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Inhibitory effect of piperine on AcrAB-TolC efflux pump in fluroquinolone resistant *Escherichia coli* isolates from bovine mastitis milk samples

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Abstract
'Antimicrobial resistance' is a global epidemic issue causing economic loss to dairy farmers especially when treatment become unresponsive to mastitis. In the recent past, role of efflux pumps in development of resistance is intervened so as to overcome bacterial resistance to broad class of chemotherapeutic agents. In the current study an attempt has been made to investigate the ability of 'piperine', a phytochemical to overcome the efflux pump, AcrAB-TolC mediated resistance in *E. coli* isolates from bovine mastitis. The study revealed piperine reduced the MIC of enrofloxacin and ciprofloxacin against *E.coli* isolates from mastitis and showed synergistic action at a concentration of 100 ppm. A 16 fold reduction in MIC of enrofloxacin and ciprofloxacin were observed upon co-exposure at concentration of 100 ppm. Gene expression studies indicated down regulation of expression of *acr* A, *acr* B and *tol C* efflux pump in *E. coli* isolates resistant to enrofloxacin and ciprofloxacin. Piperine showed increased efflux pump inhibitory activity in ciprofloxacin resistant *E. coli* isolates. *In silico* analysis revealed that piperine binds with acrB protein with least binding energy. This study revealed that piperine potentiated the action of enrofloxacin and ciprofloxacin by inhibition of AcrAB-TolC efflux pump.

Keywords: Piperine, efflux pump inhibitor, *E.coli*, mastitis

Introduction
'Antimicrobial resistance' is a global epidemic issue and a worldwide concern as there is millions of human deaths due to unsuccessful chemotherapy. An European estimate indicate annual mortality due to multiple drug resistance exceeds 25000 and costs more than 2.5 billion dollar (WHO, 2016) [1]. In order to overcome the antimicrobial resistance there is an urgent need to understand the resistance mechanisms evolved by bacteria, possible measures to overcome resistance mechanism and development of newer antimicrobial agents with novel pharmacokinetic properties (Adwan *et al.*, 2010) [2]. Efflux pump mediated resistance is one of the mechanism contributing for multiple drug resistance in both Gram positive and Gram negative organisms (Kumar and Pooja, 2016) [3]. AcrAB-tolC is a prototype multidrug efflux pump in *E. coli* and a major contributor to antibiotic resistance in clinical cases. Nikaido (2001) [4] The acrAB system is expressed in *E. coli* and is responsible for the characteristic intrinsic resistance of *E coli* to dyes, detergents, and most lipophilic antibiotics.
Inhibition of the efflux pumps increase the potency of antimicrobial agent against microbes. Efflux pump inhibitors (EPI) could be used as adjunctive therapies with antibiotics which would increase the potency of antibiotics and decrease the emergence of MDR bacteria (Opperman and Nguyen., 2015) [5]. Piperine, an alkaloid reported to inhibit efflux pumps like P-glycoprotein (P*gp*) in bacteria (Bhardwaj, 2002 [6]; Feng *et al*., 2014 [7]; Kalsi and Grewal, 2015 [8]) which aids in the bio enhancement of various drugs (Bhardwaj, 2002) [6]. It also promote intracellular concentration of anticancer agent in tumour cells (Katiyar *et al.*, 2016) [9]. In this study piperine, an alkaloid present in *piper longum* and *piper nigrum* studied for its action on AcrAB-tolC efflux pump in quinolone resistant *E. coli* isolates from bovine mastits samples.

Materials and Methods
Present research was aimed to find out the effect of piperine on fluoroqunolone resistant *E.coli* isolates in combination with enrofloxacin and ciprofloxacin based on MIC (Minimum inhibitory concentration) and to rule out possible molecular mechanisms related to mRNA expression for acrAB-tolC efflux pump. MIC values determined by microdilution assay for piperine, fluoroquinolone antibiotics (Enrofloxacin and ciprofloxacin) and combination of respective antibiotics with different concentration of piperine.

Range of antibiotic concentration of enrofloxacin and ciprofloxacin for *E. coli* selected ranged from 0.25-128 mg/l. (Petersen *et al*. 2006) [10] and piperine used in the concentarion of 20 ppm, 40ppm, 60pp, 80ppm, and 100 ppm. MIC values for respective antibiotics and piperin- antibiotic combination is calculated according to CLSI guidelines, 2018. Antibiotic stock solutions was prepared using the formula

1000 x V x C /P= W

Where
P = potency given by the manufacturer (µg/mg)
V = volume required (mL)
C = final concentration of solution (multiples of 1000) (mg/L)
W = weight of antibiotic in mg to be dissolved in volume V (mL).

Synergistic action of antibiotics and piperine was determined by Fractional Inhibitory Concentration (FIC) index which is calculated by following formula (Petersen *et al*.2006) [10]**.**

FIC index = $FIC_A + FIC_B$

Where,
FICA = MIC of drug A in combination/ MIC of drug A alone
FICB = MIC of drug B in combination/ MIC of drug B alone.

