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Screening of Indian medicinal plants as efflux pump inhibitors of fluoroquinolones

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ABSTRACT

Efflux mechanisms have become broadly recognized as major components of resistance to many classes of antibiotics. Efflux related multidrug resistance (MDR) is a significant means by which bacteria can evade the effects of antibacterial agents. This study sought to give a scientific basis to plants already used for traditional purposes as bacterial efflux pump inhibitors (EPIs). A decline in minimum inhibitory concentration (MIC) 1/2 to 1/8 was used to determine the activity of 35 Indian medicinal plant extracts as drug EPIs against various bacterial strains. Fluoroquinolones (ciprofloxacin and ofloxacin) possesses antibacterial activity and when combined with plant extracts resulted in synergistic interactions by declining its MIC to 1/2 in 10 plants and 1/4 in 6 plants. Methanolic extracts of twelve plants were found to be putative EPI hence, the compounds in these extracts can serve as templates of new antibacterial agents. Plant extracts significantly enhanced accumulation and decreased the efflux of fluoroquinolones in bacterial strains, suggesting their ability to inhibit bacterial efflux pumps. EPI reduced the ciprofloxacin and ofloxacin MIC by 1/4 in 3 and 1 strains respectively. Our data support the fact that increased fluoroquinolones usage can negatively impact susceptibility of bacterial strains. Plants should be further exploited for their potential to produce compounds capable of blocking the mechanism of efflux.

Keywords: Multidrug resistance (MDR), minimum inhibitory concentration (MIC), efflux pump inhibitors (EPIs), Fluoroquinolones.

1. Introduction

Bacteria cause serious infections in humans as well as other animals. The indiscriminate use of antibiotics has led to the development of multidrug-resistant pathogens. Around 90-95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin [1] and in most Asian countries, 70-80% of the same strains have become methicillin resistant [2]. There are reports on the development of resistance to the last line of antibiotic defense, which has led to a search for reliable methods to control vancomycin-resistant enterococci (VRE), *S. aureus* (VRSA), and methicillin resistant *S. aureus* (MRSA) [3]. The rapid spread of bacteria expressing multidrug resistance (MDR) has necessitated the discovery of new antibacterials and resistance modifying agents. Since the initial discovery of bacterial efflux pumps in the 1980s, many have been characterized in community and hospital acquired Gram-positive and Gram-negative pathogens such as, *S. aureus*, *P. aeruginosa* and *E. coli* [4]. Efflux-related multidrug resistance (MDR) has become appreciated as a significant complicating factor in the chemotherapy of bacterial infections. Most of the pumps that transport drugs in gram-positive bacteria are members of the major facilitator superfamily (MFS). Efflux pumps are able to extrude structurally diverse compounds, including antibiotics used in a clinical setting, rendering the drug therapeutically ineffective [5]. 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 [6].

Natural product medicines have come from various source materials including terrestrial plants [7]. The use of indigenous knowledge of traditional medical practitioners as leads provides a useful route employed in the search for novel drugs. This is rewarding, and since most indigenous plants used in traditional medicine have not been explored in detail, the potential for discovery of more novel therapeutic compounds through bioprospecting of the flora is tremendous [5]. The antimicrobial potential of different medicinal plants are being extensively studied all over the world, but only a few studies have been carried out in a systematic manner [8, 9]. In 1998 it was shown that plant-derived compounds have activity against Gram-positive

bacteria, in particular *S. aureus* [10]. Several compounds, such as reserpine, behave as if they inhibit efflux pumps and hence known as efflux pump inhibitors (EPIs) [11]. Reserpine is an antihypertensive plant alkaloid that was first isolated from the roots of *Rauwolfia vomitoria* and was found to reverse Nor A-conferred MDR [4].

Antibiotic resistance can develop rapidly through changes in the expression of efflux pumps. It is therefore, imperative that new antibiotics, resistance modifying agents and more specifically, efflux pump inhibitors (EPIs) are characterized [12]. The use of bacterial resistance modifiers such as EPIs could facilitate the re-introduction of therapeutically ineffective antibiotics back into clinical use and might even suppress the emergence of MDR strains [4]. In the present study 35 medicinal plants of different families were selected to assess their EPI potential. Some of the plants are known for their use as traditional medicines to cure common ailments while some of them were randomly selected with no history of use in traditional medicine. To give a scientific basis to the use of these plants, methanolic extracts from these plants were investigated for synergistic activity with fluoroquinolones (ciprofloxacin and ofloxacin) against some bacterial strains. The identification and development of safe and effective inhibitors of bacterial efflux pumps are needed.

