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Antimycobacterial activity of ferutinin alone and in combination with antitubercular drugs against a rapidly growing surrogate of *Mycobacterium tuberculosis*

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The aim of this study was to investigate the antimycobacterial activity of the major daucane constituent, ferutinin (jaeschkeandiol *p*-hydroxybenzoate, **1**), four of its natural analogues, its hydrolysis products, as well as methyl *p*-hydroxybenzoate (methylparaben) against *Mycobacterium smegmatis*, a rapidly growing surrogate of *Mycobacterium tuberculosis*. The agar dilution assay was utilised for an antimycobacterial evaluation of single compounds. A modified agar dilution assay, the checkerboard method, was utilised for evaluating the potentiating effect of **1** on different antitubercular drugs, namely isoniazid, ethionamide, rifampin and streptomycin. In the agar dilution assay, **1** exhibited higher potency (minimum inhibitory concentration [MIC] $10\,\mu g\,mL^{-1}$) than streptomycin and rifampin (MIC $20\,\mu g\,mL^{-1}$ for each). Of the natural analogues, 8,9-epoxyjaeschkeandiol *p*-hydroxybenzoate and 8,9-epoxyjaeschkeandiol benzoate exhibited marginal activity (MIC ≥ 40 and $80\,\mu g\,mL^{-1}$, respectively). The checkerboard method showed that the combination of **1** with each antitubercular drug led to mutual enhancement of the antimycobacterial activity with isoniazid and ethionamide, while no such effect was observed with rifampin or streptomycin. Based on this study and earlier studies with *Staphylococcus aureus*, the major constituent **1** may be responsible for the major part of the antimicrobial activity of the root of *Ferula hermonis*.

Keywords: Shirsh El Zallouh; *Ferula hermonis*; ferutinin; antimycobacterial; rapidly growing mycobacteria; potentiation; antitubercular drugs

1. Introduction

The resistance of pathogenic microorganisms to antimicrobial drugs has increased worldwide in the past two decades. This problem is specifically associated with the antibiotics used for the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* and fungal infections. The resistance to antimicrobial agents necessitates increasing the dose of antibiotics to high levels to attain therapeutic effect. The latter practice may result in the

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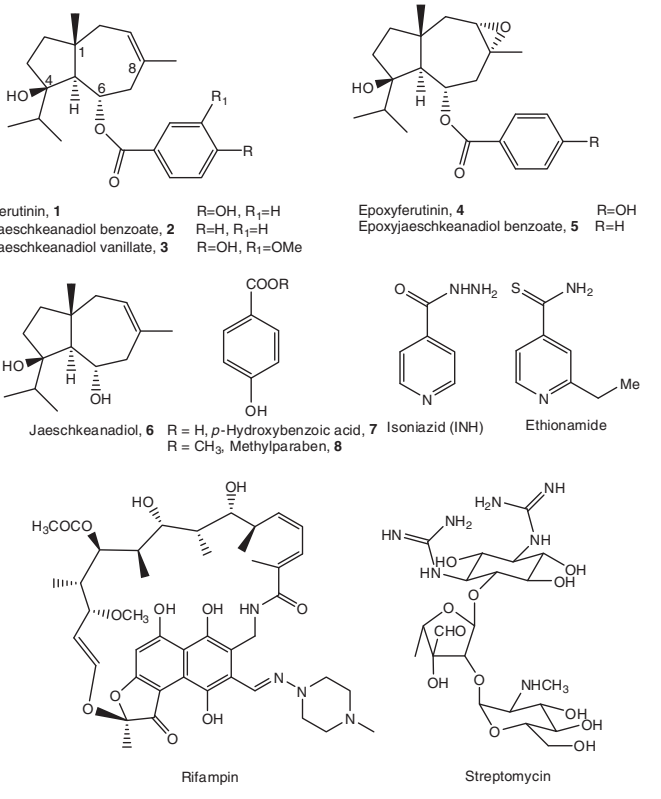
increased incidence of adverse effects and/or toxicity (Plorde, 1994). Excessive use of antibiotics is also becoming a major public health concern due to the additional costs of prescriptions, hospitalisation and increased rates of morbidity and mortality from infections. To illustrate, community-acquired MRSA infections and the numbers of MRSA isolated from blood and cerebrospinal fluid (CSF) samples have significantly risen over the last decade (Dirk, 2007; Nakamura et al., 2008). Similarly, the number of infections with vancomycin-resistant *Enterococci*, which were rare up until 1992, has also increased. Most of the reasons for this increase are accounted for by *Enterococcus faecium*, which had a 30% prevalence of resistance in 2000 (Mascini & Bonten, 2005).

