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Essential oils from Moroccan plants as potential chemosensitisers restoring antibiotic activity in resistant Gram-negative bacteria

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ABSTRACT

Bacterial drug resistance is a worrying public health problem. Antibiotic efflux is a major non-specific resistance mechanism used by bacteria, and efflux pumps are involved in the low-level susceptibility of various important Gram-negative pathogens. Use of molecules that can block bacterial pumps is an attractive strategy, but several studies report only partial efficacy owing to limits of these molecules (stability, selectivity, bioavailability, toxicity, etc.). The objective of this study was to search for natural sources of molecules able to inhibit efflux pump systems of resistant Gram-negative bacteria (*Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella enterica* serotype Typhimurium and *Pseudomonas aeruginosa*). The results indicate that the studied essential oils exhibit interesting activity against the tested bacteria. This activity was significantly enhanced in the presence of an efflux pump inhibitor such as phenylalanine arginyl β -naphthylamide (PA β N). The role of lipopolysaccharide (LPS) structure in the effect of essential oils was also reported in *Salmonella* LPS deep-rough mutants. In addition, essential oils of *Thymus maroccanus* and *Thymus broussonetii*, used at a low concentration (a fraction of the minimum inhibitory concentration), are able to significantly increase chloramphenicol susceptibility of several resistant isolates. These results demonstrate that these essential oils can alter efflux pump activity and may be attractive candidates to develop new drugs for chemosensitising multidrug-resistant strains to clinically used antibiotics.

1. Introduction

The continued emergence and spread of multidrug-resistant (MDR) bacteria strongly impairs the efficacy of antibiotics and is of growing concern for human health [1,2].

Three main mechanisms are involved in bacterial resistance to antibiotics: production of enzymes that inactivate the antibiotics [3]; modification or masking of the antibiotic target, inhibiting their antibacterial action [4,5]; and modification of the membrane organisation, which includes alteration of membrane permeability and the expression of transporters expelling the antibiotic outside the cell [6–8]. This drug transport is associated with the expression of efflux pumps [7]. These membrane transporters, classified into five families, can confer resistance to a specific class of antibiotics or to a large number of drugs [8,9]. The latter group of efflux pumps, such as the AcrAB family, can handle a wide variety of structurally unrelated compounds [8–11] and the majority of antibiotic families are recognised and expelled by efflux pumps, including quinolones, β -lactams, phenicols, tetracyclines and aminoglycosides [11,12].

With increasing evidence demonstrating the involvement of efflux pumps in the antibiotic resistance detected in clinical isolates, many studies are engaged in developing molecules capable of blocking, at least partially, the action of the efflux pumps and that therefore have the potential to circumvent antimicrobial resistance [9,11,13]. Several molecules have been characterised for their inhibitory ability, such as phenylalanine arginyl β -naphthylamide (PA β N), carbonyl cyanide *m*-chlorophenylhydrazone and reserpine [13–18]. Use of bacterial pump inhibitors is attractive because it is an original anti-resistance approach that can be active on

several efflux-producing bacteria, and these compounds may restore activity for different families of antibiotics [13,19]. However, their clinical use is difficult owing to the toxicity, stability, selectivity and bioavailability of available molecules [17,19].

Natural products such as plant oils provide a wide diversity of chemical structures that exhibit broad-spectrum activity. Over the past few years, several natural compounds acting as efflux pumps inhibitors have been investigated [10,20–23]. Some of them are able to restore the activity of usual antibiotics on resistant clinical bacteria isolated during therapeutic treatment.

The aim of this present study was (i) to evaluate the activity of some Moroccan essential oils on different MDR isolates expressing efflux mechanisms and (ii) to test their capability, when used in combination, to restore antibiotic efficacy by blocking the drug efflux pumps expressed in the isolates.

