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Lawsonia inermis: A potent efflux pump inhibitor of fluoroquinolones

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Abstract: Antibiotic resistance in bacterial strains always remains a critical concern. Mutations in antibiotic target sites, over expression of efflux pump are the major modes of development of antibiotic resistant. The present study was conducted to determine fluoroquinolones antibiotic resistance and role of efflux pumps in fluoroquinolone resistance by using efflux pump inhibitors. Fifty seven bacterial strains were tested against ciprofloxacin and ofloxacin by agar well diffusion assay. Twenty four bacterial strains were found to be resistant against ciprofloxacin and 19 against ofloxacin. Further, fluoroquinolone sensitive strains were tested to observe the decline in Minimum inhibitory concentration (MIC) levels in presence/absence of standard efflux pump inhibitors (piperine and plumbagin). Leaf extracts of *Lawsonia inermis* were tested as efflux pump inhibitor (EPI) against the selected bacterial strains. Fluoroquinolones (ciprofloxacin) possesses antibacterial activity and when combined with various subfractions resulted in synergistic interactions by declining its MIC to 1/2 and 1/4 against *K. pneumoniae*. Hence, the compounds in these extracts can serve as templates of new antibacterial agents as well as EPIs.

Keywords: Antibiotic-resistance, Efflux pump inhibitors, Fluoroquinolones, MIC, antibiotic

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The development of antibiotics in 1940s offered a powerful weapon against bacterial infections and saved the lives of many people. In many parts of world the emergence of new, rare or already forgotten infectious diseases has stimulated interest to generate new drugs against antibiotic resistant strains. There are various social and medical factors responsible for the development of antibiotic resistance among these antibiotics. In hospitals, 190 million doses of antibiotics are administered each day. More than 133 million courses of antibiotics are coursed by non hospitalized patients. About 50% of these prescriptions are unnecessary. In many cases full dosage is also not done. Such improper use and abuse of antibiotics contribute high in generating these antibiotic resistant bacterial strains. Besides direct consumption, agricultural practices account for over 60% antibiotic usage (Stuart and Levy, 2002; Bhardwaj and Mohanty, 2012; Dyar et al. 2012). The bacteria silently develop several ways to resist antibiotics. Since the bacteria multiply rapidly, this mutation quickly becomes dominant throughout the microbial population. Antibiotic resistance mechanisms can be broadly divided into three categories (Walsh, 2000) *viz.* inactivation of the antibiotic, efflux pump inhibiton and modification of antibacterial target sites. This increasing prevalence of multidrug resistance strains of bacteria and recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection fighting strategies (Sieradazki et al.1999).

Among these mechanisms, resistance in bacteria acquired by efflux pump remains crucial. Efflux pumps plays a vital role to build the multidrug resistance as they export several unrelated substances including molecules produced by host organisms as well as various types of antibiotics and chemicals such as dyes, organic solvents and detergents, molecules needed for the cell-cell communication, biocides and metabolic products (Schweizer, 2003; Vidal et al. 2009; Okandeji et al. 2011).

The use of bacterial resistance modifiers such as EPIs could facilitate the re-introduction of the-

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rapeutically ineffective antibiotics back into clinical use and might even suppress the emergence of MDR strains (Stavri et al. 2007). The identification and development of safe and effective inhibitors of bacterial efflux pumps are needed.

Plant kingdom is a gold mine for novel and affordable antimicrobials. Plant derived medicines have been part of traditional health care in most of the world for thousands of years and there is increasing interest in them as source of agents to fight microbial diseases (Ajayi and Akintola, 2010). The medicinal effects of plants are due to metabolites especially secondary compounds produced by plant species (Bharti et al. 2012). Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins and several other aromatic compounds are secondary metabolites of plants that serve a defense mechanism against predation by many micro-organisms, insects and herbivores (Bonjor et al. 2004).

Lawsonia inermis belonging to family Lythraceae is a perennial plant. It is commonly known as Henna or Mehandi. Its leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of ailments as rheumatoid arthritis, headache, ulcers, diarrheoa, leprosy, fever, leucorrhoea, diabetes, cardiac disease, hepatoprotective and colouring agent. (Chetty, 2008, Chopra et al. 1956, Reddy, 1988). Lawsone , the antimicrobial agent in henna (Malekzadeh, 1968; Sharma et al. 1995) exerted inhibitory effects upon common nosocomial urinary tract pathogens such as *E. coli, P. mirabilis, K. pneumoniae, P. aeruginosa* and *S. aureus* at certain concentrations (Bhuvaneswari et al. 2002).

