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Short communication

Berberine enhances the antibacterial activity of thymoquinone, carvacrol and thymol against multi-drug resistant nontuberculous mycobacteria

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Abstract

The aim of this study was to investigate the activity of thymoquinone (TQ), carvacrol (CAR) and thymol (TYM) against multi-drug resistant nontuberculous mycobacteria (MDR-NTM), alone and in combination with berberine (BER). Antimicrobial activity was first evaluated at concentrations from 8 to 512 µg/mL. Each of the compounds tested exhibited good activity against nontuberculous mycobacteria (NTM) isolated from fish, with MIC values of 32-128 µg/mL. In this study, we have shown for the first time the synergistic efficacy of BER with CAR, TYM or TQ against NTM strains. Thus, the combination of these compounds with BER seems to be a new approach for combating MDR-NTM strains.

Keywords: atypical *Mycobacterium*, multi-drug resistant mycobacteria, nontuberculous mycobacteria

Introduction

The genus *Mycobacterium* includes mycobacteria causing tuberculosis, mycobacteria causing leprosy, and nontuberculous mycobacteria (NTM), a widely diverse group of species ranging from saprophytes to pathogens of humans and animals, including fish (Puk and Guz 2020). NTM infections are difficult to treat due to their relative resistance to currently available drugs and low tolerance to prolonged treatment. Drug resistance in mycobacteria is conferred by their highly lipophilic cell wall and the various mechanisms that control cell wall content, a low number of porins, a broad range of efflux pumps, active biotransformation by cytosolic enzymes, and inducible resistance mechanisms (Van Ingen et al. 2012). A number of plant extracts (Puk et al. 2023) and plant secondary metabolites, such as alkaloids (e.g. berberine), quinones (e.g. thymoquinone – TQ) and terpenoids (e.g. thymol – TYM, and carvacrol – CAR), have been described as important agents exhibiting antimycobacterial activity (Gentry et al. 1998, Dey et al. 2014, Andrade-Ochoa et al. 2015, Sieniawska et al. 2017, Marini et al. 2019). It has been documented that berberine (BER) inhibits the activity of efflux pumps in resistant bacteria. Moreover, several studies have reported positive synergistic activity of BER when combined with antibiotics (Zuo et al. 2014, Zhou et al. 2016, Puk and Guz 2022). However, the combined effects of BER and plant secondary metabolites against multidrug resistant NTM (MDR-NTM) isolates have not been previously examined.

Bacteria such as *M. abscessus*, *M. marinum*, and *M. chelonae* can cause skin and soft tissues infections in humans (Lamb and Dawn 2014). Due to their high level of natural and/or acquired resistance to the chemotherapeutic agents used in treatment, it is essential to search for new, effective treatment methods. The aim of the present study was to investigate the anti-MDR-NTB activity of CAR (5-isopropyl-2-methylphenol), TYM (2-isopropyl-5-methylphenol) and TQ (2-isopropyl-5-methyl-1,4-benzoquinone) applied alone and in combination with BER.

Materials and Methods

The study was conducted on six MDR-NTM strains, i.e. *Mycobacterium abscessus* (M11), *Mycobacterium chelonae* (M2, M12, M57, and M102) and *Mycobacterium marinum* (M4), originally isolated from diseased ornamental fish (Puk and Guz 2020). The strains were stored at the Department of Biology and Fish Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin. The experiment was performed in accordance with the guidelines set out by the Local Ethics Committee. The minimum inhibitory concentrations (MICs) of carvacrol (Sigma-Aldrich), thymol (Sigma-Aldrich) and thymoquinone (Sigma-Aldrich), alone and in combination with berberine (Sigma-Aldrich), were determined for each strain using the resazurin (Sigma-Aldrich) microtiter assay, as previously described by Guz and Puk (2022). The MIC was defined as the lowest drug concentration that prevented colour change. A growth control containing BER without tested compounds, and a sterility control without inoculum were included in each plate.

The *in vitro* haemolytic activity was performed using the spectrophotometric method described by Kumar et al. (2011) with the use of carp (*Cyprinus carpio*) erythrocytes.

Table 1. Distribution of multi-drug resistant patterns among nontuberculous mycobacteria strains retrieved from diseased ornamental fish (Guz and Puk 2022).