Gene expression study
Real time – quantitative Polymerase Chain Reaction (RT-qPCR) was employed for studying the gene expression of *acr A, acr B and tol C* genes.Total RNA was isolated using RNeasy Mini Kit (74106 Qiagen, Hilden, Germany) as per manufacturer's instruction with slight modifications. Isolated RNA was immediately subjected to DNase treatment by using DNase kit (Sigma- Aldrich, USA). The purity and concentration of total RNA isolated was estimated by Nanodrop ™ spectrophotometer 2000C (Thermo scientific, USA). Complementary DNA (cDNA) synthesis was carried out from total RNA using Revert Aid first strand cDNA synthesis kit (K1622, M/s Thermoscientific, USA) as per manufacturer's protocol. After validation of primer annealing temperature, the cDNA of target genes *acr A, acr B, tol C* along with reference gene *rspL* was studied for mRNA expression by RTq- PCR (M/s Applied Biosystems, USA).The expression of *acr A, acr B, tol C* gene was studied using SYBR green chemistry (Maxima SYBR green qPCR master mix, M/s Thermo Scientific, USA).

***In silico* analysis**
Piperine structure was downloaded from pubchem (Pubchem CID: 638024, Fig. 9) which was saved in PDBQT format after 2D and 3D cleaning. Acr B structure (PDB id :10YD, Fig.10) was downloaded from protein data bank (PDB) which was saved in PDB format. Grid was prepared for acr B and saved in GPF format. After preparation of ligand and receptor in desired format, they subjected to docking by using "autodock" software.

Results
Checkerboard method
Present study was conducted to analyse the minimum inhibitory concentration of fluroquinolones resistant *E. coli* isolates from mastitis and also the effect of piperine on MIC value and antibacterial activity of respective antibiotics.

Enrofloxacin
Minimum inhibitory concentarion of enrofloxacin was 10 ± 0.1 which reduced to 0.6528 ± 0.1367 in combination with piperine at 100 ppm. Fractional inhibitory concentration (FIC) of combination of enrofloxacin and piperine was 0.0677, hence showed the synergistic action (Table 1).

Ciprofloxacin
Minimum inhibitory concentartion of ciprofloxacin was 10.66 ± 1.01 which reduced to 0.447 ± 0.07 in combination with piperine at 100 ppm. FIC (Fractional inhibitory concentartion) index of combination of ciprofloxacin and piperine was 0.0437 hence showed the synergistic action (Table.2).
There was significant down regulation of all three genes encoding efflux pumps after treatment of enrofloxacin resistant *E. coli* isolates with piperine at 20 ppm and 100 ppm (Table.3, Melt curve-Fig.1, 2, 3, 4 amplification plot- Fig. 5, 6, 7, 8). Thus piperine inhibited the effflux pump (AcrAB - TolC). At 20 ppm of piperine there was 22.75 per cent of down regulation of *acrA* gene, 44 per cent of *acr B* gene and 22.8 per cent of *tol C* gene whereas at 100 ppm level of piperine, there was further down regulation of *acr A* gene to 64 per cent, *acr B* gene to 85 per cent and *tol C* to 64 per cent.
There was significant down regulation of expression for all three genes encoding efflux pumps after treatment of ciprorofloxacin resistant *E. coli* isolates with piperine at 20 ppm and 100 ppm levels (Table 4, Melt curve-Fig.1, 2, 3, 4 amplification plot- Fig. 5, 6, 7, 8). At 20 ppm of piperine there was 44 per cent of down regulation of *acr A* gene, 77.4 per cent of *acrB* gene and 22 per cent of *tol C* gene. Piperine down regulated *acrA* gene to 84.1 per cent, *acr B* gene to 86.22 per cent and *tol C* to 60 per cent.

In silico analysis
Docking study revealed that piperine binds with AcrB protein with binding energy of -7.79 (Fig.11 and Fig.12). Piperine binded with acrB protein with 1 Hydrogen bond between Glutamine 124 and O3 atom of Piperine and 7 Hydrophobic interactions occurred with Leu 111, Gln 112, Met 115, Pro 116, Gln 123, Gly 126 and Val 127 molecules (Fig.1). *In silico* studies showed that piperine has high affinity for acrB protein of the efflux pump.

Discussion
Enrofloxacin
Minimum inhibitory concentration of enrofloxacin was 10 ± 0.1 which reduced to 0.6528±0.1367 (µg/ml) in combination with 100 ppm of piperine. (Table 1). There was twofold reductions in the MIC of enrofloxacin in combination with 40 ppm of piperine. MIC values decreased with the increasing dose of piperine (Table 1). At 100 ppm level of piperine the MIC values were reduced to 0.6528±0.1367 (µg/ml) and there was 16 fold reductions in MIC at this dose. Fractional inhibitory concentration (FIC) of combination of enrofloxacin and piperine was 0.0677, hence showed the synergistic action. Similar studies were reported by Raja *et al*. (2015) [11] in which MIC of 16 mg/l ofloxacin was reduced in to 4 mg/l by DNP, 2 mg/l for verapamil and piperine through the inhibition of efflux pumps.

Ciprofloxacin
The effect of piperine with ciprofloxacin were similar to its effect when combined with enrofloxacin. MIC of ciprofloxacin was 10.66±1.01 µg/ml, which reduced to 0.447±0.0 µg/ml in combination with 100 ppm of piperine.

(Table 1).There was twofold reduction in the MIC of ciprofoxacin on combination with 40 ppm of piperine. MIC values decreased with the increasing dose of piperine (Table 2). At 100 ppm piperine level, there was more than 16 fold reduction in MIC of ciprofloxacin. Fractional inhibitory concentration (FIC) of combination of enrofloxacin and piperine was 0.0437, hence this combination show the synergistic action.

