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Evaluating the Anti-mycobacterial Activity of *Nigella sativa* Seed Extracts

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ABSTRACT

Microbial resistance to existing antibiotics has led to an increase in the use of medicinal plants that show beneficial effects for various infectious diseases. To evaluate the susceptibility of mycobacteria to Nigella sativa extract. In the present study Nigella sativa was extracted with methanol, cholorform and water using Soxhlet extractor. The extracts were than tested against Mycobacterium sp. using mycobacteriophage propagation. The Minimal Inhibitory Concentration (MIC) of N. sativa seeds were determined against all three mycobacterial strains using Luciferase Reporter Phage (LRP) assay. The TLC profiling of N. sativa seeds was carried out. In this study, the methanolic and water extract of N. sativa seeds showed inhibition against M. tuberculosis H37Rv, All drug Sensitive M. tuberculosis, MDR M. tuberculosis respectively. The methanol extract showed least inhibition at concentration of 50 µg/ml, 250 µg/ml and 100 µg/ml against M. tuberculosis H37Rv, All drug sensitive M. tuberculosis and MDR M. tuberculosis respectively. N. sativa seed is a traditional medicine, which possesses anti-tubercular property. This plant has great potential to develop as a natural drug for the treatment of tuberculosis including drug resistant TB.

Keywords: *Nigella sativa, M. tuberculosis,* LRP assay.

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by the bacterium *Mycobacterium tuberculosis*. One third of the world population is infected with TB. It is considered as one of the deadliest infectious disease which kills approximately 1.5 million people every year [1,2]. The current treatment includes combination of antibiotics and requires longer duration of treatment. The major threat involved in the effective management of TB is the development of antibiotic resistance. New Multi-Drug Resistant (MDR) TB cases are estimated to be 450,000 per year. The drugs used in the first line treatment are more than four decades old [3,4]. Hence there is an urgent requirement for the development of new anti TB drug candidates to shorten the treatment and also to be effective against all sub populations of *M. tuberculosis* and drug resistant strains [5].

Plants and herbs have been exploited for their therapeutic properties since ancient times their importance has increased in the recent years. They play a major role in curing many diseases naturally without any side effects [6]. *Nigella sativa* is an annual herbaceous plant whose name is derived from the Latin word, nigellus meaning black. It belongs to the family Ranunculaceae. Its seeds are called black seeds or black cumin. It has been used widely by people across the globe for over 3,300 years for a healthy life. Its curative effects have been studied extensively and it possesses numerous potential therapeutic properties such as antineurodegenerative, anti-alzheimer's, antiepileptic and antiparkinson's, etc., The Black seeds regulate the immune system, maintain proper metabolism, control the blood sugar levels and treat many dermal problems [7,8]. Therefore, it is considered as a miracle herb and attains a remarkable position in the list of drugs in Indian traditional medicine system [9,10]. *N. sativa* contains many active ingredients like essential oils, proteins, alkaloids, saponin, etc., [11]. The pharmacologically important active ingredients present in the seed oil are thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ), and thymol (THY) [12]. These are effective against different cancers such as breast cancer, blood cancer, colon cancer, pancreatic cancer, cervical cancer etc., [13]. In this study, the *in vitro* anti-mycobacterial activity of various solvent crude extracts of *Nigella sativa* seeds were in evaluated by testing against three different strains of *M. tuberculosis* at different concentrations.

MATERIALS AND METHODS

Collection and identification of plant materials

Collection and identification of plant material: The plant materials *Nigella sativa* were purchased from the local Ayurvedic shop, Chennai. The seeds were identified and authenticated by the botanist.

Mycobacterial cultures and mycobacteriophage: The standard strain *M. tuberculosis* $H_{37}Rv$, Clinical isolates of SHRE sensitive M. *tuberculosis*, HR Resistant (MDR) *M. tuberculosis* and mycobacteriophage (phAE202) were kindly gifted to Dr. Vanaja Kumar, Senior Scientist, Centre for Drug Discovery and Development, Sathyabama University, Chennaiby National Institute for Research in Tuberculosis (NIRT), Chennai. All the strains were grown and maintained at 37°C on LJ media. Mycobacteriophage (phAE202) and propagated in MB buffer and stored at 4°C.

Bacteriological media and chemicals: Middlebrook 7H9 broth and D-Luciferin were obtained from Himedia (India) and Cayman Chemicals (USA) respectively.

