UNIVERSITI PUTRA MALAYSIA

ANTIMICROBIAL ACTIVITY OF NIGELLA SATIVA (BLACK SEED) OIL AGAINST LEPTOSPIRA SPECIE

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ANTIMICROBIAL ACTIVITY OF *NIGELLA SATIVA* (BLACK SEED) OIL AGAINST *LEPTOSPIRA* SPECIES

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It is hereby certified that we have read this project paper entitled "Antimicrobial Activity of *Nigella Sativa* (Black Seed) Oil against *Leptospira* Species" by Ahmad Rasul bin Radzali, and in our opinion it is satisfactory in term of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project.

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DEDICATION

"Dedicated especially to my parents, my siblings and my friends"

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

AKTIVITI ANTI-MIKROB MINYAK *NIGELLA SATIVA* (HABBATUS SAUDA) TERHADAP SPESIS *LEPTOSPIRA*

Disediakan oleh

Ahmad Rasul bin Radzali

2014

Penyelia: Prof. Madya Dr. Siti Khairani binti Bejo

Penyelia Bersama: Prof. Dato' Dr. Abdul Rani bin Bahaman

Aktiviti antimikrob minyak *Nigella sativa* telah dikaji terhadap lima serovar *Leptospira interrogans* iaitu *L. pomona , L. hardjobovis , L. australis , L. canicola* , dan *L. icterohaemorrhagiae*. Kepekatan perencatan minima (MIC) telah ditentukan dengan menggunakan kaedah ***broth microdilution*** dengan memerhatikan perencatan lengkap motilitas *Leptospira* melalui mikroskop ***dark-field*** pada pelbagai tempoh inkubasi (1 jam , 1 hari, 3 hari, dan 7 hari). Minyak *N. sativa* telah dilarutkan dalam dimetil sulfoksida (DMSO) sebelum dilarutkan dalam medium cecair Ellinghausen , McCullough , Johnson dan Harris (EMJH) dengan kepekatan akhir dari 0.1 hingga

50mg/ml. Semua serovar *Leptospira* adalah sensitif kepada minyak *N. sativa* pada setiap tempoh inkubasi dengan nilai-nilai MIC yang berbeza-beza dari 0.39 hingga 6.25 mg/ml. *Leptospira pomona* adalah lebih sensitif kepada minyak *N. sativa* berbanding dengan serovar-serovar lain, dengan nilai MIC yang paling rendah dalam setiap tempoh inkubasi (1 jam = 1.5 6mg/ml , 1 hari = 0.78 mg/ml , 3 hari = 0.78 mg/ml , 7 hari = 0.39 mg/ml), manakala *L. australis* adalah yang paling kurang sensitif terhadap minyak *N. sativa* dengan nilai MIC 6.25 mg/ml pada tempoh inkubasi 7 hari. Penicilin G dan DMSO telah dipilih sebagai kawalan positif dan negatif untuk eksperimen ini. Terdapat perbezaan yang signifikan antara serovar-serovar *Leptospira* yang dirawat dengan minyak *N. sativa* pada MIC yang diperolehi dalam setiap tempoh inkubasi; 1 jam (p = 0.014), 1 hari (p = 0.016), 3 hari (p = 0.026) dan 7 hari (p = 0.010). Walau bagaimanapun, tidak terdapat perbezaan yang signifikan di antara tempoh inkubasi yang berbeza-beza dengan nilai-nilai MIC yang diperolehi (p = 0.332).

Kata-kata kunci: *Leptospira*, *Nigella sativa*, Kepekatan perencat minima (MIC)

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Project.

ANTIMICROBIAL ACTIVITY OF *NIGELLA SATIVA* (BLACK SEED) OIL AGAINST *LEPTOSPIRA* SPECIES

By

Ahmad Rasul bin Radzali

2014

Supervisor: Assoc. Prof. Dr. Siti Khairani binti Bejo

Co-supervisor: Prof. Dato' Dr. Abdul Rani bin Bahaman

The antimicrobial activity of *Nigella sativa* oil was studied against five serovars of *Leptospira interrogans* which are *L. pomona, L. hardjobovis, L. australis, L. canicola, and L. icterohaemorrhagiae.* The minimum inhibitory concentration (MIC) was determined using broth microdilution method by observing complete motility inhibition of the *Leptospira* through dark-field microscopy at various incubation periods (1 hour, 1 day, 3 days, and 7 days). The *N. sativa* oil was dissolved in dimethyl sulfoxide (DMSO) prior to further dilution in Ellinghausen, McCullough, Johnson and Harris (EMJH) liquid medium with final concentration ranged from 0.1 to 50mg/ml. All *Leptospira* serovars were sensitive to *N. sativa* oil dilution at every incubation period with MIC values varying from 0.52 to 5.21mg/ml. *Leptospira*

