BLACK CUMIN (*Nigella sativa*) AGAINST *Mycobacterium tuberculosis* STRAIN H37RV AND MDR-TB

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Received : March 31, 2021 Accepted : June 26, 2021 Published : June 30, 2021

Abstract: Tuberculosis (TB) is a contagious infectious disease caused by *Mycobacterium tuberculosis*. 10 million people suffer from TB Every year. Although TB is a preventable and treatable disease, 1.5 million people die every year due to TB. Alternative treatments continue to be pursued, and treatment with the latest TB drugs that are continuously being encouraged. Black cumin (*Nigella sativa*) seed contains essential oils with active compounds such as thymohydroquinone, Oleoresins, flavonoids, alkaloids, saponins, tannins, and terpenoids that act as antibacterial drugs. This study aims to determine the sensitivity of *N. sativa* seed extract in inhibiting the growth of *M. tuberculosis* strain H37RV and MDR-TB (Multidrug Resistance-TB). This research using Microscopic-Observation and Drug-Susceptibility Assay (MODS) method. Extraction of *N. sativa* was carried out by the maceration method using 70% methanol as a solvent. The results showed that the *M. tuberculosis* strain H37RV and MDR-TB were sensitive to *N. sativa* extract at concentrations of 5 and 10% but resistant to *N. sativa* extract at concentrations of 1 and 3%.
Keywords: Sensitivity, *Nigella sativa* seed extract, *Mycobacterium tuberculosis*, Microscopic-Observation Drug-Susceptibility Assay (MODS).

Abstrak: Tuberkulosis (TB) adalah penyakit menular yang disebabkan oleh Bakteri *Mycobacterium tuberculosis*. Penyakit ini menimbulkan dampak kematian yang cukup mengkhawatirkan. Penyakit tersebut dapat dicegah dan diobati. Salah satu sumber pengobatannya menggunakan biji jintan hitam (*Nigella sativa*) yang mengandung minyak atsiri dengan senyawa aktif seperti timohidrokuinon, oleoresin, flavonoid, alkaloid, saponin, tanin, dan terpenoid yang berfungsi sebagai obat antibakteri. Penelitian ini bertujuan untuk mengetahui sensitivitas ekstrak biji *N. sativa* dalam menghambat pertumbuhan *M. tuberculosis* strain H37RV and MDR-TB (*Multidrug-Resistance-TB*). Penelitian ini menggunakan metode Microscopic-Observation and Drug-Susceptibility Assay (MODS). Ekstraksi *N. sativa* dilakukan dengan metode maserasi menggunakan pelarut metanol 70%. Hasil yang diperoleh menunjukkan bahwa bakteri *M. tuberculosis* strain H37RV dan TB-MDR, kedua strain tsb sensitif terhadap ekstrak *N. sativa* konsentrasi 5 dan 10%, tetapi resisten terhadap ekstrak *N. sativa* konsentrasi 1 dan 3%.

Kata kunci: Sensitivitas, Ekstrak biji *Nigella sativa*, *Mycobacterium tuberculosis*, Microscopic-Observation Drug-Susceptibility Assay (MODS).

Recommended APA Citation :

Masri, M., Muthiadin, C., Masita, Cahyanto, T., Lianah, L., Rusny, & Tridesianti, S. (2021). Black Cumin (*Nigella sativa*) Against *Mycobacterium tuberculosis* Strain H37RV And MDR-TB. *Elkawnie*, *7*(1), 182-196. https://doi.org/10.22373/ekw.v7i1.9335

Introduction

TB (tuberculosis), one of the most significant infectious causes of death, is a significant public health problem in developing countries (Dhingra et al., 2020), now exacerbated by the increasing incidence of Multidrug-Resistance (MDR) (Liu et al., 2021; Vasava et al., 2019). MDR is defined as resistance to at least isoniazid and rifampin, which appear to threaten TB (Okethwangu et al., 2019). The emergence of TB-MDR is caused by a strain of *M. tuberculosis* resistant to two first-line drugs (Isoniazid and Rifampin) (Dagne et al., 2021).