The present study aims to check the availability of resistance to fluoroquinolones among random samples of bacteria and to determine the prevalence of the resistance due to efflux pump. *L. inermis* was selected to assess the EPI potential. To give the exact source to the use of this plant, different extracts from this plant were investigated for synergistic activity with fluoroquinolones (ciprofloxacin and ofloxacin) against bacterial strains.

MATERIAL AND METHODOLOGY

Sample size and Sample Collection

A total of 57 bacterial strains from various sources were sampled in the study. Thirteen Gram positive {*Bacillus cereus* (MTCC 430), *Bacillus polymyxa* (NCDC 68), *Bacillus pumilus* (MTCC 7411), *Bacillus stearothermophilus* (MTCC 8505), *Bacillus subtilis* (MTCC 8509 and MTCC 121), *Lactobacillus brevis* (NCDC 371), *Lactobacillus plantarum* (NCDC 20), *Staphylococcus aureus* (MTCC 3160 and MTCC 109), *Staphylococcus epidermidis* (MTCC 3086 and MTCC 435), *Staphylococcus hominis* (MTCC 4435)} and Six Gram negative {*Escherichia coli* (MTCC 1885), *Klebsiella pneumoniae* (MTCC 4030), *Pediococcus acidilactici* (NCDC 252), *Proteus vulgaris* (MTCC 426), *Pseudomonas aeruginosa* (MTCC 424 and MTCC 7453)} bacterial strains were procured from Microbial Type Culture Collection Institute of Microbial Technology, Chandigarh (MTCC), National Dairy Research Institute, Karnal (NCDC). Twenty one *Staphylococcus aureus* strains (SA-1 to SA-21) and Seventeen *Enterococcus* strains (E-1 to E-17) were isolated from various nasal and milk samples of chicken and buffaloes.

Isolation and Identification of Bacteria

The different Bacterial samples were isolated and cultured by standard methods. Morphologically distinct colonies of *Staphylococcus aureus* (black coloured) and *Enterococcus* strains (Mehroon coloured) were obtained on Baird Parker agar and Slantez and Bartley Agar media (Hi-Media, Mumbai, India) respectively which are specific media for them. The bacterial isolates were identified on the basis of standard cultural, morphological and biochemical characteristics (Cowan and Steel, 1985). Samples procured from MTCC and NCDC were cultured on Nutrient agar media.

Chemicals

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

Baird parker agar media (Readymade) was used for *Staphylococcus aureus* and was procured from Hi-media Pvt. Ltd. Mumbai.

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

Antibiotics susceptibility test

The antibiotics Ciprofloxacin (Ci) and Ofloxacin (Of) belonging to fluoroquinolones class were used in this study. Bacterial strains were tested against these antibiotics (10µg/ml of 10% DMSO). Disc diffusion method was performed, with an inoculum of 10^6 CFU (Macfar Land standard) on nutrient agar media NCCLS (2000). After incubation for 24 h at 37^0 C, the plates were observed and the antibiotic susceptibility/ resistance 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.

Prevalence of Efflux pumps in bacterial samples EPI Inhibitors

Efflux Pump Inhibitors (EPI) are compounds that have ability to reduce (2 to 8 times) or reverse antibiotic resistance to given antibiotics (Escribano et al. 2007). Piperine and Plumbagin are reported as potentiating

EPI of Ciprofloxacin and Ofloxacin antibiotics (Khan et al. 2006; Stavri et al. 2007).

Minimum Inhibitory Concentration (MIC) Determination
MIC of 57 bacterial strains against Ciprofloxacin and Ofloxacin antibiotics (10µg/ml of 10% DMSO) was determined by microdilution technique. The final inoculums of approximately 10^6 CFU/ml were prepared in 5 ml nutrient broth. Positive controls without the antibiotics were taken. Tubes were incubated at 37^0 C 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 with respective medium. The tests were conducted in triplicate. The MIC was defined as the lowest concentration of compound that inhibited visible growth.