Strains	Phenotypic resistance patterns
M4	CIP, DOX, RMP, INH
M11	CIP, TOB, DOX, SMX, RMP, INH, STR
M2	CIP, TOB, DOX, SMX, RMP, INH, STR
M12	CIP, TOB, DOX, SMX, RMP, INH, STR
M57	CIP, TOB, DOX, SMX, RMP, INH, STR
M102	CIP, TOB, DOX, SMX, RMP, INH, STR

TOB – tobramycin, DOX – doxycycline, CIP – ciprofloxacin, SMX – sulfamethoxazole, RMP – rifampicin, INH – isoniazid, M4 – *Mycobacterium marinum*, M11 – *M. abscessus*, M2 – *M. chelonae*, M12 – *M. chelonae*, M57 – *M. chelonae*, M102 – *M. chelonae*

Results and Discussion

MDR-NTM strains, e.g., *M. abscessus* (M11), *M. chelonae* (M2, M12, M57, and M102), and *M. marinum* (M4) (Table 1) were used to investigate the effect of BER on the antibacterial activity of CAR,

Table 2. Carvacrol, thymol and thymoquinone minimum inhibitory concentrations (μg/mL) in the presence of different concentrations of berberine.

	BER*	Minimal inhibitory concentrations (μg/mL)					
		M4	M11	M2	M12	M57	M102
Carvacrol	0	128	64	128	128	64	128
	16	64	32	64	64	32	64
	32	32	32	64	64	32	64
	64	32	32	64	64	32	32
Thymol	0	128	64	64	128	64	64
	16	64	64	64	64	32	64
	32	32	64	64	64	32	64
	64	32	32	32	64	32	32
Thymoquinen	0	64	64	32	64	32	64
	16	32	16	32	32	32	64
	32	8	16	32	32	32	16
	64	8	16	16	32	16	16
BER		128	128	256	256	256	256

BER – berberine, BER* – concentrations of BER (μg/mL), M4 – *M. marinum*, M11 – *M. abscessus*, M2 – *M. chelonae*, M12 – *M. chelonae*, M57 – *M. chelonae*, M102 – *M. chelonae*

TYM, and TQ. The susceptibility of strains was studied by the colorimetric method using the resazurin microtiter assay plate testing. Resazurin (7-hydroxy-3H--phenoxazin-3-one-10-oxide), a blue dye, is reduced by the oxidoreductase of active bacteria to resorufin and then to hydroresorufin. Active bacterial cells reduce the resazurin to hydroresorufin, providing a direct quantitative indicator of bacterial metabolic activity used to determine MIC of substances (Li et al. 2017, Chakamsin et al. 2022).

Given the importance of drug resistance and the increasing incidence of MDR-NTM infections, the search for novel antimycobacterial agents is crucial. BER has been documented to act synergistically with antibiotics by inhibiting the activity of efflux pumps in resistant bacteria. In this regard, we have recently reported that BER potentiated the antimycobacterial activity of antibiotics (Puk and Guz 2022). In the present study, we aimed to determine whether BER has a synergistic effect with CAR, TYM or TQ against MDR-NTB isolates (Table 1) from diseased ornamental fish (Puk and Guz 2020). Previous studies have shown that CAR, TYM and TQ have antimycobacterial activity.

Jankowski et al. (2024) reported that TQ exhibited a potent growth-inhibitory effect against *M. tuberculosis*. The antimycobacterial activity of TQ is linked to the depletion of pools of NAD (Jankowski et al. 2024) and ATP (Wang et al. 2021, Jankowski et al. 2024) and the downregulation of plasma membrane lipids (Fan et al. 2021, Jankowski et al. 2024). Results presented by Mouwakeh et al. (2018) suggest that TQ acts as an efflux pump inhibitor in *Listeria*, modulating the resistance of the bacteria to antibiotics. Our present results showed that TQ exhibited an antibacterial effect against MDR-NTM isolated from diseased fish, with MIC values ranging from 32 to 64 μg/mL. Synergistic action of BER and TQ on MDR-NTB isolates was demonstrated (Table 2).

