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EXOGENOUS ADMINISTRATION OF L-ARGININE ENHANCES THE ANTI-INFLAMMATORY ACTIVITY OF *NIGELLA SATIVA* (BLACKSEED) OIL IN WISTAR RATS

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Abstract

Nigella sativa (NS) Linn. (Ranunculaceae) oil is used in Northern Nigeria for the treatment of malaria, stomach ulcer, and other inflammatory diseases. The effect of *NS* (*NS)* oil on inflammation and the nitric oxide (NO) system was investigated in this study. Chronic inflammation was induced using formaldehyde arthritic model while acute inflammation was produced using carrageenan-induced paw oedema in rats. For the chronic inflammatory model, the animals were divided into four groups of five animals each as follows: Group A, B, C, and D, they were administered normal saline, indomethacin (5 mg/Kg), *NS* oil (1 ml/Kg), and *NS* oil (2 ml/Kg) orally respectively, while the acute inflammatory model had a fifth group, E, administered N^G-nitro-L-arginine methyl ester (L-NAME, 100 mg/Kg). In order to investigate the effect of L-arginine administration, the acute inflammation experiment was repeated with intraperitoneal administration of L-arginine (300 mg/kg) to all groups 1h after the induction of inflammation. Oedema (inflammation) was measured daily for the 10-day duration of the chronic inflammatory model and hourly for the 6h duration of the acute inflammatory model. The results showed that *NS* oil has the capacity to inhibit acute and chronic inflammation; it also showed that administration of L-arginine enhanced the anti-inflammatory activity of *NS* oil. It is hereby concluded that the anti-inflammatory activity of *NS* oil may be enhanced by L-arginine.

Keywords: *Nigella sativa,* Nitric oxide, Inflammation, Carrageenan, Formaldehyde, N^G-nitro-L-arginine methyl ester (L-NAME)

Introduction

Inflammation has been known to humans for centuries, partly due to its accomplice action related to two scourges of the past, in the form of wounds and infections, and also due to its conspicuous nature. Cornelius Celsus, a Roman doctor was apparently the first to describe the clinical symptoms of inflammation in the 1st century AD. He described four symptoms that came to be known as the "four cardinal signs of inflammation";*rubor et tumor cum calore et dolore*(redness and swelling with heat and pain) [1]. In 1858, Rudolph Virchow added the "fifth cardinal sign", *functio laesa* (disturbance of function). These signs and symptoms are the resultant effects of endogenous protective mechanisms employed by the body in response to tissue injury caused by trauma, noxious chemicals or microbial agents. These help to inactivate or destroy invading organisms, to remove irritants and set the stage for tissue repair through the release of chemical mediators from injured tissues and migrating cells [2].

Among the several complicated factors involved in the molecular mechanism of inflammation are nitrogen and oxygen reactive species such as nitric oxide and peroxynitrite, hydrogen peroxide, superoxide anion, and hydroxyl radical [3]. Reactive oxygen species have been shown to be produced at the site of inflammation and they have been shown to be proinflammatory [4] while the role of nitric oxide (NO) is paradoxical. Many aspects of inflammatory response, like the release of various inflammatory mediators, blood flow, activity of different enzymes, and adhesion of leucocytes to vascular endothelium is mediated by NO [5]. During infections, NO can be anti-inflammatory or pro-inflammatory and has been described as a "double-edge sword mediator".

The successes recorded with the use of scientifically-based medicine and the constant optimization of drug use in our civilized and highly developed societies cannot be overemphasized. Despite these successes we still crave for new remedies devoid of side effects that are characteristic of orthodox medicine prompting the search for new drugs with minimal or no side effects from plant sources [6]. Many plants are implicated with therapeutic importance, *NS* Linn. (Ranunculaceae), an annual spicy, delicate and beautiful herb grown predominantly in Asia and North Africa is no exception to this. The last two decades witnessed a lot of scientific effort aimed at revealing its pharmacological actions. Research results reveal *NS* as having anti-inflammatory [7], antiulcer [8], antipyretic [9], antimicrobial [10], antidiabetic [11], hepatoprotective [12], cytotoxic [13], antihypertensive [14], antihelminthic [15], tracheal relaxant [16, 17, 18] activities among others.

