

RHINOLOGY

The value of *Nigella sativa* in the treatment of experimentally induced rhinosinusitis

Potenziale della Nigella sativa nel trattamento della rinosinusite indotta in setting sperimentale

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SUMMARY

The aim of this study was to investigate the effect of *Nigella sativa* and cephalexin in the therapy of experimental bacterial rhinosinusitis. Bacterial rhinosinusitis was induced with *Staphylococcus aureus*. Rabbits were divided into five groups; control (n = 6), *N. sativa* 50 mg/kg/d (n = 6), *N. sativa* 100 mg/kg/d (n = 6), *N. sativa* 200 mg/kg/d (n = 6), and cephalexin 20 mg/kg/d (n = 6) groups. *N. sativa* was given orally for 7 days. The same volume of normal saline (0.9% NaCl) was given as a vehicle to the control group for the same period. After treatment period, sinus mucosa samples were evaluated using stereological and histopathological methods. Half of the maxillary sinus mucosa samples were frozen at -80°C for further analysis of NO levels. Pathology revealed a severe acute inflammatory process in rabbits treated with saline. Only mild inflammation was determined in cephalexin group, *N. sativa* 100 mg/kg/d and *N. sativa* 200 mg/kg/d groups. The level of NO increased in the saline group was significantly reduced in all treatment groups. *N. sativa* may prevent histopathological changes of rhinosinusitis via decreased NO levels in a dose dependent manner and can be used in the treatment of rhinosinusitis diseases.

KEY WORDS: *Nigella sativa* • Rhinosinusitis • Nitric Oxide • Neutrophil • Rabbit

RIASSUNTO

Obiettivo del presente studio è stato quello di investigare l'effetto della Nigella sativa e della cefalexina nel trattamento della rinosinusite batterica indotta in setting sperimentale. La rinosinusite batterica è stata indotta mediante stafilococco aureo. I conigli sono stati suddivisi in 5 gruppi; uno di controllo (n = 6), N. sativa 50 mg/kg/d (n = 6), N. sativa 100 mg/kg/d (n = 6), N. sativa 200 mg/kg/d (n = 6), e cefalexina 20 mg/kg/d (n = 6). La N. sativa è stata somministrata per via orale per 7 giorni. Lo stesso volume di soluzione salina (% 0,9 NaCl) è stato quindi somministrato al gruppo di controllo per lo stesso periodo di tempo. Dopo il periodo di trattamento i campioni di mucosa dei seni mascellari sono stati valutati utilizzando metodologie istopatologiche e stereologiche. La metà dei campioni di mucosa del seno mascellare sono stati congelati a -80°C per una successiva analisi dei livelli di ossido nitrico. L'analisi patologica ha rivelato un intenso processo infiammatorio in atto nei conigli trattati con sola soluzione salina. Solo un lieve grado di infiammazione è stato invece rilevato nei conigli nei gruppi trattati con cefalexina, N. sativa 100 mg/kg/d, e N. sativa 200 mg/kg/d. Il livello di ossido nitrico, elevato nel gruppo placebo, è risultato invece essere ridotto negli altri gruppi. La N. sativa potrebbe prevenire i cambiamenti istopatologici indotti dalla rinosinusite mediante una riduzione dei livelli di ossido nitrico con andamento dose dipendente, e potrebbe essere usata nel trattamento della rinosinusite.

PAROLE CHIAVE: *Nigella sativa* • Rinosinusite • Ossido nitrico • Neutrofili • Coniglio

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Introduction

Rhinosinusitis is one of the most frequently reported chronic diseases. In contrast to acute rhinosinusitis, where bacterial or viral aetiology is well established, chronic rhinosinusitis has been defined as an inflammation of the mucous membrane of the paranasal sinuses resulting from impaired transport mechanisms¹. Local and systemic host immune responses interact under such conditions, leading to bacterial and respiratory virus effects in the pathophysiological events which is characterised by hyperaemia,

haemorrhage and submucosal oedema with polymorphonuclear infiltration of rhinosinusitis². Therefore, treatment of rhinosinusitis must break the vicious cycle of inflammation, oedema formation and mucous hypersecretion, before antibiotic treatment. Although treatment of rhinosinusitis is usually based upon use of antibiotics and/or surgery, it may result in both high medical costs and development of multiple drug resistance in sinusitis-causing pathogenic microorganisms in humans^{3,4}. In this regard, new antimicrobial substances from various sources like medicinal plants are arousing interest.

