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Nigella Sativa Seed Extract has Potential Antimicrobial Activity on Pasteurella multocida in Vitro

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ABSTRACT

Nigella sativa known as black cumin has various bioactive compounds to treat many diseases. Some of the benefits of N. sativa seed are as immune booster, antihistamin, anti-diabetic, antihypertension, antiinflammatory, antimicrobial, and antitumor. This study aimed at determining the antimicrobial activity and finding out the effective concentration of N. sativa seed ethanolic extract on Pasteurella multocida in vitro. N. sativa seed extract was divided into three groups consisting of 15% extract (T1), 25% extract (T2), 45% extract (T3), and chloramphenicol was used as positive control. For antimicrobial test, Kirby Bauer diffusion method was used, and the data were analyzed by ANOVA. The results revealed that T3 had the most effective antimicrobial activity, shown by the largest inhibition zone (17.72 mm), followed by T2 (15.93 mm) and T1 (10.75 mm). ANOVA test results showed significant differences (P<0.05) in each group. The antimicrobial effect of T3 and T2 was categorized as strong, whilst T1 as moderate. From the results it can be concluded that N. sativa seed extract had strong and moderate inhibition activity on the growth of *P. multocida*, therefore, *N. sativa* seed is a potential candidate for antimicrobial drug development againts *P. multocida*.
Keywords: antibacterial, black cumin, Kirby Bauer, Pasteurella multocida.

ABSTRAK

Nigella sativa yang juga dikenal dengan nama jintan hitam memiliki banyak senyawa bioaktif yang dapat mengobati berbagai macam penyakit. Beberapa manfaat biji *N. sativa* antara lain sebagai peningkat sistem imun, antihistamin, antidiabetes, antihipertensi, antiinflamasi, antimikroba, dan antitumor. Penelitian ini bertujuan untuk mengetahui aktivitas antimikroba dan mengetahui konsentrasi efektif ekstrak etanol biji *N. sativa* terhadap *Pasteurella multocida* secara in vitro. Ekstrak biji *N. sativa* dibagi menjadi tiga kelompok perlakuan yaitu 15% (T1), 25% (T2), 45% (T3), dan kloramfenikol sebagai kontrol positif. Uji antimikroba dilakukan menggunakan metode *Kirby Bauer*, dan data dianalisis menggunakan ANOVA. Hasil penelitian menunjukkan bahwa T3 memiliki aktivitas antimikroba yang paling efektif, ditunjukkan dengan zona hambat terbesar (17,72 mm), diikuti oleh T2 (15,93 mm) dan T1 (10,75 mm). Hasil uji ANOVA menunjukkan perbedaan yang signifikan (P<0,05) pada masing-masing kelompok perlakuan. Daya antimikroba T3 dan T2 tergolong kuat, sedangkan T1 tergolong moderat. Dari hasil penelitian dapat disimpulkan bahwa ekstrak biji *N. sativa* memiliki aktivitas antimikroba yang kuat dan moderat terhadap *P. multocida* secara in vitro, karena itu *N. sativa* merupakan kandidat yang baik untuk dikembangkan sebagai antibakteri spesifik terhadap *P. multocida.*
Kata kunci: antibakteri, jintan hitam, *Kirby Bauer*, *Pasteurella multocida.*

INTRODUCTION

In light of the current era characterized by the potential interspecies transmission of pathogens from animals to humans, the dissemination of zoonotic diseases poses a serious risk to both global public health and biosecurity (*Zhang et al.*, 2022). Bacterial infections are a matter of concern for professionals endeavoring toward the eradication of pathogenic disease caused by these microorganisms, considering the significant deleterious impact they impose. The discovery of antibiotics constituted a monumental paradigm shift toward the mitigation of infectious diseases, thereby contributing to the increased human welfare. Ultimately, an overuse of antibiotics has led to the emergence of resistance among various pathogenic microorganisms. Having this mentioned, many researchers raised significant attention to *Pasteurella multocida*.

Pasteurella multocida predominantly causes respiratory disease in mammals, including cattle and other hoofed animals. *P. multocida* was reported as a prevalent pathogen that caused bovine respiratory disease (BRD), commonly known as "shipping fever" or nonsepticemic pneumonia, in addition to other causative agents such as *Mannheimia haemolytica*, *Histophilus somni, Mycoplasma bovis*, and *Arcanobacterium pyogenes* (Wilson and Ho, 2013).

This bacterium which belongs to *Pasteurellaceae* family, is identified as Gram negative, and usually attacks the respiratory tract in livestock such as cattle, buffalo, pigs, goats and poultry, led to infection called pasteurellosis (Besung *et al*., 2016). Sandlund *et al*., (2021) stated that *Pastereulla* can also infect aquatic animals, and can even be zoonotic (Michael *et al*., 2018).