A fairly large number of plant natural products have been reported to exhibit antimicrobial activity of experimental promise rather than clinical potential. Antibiotic potentiation through synergistic interaction with natural products has been investigated as an approach towards the minimisation of side effects of, and resistance to, antibiotics (Didry, Dubreuil, & Pinkas, 1993; Gibbons, Oluwatuyi, Veitch, & Gray, 2003; Kubo, Muroi, & Himejima, 1993; Park, Kim, & Hahm, 2004; Periago, Palop, & Fernandez, 2001; Shin & Kang, 2003), especially when the natural products are not sufficiently potent as single entities. Recently, peptides derived from a *Helicobacter pylori* protein resulted in a four-fold reduction in the minimum bactericidal concentrations (MBCs) of chloramphenicol against selected Gram-positive and Gram-negative bacteria (Park et al., 2004). Diterpenes from *Lycopus europaeus* had no antibacterial activity against the resistant strains of *S. aureus*, but resulted in a two-fold potentiation of the activities of tetracycline and erythromycin against the same staphylococcal strains (Gibbons et al., 2003).

Systemic infections caused by rapidly growing mycobacteria (RGMs), *Mycobacterium avium* complex (MAC) and fungi, such as *Cryptococcus neoformans* and *Candida albicans*, are serious conditions occurring primarily in immunocompromised neutropenic patients, especially those with AIDS (Iseman, 1999). For such conditions, drug combinations that are periodically altered are routinely used in an attempt to achieve maximum efficacy while reducing the side effects and slowing the rate of the development of resistance (Rex, Sobel, & Powderly, 1999). Isonicotinic acid hydrazide (isoniazid, INH), streptomycin and isoniazrifampinid arefrontline drugs used in the combined treatment of *M. tuberculosis* (with MICs 0.01–0.25 $\mu g\,mL^{-1}$). However, the first two are not very effective against other mycobacteria (MICs $> 1\,\mu g\,mL^{-1}$; Lemke, 2002). Two more recent reports (Abourashed, Galal, Shibl, & Mossa, 2007; Mossa, El-Feraly, & Muhammad, 2004) showed that the constituents from *Juniperus procera*, *Ferula communis*, *Plumbago zeylanica*, *Syzygium aromaticum* and *Aframomum melegueta* exhibited an *in vitro* synergistic activity with INH against four atypical mycobacteria from the RGM group and against *M. tuberculosis* (TB).

The root of *Ferula hermonis* Boiss (family Apiaceae), known in Syria, Lebanon, and in the Middle East as 'Shirsh El Zallouh', has been used in local traditional medicine as an aphrodisiac for male erectile dysfunction and as a general tonic. Zallouh is also believed to possess antimicrobial and antioxidant activities (Abourashed, Galal, El-Feraly, & Khan, 2001; Fitzsimmons, 2009; Hilan et al., 2007). Ferutinin (**1**) is a major daucane sesquiterpene ester, isolated from *F. hermonis* and other species of *Ferula* (Abourashed et al., 2001; Galal, 2000). Ferutinin exhibited antimicrobial activity against *S. aureus* (MIC 3.5 $\mu g\,mL^{-1}$) and a weak

antifungal effect against *Aspergillus niger* (Galal et al., 2001). Other natural analogues of **1** include jaeschkeandiol benzoate (**2**), jaeschkeandiol vanillate (**3**), epoxyferutinin (**4**), epoxyjaeschkeandiol benzoate (**5**), and others (Diab, Dolmazon, & Bessiere, 2001; Diab, Dolmazon, & Fenet, 2001; Lhuillier et al., 2005). Since none of **1** or its natural analogues (**2**–**5**) have been tested against *Mycobacteria*, the purpose of this work was to investigate the antimycobacterial effect of compounds **1**–**5**, the hydrolysis products of **1** (**6**, jaeschkeandiol; **7**, *p*-hydroxybenzoic acid), as well as methyl *p*-hydroxybenzoate (**8**, methylparaben) in an *in vitro* assay utilising *Mycobacterium smegmatis* as a representative of RGMs and as a surrogate microorganism to TB. The potentiating and synergistic effects of **1** on a number of antitubercular drugs were also investigated in the same microorganism, and the results are presented in this article.