2. Materials and Method

2.1 Selection of plant material

Medicinal Plants belonging to different families (Table 2) were selected on the basis of traditional applications and pharmacological reports. The plant materials were collected from herbal gardens, surroundings and local market. Voucher plant specimen was deposited at the Wild Life Institute of India, Dehradun, under specimen numbers GS-401 to GS- 437 for future reference (Table 2).

2.2 Preparation of Plant Extracts

The samples were carefully washed under running tap water followed by sterile water and shade dried for 4-5 days. The dried plant materials were ground to powder and stored in airtight containers. Methanol was used for extraction. 10 g of powdered sample was soaked in conical flask containing 100ml of methanol for 24 hrs. Conical flask was allowed to stand for 30 mins in a water bath (at 100 °C) with occasional shaking followed by keeping all the flasks on rotary shaker at 200 rpm for 24 h [13]. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40 °C using a rotary evaporator. The dried extract, thus, obtained was sterilized by overnight UV-irradiation, checked for sterility on nutrient agar plates and stored at 4 °C in refrigerator for further use [14]. The dried extracts were reconstituted to 10% in dimethylsulphoxide (DMSO) for the antibacterial analysis.

2.3 Microbial strains:

The microorganisms used in present study were procured from Institute of Microbial Technology, Chandigarh (IMTECH), National Dairy Research Institute, Karnal (Table 1). Bacterial strains [*Staphylococcus aureus* (SA-1 to 21) and *Enterococcus* species (E-1 to 17)].

2.4 Chemicals

Beef extract, yeast extract, peptone, sodium chloride, agar agar, DMSO and methanol were purchased from Hi-media Pvt. Ltd. Mumbai. Efflux pump inhibitor of ofloxacin i.e. Plumbagin (EPI_o) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Efflux pump inhibitor of ciprofloxacin i.e. Piperine (EPI_c) was purchased from Natural remedies Pvt. Ltd. Bangalore. All other antibiotics and chemicals used were of highest purity grade available commercially.

*__Slantez and Bartley Agar media__ (Readymade) was used for *Enterococcus species* and was procured from Hi-media Pvt. Ltd. Mumbai.

2.5 Antibacterial susceptibility testing

Agar well diffusion method was carried out by allowing perforation of various antibiotics (10 µg/well)/plant extracts (30 mg/well) dissolved in 10% DMSO. Petri plates containing 30 ml nutrient agar medium were kept for solidification followed by spreading 100 µl (10^6 CFU/ml) of microorganism (24 hour broth culture). Desired number of wells of uniform diameter of 8 mm was made after solidification, using sterile aluminium borer. 0.1 ml of each test sample and negative (solvent blank) controls were poured into wells. After incubation for 24 h at respective incubation temperature, the plates were observed and the antibacterial activity was evaluated by presence or absence of zone of inhibition (diameter in mm). The tests were conducted in triplicate. The negative control was 10% DMSO.

2.6 Minimum Inhibitory Concentration (MIC)

MIC of fluoroquinolones (ciprofloxacin and ofloxacin) was determined by microdilution technique as described by the National Committee for Clinical Laboratories standards [15]. The bacterial inoculums were prepared in 5 ml nutrient broth and incubated at respective incubation temperature. The final inoculums were of approximately 10^6 CFU/ml. Controls with 0.5 ml of culture medium without the samples and other without microorganisms were used in the tests. Tubes were incubated for 24 h. The activity was measured as a function of turbidity at 660 nm. Lack of turbidity was further confirmed by pouring suspension aliquot of 0.1 ml into pre-sterilized Petri dishes containing nutrient agar medium. The tests were conducted in triplicate.

2.7 Screening of Bacteria possessing EPI

2.7.1 Two fluoroquinolones efflux pumps (Ciprofloxacin and Ofloxacin) were studied. Reported commercially available Efflux pump inhibitor of Ciprofloxacin (EPI_c) i.e. piperine and Ofloxacin (EPI_o) i.e. plumbagin were studied, to check decline in MIC of Ciprofloxacin and Ofloxacin respectively among studied bacterial strains.

2.7.2 EPI_c and EPI_o (30 µg/ml of 3% DMSO) has no direct antibacterial activity but when they were synergistically tested with ciprofloxacin and ofloxacin (10 µg/ml of 10% DMSO), their MIC tend to decline by 1/2, 1/4, 1/8 and 1/16.

