ORIGINAL ARTICLE
IN VITRO ANTITUBERCULOUS ACTIVITY OF THYMOQUINONE, AN ACTIVE PRINCIPLE OF *NIGELLA SATIVA*

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Background: *Nigella sativa* seed has been used in folk medicine against many ailments including infections. The present study was aimed to investigate activity of thymoquinone, *in vitro*, against clinical isolates of *Mycobacterium tuberculosis*. **Methods:** Mycobacteria obtained from patients of King Fahd Hospital of University, Alkhobar, Saudi Arabia were subcultured at 37 °C in MGIT tubes containing Middlebrook broth and OADC growth supplement and growth detected by BACTEC MicroMGIT fluorometer on day 10. Mycobacteria were then inoculated in MGIT tubes containing thymoquinone 2.5, 5, 10, 20, 40, 80 µg/ml, streptomycin 1.25 µg/ml or controls in Middlebrook broth plus supplement, incubated at 37 °C for 14 days and read daily for fluorescence. In addition, isolates were inoculated in culture tubes containing Middlebrook agar (plus supplement) in presence of thymoquinone 2.5, 5, 10, 20, 40, 80 µg/ml, streptomycin 1.25 µg/ml or controls, and incubated at 37 °C for 4 weeks. **Results:** In Middlebrook broth, fluorescence test for tuberculosis was negative with thymoquinone 20, 40 and 80 µg/ml and streptomycin 1.25 µg/ml up to day 14th. With controls, thymoquinone 2.5, 5 and 10 µg/ml fluorescence was detectable from day 10 to 14. In Middlebrook agar, there was no visible growth of tubercle bacillus with thymoquinone 20, 40 and 80 µg/ml and streptomycin 1.25 µg/ml, however, with controls, thymoquinone 2.5 and 5 µg/ml abundant and with 10 µg/ml few colonies were observed. **Conclusions:** Thymoquinone possesses activity against *M. tuberculosis* with MIC of 20 µg/ml and has potential for further investigation. Our study confirms the benefit of *N. sativa* in native medicine against chest infection.
Keywords: *Nigella sativa*, thymoquinone, *Mycobacterium tuberculosis*, Micro MGIT fluorometer, Middlebrook broth, Middlebrook agar

INTRODUCTION

Thymoquinone is an active principle of *Nigella sativa* (*N. sativa*), which is a member of the *Ranunculaceae* family of plants. This plant is grown in several parts of the world, particularly Middle East, Middle Asia and Far Eastern countries; and its seeds have been used as a natural remedy for many diseases over centuries as well as flavouring agent in bakery products and pickles.[1,2]

Recently many active principles have been isolated from *N. sativa*, including thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellimine N oxide, nigellicine, nigellidine and alphahedrin.[3–7] In addition, using modern scientific techniques, many pharmacological effects of *N. sativa* and its active principles have been identified e.g. immune stimulation, antiinflammatory, anticancer, hypoglycemic, antihypertensive, anticestodal and antimicrobial effects; reviewed in Randhawa and Alghamdi, Ali and Blunden ans Salem.[8–10] The antibacterial effect of the phenolic fraction of *N. sativa* oil was first reported by Topozada in 1965.[11] Since then various extracts of *Nigella sativa* and thymoquinone were shown to possess antibacterial, antifungal and antiviral activities.[12]

Mycobacterium tuberculosis (*M. tuberculosis*) is a serious worldwide health threat, killing 3 million people annually, with 8 million active cases per year and the World health Organization estimates that a staggering 1/3 of the world's population is latently infected. Other Mycobacterial species, *Mycobacterium avium* are the emerging pathogens in the immunocompromised population, most notably AIDS patients. The treatment options are limited, and no new antibiotics have been introduced against mycobacteria since the 1970s. The existing drugs have many adverse effects and, moreover, the mycobacteria are becoming resistant to them. Therefore, there is an urgent need for the development of new drugs for the treatment and prevention of mycobacterial infections.[13]

Considering the wide spectrum of antimicrobial effects of *N. sativa* and the emerging need to develop new remedies for mycobacterial infections, the present study was aimed to investigate *in vitro* activity of thymoquinone against clinical isolates of *Mycobacterium tuberculosis*.