Nitric oxide (NO) is one of the most abundant free radicals in the body and acts as a signalling or toxic agent between cells. Excess NO production causes mitochondrial respiratory enzyme inhibition⁵. NO has been described in many cell types in a variety of tissues, where it acts as a regulator of vascular tone, neurotransmitters, acute and chronic inflammation and host defence mechanisms^{6,7}. It is involved in innate immunity, which is generated during immune and inflammatory actions as a toxic agent towards infectious organisms^{8,9}. Neutrophils are capable of sustained release of high levels of NO, initiated by inflammatory cytokines and bacterial products¹⁰. As a result, many types of infections may cause an increase in levels of NO.

Nigella sativa (*N. sativa*), belonging to the Ranunculaceae family, is an annual herbaceous plant native to Asian and Mediterranean countries. The plant is known as "black cummin" or "black seed". There are numerous reports on the phytochemical, pharmacological and toxicological properties of black cummin^{11,12}. Recent studies have shown that *N. sativa* seeds possess various pharmacological effects including analgesic, antipyretic, anti-inflammatory and anti-nociceptive, antioxidant, anti-tumour and cytotoxic, anti-diabetic and anti-ulcer properties^{9,13-21}. Many of these properties have been attributed to its quinone constituents, and especially the thymoquinone of black cummin seeds¹⁷. Many reports have also been cited the actions of *N. sativa* seed extracts or its oil on different bacterial isolates¹¹. The oil or its extracts have been found to have a broad spectrum of antibacterial activity¹³. It has also been demonstrated that both crude alkaloid extracts and water extracts of *N. sativa* seeds were effective against a variety of organisms, isolated from human patients suffering from septic arthritis, and even many organisms that were resistant to antibiotics²².

The aim of this study was to document the effects of three different doses of oral administration of *Nigella sativa* in a well-characterised rabbit model of rhinosinusitis, compared with cephalexin treatment, by analysing the number of neutrophils, histopathology and level of NO of the nasal mucosa following treatment.

Materials and methods

Animals

Thirty adult male albino rabbits weighing an average of 3 kg were obtained from Ataturk University Experimental Animal Laboratory of Medicinal and Experimental Application and Research Centre. The rabbits were maintained in our laboratory under controlled environmental conditions and fed *ad libitum* consumption of pelleted feed mixture that was formulated to meet or exceed National Research Council recommendations (National Research Council, 1995). Animal experiments were performed in accordance with the national guidelines for the use and

care of laboratory animals and were approved by the local animal care committee of Ataturk University.

Drugs

The seeds of *N. sativa* were purchased from a local market. *N. sativa* seed samples (200 g) were separately extracted four times with 600 ml of chloroform (CHCl₃) or ethanol (EtOH) at room temperature. After the organic solvents were evaporated to dryness under vacuum at low temperature using a rotary evaporator, 59.6 g liquid CHCl₃ extract (29.8% yield) and 51.8 g liquid ethanol extract (51.8% yield) were obtained.

Experimentally induced rhinosinusitis and treatment

The animals were inoculated with *Staphylococcus aureus* (*S. aureus*) after the right nasal cavity was packed with Meroce^lR (Medtronic Xomed, Jacksonville, FL, USA). Twenty-four hours after bacterial inoculation, the Meroce^lR in the right nasal cavities was removed. Thirty animals were assigned randomly to receive saline (per-oral, group 1), 50 mg/kg/d *N. sativa* per-oral (group 2), 100 mg/kg/d *N. sativa* per-oral (group 3); 200 mg/kg/d *N. sativa* per-oral (group 4) and 50 mg/kg/d cephalexin (i.m.) (group 5) daily for 7 days. Twenty-four hours after the treatment period, the animals were sacrificed with injection of sodium pentobarbital via the auricular vein and then decapitated after ensuring death.

Preparation of bacterial suspension and induction of rhinosinusitis

S. aureus strain ATCC 25923 was suspended at a concentration of 900x10⁶ cells/ml using a McFarland Nephelometer Standard III at the Department of Microbiology, School of Medicine, Ataturk University, Erzurum, Turkey. After administration of a sedative, the nasal cavities were packed with Meroce^lR for indirect obstruction of the maxillary sinus ostium²³. The nasal dorsum was aseptically prepared with povidone-iodine before administration of a local anaesthetic to the skin and adjacent soft tissue. Next, 0.5 ml of bacterial suspension was injected to the right maxillary cavity using a hypodermic syringe.

