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Medicinal plant extracts with efflux inhibitory activity against Gram-negative bacteria

Mark I. Garvey ^a, Mukhlesur Rahman ^b, Simon Gibbons ^{b,*}, Laura J.V. Piddock ^{a,*}

^a *Antimicrobial Agents Research Group, School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK*

^b *Department of Pharmaceutical and Biological Chemistry, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK*

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* Corresponding authors. Tel.: +44 121 414 6966; fax: +44 121 414 6819 (L.J.V.

Piddock); tel.: +44 207 753 5913; fax: +44 207 753 5964 (S. Gibbons).

E-mail addresses: l.j.v.piddock@bham.ac.uk (L.J.V. Piddock);

simon.gibbons@pharmacy.ac.uk (S. Gibbons).

ABSTRACT

It was hypothesised that extracts from plants that are used as herbal medicinal products contain inhibitors of efflux in Gram-negative bacteria. Extracts from 21 plants were screened by bioassay for synergy with ciprofloxacin against *Salmonella enterica* serotype Typhimurium, including mutants in which *acrB* and *tolC* had been inactivated. The most active extracts, fractions and purified compounds were further examined by minimum inhibitory concentration testing with five antibiotics for activity against Enterobacteriaceae and *Pseudomonas aeruginosa*. Efflux activity was determined using the fluorescent dye Hoechst 33342. Eighty four extracts from 21 plants, 12 fractions thereof and 2 purified molecules were analysed. Of these, 12 plant extracts showed synergy with ciprofloxacin, 2 of which had activity suggesting efflux inhibition. The most active extract, from *Levisticum officinale*, was fractionated and the two fractions displaying the greatest synergy with the five antibiotics were further analysed. From these two fractions, faltarindiol and the fatty acids oleic acid and linoleic acid were isolated. The fractions and compounds possessed antibacterial activity especially for mutants lacking a component of AcrAB–TolC. However, no synergism was seen with the fractions or purified molecules, suggesting that a combination of compounds is required for efflux inhibition. These data indicate that medicinal plant extracts may provide suitable lead compounds for future development and possible clinical utility as inhibitors of efflux for various Gram-negative bacteria.

1. Introduction

Over the last decade there has been a dramatic reduction in the number of pharmaceutical companies developing new antimicrobial agents [1]. In parallel, the number of antibiotic-resistant Gram-negative bacteria has increased [2]. With the reduction in the number of new agents and in antibiotic development, there has been a resurgence of interest in the search for compounds that will restore the activity of licensed antimicrobial agents that until recently had excellent activity against Gram-negative bacteria. In 1998 it was shown that plant-derived compounds have activity against Gram-positive bacteria, in particular *Staphylococcus aureus* [3]. Several compounds, such as reserpine, behave as if they inhibit efflux pumps and hence have become known as efflux pump inhibitors (EPIs) [4]. Since that time, numerous phytochemicals have been shown to have activity against *S. aureus* or other Gram-positive bacteria, or to act as potential EPIs with antimicrobials for Gram-positive bacteria [5–11]. Unfortunately, most of the plant-derived compounds have little or no activity with antibiotics against Gram-negative bacteria and it was suggested that, as many plant pathogens are Gram-negative bacteria, plants may not produce molecules effective against these organisms [5]. None the less, there are many medicinal plants that have traditional usage in various parts of the world in the treatment of infections caused by Gram-negative bacteria [12,13].

Gram-negative bacteria have innate multidrug resistance to many antimicrobial compounds owing to the presence of efflux pumps. In the Enterobacteriaceae, the efflux pump most commonly associated with this innate multidrug resistance is the AcrAB–TolC efflux system.

Homologues of this pump are found in other Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Campylobacter jejuni* [14]. The presence of these pumps and their broad substrate profile is the cause of the innate resistance to many of the agents that have good antimicrobial activity against Gram-positive bacteria. Overproduction of these efflux pumps confers clinically relevant resistance to many antimicrobial agents, including ciprofloxacin and tigecycline, in Enterobacteriaceae [14].

We hypothesised that extracts from plants that are used as natural herbal medicines contain molecules that act as EPIs against the efflux pumps of Gram-negative bacteria. Therefore, 84 extracts from 21 medicinal plants were investigated for synergistic activity with ciprofloxacin against *Salmonella enterica* serotype Typhimurium. These plants were chosen as they all have a use in various systems of medicine and some are used as topical antimicrobials, for example *Melissa officinalis*. Extracts were screened for differential activity against strains that either expressed or overproduced the AcrAB–TolC pump but had no activity for mutants in which a component of this pump had been inactivated. In this way, it was hoped to identify extracts that contained an EPI specific for this pump. For those extracts where data suggested active components, the effect upon growth of *Salmonella* spp. as well as inhibition of efflux activity was determined. Two extracts showing the greatest synergy with ciprofloxacin were fractionated and the active molecules were identified.