MATERIAL AND METHODS

Thymoquinone was obtained from Aldrich, a division of SIGMA, USA. The stock solution (1 mg/ml) was prepared by dissolving 50 mg of thymoquinone in 1 ml of ethyl-alcohol and made up to 50 ml with sterile distilled water. The streptomycin was from Grunenthal, Germany and to 1g streptomycin base was added 4 ml of sterile distilled water to prepare the stock solution.

M. tuberculosis were obtained from the sputum of patients reporting to chest clinic of the King Fahd Hospital of the University, Alkhobar, Saudi Arabia in November 2009 and found positive for Mycobacterium tuberculosis with Ziehl Neelsen stain and Lowenstein-Jensen (L J) medium.

MGIT (Mycobacterium Growth Indicator Tubes) tubes, BBL Middlebrook broth, BBL Middlebrook agar and BBL OADC growth supplement (Oleic acid 0.6 g, Bovine Albumin 50 g, Dextrose 20 g and Catalase 0.03 g) were purchased from local companies.

The surface scrapings from the LJ medium, used for the culture of clinical samples and positive for *Mycobacterium tuberculosis*, were aseptically removed and added to Middlebrook broth in sterile culture tubes, vortexed for 2 to 3 minutes and allowed to stand for 15 minutes. The supernatant was transferred to another tube and allowed to stand for 15 minutes again. The suspension was transferred to a third tube and the turbidity adjusted to 0.5 Macforland standard with sterile water. Then 0.5 ml of this suspension was added to MGIT tube containing 7 ml of Middlebrook broth and 0.8 ml of OADC growth supplement, incubated at 37 °C and read daily for the fluorescence by BACTEC Micro MGIT fluorometer till positive for the growth of mycobacteria. The tube was vortexed for 2 to 3 minutes, 0.1 ml of its suspension added to 10 ml of the Middle brook broth to make a 1:100 dilution and used for the antimycobaterial assay, which was performed in two sets of experiments: In the Middle brook broth and the Middle brook agar.

The Middlebrook broth assay was performed in MGIT tubes containing 7 ml of broth and 0.8 ml of OADC growth supplement. From the freshly prepared stock solutions, the drug dilutions of thymoquinone and streptomycin were prepared in sterile water in such a way that the addition of 0.5 ml of the drug solution to the corresponding MGIT tubes will provide thymoquinone 2.5, 5, 10, 20, 40, 80 μg/ml or streptomycin 1.25 μg/ml. The controls were prepared by the addition of 0.5 ml of sterile water in the corresponding MGIT tubes to equalise the volume. Four tubes were prepared for each concentration of thymoquinone, streptomycin and the controls. To each MGIT tube was added 0.5ml of the mycobacterial suspension (1:100 dilution), incubated at 37 °C and read daily for fluorescence by BACTEC Micro MGIT fluorometer from day 3rd to 14th.

The Middle brook agar assay was performed in sterile culture tubes containing 10 ml of agar and 1 ml of OADC growth supplement. From the freshly prepared stock solutions, the drug dilutions of thymoquinone and streptomycin were prepared in sterile water in such a way that the addition of 0.5 ml of the drug solution to the corresponding culture tubes will provide thymoquinone 2.5, 5, 10, 20, 40, 80 μg/ml or streptomycin 1.25 μg/ml. To prepare the controls, 0.5 ml of sterile water was added in culture tubes to equalise the volume. Four tubes were prepared for each concentration of thymoquinone, streptomycin and the control; and left at room temperature for 24 hours, tilted at about 30 degree, for solidification. Then to each culture tube was added 0.5 ml of the mycobacterial suspension (1:100 dilution), spread over the surface, incubated at 37 °C for 4 weeks and observed for the colonial growth of mycobacteria.