The anti-inflammatory activity of *NS* oil and its active constituent, thymoquinone has been extensively studied employing different models for inflammation including carrageenan-induced oedema and formaldehyde-induced arthritic models. Study also shows that NSAIDs (indomethacin) may exact their effect in addition to other known mechanisms by increasing endogenous production of NO [19]. To this effect NO-releasing NSAIDs are currently under clinical trial and is potentially seen as a better alternative to the current NSAIDs. This study was carried out to confirm the effect of *NS* in acute and chronic inflammation, to investigate the effect of exogenous administration of NO precursor, L-arginine in acute inflammation and also to determine its (L-Arginine) effect on the anti-inflammatory activity of *NS* oil (in acute inflammation) in order to confirm the potential efficacy of combining substances with anti-inflammatory activity and NO-releasing compounds in the treatment of inflammation.

Materials and Methods

Drugs and Reagents

Black seed (*NS*) oil employed in this study was a product of Mustafa Enterprises Karachi, Pakistan. Formaldehyde was obtained from BDH Chemicals Ltd. Poole, England. Indomethacin used for this study was a product of TUYIL pharmaceuticals, Ilorin, Nigeria. Carrageenan, L-arginine, and NG –nitro-L-arginine methyl ester (L-NAME), were products of Sigma-Aldrich.

Experimental Animals and Groupings

Male and female rats weighing between 120-150 g were used for the experiment. Standard conditions of temperature, humidity and light-dark cycle were maintained throughout the duration of the experiment. Animals also had free access to water and food. Ethical guidelines in experimental animal use stipulated by the ethical committee of the College of Health Sciences, University of Ilorin, Nigeria was adhered to during this investigation. Animals for the various anti-inflammatory studies were divided into five groups (except in the

formaldehyde-induced arthritic model that had four groups) comprising of five animals each.
These groups are: (A) Normal saline, (B) Indomethacin (5 mg/Kg), (C) L-NAME (100 mg/Kg), (D) *NS* oil (1 ml/Kg) and (E) *NS* oil (2 ml/Kg).
For the formaldehyde-induced arthritic model, the four groups are: (A) Normal saline, (B) Indomethacin (5 mg/Kg), (C) *NS* oil (1 ml/Kg), (D) *NS* oil (2 ml/Kg). Normal saline, indomethacin, L-NAME, and the oil extract were administered orally to respective groups using an oral cannula.

Carrageenan-induced paw Oedema
Animals were fasted overnight for 12h before paw inflammation. Paw inflammation was produced in all groups according to the method described by Winter et al., 1962 [20] and Owoyele et al., 2011 [21]. It involved injecting 0.1 ml of 1% solution of carrageenan into the sub-plantar aponeurosis of the left hind-paw of each animal. This inflammation was produced 1h after the administration of normal saline, indomethacin, L-NAME, and different doses of the oil extract to corresponding groups. Measurement of paw size was carried out by wrapping a piece of cotton thread round the paw and measuring the circumference with a metre rule. Paw size was measured immediately before carrageenan injection and 1 to 6h after. Changes in paw size of the treated and experimental groups were compared with that of the control group.

Effect of Exogenous L-arginine administration on carrageenan-induced oedema
This study was carried out to investigate the effect of L-arginine administration on the inflammatory effect of NG oil in carrageenan-induced oedema. The same method was employed as described above. In this model L-arginine (300 mg/Kg) was administered intraperitonially to all groups one hour after paw inflammation was induced according to the method used by Foyet et al. 2008 [22]. Hourly changes in paw size in each of the five groups were compared with corresponding groups in the model not involving L-arginine administration.

Formaldehyde-induced Arthritis
Animals were fasted overnight for 12h before paw inflammation. This study was carried out as previously described by Owoyele et al., 2010 [23]. The left paws of all the animals in all groups were injected with 0.1 mL of 4% formaldehyde on the first and third day of the experiment. Normal saline, indomethacin, and the different extract doses were administered to corresponding groups orally once daily for 10 consecutive days starting from the first day of formaldehyde administration. Daily changes in the paw size measured using cotton thread and metre rule was taken as a measure for oedema. Changes in paw size of the treated and experimental groups were compared with those in the control group.