2. Material and methods

2.1. Bacterial strains

The Gram-negative bacteria used in this study are listed in Table 1. *Enterobacter aerogenes* ATCC 13048 [18], *Escherichia coli* AG100, *Pseudomonas aeruginosa* PA01 and *Salmonella enterica* serotype Typhimurium SL696 were used as control strains. *Enterobacter aerogenes* EAEP289 is a kanamycin-sensitive derivative of EA27 (MDR isolate that exhibits active efflux of norfloxacin and chloramphenicol). EAEP294 is an *acrA* mutant constructed from EAEP289 [24]. *Escherichia coli* AG100A is kanamycin-resistant deleted of AcrAB and hypersusceptible to

chloramphenicol, tetracycline, ampicillin and nalidixic acid [25]. AG102 overproducing AcrAB is an AG100 derivative [26]. *Klebsiella pneumoniae* KPBj1E+ is a clinical isolate showing resistance to ceftiofur, quinolones and chloramphenicol associated with the expression of an efflux system. KPBj1Rev is a reverted strain derivative of KPBj1E+ that spontaneously becomes susceptible to the three antibiotic families [27]. *Salmonella* Typhimurium SL1069 and SL1102 are derivative strains from SL696 with deletion of a small and a large part of lipopolysaccharide (LPS), respectively [28]. *Pseudomonas aeruginosa* PA124 is a clinical isolate that overproduces OprM [21].

Strains were preserved at 4 °C and were grown on Luria–Bertani agar 24 h prior to any assay. Mueller–Hinton broth (MHB) was used for the antibiotic susceptibility tests.

2.2. Plant material and essential oils

Aerial parts of *Thymus broussonetii*, *Thymus maroccanus*, *Thymus pallidus* and *Rosmarinus officinalis*, belonging to the Lamiaceae family, were collected at full flowering stage in 2009 from different regions of Morocco. Identification of plant material was confirmed by Prof. Ahmed Ouhammou, a plant taxonomist in the Laboratory of Ecology and Environment of the University Cadi Ayyad (Marrakech, Morocco), where the voucher specimens were deposited. Dry aerial material of plants was subjected to hydrodistillation for 4 h. The oil recovered was stored in darkness at 4 °C until use in bioassays.

The method for gas chromatography/mass spectroscopy (GC/MS) has been described previously [29]. Briefly, analysis of the essential oils was carried out using GC/MS (Agilent Technologies, Palo Alto, CA) and the appropriate conditions (carrier gas, injector, times and temperatures). Identification of components was assigned by matching their mass spectra with Wiley and NIST library data, standards of the main components and by comparing their Kovats retention indices with reference libraries as described [29]. The component concentration was obtained by semiquantification by peak area integration from GC peaks and by applying the correction factors [29,30].

2.3. Minimum inhibitory concentration (MIC) determination

MIC assays were performed by microdilution assay. Each compound was analysed using a two-fold dilution series prepared in dimethyl sulphoxide (DMSO) (4%). Microwells containing 100 μ L of oil dilution were inoculated with 100 μ L of cell suspension prepared by diluting an overnight culture in MHB twice concentrated to obtain viable counts of ca. 10^6 colony-forming units/mL at a final DMSO concentration of 2%. At this concentration, no significant effect has been observed on the bacteria tested as control under these conditions. The inoculated microplates were incubated at 37 °C for 18–24 h. The MIC was defined as the lowest concentration of essential oil inhibiting visible growth of the test strain. Chloramphenicol, cloxacillin and norfloxacin were used as usual antibiotics.

The MIC of essential oils was determined in combination with PA β N at a final concentration of 20 mg/L, whereas synergy between antibiotics and essential oils was studied using essential oils at a low concentration (fraction of the MIC). Assays

were repeated three times on separate days and similar results were obtained in each experiment.

3. Results

Previous GC/MS analysis had indicated that carvacrol (76.35%) was the major constituent in *T. maroccanus* essential oil in addition to other constituents exhibiting relatively low concentrations. *Thymus broussonetii* was found to be richer qualitatively and it is mainly composed of carvacrol (39.77%), borneol (12.03%), bicyclogermacrene (7.95%), thymol (4.98%) and terpinene (4.09%) [30]. The chemical composition of *R. officinalis* and *T. pallidus* essential oils (Table 2) shows that seven compounds representing 98.41% were identified in *T. pallidus* essential oil, with *O*-cymene (47.05%) as the major constituent, in addition to thymol (15.63%), γ -terpinene (15.51%), linalyl acetate (7.95%), borneol (6.93%) and caryophyllene (3.9%). Thirty three compounds were identified in *R. officinalis* essential oil, mainly composed by 1,8-cineole (26.07%), camphor (11.2%), pinene (8.4%), β -pinene (7.27%) caryophyllene (6.89%) and borneol (6.62%).