In contrast to cases in mammals where transmission occurs in the respiratory tract, cases of *P. multocida* in human are mainly related to animal exposure particularly in soft tissue after animal bites or scratches. In the United States, *Pasteurella* species are isolated from some 50% of dog bites and 75% of cat bites (Breen Breen *et al.,* 2000). Although this disease rarely cause death, but it is quite dangerous to human (Giordano *et al.*, 2015). Moreover, this microbes shows high resistance to a few classes of anti-microbials, including beta-lactams, tetracyclines, aminoglycosides, and quinolones which makes it more difficult to treat.

Anti-microbial resistance (AMR) occurs against all types of microorganisms, including fungi, viruses, bacteria, and protozoa. This happens because microorganisms evolve and mutate. Bacteria that are already resistant to antibiotics will be more difficult to treat, because higher doses of antibiotics are needed, or other types of antibiotics that are generally more toxic to kill the bacteria. There are even several types of bacteria that show resistance to several types of antibiotics, known as multidrug resistance (MDR) (Magiorakos *et al*., 2012; Marston *et al*., 2016). Bacterial resistance to antibiotics is a danger for the survival of animals and human beings.

The studies on the pathogenesis and epidemiology of *P. multocida* serogroup classification was based on the capsular type. It suggests that the capsule is related to the pathogenesis of the individual diseases and to the host's preference for certain serotypes (Chung *et al.*, 1998). A study by Furian et al. (2016) detected 22 virulence-associated genes of. *P. multocida* in poultry and swine strains which were related to antimicrobial susceptibility, including capsular serogroups A, B, and D. The results revealed that gentamicin and amoxicillin were effective with susceptibility higher than 97%, however, 77% of poultry strains and 85% of swine strains were resistant to sulphonamides. Moreover, 19.6% of avian strains and 36.6% of swine strains were multi-resistant.

For this reason, it is necessary to take an alternative approach so that the resistance of *P. multocida* can be solved. Historically, people are turning to natural products that have been proven to have antimicrobial effects. Natural products have been used as potent therapeutics against pathogenic bacteria since the golden age of antibiotics of the mid-20th century (Rossiter *et al.,* 2017). The escalating of antibiotic-resistant microorganism clearly demonstrates that new antibiotics are critical for modern medicine. Instead of developing new antibiotics, exploring potential antimicrobial agents from plant sources is more cost-effective, and one of the plants that is widely used as natural antibiotic is *Nigella sativa* seed, known as black cumin seed (Hossain *et al*., 2021)

Black cumin is a plant that has long been used as traditional medicine in the Middle East and South Asia and is commonly used to treat disorders of the respiratory tract, digestive tract, kidneys, liver function, cardiovascular system, and immune system (Tavakkoli *et al.,* 2017). The therapeutic effects of black cumin seed are due to its richness in phytochemicals, nutritionally vital constituents, and polyunsaturated fatty acids (PUFA). It has several bioactive compounds, including thymoquinone, *p*-cymene, α-thujene, carvacrol, β-pinene, limonene, sabinene, D- limonene, methyl linoleate 4,5-epoxy-1-isopropyl-4-methyl-1-cyclohexene, and 4- terpineol (Kabir *et al.,* 2020; Leisegang *et al,.* 2021). From all of these bioactive compounds, thymoquinone exerts many pharmacological activities, including antimicrobial, anticancer, anticonvulsant, anti-diabetic, antihistaminic, anti-inflammatory, and antioxidant. It also significantly inhibits the formation of biofilm, which is responsible in increasing AMR in antibiotic-susceptible microorganisms (Goel and Mishra, 2018; Hossain *et al,.* 2021).

Many research has been carried out to investigate the antimicrobial and anthelmintics activity of black cumin seed against various bacteria and parasites, including *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* (Hanafy and Hathem, 1991; Forouzanfar *et al.,* 2014), and *Paramphistomum* spp. (Hambal *et al*., 2021). A study by Nair *et al*., (2005) indicated that the oil of black cumin had strong antimicrobial activity against *Listeria monocytogenes*, and Hannan *et al,.* (2008) reported that the ethanolic extract of black cumin seed had the ability to inhibit the growth of methicillin resistance *Staphylococcus aureus* (MRSA) (Nair *et al.*, 2005; Hannan *et al.,* 2008).

However, not much information is available about the effectiveness of this plant on *P. multocida* as this bacterium has important impact in veterinary field. In this study, we investigated the antimicrobial activity of ethanolic extract of *N. sativa* seed againts *P. multocida* in vitro and this data could give contribution to suppress AMR.

