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Geraniol Restores Antibiotic Activities against Multidrug-Resistant Isolates from Gram-Negative Species▽†

Vannina Lorenzi,[1]§ Alain Muselli,[2] Antoine François Bernardini,[2] Liliane Berti,[3] Jean-Marie Pagès,[1] Leonard Amaral,[4] and Jean-Michel Bolla[1]*

Université de la Méditerranée, UMR-MD1, IFR88, Faculté de Médecine, 13385 Marseille Cedex 05, France[1]; Université de Corse, Equipe Chimie des Produits Naturels, UMR-CNRS 6134, Quartier Grossetti, BP 52, 20250 Corte, France[2]; Université de Corse, Laboratoire de Biochimie et de Biologie Moléculaire du Végétal, UMR-CNRS 6134, Quartier Grossetti, BP 52, 20250 Corte, France[3]; and Unit of Mycobacteriology, Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisbon, Portugal[4]

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The essential oil of *Helichrysum italicum* significantly reduces the multidrug resistance of *Enterobacter aerogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Combinations of the two most active fractions of the essential oil with each other or with phenylalanine arginine β-naphthylamide yield synergistic activity. Geraniol, a component of one fraction, significantly increased the efficacy of β-lactams, quinolones, and chloramphenicol.

The constant use of antibiotics in the hospital environment has selected bacterial populations that are resistant to many antibiotics. This multidrug resistance (MDR) results in part because of the activation of efflux pumps (8). The vast majority of efflux pump inhibitors (EPIs) identified so far are active against gram-positive bacteria, particularly *Staphylococcus aureus*. The very few EPIs that are active against gram-negative bacteria are toxic, and gram-negative bacteria such as *Pseudomonas*, *Acinetobacter*, *Escherichia*, and *Enterobacter* spp. are rapidly becoming the most problematic bacteria to treat due to the expression of MDR phenotypes and nosocomial status (1, 3, 4). There is a dire need to search for EPIs that are effective in rendering MDR gram-negative bacteria susceptible to antibiotics to which they are initially resistant (6, 7, 10, 12). The aim of this study was to discover EPIs in essential oils obtained from Corsican plants that are effective against efflux systems of gram-negative bacteria.

Among the 15 plant extracts tested, the essential oil derived from *Helichrysum italicum* when used at a concentration of 2.5% reduces the MIC of chloramphenicol from 1,024 to 128 mg/liter (eightfold) for the *Enterobacter aerogenes* MDR strain EA27 (8); the remainder of the plants are less active and reduce the MIC of chloramphenicol from between two- and fourfold (see the supplemental material). Due to the ability of the essential oil derived from *H. italicum* to reduce the chloramphenicol resistance of EA27 to a level that is close to that of the control phenylalanine arginine β-naphthylamide (PAβN) (5), it was selected for further study that would evaluate its ability to reduce the antibiotic resistance of other gram-negative bacteria. As demonstrated in Table 1, the essential oil derived from *H. italicum* is more effective than PAβN in reducing the resistance of *Acinetobacter baumannii* strains to chloramphenicol. In the case of the highly chloramphenicol-resistant *Pseudomonas aeruginosa* strains, although it reduces resistance to essentially the same degree as that produced in *A. baumannii*, it is not as effective as PAβN. The ability of the *H. italicum* essential oil to significantly reduce the chloramphenicol resistance of three very pathogenic MDR gram-negative bacteria (1, 12) supports the prediction of similar activities against other gram-negative pathogens.

Evaluation of potential EPIs is best conducted with bacteria that overexpress a specific efflux pump that renders the bacterium MDR. The ability of the *H. italicum* essential oil to reduce chloramphenicol resistance of EA27, a strain that overexpresses its AcrAB efflux pump (11), suggests that this essential oil contains an agent with the activity of an EPI. That this suggestion is correct is evident from the data summarized in Table 2. The essential oil not only reduces chloramphenicol resistance of the MDR *E. aerogenes* strain EAEP289 that overexpresses efflux pumps (11) but also reduces intrinsic chloramphenicol resistance of the wild-type controls *E. aerogenes* ATCC 13048 and *Escherichia coli* AG100. Moreover, a strong restoration was observed for *E. aerogenes* strain CM-64 that overproduced AcrAB (13) and also for the MDR *E. aerogenes* strain EAEP294 that has its *acrAB* operon deleted (11) but still has other active efflux pumps (2). In contrast to these effects, the *E. coli* mutant AG100A, which has its *acrAB* operon deleted (9), is not affected by the essential oil. Because the resistance of the *acrAB*-deleted progeny, which has been induced to high-level resistance to chloramphenicol, is due to the overexpression of the AcrEF system (13), the reduced resistance to chloramphenicol noted for the *acrAB* Tet^r mutant suggests that the essential oil is active against AcrEF. Regardless, from the data summarized in Table 2, it is clear that the essential oil derived from *H. italicum* contains one or more agents that have

