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 antitumor, 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 in 100 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 ≤8 µg/ml is considered as sensitive, 16 µg/ml as intermediate, and ≥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 µg/ml, or metronidazole, 25-0.195 µg/ml), the standard inoculum of (1 µl) of 0.5 MacFarland (10⁴ 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   | 10   | 5    | 2.5  | 1.25 |
| C. perfringens ATCC 13124 | S    | I    | R    | R    | R    | R    | R    |
| C. difficile ATCC 700057 | S    | S    | S    | I    | R    | R    | R    |
| B. fragilis ATCC 25285 | S    | R    | R    | R    | R    | R    | R    |
| B. thetaiotaomicron ATCC 29741 | S    | 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
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...
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The study also supports the use of black seed in the treatment of diarrhea in folk medicine.

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