Histological and stereological procedures

For analyses, dissected tissue samples were fixed in 10% formalin. After fixation, the tissue samples were decalcified with EDTA. The decalcified tissue samples were then washed, dehydrated in ascending graded alcohols, cleared in xylene, and embedded in paraffin. For stereological examination, 20 micron sections were taken from the blocked tissues using a systematically randomised sampling method^{24,25}. For histological examination, 5 micron sections were taken from tissue blocks. After deparaffinisation and rehydration, serial sections were stained with haematoxylin and eosin.

An unbiased stereological technique, the optical dissector/fractionator method, was used to estimate neutrophil

numerical density the serial sections of the sinus mucosa^{26 27}. The neutrophils in 20 micron sections were counted using a system for image analysis consisting of a light microscope (Leica DM4000 B, Cambridge, UK), equipped with an X-Y-Z movement-sensitive stage (Bio-Precision MAC 5000 controller system; Ludl Electronic Products, Hawthorne, NY, USA) and a video camera (Optronics MicroFire, Goleta, CA, USA) coupled to a computer containing Stereo Investigator software (Micro-BrightField, Williston, VT, USA).

Extraction of maxillary sinus mucosa and measurement of nitric oxide level

Sinus mucosa tissue NO levels were measured using the Griess reagent as previously described^{28 29}. Griess reagent, a mixture (1:1) of 0.2% N-(1-naphthyl)ethylenediamine and 2% sulphanilamide in 5% phosphoric acid gives a red-violet diazo dye with nitrite, and the resultant colour was measured at 540 nm. First, nitrate was converted to nitrite using nitrate reductase. The second step was the addition of Griess reagent, which converts nitrite to a deep purple azo compound; photometric measurement of the absorbance at 540 nm then determines the nitrite concentration. Protein interference was eliminated by treatment of the reacted samples with zinc sulphate and centrifugation for 5 min 10,000xg. Biochemical measurements were carried out using a Cecil CE 3041 (Cambridge, UK) spectrophotometer.

Statistical analysis

For all parameters studied, means and standard deviations were calculated. Statistical analysis was performed using SPSS 13 for Windows (SPSS Inc., Chicago, IL, USA). The analysis of variance with the post-hoc LSD (least significant difference) test was used to compare results for all the groups. A p < 0.05 was considered statistically significant.

Results

Physical examination

There was nasal discharge in all animals at the time of the removal of Merocel. Macroscopically, there was also oedema at varying degrees in the right maxillary sinus of all animals when sinus cavities were opened after the treatment period.

Histopathological lesions

In our study, the numerical density of neutrophils was also estimated. For all groups (control, cephalixin, *N. sativa* 50 mg/kg/d, *N. sativa* 100 mg/kg/d and *N. sativa* 200 mg/kg/d) the numerical density of neutrophils was 0.000351/ μm^3 , 0.000020/ μm^3 , 0.000072/ μm^3 , 0.000058/ μm^3 , 0.000068/ μm^3 respectively (Table I and Fig. 1). Severe neutrophil infiltration was detected in control rabbits treated with saline. Pathology revealed a severe acute inflammatory process, with neutrophil and oedema formation that permeated the maxillary sinus and surrounded a vasodilatation mucosal capillary. Several instances of vascular dilatation and haemorrhage were determined in section profiles.

Although the general architecture of the maxillary sinus in cephalixin groups was partly protected, no important pathological findings at the structural level were observed, but only mild inflammation (Fig. 2A). Neutrophil infiltration of the maxillary sinus was lower than in the saline group (Fig. 2A). Similar histopathological findings were observed in the *N. sativa* 100 mg/kg/d and *N. sativa* 200 mg/kg/d groups (Fig. 2C and 2D). In the *N. sativa* 50 mg/kg/d group, neither maxillary sinus nor vascular