In the end of both anti-microbial assays, slides were prepared from the broth suspensions and from the surface scrapings of the agar from all MGIT/culture tubes containing the test drugs as well as controls. The slides were impregnated with Ziehl Neelsen stain and observed under the microscope (X100) for AFB.

RESULTS

In the case of Middlebrook broth assay, the fluorescence test remained negative with thymoquinone 20, 40 and 80 μg/ml and streptomycin 1.25 μg/ml up to day 14th. With controls and thymoquinone 2.5 and 5 μg/ml fluorescence test became positive on day 10th, whereas with thymoquinone 10 μg/ml the fluorescence test became positive on day 14th (Table-1).

Table-1: Results of fluorescence test for growth (+) of *M. tuberculosis* in Middlebrook broth in presence of thymoquinone (TQ) 2.5, 5, 10, 20, 40 and 80 μg/ml, streptomycin (STR) 1.25 μg/ml and controls on day 1–14
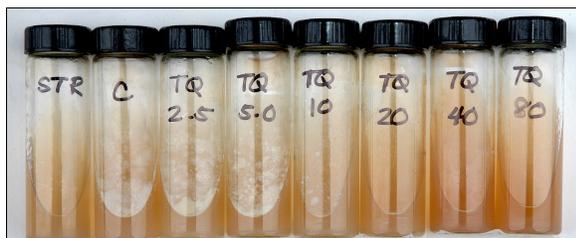
Observation day	TQ 2.5	TQ 5	TQ 10	TQ 20	TQ 40	TQ 80	STR 1.25	Controls
1–9	-	-	-	-	-	-	-	-
10	+	+	-	-	-	-	-	+
11	+	+	-	-	-	-	-	+
12	+	+	-	-	-	-	-	+
13	+	+	-	-	-	-	-	+
14	+	+	+					+

In the case of Middlebrook agar, there was no visible growth of tubercle bacillus with thymoquinone 20, 40 and 80 μg/ml and streptomycin 1.25 μg /ml. With controls, thymoquinone 2.5 and 5 μg/ml abundant colonies were observed, but much less with 10 μg/ml (Table-2, Figure-1). There was uniform growth in all 4 tubes for each concentration of the drug and controls.

Table-2: Growth (+) of *M. tuberculosis* in Middlebrook agar in presence of thymoquinone (TQ) 2.5, 5, 10, 20, 40 and 80 µg/ml, streptomycin (STR) 1.25 µg /ml, and controls

Tube No.	TQ 2.5	TQ 5	TQ 10	TQ 20	TQ 40	TQ 80	STR 1.25	Controls
1	++	++	+	-	-	-	-	++
2	++	++	+	-	-	-	-	++
3	++	++	+	-	-	-	-	++
4	++	++	+	-	-	-	-	++

No growth of tubercle bacillus with thymoquinone (TQ) 20, 40 and 80 g/ml and streptomycin 1.25 µg/ml; abundant colonies with control (C), thymoquinone 2.5 and 5 µg/ml and much less with 10 µg/ml

Figure-1: Growth of *M. tuberculosis* in Middlebrook agar with OADC growth supplement

The slides prepared from the MGIT tubes with positive fluorescence test and culture tubes showing growth contained abundant colonies of AFB. Whereas, no AFB were detected from slides prepared from MGIT tubes with negative fluorescence test and the culture tubes without growth.