pomona was more sensitive to *N. sativa* oil compared to the other strains, with the lowest MIC value obtained in every incubation period (1 hour = 1.56mg/ml, 1 day = 1.56mg/ml, 3 days = 0.78mg/ml, 7 days = 0.52mg/ml), while *L. australis* was the least sensitive towards *N. sativa* oil which was 5.21mg/ml at 7-day incubation period. Penicilin G and DMSO were chosen as positive and negative controls for the experiment respectively. There were significant differences among the *Leptospira* serovars treated with *N. sativa* oil on MIC values at 1-hour ($p = 0.014$), 1-day ($p = 0.016$), 3-day ($p = 0.026$) and 7-day ($p = 0.010$) incubation periods. However, there was no significant difference among different incubation periods on the MIC values of *N. sativa* oil ($p = 0.332$).

Keywords: *Leptospira*, *Nigella sativa*, Minimal inhibitory concentration (MIC)

1.0 INTRODUCTION

Leptospirosis is a zoonotic disease caused by any of the pathogenic members of the genus *Leptospira*. The disease occurs worldwide and is more common in tropical and subtropical areas with high rainfall (WHO, 2003). In Malaysia, the first diagnosed case of leptospirosis in human was in 1926 by Fletcher (Lim *et al.*, 2011). The leptospirosis cases in human have increased significantly starting from 263 cases in year 2004 to 1418 cases in 2009, and continued to rise with 1876 cases in 2010, followed by 2268 cases in 2011 and 3665 cases in 2012 (Suhailah *et al.*, 2014).

There are nearly 300 serovars of *Leptospira* distributed worldwide among both pathogenic and non-pathogenic species (Adler *et al.*, 2010). Most of them have their primary reservoirs in wild and domestic animal or livestock. In humans, leptospirosis may be presented with broad range of clinical manifestations from mild influenza-like illness to severe infection with multiple-organ failure (WHO, 2003). In livestock animals, leptospirosis could lead to abortion, pre-mature birth, infertility, low milk production and death that would cause great loss to the livestock industry (JPV, 2011).

Various antibiotics have been suggested to be used in treating leptospirosis. In severe cases of leptospirosis, high doses of intravenous penicillin is recommended, while less severe cases can be treated with oral antibiotics like amoxicillin, ampicillin, doxycycline or erythromycin (WHO, 2003). Nevertheless, the

development of antibiotic resistance should be concerned especially with the use of the same antibiotics over a period of time, as well as improper antibiotic administration. Thus, there is a need to find alternatives to the antibiotic therapy, and herbal medicine may be one of them.

Nigella sativa, commonly known as black seed or Habbatussauda, is herbaceous plant that is well recognised for its medicinal properties in treating various diseases in humans. Its seed extract as well as its essential oil has been reported to have significant antimicrobial properties to variety of pathogenic bacteria including some bacterial strains that have multidrug resistance (Salman *et al.*, 2007). However, there is lack of information on the antimicrobial activity that *N. sativa* seed on *Leptospira* species. Hence, the objectives of this study were

1. to investigate the antimicrobial activity of *N. sativa* oil against *Leptospira* species.
2. to determine the Minimum Inhibitory Concentration (MIC) of *N. sativa* oil on *L*eptospira species.

2.0 LITERATURE REVIEW

2.1 *Leptospira sp* and Leptospirosis

Leptospira sp is gram negative spiral shape aerobic bacteria. It is fastidious and slow-growing bacteria that requires a special media and conditions for their growth. The bacteria are motile and can be observed under dark-field microscope. The genus *Leptospira* falls under the family of Leptospiracae and the order of Spirochetales. Currently there are 20 species of *Leptospira* that are categorized into 3 groups; 9 pathogenic, 5 intermediate, and 6 saprophytes. Other than that, there are more than 300 serovars of *Leptospira* which are grouped into 20 serogroups (Bolin, 2012; Picardeau, 2013).