2. Materials and methods

2.1. Bacterial strains, storage and growth

All bacteria used in this study are listed in Table 1. Construction of mutants derived from *S. Typhimurium* has been described previously [16,19,21,22]. *Salmonella* Typhimurium L3 is a pre-therapy human clinical isolate and L10 is a post-therapy human clinical isolate that overexpresses *acrAB*, both of which have been previously described [20,23]. All bacteria were stored on ProtectTM beads (Technical Service Consultants Ltd., Heywood, UK) at $-80\text{ }^{\circ}\text{C}$. Identification of each species was confirmed by Gram-stain and analytical profile index (API 20E; bioMérieux, Marcy-l'Étoile, France).

2.2. Media and chemicals

Bacteria were grown on Luria–Bertani (LB) agar plates (Oxoid Ltd., Basingstoke, UK) and in LB broth (Oxoid Ltd.). All antibiotics and other compounds were obtained from Sigma (Poole, UK).

2.3. Extraction of plants

In total, 21 plants were used in this study (Table 2). All plants except *Catha edulis* (purchased locally in London, UK) were purchased from Proline Botanicals (Essendine, UK). Each plant (200–400 g) was extracted at $21\text{ }^{\circ}\text{C}$ sequentially with 300–600 mL of chloroform, methanol and water.

2.4. Fractionation of extracts

Following assessment of bioactivity, the chloroform extract of *Levisticum officinale* (10 g) was fractionated by vacuum liquid chromatography (VLC) on silica gel 60G (Merck Ltd., Lutterworth, UK) using solvent systems of increasing polarity. Fractions were eluted with hexane containing increasing 10% or 20% increments of ethyl acetate (EA) to give fractions A–H (A, 10% EA; B, 20% EA; C, 30% EA; D, 40% EA; E, 50% EA; F, 60% EA; G, 80% EA; and H, 100% EA). Fraction I was eluted with 5% methanol in EA, fraction J with 10% methanol in EA, fraction K with 20% methanol in EA and finally fraction L with 50% methanol in EA.

2.5. Purification of active compounds

Bioassay-directed isolation indicated that the VLC fraction eluted with 30% EA was active. This was purified by preparative thin-layer chromatography (Si Gel; Merck) (mobile phase 20% EA in toluene; double development) yielding falcariindiol (**1**; 450 mg) and levistolide A (**2**; 69 mg) (Fig. 1), which were identified by comparison with literature data [24,25].

2.6. Bioassay screening

Screening with ciprofloxacin in the absence or presence of plant extracts (Table 2), fractions of *L. officinale* and *M. officinalis*, and purified compounds from fractions of the chloroform extract of *L. officinale* for synergistic activity was carried out by bioassay essentially as described previously [26,27]. Six rows of 6 wells (total 36 wells) each of 8-mm diameter were cut out of each agar plate using an agar cutter (Fisher Scientific, Loughborough, UK). Then, 200 μ L of sterile Iso-Sensitest™ broth

(Oxoid Ltd.) containing ciprofloxacin (0.5, 1 and 2 mg/L) was added to three sets of wells. This volume and concentration gave a zone of inhibition ≥ 15 mm in diameter. To determine test compound synergism with ciprofloxacin, 200 μ L of a solution containing ciprofloxacin (0.5, 1 and 2 mg/L) and the plant extract (100 mg/L) in broth was added to three separate wells. The plates were incubated overnight at 37 °C and the diameters of the inhibition zones were measured. Each experiment contained three technical repeats and was repeated on three separate days. A zone of inhibition of the antibiotic plus test plant extract larger than that of the antibiotic alone was taken to suggest synergy. Positive controls were ciprofloxacin plus L-phenylalanyl-L-arginyl- β -naphthylamide (PA β N) (100 mg/L) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (100 μ M), and negative controls were Iso-Sensitest broth alone and plant extract alone to determine whether the plant extract had inherent antimicrobial activity.

2.7. Determination of the minimum inhibitory concentration (MIC) of antibiotics \pm synergising extracts, fractions and compounds

The MICs of ciprofloxacin, tetracycline, chloramphenicol, erythromycin and ethidium bromide (EtBr) in the absence or presence of plant extracts and fractions (at 100 mg/L) were determined by the agar doubling dilution method and by the microbroth dilution method [28,29]. MIC determinations were repeated at least three times in independent experiments. Antibiotics, compounds and EPIs were made up and used according to the manufacturer's instructions.

2.8. Determination of the fractional inhibitory concentration index (FICI)

Activity of antibiotics plus the plant extract/fraction/purified compound was determined using the checkerboard technique [30]. The first antibiotic (ciprofloxacin) of a combination was serially diluted along the ordinate of a 96-well microtitre tray; the second agent (e.g. plant extract) was diluted along the abscissa. An inoculum equal to a 0.5 McFarland turbidity standard was prepared from each strain in LB broth. Each well in the microtitre tray was inoculated with 100 μ L of the bacterial suspension (at 5×10^5 colony-forming units/mL) and the plates were incubated at 37 °C for 24 h under aerobic conditions. The resulting checkerboard contained each combination of ciprofloxacin and test agent with wells that contained the highest concentration of each agent at opposite corners. The MIC was defined as the lowest concentration of antibiotic that completely inhibited growth of the organism as detected with the naked eye [28]. Synergy was defined as an FICI (FICI) of ≤ 0.5 , no interaction was defined as an FICI of $>0.5-4$ and antagonism was defined as an FICI >4 [31].