2.7.3 The short listed bacterial strains (Table 1) in which the MIC level was declined due to respective efflux pump inhibitors presence were carried for further studies with medicinal plants.

2.8 Direct antibacterial activity studies and EPI Evaluation Assay

2.8.1 Direct antibacterial activity of methanolic extracts of 35 Indian medicinal plants (Table 2) against the selected bacterial strains possessing ciprofloxacin efflux pump and ofloxacin efflux pump (Table 1) was checked according to the standard protocols of NCCLS [15]. The short listed plants which possessed no or little direct antibacterial activity were carried out for further studies.

2.8.2 Potentiation for efflux pump inhibition experiment was performed by testing synergism of methanol extracts of plants (Table 2) with ciprofloxacin/ofloxacin against selected bacteria (Table 1). [50 µl of plant extract (30 mg/ml) + 50 µl of Ciprofloxacin/Ofloxacin (10 µg/ml) at its MIC, 1/2 MIC, 1/4 MIC]

2.8.3 The methanolic plant extracts which declined the MIC levels of particular antibiotic were noted.

3. Results

3.1 Screening of Bacteria possessing EPI

MICs of antibiotics determined in absence of efflux inhibitors were compared with those determined in presence of standard efflux pump inhibitors i.e. piperine for ciprofloxacin and plumbagin for ofloxacin. It was observed that the MIC levels of ciprofloxacin were lowered in 16 of 57 (Table 1) bacteria in the presence of piperine. The results indicate that 28.07% of bacterial strains attained antibiotic resistance due to active efflux pump of ciprofloxacin. 16 bacterial strains declined the ciprofloxacin MIC to 1/2. Among 16, 11 were Gram positive and 5 were Gram negative.

Similarly, MIC levels of ofloxacin were declined in 11 of 57 (Table 1) strains in the presence of plumbagin. In the presence of plumbagin the MIC values for ofloxacin were found to decrease upto 1/4 in 1 strain and upto 1/2 in 10 strains. Out of 11 bacterial strains, 10 were Gram positive and 1 was Gram negative.

Table 1: List of bacterial strains possessing EPI.

Sr. No.	Bacterial strains	Ci + Piperine	Of + Plumbagin
		Decline in Ci MIC	Decline in Of MIC
1	*Bacillus cereus* (MTCC 430)	1/2	NE
2	*Bacillus polymyxa* (NCDC 68)	NE	1/2
3	*Bacillus pumilus* (MTCC 7411)	NE	NE
4	*Bacillus stearothermophilus* (MTCC 8505)	1/2	NE
5	*Bacillus subtilis* (MTCC 8509)	NE	1/2
6	*Bacillus subtilis* (MTCC 121)	1/2	1/2
7	*Lactobacillus brevis* (NCDC 371)	NE	NE
8	*Lactobacillus plantarum* (NCDC 20)	NE	NE
9	*Staphylococcus aureus* (MTCC 3160)	1/2	NE
10	*Staphylococcus aureus* (MTCC 109)	1/2	NE
11	*Staphylococcus epidermidis* (MTCC 3086)	NE	NE
12	*Staphylococcus epidermidis* (MTCC 435)	NE	NE
13	*Staphylococcus hominis* (MTCC 4435)	NE	1/2
14	*Escherichia coli* (MTCC 1885)	1/2	1/2
15	*Klebsiella pneumoniae* (MTCC 4030)	1/2	NE
16	*Pediococcus acidilactici* (NCDC 252)	NE	NE
17	*Proteus vulgaris* (MTCC 426)	1/2	NE
18	*Pseudomonas aeruginosa* (MTCC 424)	1/2	NE
19	*Pseudomonas aeruginosa* (MTCC 7453)	1/2	NE
20	SA-1	NE	NE
21	SA-2	NE	1/2
22	SA-3	NE	NE
23	SA-4	NE	NE
24	SA-5	NE	NE
25	SA-6	NE	NE
26	SA-7	1/2	NE
27	SA-8	NE	1/2
28	SA-9	NE	NE
29	SA-10	NE	1/2
30	SA-11	NE	NE
31	SA-12	NE	NE
32	SA-13	1/2	NE
33	SA-14	NE	NE
34	SA-15	NE	NE
35	SA-16	1/2	1/2
36	SA-17	NE	NE
37	SA-18	NE	NE
38	SA-19	NE	NE
39	SA-20	NE	1/4
40	SA-21	NE	1/2
41	E-1	NE	NE
42	E-2	NE	NE
43	E-3	NE	NE

Continue Table 1.