3.1. Activity of essential oils against various Gram-negative resistant strains

The activity of essential oils and standard antibiotics against *E. coli*, *E. aerogenes*, *K. pneumoniae* and *P. aeruginosa* is summarised in Table 3. *Thymus maroccanus* and *T. broussonetii* had comparative activity against the tested bacteria, with MICs ranging from 0.936 mL/L to 0.468 mL/L for AG102 and EA27 overexpressing the AcrAB pump and from 0.234 mL/L to 0.117 mL/L for EAEP294 and AG100A, respectively, deleted of AcrAB. The activity of *T. pallidus* was reduced and the *R.*

officinalis essential oil demonstrated poor efficacy, with MICs of 3.75 mL/L for EAEP294 and AG100A. For strains overexpressing efflux pumps (EA27 and AG102), MICs ranged from 30 mL/L to 60 mL/L. Regarding the *K. pneumoniae* strains, KPBJ1E+ and KPBJ1Rev presented the same susceptibility to *T. maroccanus* and *T. broussonetii*; only *T. pallidus* and *R. officinalis* showed a difference between the two strains.

Regarding *P. aeruginosa*, essential oils had the same activity against the two strains (PA01 and PA124). The MIC was 15 mL/L for *T. maroccanus* and ≥ 30 mL/L for *T. broussonetii*, the highest values obtained for these two essential oils.

In combination with PA β N (Table 4), the MIC of EA27 and AG102 that overexpress the AcrAB pump decreased 4–8 times for *T. maroccanus*, *T. broussonetii* and *T. pallidus*, whereas for *R. officinalis* the decrease was ca. 32 times. The chemosensitising effect of PA β N was less important for EAEP294 and AG100A that are deleted of *acrAB*, with the reduction corresponding to only two times the MIC for *R. officinalis* and no reduction for the other tested essential oils. The MIC decrease for *K. pneumoniae* strains was greater (8–16 times), except for KPBJ1Rev where it was approximately two times for *T. broussonetii* and *T. pallidus*. Interestingly, for the *P. aeruginosa* strains, PA β N significantly reduced resistance to essential oils in the same way; the MIC dropped from mL/L 15 to 0.234 mL/L and from ≥ 30 mL/L to 0.117 mL/L for *T. maroccanus* and *T. broussonetii*, respectively.

3.2. Activity of essential oils on lipopolysaccharide deep-rough mutants

In Gram-negative bacteria, LPS constitutes the outer leaflet of the outer membrane and strongly controls the penetration of antibacterial agents [31]. Recently, involvement of intact LPS in resistance to new antibacterial molecules has been described [32]. The activity of essential oils against *S. Typhimurium* strains (Table 5) indicated that SL696 is more resistant to *T. maroccanus* and *T. broussonetii* essential oils than SL1069 deleted of a distal part of LPS, which is itself more resistant than SL1102 in which LPS was deleted of a major part. The MICs obtained for *T. maroccanus* and *T. broussonetii* on LPS-truncated mutants are quite similar to those observed with *acrAB E. coli* mutants (Table 4). Interestingly, in the presence of PA β N the MIC for SL696, SL1102 and SL1069 was reduced by 2-, 16- and 32-fold, respectively, for *T. broussonetii* and by 2-, 8- and 16-fold, respectively, for *T. maroccanus*.

3.3. Effect of *Thymus maroccanus* and *Thymus broussonetii* oils on chloramphenicol susceptibility of various resistant strains

To evaluate the ability of essential oils to reduce chloramphenicol resistance of different strains, the effect of *T. maroccanus* and *T. broussonetii* was investigated on *E. aerogenes* (ATCC 13048 and EA27) and *E. coli* (AG100 and AG102) (Table 6). For *E. aerogenes* ATCC 13048 used as susceptible strain, *T. maroccanus* and *T. broussonetii* decreased the MIC of chloramphenicol from 8 mg/L to 2 mg/L and a similar reduction was observed with PA β N. Regarding the strains that overexpress efflux pumps (EA27 and AG102), the MIC decreased ca. 8–16-fold and 32-fold, respectively, comparable with that obtained with PA β N.