Statistical Analysis
The results are expressed as mean ± standard error of mean. Data were subjected to analysis of variance (ANOVA) followed by Waller-Duncan post hoc test. Comparison between corresponding groups was carried out using T-test. SPSS 16.0 statistical package was employed. $P<0.05$ was considered statistically significant.

Results

Effect of NS oil in carrageenan-induced oedema
The result for carrageenan-induced paw oedema without the administration of L-arginine is shown in table 1. This result shows that the oil extract of *NS* at doses of 1 ml/Kg and 2 ml/Kg body weight, L-NAME and indomethacin significantly ($P< 0.05$) inhibited paw oedema when compared with the control group that received normal saline.

Effect of NS oil in carrageenan-induced oedema after L-arginine administration
The result for carrageenan-induced paw oedema with the administration of L-arginine is shown in table 2. The result shows significant ($P< 0.05$) reduction in oedema of rats in the treated and experimental groups compared to the control group. The results show that L-arginine administration produced no abolishment of the anti-inflammatory activities of the oil extract of *NS,* L-NAME, nor indomethacin.

Effect of L-arginine on the activity of NS oil in carrageenan-induced oedema
To check for the role of exogenous administration of nitric oxide precursor, L-arginine, results of corresponding groups not receiving L-arginine (table 1) and those that received L-arginine in table 2 in Fig. 1-5. The comparison shows that administration of L-arginine produced significant ($P< 0.05$) inhibition in oedema response in the control group receiving normal saline. L-arginine produced no significant

change in the inhibitory activity of indomethacin (Fig. 2) and dampened the inhibitory effect of L-NAME in the first phase of oedema response but produced significant (P< 0.05) inhibition of oedema in the 4th and 5th hr of the second phase of oedema response.

Formaldehyde-induced Arthritis

The result for formaldehyde-induced arthritis is shown in Fig.6. The figure compares daily changes in hind-paw oedema in the control group (normal saline) with the treated group (5 mg/Kg body weight of indomethacin), and the experimental groups receiving 1 ml/Kg and 2 ml/Kg body weight doses of NS oil extract. The results show that indomethacin and the oil extract at the dose of 2 ml/Kg body weight produced significant (P< 0.05) inhibition of arthritis for the ten day period of the experiment while the significant (P< 0.05) inhibition of arthritis was observed from the second day of the experiment in the group receiving 1 ml/kg body weight dose of the oil extract.

Discussion

The anti-inflammatory effect of NS oil was examined using a model of chronic (formaldehyde-induced arthritis) and acute inflammation (carrageenan-induced oedema) in this study.

One of the most appropriate modus operandi to screen for anti-arthritic and anti-inflammatory agents is the inhibition of formaldehyde-induced arthritis, as this model closely mimics arthritis in humans [24]. Sub-plantar injection of formaldehyde produces localised inflammation and pain due to release of prostaglandin-like substances, serotonin and histamine [25]. Agents with probable anti-proliferative activity can be tested using this model [26]. The results obtained for chronic inflammation show that NS oil and the reference drug (indomethacin) significantly suppressed formaldehyde-induced arthritis. This conforms to previous studies where thymoquinone, an active ingredient in this oil was reported to possess anti-inflammatory activity due to its potent inhibitory action on eicosanoid generation [27, 28].

A widely used phlogistic agent which shows signs and symptoms of inflammation, which can be assessed by increase in paw circumference is carrageenan. Carrageenan-induced inflammation like other acute inflammations is biphasic; the first phase involving histamine, serotonin and kinins [29], and the second phase, which is sensitive to steroidal and non-steroidal anti-inflammatory drugs involve the release of prostaglandin like substances [30].From the results obtained, NS oil produced a dose dependent inhibition of oedema in the carrageenan model of acute inflammation which is comparable to that of standard anti-inflammatory drug, indomethacin. This inhibition was higher in the second phase of inflammatory response suggesting that the anti-inflammatory effect of NS may be due in part to inhibition of prostaglandin like substances. L-NAME, an inhibitor of nitric oxide synthase (NOS) also inhibited oedema and its anti-inflammatory activity have been shown to be as a result of its inhibition of the calcium-independent inducible nitric oxide synthase (iNOS).