2.9. Accumulation of Hoechst 33342 \pm plant extracts by *Salmonella Typhimurium*

Efflux activity of five *S. Typhimurium* strains [L3, L10, SL1344, ATCC 15277 (LT2) and ATCC 14028s] was determined by measuring accumulation of the fluorescent dye Hoechst 33342 (bisbenzimidazole; 2.5 μ M) in the absence or presence of known efflux inhibitors (CCCP 100 μ M and PA β N 100 mg/L) and in the absence or presence of those plant extracts (at 100 mg/L) revealed to have activity in the bioassay and MIC tests. Measurements were taken at excitation and emission wavelengths of 350 nm and 460 nm, respectively, over 30 min using a FLUOstar

OPTIMA (BMG Labtech, Aylesbury, UK) as previously described [32]. Differences in accumulation in the absence of the plant extracts were analysed for statistical significance using the two-tailed Student's *t*-test. *P*-values ≤ 0.05 were considered significant.

2.10. Growth kinetics \pm plant extracts

The growth kinetics of L3, L10, SL1344, ATCC 15277 (LT2) and ATCC 14028s in the absence or presence of plant extracts was determined by monitoring the optical density at 600 nm every 10 min at 37 °C for 24 h using a FLUOstar OPTIMA (BMG Labtech). *Levisticum officinale* chloroform extract and *M. officinalis* methanol extract (at 100 mg/L) were added to the bacterial cultures at mid-logarithmic growth phase (2 h). Samples were also removed and visualised microscopically to detect any gross changes to cell morphology, i.e. filamentation. A two-tailed Student's *t*-test was used to compare the generation times. *P*-values ≤ 0.05 were considered significant.

3. Results

3.1. Medicinal plant extracts synergise with ciprofloxacin

Plants were extracted with chloroform, methanol and water and were screened to identify those that showed synergy with ciprofloxacin and could be putative EPIs.

Ciprofloxacin was chosen as increased MICs of this agent are often associated with overproduction of the AcrAB–TolC pump. The positive control was PA β N at 100 mg/L, which was the lowest concentration to elicit an effect in this assay. Initial

experiments were performed with *Salmonella* Typhimurium as a model Gram-negative bacterium and because a defined isogenic set of mutants that lack or overproduce the AcrAB–TolC pump was available. Eighty-four extracts from 21 plants were screened for synergistic activity with ciprofloxacin against *S.*

Typhimurium SL1344, ATCC 15277 (LT2), ATCC 14028s, L3 and L10. Of these, 12 extracts showed synergy with ciprofloxacin for these strains, whereas 72 showed little or no activity.

The most active antibiotic-potentiating extracts were those from *L. officinale* (extracted with chloroform, methanol and water), *M. officinalis* (chloroform and methanol), *Cinnamomum aromaticum* (chloroform), *Pimpinella anisum* (chloroform) and *Pulmonaria officinalis* (chloroform and methanol) (Table 3) as well as the chloroform extracts of *Angelica sinensis*, *Tussilago farfara* (methanol) and *Commiphora molmol* (chloroform) (data not shown). Of the extracts investigated, those from *M. officinalis* and *L. officinale* were the most active against strains that either had wild-type levels of AcrAB–TolC or that overproduced AcrAB (Table 4). Chloroform and methanol extracts of *L. officinale* and *M. officinalis* (both 100 mg/L) had no effect on the growth kinetics of the five *Salmonella* strains tested (data not shown), and by MIC determination the extract of *L. officinale* had synergistic activity with ciprofloxacin against most of the strains (Table 4).

3.2. Plant extracts synergise with several antibiotics

Owing to their promising activity, the MICs of the chloroform extract of *L. officinale* and the methanol extract of *M. officinalis* (i.e. those extracts that gave the largest zones of inhibition in the bioassay) were determined in combination with tetracycline,

chloramphenicol, erythromycin and EtBr. All extracts showed better activity in liquid media than in agar, with MIC values one to two dilutions lower from the microbroth dilution procedure than with agar (data not shown). No antimicrobial activity was detected at 100 mg/L of either extract; likewise, chloroform and methanol had no activity at the concentration used. Synergy between the extracts from *M. officinalis* and *L. officinale* with tetracycline, chloramphenicol, erythromycin or EtBr was seen for fewer strains than when the plant extracts were combined with ciprofloxacin. None the less, EPI-like activity was detected for both extracts combined with tetracycline, erythromycin and EtBr against wild-type *Salmonella* and the strain in which AcrAB was overproduced. The MIC experiments with the chloroform extract of *L. officinale* indicated that it had synergistic activity with ciprofloxacin and tetracycline for the salmonellae and two *Klebsiella pneumoniae* strains (Table 4). Of interest, the chloroform extract of *L. officinale* also had synergistic activity with ciprofloxacin and EtBr for *Salmonella* Typhimurium in which TolC was not produced and with EtBr for *S. Typhimurium* in which AcrAB was not produced (Table 4). The *M. officinalis* extract had synergistic activity with most antibiotics for the salmonellae and *K. pneumoniae* strains (Table 4).