For *P. aeruginosa* strains, the two essential oils were used at 7.5 mL/L, 3.75 mL/L and 1.87 mL/L in combination with chloramphenicol. These concentrations corresponded to 1/2, 1/4, 1/8 and 1/16 of the MICs determined for *T. maroccanus* and *T. broussonetii*, respectively (Table 3). At 7.5 mL/L (MIC/2) for *T. maroccanus* and at 7.5 mL/L (MIC/4) and 3.75 mL/L (MIC/8) for *T. broussonetii*, these essential oils greatly increased the chloramphenicol susceptibility of *P. aeruginosa* strains (Table 7). The MIC reduction was better, or at least similar, to that observed in the presence of PA β N. It is interesting to note that the decrease of chloramphenicol MIC appears to be dose-dependent for *T. maroccanus* and *T. broussonetii* (Table 7).

4. Discussion

The intensive and sometimes inappropriate use of antibacterial agents, including antibiotics, disinfectants, etc. leads to the selection of MDR bacterial populations [2,4]. To reduce this emergence and the dissemination of MDR bacteria, new molecules are urgently needed [33]. With original chemically synthesised molecules, natural compounds present interesting antibacterial activities. To assess the involvement of efflux and LPS structure in the activity of essential oils of some Moroccan plants and their possible potency to restore antibiotic efficacy through specific interaction with drug efflux pumps, a set of MDR Gram-negative strains and their derivatives (*E. coli*, *E. aerogenes*, *K. pneumoniae*, *S. Typhimurium* and *P. aeruginosa*) were studied.

First, the direct activity of essential oils on the bacteria was determined. For all tested essential oils, MICs obtained for EA27 and AG102 that overexpress AcrAB

were greater than the MIC obtained for isogenic strains deleted of this pump (EAEP294 and AG100A) [24–26]. This demonstrates that the active compounds of tested essential oils can be substrates of the efflux pumps involved in the antibiotic resistance of these bacteria (AcrAB–TolC or MexAB–OprM). These results agree with those recently reported regarding some natural compounds [34]. However, no difference was recorded between the susceptibility of the two strains of *K. pneumoniae*. This suggests that the presence of a basal level of efflux could be sufficient to expel the active components of the oils or, alternatively, the resistance–nodulation–cell division (RND) efflux pump (e.g. AcrAB) could not be the main mechanism of resistance involved in these MDR bacteria against such compounds [27,34]. In addition, the natural resistance of Gram-negative bacterial strains to essential oils also involves LPS and there was a decreased level of resistance in strains producing truncated LPS molecules.

The results suggest that the truncated LPS increased the bacterial susceptibility to essential oils probably by facilitating diffusion through the outer membrane or, alternatively, the alteration of LPS structure presensitises the envelope to the effects of natural products. The outer membrane is the first barrier impairing the penetration of toxic molecules and the LPS organises an effective protection against external molecules [31,35–37].

In the presence of PA β N, a well known efflux pump inhibitor [17,19], the antibacterial activity of essential oils increased significantly. This improvement was specifically observed with bacterial strains that overexpress efflux pumps (EA27, EAEP289, AG102 and KP Bj1E+). In contrast, no significant change was observed with the

mutants exhibiting an *acrAB* deletion in the presence of PA β N. Regarding *Klebsiella* strains, the effect induced by PA β N on KPBJ1Rev is probably due to the presence of basal expression of a PA β N-sensitive efflux pump. These data agree with several recent studies that have reported the involvement of efflux pumps in the resistance of Gram-negative bacteria towards many natural products [21,34,37–39]. It is important to note that *R. officinalis* exhibits a high MIC against the various tested Enterobacteriaceae. When PA β N was added the susceptibility increased, suggesting that PA β N-sensitive efflux is a main barrier for this essential oil.