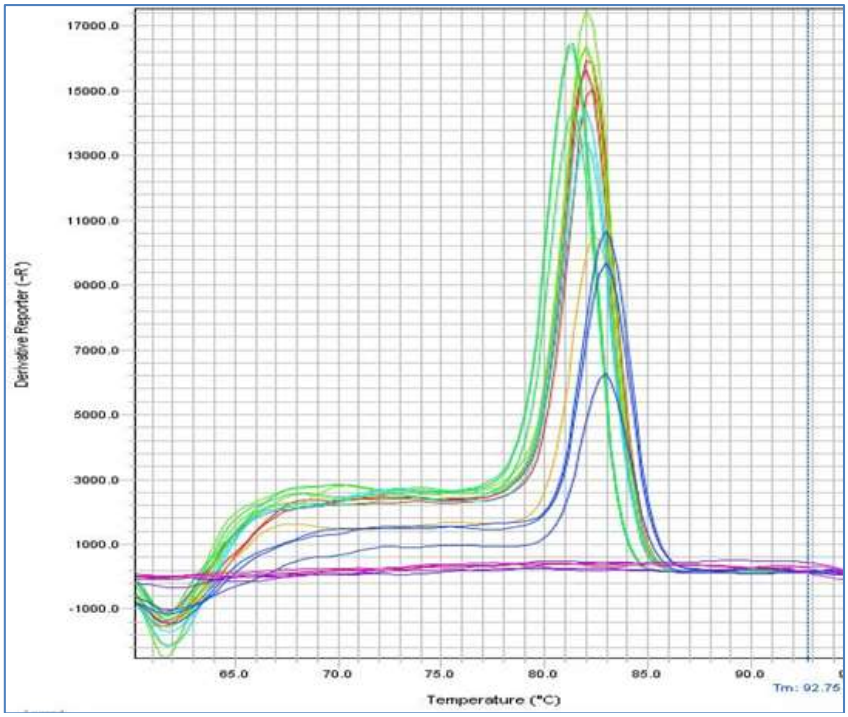

Fig 1: Melt curve for *acrA*

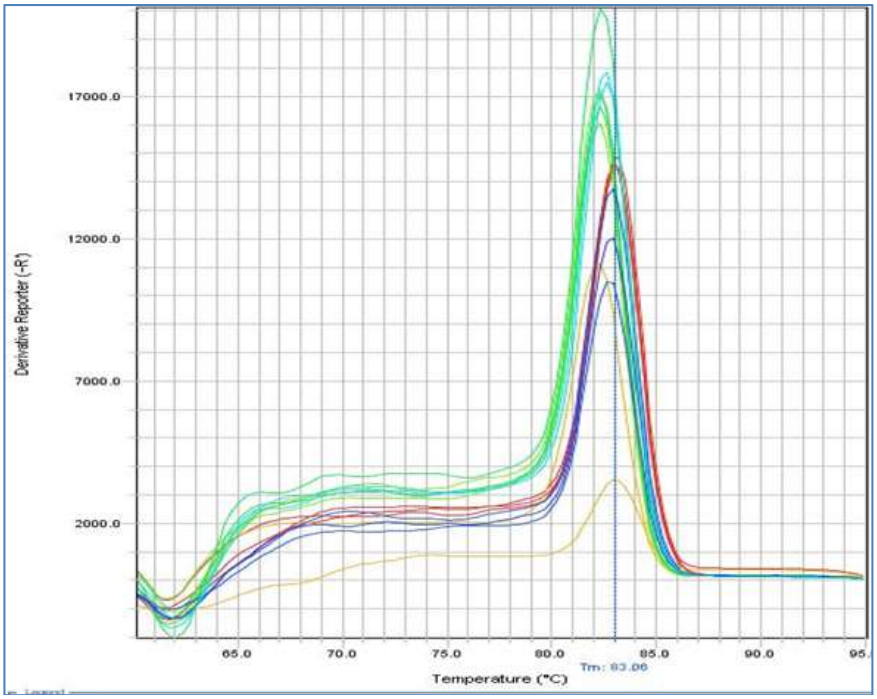

Fig 2: Melt curve for *acrB*

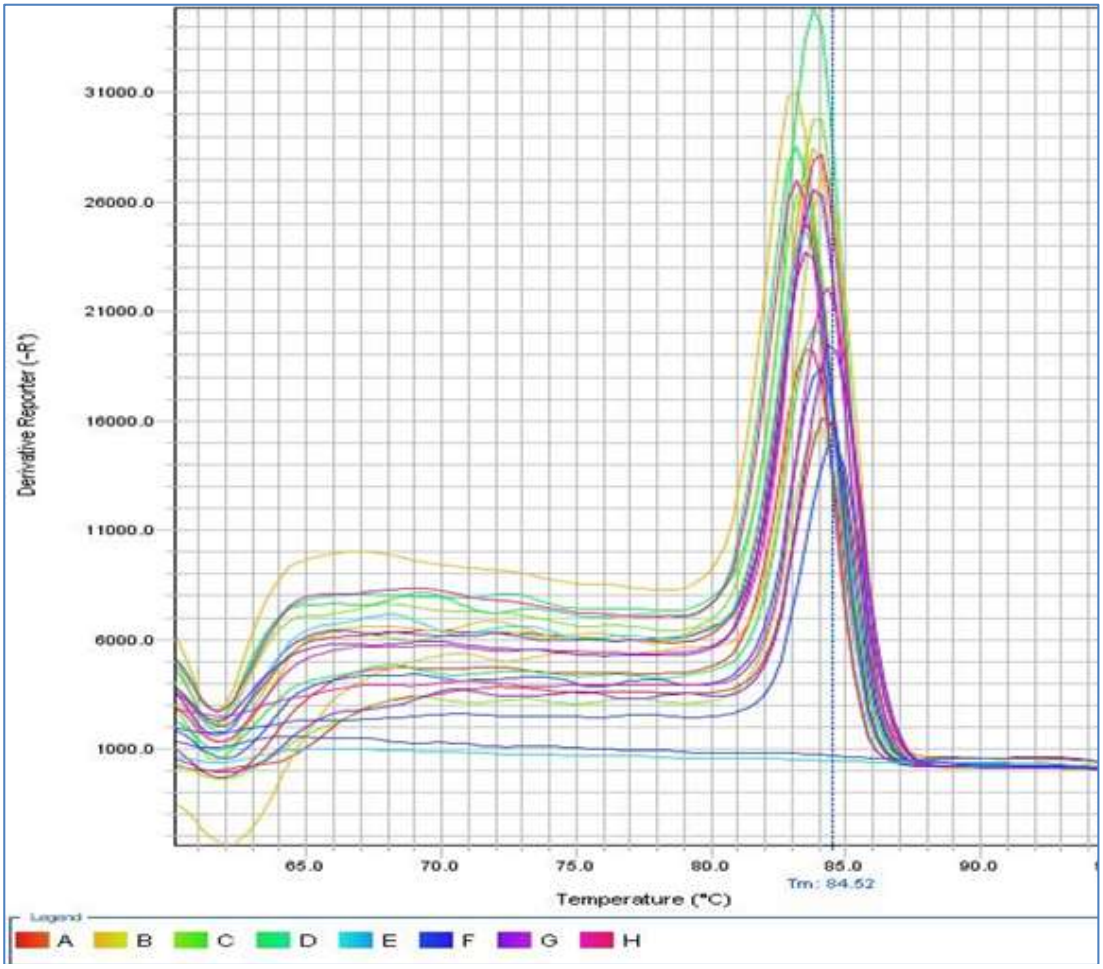