Leptospirosis is a transmissible disease of humans and animals caused by pathogenic species of *Leptospira* with a worldwide distribution. In humans, the case fatality rates have been reported to range from less than 5% to 30% while the incidences reported to be approximately 0.1 – 100,000 per year in temperate climates and 10 – 100 per 100,000 per year in the humid tropics (WHO, 2003). Leptospirosis can infect many species of animals, and among domestic animals, the disease is commonly recognized in cattle, swine, dogs, and horses. When an animal is infected, it can either become maintenance host or incidental host. In maintenance hosts, there would be high prevalence of infection, however the clinical signs would be mild and acute, and the bacteria would persistently infect the kidney and genital tract. On the

other hand, incidental host would have low prevalence of infection, but with severe clinical signs, and short-lived urogenital tract infection (Bolin, 2012).

2.2 Treatment and Prevention of Leptospirosis

The early antimicrobial therapy is required to clear the leptospiremic phase as well as to eradicate the bacteria from the urine of infected animals thus preventing the spread of the *Leptospira* bacteria to other animals or humans (Goldstein, 2013). Various antibiotics have been used to treat leptospirosis. Penicillin or doxycycline have been known to be the antibiotic choices in humans and dogs infected with leptospirosis. Other antibiotics like ceftriaxone, cefotaxime and azithromycin are also effective in human leptospirosis. Leptospires are less susceptible to cephalosporins, and are resistant to chloramphenicol. There has been a controversial on the use of fluoroquinolones as it was proven to be inefficient in clearing out leptospires from the kidneys and blood of a hamster model, however, doxycycline abled to clear the organisms from all the sites within 3 days of infection. Consensus Statements of the American College of Veterinary Internal Medicine suggested for canine leptospirosis to be treated with doxycycline, 5 mg/kg PO or IV every 12 hours for 2 weeks. If the dog shows any side-effects of doxycycline administration like vomiting, it is wise to change the treatment with ampicillin,20 mg/kg IV every 6 hours, with dose reduction for azotemic dogs. Penicillin G (25,000–40,000 U/kg IV q12h) can also be given. Nevertherless, in order to eradicate the organisms from the renal tubules, the dogs should receive doxycycline for 2 weeks after gastrointestinal signs subside. It is not recommended to use fluoroquinolonein dogs with

leptospirosis as it may contributes to antimicrobial resistance in other bacteria (Sykes *et al.,* 2011).

Vaccination has been used in many domestic animals as a step to prevent leptospirosis. In livestock herds, vaccines helps to avoid further abortions in leptospirosis outbreak. As these vaccines are only useful against some *Leptospira* serovars, their content may need to be altered from time to time based on the current prevalence of *Leptospira* serovars infecting the animal. Vaccines in cattle usually contain serovar hardjo, while in pigs, serovar pomona is mostly used. In dogs, traditionally, the vaccines contained serovar icterohaemorrhagiae, however, there are other serovars being included in some vaccines in North America. Other than vaccination, prophylactic antibiotics treatment can also be used to prevent disease in animals exposed to the bacteria. Contact with infected animals as well as contaminated environment should be minimized whenever possible. Besides that, good sanitation as well as quarantine and testing of new animal into the farm are also essential to reduce the infection risk and prevent introduction of the disease (Iowa State University, 2013)

2.3 *Nigella sativa*

Nigella sativa is an annual herb from Ranunculaceae family. The seeds of *N. sativa* was called as the black seeds or black cumin (in English), ‘kalonji’ (in Urdu), and ‘habbatus sauda’ or ‘habbatul barakah’ which means the blessed seeds (in Arabic) (Hurairah, 2014). *Nigella sativa* seeds have been used in the Middle East,

Northen Africa and India, to treat many ailments like asthma, cough, bronchitis, headache, rheumatism, fever, influenza, and eczema. Besides that, the black seeds are also used in bread and other dishes as spice or carminative (Burits 2000).