Pseudomonas aeruginosa is an opportunistic pathogen well described for its high level of resistance towards antimicrobial agents [40], mediated by a combination of different mechanisms including reduced membrane permeability and tripartite multidrug efflux systems such as constitutive MexAB–OprM [8,9,41,42]. To determine the role of MexAB–OprM in *P. aeruginosa* sensibility to Moroccan essential oils, PA β N was used in combination with essential oils and antibiotics. Results showed that PA β N strongly increased the susceptibility to *T. maroccanus* and *T. broussonetii* for the two strains of *P. aeruginosa*. Interestingly, no difference was observed in the level of MIC reduction with the two strains PA01 and PA124 (overexpressing OprM).

This suggests that active compounds of essential oils can be substrates of efflux pumps existing in the reference strain of *P. aeruginosa* PA01. These efflux pumps are PA β N-sensitive and could be different from those involved in the resistance against usual antibiotics.

Thymus maroccanus and *T. broussonetii* were evaluated for their ability to reduce resistance to chloramphenicol in certain MDR Gram-negative bacteria. The two essential oils significantly increased the susceptibility of efflux pump-overexpressing strains (EA27 and AG102). At concentrations below the MIC, *T. maroccanus* and *T. broussonetii* strongly increased the susceptibility of *P. aeruginosa* strains (PA01 and PA124) to chloramphenicol. This result supports the hypothesis that some compounds in the various essential oils are able to block the efflux of usual antibiotics, as previously reported [21]. This active compound can restore susceptibility to chloramphenicol by producing a competition to drug efflux by a mechanism similar to 5'-methoxyhydnocarpin that inhibits efflux of hydrophobic alkaloid berberine in resistant bacteria [23,43]. More interestingly, this study demonstrated the ability of low concentrations of *T. maroccanus* and *T. broussonetii* essential oils to restore antibiotic susceptibility in resistant effluxing bacteria.

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Competing interests

None declared.

Ethical approval

Not required.

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Table 1

Bacterial strains used in this study

Bacterial strain	Major characteristics	Origin
<i>Enterobacter aerogenes</i>		
ATCC 13048	Reference strain	[18]
EA27	MDR clinical isolate; Kan ^r Amp ^r Chl ^r Nal ^r Str ^r Tet ^r	[18]
EAEP289	Kan ^s derivative of EA27	[24]
EAEP294	EAEP289 <i>acrA::Kan^r</i> (pEP755 integration)	[24]
<i>Escherichia coli</i>		
AG100	Wild-type <i>E. coli</i> K-12	[25]
AG100A	AG100 Δ <i>acrAB::Kan^r</i>	[25]
AG102	AG100 overproducing AcrAB pump	[26]
<i>Klebsiella pneumoniae</i>		
KPBj1E+	Clinical isolate resistant to ceftiofloxacin, quinolones and chloramphenicol related to an overexpressed efflux system	[27]
KPBj1Rev	Spontaneous reverted strain derivative of KPBj1E+, ertapenem sensitive	[27]
<i>Salmonella enterica</i> serotype Typhimurium		
SL696	Wild-type, metA22, trpB2, strAi20	[28]
SL1069 Rc	SL696 Rc derivative	[28]
SL1102 Re	SL696 Re derivative	[28]
<i>Pseudomonas aeruginosa</i>		
PA01	Reference strain	[21]
PA124	Clinical MDR isolate overexpressing OprM	[21]

MDR, multidrug-resistant; Kan^r, Amp^r, Chl^r, Nal^r, Str^r and Tet^r, resistance to

kanamycin, ampicillin, chloramphenicol, nalidixic acid, streptomycin and tetracycline,

respectively; Kan^s, susceptible to kanamycin.

