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Short communication

Combined antimicrobial action of streptomycin and terpenes against atypical mycobacteria isolated from fish

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Abstract

The aim of this study was to investigate the antimycobacterial activity of 39 free terpenes and their activity in combination with streptomycin. Antimicrobial activity was first evaluated by screening 39 free terpenes at concentrations from 1.56 to 400 µg/mL. None of these exhibited positive effects against any of the nontuberculous mycobacteria (NTM) strains tested. However, six of the 39 terpenes (isoeugenol, nerol, (+)-α-terpineol, (1R)-(−)-myrtenol, (+)-terpinen-4-ol, and eugenol) were shown to enhance the activity of streptomycin against the NTM strains isolated from diseased ornamental fish.

Keywords: antibacterial, atypical *Mycobacterium* sp., terpenes

Introduction

Fish mycobacteriosis, which is one of the most common bacterial diseases, is caused by various atypical mycobacteria (nontuberculous mycobacteria – NTM), a ubiquitous group of environmental organisms that have the potential to cause diseases in humans (Szmygin-Milanowska et al. 2016) and animals, including over 150 species of fish (Decostere et al. 2004, Guz et al. 2013, Puk et al. 2018, Puk and Guz 2020). *Mycobacterium marinum*, *M. chelonae*, *M. gordonae*, *M. peregrinum* and *M. fortuitum* are the predominant mycobacterial species in infected fish (Puk and Guz 2020). A number of plant extracts (Puk et al. 2023) and plant secondary metabolites, such as alkaloids (Puk and Guz 2022) and terpenoids (Sieniawska et al. 2017, 2018), have been described as important agents exhibiting antimycobacterial activity (Cantrell et al. 2001). In recent years terpenoids have been shown to play an increasingly important role in antimycobacterial activity (Cantrell et al. 2001). Results presented in reviews by Mahizan et al. (2019) and Dias et al.

(2022) suggest that terpenes are significant efflux pump inhibitors and can be used in drug development targeting antibacterial resistance. According to the literature, farnesol (Jin et al. 2010), uvaol, β-amyrin, oleanolic acid (Martins et al. 2011), and carvacrol (Vasconcelos et al. 2018) decreased the MIC of ethidium bromide and antibiotics and increased the accumulation of ethidium bromide in *Mycobacterium* sp.

Plant secondary metabolites are considered potential agents that can be used alone or in combination with commonly used antimycobacterial drugs to avert toxicity and reduce the ineffectiveness of current antimycobacterial drugs (Sieniawska et al. 2018, Kumar et al. 2021, Puk and Guz 2022). The aim of the present study was to investigate the antimycobacterial activity of 39 terpenes, applied alone and in combination with streptomycin, a first-line tuberculostatic aminoglycoside antibiotic which inhibits protein synthesis by binding to the 16S rRNA of the bacterial 30S ribosome subunit.

Materials and Methods

Resazurin, streptomycin, and 39 terpenoids were purchased from Sigma-Aldrich (St Louis, MO, USA). The terpenoids included monoterpenes, i.e. (+)-aromadendrene, (−)-borneol, (−)-bornyl acetate, (+)-camphene, (-)-camphene, (1R)-(+)-camphor, (R)-(−)-carvone, (+)-1,4-cineole, (S)-(−)-citronellal, (+)-β-citronellene, (S)-(−)-β-citronellol, eucalyptol, eugenol, (+)-fenchone, (1R)-(−)-fenchone, isoborneol, isoeugenol, (1R)-(−)-menthyl acetate, methyl jasmonate, (R)-(+)-limonene, (1R,2S,5R)-(−)-menthol, (1S,2R,5S)-(+)-menthol, (−)-menthone, (1R)-(−)-myrtenol, nerol, nerolidol, (1R)-(−)-nopol, (+)-β-pinene, α-pinene, (R)-(+)-pulegone, (+)-terpinen-4-ol, (+)-α-terpineol, and (−)-terpinen-4-ol, as well as sesquiterpenes: (−)-α-bisabolol, (−)-trans-caryophyllene, (+)-cedrol, α-ionone, β-ionone, and nerolidol.

Eleven fish pathogenic bacteria were used in this study: one *Mycobacterium abscessus* strain (M11), five *Mycobacterium chelonae* strains (M2, M12, M34, M57 and M102), three *Mycobacterium fortuitum* strains (M7, M17 and M88), and two *Mycobacterium marinum* strains (M4 and M72). *Mycobacterium* species were isolated from diseased ornamental fish.