Many studies have demonstrated effective activity of carvacrol against mycobacterial isolates (Al-Ani et al. 2015, Nakamura de Vasconcelos et al. 2018, Marini et al. 2019, Fermiano et al. 2024). *In silico* approaches (Fermiano et al. 2024) showed that CAR targets the mycobacterial enzyme chorismate mutase and heat shock protein 16.3 (sHSP 16.3 Rv2031c). In a study by Nakamura de Vasconcelos et al. (2018), CAR showed good anti-*M. tuberculosis* activity and synergism with rifampicin. High antimycobacterial activity of CAR has been also described against *M. tuberculosis* and *M. bovis* by Andrade-Ochoa et al. (2015). In our study, CAR exhibited an antibacterial effect against MDR-NTM isolates, with MIC values ranging from 64 to 128 μg/mL. Synergistic action of BER and CAR against MDR-NTM isolates was also demonstrated (Table 2).

High antimycobacterial activity of TYM, a positional isomer of CAR, has been described against *M. tuberculosis* and *M. bovis* (Andrade-Ochoa et al. 2015). The TYM structure disintegrates the external membrane of G(-) bacteria, releasing lipopolysaccharide and increasing the permeability of the cytoplasmic membrane to ATP (Helander et al. 1998). Dos Santos Barbosa et al. (2021) suggested that TYM acts as

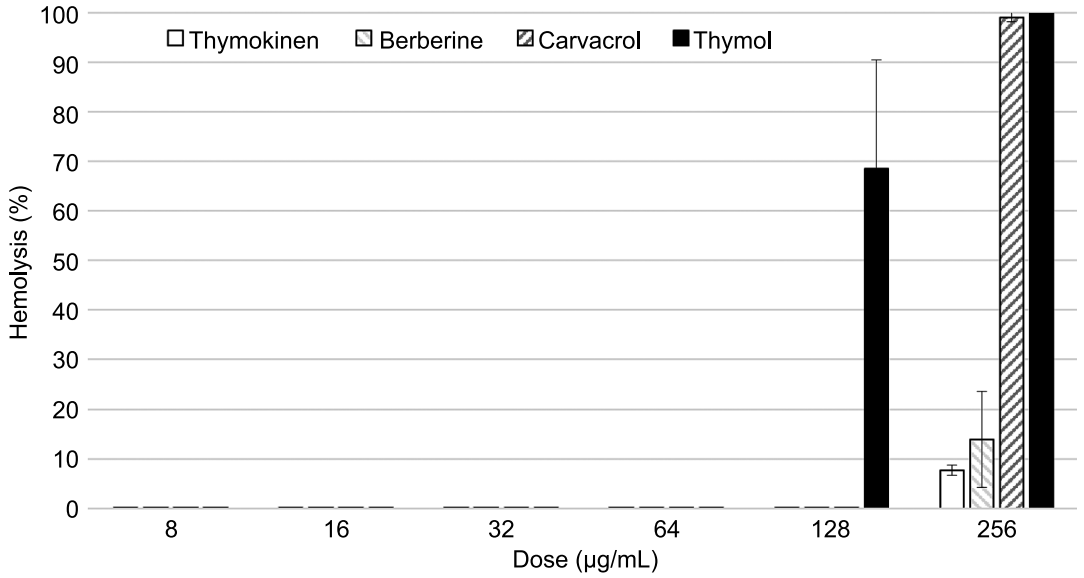

Fig. 1. Haemolytic activity of berberine, carvacrol, thymol, and thymoquinone against carp erythrocytes. Data are represented as mean ± standard deviation (n=3).

a competitive inhibitor of NorA (MFS family efflux pumps, expressed by *Staphylococcus aureus*). In our study, TYM exhibited an antibacterial effect against the MDR-NTM isolates, with MIC values ranging from 64 to 128 µg/mL. Synergistic action of BER and TYM against MDR-NTM isolates was also demonstrated (Table 2).

The haemolytic effects induced by the different concentrations of BER, CAR, TYM, and TQ indicated that all tested compounds exhibited very week haemolytic activity in a dose dependent manner. At doses ranging from 8 to 64 µg/mL, no haemolysis was observed in any of the tested compounds (Fig. 1).

In conclusion, we have shown for the first time the synergistic efficacy of BER with CAR, TYM or TQ against NTM strains. Thus, the combination of these compounds with BER seems to be a new approach for combating MDR-NTM strains. However, further studies through *in vivo* experiments should be considered in the future in order to best justify its safety and therapeutic efficacy.

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