Fig 3: Melt curve for *tolC*

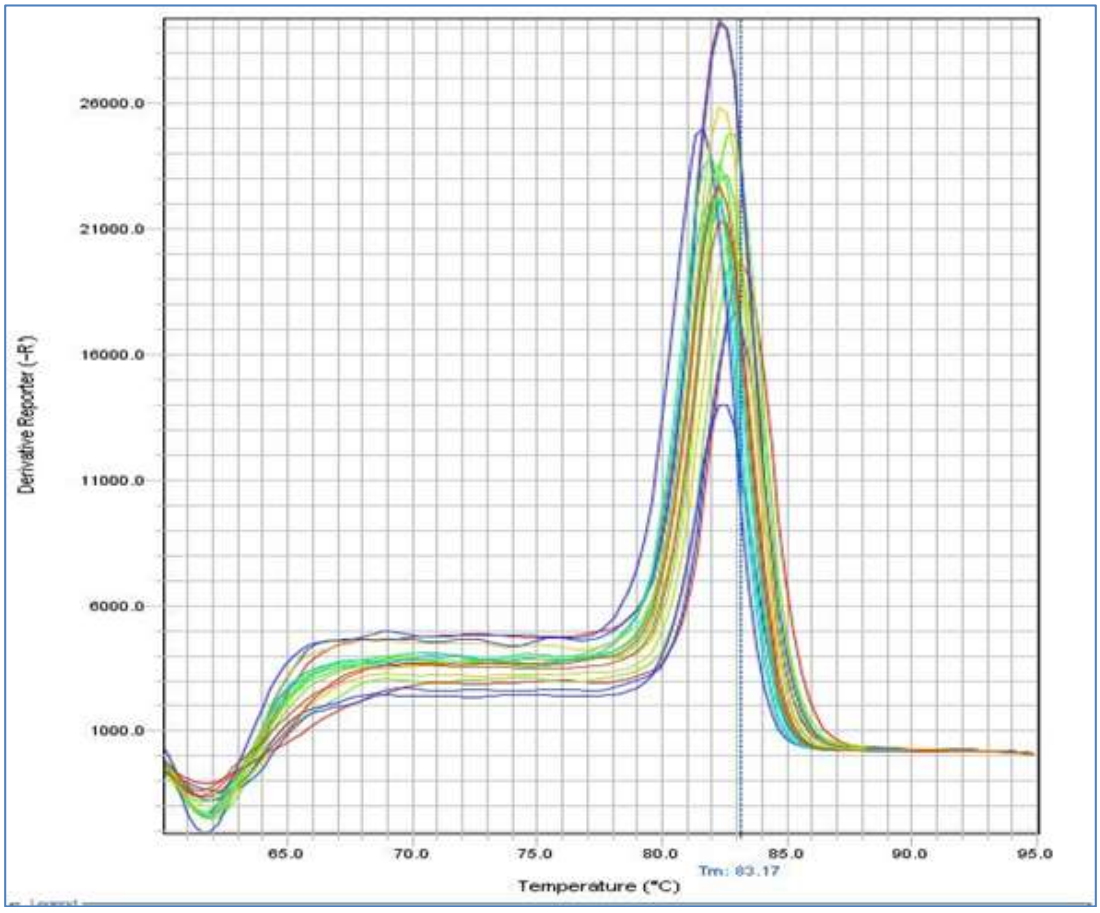

Fig 4: Melt curve for *rspL*

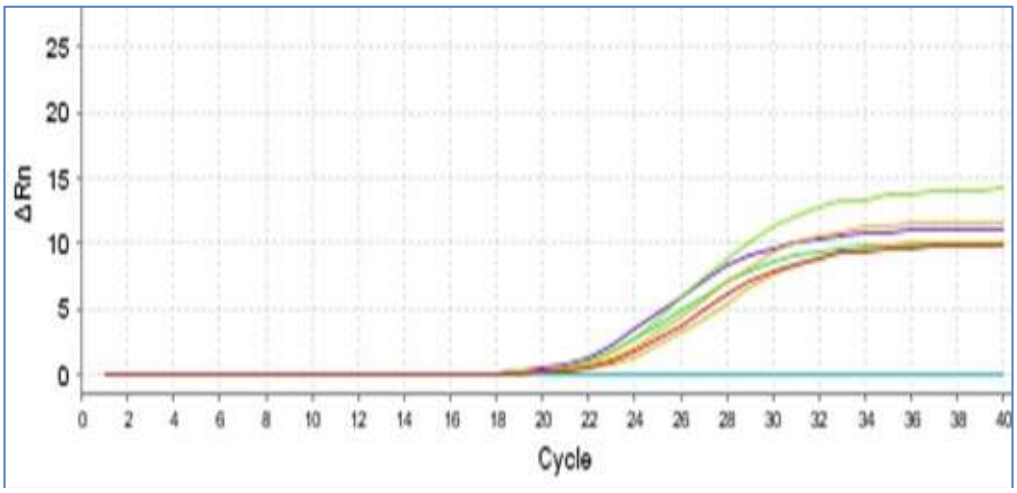

Fig 5: Amplification plot for *acrA*

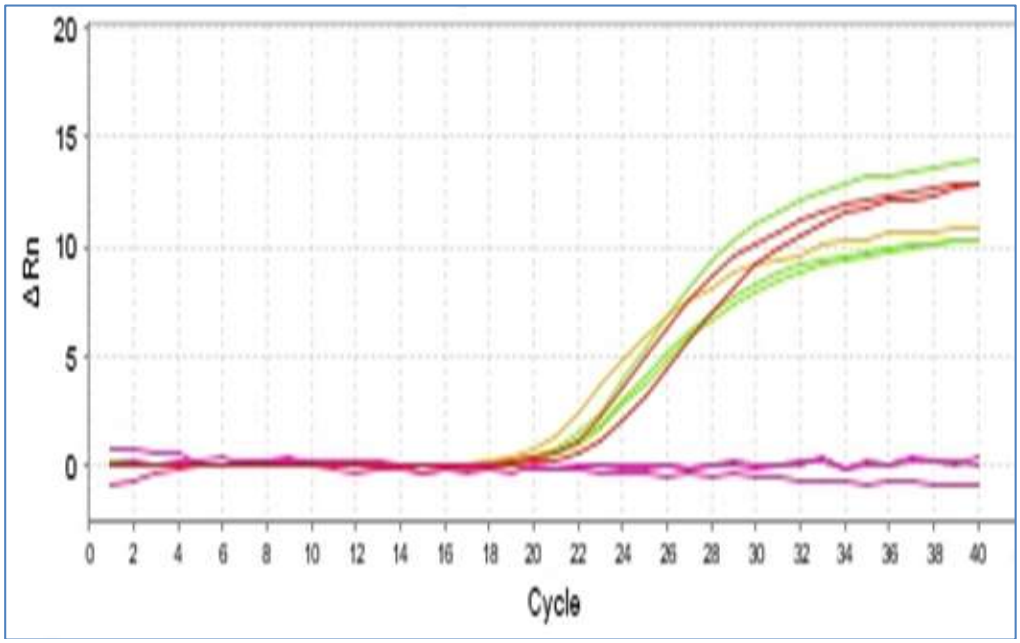