2. Results

2.1. *Antimycobacterial activity of individual compounds*

In the agar dilution technique, **1** was the most active antimycobacterial (against *M. smegmatis*) of all the tested compounds, with an MIC of $10\,\mu g\,mL^{-1}$. The MIC of **1** was even lower than those of streptomycin and rifampin (MICs $20\,\mu g\,mL^{-1}$ for each). All other compounds were either inactive or marginally active, such as **4** and **5**

Table 1. Checkerboard charts[a] and FIC indices for ferutinin in combination with (A) isoniazid and (B) ethionamide.

		Ferutinin ($\mu g\,mL^{-1}$)			
		5	2.5	1.25	0.625
Panel A: isoniazid (FIC index = 0.250)					
Isoniazid ($\mu g\,mL^{-1}$)	10	+	+	+	−
	5	+	+	+	−
	2.5	+	+	+	−
	1.25	−	−	−	−
Panel B: ethionamide (FIC index = 0.125)					
Ethionamide ($\mu g\,mL^{-1}$)	10	+	+	+	+
	5	+	+	+	−
	2.5	+	+	−	−
	1.25	+	−	−	−

Note: [a]Highest concentration of each tested compound = ½MIC.

(MICs $\geq$ 40 and 80 $\mu g\,mL^{-1}$, respectively). The hydrolysis products of **1** were also inactive.

2.2. *Potentiation of antimycobacterial activity*

Based on its significant antimycobacterial activity, **1** was selected for the potentiation assays in combination with each of the known antitubercular agents: streptomycin, rifampin, ethionamide and isoniazid. Strong antimycobacterial synergism (FIC index $<$ 0.50) was exhibited between **1** and both ethionamide and isoniazid (Table 1). At 1.25 $\mu g\,mL^{-1}$ (1/8 MIC), **1** increased the antimycobacterial activity of isoniazid eight-fold, with an FIC index of 0.25 (Panel A). Similarly, at 5 $\mu g\,mL^{-1}$ (1/2 MIC), **1** increased the antimycobacterial activity of ethionamide 16-fold, with an FIC index of 0.125 (Panel B). No potentiation was observed between **1** and either streptomycin or rifampin.

3. Discussion

Comparison of the antimycobacterial effect of the natural esters and the hydrolysis products of **1** showed that the *p*-hydroxybenzoyl moiety was a prerequisite for activity and that **1** was the most active of the natural esters. Thus, **1** was chosen for further evaluation as a potentiating agent. It is interesting to note that *p*-hydroxybenzoic acid, which is commonly used as a starting material for preparation of cosmetic preservatives (parabens), in addition to its methyl ester (methylparaben), did not display any antimycobacterial activity against *M. smegmatis*. Also, the high MIC values observed for rifampin and streptomycin may be attributed to the reported natural resistance of *M. smegmatis* towards rifampin, which may also be applicable to streptomycin (Hetherington, Watson, & Patrick, 1995; Quan, Venter, & Dabbs, 1997).

The synergistic effect between **1** and both isoniazid and ethionamide, with FIC indices of 0.25 and 0.125, respectively, correlates with our previously reported synergism between paradol, a natural phenolic ketone, and isoniazid (Abourashed et al., 2007; Galal, 1996). The observed potentiation may be due to an enhancing effect on the permeability of the mycobacterial cell membrane to antimycobacterial drugs (Fitzgerlad et al., 2004). Since isoniazid and ethionamide are chemically analogous, having a pyridine ring and an amide (acetamide and thionamide in isoniazid and ethinoamide, respectively), it is rational to expect a similar trend in their interaction with **1**. The lack of synergism exhibited by ferutinin with streptomycin, an aminocyclitol glycoside, and isoniazrifampinid, an ansamacrolide, may be due to their relative bulkiness and structural dissimilarity as compared to isoniazid and ethionamide. Thus, synergism based on enhanced permeability may not be operational in this case.

