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In vitro and *in vivo* study of the antibacterial effects of *Nigella sativa* methanol extract in dairy cow mastitis

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Abstract

Objective: *Nigella Sativa (N. Sativa)* seeds were used in traditional medicine for the treatment of a variety of diseases. The seed extracts and oil of this plant have shown various pharmacological properties including antimicrobial actions. In this study, the *in vitro* and *in vivo* antibacterial effects of methanol extract of the seeds against pathogenic bacteria causing mastitis in cows have been investigated.
Materials and methods: in *in vivo* experiments, 10 cows with mastitis were treated by local injection of different concentrations of methanol extract of the seeds into the infected breasts. In *in vitro* experiments, the microorganisms were collected from the same infected breasts and used for the assessment of the antimicrobial effects of the extract by means of agar dilution and disk diffusion methods.
Results and conclusion: The extract showed significant *in vitro* and *in vivo* inhibitory effects on causative organisms compared to standard drugs and also induced healing of the disease. This is the first veterinary experiment, to our knowledge, that investigated the antibacterial effects of *Nigella sativa*.

Keywords: *Nigella sativa*, Blackseed, Antibacterial activity, Plant extracts, Mastitis

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Introduction

Nigella sativa (blackseed) is an annual Ranunculaceae herbaceous plant growing to 30 cm and has an upright branching stem, fine deeply cut leaves, gray-blue flowers and toothed seedpots. The plant is native to Western Asia and the Mediterranean region. The seeds contain 40% fixed oil, a saponin (melantin) and up to 1.4% volatile oil (Chevallier, 1996).

Dioscorides, a Greek physician of the 1st century AD, recorded that black cumin seeds were taken to treat headaches, nasal catarrh, toothache, and intestinal worms (Chevallier, 1996). The seeds of *N. sativa* have been used traditionally for centuries in the Middle East, Northern Africa and India for the treatment of various diseases (Brutis and Bucar, 2000; Gilani et al., 2004).

The plant extracts and essential oil showed a broad range of pharmacological effects such as antidiabetic (Farah et al., 2002; Benhaddou-Andaloussi et al., 2010), spasmolytic and bronchodilator (Gilani et al., 2001; Boskabady et al., 2008), hepatoprotective (Al-Ghamdi, 2003; Coban et al., 2010), analgesic and anti-inflammatory (Hajhashemi et al., 2004; Bashir and Qureshi, 2010), antitumor (Khan et al., 2003; Majdalawieh et al., 2010) and gastroprotective (El-Dakhakhny et al., 2000; Kanter et al., 2006) effects in various studies. The extracts also showed *in vitro* and *in vivo* antimicrobial (Mashhadian and Rakhshandeh, 2005; Salem et al., 2010), and anticestodal effects (Akhtar and Riffat, 1991). It is used traditionally in Iran as laxative, carminative and intestinal antiprotozoal drug (Amin, 1990).

Mastitis is an inflammation of mammary gland regardless of the cause, with economical and health consequences. Many infective agents have been implicated as causes of mastitis; among the major pathogens are *S. aureus*, *E. coli*, streptococcus strains and *enterobacter aerogens* (Radostits et al., 1994). There is an increasing problem with antimicrobial drug resistance and so increasing demand for the development of new antimicrobial agents, especially in common veterinary and human infections. As there is no previous study on veterinary infections due to pathogenic microbes, we decided to study the in vitro and in vivo effects of methanol extract of blackseed on cows mastitis. This is the first veterinary study, to our knowledge, of antibacterial effects of *N. sativa*.

Materials and methods

Nigella sativa seeds

The seeds were collected from local herbal drugs shops.

Chemicals

Methanol was purchased from Arastoo Chemical and Pharmaceutical Company (Tehran, Iran). Streptomycin and penicillin G were kindly provided by Jaber-ibn-Hayan pharmaceutical company (Tehran, Iran).

Reflux extraction

50 g of blackseed powder were used with 300 ml of methanol with an extraction period of 10-12 hours. The extract was filtered using filter paper and the solvent was evaporated using rotary distillation apparatus. In order to obtain a completely dry extract, the resultant extract was transferred to glass dishes and was left in a 50°C oven for 24 hours. Then, it was stored at 4°C until assessment of its antimicrobial activity.

Clinical experiments

Treatment of mastitis in cows was carried out in two separate farms in Quchan (a town 140 km west of Mashhad, Iran, Farm 1) and Mashhad (Farm 2). Mastitis

and the results of treatment were confirmed by a veterinary surgeon. In farm 1, 5 ml of 1% and 3% solutions of methanol extract of the seeds in liquid paraffin were injected into the breast quarters once daily for 5 consecutive days for the treatment of clinically-confirmed mastitis in cows. In farm 2, 10 ml of 6% solutions of the extract were injected twice daily for the same duration. Negative control groups in both farms were treated with liquid paraffin only. Milk samples were collected before and 2 days after treatment and assayed for the presence of pathogenic bacteria. To avoid any contamination, sampling was carried out after careful washing of the breasts and disinfecting with alcohol.