MATERIALS AND METHODS

Plant extraction

Nigella sativa seed was purchased from local market in Banda Aceh, imported from India. The seed was dried, ground, and extracted by cool maceration using ethanol 96% for three days in room temperature. The filtrate was collected and then concentrated by vacuum rotary evaporator. Further the extract was diluted with phosphate buffer saline (PBS) to 15%, 25%, and 45% concentration.

Antimicrobial Test

Antimicrobial test was carried out according to Kirby-Bauer diffusion method (Qin *et al*., 2004). Treatment groups were consisted of three groups, namely T1, T2 and T3. *Pasteurella multocida* (ATCC 43137) was inoculated on nutrient broth and incubated at 37 °C for 24 hours. The bacterium was then transferred into a test tube containing sterile NaCl, adjusted to 0.5 McFarland (1.5×10^8 CFU/ml) standard suspension and shaken until homogeneous. Then the bacteria were smeared on the Mueller Hinton agar and left for five minutes to dry. Paper discs that have been dripped with 15% (T1), 25% (T2), and 45% (T3) of the extract were placed on the media. Chloramphenicol disc was used as positive control (C1) with a concentration of 30 g/L, and PBS disc as negative control (C0). The plate was incubated at 37 °C for 24 hours. Observations were made after 24 hours by measuring the inhibition zone formed.

RESULTS

This study was conducted to investigate the antimicrobial property of *N. sativa* seed extract on *P. multocida* in vitro. The extract was diluted into three different concentrations and tested on the bacteria in triplicate. The results showed that each concentration formed inhibition zone on the plate (Table 1). T3 had the largest inhibition zone with the average of 17.72 mm, while T2 showed the average inhibition zone of 15.93 mm, and T1 had 10.75 mm inhibition zone. The average inhibition zone of each treatment group showed significant differences in each group.

Table 1. Average inhibition zone of all *Nigella sativa* Seed extract treatment groups

Group	Average inhibition zone ± SD (mm)
C0	0 ± 0 [e]
C1	31,22± 0,22 [a]
T1	10,75 ± 0,31 [d]
T2	15,93 ± 0,15 [c]
T3	17,72 ± 0,38 [b]

[a,b,c,d,e] Different superscripts in the same column show significant differences in each treatment group ($P<0.05$).

C0 = Negative control
C1 = Positive control
T1 = 15% of Nigella sativa seed extract
T2 = 25% of Nigella sativa seed extract
T3 = 45% of Nigella sativa seed extract

The results of Duncan's test showed significant differences in each treatment group with the best level of inhibition was observed on C1 group, followed by T3, T2, T1 and C0 groups. The image of inhibition zone formed on every treatment group is shown in Figure 1.

Chloramphenicol was used as positive control in this study because this drug is a broad-spectrum antibiotic and has been administered to treat several diseases including infection by Gram negative bacteria.

DISCUSSION

Inhibition zone is a clear area that does not show any bacterial growth around the antibacterial discs at the time after incubation as shown in Figure 1. The ethanolic extract of *N. sativa* seed extract was

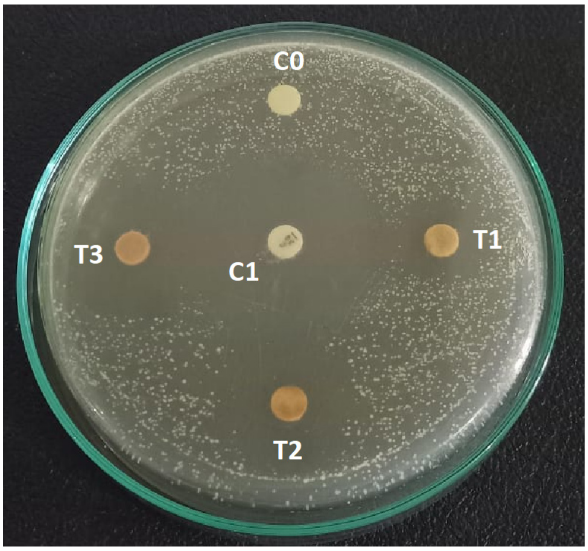

Figure 1. Inhibition zone formed on all treatment groups of *Nigella sativa* seed extract. C1= positive control. C0= negative control. T1= 15% of *Nigella sativa* seed extract. T2= 25% of *Nigella sativa* seed extract. T3= 45% of *Nigella sativa* seed extract.