The minimum inhibitory concentrations (MIC) of the terpenoids and streptomycin (in the presence and absence of terpenoids) for each strain were determined using the resazurin microtiter assay (REMA), as previously described by Guz and Puk (2022). A growth control containing terpene without antibiotics and a sterility control without inoculum were included in each plate. A fourfold or higher reduction in MIC was considered an indication of a significant synergistic effect between the antibiotic and the terpene.

In vitro haemolytic activity was assessed by spectrophotometry (Kumar et al. 2011). Briefly, a 0.5 mL volume of common carp (*Cyprinus carpio*) erythrocyte suspension was mixed with 0.5 mL of the terpenes (128, 64 and 32 μg/mL concentration in phosphate buffer saline). The mixtures were incubated for 30 min at 30°C and centrifuged at 180 x g for 10 min. The free haemoglobin in the supernatant was measured in a spectrophotometer at 540 nm. Phosphate buffer saline and distilled water were used as minimal and maximal haemolytic controls. The percentage of haemolysis by the terpenes was calculated according to the formula H(%) = (At – An / Ac – An) x 100, where At – absorbance of test sample, An – absorbance of the saline control, Ac – absorbance of the water control. Blood for testing was sampled once (fish were anesthetized with tricaine methanesulfonate (MS-222) at 100 mg/mL in a water bath), and the fish were then euthanized with MS-222 at 200 mg/mL in a water bath. For this reason, the approval of the Ethics Committee was not required.

Results and Discussion

We tested the antimycobacterial activity of 39 terpenes against 11 mycobacterial strains (*M. abscessus*, n=1; *M. chelonae*, n=5; *M. fortuitum*, n=3; and *M. marinum*, n=2) isolated from diseased fish. MICs were not determined for any of the free terpenes, as they were above the tested range (MIC > 400 μg/mL) (data not shown). Streptomycin inhibited strains M4 (at 1 μg/mL), M72 (at 0.5 μg/mL), M7, M17, M88 (32 μg/mL), M2, M11, M12, M34, M57, and M102 (at > 128 μg/mL). When streptomycin was tested in the presence of terpenes, isoeugenol, nerol, (+)-α-terpineol, (1R)-(−)-myrtenol, (+)-terpinen-4-ol, and eugenol influenced the activity of the antibiotic against all strains (Table 1). The *in vitro* cytotoxicity determination for six active terpenes showed a dose-dependent increase in haemolysis against the common carp red blood cells. The results of the study indicate that at lower concentrations, terpineol, terpinen-4-ol, eugenol, and isoeugenol are nontoxic or less toxic to carp erythrocytes.

Recent reports have shown that efflux pumps can decrease intracellular drug concentrations (De Rosi et al. 2006, Gupta et al. 2006). Bacterial efflux pump inhibitors increase the intrinsic resistance of bacteria to antibiotics (Marquez 2005). A broad range of plant terpenoids of various classes have been evaluated for their *in vitro* antimycobacterial activity (Cantrell et al. 2001, Sieniawska et al. 2017, 2018). In this experiment, the 39 terpenes were not active against NTM (i.e. *M. abscessus*, *M. chelonae*, *M. fortuitum*, and

Table 1. Streptomycin minimum inhibitory concentrations (MIC) in presence of different concentrations of terpenoids (μg/mL).

Compounds	T.C. mg/mL	M. m. M4	M. m. M72	M. f. M7	M. f. M17	M. f. M88	M. a. M11	M. ch. M2	M. ch. M12	M. ch. M34	M. ch. M57	M. ch. M102
Streptomycin	-	1	0.5	32	32	32	>128	>128	>128	>128	>128	>128
Streptomycin and isoeugenol	32	0.5	0.25	16	16	16	64	64	64	64	64	64
	64	0.062	0.031	0.062	0.5	0.031	0.125	0.062	0.062	0.062	0.062	0.062
	128	0.062	0.031	0.062	0.5	0.031	0.125	0.062	0.062	0.062	0.062	0.062
Streptomycin and nerol	32	0.5	0.5	16	16	32	>128	>128	>128	>128	>128	>128
	64	0.5	0.5	16	16	16	>128	>128	>128	>128	>128	>128
	128	0.5	0.25	8	8	8	64	64	32	64	64	32
Streptomycin and (+)-α-terpineol	32	1	0.5	32	32	32	>128	>128	>128	>128	>128	>128
	64	0.5	0.25	16	16	16	64	64	64	64	64	64
	128	0.5	0.25	8	16	8	32	32	32	32	32	32
Streptomycin and (1R)-(−)-myrtenol	32	1	0.25	16	16	32	32	>128	>128	>128	>128	>128
	64	0.25	0.25	4	16	16	16	>128	>128	>128	>128	>128
	128	0.25	0.25	4	8	4	16	64	64	64	64	64
Streptomycin and (+)-terpinen-4-ol	32	1	0.5	32	32	32	>128	>128	>128	>128	>128	>128
	64	0.5	0.5	32	32	32	>128	>128	>128	>128	>128	>128
	128	0.25	0.25	4	8	8	64	128	128	128	128	128
Streptomycin and eugenol	32	1	0.25	16	32	32	>128	>128	>128	>128	>128	>128
	64	0.5	0.25	4	16	4	>128	>128	>128	>128	>128	>128
	128	0.5	0.25	4	8	4	64	64	32	64	64	32
Streptomycin and 1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*, 24*, 25*, 26*, 27*, 28*, 29*, 30*, 31* or 32*	32	1	0.5	32	32	32	>128	>128	>128	>128	>128	>128
	64	1	0.5	32	32	32	>128	>128	>128	>128	>128	>128
	128	1	0.5	32	32	32	>128	>128	>128	>128	>128	>128