The extracts of the other plants showing synergistic activity with ciprofloxacin in the bioassay did not reduce the MIC of ciprofloxacin as much as the extracts from *L. officinale* or *M. officinalis*, and when synergy was observed it was seen for fewer bacterial strains. For these reasons, no further experiments were performed with these extracts.

3.3. Activity of fractions derived from the chloroform extract of *Levisticum officinale*

As the chloroform extract of *L. officinale* showed synergistic activity with more agents and bacteria than that from *M. officinalis*, it was fractionated as the first step to the identification of the active component(s). The chloroform extract of *L. officinale* was subjected to chromatography and 12 fractions (A–L) were obtained. MICs of ciprofloxacin and tetracycline in the absence or presence of each fraction (100 mg/L) were determined. Compared with the chloroform extract of *L. officinale*, few of the fractions had the same synergistic activity with ciprofloxacin. Of interest, fractions A, C, F, G and K synergised with ciprofloxacin for the *Salmonella* mutants in which AcrAB and TolC were not produced, and A, C, G and K also synergised with ciprofloxacin for the two *K. pneumoniae* strains. Fractions A, G and K also had synergistic activity with ciprofloxacin for wild-type *Salmonella* and the clinical isolate that overproduced AcrAB (Table 5). Two fractions (C and F) also had antimicrobial activity alone for the mutant *Salmonella* in which a component of AcrAB–TolC was not produced (Table 5); this was most marked for fraction C.

3.4. Activity of compounds purified from fraction C of *Levisticum officinale*

As fraction C showed synergistic activity in combination with ciprofloxacin and also antimicrobial activity alone especially against the strains in which *acrB* and *tolC* had been inactivated, this fraction was analysed to identify the active compounds. Nuclear magnetic resonance (NMR) revealed that it contained predominantly the antibacterial fatty acids oleic acid and linoleic acid plus falcarindiol and levistolide A (Fig. 1). These compounds were further purified and the spectral data were in close agreement to those published [24,25]. The absolute stereochemistry of falcarindiol

was determined using a modified Mosher's ester method as the 3(S), 8(S) diastereomer (Fig. 1).

MICs of linoleic acid and falcarindiol were >500 mg/L for the wild-type *Salmonella* SL1344 and ATCC 14028s. For the TolC mutants of these strains the MIC of linoleic acid was 500 mg/L, and the MIC of falcarindiol was 75 mg/L and 125 mg/L, respectively. When linoleic acid was combined with ciprofloxacin MICs of 0.008 mg/L and 0.004 mg/L were obtained for the TolC mutants, and for falcarindiol in combination with ciprofloxacin MIC values <0.001 mg/L were obtained.

Synergistic activity between ciprofloxacin and the compounds for the mutants was confirmed in checkerboard assays, with FICI values of 0.14–0.27 for the combination and the TolC mutants (Table 6). The FICI for ciprofloxacin in combination with the methanol-soluble extract from *M. officinalis* against L3 (pre-therapy isolate) was lower than that for L10 (that overexpresses *acrB*). This suggests that a higher concentration of the putative EPI is required to inhibit growth of L10 owing to the greater production of AcrB by L10. No synergy was seen with this combination for the parental strains, despite it being clearly demonstrated with the extract itself. These data suggest that an EPI-like molecule(s) was lost during the separation process or was inactivated during purification.

3.5. Efflux activity of plant extracts

Accumulation of Hoechst 33342 has been used as a marker for efflux as it is fluorescent and is a substrate of several efflux pumps including AcrAB–TolC [17,32]. An increase in the amount of Hoechst 33342 accumulated in the presence of an EPI

such as PA β N indicates inhibition of efflux [17]. To determine whether synergistic activity displayed by the extracts of *L. officinale* with various antibiotics was due to inhibition of efflux, accumulation of Hoechst 33342 in the presence and absence of 100 mg/L of the chloroform and methanol extracts of *L. officinale* was carried out for five strains of *S. Typhimurium* [SL1344, ATCC 15277 (LT2), ATCC 14028s, L3 and L10]. For the four antibiotic-susceptible wild-type strains of *S. Typhimurium*, both extracts of *L. officinale* increased the amount of Hoechst 33342 accumulated (i.e. reduced efflux) by 1.5–2-fold (Fig. 2). The efflux activity of the strain that overexpressed AcrB (L10) was also reduced by 1.5-fold.

