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Original Research

An active principle of *Nigella sativa* L., thymoquinone, showing significant antimicrobial activity against anaerobic bacteria

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ABSTRACT

Aim/Background: Thymoquinone (TQ) is the major active principle of *Nigella sativa* seed (black seed) and is known to control many fungi, bacteria, and some viruses. However, the activity of TQ against anaerobic bacteria is not well demonstrated. Anaerobic bacteria can cause severe infections, including diarrhea, aspiration pneumonia, and brain abscess, particularly in immunodeficient individuals. The present study aimed to investigate the *in vitro* antimicrobial activity of TQ against some anaerobic pathogens in comparison to metronidazole. **Methods:** Standard, ATCC, strains of four anaerobic bacteria (*Clostridium difficile, Clostridium perfringens, Bacteroides fragilis,* and *Bacteroides thetaiotaomicron*), were initially isolated on special *Brucella* agar base (with hemin and vitamin K). Then, minimum inhibitory concentrations (MICs) of TQ and metronidazole were determined against these anaerobes when grown in *Brucella* agar, using serial agar dilution method according to the recommended guidelines for anaerobic organisms instructed by the Clinical and Laboratory Standards Institute. **Results:** TQ showed a significant antimicrobial activity against anaerobic bacteria although much weaker than metronidazole. MICs of TQ and metronidazole against various anaerobic human pathogens tested were found to be between 10-160 mg/L and 0.19-6.25 mg/L, respectively. **Conclusions:** TQ controlled the anaerobic human pathogenic bacteria, which supports the use of *N. sativa* in the treatment of diarrhea in folk medicine. Further investigations are in need for determination of the synergistic effect of TQ in combination with metronidazole and the activity of derivatives of TQ against anaerobic infections.

KEY WORDS: *Brucella* blood agar, metronidazole, minimum inhibitory concentrations, *Nigella sativa*, thymoquinone

INTRODUCTION

Thymoquinone (TQ) is the major active principle of *Nigella sativa* L. seed. This seed is commonly named as "Al-Habbah Al-Sawda" in Arabic and "black seed" in English language [1]. Black seed is a commonly used herbal medicine for many ailments in Arab countries, Middle Asia, and the Indian Subcontinent [2].

TQ is known to have many pharmacological activities, to include anticancer, anti-inflammatory, antiasthmatic, antidiabetic, antihypertensive, and hypolipidemic, and antimicrobial effects [2-4]. The antimicrobial activity of TQ and various extracts of *N. sativa* has been reported against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, and *Listeria monocytogenes* [5-8].

Microorganisms are becoming resistant to many antibiotics. Therefore, there is need to find new remedies against pathogenic microbes [9]. Black seed extracts were found to be effective against some resistant microorganisms, such as *S. aureus* and *P. aeruginosa* [9,10].

Anaerobic bacteria can cause serious infections, particularly in immunocompromised individuals, for example, elderly, diabetics, and those suffering from HIV infection and using anticancer chemotherapy, immune suppressant drugs, or broad spectrum antibiotics. Anaerobes have been reported to cause aspiration pneumonia, lung abscess, and emphysema [11-13]. Moreover, they have been shown to cause brain abscess and bacterial meningitis [14-16]. They are generally resistant to many antibacterial drugs and are known to develop biofilm around them [17]. Metronidazole is considered as a drug of choice for the treatment of anaerobic infections but can cause agranulocytosis [18].

Because of the scarcity of studies for the activity of black seed or its active components against anaerobic bacteria, the present study has been designed to investigate the activity of TQ, *in vitro*, against anaerobic human pathogenic strains, including *Clostridium difficile*, *Clostridium perfringens*, *Bacteroides fragilis*, and *Bacteroides thetaiotaomicron* by standard antimicrobial assay and compare it with that of metronidazole.

METHODS

Microorganisms

Standard, ATCC, strains of *C. difficile*, *C. perfringens*, *B. fragilis*, and *B. thetaiotaomicron* were purchased from Danat Alajiyal for Medical and Scientific Equipment (Saudi Arabia). These strains were initially grown on special *Brucella* agar base (with hemin and vitamin K), supplemented with 5% laked or defibrinated sheep blood in Petri plates and identified by conventional methods.