Current MDR-TB rapid detection methods use Microscopic-Observation Drug-Susceptibility Assay (MODS). This method detects resistant *M. tuberculosis* to isoniazid and rifampin only for 13 days (Owusu & Newman, 2020). The control program to solve the problem uses the method MODS because the MODS method is for detecting MDR-TB and initiating therapy in patients (Alcántara et al., 2019).

The increasing resistance of bacteria to existing drugs is a serious problem resulting in an urgent need for TB and shorter treatment regimens (Dadgostar, 2019; Tiberi et al., 2018). Unlike synthetic drugs, antibacterial in origin herbs show fewer side effects and can potentially treat various infectious diseases (da Silva et al., 2021; Mani et al., 2021). Traditional medicine is a type of medicine in which Knowledge of these medicinal uses comes from contributions of traditional knowledge holders in specific cultures (Crawford, 2019). The use of traditional medicines, in general, is prioritized as an effort to maintain health (Redvers & Blondin, 2020). With the development of traditional medicine, it is added with the echo of back to nature, has increased the popularity of the drug traditional (Crawford, 2019; Jaiswal & Williams, 2017). One of the plants that can be used as a medicinal ingredient Traditional is Black cumin (*Nigella sativa*) (Kulyar et al., 2020; Majeed et al., 2020).

N. sativa is reported to have been exhibited pharmacological effects, which include antimicrobial (Bakal et al., 2017), antibacterial (Ugur et al., 2016), antidiabetic (Vijayakumar et al., 2021), antifungal (Kul'Ko et al., 2016), antiviral (Maideen, 2020), anti-inflammatory (Georgescu et al., 2018), antioxidant (Toma et al., 2015; Vijayakumar et al., 2021).

The efficacy of *N. sativa* lies in its chemical content in the seed (Dajani et al., 2016). The chemical constituents of black cumin *N. sativa* consists of Thymoquinone (TQ) (up to 50%), p-cymene (40%), carvacrol (6%–12%), 4-terpineol (2%–7%), *t*-anethole (1%–4%), sesquiterpene longifolene (1%–8%), dithymoquinone (nigellone), thymol, α-pinene, and thymohydroquinone (Mollazadeh et al., 2017; Srinivasan, 2018).

It is necessary to research the growth inhibition of *M. tuberculosis* that causes TB disease. Therefore, the test's research has conducted the sensitivity of *N. sativa* seed extract to *M. tuberculosis*.

Materials and Methods

Preparing Stage

The stages of making *N. sativa* seed extract

The stage of preparing N. sativa simplicia

The manufacture of excellent and fulfilling simplicia consists of selecting, washing, drying, milling, and sieving. At this stage, *N. sativa* seeds are sorted first, choosing the solid color. The seed is not damaged, a little dry, and not rot. The next step is washing *N. sativa* with water 3 times and then using distilled water 2 times, then dried using an oven with a temperature of 45^{o}C for 3 hours. After *N. sativa* seed dry, the following process is to grind it with a grinding machine until it becomes powder (Khan, 1999; Shabnam Javed, 2012; Singh et al., 2014).

Preparation of N. sativa seed extract

The powder of *N. sativa* seed is weighed 500 grams, then put into a maceration container, and 2000 ml of 70% methanol is added so that all samples are completely immersed, then stir for 30 minutes and let stand for 24 hours. Then the sample is filtered, and the pulp and filtrate are separated, then the dregs are macerated again using the same solvent. The 70% methanol solvent used is replaced for 1 × 24 hours so that there is no saturation point in the solvent. This is done 3 times in a row. The filtrate obtained is evaporated with a vacuum rotary evaporator (BIO-RAD). From this process, a thick extract of *N. sativa* seed was obtained. This thick extract was then poured into a porcelain dish and heated with a water bath (BIO-RAD) at 70^{o}C while continuing to stir, and it was obtained that the *N. sativa* seed extract was ready for use (Khan, 1999; Shabnam Javed, 2012; Singh et al., 2014).