4. Discussion

Many conventional drugs have arisen from natural products, including plants. However, few antimicrobial agents have come from this source, with the vast majority in clinical use derived from products naturally produced by microorganisms [33]. None the less, the use of medicinal and herbal plant remedies to treat infectious diseases is common in many countries [34]. Owing to the continued clinical pressure for novel approaches to combat antibiotic-resistant bacterial infections, there is a need to identify new agents to treat such infections. One approach is to screen for natural products from plants. Plants have already successfully yielded compounds with activities suggesting that they inhibit efflux pumps of Gram-positive bacteria [3,5]. Therefore, in this study we sought to identify medicinal plants that could provide compounds for further antimicrobial drug development. In addition, as there are many clinically licensed antibacterial agents for the treatment of infections by Gram-negative bacteria, but which are effluxed by the various pumps possessed by these bacteria, we sought to screen for activity that suggested efflux inhibition. One

desirable property of a putative EPI is that it should synergise with antibiotics for bacteria with wild-type or overproduction of AcrAB–TolC but have no effect on a strain in which AcrAB or TolC are produced. As ciprofloxacin is a substrate of many bacterial efflux pumps [14], the initial experiments used a simple bioassay to identify plant extracts that synergised with this agent [27]. This allowed many plant extracts made under different conditions to be screened with speed. In a prior study, we sought to identify compounds with EPI-like activity and to use this as an aid to predict the common structural features of EPIs and so guide the type of plant from which to make extracts for this study [27]. Of the 84 extracts from the 21 plants, 2 extracts, an ethanol extract from *M. officinalis* (lemon balm) and a chloroform extract of *L. officinale* (lovage root), had the greatest activity in terms of antibiotic potentiation with either the antibiotics ciprofloxacin or tetracycline or the dye EtBr and these data suggest that these extracts contain an inhibitor of efflux. The extract from *L. officinale* had the greatest EPI-like activity and so this was taken forward and fractionated as an initial step to identify the compounds with EPI-like activity. It was surprising to find that none of the 12 fractions of the chloroform extract of *L. officinale* had EPI-like activity, as those fractions that displayed synergy with ciprofloxacin did so in all strains irrespective of the presence or absence of a functional AcrAB–TolC system. These data suggest an interaction with other pumps or another mechanism of synergy. Most interesting was that two fractions (C and F) had some antimicrobial activity alone against all strains including those that lacked TolC. This is highly unusual as the majority of plant extracts display activity only towards Gram-positive bacteria. Fraction C, which had the greatest antimicrobial activity, was further fractionated to identify the compounds conferring the observed antimicrobial activity. Of these, falcarindiol (**1**) had the greatest antimicrobial activity and a strong additive

effect to the activity of ciprofloxacin. Falcarindiol has previously been shown to possess antibacterial activity, but for mycobacteria and Gram-positive bacteria and not Gram-negative bacteria [24,35–37]. It is possible that the activity in the fraction was potentiated by the co-isolated fatty acids, and these could have membrane permeabilising activity. As these are ubiquitous in all organisms, this does not entirely explain the data or why so few plant extracts are active against Gram-negative bacteria. Rather, the data suggest that falcarindiol is a substrate of TolC and this may be why it has not been previously identified as having antimicrobial activity for Gram-negative bacteria.

Despite not successfully obtaining the compound(s) that conferred the EPI-like activity in the original plant extract, we postulate that the putative inhibitor of efflux was either lost or was inactivated during fractionation or that it acts in combination with another compound in the plant extract from which it was separated during the fractionation process. Further work to examine reconstituting fractions is required to understand whether a synergistic phenomenon is associated with selected fractions. This will also allow us to conclude whether a putative EPI is being lost in the separation process or is unstable.

Gram-negative plant pathogens such as *Pseudomonas syringae* can cause enormous damage to crop plants [38], and *P. syringae* has been shown to possess a homologue of the tripartite resistance–nodulation–cell division (RND) efflux system MexAB–OprM [39]. This would suggest that plants may not produce chemicals that are active against efflux pumps of Gram-negative plant pathogens. However, the current data suggest that plants do produce molecules with activity against Gram-

negative bacteria. Furthermore, we postulate that these molecules are substrates of TolC (and homologues thereof) and associated efflux pumps. Therefore, for plants to withstand infection by these pathogens they need to produce EPIs for the natural antimicrobial agents to be active. It is tempting to speculate that falcarindiol is produced alongside a compound(s) that inhibits efflux.

Lorenzi et al. workers [40] have recently shown that extracts from plants can also provide compounds with EPI-like activity or antibacterial activity per se. They also found that an extract of an essential oil from a Corsican plant, *Helichrysum italicum*, was able to reduce the MIC of chloramphenicol for *Enterobacter aerogenes*, *Acinetobacter baumannii* and *P. aeruginosa*. Two fractions of this essential oil and one compound within this oil, geraniol, synergised with chloramphenicol both for the wild-type strain of *E. aerogenes* and its AcrAB mutant. Another recent study by Adonizio et al. [41] showed that medicinal plant extracts can attenuate the virulence of *P. aeruginosa* when explored in the *Caenorhabditis elegans* model system. Although the extracts were from medicinal plants previously used in herbal medicine, the active compounds were not identified. The current data taken together with that of Adonizio et al. [41] and Lorenzi et al. [40] indicate that plants as a source of new and/or novel antimicrobial compounds with activity for Gram-negative bacteria are an underexplored resource. Plant extracts such as those described by ourselves and others provide lead compounds for further exploration and development as antimicrobial agents, as agents to inhibit efflux or for combination with licensed antimicrobials.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Structures of falcarindiol (**1**) and levistolide A (**2**).