Effect of potentiative EPI on MIC levels of Antibiotics
To determine the extent of the efflux pump mediated antibiotic resistance in various bacterial isolates, MIC levels for ciprofloxacin and ofloxacin were determined in the presence and absence of Piperine (30 µg/ml in 10% DMSO) and plumbagin (30 µg/ml of 10% DMSO) respectively. Per cent Efflux Pump prevalence was determined.

Selection of plant material
Lawsonia inermis belonging to family Lythraceae was selected on the basis of traditional applications and pharmacological reports. *The plant material (leaves) was collected from Botanical Garden Kurukshetra University. Authentication of plant material was done from Wild Life Institute of India, Dehradun with specimen number GS-420.*

Preparation of Plant Extract
The leaves were carefully washed under running tap water followed by sterile water and shade dried for 4-5 days. The dried leaves were ground to powder and stored in airtight containers. Plants secondary metabolites possess various biological activities and methanol is capable of extracting a wide range of polar and rather non-polar compounds such as alkaloids, sterols, flavonoids and carbohydrates due to its high polarity therefore it was used for extraction. 10g of powdered leaves 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 24h (Ogundiya, 2006). 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. (Aneja et al. 2010). The dried extracts were reconstituted to 10% in dimethylsulphoxide (DMSO) for the antibacterial analysis.

Direct antibacterial activity studies and EPI Evaluation Assay:
- Direct antibacterial activity of methanolic extracts of *L. inermis* against the selected bacterial strains possessing ciprofloxacin efflux pump and ofloxacin efflux pump was checked according to the standard protocols of NCCLS, 2000.
- The bacterial strain in which the MIC level was declined due to respective efflux pump inhibitors (piperine and plumbagin) presence were carried for further studies with methanol extract.
- Potentiation for efflux pump inhibition experiment was performed by testing synergism of methanol extract with ciprofloxacin/ofloxacin against selected bacteria. [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]
- Decline in the MIC levels of particular antibiotic were noted.

Preparation of sub fractions
Sub fractions of the methanol extracts of *L. inermis* were prepared in three solvents on the basis of increasing order of their polarity i.e. n-hexane, chloroform and ethyl acetate (Bai et al. 2014). To prepare the sub fractions the methanol extracts of the plants were dissolved in hot water. The aqueous solution of methanol extract was transferred into a separating funnel for partitioning with n- hexane, chloroform and ethyl acetate successively. Each sub fraction was dried in rotary evaporator and stored in refrigerator for further use. EPI assay of the three sub fractions: n-hexane, chloroform and ethyl acetate along with methanol extract were determined.
EPI Evaluation Assay of fractions was performed as discussed above.

RESULTS

Resistance to Fluoroquinolones
All the fifty seven bacterial strains from various sources were tested for resistance against the 2 fluoroquinolones i.e. ciprofloxacin and ofloxacin. The studied strains showed high resistance to ciprofloxacin (42.11%) and ofloxacin (33.33%). The detailed observations of antibiotic resistance are presented in Table 1.

Effect of EPIs on Antibiotic MICs
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 2) 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 (Table 3).

Table 1 Resistance of various bacterial strains against ciprofloxacin and ofloxacin