Chemicals

The materials for the culture media used in the study were purchased from Micromaster and Himedia (Saudi Arabia), TQ from Sigma-Aldrich (Saudi Arabia) and anaerobic jar, anaerobic gas pack and indicator from Becton Dickinson (Saudi Arabia). Metronidazole IV fluid (Flagyl from Pfizer, USA) was obtained from the Pharmacy Department of Prince Abdulaziz Bin Mosad Hospital, Arar, Saudi Arabia.

Stock Solutions and Serial Dilutions

Stock solution of TQ 64 mg/ml was prepared in DMSO and water. From the stock solution, serial dilutions of TQ 32, 16, 8, 4, 2, 1, 0.5, and 0.25 mg/ml were prepared in 5 ml sterile test tubes. Then, 100 μl from each diluted concentration of TQ was added to 20 ml of molten *Brucella* agar base (with hemin and vitamin K), supplemented with 5% defibrinated sheep blood, giving final concentrations of TQ 160-1.25 μg/ml (160, 80, 40, 20, 10, 5, 2.5, and 1.25 μg/ml) in the Petri plates (three plates for each concentration level).

Stock solution of metronidazole (Flagyl) contained 500 mg of metronidazole in100 ml of water (5 mg/ml), which was serially diluted down to 0.035 mg/ml (5, 2.5, 1.25, 0.625, 0.31, 0.15, 0.07, and 0.035 mg/ml) in 5 ml sterile test tubes. Then, 100 μl from each diluted concentration of metronidazole was added to 20 ml of molten *Brucella* agar base (with hemin and vitamin K), supplemented with 5% defibrinated sheep blood, giving final concentrations of metronidazole 25-0.195 μg/ml (25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.195 μg/ml) in the Petri plates (three plates for each concentration level).

The ranges of serial dilutions of TQ and metronidazole in *Brucella* agar given above were chosen from the results of the pilot study. According to the Clinical and Laboratory Standards Institute (CLSI) guidelines for the *Brucella* agar method, metronidazole $\leq$8 μg/ml is considered as sensitive, 16 μg/ml as intermediate, and $\geq$32 μg/ml as resistant.

Minimum Inhibitory Concentration (MIC) Value Determination Assay

The MICs of TQ and metronidazole against the tested strains were determined by the standard method recommended by the CLSI. In each Petri plate (Either containing TQ 160- 1.25 ug/ml, or metronidazole, 25-0.195 ug/ml), the standard inoculum of (1 μl) of 0.5 MacFarland (10^5 CFU) was spot inoculated. Three Petri plates containing 20 ml *Brucella* agar (with supplements) without TQ or metronidazole were also inoculated with the standard inoculum of each test strain as controls. All plates were incubated anaerobically for 42-48 h and the bacterial growth was observed.

RESULTS

The results of the antibacterial activity of various concentrations of TQ are depicted in Table 1 and Figures 1-4, which reveal that *C. difficile* was the most sensitive among the anaerobes tested, with intermediate sensitivity to TQ 10 and 20 μg/ml and completely sensitive to TQ 40 μg/ml, giving an MIC of 40 μg/ml. Whereas, *C. perfringens*, *B. fragilis* and *B. thetaiotaomicron* were relatively less sensitive to TQ, with MICs of 160 μg/ml.

The results of the antibacterial activity of various concentrations of metronidazole are given in Table 2, which reveal that *C. difficile* was again the most sensitive to metronidazole (MIC 0.78 μg/ml), followed by *B. fragilis* and *B. thetaiotaomicron* (MICs 3.12 μg/ml), while *C. perfringens* was least sensitive (MIC 6.25 μg/ml).