Table 2Chemical composition of *Rosmarinus officinalis* and *Thymus pallidus* essential oils

KI	Component	<i>R. officinalis</i> (%)	<i>T. pallidus</i> (%)
929	Tricyclene	0.20	N/D
937	α -Thujene	0.32	N/D
947	Pinene	8.40	N/D
963	Camphene	5.14	N/D
999	β -Pinene	7.27	N/D
1009	Myrcene	2.17	N/D
1019	1-Phellandrene	0.41	N/D
1028	α -Terpinene	1.32	N/D
1041	1,8-Cineole	26.07	N/D
1050	O-cymene	0.17	47.05
1057	γ -Terpinene	2.20	15.51
–	Linalyl acetate	N/D	7.95
1076	Terpinolene	0.96	N/D
1105	Fenchol	0.07	N/D
1151	Camphor	11.20	N/D
1198	Borneol	6.62	6.93
1227	α -Terpineol	4.76	N/D
1238	Verbenone	0.42	N/D
1313	L-Bornyl acetate	2.64	N/D
1347	Thymol	0.31	15.63
1358	Carvacrol	1.04	1.45
1378	α -Cubebene	0.15	N/D
1402	Ylangene	0.35	N/D
1408	Copaene	0.95	N/D
1441	Methyl eugenol	0.18	N/D
1456	Caryophyllene	6.89	3.9
1488	Humulene	1.31	N/D
1510	α -Amorphene	0.89	N/D
1524	Aromadendrene	0.07	N/D

1535	α -Muurolene	0.89	N/D
1542	Δ -Cadinene	0.23	N/D
1553	γ -Cadinene	0.35	N/D
1627	Oxide de caryophyllene	0.25	N/D
–	Linoleic acid	0.15	N/D
Total		94.35%	98.41

KI, Kovats index; N/D, not detected.

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Table 3

Minimum inhibitory concentrations (MICs) of essential oils and antibiotics against tested Gram-negative bacteria

Strain	MIC of essential oils (mL/L)				MIC of antibiotics (mg/L)		
	TM	TB	TP	RO	NOR	CLX	CHL
<i>Enterobacter aerogenes</i>							
ATCC 13048	0.468	0.936	1.875	30	0.06	512	8
EA27	0.468	0.468	1.875	30	64	2048	1024
EAEP289	0.936	1.875	7.5	60	64	2048	1024
EAEP294	0.234	0.234	0.468	3.75	8	1024	64
<i>Escherichia coli</i>							
AG100	0.936	0.936	3.75	15	0.06	512	8
AG102	0.468	0.936	7.5	30/60 ^a	0.125	512	32
AG100A	0.117	0.117	0.234	3.75	0.015	2	0.5
<i>Klebsiella pneumoniae</i>							
KPBj1E+	1.875	1.875	3.75	>15	4	1024	128
KPBj1Rev	1.875	1.875	1.875	15	0.25	512	4
<i>Pseudomonas aeruginosa</i>							
PA01	15	30	N/D	>30	0.5	4096	32
PA124	15	>30	N/D	>30	64	>4096	256

TM, *Thymus maroccanus*; TB, *Thymus broussonetii*; TP, *Thymus pallidus*; RO,

Rosmarinus officinalis; NOR, norfloxacin; CLX, cloxacillin; CHL, chloramphenicol;

N/D, not determined.

^a Variation of one dilution when MICs determined separately in different experiments.

Table 4

Minimum inhibitory concentrations (MICs) of essential oils and antibiotics in combination with phenylalanine arginyl β -naphthylamide (PA β N) (20 mg/L) against tested Gram-negative bacteria

Strain	MIC of essential oils (mL/L)								MIC of antibiotics (mg/L)					
	TM		TB		TP		RO		NOR		CLX		CHL	
	-	+PA β N	-	+PA β N	-	+PA β N	-	+PA β N	-	+PA β N	-	+PA β N	-	+PA β N
	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N	PA β N
<i>Enterobacter aerogenes</i>														
ATCC 13048	0.468	0.234	0.936	0.468	1.875	0.468	30	7.5	0.06	0.06	512	256	8	2
EA27	0.468	0.117	0.468	0.117	1.875	0.468	30	0.936	64	64	2048	1024	1024	64
EAEP289	0.936	0.234	1.875	0.234	7.5	0.936	60	1.875	64	64	2048	1024	1024	256
EAEP294	0.234	0.234	0.234	0.234	0.468	0.468	3.75	1.875	8	8	1024	256	64	32
<i>Escherichia coli</i>														
AG100	0.936	0.015	0.936	0.015	3.75	0.936	15	1.875	0.06	0.06	512	32	8	2
AG102	0.468	0.117	0.936	0.117	7.5	0.936	30/60 ^a	1.875	0.125	0.015	512	64	32	1
AG100A	0.117	0.117	0.117	0.117	0.234	0.234	3.75	1.875	0.015	0.002	2	<0.06	0.5	0.25
<i>Klebsiella pneumoniae</i>														
KPBj1E+	1.875	0.117	1.875	0.234	3.75	0.468	>15	1.875	4	0.5	1024	64	128	1