effective in inhibiting the growth of *P. multocida.* According to Davis and Stout (1971), supported by report of Pratiwi *et al.*, (2022), the criteria for antibacterial inhibitory property was as follows; a diameter of >5 mm was categorized as weak, 5-10 mm was moderate, 11-20 mm was strong and >20 mm was very strong. Thus, the diameter of the inhibition zone formed by C1 (31.22 mm) was categorized as very strong, T3 (17.72 mm) and T2 (15.93 mm) were categorized as strong, and T1 (10.75 mm) was categorized as moderate. Meanwhile, according to Greenwood (1995), the antibacterial effect of C1 was categorized as strong, T3 was moderate, and T2 and T1 had weak antibacterial property. In this study, the ability of *N. sativa* seed extract to inhibit the viability of microorganisms depends on the concentration of the extract. The authors were convinced that the high extract concentrations do not necessarily correlate with high inhibitory property of *N. sativa*, therefore further study is substantial.

In addition to the concentration factor, the chemical substances produced by the plant also determine the ability to inhibit bacterial growth. The main active compounds in *N. sativa* seed are thymoquinone, thymohydroquinone, dithymoquinone (nigellone), p-cymene, carvacrol, thymol, and tannins (Ijaz *et al.*, 2017; Srinivasan, 2018). It also contained alkaloids (nigellicine and nigelledine), saponins (alpha-hederin), and sterols, especially beta sitosterol which has the ability to prevent cancer. *N. sativa* seed has antioxidant activity through decreased strength and inhibition of peroxidation process (Tiwari *et al.*, 2019; Habib *et al.*, 2020). Pharmacological property of *N. sativa* predominantly due to quinine substances, especially thymoquinone as being the most abundant metabolite in *N. sativa* seed (Srinivasan, 2018).

Gram-negative and Gram-positive bacteria have special pumps that help them survive when they are attacked by medicines. These pumps are called multidrug efflux pumps (EPs). EPs are transporter that take drugs and toxic chemicals out of the bacteria cell and throw them away. This EPs are the cause of MDR in bacteria (Van Bambeke and Lee, 2006; Chaieb *et al.,* 2011). Various efflux pumps have been reported to cause macrolide resistance in *P. multocida* and other respiratory pathogens (Dayao *et al.*, 2016). The first antibiotic-specific drug transporter described in Gram-negative bacteria is MacAB, a macrolide-specific ATP-binding casette (ABC)-type efflux carrier (Kobayashi *et al.*, 2001). A study by Mouwakeh *et al.*, (2018) stated that thymoquinone had the ability to inhibit efflux pump, therefore increasing the amount of antibiotics present in bacterial cells at a lower dose and improving their efficacy. Moreover, thymoquinone also intercedes in its antibacterial impact by producing ROS (Reactive Oxygen Species) which stimulates oxidative stress and cell apoptosis for the bacteria. However, p-cymene does not have the ability to kill bacteria or other microorganisms, but it makes it easier for antimicrobial substances to get inside the cell by increasing membrane permeability (Mouwakeh *et al.*, 2018; Hossain *et al.*, 2021).

The research on biofilm formation of *P. multocida* have become crucial because it is a respiratory pathogen and its biofilm formation might be one of its virulence factors for survival inside the host. Bacteria in biofilm formation have been appeared to be much more resistant to antibiotics (Emery *et al.*, 2017). The effectiveness of bioactive compounds from natural resources in inhibiting cell attachment is a favorable appliance for decreasing microbial colonization (Bavington and Page, 2005). Utilization of anti-adhesion agents appears to be a very interesting approach in the prevention of microbial infection. A study by Chaieb *et al.*, (2011) revealed that thymoquinone had the capability of inhibiting biofilm production in some bacteria, thus enhancing its antibacterial activity.

More study on the antibacterial property of black cumin seed have been reported on various bacteria, e.g. *E. coli* (Asniyah, 2009; Bakal *et al.*, 2017), *Staphylococcus* sp. (Ugur *et al.*, 2016; Saleh *et al.,* 2018), *Streptococcus pyogenes*, and *Bacillus subtilis* (Morsi, 2000). A study of Ugur *et al.* (2016), suggested that *N. sativa* oil had potential activity against methicillin resistant *S. aureus* (MRSA) and methicillin resistant

Coagulase-negative *Staphylococci* (MRCoNS) and showed no cytotoxicity at relevant concentrations. A report by Asharf *et al*., (2018) also confirmed that *N. sativa* seed extract was potential in inhibiting the growth of *S. enterica* in vitro.

In this study, the results showed that the ethanolic extract of *N. sativa* seed had strong and moderate inhibition activity on the growth of *P. multocida*. Therefore, *N. sativa* seed is a potential candidate for antimicrobial drug development. However, more studies including in vivo and in silico approach are necessary to confirm this result.

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