain showed that single treatment with *N. sativa* oil and honey in the dose 1 gm/kg, p.o, each, led to significant ($p<0.05$) increase in the antinociceptive latency of the tail immersion test. The effect started 30 min after treatment (fig. 2B) with *N. sativa* oil, while started after 60 min with honey and started to decrease 120 and 180 min after treatment (fig. 2D and 2E). Combined treatment of *N. sativa* oil with honey significantly ($p< 0.01$) increased the antinociceptive effect via increasing the latency of the tail immersion test compared with control and compared with single *N. sativa* oil treated group. The antinociceptive effect of the combination treatment also appeared earlier, after 30 min of treatment (fig. 2B) and prolonged for 180 min (fig. 2E) of treatment. The time profile of the antinociceptive effect of the tested drugs in tail immersion test is shown in fig. 2F.

Effect of N. sativa oil, honey and its combination on antioxidant in-vitro

Free radical scavenging activity (DPPH$^{\bullet}$)

The scavenging effect of honey and *N. sativa* oil on DPPH$^{\bullet}$ increased linearly as concentration increased from 20-200µg mL^{-1} (table 1). The highest scavenging activity was seen at 200µg mL^{-1} for honey (33.73% inhibition) and *N. sativa* oil (39.25% inhibition). Moreover, combination of honey and *N. sativa* oil enhanced the DPPH$^{\bullet}$ scavenging activity (45.85% inhibition) at 200µg mL^{-1} (fig. 3A). The IC50 values for honey, *N. sativa* oil and their combination was found to be 380.06µg/ml, 249.327µg/ml and 210.192 µg/ml, respectively (table 1).

Reducing power activity

It is routinely used for evaluating the antioxidant potential of natural antioxidants. The reducing capacity ("Fe^{3+} to Fe^{2+} transformation" in terms of increasing absorbance) increased proportionally with increasing concentrations of test samples. Honey, *N. sativa* oil and their combination showed highest reducing power at a concentration of 450µg mL^{-1} (table 2). Combination of honey and *N. sativa* oil potentiated the reducing power of honey and *N. sativa* oil, which was comparable with standard ascorbic acid. (fig 3C).

Hydroxyl radical scavenging activity

Honey, *N. sativa* oil and their combination inhibited hydroxyl radical-mediated deoxyribose degradation in a concentration dependent manner. The EC50 value of honey, *N. sativa* oil and their combination was found to be 410.64 µg mL^{-1}, 420.14µg mL^{-1} and 320.71 µg mL^{-1} respectively. The EC50 value of reference standard mannitol was found to be 74.57µg mL^{-1} (table 3 and fig 3D).

DISCUSSION

The present study investigated the antinociceptive effect of *N. sativa* oil and honey in relatively large dose in its single and combined treatment using different The

Table 1: *In-vitro* DDPH Scavenging activity.

S. No.	Standard	Concentration (µg/ml)	% of inhibition	% of inhibition	% of inhibition
	Ascorbic Acid		Honey	*N. Sativa* Oil	Mixture (Honey + *N. Sativa* Oil)
1)	4.26	20	8.15	9.26	13.11
2)	21.63	40	15.45	11.36	16.52
3)	51.24	60	17.660	16.84	19.47
4)	58.63	80	19.95	19.42	25.49
5)	71.63	100	20.16	23.48	29.47
6)	-	120	21.54	29.84	34.65
7)	-	140	23.74	32.55	38.85
8)	-	160	24.59	34.15	41.52
9)	-	180	26.36	37.42	42.74
10)	-	200	33.73	39.25	45.85
IC-50 values	69.97 µg /ml	IC-50 values	380.065 µg /ml	249.327 µg /ml	210.192 µg /ml
Y=mX+C	Y=0858X-10.04	Y=mX+C	Y=0.107X+9.333	Y=0.177X+5.869	Y=0.192X+9.643
R^2	0.958	R^2	0.903	0.982	0.982

Table 2: *In-vitro* Reducing power activity.