Solvent extraction of *Nigella sativa* seeds: The authenticated *Nigella sativa* seeds are washed thrice with sterile distilled water and then shade dried. The dried seeds were crushed using a pestle and mortar. Soxhlet extractor was used to obtain different solvent extracts from the powdered seeds. Briefly, 250 ml of methanol were added to a round bottom flask and powdered seed material was loaded into the thimble, which is placed inside the soxhlet extractor. Methanol was then heated using the isomantle for 2 hours at 55°C. The condensate was collected and solvent (methanol) was evaporated using a rotary evaporator at 45°C, till a yield of about 3 ml of extract was left in the glass bottom flask. The obtained extracts were dried at 45°C. The extract thus obtained was named as NI-M. The above mentioned procedure was applied for extraction with chloroform and water, but the temperature and time were altered for extraction with water. For extraction with chloroform, the temperature and time were same as methanol extraction whereas for extraction with water, the extraction was done at 80°C for 2 hours. The chloroform extracts and water extracts were named as NI-C and NI-W respectively. All the obtained extracts were subjected to anti-mycobacterial activity using Luciferase Reporter Phage (LRP) assay.

Anti-mycobacterial activity Luciferase Reporter Phage (LRP) assay: The *in vitro* anti-mycobacterial activity of the different extracts of *N. sativa* seeds have been screened by Luciferase Reporter Phage (LRP) assay. The dried crude extracts of NI-C, NI-W, NI-M at concentration of 500 µg/ml were tested against *M. tuberculosis* H37Rv, all Drug Sensitive *M. tuberculosis* and Multi-Drug Resistant *M. tuberculosis*.

Extract preparation

One ml of 10% DMSO was added to 10mg of the dried methanol extract of *N. sativa* seed (10 mg/ml concentration). The solution was filtered through 0.45 µ filter and named as stock (S). From the stock solutions, 500 µl was taken and added to 500 µl of

sterile distilled water to get 500 µg/ml concentrations (S1). The same dilution method was followed for remaining chloroform and water extracts of *N. sativa* seed.

Preparation of rifampicin drug control

Rifampicin standard drug concentration of 2 µg/ml in the assay was prepared by standard protocol and labeled as 'Rif-7H9'

Preparation of cell suspension

The cell suspension of *Mycobacterium tuberculosis* H37Rv, All Drug Sensitive *M. tuberculosis* and Multi-Drug Resistant *M. tuberculosis* were prepared to equivalent of McFarland No. 2 in Middlebrook 7H9 broth as per the procedure mentioned.

Mycobacteriophage propagation

About 500 µl of Mycobacteriophage Buffer (MP Buffer) was dispensed into each of the five cryovials and labeled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Fifty microlitre of phAE202 (stock) were added to the first 10^{-1} cryovial and serially diluted upto the 10^{-5} dilutions. All cryovials were inoculated with 500 µl of *M. smegmatis* suspension equivalent to 2-3 McFarlands standard and incubated at 37°C for 30 minutes. After incubation, 200 µl from each dilution were mixed separately with 5 ml of agar (1%) at 45°C. Then immediately the mixture was poured on middlebrook 7H9 agar plate and incubated at 37°C. After 18 hours, the plates which showing a lacey pattern of plaque formation was added with 5 ml of MP buffer and kept in shaker for 2 hours at room temperature. Then the phage filtrate was obtained by filtering the buffer using 0.45 micron membrane filter. The phage was stored in 4°C for future use.

Assay

The cryovials were taken and labeled as two growth control, solvent control, drug control and tests namely NI-C, NI-W and NI-M. About 400 µl of 7H9 broth was added to two growth control vials, 400 µl of 7H9 broth containing rifampicin (2 µg/ml) to the drug control vial, 350 µl of 7H9 broth to each solvent control vial and test vials. About 50 µl of 10% DMSO was added to the solvent control vial and each 50 µl extract from NI-M, NI-C and NI-W was added separately to the respective test vials. Then 100 µl of *M. tuberculosis* H37Rv suspension (Mcfarland no. 2) was added to all the vials. The same above mentioned procedure was carried out for all Drug Sensitive *M. tuberculosis* and Multi-Drug Resistant (MDR) *M. tuberculosis*. All the vials were incubated at 37°C for 72 hours. After incubation, the phage infection was done by adding 40 µl of 0.1 M $CaCl_2$ and 50 µl of

mycobacteriophage (phAE202) to all the vials and incubated at 37°C. After 4 hours incubation, 100 µl of mixture from each vial was pipette out and added with 100 µl of D-Luciferin in a star tube. Then the tube was immediately placed in Luminometer (Lumat 9508, Berthold, Germany) to measure Relative Light Unit (RLU) at 10 seconds integration time. The percentage of Reduction was calculated using the below formula,

$$\% \ RLU \ Reduction \ = \frac{Control \ RLU \ - \ Test \ RLU}{Control \ RLU} X \ 100$$