Fig. 2. Accumulation of Hoechst 33342 in the absence or presence of extracts of *Levisticum officinale*. White bars, Hoechst 33342 alone; dotted bars, Hoechst 33342 with chloroform (CHCl₃) extract of *L. officinale*; dark grey bars, Hoechst 33342 with methanol (MeOH) extract of *L. officinale*. A value of 1 represents accumulation of Hoechst 33342 in the absence of plant extract; a value >1 indicates increased accumulation of Hoechst 33342 (i.e. reduced efflux). Error bars indicate standard deviations.

Table 1

Bacterial strains used in this study

Strain	Species	Description	Reference/source
L354	<i>S. Typhimurium</i>	SL1344	[15]
L109	<i>S. Typhimurium</i>	SL1344 <i>tolC::aph</i>	[16]
L110	<i>S. Typhimurium</i>	SL1344 <i>acrB::aph</i>	[16]
L884	<i>S. Typhimurium</i>	SL1344 <i>acrA::aph</i>	[17]
L785	<i>S. Typhimurium</i>	ATCC 15277 (LT2)	[18]
L828	<i>S. Typhimurium</i>	ATCC 14028s	[18]
L829	<i>S. Typhimurium</i>	ATCC 14028s $\Delta tolC$	[19]
L830	<i>S. Typhimurium</i>	ATCC 14028s $\Delta acrB$	[19]
L831	<i>S. Typhimurium</i>	ATCC 14028s $\Delta acrAB$	[19]
L3	<i>S. Typhimurium</i>	Human pre-therapy clinical isolate, antibiotic-susceptible	[20]
L10	<i>S. Typhimurium</i>	Human post-therapy clinical isolate; MDR and overproducing <i>acrAB</i>	[20]
A1	<i>Enterobacter cloacae</i>	NCTC 10005	NCTC
B14	<i>Serratia marcescens</i>	NCTC 2847	NCTC
G1	<i>Pseudomonas aeruginosa</i>	NCTC 10662	NCTC
H42	<i>Klebsiella pneumoniae</i>	NCTC 10896	NCTC
H43	<i>K. pneumoniae</i>	NCTC 9633	NCTC
I114	<i>Escherichia coli</i>	NCTC 10538	NCTC
J29	<i>Morganella morganii</i>	NCTC 235	NCTC

S. Typhimurium, *Salmonella enterica* serotype Typhimurium; ATCC, American Type Culture Collection; MDR, multidrug-resistant; NCTC, National Culture Type Collection (Health Protection Agency, Colindale, London, UK).

Table 2

List of medicinal plants used in this study

Plant name	Common name	Family
<i>Anemone nemorosa</i>	Wood anemone	Ranunculaceae
<i>Angelica sinensis</i>	Female ginseng	Apiaceae
<i>Apium graveolens</i>	Dill	Apiaceae
<i>Artemisia abrotanum</i>	Southernwood	Asteraceae
<i>Asclepias tuberosa</i>	Butterfly weed	Apocynaceae
<i>Boswellia serrata</i>	Salai	Burseraceae
<i>Catha edulis</i>	Khat	Celastraceae
<i>Centella asiatica</i>	Gotu kola	Apiaceae
<i>Cinnamomum aromaticum</i>	Cassia cinnamon	Lauraceae
<i>Citrus aurantium</i>	Bitter orange	Rutaceae
<i>Commiphora molmol</i>	Myrrh	Burseraceae
<i>Daucus carota</i>	Wild carrot	Apiaceae
<i>Ephedra funereal</i>	Death Valley ephedra	Ephedraceae
<i>Glycyrrhiza glabra</i>	Liquorice	Leguminosae
<i>Levisticum officinale</i>	Lovage	Apiaceae
<i>Melissa officinalis</i>	Lemon balm	Lamiaceae
<i>Papaver somniferum</i>	Opium poppy	Papaveraceae
<i>Pimpinella anisum</i>	Anise	Apiaceae
<i>Pulmonaria officinalis</i>	Lungwort	Boraginaceae
<i>Thymus vulgaris</i>	Thyme	Lamiaceae
<i>Tussilago farfara</i>	Coltsfoot	Asteraceae

Table 3

Plant extracts with greatest synergy with ciprofloxacin

Strain ^a	PAβN	Lo	Lo	Lo	M	M	C	Pi	Pu	Pu
		MeOH	CHCl ₃	Water	MeOH	CHCl ₃	CHCl ₃	CHCl ₃	CHCl ₃	MeOH
S. Typhimurium SL1344	S	NE	NE	NE	NE	NE	NE	NE	NE	NE
S. Typhimurium ATCC 15277 (LT2)	S	S	S	S	S	NE	S	S	S	S
S. Typhimurium ATCC 14028s	S	S	S	S	S	S	S	S	S	S
Human pre-therapy isolate L3	S	S	S	S	S	S	S	S	S	S
Human post-therapy isolate L10	S	S	S	S	S	S	NE	S	S	S

^a Details of strains are given in Table 1.