In order to calculate the reduction of colony count due to treatments, the following formula was used: Percent of reduction in colony count = 100 – Colony count after treatment / Colony count before treatment × 100

In vitro microbiological assessments

In vitro experiments were carried out only in farm 2.

Agar dilution method

Pre-defined amounts (Table 3) of the extracts were added to 100 ml of Muller-Hinton agar culture media (Merck) to obtain different concentrations in the media. Plates containing 15 ml of these media were incubated in 25^{o}C for 24 h to assure that they have not been contaminated during preparation. The microorganisms were cultured on these plates, incubated as mentioned above and the results were recorded for analysis.

Disk diffusion method

Previously weighed paper disks were immersed for half an hour in the solutions of different concentrations of the extracts and were dried out under a laminar flow cabinet. By weighing the dried disks and comparing with before immersion weights, the amount of extracts in disks were calculated. Negative control disks were prepared using the solvent of the extracts in the same way. These (together with positive control disks, containing penicillin G and streptomycin) have been used for microbiological assay in plates containing the appropriate microorganisms.

Cylinder plate method

These experiments were carried out just to confirm significant penetration of the extract in disk diffusion method. Data was not shown.

Statistical analysis

Paired t-test was used for the statistical analysis of the results of clinical experiments. Analysis of in vitro experiments was carried out using ANOVA and Tukey tests.

Results

Clinical experiments Collected samples of microorganism from the two farms are shown in Table 1.

Farm 1

The results of colony count before and after once daily treatment of mastitis with 5 ml of 1% and 3% solutions of methanol extract of *N. sativa* seeds in paraffin are shown in Tables 2. The results indicate that the extract had a stronger effect against *S. aureus* than against group B □*-hemolytic streptococci.*

Farm 2

The results of colony count before and after twice daily treatment of mastitis with 10 ml of 6% solution of methanol extract of the seeds in paraffin are shown in Table 2. The results indicate that the extract had a strong effect against group D *streptococci*, *E. coli* and *S. aureus* in this

concentration.The inhibitory effect was weaker against *E. coli* than against the other two organisms.

Table 1. Collected samples of milk from the affected cows.

Sample No.	Organism	No. of cases	
		Farm 1	Farm 2
1	S. aureus	10	5
2	Group B streptococci	12	0
3	S. epidermis	4	2
4	E. coli	3	15
5	P. aeroginosa	2	2
6	Group A streptococci	3	0
7	Corinebacterium	2	0
8	Enterococci (Group D streptococci)	0	30
9	Klebsiella	0	2
10	Citrobacter	0	1
11	Sterile	*	8
	Total	36	65

*Not identified

In vitro microbiological experiments

As stated above, these experiments were carried out only on farm 2 samples.

In this part of study, the antibacterial effects of methanol extract of *N. sativa* seeds on microorganisms isolated from the breast of the affected cows were investigated. As the most common organisms in these experiments were *Enterococci* (group D *Streptococci*), *E. coli* and *S. aureus*, microbiological assessment was focused on these organisms. Table 1 shows the number of collected samples for the microorganisms.

Agar dilution method

As shown in Table 3, the minimum inhibitory concentration (MIC) of the extract was 20 mg/ml for all samples of the organisms.

Disk diffusion method

Table 4 shows the zones of inhibition for different concentrations of the extract against collected samples of *S. aureus*, *E. coli* and *Enterococci*. The extract did not show any effect against *E. coli* in this method.The amount of the extract in the disk for zone of inhibition of 10-20 mm for the collected samples of *S. aureus* and *Enterococci* were 1.5 mg and 5 mg, respectively. The extract did not show any activity against *E. coli*.

Zone of inhibition of more than 20 mm was formed only for collected strains of *CPSA* in 8 mg disks

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Table 2**.** The *in vivo* antibacterial effect of methanol extract of *Nigella sativa* seeds against different infecting bacteria (Colony count in 1 ml of milk × 1000).

Organism	Collected from	Extract concentration	n	Before treatment	After treatment	p value
S. aureus	Farm 1	1%	10	62±10.8	34±9.1	ns
	Farm 1	3%	10	51.5±11.9	17.3±7.8	*p<0.05
β-hemolytic streptococci	Farm 2	1%	12	45±8.3	42.7±7.6	ns
	Farm 2	3%	11	38.2±9.1	18.7±6.8	*p<0.05
***Enterococci* (Group D *streptococci*)**	Farm 2	6%	15	45.87±8.98	10.7±4.4	** p< 0.01
E. coli	Farm 2	6%	12	19.2±8.4	0±0	*p<0.05
S. aureus	Farm 2	6%	5	42±12	0±0	** p< 0.01

Data are presented as mean± SEM.