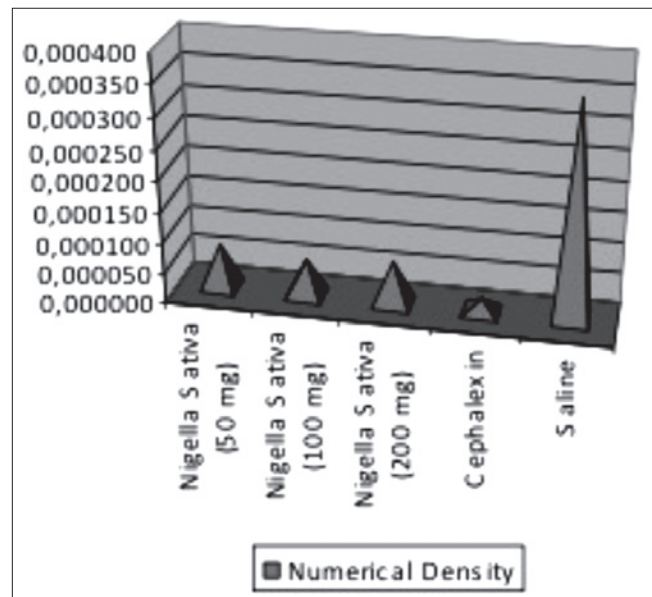


Fig. 1. Changes in mean neutrophil numerical density during treatment for 7 days in each group.

Table I. Neutrophil density in all the groups.

Groups	Numerical density of neutrophils (n/ μm^3)	Standard deviation	Standard error mean	p
<i>N. sativa</i> (50 mg/kg/d, N = 6)	0.000072	0.00003950	0.00001613	< 0.001
<i>N. sativa</i> (100 mg/kg/d, N = 6)	0.000058	0.00004831	0.00002416	< 0.001
<i>N. sativa</i> (200 mg/kg/d, N = 6)	0.000068	0.00001058	0.00001067	< 0.001
Cephalixin (20 mg/kg/d, N = 6)	0.000020	0.00000496	0.00000202	< 0.001
Saline (N=6)	0.000351	0.00003726	0.00001862	< 0.001

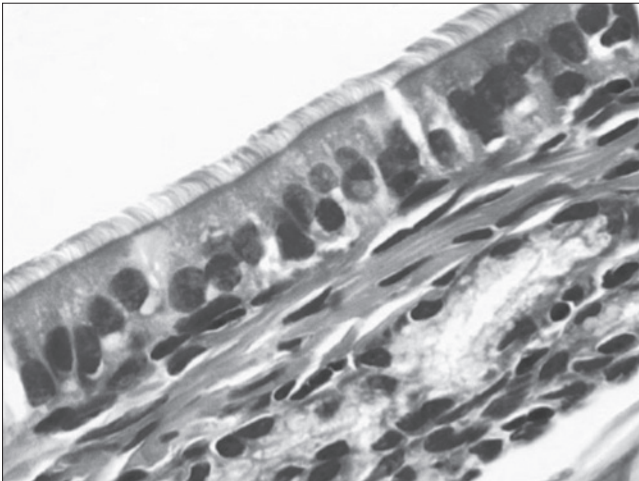


Fig. 2A. Histopathological section of rabbit maxillary sinus mucosa of treatment with cephalexin (H&E X200).

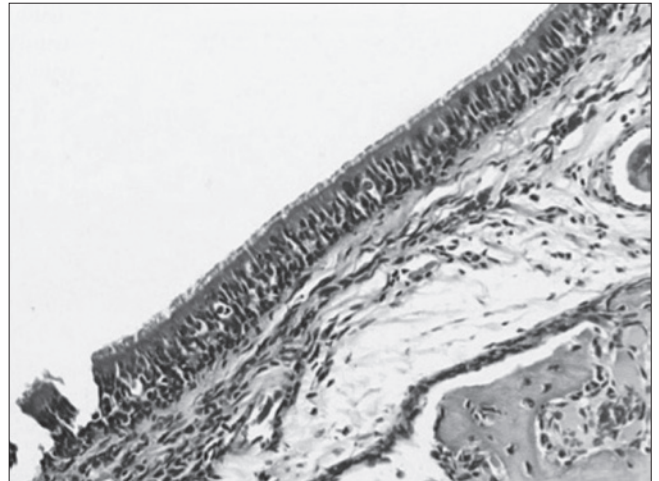


Fig. 2D. Histopathological section of rabbit maxillary sinus mucosa treated with *N. sativa* 200 mg/kg/d (H&E X50).