* Corresponding author. Mailing address: UMR-MD1, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cedex 05, France. Phone: (33) 4 91 32 44 40. Fax: (33) 4 91 32 46 06. E-mail: Jean-Michel.Bolla@medecine.univ-mrs.fr.

§ Present address: Université de Corse, Laboratoire de Biochimie et de Biologie Moléculaire du Végétal, UMR-CNRS 6134, Quartier Grossetti, BP 52, 20250 Corte, France.

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TABLE 1. Effect of *Helichrysum italicum* essential oil on the susceptibilities of gram-negative species to chloramphenicol

Substance[a]	Chloramphenicol MIC (mg/liter) for indicated strain[b]					
	E. aerogenes		*A. baumannii*		*P. aeruginosa*	
	ATCC 13048	EA27	ATCC 19606	AB1	PAO1	PA124
None	8	1,024	32	32	512	128
PAβN	2	64	16	16	2	4
H. italicum essential oil	4	128	4	4	32	16

[a] *H. italicum* essential oil was used at a concentration of 2.5%, and PAβN was used at a concentration of 20 mg/liter.
[b] For a description of characteristics and origins of the strains, see Table S1 in the supplemental material.

TABLE 3. EPI activity of *H. italicum* fractions alone or in combination with each other or with the EPI PAβN against *E. aerogenes* strains

Compound or fraction[a]	Chloramphenicol MIC (mg/liter) for indicated *E. aerogenes* strain[b]	
	EAEP289	EAEP294M (*acrAB* mutant)
None	1,024	64
F2	512	32
F3	512	0.5
F2+F3	128	<0.25
PAβN	64	32
F2+PAβN	4	4
F3+PAβN	<0.25	<0.25
Geraniol	64	<0.25
Geraniol+PAβN	<0.25	<0.25

[a] Each compound or fraction was used at a concentration corresponding to the MIC/4. For a description of fractions, see Table S3 in the supplemental material.
[b] For a description of characteristics and origins of the strains, see Table S1 in the supplemental material.

EPI activity, and hence, an attempt to isolate that agent or agents was made (see the supplemental material).

To our knowledge, none of the compounds identified in the essential oil (see the supplemental material) have been previously evaluated for EPI activity. Therefore, the evaluation of the main fractions of the essential oil derived from *H. italicum* for EPI activity against the MDR EAEP289 strain was conducted, and Table 3 shows a summary of the results obtained. Briefly, whereas the hydrocarbon and F1 fractions are devoid of any EPI activity (i.e., reduction of chloramphenicol resistance) when used alone against the MDR EAEP289 strain (data not shown), fractions F2 and F3 produce twofold reductions of chloramphenicol resistance. However, when fractions F2 and F3 are combined, they reduce chloramphenicol resistance from an initial MIC of 1,024 to 128 mg/liter. Reduction of resistance can also be achieved with combinations of PAβN with either the F2 or F3 fraction, with combinations of the latter producing the greatest reduction that is comparable to a complete reversal of chloramphenicol resistance expected of a chloramphenicol hypersusceptible strain (i.e., a MIC of less than 0.25 mg/liter). Identical evaluation of each fraction alone or in combination with each other or with PAβN for EPI activity against the *acrAB* derivative EAEP294 strain demonstrated the very strong activity of fraction F3. We thus decided to perform chloramphenicol susceptibility testing in the presence of various compounds of F3 that were available (see the supplemental material). Among the compounds tested, geraniol produced significant restoration of susceptibility of the MDR strain EAEP289 to chloramphenicol by as much as 16-fold. When combined with PAβN, it rendered the organism fully susceptible to chloramphenicol, i.e., it completely reversed initial resistance (Table 3). Interestingly, geraniol restored the susceptibility of the *acrAB* derivative EAEP294 to the same extent as the combination of F3 and PAβN (Table 3). The other compounds identified in F3 did not significantly reduce the resistance to chloramphenicol (data not shown).