DISCUSSION

Tuberculosis (TB) is a growing international health concern, since it is the leading infectious cause of death in the world today. In particular, the increasing prevalence of multidrug resistant (MDR) TB has greatly contributed to the increased difficulties in the control of TB.[19] A recent study for the detection of MDR *Mycobacterium tuberculosis* reported that amongst the 138 clinical isolates tested, 55 were resistant to at least one drug, 34 of 38 (89.5%) resistant to isoniazid, 28 out of 28 (100%) resistant to rifampicin, 15 of 18 (83.3%) resistant to ethambutol, 18 out of 30 (60%) resistant to streptomycin and 17 of 17 (100%) resistant to pyrazinamide.[20]

The development of potent new anti-TB drugs is urgently needed, but so far only few compounds are in the process of investigation, such as oxazolidinones (linezolid, PNU 100480), nitroimidazoles (nitroimidazopyran PA 824, metronidazole), 2-pyridone, riminophenazines and diarylquinolines.[19] Many herbal medicines are claimed to possess anti-TB effect but only a few have been tested by microbiological techniques., e.g., extracts of *Croton pseudopulchellus*, *Ekebergia capensis*, *Euclea natalensis*, *Nidorella anomala* and *Polygala myrtifolia*, *Cryptocarya latifolia*, *Euclea natalensis*, *Helichrysum melanacme*, *Nidorella anomala Thymus vulgaris*, *Chenopodium ambrosioides*, *Ekebergia capensis*, *Euclea natalensis*, *Helichrysum melanacme*, *Nidorella anomala* and *Polygala myrtifolia* were shown to inhibit the growth of *M. tuberculosis*, giving an MIC of 0.1 to 1.0 mg/ml by agar plate method and rapid radiometric method.[21] Some quinones have also been tested against Mycobacteria using agar plate and fluorescent assay and amongst these plumbagin and juglone were the most potent, with an MIC of 66 µM and 72 µM, respectively, against *Mycobacterium avium* complex.[22] In the present study thymoquinone inhibited *M. tuberculosis* in reasonably low concentration, MIC being 20 µg/ml using the manual MGIT method.

In a recent study aimed to evaluate the manual mycobacterium growth indicator tube (MGIT) system for the testing of *M. tuberculosis* susceptibility to second-line drugs compared to the proportion method , the results showed that the manual MGIT is an accurate method for the rapid susceptibility testing of *M. tuberculosis* to second-line drugs and the authors concluded that there is no need for a machine when using the manual MGIT and results can be read with a simple UV lamp or with a semi quantitative reader.[23] The manual MGIT (MMGIT) method is also reported to be cost effective when compared with Lowenstein-Jensen culture (HLJ and CLJ) as well as automated MGIT (AMGIT) liquid culture methods. Costs per *M. tuberculosis* specimen detected were, respectively, US$197, $202, $312 and $340 for MMGIT, AMGIT, CLJ and HLJ.[24]

Thymoquinone has been reported to possess potent anti-inflammatory effects observed on several inflammation-based models including experimental encephalomyelitis, colitis, peritonitis, oedema, and arthritis through suppression of the inflammatory mediators: prostaglandins and leukotriens; as well as immunomodulatory properties as it augmented T cell and natural killer cell mediated immune responses, which would perhaps contribute to the overall improvement of the patients suffering from TB.[25]

Regarding the toxicity, oral thymoquinone was found to be quite safe, LD$_{50}$ of 2.4 g/Kg in mice.[26] However, there were controversial reports for the LD$_{50}$ of thymoquinone given intraperitoneally, varying from 10 mg/Kg[27] to 90.3 mg/Kg[28]. In a recent report the LD$_{50}$ in mice after intraperitoneal injection and oral ingestion were 104.7 mg/Kg and 870.9 mg/Kg, respectively, whereas, LD$_{50}$ in rats after intraperitoneal injection and oral ingestion were 57.5 mg/Kg and 794.3 mg/Kg

respectively.[29] Lower intraperitoneal LD_{50} values are probably due to local irritation caused by thymoquinone, as seen after autopsy.[30]

CONCLUSION

Thymoquinone possesses anti-*Mycobacterium tuberculous* activity in reasonably low concentrations *in vitro* with an MIC of 20 µg/ml, and has potential for development as a new, effective and safe drug for tuberculosis.

ACKNOWLEDGEMENTS

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