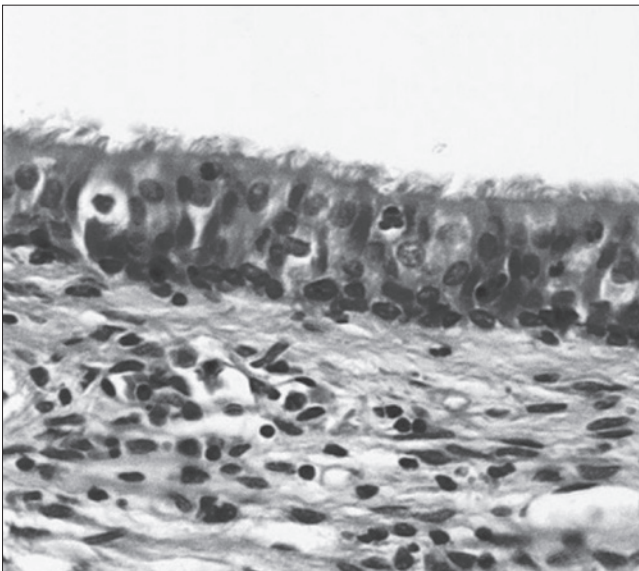


Fig. 2B. Histopathological section of rabbit maxillary sinus mucosa treated with *N. sativa* 50 mg/kg/d (H&E X100).

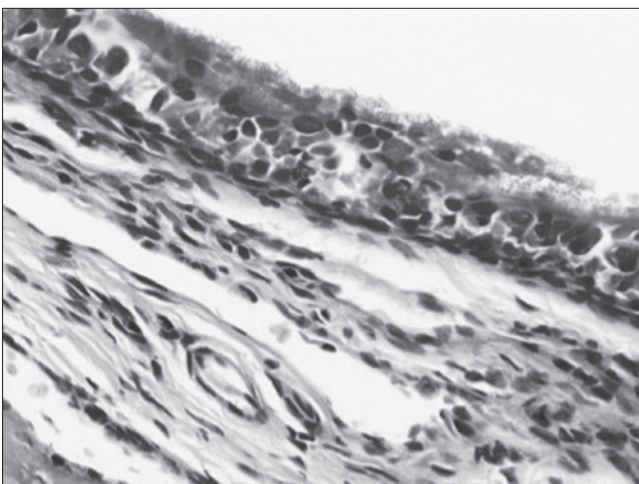


Fig. 2C. Histopathological section of rabbit maxillary sinus mucosa treated with *N. sativa* 100 mg/kg/d (H&E X100).

structures were normal in appearance compared with the cephalexin group (Fig. 2B).

Nitric oxide level

Table II shows that the level of NO increased in the saline treated group in comparison to the cephalexin 20 mg/kg/d, *N. sativa* 50 mg/kg/d, *N. sativa* 100 mg/kg/d and *N. sativa* 200 mg/kg/d groups. The mean tissue total NO levels were significantly ($p < 0.001$) higher in the saline group and correlated well with disease acuteness. Cephalexin 20 mg/kg/d, *N. sativa* 50 mg/kg/d, *N. sativa* 100 mg/kg/d and *N. sativa* 200 mg/kg/d had a similar effect on the levels of NO, all reducing its value, which were 163.31 $\mu\text{mol/ml}$, 166.40 $\mu\text{mol/ml}$, 165.20 $\mu\text{mol/ml}$ and 166.43 $\mu\text{mol/ml}$ in experimental rhinosinusitis, respectively. In the saline control group, the value of NO level was 184.17 $\mu\text{mol/ml}$.

Discussion

Nasal and paranasal sinus mucosa have a highly efficient system for the physiologic functions of olfaction, respiration, and protection³⁰. The respiratory epithelial cell layer presents a physical barrier that prevents invasion by micro-

Table II. Effects of cefalexin and *N. sativa* on changes in the level of NO in sinus mucosa samples of rabbits in an experimental rhinosinusitis model. The groups treated with three different doses of *N. sativa* and cefalexin were compared with the saline group.