44	E-4	NE	NE
45	E-5	1/2	NE
46	E-6	NE	NE
47	E-7	NE	NE
48	E-8	NE	NE
49	E-9	NE	NE
50	E-10	NE	NE
51	E-11	NE	NE
52	E-12	NE	NE
53	E-13	NE	NE
54	E-14	NE	NE
55	E-15	NE	NE
56	E-16	NE	NE
57	E-17	NE	NE

SA: *Staphylococcus aureus*; E: *Enterococcus*; Ci: Ciprofloxacin; Of: Ofloxacin; NE: No effect

3.2 Screening of medicinal plants as potential efflux pump inhibitor

Bacterial efflux pumps clearly contribute to the increasing problem of multi-drug resistance (MDR). Identification of inhibitors of efflux pumps for which antimicrobial agents are substrates is an active area of research in both the pharmaceutical and academic sectors [16, 17, 18, 19]. When the sixteen bacteria (Table 1) were tested for antibacterial studies for 35 medicinal plants (Table 2), only ten plants possessed potential of declining MIC of ciprofloxacin upto 1/2 and 1/4 (Table 2). Only those plants which didn't possess direct antibacterial activity were taken for this study. Among the ten medicinal plants screened which possessed latent of declining MIC of ciprofloxacin, *C. annuum* covered 25% of bacterial strains, followed by *C. longa* (18.75%). Similarly, when 11 bacteria (Table 1) were tested for antibacterial studies for 35 medicinal plants, only two plants (viz., *C. fistula* and *M. indica*) possessed potential of declining MIC of ofloxacin upto 1/2 and 1/4 (Table 2).

Table 2: List of methanol extracts of plants possessing potentiation as EPI of ciprofloxacin and ofloxacin.

Sr. No.	Plant name (Part used) (Plant Specimen Voucher No.)*	Bacterial strain	Ciprofloxacin MIC decline	Ofloxacin MIC decline
1	*Acacia nilotica* (Leaf) (GS-440)*	-	-	-
2	*Aegle marmelos* (leaf) (GS-441)*	-	-	-
3	*Amomum subulatum* (Dried fruit) (GS-405)*	-	-	-
4	*Azadirachta indica* (leaf) (GS-415)*	*K. pneumoniae* 4030 *E. coli* 1885	1/2 **1/4**	-
5	*Brassica campestris* (Seeds) (GS-438)*	-	-	-
6	*Calotropis procera* (leaf) (GS-422)*	*S. aureus*-13	**1/4**	-
7	*Capsicum annuum* (Fruit) (GS-408)*	*S. aureus* 109 *S. aureus* 3160 *S. aureus*-16 *S. epidermidis* 435	1/2 1/2 1/2 1/2	-
8	*Cassia fistula* (leaf) (GS-414)*	*E. coli* 1885 *Bacillus subtilis* 8509 *Bacillus polymyxa*	-	1/2 1/2 **1/4**
9	*Catharanthus roseus* (leaf) (GS-418)*	*S. aureus* 3160 *K. pneumoniae* 4030	1/2 1/2	-
10	*Cinnamomum zeylanicum* (Bark) (GS-411)*	-	-	-
11	*Coriandrum sativum* (Seeds) (GS-407)*	-	-	-

Continue Table 2.