Many research have been done to discover the medicinal properties that the black seeds contain. The black seeds has been proven to have anti-inflammatory, analgesic, immune enhancer, anti-bacterial, anti-fungal, anti-tumour, anti-cestodal and anti-diabetic effects. Many strains of microorganisms have been tested to be susceptible to the *N. sativa* oil or its extract, and some of these are known to be highly resistant to drugs. Among microbes that have been tested were *V. cholera, E. coli*, most strains of *Shigella* spp., *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus* spp. and *Streptococcus pyogenes* (Hurairah, 2014, Salman *et al.*, 2007). However, there is lack of research that have been done on the antimicrobial effect of *N. sativa* seeds on *Leptospira sp.*

3.0 MATERIALS AND METHODS

3.1 *Leptospira* isolates

Five pathogenic *Leptospira interrogans* serovars were used in this study, which are *L. pomona, L. hardjobovis, L. australis, L. canicola,* and *L. icterohaemorrhagiae.* The *Leptospira* sp were obtained from Bacteriology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The organisms were grown in Ellinghausen, McCullough, Johnson, and Harris (EMJH) liquid medium at 30˚C for 7 days.

3.2 Preparation of *Nigella sativa* oil dilution

Figure 1 Commercial cold-pressed *N. sativa* oil

A commercially available cold-pressed *N. sativa* oil was used in this study. Two hundred (200) milligram of *N. sativa* oil was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to prepare a stock solution with 200 mg/ml in concentration. The working

solution was prepared by mixing 500 µl of the stock solution with 1ml of EMJH liquid medium to a concentration of 100 mg/ml.

3.3 Preparation of negative and positive controls

Dimethyl sulfoxide was selected to be the negative control for the experiment. Five hundred (500) µl DMSO was diluted in 1 ml of EMJH medium to a concentration of 50% as the working solution. Penicillin G was selected as the positive control for the experiment, where the stock solution (1000 µg/ml) of Penicillin G was prepared by dissolving 2 mg of Penicillin G powder in 2 ml sterile distilled water, and then 50 µl of the stock solution was mixed with 1 ml of EMJH liquid medium to prepare the working solution of 50 µg/ml.

3.4 Minimum Inhibitory Concentration (MIC) determination

Broth microdilution method was used to determine the minimum inhibitory concentration. Two fold serial dilution of the working solution of *N. sativa* oil, DMSO and Penicillin G was done with EMJH medium in the 96-well microtitre plate up to 10 dilutions. The initial volume in every well was 50µl. Then, a 50µl volume of *Leptospira* suspension was added to each well leading to further dilution of the samples resulting in final concentration range of 0.10-50mg/ml for *N. sativa* oil, 0.05-25% for DMSO, and 0.05-25µg/ml. Another negative control (EMJH medium and leptospires without *N. sativa* oil) was also included in every plate. The plate was mixed well and covered in clean aluminium foil prior to incubation at

30°C for 7 days. During the incubation period, the plate was observed for complete motility inhibition of the leptospires under dark field microscope for 4 times at different time of incubation; after 1 to 3 hours, after 1 day, after 3 days and after 7 days of incubation. The MIC was defined as the lowest concentration of the *N. sativa* oil that exhibited complete motility inhibition of the leptospires. All tests were carried out in triplicate for *N. sativa* oil, and duplicate for DMSO and penicillin G.

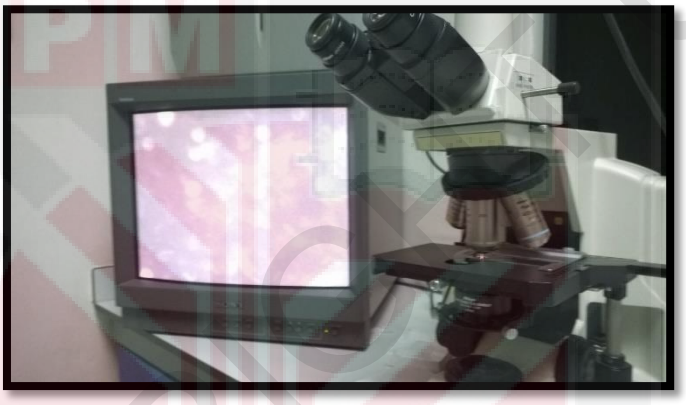

Figure 2. Dark-field microscope

3.5 Statistical Analysis

Statistical analysis was done by using software IBM SPSS version 21. Kruskal-Wallis test was used to test differences among the *Leptospira* serovars treated with the *N. sativa* oil on MIC values obtained in every incubation. Similar test was also used to test the difference among different incubation periods on distribution of the MIC values of *N. sativa* oil.