Determination of the concentration of N. sativa seed extract

The test solution's concentration was determined based on the predetermined orientation results using four concentrations; 10%, 5%, 3%, and 1%. The stock concentration used was taken from a concentration of 20%. The seed extract of *N. sativa* was weighed 2 grams in 10 ml double-distilled water (ddH_2O). After getting a concentration of 20% then it was diluted into four concentrations; 10%, 5%, 3%, and 1%.

Mycobacterium tuberculosis (H37RV and MDR-TB) Subcultures

Added the Middlebrook culture medium (Fisher Scientific, USA) into each tube as much as 10 ml, then added 5 ml the polymixin-B-amphotericin-B-nalidixic-acid-trimethoprim-azlocillin (PANTA; BD, USA) enrichment and oleic acid-albumin-dextrose-catalase (OADC; Fisher Scientific, USA), then added 1 ml of H37RV and MDR-TB isolates respectively into each tube (Alcántara et al., 2019).

c. Dilution of *Mycobacterium tuberculosis* (H37RV and MDR-TB)

At standard dilutions to reach 0.5 Mc Farland, at the strain of H37RV and MDR-TB, each pipette 1 ml from the subculture results, which is then inserted into a tube containing 10 ml of Middlebrook media, PANTA supplement, and OADC 5 ml (Alcántara et al., 2019).

Testing Stage

Implementation of the sensitivity test of N. sativa extract

Microscopic-Observation Drug-Susceptibility Assay (MODS) was used to test the *N. sativa* seed extract. There are two plates, and the first plate is for the H37RV strain. Each plate consisted of 21 wells, 12 wells contained 2 ml, namely 1 ml from the dilution tube containing the H37RV strain, Middlebrook media, OADC, and PANTA. And another 1 ml of black cumin seed extract (*N. sativa*) with each concentration of 10%, 5%, 3%, 1%. 6 wells for the positive control (3 wells for Rifampicin and Isoniazid respectively) contained 2 ml, namely 1 ml from the dilution tube containing the H37RV strain, Middlebrook media, OADC and PANTA. And another 1 ml of rifampicin and 1 ml of isoniazid, respectively. 3 wells for negative control containing only 1 ml, namely 1 ml from the dilution tube containing the H37RV strain, Middlebrook media, OADC, and PANTA. The second plate is for the MDR-TB strain. The treatment was the same as the first plate of the H37RV strain, which differed only in the strained content in each well, each containing the MDR-TB strain for the second plate. There are 3 tests for each consentration of *N.sativa* (Alcántara et al., 2019; Bakal et al., 2017).

Data Processing and Analysis Techniques

Data processing techniques were performed using a presentation model in tabular form, and the data was calculated by testing the sensitivity of drugs (isoniazid and Rifampicin) or extracts (*N. sativa*) to the growth of *M. tuberculosis* strain H37RV and MDR-TB. Sensitive means no bacterial growth after testing with *N.sativa*. Resistant means there is bacterial growth after testing with *N.sativa.* Growth means there is bacterial growth. No Growth means no bacterial growth. Growth and No Growth are terms for the positive and negative control, which are no testing with *N. sativa*.

Result And Discussion

Sensitivity Test of Black Cumin Seed Extract (*Nigella sativa*) to *Mycobacterium tuberculosis*

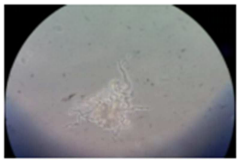
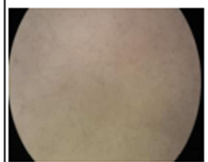
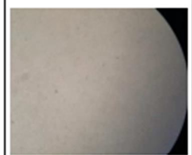
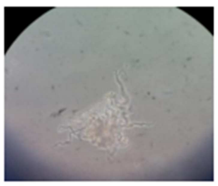
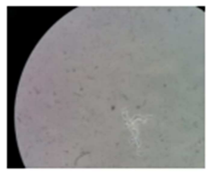
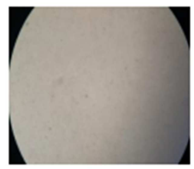
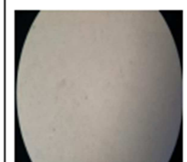
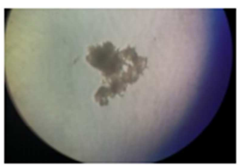
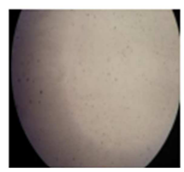
This study used 7 treatment groups: the first group is a negative control, 2 positive controls; isoniazid and rifampin, while the treatment group consisted of four different concentrations of *N. sativa* seed extract, 1%, 3%, 5%, and 10%.