The test shows more than 50% of reduction considered as Inhibition whereas the test showing less than 50% of reduction considered as no Inhibition.

Determination of Minimal Inhibitory Concentration (MIC)

Minimal Inhibitory Concentration (MIC) of NI-W and NI-M extracts was determined against *M. tuberculosis* H37Rv, All Drug Sensitive *M. tuberculosis* and Multi-Drug Resistant *M. tuberculosis* whereas NI-C extract was not subjected to MIC determination as lack of anti-mycobacterial activity. NI-W and NI-M extracts concentration at 250 µg/ml, 100 µg/ml and 50 µg/ml was prepared separately as mentioned earlier. Luciferase Reporter Phage (LRP) assay was performed as mentioned above. Finally, the calculation was made using the RLU obtained from each concentration by using formula and interpreted.

Time kill evaluation of methanol and water extracts of Nigella sativa seeds

Assessment of the killing rate of *M. tuberculosis* H37Rv by the NI-W and NI-M extracts were carried out using LRP assay as mentioned earlier. In this assay, the extract concentration for both NI-W and NI-M was set at 250 µg/ml concentration. All the vials were incubated at 37°C. Aliquot from the culture medium was taken at 4, 8 and 16 hrs and phage infection was done by adding 40 µl of 0.1M $CaCl_2$ and 50 µl of mycobacteriophage (phAE202) to all the vials and incubated at 37°C. After 4 hours incubation, RLU reduction was estimated by Luminometer (Lumat 9508, Berthold, Germany) and percentage of reduction was determined by using the formula as mentioned above in LRP assay.

Thin layer chromatography

The methanol extract of *Nigella sativa* was subjected to Thin Layer Chromatography (TLC) to separate the compounds on silica gel 60 F254 plates (Merck, 0.25 mm). The extract was dissolved in methanol and it was spot on the silica plate using capillary

tube. The strips were placed in the tank containing ethyl acetate:hexane (20:80) solvent. After development, the strips were placed in an iodine vapor tank to visualize the spot then Rf value was calculated.

RESULT AND DISCUSSION

The *in vitro* anti-mycobacterial activity results were summarized in Table 1.

Table 1: Anti-mycobacterial Activity of *Nigella sativa* seeds extracts by different solvent extractions.

S. No	Extracts & Concentration		*M. tuberculosis* $H_{37}Rv$		SHRE Sensitive *M. tuberculosis*		R Resistant *M. tuberculosis*	
	Reference Name	Test Conc.	% of Reduction	Result	% of Reduction	Result	% of Reduction	Result
1	NI-C	500 µg/ml	9.51	NI	20.25	NI	31.30	NI
2	NI-M	500 µg/ml	71.73	I	90.37	I	87.82	I
3	NI-W	500 µg/ml	80.83	I	93.50	I	84.83	I
4.	Rifampicin drug	2 µg/ml	99.02	S	99.41	S	22.95	R

Note: S-Streptomycin, H-Isoniazid, R- Rifampicin, E-Ethambutol; NI- No Inhibition, I- Inhibition

In our study, the methanolic extract of *N. sativa* seeds showed inhibition in view of 71.73%, 90.37% and 87.82% RLU reduction at a concentration of 500 µg/ml against *M. tuberculosis* H37Rv, All drug Sensitive *M. tuberculosis*, MDR*M. tuberculosis* respectively. All the three strains showed inhibition by aqueous extracts of *N. sativa* seeds. We observed the RLU reduction 80.83%, 93.50% and 84.83% for *M. tuberculosis* H37Rv, All drug Sensitive *M. tuberculosis* and MDR *M. tuberculosis* respectively. The chloroform extracts of *N. sativa* seeds have not showed inhibition against any of the three strains A study reported that different extracts of *N. sativa* as well as T Thymoquinone (TQ) have shown broad antimicrobial activity against both Gram-negative, Gram-positive bacteria [14]. TQ has been considered as one of the most active constituent which is present in *N. sativa* seeds. It was reported that TQ possesses anti-TB activity at 20 µg/ml concentration [15].