Fig 6: Amplification plot for *acrB*

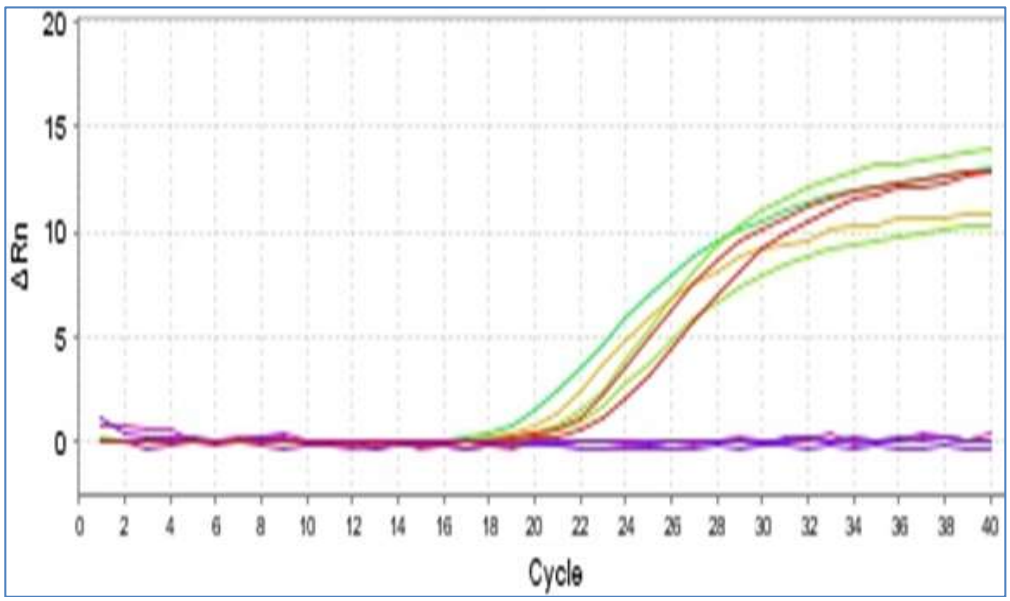

Fig 7: Amplification plot for *tolC*

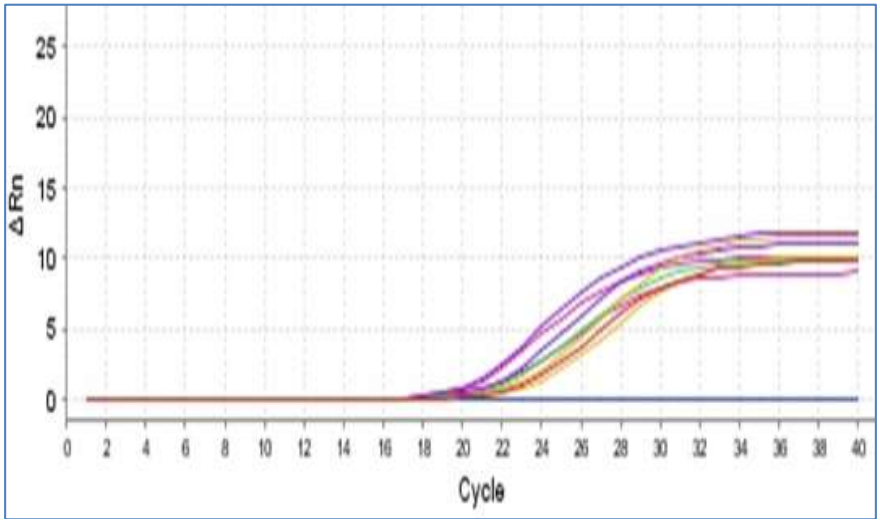

Fig 8: Amplification plot for *rspL*