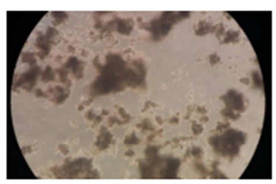
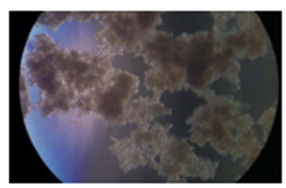
In this study, the ability of black cumin seed extract *N. sativa* in inhibiting the test, bacteria can be seen under a microscope by observing bacterial growth per well on the plate. After observing the sensitivity test to the tested bacteria using the MODS method. Results of his observations can be seen in table 1 and table 2.

Table 1. Sensitivity Test of *N. sativa* seed extract against *M. tuberculosis* Strain H37RV

Treatment	Consentration	Test 1	Test 2	Test 3
N. sativa seed extract	1%	Resistant	Resistant	Resistant
	3%	Resistant	Resistant	Resistant
	5%	Sensitive	Sensitive	Sensitive
	10%	Sensitive	Sensitive	Sensitive
Isoniazid (Positive control)		No Growth	No Growth	No Growth
Rifampicin (Positive control)		No Growth	No Growth	No Growth
Negative control		Growth	Growth	Growth

Description :
Sensitive : No bacterial growth after testing with *N.sativa*
Resistant : There is bacterial growth after testing with *N.sativa*
Growth : There is bacterial growth
No Growth : No bacterial growth

H37RV	Concentration of *Nagella sativa* seed extracts			
	1%	3%	5%	10%
Test 1				
Test 2				
Test 3				

Negative Control		
		

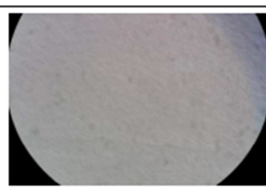
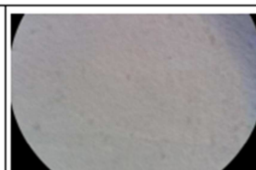
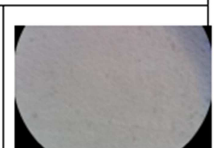
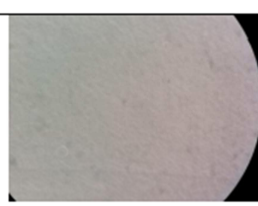
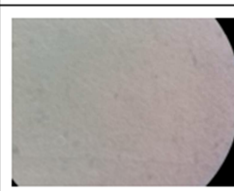
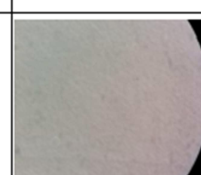
Positive Control			
Isoniazid			
Rifampisin			

Figure 1. Sensitivity Test of *N. sativa* seed extract against *M. tuberculosis* Strain H37RV (microscopic view)

In table 1 and figure 1 indicates that at a concentration of 1% and 3%, it is not sensitive to the growth of *M. tuberculosis* strain H37RV, while for 5% and 10% concentration are sensitive against the growth of *M. tuberculosis* strain H37RV, for the negative control there is a growth of bacteria, and positive control there is no growth of bacteria.