Chloramphenicol was used in this study to reveal efflux inhibition. However, this antibiotic is not currently used as therapeutic agent against gram-negative bacteria. β-Lactams and quinolones are actually mostly used, even though resistance to these agents is becoming a major concern (1). Herein we have assayed the efficacy of geraniol in restoring antibiotic susceptibility, or at least increasing the susceptibility of EAEP289 and EAEP294 to the β-lactams ampicillin and penicillin and to the fluoroquinolone norfloxacin. The results are summarized in Table 4). As shown, the MICs of these antibiotics are very high, especially for the two β-lactams. When geraniol was added, we were not able to observe a change in the susceptibility of strain EAEP289. However, for EAEP294, the AcrAB-deficient strain, a very strong increase in susceptibility was observed in the presence of geraniol and a β-lactam. This indicated that the geraniol had a synergistic effect with β-lactams. Moreover, the same results were obtained with the fluoroquinolone norfloxacin, thus demonstrating a pleiotropic effect of geraniol against the MDR phenotype in *E. aerogenes*. Taken together, these results suggest that PAβN and geraniol have an

TABLE 2. Effect of *H. italicum* essential oil on susceptibilities of *E. aerogenes* and *E. coli* efflux mutant strains to chloramphenicol

Substance[a]	Chloramphenicol MIC (mg/liter) for indicated strain[b]						
	E. aerogenes				*E. coli*		
	EAEP289	EAEP294	ATCC 13048	CM-64	AG100	AG100A	AG100A Tet^r
None	1,024	64	8	512	8	0.5	64
PAβN	64	32	2	16	2	0.5	2
H. italicum essential oil	128	0.5	4	64	2	1	0.25
H. italicum essential oil+PAβN	4	–	–	–	–	–	–

[a] *H. italicum* essential oil and PAβN were used as described in Table 1.
[b] For a description of characteristics and origins of the strains, see Table S1 in the supplemental material. –, not tested.

TABLE 4. EPI activity of geraniol on susceptibilities of two β-lactams and a quinolone

Strain	MIC (mg/liter) of indicated antibiotic[a]					
	Ampicillin		Penicillin		Norfloxacin	
	−	+	−	+	−	+
EAEP289	>1,024	>1,024	>1,024	>1,024	256	128
EAEP294	512	<0.07	>1,024	<0.07	64	<0.07

[a] +, Geraniol was added at a concentration corresponding to the MIC/4; −, no geraniol was added.

inhibitory effect on different mechanisms that are altogether involved in resistance.

In this study, we demonstrated that the essential oil from *Helichrysum italicum* contains compounds that modulate drug resistance in several gram-negative bacterial species by targeting efflux mechanisms. This conclusion is based on the following evidence. First, *H. italicum* decreases the chloramphenicol MIC for *E. aerogenes* isolates (Tables 1 and 2) in addition to *A. baumannii* and *P. aeruginosa* strains (Table 1). Second, *H. italicum* decreases the MIC of chloramphenicol for a strain that overproduced the tripartite efflux pump AcrAB-TolC (the *E. aerogenes* strain CM-64) (Table 2), and in addition it is able to restore susceptibility in a strain that overexpresses efflux pumps different from AcrAB, the *E. coli* strain AG100A Tet^r (Table 2). The two fractions (Table 3) that are the most active contain compounds that have not been previously described as modulators (see the supplemental material). Among these compounds, geraniol appeared to be a potent inhibitor of efflux mechanisms (Tables 3 and 4). Interestingly, the inhibition efficiency of geraniol that was first assayed for chloramphenicol resistance (Table 3) was also observed for resistance to β-lactams and to the fluoroquinolone norfloxacin that are more clinically important antibiotics (Table 4). Moreover, a ranking of the EPI activity demonstrated that geraniol is a very potent inhibitor of resistance in an *acrAB* mutant compared to PAβN (see the supplemental material), thus suggesting that these two molecules have different targets. Together, these findings provide a new source of drugs that may help in therapy, and also geraniol may help to create a better understanding of MDR in gram-negative bacteria that continue to pose a threat to public health.

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