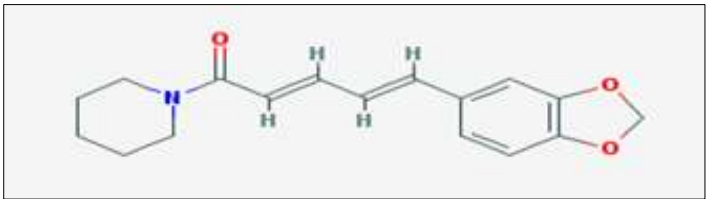

Fig 9: Ligand: Piperine (Pubchem CID: 638024)

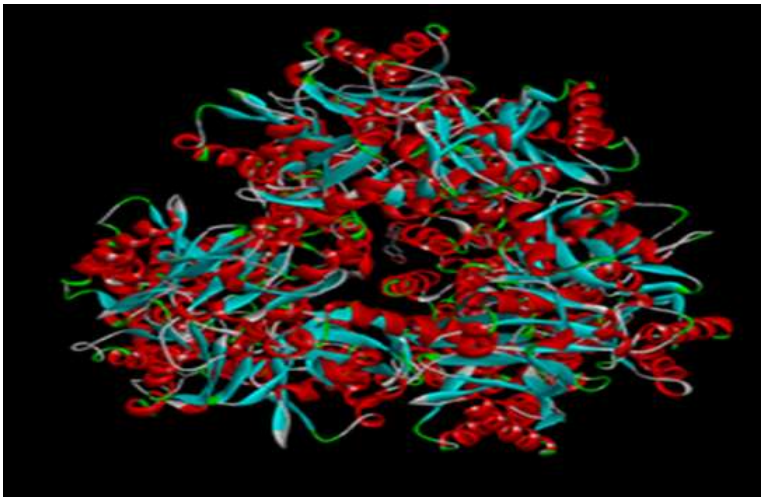

Fig 10: Macromolecule: AcrB Protein (PDB id :10YD)