The sporadic reports on the use of zallouh root as a traditional remedy for certain infections in humans and animals may have some validity in view of our current results with ferutinin, which is one of the major constituents of the root. The antimicrobial indication of zallouh is also supported by earlier reports regarding the activity of ferutinin against Gram-positive bacteria (Galal, 2000). The potentiating and synergistic effects exhibited by ferutinin are a possible approach with which to improve the safety and/or antimycobacterial efficacy of isoniazid and ethionamide, as well as for reducing the development of microbial resistance to these drugs. Further evaluation of ferutinin, and other small molecules, against an extended spectrum of microorganisms and in appropriate animal models should be the subject of further research.

4. Experimental

4.1. *Plant material and chemicals*

Roots of *F. hermonis* were purchased from the local herbal market in Syria in 2000 and were identified by Dr Sultan-ul-Abdeen, College of Pharmacy, King Saud University, Saudi Arabia. A voucher specimen was deposited at the herbarium of the college. Authenticity of the sample was later verified by TLC and HPLC fingerprinting of the total methanolic extract of the powder (Abourashed et al., 2001). The antitubercular drugs and *m*-chloroperbenzoic acid (MCPBA) were purchased from Fluka AG (Buchs SG, Switzerland) and Sigma–Aldrich (St. Louis, MO), respectively. Methylparaben was purchased from Sigma–Aldrich. Ferutinin and the natural derivatives were isolated from the *n*-hexane extract of *F. hermonis* roots.

4.2. *Extraction and isolation*

Isolation of the tested compounds followed a published procedure (Galal, 2000). In brief, powdered root of *F. hermonis* (50 g) was exhaustively extracted with hot *n*-hexanes, using a Soxhlet apparatus, and the extract was concentrated at 40°C under vacuum to an oily residue (4 g). This residue was partitioned between MeCN and *n*-hexanes. The MeCN fraction was subjected to silica gel column chromatography, eluted with mixtures of *n*-hexanes/EtOAc with increasing polarity, to isolate

the target constituents. The structures of the isolated compounds were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy methods. The purity of the compound was found to be more than 98% by HPLC.

4.3. *Hydrolysis of 1*

Compound **1** was hydrolysed by refluxing with 10% KOH in MeOH for 2 h. The liberated alcohol was back-extracted with ether followed by the removal of the solvent to obtain jaeschkeanadiol (**6**, 74% yield). The aqueous phase was acidified with 1N HCl and extracted with diethylether to afford *p*-hydroxybenzoic acid (**7**) after evaporation of the solvent. The identities of **6** and **7** were confirmed by comparing their NMR data with that in the literature.

4.4. *Preparation of 4 and 5*

The epoxy derivatives were prepared from the corresponding parent compounds by epoxidation at $\Delta^{8,9}$ using MCPBA, following a procedure given in the literature (Galal, 2000).

4.5. *Evaluation of the antimycobacterial activity*

The atypical mycobacteria, *M. smegmatis* (NCTC RR10), was obtained from the National Collection of Type Culture, Central Public Health Laboratory, London, UK. The agar serial dilution technique was utilised for determining the MIC values of the tested compounds against the selected microorganisms (Mitscher, Leu, Bathala, & Beal, 1972). Each compound was evaluated at different concentration levels (200–10 $\mu g\,mL^{-1}$) by mixing decreasing volumes of a DMSO stock solution of each compound with 10 mL of molten Mueller–Hinton agar (45–50°C) to obtain the final concentration. The inocula were prepared by growing the culture overnight in Mueller–Hinton broth to yield a viable count of 10^{9} cfu mL^{-1}. The culture was diluted 1 : 1000 in a pre-warmed broth to give a final density of 10^{4} cfu per spot. Test organisms were streaked in a radial pattern and then the plates were incubated at 35°C for 16–20 h. Complete suppression of growth was observed prior to declaring the compound to be active at the respective concentration.