Concentration (µg/ml)	Ascorbic Acid (Standard)	Honey	*N. Sativa* Oil	Honey + *N. Sativa* Oil
	Absorbance	Absorbance	Absorbance	Absorbance
50	0.52	0.1	0.2	0.2
100	0.61	0.3	0.28	0.31
150	0.75	0.34	0.31	0.39
200	0.86	0.38	0.41	0.43
250	0.92	0.41	0.46	0.48
300	1.14	0.51	0.53	0.55
350	1.26	0.55	0.62	0.68
400	1.39	0.59	0.66	0.73
450	1.45	0.68	0.71	0.88

Table 3: *In-vitro* Hydroxyl radical scavenging activity.

Standard	Mannitol	Honey	*N. Sativa* Oil	Honey + *N. Sativa* Oil
Concentration (µg/ml)	% Hydroxyl Scavenging	% Hydroxyl Scavenging	% Hydroxyl Scavenging	% Hydroxyl Scavenging
100	51.23	28.63	31.88	35.84
200	58.42	31.56	32.36	41.52
300	62.49	39.74	39.76	47.96
400	69.52	48.65	47.42	52.98
500	75.33	59.48	58.64	65.42
IC-50 values	74.57µg /ml	410.64µg /ml	420.14 µg /ml	320.71 µg /ml
Y=mX+C	Y=0.059X+45.60	Y=0.078X+17.97	Y=0.068X+21.43	Y=0.070X+27.55
R^2	0.994	0.967	0.928	0.967

mechanisms by which *N. sativa* elicits its analgesic effect is not fully understood. In a previous study, *N. sativa* oil (50-400 mg/kg, p.o.) enhanced the antinociceptive tests. *N. sativa* showed an antinociceptive effect, in the form of significant increase in the analgesic latency. This effect appeared after 30 min of administration and was maximum after 60 min and started to decrease after 120 and 180 min of its administration. Combined administration of *N. sativa* with honey in 1:1 dose ratio increased the antinociceptive effect of *N. sativa*, the fact that was reflected in the form of increase in the analgesic latency of the employed tests of nociception, the results reflected the synergistic effect of honey to the antinociceptive effect of *N. sativa*. Moreover, the analgesic effect of combined treatment started in earlier time interval (30min) after treatment compared with (60min) with *N. sativa* alone, the fact that reflect the decrease in the antinociceptive onset of combination treatment of *N. sativa* with honey. Furthermore, the analgesic effect of combination treatment extended to 120 and 180 min after treatment, the fact that reflected an increase in the duration of the antinociceptive effect of the combined treatment of *N. sativa* with honey relative to the antinociceptive effect of *N. sativa* alone.