S.No	Bacterial strains	Ciprofloxacin	Ofloxacin	S.No	Bacterial strains	Ciprofloxacin	Ofloxacin
1	*B. cereus* (MTCC 430)	S	S	30	*S. aureus* SA-11	S	R
2	*B. polymyxa* (NCDC 68)	S	S	31	*S. aureus* SA-12	R	S
3	*B. pumilus* (MTCC 7411)	S	S	32	*S. aureus* SA-13	S	S
4	*B. stearothermophilus* (MTCC 8505)	S	S	33	*S. aureus* SA-14	S	S
5	*B. subtilis* (MTCC 8509)	S	S	34	*S. aureus* SA-15	S	S
6	*B. subtilis* (MTCC 121)	S	S	35	*S. aureus* SA-16	S	S
7	*L. brevis* (NCDC 371)	S	S	36	*S. aureus* SA-17	R	S
8	*L. plantarum* (NCDC 20)	R	R	37	*S. aureus* SA-18	S	S
9	*S. aureus* (MTCC 3160)	S	S	38	*S. aureus* SA-19	R	S
10	*S. aureus* (MTCC 109)	S	R	39	*S. aureus* SA-20	R	S
11	*S. epidermidis* (MTCC 3086)	R	S	40	*S. aureus* SA-21	S	S
12	*S. epidermidis* (MTCC 435)	S	S	41	*Enterococcus* E-1	S	S
13	*S. hominis* (MTCC 4435)	S	S	42	*Enterococcus* E-2	R	R
14	*E.coli* (MTCC 1885)	S	S	43	*Enterococcus* E-3	R	R
15	*K. pneumoniae* (MTCC 4030)	S	S	44	*Enterococcus* E-4	S	S
16	*P. acidilactici* (NCDC 252)	S	S	45	*Enterococcus* E-5	S	S
17	*P. vulgaris* (MTCC 426)	S	S	46	*Enterococcus* E-6	S	S
18	*P. aeruginosa* (MTCC 424)	R	S	47	*Enterococcus* E-7	S	R
19	*P. aeruginosa* (MTCC 7453)	S	S	48	*Enterococcus* E-8	R	R
20	*S. aureus* SA-1	S	S	49	*Enterococcus* E-9	R	R
21	*S. aureus* SA-2	R	S	50	*Enterococcus* E-10	R	R
22	*S. aureus* SA-3	R	R	51	*Enterococcus* E-11	R	R
23	*S. aureus* SA-4	S	S	52	*Enterococcus* E-12	R	R
24	*S. aureus* SA-5	R	R	53	*Enterococcus* E-13	R	R
25	*S. aureus* SA-6	S	R	54	*Enterococcus* E-14	R	R
26	*S. aureus* SA-7	S	S	55	*Enterococcus* E-15	R	R
27	*S. aureus* SA-8	S	S	56	*Enterococcus* E-16	R	R
28	*S. aureus* SA-9	R	S	57	*Enterococcus* E-17	R	S
29	*S. aureus* SA-10	R	R				

R: Resistant; S: Sensitive;

Similarly, MIC levels of ofloxacin were declined in 11 of 57 (Table 2) 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 4).

Lawsonia inermis extracts 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 (Markham, *et al.*, 1999; Aeshlimann et al.1999; Lomovskaya et al.2001; Guz et al.2001). *L. inermis* methanol extract obssessed latent of declining MIC of ciprofloxacin to 1/2 covering, 6.25% of bacterial strains *i.e* against *K. pneumoniae*. But, there was no effect of *L. inermis* methanol extract on MIC of ofloxacin against 11bacteria (Table 4).

The fraction based studies show that there was no effect of hexane extracts and chloroform extracts in declining the MIC of ciprofloxacin. While ethyl acetate extract declined the MIC of ciprofloxacin to 1/4 (Table 5). The difference in the activity of extracts may be described by the difference in total phenolic and flavonoid content. Flavonoids are not very polar, they tend to accumulate in the ethyl acetate rather than the more polar methanol extract. Methanol is capable

Table 2 Decline in antibiotics MIC of Bacterial strains (57) in presence of efflux inhibitors

Efflux inhibitors (No. of isolates)	Fold changes in presence of efflux inhibitor in ciprofloxacin resistant isolates (%)	Fold changes in presence of efflux inhibitor in ofloxacin resistant isolates (%)	
	2 fold	2 fold	4 fold
Piperine (n=16; 28.07%)	16 (28.07%)	-	-
Plumbagin (n=11; 19.29%)	-	10 (17.54%)	1 (1.75%)

Table 3 List of bacterial strains possessing declined MIC levels of ciprofloxacin in presence of piperine

S. No	Bacterial strains	MIC of Ciprofloxacin (µg/ml)	Declined MIC of Ciprofloxacin (µg/ml)
1.	*B. cereus*	5.000	2.500
2.	*B. stearothermophilus*	2.500	1.250
3.	*B.subtilis*121	5.000	2.500
4.	*E. coli*	2.500	1.250
5.	*K. pneumoniae*	1.250	0.625
6.	*P. vulgaris*	0.312	0.156
7.	*P. aeruginosa* 424	1.250	0.625
8.	*P. aeruginosa*7453	1.250	0.625
9.	*S. aureus* 109	5.000	2.500
10.	*S. aureus* 3160	2.500	1.250
11.	*S. aureus* 7	5.000	2.500
12.	*S.aureus*13	1.250	0.625
13.	*S. aureus* 16	2.500	1.250
14.	*S. epidermidis*1435	2.500	1.250
15.	*S. epidermidis* 3086	5.000	2.500
16.	*Enterococcus* 5	5.000	2.500