12	*Cuminum cyminum* (Seeds) (GS-401)*	-	-	-
13	*Curcuma longa* (Rhizome) (GS-409)*	*S. aureus* 109 *S. epidermidis* 435 *S. epidermidis* 3086	1/2 1/2 **1/4**	-
14	*Cyanodon dactylon* (Whole plant) (GS-435)*	-	-	-
15	*Diospyros melanoxylon* (leaf) (GS-421)*	-	-	-
16	*Elettaria cardamomum* (Dried fruit) (GS-404)*	*E. coli* 1885	1/2	-
17	*Foeniculum vulgare* (Seed) (GS-431)*	-	-	-
18	*Lawsonia inermis* (leaf) (GS-420)*	*K. pneumoniae* 4030	1/2	-
19	*Mangifera indica* (leaf) (GS-432)*	*Bacillus polymyxa*	-	**1/4**
20	*Murraya koenigii* (leaf) (GS-419)*	-	-	-
21	*Myristica fragrans* (Fruit) (GS-412)*	-	-	-
22	*Nicotiana tabacum* (leaf) (GS-424)*	-	-	-
23	*Ocimum sanctum* (leaf) (GS-433)*	-	-	-
24	*Phyllanthus emblica* (leaf) (GS-426)*	-	-	-
25	*Piper nigrum* (Fruit) (GS-406)*	-	-	-
26	*Psidium guajava* (leaf) (GS-417)*	-	-	-
27	*Rosa indica* (Flower) (GS-423)*	-	-	-
28	*Sesame indicum* (Seed) (GS-436)*	*S. epidermidis* 3086 *B. stearothermophilus* 8505	**1/4** 1/2	-
29	*Syzygium aromaticum* (Dried buds) (GS-402)*	-	-	-
30	*Tagetes erecta* (Flower) (GS-416)*	-	-	-
31	*Tectona grandis* (leaf) (GS-425)*	-	-	-
32	*Terminalia chebula* (Fruit) (GS-410)*	-	-	-
33	*Trigonella foenum graecum* (Seeds) (GS-413)*	*S. aureus*-16 *B. stearothermophilus* 8505	1/2 1/2	-
34	*Zingiber officinale* (Rhizome) (GS-403)*	*S. aureus*-13	1/2	-
35	*Ziziphus mauritiana* (leaf) (GS-437)*	-	-	-

-: No effects

4. Discussion

The EPI evaluation assay studies suggest that some moderate or strong polar components play a role in inhibiting bacteria. The basis of varying degree of sensitivity of bacteria may be due to varied efflux pump availability in different strains and the nature and combination of phytocompounds present in crude extract. Out of the 35 plants studied the methanol extracts of 12 plants were more effective in blocking drug efflux pumps. A possible reason for this would be the presence of abundant chemical compounds in the extracts with efflux pump inhibitory activity. Hence, these extracts have a promising future for the development of effective EPIs which would augment the antibacterial activities of standard antibiotics. The identification of plants able to inhibit efflux pumps is important as they provide a potential lead optimization and future use with an existing antibacterial rendered ineffective due to MDR pumps in both Gram-positive and Gram-negative bacteria.

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. As ciprofloxacin is a substrate of many bacterial efflux pumps [6], the experiments used a simple assay to identify plant extracts that synergised with fluoroquinolones [20]. This allowed many plant extracts made under different conditions to be screened with speed. Our studies are supporting Stavri *et al*. [4], as the majority of plant extracts display EPI activity towards Gram-positive bacteria.

Tariro *et al*., [21] reported *M. indica* ethanolic extracts of stem and twigs as potent EPI against *B. cereus*, *B. subtilis* and *P. aeruginosa* using Rhodamine 6G accumulation experiments. Our findings also support them in methanol extracts against *B. polymyxa* by declining the MIC of ofloxacin to 1/4.

Our study revealed that methanol extracts of *C. longa* exhibit resistance modifying activity (EPI activity) against *S. epidermidis* by declining the MIC of ciprofloxacin to 1/4 contradicting Pinanong *et al*., [22] (novobiocin synergy studies against *Acinetobacter baumannii*).

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.

5. Conclusion

Efflux mechanisms have become broadly recognized as major components of resistance to many classes of antibiotics. Among all studied strains the MIC decline potential was only 1/2 with piperine (EPI_c) and plumbagin (EPI_o). But in the present study six medicinal plants revealed upto 1/4 MIC decline, indicating more potentiation as efflux pump inhibitor as compared to standard EPI_c and EPI_o. Present studies are also in accordance with the earlier reports, that the vast majority of EPI, are active against gram positive bacteria and particularly in *Staphylococcus* strain. There are numerous potentially beneficial consequences of the inhibition of efflux pumps in improving the clinical performance of various antibiotics. The search of potential efflux pump inhibitors provides an approach to generate therapy by interaction between different mechanisms of resistance. The present study indicates a high potential of EPI in Indian medicinal plants. The activity of some of the extracts is appreciable and warrant further study as possible candidates for lead optimization. This kind of approach decreases the frequency of emergence of resistant strains also. The extracts from twelve plants showed activity as efflux pump inhibitors. Lead compounds from such plant extracts need to be isolated so that they can serve as templates for the production of new antibiotics as well as efflux pump inhibitors. Inhibition of MDR efflux pumps can restore the activities of antibacterial agents.

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