The checkerboard method for evaluating synergistic antimycobacterial effect was employed to determine the MIC values of differently paired combinations of the tested compounds (Beale & Sutherland, 1983; Mackay, Milne, & Gould, 2000). A protocol similar to the one described for MIC determination was adopted with the difference that serial two-fold dilutions of each compound were tested in combination with two-fold dilutions of another compound from the selected group. Each evaluation was run in triplicate. The fractional inhibitory concentration (FIC) indices were calculated from the following formula (Beale & Sutherland, 1983):

$$\text{FIC index} = (A/MIC_A) + (B/MIC_B) = FIC_A + FIC_B,$$

where A is the lowest inhibitory concentration of the first component of a binary mixture in its row, MIC_A is the MIC of the component alone, B is the lowest inhibitory concentration of the second component of the mixture in its row and

MIC_B is the MIC of the second component alone. Total synergism ($FIC \leq 0.5$), partial synergism ($0.5 < FIC \leq 0.75$) and additive effect ($0.75 < FIC \leq 2$) or antagonism ($FIC > 2$) are the possible interpretations based on FIC index values (Didry et al., 1993).

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References

Abourashed, E.A., Galal, A.M., El-Feraly, F.S., & Khan, I.A. (2001). Separation and quantitation of the major daucane esters of *Ferula hermonis* by HPLC. *Planta Medica, 67*, 681–682.

Abourashed, E.A., Galal, A.M., Shibl, A.M., & Mossa, J.S. (2007). Enhancing effect of isoeugenol on the antimicrobial activity of isoniazid, 6-paradol and 6-shogaol. *Journal of Herbs, Spices and Medicinal Plants, 13*(4), 92–100.

Beale, A.S., & Sutherland, R. (1983). Measurement of combined antibiotic action. In A.D. Russell & L.B. Quensel (Eds.), *Antibiotics: Assessment of antimicrobial activity and resistance* (pp. 299–315). London: Academic Press.

Diab, Y., Dolmazon, R., & Bessiere, J.-M. (2001). Daucane aryl esters composition from the Lebanese *Ferula hermonis* Boiss. (zalloh root). *Flavour and Fragrance Journal, 16*(2), 120–122.

Diab, Y., Dolmazon, R., & Fenet, B. (2001). 2,3-α-Epoxyjaeschkeandiol 5-benzoate from *Ferula hermonis* Boiss. *Flavour and Fragrance Journal, 16*(6), 397–400.

Didry, N., Dubreuil, L., & Pinkas, M. (1993). Microbiological properties of protoanemonin isolated from *Ranunculus bulbosus*. *Phytotherapy Research, 7*, 21–24.

Dirk, E. (2007). Community-acquired methicillin-resistant *Staphylococcus aureus*. *Journal of the American Academy of Dermatology, 56*(1), 1–16.

Fitzgerlad, D.J., Stratford, M., Gasson, M.J., Ueckert, J., Bos, A., & Narbad, A. (2004). Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. *Journal of Applied Microbiology, 97*(1), 104–113.

Fitzsimmons, S. (2009). *Ferula hermonis* – the Lebanese viagra. Retrieved November 19, 2009, from http://www.gcwhite.co.uk/Ferula.htm.

Galal, A.M. (1996). Antimicrobial activity of 6-paradol and related compounds. *International Journal of Pharmaconosy, 34*(1), 64–69.

Galal, A.M. (2000). Sesquiterpenes from *Ferula hermonis* Boiss. *Pharmazie, 55*, 961–962.

Galal, A.M., Abourashed, E.A., Ross, S.A., ElSohly, M.A., Al-Said, M.A., & ElFeraly, F.S. (2001). Daucane sesquiterpenes from *Ferula hermonis*. *Journal of Natural Products, 64*, 399–400.

Gibbons, S., Oluwatuyi, M., Veitch, N.C., & Gray, A.I. (2003). Bacterial resistance modifying agents from *Lycopus europaeus*. *Phytochemistry, 62*, 83–87.