The Minimal Inhibitory Concentration (MIC) of NI-M and NI-W extracts of *N. sativa* seeds were determined against all three mycobacterial strains using LRP assay. The results were summarized in Table 2.

Table 2: Minimal Inhibitory Concentration (MIC) Determination of NI-W and NI-M.

S. No	Extracts & Concentration		*M. tuberculosis* $H_{37}Rv$		SHRE Sensitive *M. tuberculosis*		R Resistant *M. tuberculosis*	
	Reference Name	Test Conc.	% of Reduction	Result	% of Reduction	Result	% of Reduction	Result
1	NI-M	250 µg/ml	66.84	I	73.61	I	83.32	I
		100 µg/ml	53.98	I	30.23	NI	50.38	I
		50 µg/ml	51.15	I	24.12	NI	38.45	NI
2	NI-W	250 µg/ml	65.90	I	70.03	I	83.92	I
		100 µg/ml	60.84	I	36.58	NI	65.88	I
		50 µg/ml	51.35	I	35.12	NI	53.88	I

Note: S-Streptomycin, H-Isoniazid, R- Rifampicin, E-Ethambutol; NI- No Inhibition, I- Inhibition

NI-M extract showed least inhibition at concentration of 50 µg/ml, 250 µg/ml and 100 µg/ml against *M. tuberculosis* H37Rv, All drug sensitive *M. tuberculosis and* MDR *M. tuberculosis* respectively. MIC at 50 µg/ml, 250 µg/ml and 50 µg/ml concentration was showed Inhibitory activity against *M. tuberculosis* H37Rv, All drug sensitive *M. tuberculosis and* MDR *M. tuberculosis* respectively. In a study, the effects of commercial TQ on intracellular mycobacterial replication were examined in macrophages (RAW 264.7 cells). The results showed the reduction of intracellular *M. tuberculosis* H37Rv and extensively drug-resistant *M. tuberculosis* at concentration of 12.5 to 25 µg/ml and 6.25 to 12.5 µg/ml respectively [16].

Time Kill assay of methanol and water extracts of *Nigella sativa* seeds was performed at 250 µg/ml concentration of the MIC inhibiting all three mycobacterial strains. After 4 h of NI-M and NI-W extract administration, we observed more than 50% RLU reduction in view of bacterial inhibition.

TLC profiling of Methanol extracts of *N. sativa* seeds provided the presence of six phytochemicals (Figure 1).

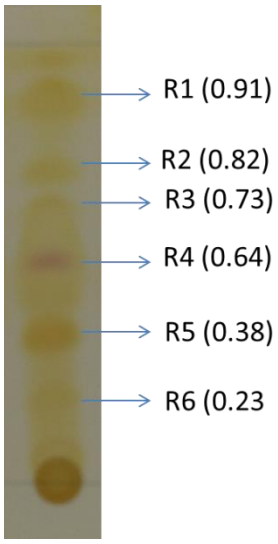

Figure 1: TLC profiling of Methanol extracts of *N. sativa* seeds.

The Rf values for each phytochemicals having 0.23, 0.38, 0.64, 0.73, 0.82 and 0.91. A study reported that the solvent system (Petroleum ether: Ethyl acetate: Methanol (6:2:2) gave 4 spots and Rf values having 0.09, 0.25, 0.81 and 0.92. Our study has agreed with two Rf values which were identical. Each phytochemicals gave different Rf values which provide their polarity and helps in selection of appropriate solvent system for separation of pure compounds [17].

CONCLUSION

N. sativa seed is a traditional medicine, which possesses anti-tubercular property. The plant was used for to cure several ailments and as they are well tested for safety efficacy window in humans. Therefore, this plant has great potential to develop as a natural drug for the treatment of tuberculosis including drug resistant TB. Further investigations like IR, NMR and MS are needed in order to determine the active metabolite. However, the additional studies including the *in vivo* toxicity dosage and mechanism of action also need to be deciphered. The synergistic activities of purified compounds of *N. sativa* seeds with known TB drugs has to be explored in future too.

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