According to Knowles & Moncada 1994 [31] “NO is a dynamic compound affecting various physiological and cellular processes in the body and in particular inflammatory processes” and its anti-inflammatory or pro-inflammatory effect is dependent on its concentration in the tissue-microenvironment and the stage of inflammation. Three isoforms of nitric oxide synthase (NOS) have been identified. They are the neuronal NOS, endothelial NOS (eNOS), and the inducible NOS (iNOS). Neuronal NOS and eNOS are constitutive and calcium dependent while iNOS is stimulated by endotoxins and inflammatory cytokines. The action of NO on vascular endothelium, leucocytes, mast cells, macrophages, platelets, some enzymes and its anti-oxidant activity contribute to its modulatory action in inflammatory processes [5].

Administration of L-arginine, a precursor of nitric oxide to the groups that received normal saline was observed to produce significant inhibition of oedema formation in the carrageenan model. The anti-inflammatory activity of NS oil was also enhanced significantly in all phases of oedema response in the carrageenan model upon L-arginine administration. These observations may be due to the effect of NO on vascular endothelium, leucocytes, mast cells, macrophages, platelets, some enzymes and its anti-oxidant property. NO produces vasodilation (relaxation of vascular smooth muscle) by activating guanylate cyclase and also by inhibiting the action of mediators, such as histamine, leukotriene C4, and platelet-activating factor that increase contraction of vascular smooth muscle, thereby inhibiting oedema [32]. Adhesion and infiltration of leucocytes, especially neutrophil is due to expression of β-2 adhesion molecules and NO have been shown to down regulate β-2 adhesion molecules and neutrophil aggregation and secretion [33, 34].

NO also inhibits production of pro-inflammatory mediators from mast cells (by increasing endogenous release of NO from itself) and macrophages and also down-regulates platelet aggregation and adhesion [35, 36, 37]. NO has also been reported to be a free radical scavenger [38] and also interacts with variety of enzymes (iron-containing) by inhibiting their activity such as cytochrome P-450, cyclooxygenase and iNOS [39, 40]. The above mechanisms by which NO exact its anti-inflammatory activity may explain the effect observed in the control animals when administered L-arginine after induction of inflammation and the enhanced anti-inflammatory activity of NS oil after NO administration suggest that the mechanism of action of NS oil may be similar to that of NO.

The inhibitory action of L-NAME was observed to be initially dampened upon the administration of L-arginine. This may be due to the down-regulation of its activity as was reported by Foyet et al. 2008. In contrast to the report by Foyet et al. 2008, L-arginine did not abolish the anti-inflammatory effect of L-NAME. The observed enhancement of the anti-inflammatory activity of L-NAME in the second phase of inflammatory response may be due to the anti-inflammatory activity of NO as observed in the group receiving normal saline or combined effects of L-NAME and L-arginine. Cyclo-oxygenase inhibitors, like indomethacin have been shown to significantly increase NO production in murine macrophages in vitro and this may be one of the mechanisms by which they exert their anti-inflammatory effects. Significant change in the anti-inflammatory activity of indomethacin was not observed in this study.

Conclusion

This study has further confirmed the anti-inflammatory effects of NS oil as well as probable role of exogenously administered nitric oxide. Specifically, nitric oxide administration has the capacity to enhance the anti-inflammatory activity of NS oil. Further studies can focus on other mechanisms involved in nitric oxide induced NS oil anti-inflammatory activities.

Declaration of Conflict of Interest

The authors report no conflict of interest

Acknowledgement

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pig tracheal chains. Iran J Med Sci 1997; 22:127-133.
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Fig 1: Comparing changes in hind-paw circumference of wistar rats in the control group (normal saline) that received L-arginine (300mg/Kg body weight) and the control group (normal saline) that received no L-arginine. *P< 0.05 vs control without L-arginine administration.