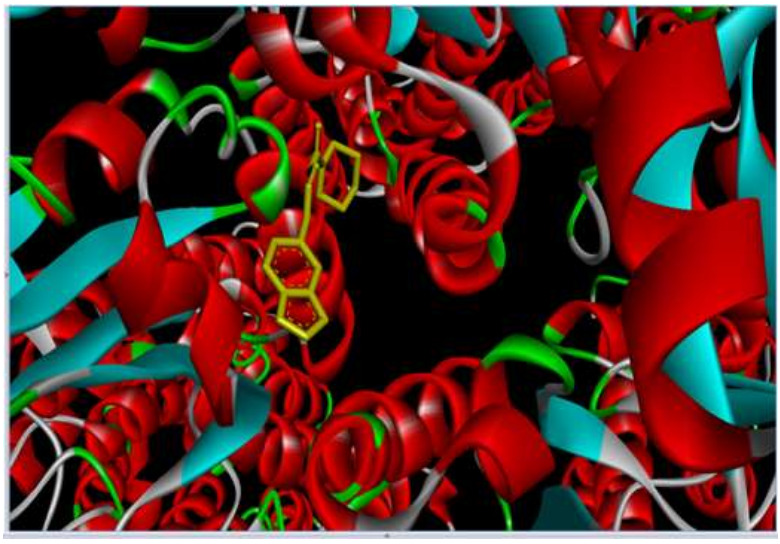

Fig 11: Macromolecule: AcrB Protein (PDB id : 2F1M) docked with piperine

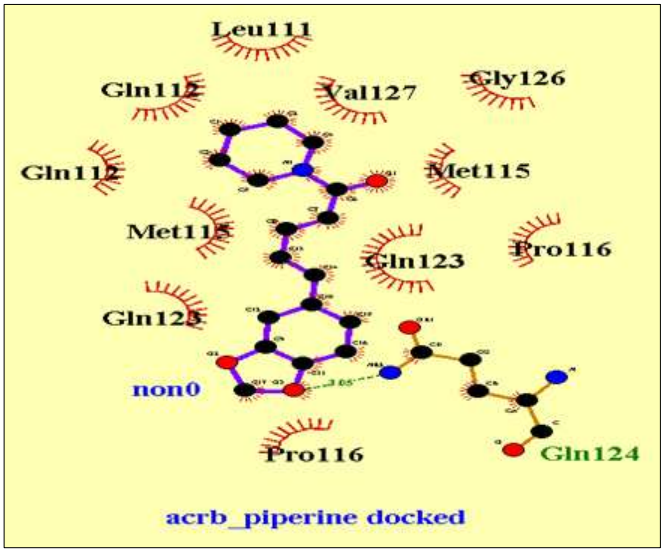

Fig 12: Hydrophobic and hydrophilic interactions of AcrB and piperine

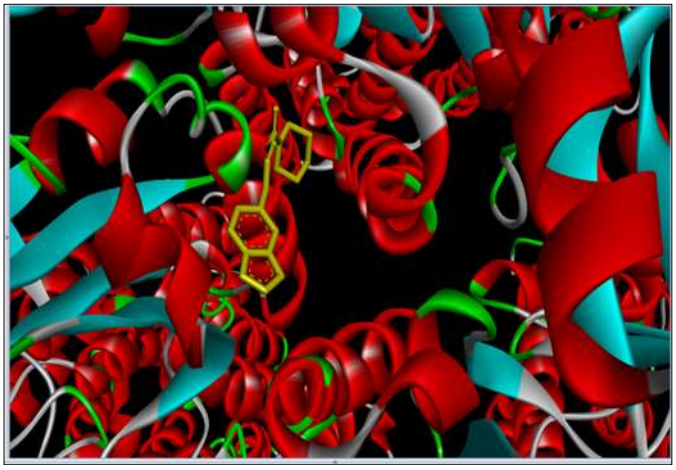

Fig 13: Macromolecule 18: AcrB Protein (PDB id : 2F1M) docked with piperine

Table 1: MIC values of enrofloxacin and combination of enrofloxacin with different doses of piperine in resistant *E.coli* isolates from mastitis

Treatment	MIC (µg/ml)
Enrofloxacin	10 ± 1.01^{b}
Enrofloxacin + piperine 20 ppm	7.78 ± 1.45^{bc}
Enrofloxacin + piperine 40 ppm	5.44 ± 0.94^{cd}
Enrofloxacin + piperine 60 ppm	2.944 ± 0.696^{de}
Enrofloxacin + piperine 80 ppm	1.75 ± 0.475^{e}
Enrofloxacin + piperine 100 ppm	0.6528 ± 0.1367^{e}
Piperine	236.10 ± 15^{a}
F- value	9.83**
P –value	0.01

**significant at 0.01 level, n=3, r = 6 replicates maintained each treatment, values are Mean ± SE Values bearing different superscriopts vary significantly ($p < 0.01$)

Table 2: MIC values of ciprofloxacin and combination of ciprofloxacin with different doses of piperine in resistant *E. coli* isolates from mastitis

Treatment	MIC (µg/ml)
Ciprorofloxacin	10.66 ± 1.01^{b}
Ciprofloxacin + piperine 20 ppm	8.66 ± 1.15^{bc}
Ciprofloxacin + piperine 40 ppm	5.22 ± 0.72^{cd}
Ciprofloxacin + piperine 60 ppm	2.611 ± 0.36^{de}
Ciprofloxacin + piperine 80 ppm	1.305 ± 0.18^{e}
Ciprofloxacin + piperine 100 ppm	0.447 ± 0.07^{e}
Piperine	222.22 ± 24.71^{a}
F-value	9.23**
P-value	0.0010