KPBj1Rev	1.875	0.234	1.875	0.936	1.875	0.936	15	1.875	0.25	0.125	512	256	4	0.5
<i>Pseudomonas aeruginosa</i>														
PA01	15	0.234	30	0.117	N/D	N/D	>30	7.5	0.5	0.125	4096	512	32	1
PA124	15	0.234	>30	0.117	N/D	N/D	>30	7.5	64	32	>4096	2048	256	4

TM, *Thymus maroccanus*; TB, *Thymus broussonetii*; TP, *Thymus pallidus*; RO, *Rosmarinus officinalis*; NOR, norfloxacin; CLX, cloxacillin; CHL, chloramphenicol; N/D, not determined.

^a Variation of one dilution when MICs determined separately in different experiments.

Table 5

Minimum inhibitory concentrations (MICs) of essential oils and antibiotics in combination with phenylalanine arginyl β -naphthylamide (PA β N) (20 mg/L) against *Salmonella enterica* serotype Typhimurium strains

Strain	MIC of essential oils (mL/L)				MIC of antibiotics (mg/L)					
	TM		TB		NOR		CLX		CHL	
	-PA β N	+PA β N	-PA β N	+PA β N	-PA β N	+PA β N	-PA β N	+PA β N	-PA β N	+PA β N
SL696	0.468	0.234	1.875	0.93	0.25	0.015	>2048	1024	4	0.5
SL1069 Rc	0.234	<0.015	0.468	<0.015	0.06	<0.015	2048	<0.5	1/0.5 ^a	<0.015
SL1102 Re	0.117	<0.015	0.234	<0.015	0.06	<0.015	32	<0.5	1/0.5 ^a	<0.015

TM, *Thymus maroccanus*; TB, *Thymus broussonetii*; NOR, norfloxacin; CLX, cloxacillin; CHL, chloramphenicol.

^a Variation of one dilution when MICs determined separately in different experiments.

Table 6

Minimum inhibitory concentrations (MICs) of chloramphenicol in combination with *Thymus maroccanus* and *Thymus broussonetii* against *Enterobacter aerogenes* and *Escherichia coli* strains

Combination	MIC of chloramphenicol (mg/L)			
	<i>E. aerogenes</i> ATCC 13048	<i>E. aerogenes</i> EA27	<i>E. coli</i> AG100	<i>E. coli</i> AG102
Chloramphenicol	8	1024	8	32
Chloramphenicol + PA β N	2	64	2	1
Gain	4	16	4	32
Chloramphenicol + <i>T. maroccanus</i> 0.31 mL/L	2	128/64	2/1	1
Gain	4	8/16	4/8	32
Chloramphenicol + <i>T. broussonetii</i> 0.31 mL/L	2	128/64	2	1
Gain	4	8/16	4	32

PA β N, phenylalanine arginyl β -naphthylamide.

Table 7

Minimum inhibitory concentrations (MICs) of chloramphenicol (CHL) in combination with *Thymus maroccanus* and *Thymus broussonetii* against *Pseudomonas aeruginosa* strains

Strain	MIC (mg/L) of CHL	MIC (mg/L) of CHL + PA β N		MIC (mg/L) of CHL + <i>T. maroccanus</i>			MIC (mg/L) of CHL + <i>T. broussonetii</i>								
		20 mg/L	Gain	7.5 mL/L	Gain	3.75 mL/L	Gain	1.87 mL/L	Gain	7.5 mL/L	Gain	3.75 mL/L	Gain	1.87 mL/L	Gain
PA01	32	1	32	<0.5	>64	4	8	8	4	<0.5	>64	4	8	16	2
PA124	256	32	8	32	8	32	8	64	4	32	8	32	8	64	4

PA β N, phenylalanine arginyl β -naphthylamide.