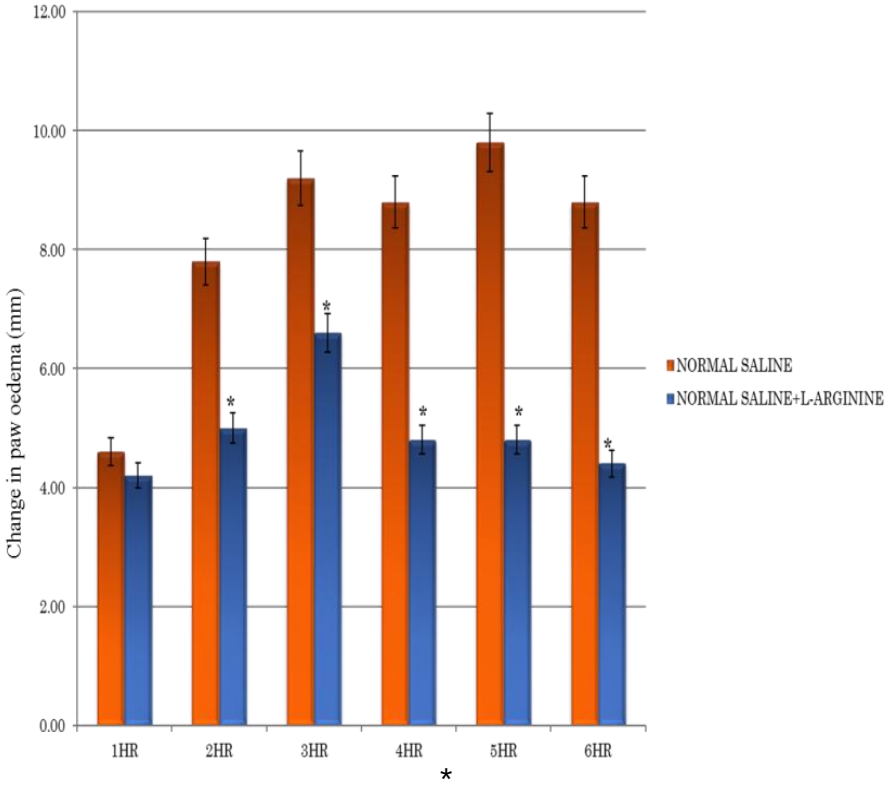

Fig 2: comparing changes in hind-paw circumference of wistar rats in the treated group receiving indomethacin (5mg/Kg body weight) and L-arginine (300mg/Kg body weight) with the treated group that received indomethacin (5mg/Kg body weight) only. *P< 0.05 vs treated group with indomethacin only.