TC, concentrations of terpenoids; M. m., *M. marinum*; M. f., *M. fortuitum*; M. a., *M. abscessus*; M. ch., *M. chelonae*; 1*, (−)-α-bisabolol; 2*, (-)-camphene; 3*, (1R)-(−)-nopol; 4*, (+)-camphene; 5*, β-ionone; 6*, isoborneol; 7*, eucalyptol; 8*, (1R)-(−)-fenchone; 9*, (R)-(+)-limonene; 10*, α-ionone; 11*, (−)-trans-caryophyllene; 12*, (1R,2S,5R)-(−)-menthol; 13*, (1R)-(+)-camphor; 14*, (+)-cedrol; 15*, (+)-aromadendrene; 16*, nerolidol; 17*, (−)-terpinen-4-ol; 18*, (−)-menthone; 19*, (1R)-(−)-menthyl acetate; 20*, (1S,2R,5S)-(+)-menthol; 21*, (−)-borneol; 22*, (R)-(−)-carvone; 23*, (−)-bornyl acetate; 24*, methyl jasmonate; 25*, (S)-(−)-citronellal; 26*, (+)-α-pinene; 27*, 1,4-cineole; 28*, (+)-β-citronellene; 29*, (R)-(+)-pulegone; 30*, (+)-β-pinene; 31*, (S)-(−)-β-citronellol; 32*, (+)-fenchone

Table 2. Haemolytic activity of terpenes against carp erythrocytes.

Concentration (μg/mL)	Percentage of hemolysis (average ± SD)					
	Terpineol	Terpinen-4-ol	Myrtenol	Eugenol	Isoeugenol	Nerol
128	43.33±1.53	99.67±2.52	94.00±2.65	107.67±0.58	96.83±1.73	54.81±0.64
64	2.50±0.41	5.41±1.16	46.20±2.46	37.83±1.70	16.47±0.60	34.17±0.42
32	0.53±0.47	3.98±1.48	0.64±0.56	1.37±1.00	10.20±0.30	16.73±1.45
16	0.30±0.52	0.74±0.72	0.32±0.30	1.03±0.90	0.09±0.16	7.17±1.47

M. marinum) isolated from diseased fish, but six of them increased the antimycobacterial activity of streptomycin by inhibiting the mechanisms of mycobacterial resistance (Table 1).

It has been documented that terpenes can enhance the activity of antibiotics by inhibiting the activity of efflux pumps in resistant bacteria (Barbosa et al. 2021). Coêlho et al. (2016) and Muniz et al. (2021) reported that eugenol and its derivatives enhanced the effectiveness of norfloxacin and reduced the MIC against

NorA-expressing *Staphylococcus aureus*. A study by Sieniawska et al. (2017) revealed that S-limonene had a strong synergistic effect with all tested first-line tuberculostatic drugs, i.e. isoniazid, rifampicin and etambutol. Moreover, the combination of myrcene, R-limonene, β-elemene, and sabinene with tuberculostatic antibiotics resulted in a decrease in the MIC of the antibiotics. The results show that terpenes act as possible efflux pump inhibitors and can be investigated as adjuvants in combined therapies aimed at reducing antibiotic resistance.

Conclusions. Our study showed that among 39 terpenes tested, six of them, i.e. isoeugenol, nerol, (+)-α-terpineol, (1R)-(−)-myrtenol, (+)-terpinen-4-ol and eugenol, enhanced the activity of streptomycin against the tested NTM strains.

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