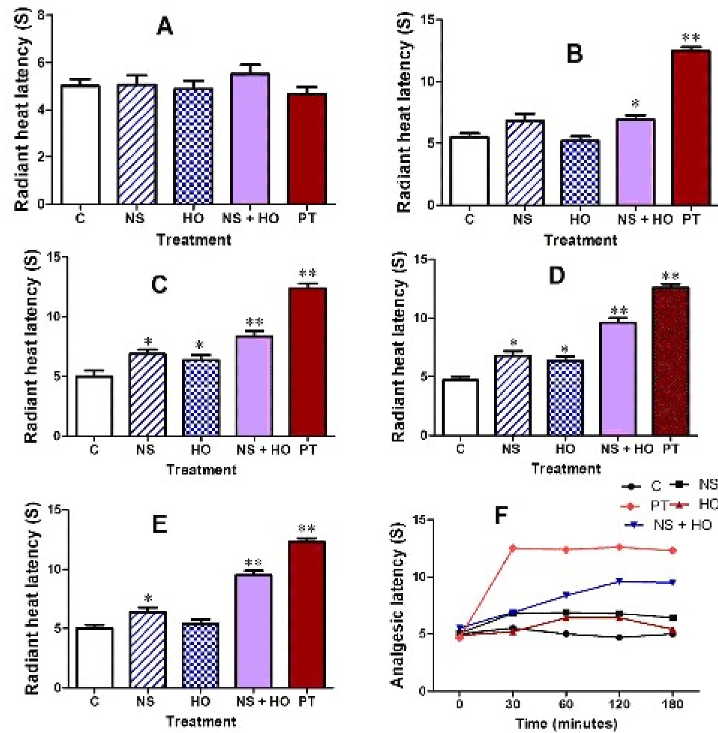

Fig. 1: Protective effect of *N. sativa* oil (NS), Honey (HO) and its combination against pain response in radiant heat test after 0 min (A); 30 min (B); 60 min (C); 120 min (D); and 180 min (E); time-course (F) of treatment using pethidine (PT) as a standard reference analgesic. Values are mean ± SEM (n=6). Differences between groups were analyzed by one-way analysis of variances (one-way ANOVA) followed by Dunnet's post test. *p<0.05 vs control (C), **p < 0.01 vs control.

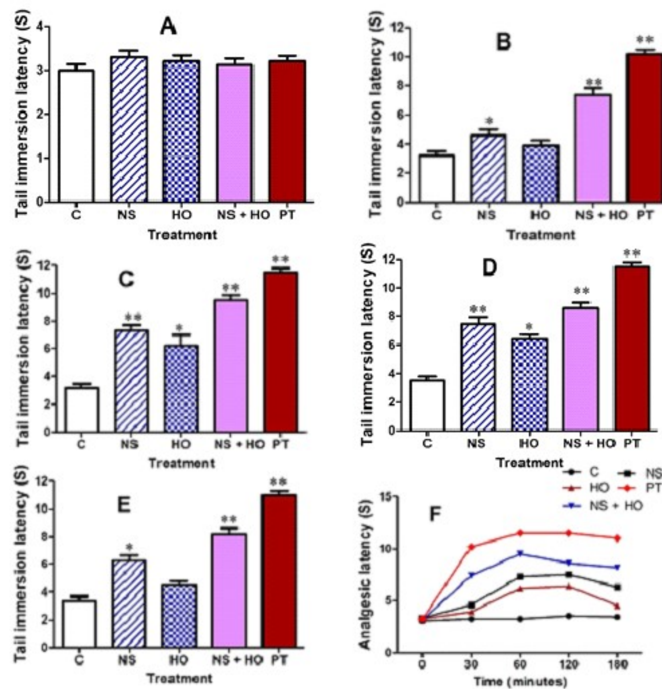

Fig. 2: Protective effect of *N sativa* oil (NS), Honey (HO) and its combination against pain response in tail immersion test after 0 min (A); 30 min (B); 60 min (C); 120 min (D); and 180 min (E); time-course (F) of treatment using pethidine (PT) as a standard reference analgesic. Values are mean ± SEM (n=6). Differences between groups were analyzed by one-way analysis of variances (one-way ANOVA) followed by Dunnet's post test.*p < 0.05 vs control (C), **p < 0.01 vs control.