Table 1: Antibacterial activity of thymoquinone against anaerobic human pathogenic strains

Reference strains	Thymoquinone (μg/ml)							
	160	80	40	20	10	5	2.5	1.25
C. perfringens ATCC 13124	S	I	R	R	R	R	R	R
C. difficile ATCC 700057	S	S	S	I	I	R	R	R
B. fragilis ATCC 25285	S	R	R	R	R	R	R	R
B. thetaiotaomicron ATCC 29741	S	R	R	R	R	R	R	R

C. perfringens: Clostridium perfringens, C. difficile: Clostridium difficile, B. fragilis: Bacteroides fragilis, B. thetaiotaomicron: Bacteroides thetaiotaomicron, S: Sensitive, I: Intermediate sensitivity, R: Resistant

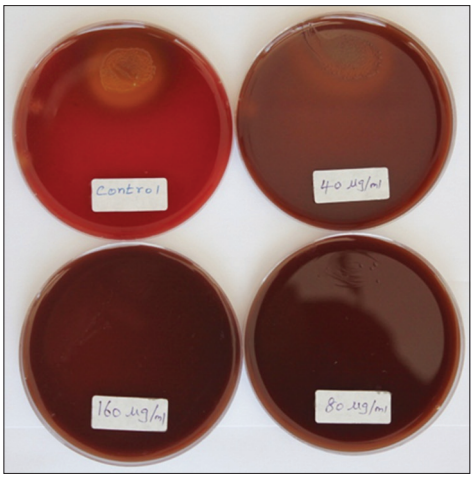

Figure 1: Growth of *Clostridium perfringens* in different concentrations of thymoquinone in Brucella agar

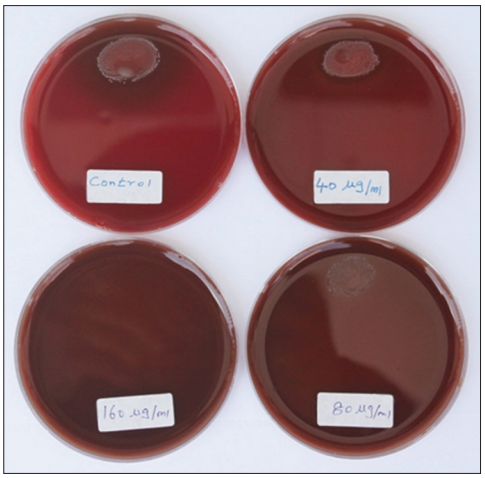

Figure 3: Growth of *Bacteroides fragilis* in different concentrations of thymoquinone in *Brucella* agar

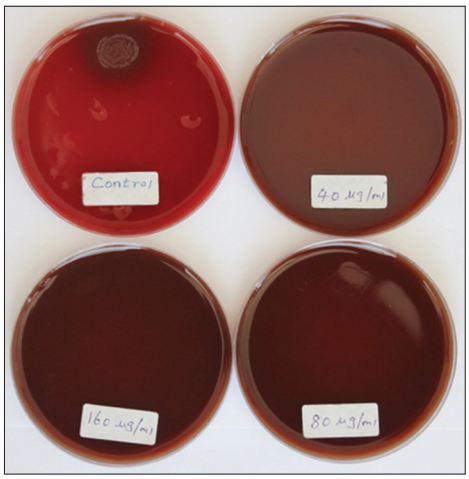

Figure 2: Growth of *Clostridium difficile* in different concentrations of thymoquinone in *Brucella* agar

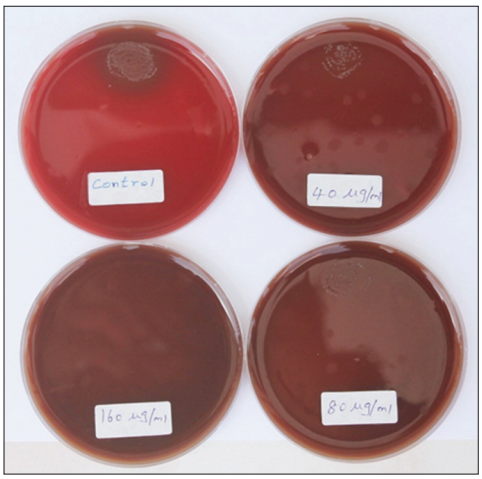

Figure 4: Growth of *Bacteroides thetaiotaomicron* in different concentrations of thymoquinone in *Brucella* agar

A summary of the results of estimated MICs for TQ and metronidazole against test anaerobes is given in Table 3. TQ showed significant antibacterial activity against anaerobic bacteria used in the study, particularly against *C. difficile*, although much weaker than metronidazole.