4.0 RESULTS AND DISCUSSION

The present study showed that *N. sativa* oil is effective against all five *Leptospira* serovars which are *L. pomona, L. hardjobovis, L. australis, L. canicola, and L. icterohaemorrhagiae,* with the MIC values ranged from 0.39mg/ml to 6.25mg/ml (Table 1). *Leptospira pomona* was the most sensitive to *N. sativa* oil in every incubation period (1 hour = 1.56mg/ml, day 1 = 0.78mg/ml, day 3 = 0.78mg/ml, day 7 = 0.39mg/ml) , while *L. australis* was the least sensitive in most incubation periods (1 hour = 3.13mg/ml, 1 day = 6.25mg/ml, 3 day = 3.13mg/ml, 7 day = 6.25mg/ml).

Statistical analysis revealed significant differences among the *Leptospira* serovars treated with *N. sativa* oil on MIC values at every incubation period [1 hour (p=0.014), 1 day (p=0.016), 3 day (p=0.026), 7 day (p=0.010)]. On the other hand, there was no significant difference among different incubation periods on the MIC values (p=0.332).

The present study demonstrated that *N. sativa* oil has some degree of antimicrobial effects on the *Leptospira* sp. The antimicrobial effects that the black seed oil displayed could be due to the presence of its natural compounds like thymoquinone, thymohydroquinone, and thymol (Salman *et al.*, 2007).

From the experiment, it was found that DMSO has inhibitory effect on *Leptospira* serovars with MIC ranged from 12.5% to 25%. This result was supported

by Wadhwani (2008) showing that DMSO did have inhibitory effect on bacterial growth, however she suggested that DMSO is generally safe at 3% for some microorganisms without denying that the inhibitory effects may differ for other microorganisms.

In comparison to other study, Penicillin G revealed higher MIC values on the *Leptospira* serovars (MIC = 25µg/ml). Seesom (2003) reported the MIC values of Penicillin G against different *Leptospira* serovars ranging from 0.39 to 6.25 µg/ml. The difference in the result may be due to the different method of preparing the antibiotic dilution. In the present study, the Penicillin G was diluted with EMJH medium instead of using sterile distilled water like in a similar study conducted by Seesom (2003).

Table 1. Minimum Inhibitory Concentration of *N. sativa* oil against *Leptospira* serovars

SAMPLE	MIC (mg/ml)			
	1 hour incubation	**1 day incubation**	**3 day incubation**	**7 day incubation**
N. sativa* oil + *L. pomona	1.56	0.78	0.78	0.39
N. sativa* oil + *L. hardjobovis	1.56	3.13	1.56	1.56
N. sativa oil + *L. australis*	3.13	6.25	3.13	6.25
N. sativa* oil + *L. canicola	6.25	3.13	3.13	3.13
N. sativa oil + *L. icterohaemorrhagiae*	6.25	3.13	1.56	1.56

Table 2. Minimum Inhibitory Concentration of DMSO and Penicillin G against *Leptospira* serovars

SAMPLE	MIC			
	1 hour incubation	**1 day incubation**	**3 day incubation**	**7 day incubation**
DMSO +*L. pomona*	-	25%	25%	12.5%
DMSO + *L. hardjobovis*	-	25%	12.5%	12.5%
DMSO + *L. australis*	25%	25%	25%	12.5%
DMSO +*L. canicola*	-	25%	25%	25%
DMSO + *L. icterohaemorrhagiae*	-	25%	25%	25%
Penicillin G + *L. pomona*	-	-	-	25 µg/ml
Penicillin G + *L. hardjobovis*	-	-	-	-
Penicillin G + *L. australis*	-	-	-	25 µg/ml
Penicillin G + *L. canicola*	-	-	-	-
Penicillin G + *L. icterohaemorrhagiae*	-	-	-	25µg/ml

5.0 CONCLUSION

Nigella sativa oil has some degrees of antimicrobial property against leptospires, however the inhibitory effect differs significantly according to *Leptopira* serovars (MIC ranged from 0.39mg/ml to 6.25mg/ml). With the emergence of antibiotic resistant organisms, natural herbs like *N. sativa* seeds may be used as alternative treatment to Leptospirosis. However, more research like toxicity testing and *in vivo* experiment need to be done to ensure the efficacy of *N. sativa* seeds in treating the disease.

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