Table 2. Sensitivity Test of *N. sativa* seed extract against *M. tuberculosis* MDR-TB sample

Treatment	Consentration	Test 1	Test 2	Test 3
N. sativa seed extract	1%	Resistant	Resistant	Resistant
	3%	Resistant	Resistant	Resistant
	5%	Sensitive	Sensitive	Sensitive
	10%	Sensitive	Sensitive	Sensitive
Isoniazid (Positive control)		Growth	Growth	Growth
Rifampicin (Positive control)		Growth	Growth	Growth
Negative control		Growth	Growth	Growth

Description :
Sensitive : No bacterial growth after testing with *N.sativa*
Resistant : There is bacterial growth after testing with *N.sativa*
Growth : There is bacterial growth
No Growth : No bacterial growth

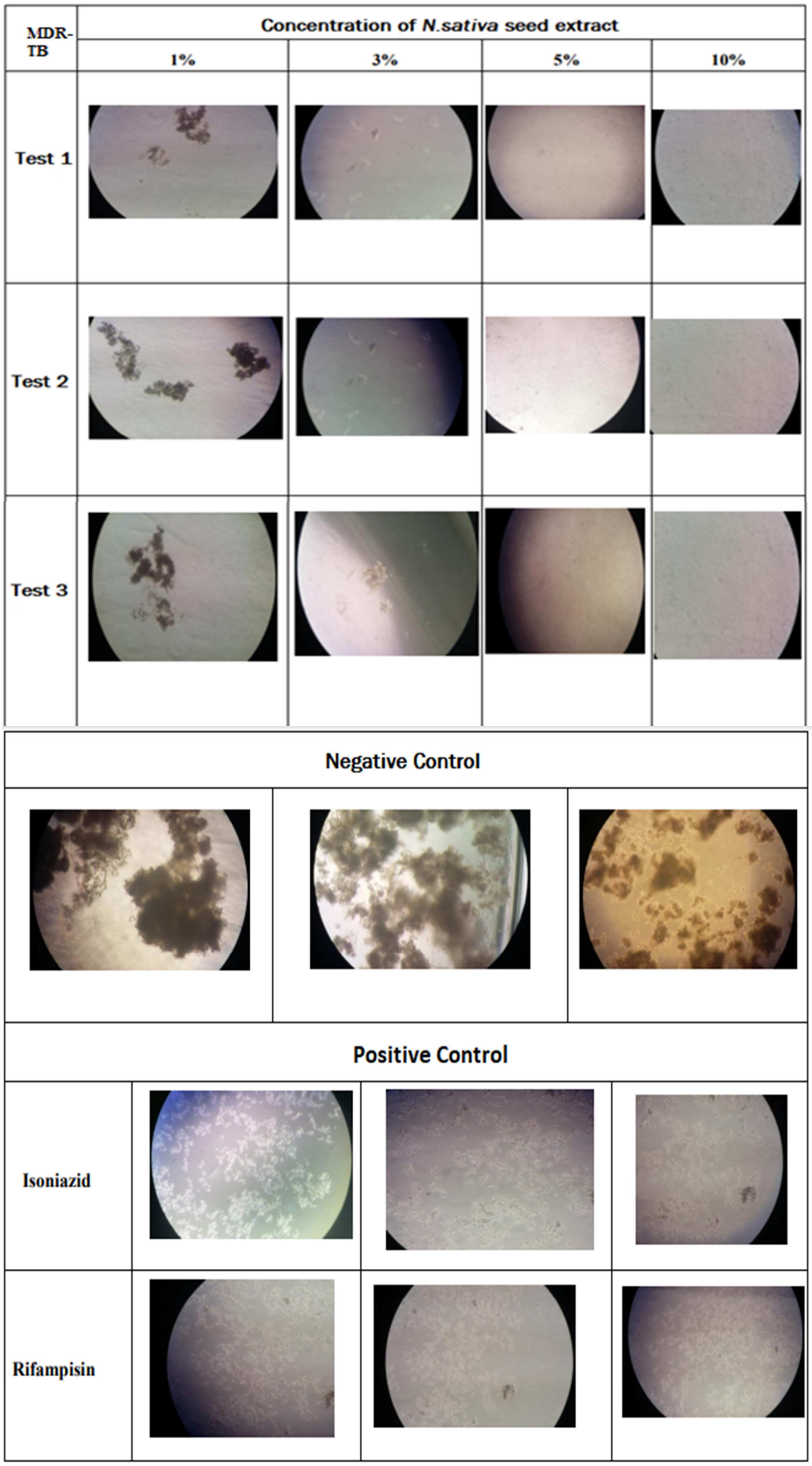


Figure 2. Sensitivity Test of *N. sativa* seed extract against *M. tuberculosis* MDR-TB sample.