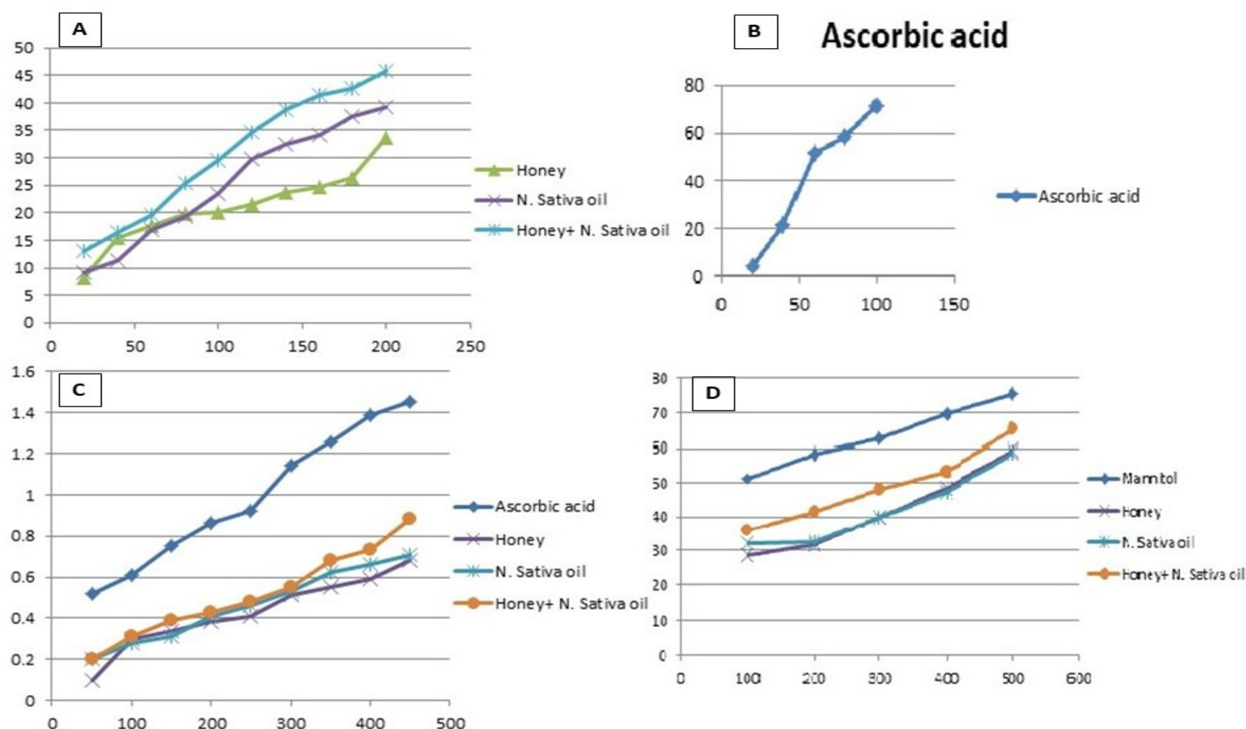

Fig. 3: Fig. 3(A) DPPH radical scavenging activity; fig. 3(B) Standard Curve for ascorbic acid; fig. 3(C) Reducing power activity; fig. 3(D) Hydroxyl radical scavenging activity.

N. sativa possesses a wide spectrum of activities including antinociceptive and anti-inflammatory effects. The mechanism by which *N. sativa* elicits its analgesic effect is not fully understood. In a previous study, *N. sativa* oil (50-400 mg/kg, p.o. enhanced the antinociceptive response in different analgesic screening models (Abdel-Fattah *et al*., 2000). In this study, the authors suggested that the antinociceptive effect is mediated via opioid receptors. The antinociceptive effect of *N. sativa* oil and thymoquinone was blocked by opioid antagonist naloxone significantly (initial phase of formalin test). In addition, naloxone injection (10 mcg/mouse), the kappa receptor antagonist nor-binaltorphimine (1-5mcg /mouse), or the mu(1)- receptor antagonist naloxonazine (1-5mcg /mouse), all administered by intra-cerebroventricular injection (i.c.v), significantly reversed the initial phase of thymoquinone-induced antinociception, but had no effect on the delayed phase of formalin test, while, the delta-opioid receptor antagonist naltrindole (1-5 ng/mouse, i.c.v.), had no effect on either phase. The above results indicate that the antinociception of *N. sativa* oil and thymoquinone is mediated through indirect activation of the kappa-opioid receptor subtypes and supraspinal mu(1)-receptors (AbdelFattah *et al*., 2000). However, in another study, naloxone failed to block the antinociceptive effect of *N. sativa* and it is more likely that multiple mechanisms other than the activation of opioid receptors may be responsible (Ghannadi *et al*., 2005).