PAβN, L-phenylalanyl-L-arginyl-β-naphthylamide; Lo, *Levisticum officinale*; M, *Melissa officinalis*; C, *Cinnamomum aromaticum*; Pi, *Pimpinella anisum*; Pu, *Pulmonaria officinalis*; MeOH, methanol extraction; CHCl₃, chloroform extraction; water, water extraction; S, synergy (zone of inhibition reproducibly larger than that of antibiotic alone); NE, no effect.

Table 4

Minimum inhibitory concentrations (MICs) of ciprofloxacin (CIP), tetracycline (TET), chloramphenicol (CHL), erythromycin (ERY) and ethidium bromide (EtBr) in the absence and presence of *Levisticum officinale* and *Melissa officinalis* extracts against Gram-negative bacteria

Strain ^a	MIC (mg/L) ^b														
	CIP	CIP + Lo	CIP + M	TET	TET + Lo	TET + M	CHL	CHL + Lo	CHL + M	ERY	ERY + Lo	ERY + M	EtBr	EtBr + Lo	EtBr + M
S. Typhimurium L354	0.03	0.008	0.015	2	1	1	8	8	8	512	512	512	2048	2048	2048
S. Typhimurium L828	0.03	0.008	0.008	16	0.5	0.5	8	8	8	512	256	512	2048	1024	2048
S. Typhimurium L829	0.008	0.004	0.008	4	0.5	0.5	1	1	1	8	8	8	16	8	16
S. Typhimurium L831	0.008	0.008	0.008	4	0.5	0.5	2	8	8	64	64	64	64	32	64
S. Typhimurium L3	0.008	0.002	0.008	4	0.5	0.25	8	4	8	512	512	512	512	512	512
S. Typhimurium L10	0.06	0.03	0.06	8	4	4	32	16	32	512	256	512	2048	1024	2048

<i>Enterobacter cloacae</i> A1	0.12	0.06	0.12	8	4	8	4	2	4	512	256	512	2048	2048	2048
<i>Serratia marcescens</i> B14	0.06	0.06	0.06	64	64	64	4	4	4	256	256	256	2048	2048	2048
<i>Pseudomonas aeruginosa</i> G1	1	1	1	64	64	64	64	64	64	512	512	512	2048	2048	2048
<i>Klebsiella pneumoniae</i> H42	0.06	0.03	0.03	16	2	4	128	64	128	512	256	512	2048	1024	1024
<i>K. pneumoniae</i> H43	0.12	0.06	0.06	8	1	2	4	4	4	512	512	512	2048	1024	2048
<i>Escherichia coli</i> I114	0.06	0.03	0.06	4	2	4	4	4	4	512	512	512	256	256	256
<i>Morganella morganii</i> J29	0.015	0.008	0.015	8	8	8	16	16	16	512	512	512	2048	2048	2048

S. Typhimurium, *Salmonella enterica* serotype Typhimurium; Lo, chloroform extract of *L. officinale* extract; M, methanol extract of *M. officinalis*.

^a Details of strains are given in Table 1.

^b Bold text indicates synergistic combinations (i.e. MICs for the combination lower than for the antibiotic alone).

Table 5

Minimum inhibitory concentrations (MICs) of ciprofloxacin (CIP) in the absence and presence of the six most active fractions derived from the plant extract from *Levisticum officinale* against a variety of *Salmonella* and Gram-negative bacteria

Strain ^b	MIC (mg/L) ^b									
	CIP	CIP + Lo	CIP + A	CIP + C	C alone	CIP + F	F alone	CIP + G	CIP + J	CIP + K
<i>S. Typhimurium</i> L354	0.03	0.008	0.03	0.03	250	0.03	250	0.03	0.03	0.03
<i>S. Typhimurium</i> L884	0.008	0.008	0.002	0.002	64	0.008	250	0.004	0.008	0.004
<i>S. Typhimurium</i> L110	0.008	0.008	0.002	0.004	128	0.008	250	0.004	0.008	0.004
<i>S. Typhimurium</i> L109	0.015	0.008	0.002	0.002	8	0.008	128	0.004	0.0015	0.004
<i>S. Typhimurium</i> L828	0.03	0.008	0.015	0.03	250	0.03	250	0.03	0.03	0.03
<i>S. Typhimurium</i> L829	0.008	0.004	<0.001	<0.001	8	0.004	128	0.004	0.015	0.004
<i>S. Typhimurium</i> L830	0.008	0.008	<0.001	<0.001	128	0.004	250	0.004	0.008	0.004
<i>S. Typhimurium</i> L831	0.008	0.008	<0.001	<0.001	64	0.004	250	0.004	0.008	0.004
<i>S. Typhimurium</i> L3	0.008	0.002	<0.001	<0.001	250	0.008	250	0.008	0.008	0.008
<i>S. Typhimurium</i> L10	0.06	0.03	0.03	0.12	250	0.12	250	0.03	0.12	0.03
<i>Enterobacter cloacae</i> A1	0.12	0.06	0.12	0.12	250	0.12	250	0.12	0.12	0.12
<i>Serratia marcescens</i> B14	0.06	0.06	0.06	0.06	250	0.06	250	0.06	0.06	0.06
<i>Pseudomonas aeruginosa</i> G1	1	1	1	1	250	1	250	1	1	1
<i>Klebsiella pneumoniae</i> H42	0.06	0.03	0.06	0.015	250	0.015	250	0.015	0.015	0.015
<i>K. pneumoniae</i> H43	0.12	0.06	0.12	0.03	250	0.03	250	0.06	0.03	0.03