Table 4 List of bacterial strains possessing declined MIC levels of ofloxacin presence of plumbagin

S. No	Bacterial strains	MIC of Ofloxacin (µg/ml)	Declined MIC of Ofloxacin (µg/ml)
1.	*B.subtilis*121	5.0	2.500
2.	*B. polymyxa*	5.0	2.500
3.	*B. subtilis* 8509	5.0	2.500
4.	*E. coli*	5.0	2.500
5.	*S. hominis*	2.5	1.250
6.	*S. aureus* 2	5.0	2.500
7.	*S. aureus* 8	5.0	2.500
8.	*S. aureus* 10	Resistant	10.00
9.	*S. aureus* 16	5.0	2.500
10.	*S. aureus* 20	2.5	0.625
11.	*S. aureus* 21	5.0	2.500

of extracting a wide range of polar and rather non-polar compounds such as alkaloids, sterols, flavonoids and carbohydrates. Flavonoids with hydroxyl group are soluble in methanol. Monoglycosylated compounds are extracted by ethyl acetate (Lin Ma et al. 2009, Koruthu et al. 2011, Anwar et al. 2012).Hence, the ethyl acetate and methanol extract containing high polar compounds possessed the best EPI activity because active principles might have eluted in high polarity solvent like ethyl acetate as compared to n-hexane and chloroform (low polarity). 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 bacterial strains.

DISCUSSION

Data from the present study revealed that the MICs of 19.29 % and 28.07 % bacterial strains were affected in the presence of plumbagin and piperine respectively suggesting the importance of efflux pumps in ofloxacin and ciprofloxacin resistant bacterial strains respectively. Reduction in MIC level in presence of plumbagin and piperine inhibitors provide evidence for the presence of both type proton motive force and ATP dependent extrusion system involved in fluoroquinolones resistance (Singh et al. 2011).

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 (Stavri et al. 2007). Singh and co-workers, (2011) have also reported the reversal of resistance to ofloxacin in presence of efflux pump inhibitors {(CCCP (35.5%), DNP (46.6%) and verapamil (53.3%)} in *M. tuberculosis* isolates. The present study also revealed the same in variable bacterial strains.
The potentiating effect of piperine with ciprofloxacin in *in vitro* combination studies against *S. aureus* and suggested its role as an EPI in *S. aureus* has been shown earlier by Khan et al. (2006). These result motivated the current study of piperine and plumbagin for use as an EPI in various bacterial strains.

Efflux mediated resistance appears to contribute significantly to fluoroquinolone resistance and multi drug resistance in organisms, our data support the fact that increased fluoroquinolone usage can negatively impact susceptibility of organisms to multiple classes of antibiotics.

Methanol extract of *L. inermis* has a good activity in terms of antibiotic potentiation with ciprofloxacin and the data suggest that these extracts contain an inhibitor of efflux. So this was taken forward and fractionated as an initial step to identify with EPI-like activity. It was surprising to find that hexane and chloroform extracts had no EPI-like activity. While ethyl acetate extract possessed more EPI activity than methanol extract by declining the ciprofloxacin MIC to one fourth. These data suggest an interaction with other pumps or another mechanism of synergy.

Conclusion

Out of 57 bacterial strains tested 24 bacterial strains were found to be resistant against ciprofloxacin and 19 against ofloxacin. 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). In the present study ethyl acetate sub fraction revealed upto 1/4 MIC decline, indicating more potentiation of *L. inermis* as efflux pump inhibitor as compared to standard EPI_c and EPI_o. The activity of methanol and ethyl acetate 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.

Table 5 List of plant extract fractions possessing potentiation as EPI of Ciprofloxacin

Plant name	Bacterial strains	Ciprofloxacin MIC decline			
		Methanol extract	Hexane Extract	Chloroform Extract	Ethyl acetate Extract
Lawsonia inermis (Henna)	*K. pneumoniae* 4030	1/2	-	-	**1/4**

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