Hetherington, S.V., Watson, A.S., & Patrick, C.C. (1995). Sequence and analysis of the rpoB gene of *Mycobacterium smegmatis*. *Antimicrobial Agents and Chemotherapy, 39*(9), 2164–2166.

Hilan, C., Sfeir, R., El Hage, R., Jawich, D., Frem, M.E., & Jawhar, K. (2007). Evaluation of the antibacterial activities of *Ferula hermonis* (Boiss.). *Lebanese Science Journal, 8*(2), 135–151.

Iseman, M.D. (1999). Rapid-growing mycobateria. In V.L. Yu, T.C. Merigan, & S.L. Barriere (Eds.), *Antimicrobial therapy and vaccines* (pp. 494–497). Baltimore: Williams & Wilkins.

Kubo, I., Muroi, H., & Himejima, M. (1993). Combination effects of antifungal nagilactones against *Candida albicans* and two other fungi with phenylpropanoids. *Journal of Natural Products, 56*(2), 220–226.

Lemke, T.L. (2002). Antimycobacterial agents. In D.A. Williams & T.L. Lemke (Eds.), *Foye's principles of medicinal chemistry* (pp. 904–920). Philadelphia: Lippincott Williams & Wilkins.

Lhuillier, A., Fabre, N., Cheble, E., Oueida, F., Maurel, S., Valentin, A., et al. (2005). Daucane sesquiterpenes from *Ferula hermonis*. *Journal of Natural Products, 68*(3), 468–471.

Mackay, M.L., Milne, K., & Gould, I.M. (2000). Comparison of methods for assessing synergic antibiotic interactions. *International Journal of Antimicrobial Agents, 15*, 125–129.

Mascini, E.M., & Bonten, M.J.M. (2005). Vancomycin-resistant enterococci: consequences for therapy and infection control. *Clinical Microbiology and Infection, 11*(Suppl. 4), 43–56.

Mitscher, L.A., Leu, R., Bathala, M.S., & Beal, J.L. (1972). Antimicrobial agents from higher plants. Part 1: introduction, rationale and methodology. *Lloydia, 35*, 157–166.

Mossa, J.S., El-Feraly, F.S., & Muhammad, I. (2004). Antimycobacterial constituents from *Juniperus procera*, *Ferula communis* and *Plumbago zeylanica* and their *in vitro* synergistic activity with isonicotinic acid hydrazide. *Phytotherapy Research, 18*, 934–937.

Nakamura, Y., Yokoyama, I., Hashimoto, N., Hasegawa, A., Nakasa, H., Nakamura, H., et al. (2008). Appropriate use of anti-methicillin-resistant *Staphylococcus aureus* agents in a retrospective study. *Yakugaku Zasshi, 128*(7), 1073–1079.

Park, Y., Kim, H.J., & Hahm, K.-S. (2004). Antibacterial synergism of novel antibiotic peptides with chloramphenicol. *Biochemical and Biophysical Research Communications, 321*, 109–115.

Periago, P.M., Palop, A., & Fernandez, P.S. (2001). Combined effect of nisin, carvacrol and thymol on the viability of *Bacillus cereus* heat-treated vegetative cells. *Food Science and Technology International, 7*(6), 487–492.

Plorde, J.J. (1994). Antimicrobics and chemotherapy of bacterial and viral infections. In K.J. Ryan (Ed.), *Sherris medical microbiology* (pp. 189–219). Norwalk, CT: Appleton & Lange.

Quan, S., Venter, H., & Dabbs, E.R. (1997). Ribosylative inactivation of rifampin by *Mycobacterium smegmatis* is a principal contributor to its low susceptibility to this antibiotic. *Antimicrobial Agents and Chemotherapy, 41*(11), 2456–2460.

Rex, J.H., Sobel, J.D., & Powderly, W.G. (1999). *Candida* species. In V.L. Yu, T.C. Merigan, & S.L. Barriere (Eds.), *Antimicrobial therapy and vaccines* (pp. 1054–1076). Baltimore: Williams & Wilkins.

Shin, S., & Kang, C.-A. (2003). Antifungal activity of the essential oil of *Agastache rugosa* Kuntze and its synergism with ketoconazole. *Letters in Applied Microbiology, 36*(2), 111–115.