Table 3**.** The *in vitro* antibacterial effect of methanol extract of *Nigella sativa* seeds against organisms collected in farm 2 in agar dilution method.

Extract Concentration (mg/ml)	*Enterococci* (Group D *streptococci*)		*E. coli*		*S. aureus*	
	Growth (%)	Growth inhibition (%)	Growth (%)	Growth inhibition (%)	Growth (%)	Growth inhibition (%)
0	100	0	100	0	100	0
20	25	75	90	0	30	70
40	0	100	30	70	0	100
80	0	100	0	100	0	100
160	0	100	0	100	0	100

Table 4. Zones of inhibition produced by the extract against organisms collected in farm 2 in disk diffusion method.

Organism	Penicillin G	Dose	N	Zones of inhibition (mm)
Enterococci	Penicillin G	(10 µg/disk)	21	13.05 ± 1.39
	Streptomycin	(10 µg/disk)	21	10.04 ± 1.9
S. aureus	Penicillin G	(10 µg/disk)	5	9.8 ± 4.3
	Streptomycin	(10 µg/disk)	5	21 ± 2.32
Enterococci	Extract	(5 mg/disk)	21	11.28 ± 1.28
		(8 mg/disk)	21	13.23 ± 1.27
S. aureus	Extract	(1.5 mg/disk)	5	10.4 ± 2.6
		(4 mg/disk)	5	17 ± 1.34
		(8 mg/disk)	5	20.2 ± 1.32

There was not any significant response with concentrations below 4 mg/disk and 2 mg/disk against *Enterococci* or *S. aureus* respectively. Also, methanol did not produce any zone of inhibition. Data presented as mean ± SEM.

Discussion

Statistical analysis of the results showed that the methanol extract of *N. sativa* seeds had significant *in vivo* antimicrobial activity in farm 1 and farm 2 experiments and this effect was dose-dependent. Treatment of clinically-confirmed mastitis with injection of 10 ml of 6% paraffin solution of the extract twice daily for 5 days induced complete response against *E. coli* and *S. aureus* and an excellent response against *Enterococci* (group D *Streptococci*) in farm 2 (Table 2). Injection of 5 ml of 1% and 3% paraffin solution of the extract once daily also produced significant antimicrobial activity against *S. aureus* in farm 1 but only 3% solution produced significant activity against group B β-hemolytic *Streptococci* in that farm (Table 2). It is obvious that the use of higher dose of the extract in farm 2 (10 ml of 6% solution twice daily compared with 5 ml of 1% and 3% twice daily in farm 1) produced stronger effects against the organisms, as shown for *S. aureus* in Table 2.

Results of *in vitro* experiments also showed significant activity of the extract against *S. aureus* and *Enterococci* in all methods and against *E. coli* in agar dilution method only (Table 3). Previously, we have

shown the antimicrobial activity of methanol and chloroform extracts of *Nigella sativa* seeds on standard and hospital microorganisms *C. albicans*, *S. aureus* and *P. aeroginosa* (Mashhadian and Rakhshandeh, 2005). Hanafy and Hatem (1991) also observed antimicrobial activity of diethyl ether extract of the plant against *S. aureus*, *P. aeroginosa*, *E. coli* and *C. albicans*. Our results did not show any activity against *E. coli* in disk diffusion method. There might be some explanations for this difference: first of all, we used methanol extract and this may explain, to some extent, the difference between the results. Secondly, the amount of ingredients of the same plant can be affected by the area and the season of collection. Thirdly, it is possible that more resistant strains of some organisms exist in animal setting compared with standard or human ones. This extract was active against *E. coli* in our clinical experiments. Finally, *in vivo* effects of antimicrobial agents may be different from their *in vitro* effects, because of the effects of immune system. It seems that immunological and environmental factors affect the activity of the extract against *E. coli*.

N. sativa seed extracts also showed anticestodal (Akhtar and Riffat, 1991) and antileishmania (unpublished data) effects. The mechanism of action of antimicrobial effects of the extracts is not clear but their broad spectrum of activity implies that they should affect basic and common key processes in the organisms.

Finally, our results are in agreement with others who showed that *N. sativa* extracts produce antimicrobial activity against a broad range of microbes and especially against multiple-antibiotic resistant bacteria (Morsi, 2000).

To our knowledge, this is the first *in vivo* study of antibacterial effects of *N. sativa* seed extracts against a common and important disease of cattle. Considering the wide margin of safety of the extracts of the seeds (Gilani et al., 2004; Vahdati-Mashhadian et al., 2005), they may be of promising veterinary uses in the future. Further studies on the activity-directed fractionation for the isolation of respective pure compounds may result in interesting outcomes.

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