In table 2 and figure 2 shows that the concentrations of 1% and 3% are resistant to the growth of *M. tuberculosis* strain MDR-TB. In comparison, 5% and 10% concentrations are sensitive against *M. tuberculosis* strain MDR-TB growth.

There is the bacteria's growth for both negative and positive control (isoniazid and rifampin). It means strain MDR-TB resistant to first-line drugs, Isoniazid and Rifampin.

In the previous test, the *N. sativa* seed extract was used concentrations of 20%, 40%, 60% and, 80%, which at a concentration of 20% is sensitive against the growth of *M. tuberculosis* strain H37RV and TB-MDR, as well with a concentration of 40%, 60% and, 80%, then from a concentration of 20%, it was diluted to get even lower concentrations, which is the MODS method these concentrations of 40%, 60%, and 80% are too concentrated so that bacterial growth is not can be observed or seen under a microscope. So that The final concentrations used were 1%, 3%, 5% and, 10%. This study showed that the higher the extract concentration was used, the greater the ability to inhibit the growth of *M. tuberculosis* strain H37RV and TB-MDR.

The mechanism of inhibition of microorganisms by antibacterial compounds contained in the seed of *N. sativa* can cause several disturbances in the compounds that make up the walls of bacteria, increase in cell membrane permeability which can cause loss of constituent components of cells, inactivate enzymes, and destruction or damage to the function of genetic material (Bakal et al., 2017; Dagne et al., 2021; Hussain & Hussain, 2016; Tariq et al., 2019). Oleoresins (extracted in n-hexane, ethyl acetate, and ethanol) *N. sativa* has a high concentration of unsaturated greasy acids and thymohydroquinone in a bit of sum responsible for its direct antimicrobial impacts. Long-chain greasy acids like linoleic corrosive and oleic corrosive were already reported to have antibacterial and antifungal action (Khan, 1999; McGaw et al., 2002; Shabnam Javed, 2012; Singh et al., 2014). p-cymene is not a proficient antimicrobial compound when used alone, but it potentiates the action of compounds like carvacrol (Rattanachaikunsopon & Phumkhachorn, 2010; Singh et al., 2014). The antimicrobial action of essential oils can often be connected to its substance of phenolic compounds. The sort of microscopic organisms moreover has an impact on the effectiveness of the unstable oil and oleoresins. Gram-negative bacteria were, by and large, less vulnerable than Gram-positive (Gilles et al., 2010).

In this study, the MODS method used has three principles; first, *M. tuberculosis* grows faster in Middlebrook media. Secondly, in media or fluid, *M. tuberculosis* grows in visual characteristics (creasing, cording), which can be observed under a microscope long before the naked eye can, imagined colonies on the media. The third, incorporation of anti TB drugs in culture MODS methods allows direct susceptibility testing of sputum samples, besides the MODS method has a higher level of safety (Huang et al., 2015; Kirwan et al., 2016; Wikman-Jorgensen et al., 2014).

The MODS method is a culture-based plate tissue test by utilizing the Middlebrook and making observations under a microscope light to detect the characteristics of *M. tuberculosis* in liquid media (Alcántara et al., 2019; Zadbuke et al., 2017), the susceptibility of rifampin and isoniazid drugs (Minh Ha et al., 2012), besides media Middlebrook is equipped with antimicrobial and nutritional supplements (PANTA and OADC) (Alcántara et al., 2020; Florentini et al., 2020).

Conclusion

M. tuberculosis strain H37RV and MDR-TB were sensitive to *N. sativa* extract at concentrations of 5, and 10% but resistant to *N. sativa* extract at concentrations of 1 and 3%.

Acknowledgement

Authors would like to thank NECHRI (Novartis-Eijkman Hasanuddin Research Initiative) laboratory Makassar for culture bacteria *M. tuberculosis* strain H37RV and MDR-TB.

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