Other studies refer to the role of prostaglandins in the analgesic effect of *N. sativa*. Indeed, Houghton et al., demonstrated that *N. sativa* fixed oils and thymoquinone hinder membrane lipid peroxidation and inhibit eicosanoid synthesis in leukocytes (Houghton *et al*., 1995). In addition, thymoquinone showed a potent inhibitory effect on cyclooxygenase-1 and cyclooxygenase-2, the two enzymes responsible for biosynthesis of prostaglandins, the important autacoid which mediates pain and inflammation (Marsik *et al*., 2005).

In our study, combined treatment of *N. sativa* with honey showed synergistic antinociceptive effect. The mechanism of this synergistic effect is not clearly understood. The anti-inflammatory and analgesic potential of honey have been previously reported (Gunduz *et al*., 2014; Alvarez-Suarez *et al*., 2013; Owoyele *et al*., 2011). Another study reported that Yemeni Sidr honey significantly blocked inflammation and pain, and the effects were dose-dependent (Alzubier and Okechukwu, 2011). Similarly, Kassim et al. (2010) reported that Gelam honey inhibited inflammation and pain by inhibition of nitric oxide (NO) and prostaglandin E2 (Kassim *et al*., 2010). Hence, the synergistic effect of inhibition of prostaglandins and NO, and modulation of opioid receptors may explain the synergistic antinocieptive effect of *N. sativa* and honey observed in our study.

However, involvement of other mechanisms cannot be omitted. Some studies reported the role of autonomic receptors in the antinociceptive action of honey. Concomitant administration of honey with tamsulosin (alpha blocker) or propranolol (beta blocker) blocked the anti-inflammatory activity of honey but had no effect on analgesia. The analgesic effect of honey on neurogenic pain was unhindered after co-administration of honey with muscarinic blocker (atropine) and propranolol, whereas, its analgesic effect on inflammatory pain was inhibited after co-administration with atropine. Hexamethonium and atropine had no effect on the anti-inflammatory effect of honey (Owoyele *et al*., 2014). These findings suggest that honey produces its anti-inflammatory effects through modulation of adrenergic receptors, as well as inhibition of NO and prostaglandins synthesis. Whereas, its analgesic effect is produced through modulation of muscarinic receptors. Therefore, too much intake of honey along with adrenergic antagonists is not advisable, especially if honey intake is indicated for inflammatory conditions.

Furthermore, combination of *N. sativa* with honey enhanced its antioxidant potential substantially, which was evident by the results of antioxidant assays in our study. The results of the present study not only revealed the increased antinociceptive effect of *N. sativa* after its combined administration with honey, but also showed faster onset and increased duration of the antinociceptive effect of the combination. These results refer to possible involvement of pharmacokinetic aspects in the observed effects. Faster onset of antinociceptive action of the *N. sativa* by honey, after their oral administration may refer to enhanced gastrointestinal absorption and more rapid achievement of plasma therapeutic concentration of *N. sativa* oil by concurrent administration with honey. Moreover, increase in the duration of antinociceptive effect of the combination, refer to possible delays in the termination of action of *N. sativa* and its elimination by honey. Indeed, approval of such assumptions needs further studies which will be our target in the future researches.

CONCLUSION

From the results of this study, we can conclude that, both *N. sativa* oil and honey have analgesic activity against thermal pain. Combined treatment with the two synergistically increases the analgesic effect of the mixture compared with the analgesic effect of single *N. sativa* oil or honey treatment. These results indicate the beneficial use of combined treatment of the tested drugs in the treatment of pain in humans. Additional studies are warranted to elucidate the possible mechanisms behind the observed pharmacological effects.

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