<i>Escherichia coli</i> I114	0.06	0.03	0.06	0.06	250	0.06	250	0.06	0.06	0.06
<i>Morganella morganii</i> J29	0.015	0.008	0.015	0.015	250	0.015	250	0.015	0.015	0.015

S. Typhimurium, *Salmonella enterica* serotype Typhimurium; Lo, chloroform extract of *L. officinale*; CIP + letter, indicates the fraction of the chloroform extract of *L. officinale*.

^a Details of strains are given in Table 1.

^b Bold text indicates synergistic combinations (i.e. MICs for the combination lower than for the antibiotic alone).

Table 6

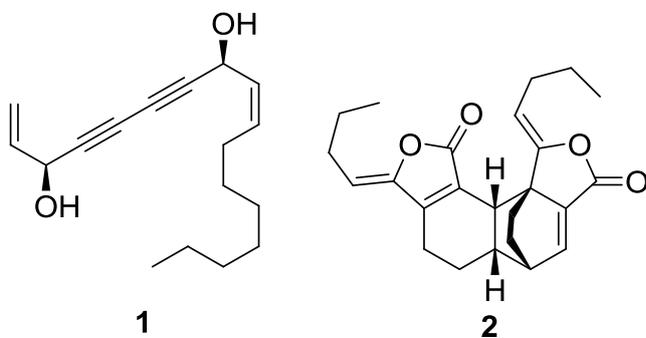
Fractional inhibitory concentration index (FICI) values of ciprofloxacin (CIP) with plant extracts, fractions and purified molecules for *Salmonella enterica* serotype Typhimurium

Strain ^a	FICI ^b					
	CIP + Lo	CIP + M	CIP + FC	CIP + FF	CIP + Lin	CIP + Fal
SL1344	0.94	0.97	0.99	0.97	1.34	0.97
SL1344 <i>tolC::aph</i>			0.27	0.38	0.17	0.26
ATCC 15277 (LT2)	0.46	0.59				
ATCC 14028s	0.48	0.41	1.01	0.98	1.09	1.05
ATCC 14028s $\Delta tolC$			0.32	0.33	0.14	0.14
Human pre-therapy isolate L3	0.32	0.13				
Human post-therapy isolate L10	0.31	0.22				

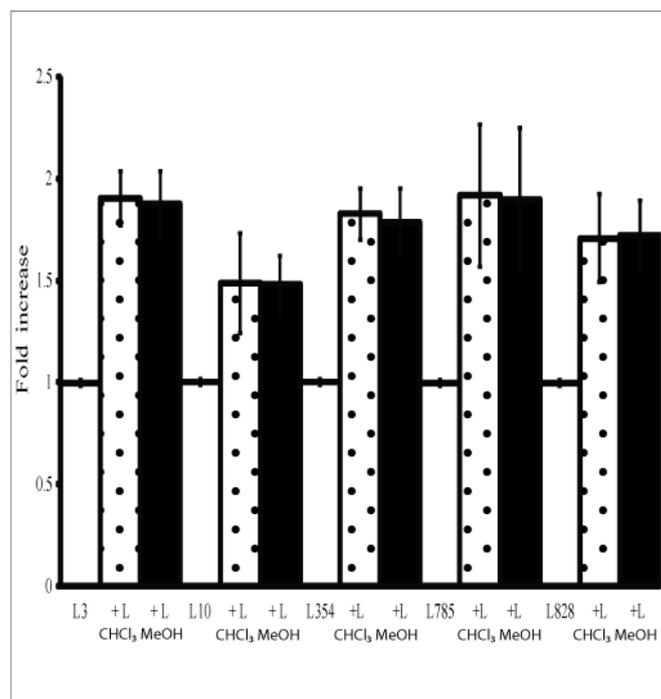
Lo, *Levisticum officinale* chloroform extract; M, *Melissa officinalis* methanol extract; FC, fraction C from *L. officinale*; FF, fraction F from *L. officinale*; Lin, linoleic acid; Fal, falcarindiol.

^a Details of strains are given in Table 1.

^b Bold text indicates synergy [31].



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