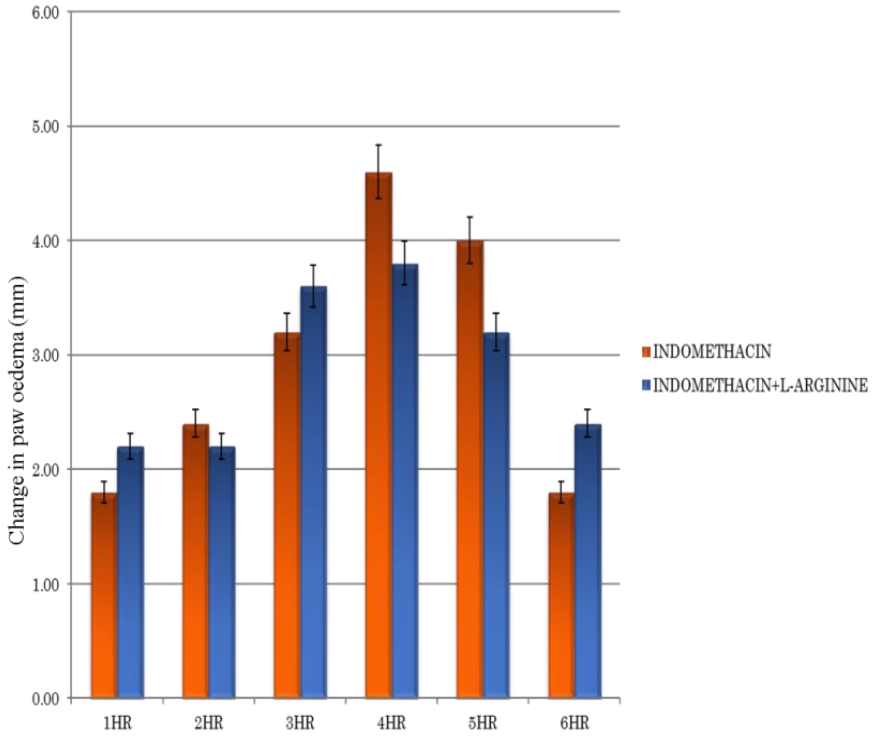

Fig 3: comparing changes in hind-paw circumference of wistar rats in the treated group receiving L-NAME (100mg/Kg body weight) and L-arginine (300mg/Kg body weight) with the treated group that received L-NAME (100/Kg body weight) only. *P< 0.05 vs treated group with L-NAME only.

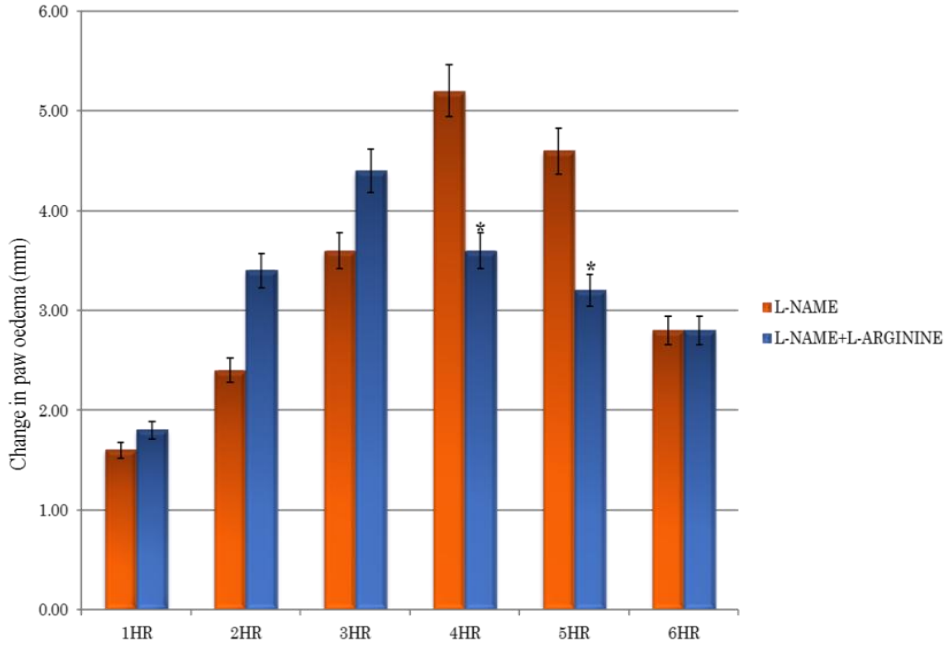

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Fig 4: comparing changes in hind-paw circumference of wistar rats in the experimental group receiving *Nigella sativa* oil extract (1ml/Kg body weight) and L-arginine (300mg/Kg body weight) with the treated group that received *Nigella sativa* oil extract (1ml/Kg body weight) only. *P< 0.05 vs experimental group receiving *Nigella sativa* oil extract only.