Groups	Number of Animals	NO ($\mu\text{mol/ml}$)	p
Control (Saline)	6	184.17 \pm 3.09	-
Cefalexin 20 mg/kg/d	6	163.31 \pm 2.98	< 0.001
<i>N. sativa</i> 50 mg/kg/d	6	166.40 \pm 0.22	< 0.001
<i>N. sativa</i> 100 mg/kg/d	6	165.20 \pm 0.43	< 0.001
<i>N. sativa</i> 200 mg/kg/d	6	166.43 \pm 0.43	< 0.001

* $p < 0.001$; ** $p < 0.01$.

organisms, and the mucociliary action prevents bacterial infection and protects the mucosa from injury and drying³¹. Sinusitis is one of the most frequently reported acute or chronic and heterogeneous diseases, which shows several types of aetiology. Where a bacterial or viral aetiology is well established, this has been defined as an inflammation of the mucous membrane of the paranasal sinuses resulting from impaired transport mechanisms¹. Various systemic and local factors are known to be associated with nasal and sinus infections^{32,33}. To maintain the physiologic condition of the nasal cavity and sinuses, it is known that nasal air-flow, anatomical conditions, patency of the natural ostium, oxygen saturation in sinuses and mucociliary clearance all play important roles. When one of these physiologic conditions is changed, these abnormal conditions cause inflammatory reactions, due to an abnormal mucous membrane immunity, phagocytosis and bacteriologic action of the nasal secretion enzymes. The local and systemic host immune responses interact under such conditions, leading to bacterial and respiratory virus effects in the pathophysiological events, which are characterised by hyperaemia, haemorrhage and submucosal oedema with polymorphonuclear infiltration of rhinosinusitis³².

The aim of this study was to compare the effectiveness of three different doses of *N. sativa*, which was of natural herbal origin, with that of cephalexin. With this therapy, we aimed to ameliorate neutrophil numbers and inhibit the inflammation that was a consequence of the NO level in the sinuses. Cingi et al. reported no significant difference histopathologically, among an antibiotic treated group, a thymoquinone treated group and a negative control group in their experimental study in which they investigated the role of thymoquinone, a phytochemical component of *N. sativa*, for sinusitis therapy³⁴. They proposed that thymoquinone can be a bioactive agent used for treatment of rhinosinusitis and have the same histopathological effect with an antibiotic. We obtained similar results herein.

The reduction of the neutrophil numbers was histopathologically relevant and statistically significant in both treatment groups, compared with the saline group. It similar in the group of rabbits treated with *N. sativa* (100 mg/kg/d and 200 mg/kg/d) and in the group treated with cephalexin. The results of our study have shown that rhinosinusitis with cephalexin eradicate infection and reduce inflammation. The effectiveness of *N. sativa* is based on its known mechanisms of action, which are anti-inflammatory, antioxidant and antimicrobial^{8,13,15-17}.

NO may play a part in tissue damage, and its roles in the control of a variety of intracellular organisms have been described in some viral and fungal infections or protozoal infestations as a toxic agent towards infectious organisms³⁵⁻³⁸. However, the role of NO in bacterial infection has not been clearly defined; it may be cytostatic or cytotoxic, not only for invading microorganisms, but also for the cells that produce it and also for neighbouring cells.

For example, NO is a mediator of NK cell killing of target cells and regulates NK cell function; it inhibits activation of mast cells and can enhance or inhibit neutrophil activation, depending on its concentration³⁹⁻⁴¹. Another potential mechanism includes a direct microbiocidal effect, via the reaction of NO with iron or thiol groups on proteins that inactivate enzymes systems in mitochondria. It may also interact with oxygen-derived radicals to generate molecules that may enhance its cytotoxicity. In addition, NO has been found to react with superoxide to form reactive oxidants capable of damaging target cells⁴².

Our results for tissue NO levels are in agreement with these data. Our results suggest that oxidative stress in tissue of rabbits with rhinosinusitis may cause an increase in NO, which arises because of tissue damage. *N. sativa* may be given to decrease the NO in patients with rhinosinusitis, with the hope of alleviating the observed toxicities. Since NO seems to have a dual role in bacterial rhinosinusitis, the high concentrations of NO could result in damage in sinus mucosa and *N. sativa* decreases the tissue level of NO via anti-inflammatory, microbiocidal or antioxidant functions.

Conclusions

Our findings underscore that acute bacterial rhinosinusitis can increase the level of NO and that high concentrations of NO may play an important role in the pathogenesis of rhinosinusitis of the impaired sinus tissue of rabbits. The results of our study suggest that *N. sativa* may prevent histopathological changes of rhinosinusitis via decreased NO levels in a dose dependent manner.

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