Arabian Journal of Chemistry (2015) **xxx**, xxx–xxx

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Determination of anti-staphylococcal activity of thymoquinone in combinations with antibiotics by checkerboard method using EVA capmat™ as a vapor barrier

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Received 4 December 2014; accepted 19 April 2015

KEYWORDS

Nigella sativa;
Benzoquinone;
Antibiotic resistance;
Synergistic effect;
Volatility

Abstract Thymoquinone (Tq) has been reported to potentiate the *in vitro* growth-inhibitory activity of some antibiotics especially against *Staphylococcus aureus*. However, it has been shown that Tq vapors can affect the results of susceptibility testing by standard broth microdilution method. Therefore, we made a comparative experiment with and without ethylene vinyl acetate cap mats (EVA capmat™) on microplates. The results showed significant differences in the minimum inhibitory concentration values and proved this capmat as an effective vapor barrier. Therefore further experiments focused on the *in vitro* anti-staphylococcal combinatory effect of Tq with oxacillin, penicillin, and tetracycline against various *S. aureus* strains have been performed by checkerboard method using EVA capmat™. The combined effect was evaluated according to the sum of fractional inhibitory concentrations (ΣFIC). Synergy was obtained for combination with oxacillin against 3

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2015.04.021

(ΣFIC 0.263–0.450), with penicillin against 1 (ΣFIC 0.466) and with tetracycline against 2 strains tested (ΣFIC 0.400–0.475). Our results confirm previous reports on the Tq enhancement of anti-staphylococcal activity of antibiotics. Moreover, this is the first report on Tq synergy with oxacillin and penicillin against *S. aureus*. Our experiments also showed that Tq vapors can affect evaluation of combined effect by checkerboard assay, whereas the use of EVA capmat™ can avoid this.

1. Introduction

Since their discovery, antimicrobial drugs have proved to be remarkably effective for the control of bacterial infections. However, it was soon evident that these pathogens are able to become resistant to many of the first effective drugs (Gold and Moellering, 1996). *Staphylococcus aureus* strains cause a broad spectrum of diseases, ranging from minor skin diseases to serious bloodstream infections (Daum, 2008). In the past, this bacterium has been successfully treated with penicillins, but many strains build up the resistance to ß-lactam antibiotics by the production of ß-lactamase (Andremont et al., 2011). Initially, the problem of bacterial resistance to antimicrobial drugs was solved by the discovery of new classes of antibiotics, such as the aminoglycosides, macrolides, and glycopeptides, as well as by the chemical modification of previously existing drugs (Gold and Moellering, 1996). Nevertheless, *S. aureus* has the ability to acquire resistance to practically all useful antibiotics and become multidrug-resistant (Gibbons, 2004).

One of the potential strategies how to overcome this problem in the treatment of staphylococcal infections is the use of antibiotics in combinations with other compounds (Drago et al., 2007). The best-known example of such a combination is the comedication of the ß-lactam antibiotics with clavulanic acid, which successfully aborts gained resistance (Wagner, 2011). In a number of previous studies, for example as reviewed by Hemaiswarya et al. (2008), plant substances were also observed to inhibit bacterial resistance and to potentiate synergistically the effect of conventional antimicrobial agents.

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) (Tq) (Fig. 1) is one of the main bioactive constituent of *Nigella sativa* L. seeds (black seed or black cumin), which have been used traditionally for thousands years in the Middle East, Northern Africa and India as a natural remedy for various infectious diseases such as cough, bronchitis, pulmonary infection, fever, and influenza (Kokoska, 2011). Tq is also found in several medicinal plant species belonging to the other genera such as *Monarda* and *Thymus* (Taborsky et al., 2012). Besides its antimycotic, anti-oxidant, anti-inflammatory and anticancer effect, Tq exhibits significant antimicrobial activity against both Gram-negative and Gram-positive bacteria (Kokoska, 2011), including methicillin resistant *S. aureus* (MRSA) (Liu et al., 1996). Furthermore, it has recently been reported to potentiate the activity of some antibiotics. Halawani (2009) demonstrated synergism between Tq and common antibiotics (ampicillin, cephalexin, chloramphenicol, tetracycline, gentamicin, and ciprofloxacin) against *S. aureus* strains used disk diffusion technique. However, the general procedure for detecting synergy is the standard checkerboard method, in which two compounds are tested in serial dilutions and in all combinations of these dilutions together to find the concentrations of each compound, both alone and in combination, that produce some specified, easily determined effect. The interaction is then determined algebraically (according to its fractional inhibitory concentrations, FICs) or geometrically (Berenbaum, 1978; EUCAST, 2000). In another study, Kouidhi et al. (2011) reported increasing activity of tetracycline and benzalkonium chloride when combined with Tq at ½ of its minimum inhibitory concentration (MIC) using the microtiter plates assay. Nevertheless, our previously reported experiments suggest that the results of standard microdilution test can be influenced by Tq vapors that are able to strongly affect concentrations in adjoining wells on microtiter plate, which was confirmed by GC/MS analysis (Novy et al., 2014). With the aim of preventing this phenomenon, in this study we examined *in vitro* anti-staphylococcal effect of Tq in combination with oxacillin, penicillin, or tetracycline against nine strains of *S. aureus* including resistant strains by the broth microdilution method, where the microtiter plates were especially covered by ethylene vinyl acetate (EVA) cap mats.

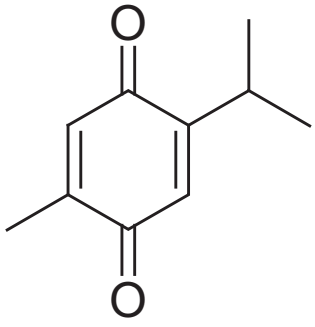

Figure 1 Chemical structure of thymoquinone.

2. Materials and methods

2.1. Chemicals

Tq, oxacillin, penicillin, and tetracycline were purchased from Sigma-Aldrich (Prague, CZ). Solvents, such a dimethyl sulfoxide (Penta, Prague, CZ), ethanol (Sigma-Aldrich, Prague, CZ), and deionized water, used as the negative control did not inhibit any strain tested.

2.2. Bacterial strains and growth media

Standard strains ATCC 29213 and ATCC 43300 were purchased from Oxoid (Basingstoke, UK). Seven clinical isolates of *S. aureus* including antibiotic resistant strains were obtained from The Motol University Hospital, Prague, Czech Republic. Cation-adjusted Mueller–Hinton broth (Oxoid, Basingstoke, UK) equilibrated with Tris-buffered saline (Sigma–Aldrich,

Please cite this article in press as: Rondevaldova, J. et al., Determination of anti-staphylococcal activity of thymoquinone in combinations with antibiotics by checkerboard method using EVA capmat™ as a vapor barrier. Arabian Journal of Chemistry (2015), http://dx.doi.org/10.1016/j.arabjc.2015.04.021