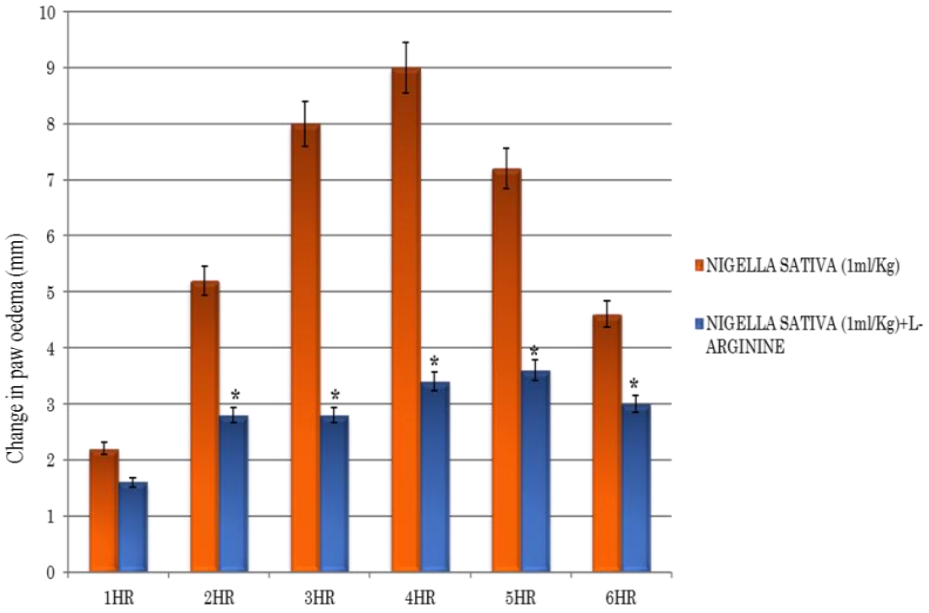

Fig 5: comparing changes in hind-paw circumference of wistar rats in the experimental group receiving *Nigella sativa* oil extract (2ml/Kg body weight) and L-arginine (300mg/Kg body weight) with the treated group that received *Nigella sativa* oil extract (2ml/Kg body weight) only. *P< 0.05 vs experimental group receiving *Nigella sativa* oil extract only.

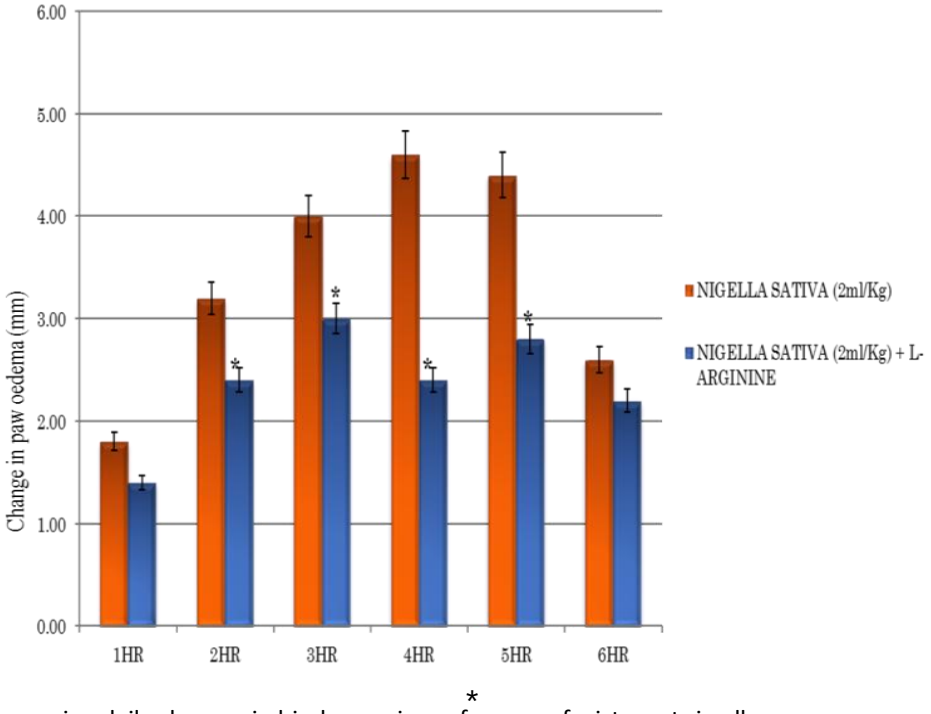

Fig. 6: Comparing daily changes in hind-paw circumference of wistar rats in all groups. *P<0.05 significantly different from control (normal saline)