DISCUSSION

Anaerobic bacteria are normal commensals and reside in human skin and mucous membranes, thus may cause endogenous infections, such as diarrhea, aspiration pneumonia, lung abscess, brain abscess, and meningitis [11-16]. Metronidazole is very effective and commonly used for the treatment of anaerobic infections but unfortunately is relatively more toxic and can cause serious adverse effects, including agranulocytosis [18]. Besides metronidazole, other effective antibiotics against anaerobic bacteria are the carbapenems (imipenem and meropenem), chloramphenicol, the combinations of penicillin and beta-lactamase inhibitor (ampicillin plus sulbactam, ticarcillin plus clavulanate, and piperacillin plus tazobactam), tigecycline, and clindamycin [19]. Unfortunately, like other bacteria, anaerobes are gradually becoming more resistant to antibiotics. The most frequently isolated antibiotic-resistant anaerobe is *B. fragilis*, but the *Clostridium* species and other anaerobes are also becoming increasingly resistant [20]. In the present study, also, MICs of both TQ and metronidazole against *B. fargilis* were relatively higher than against *C. difficile*.

Because of the limited published work for the antibacterial activity of TQ against anaerobic human pathogens, we could not find similar studies to compare our results. However, there was one study reported in the literature regarding the effect of TQ on foodborne anaerobic bacteria and the results of our study were not much different from that (MIC of TQ against *Clostridium* species was from 5 to 10 μg/ml in the former study while, in our study, it was from 10 to 40 μg/ml for *C. difficile* [21].

The activity of TQ against anaerobic human pathogens is much less than metronidazole. However, derivatives of TQ could be prepared and tested for their activity against

Table 2: Antibacterial activity of metronidazole against anaerobic human pathogenic strains

Reference strains	Metronidazole (μg/ml)							
	25	12.5	6.25	3.12	1.56	0.78	0.39	0.19
C. perfringens ATCC 13124	S	S	S	I	R	R	R	R
C. difficile ATCC 700057	S	S	S	S	S	S	I	R
B. fragilis ATCC 25285	S	S	S	S	I	R	R	R
B. thetaiotaomicron ATCC 29741	S	S	S	S	I	R	R	R

C. perfringens: Clostridium perfringens, C. difficile: Clostridium difficile, B. fragilis: Bacteroides fragilis, B. thetaiotaomicron: Bacteroides thetaiotaomicron, S: Sensitive, I: Intermediate sensitivity, R: Resistant

Table 3: MIC of thymoquinone and metronidazole against anaerobic human pathogens

Microorganisms	Thymoquinone (μg/ml)	Metronidazole (μg/ml)
	MIC	MIC
C. perfringens ATCC 13124	80-160	1.56-6.25
C. difficile ATCC 700057	10-40	0.19-0.78
B. fragilis ATCC 25285	80-160	0.78-3.12
B. thetaiotaomicron ATCC 29741	80-160	0.78-3.12

C. perfringens: Clostridium perfringens, C. difficile: Clostridium difficile, B. fragilis: Bacteroides fragilis, B. thetaiotaomicron: *Bacteroides thetaiotaomicron*, MIC: Minimum inhibitory concentrations

anaerobes and might be as effective as metronidazole, because some derivatives of TQ were demonstrated to be much more effective than TQ itself against cancer cell lines. For example, some analogs of TQ, when tested for their biological activity against pancreatic cancer cell lines, were found to be more potent than TQ in terms of inhibition of cell growth, induction of apoptosis, and modulation of transcription factor [22].

C. difficile is one of the most important microorganisms causing health-care-associated diarrhea, i.e., diarrhea secondary to the use of broad-spectrum antibiotics, cancer chemotherapy, and immune suppressant drugs. Unfortunately, data reporting the decreased effectiveness of metronidazole in the treatment of severe disease due to *C. difficile* have also been published [23]. In the present work, TQ was found to be relatively more active against *C. difficile* and further studies could be designed to investigate effectiveness of the combination of TQ with metronidazole for the control of health-care-related diarrhea.

CONCLUSIONS

TQ, the most abundant active principle of *N. sativa*, was shown to possess a reasonable activity against anaerobic human pathogens. TQ was, particularly, more effective against *C. difficile*, which is relatively common to cause diarrhea in immunocompromised individuals and those taking broad-spectrum antibiotics. Regular use of black seed could prevent infection from *C. difficile*. The study also supports the use of black seed in the treatment of diarrhea in folk medicine.

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