**significant at 0.01 level, n=3, r = 6 replicates maintained each treatment, values expressed as Mean ± SE. Values bearing different superscripts vary significantly ($p < 0.01$)

Table 3: Relative expression for *acrA*, *acrB*, and *tolC* gene in enrofloxacin combined with piperine

Treatment	fold of expression (*acr A*)	fold of expression (*acrB*)	fold of expression (*tolC*)
Enrofloxacin (Treatment I)	1^{a}	1^{a}	1^{a}
Enrofloxacin + piperine 20 ppm (Treatment II)	0.7725 ± 0.17^{a}	0.56 ± 0.27^{ab}	0.77204 ± 0.17^{b}
Enrofloxacin + piperine 100 ppm (Treatment VI)	0.36 ± 0.09^{b}	0.159 ± 0.08^{b}	0.36 ± 0.97^{b}
F value	7.777*	6.448*	32.639**
P value	0.022	0.032	0.001

**significant at 0.01 level *significant at 0.05 level

n=3, r = 6 replicates maintained each treatment, values expressed as Mean ± SE, for each mean with different superscript indicate significant difference

Table 4: Relative expression for *acr A*, *acr B* and *tol C* gene in ciprofloxacin combined with piperine

Treatment	fold of expression(*acrA*)	fold of expression(*acrB*)	fold of expression(*tolC*)
Ciprofloxacin(treatment I)	1^{a}	1^{a}	1^{a}
Ciprofloxacin+ piperine 20 ppm(treatment II)	0.56 ± 0.27^{a}	0.2265 ± 0.19^{b}	0.78 ± 0.05^{b}
Ciprofloxacin + piperine 100 ppm(treatment VI)	0.159 ± 0.08^{b}	0.1378 ± 0.098^{b}	0.40 ± 0.015^{c}
F –value	7.189*	23.072**	86.309**
P- value	0.026	0.002	0.0001

** significant at 0.01 level * significant at 0.05 level n=3, r = 6 replicates maintained each treatment, values expressed as Mean ± SE, for each mean with different superscript indicate significant difference

Gene Expression Study

Real time PCR was conducted to measure the mRNA level of *acr A*, *acr B* and *tol C*. Gene expression study was conducted in *E. coli* isolates co treated with antibiotics and piperine.

Enrofloxacin

There was down regulation of *acr A*, *acr B* and *tol C* genes in *E. coli* isolates co treated with piperine. There was 23 per cent down regulation of *acr A* and *tol C* genes and 44 per cent down regulation of *acr B* genes at 20 ppm of piperine. When 100 ppm piperine treated along with antibiotic, there was 64 per cent down regulation of *acr A* and *tol C* genes and *acr B* down regulated up to 85 per cent. Among three genes *acr B* down regulated more compared to *acr A* and *tol C*.A significant difference between treatment I (antibiotic alone) and treatment VI (enrofloxacin +100 ppm piperine) at 5 per cent level for *acr A* and *acr B* gene observed, but no significant difference between treatment I (Enrofloxacin) and treatment II (Enrofloxacin). There was down regulation in the expression of *tol C* genes at 1 per cent level. Results were similar to the study conducted by Bohnert, (2013) [12], where pimozide inhibited the AcrAB-TolC pump in *E. coli* in ethidium bromide assay and there by increased the antibacterial activity of oxacillin.

Ciprofloxacin

Piperine inhibited *acr A*, *acr B*, and *Tol C* genes of the effflux pump (AcrAB - TolC) when used in combination with ciprofloxacin. At 20 ppm of piperine there was 44 per cent of down regulation of *acr A* gene, 77.4 per cent of *acr B* gene and 22 per cent of *tol C* gene whereas at 100 ppm piperine. There was further down regulation of *acr A* gene to 84.1 per cent, *acr B* gene to 86.22 per cent and *tol C* to 60 per cent. There was highest down regulation of *acrB* gene in both 20 ppm and 100 ppm piperine treatment group. Even with the 20 ppm of piperine treatment there was significant down reguation of *acrB* gene. A significant difference exist between treatment I (Ciprofloxacin alone) and treatment VI (ciprofloxacin+100 ppm piperine) at five per cent level for *acr A* but no significant difference between treatment I (Ciprofloxacin alone) and treatment II (ciprofloxacin with 20 ppm piperine). For the expression of *acr B* gene, there was a significant difference between treatment I and treatment II at five per cent level, but there is no significant difference between treatment II and treatment VI. There was also down regulation of *tol C* genes and exists a significant difference between all the three treatments at one per cent level. The results were similar to study conducted by Asgarshirazi *et al*. (2014) [13]. There was down regulation of *nor E* gene in MRSA isolates after treatment with reserpine. Down regulation of *nor E* efflux pump by reserpine increased the antibacterial activity of ciprofloxacin in *S. aureus*. Thus, the present study can be interpreted that, the inhibition of *acr A*, *acr B* and *tol C* genes of the efflux pump by piperine enhance antibacterial activity of ciprofloxacin.

***In Silico* Studies**

In silico studies showed that piperine binds with the acr B protein of the pump with one hydrogen bond and seven hydrophobic interactions. Piperine bind with the acrB protein of the pump with the least binding energy of -7.79 kcal/ mol and is having more affinity with the acrB protein. Vargiu *et al*. (2014) [14] reported the computational binding of MBX2319 with AcrB. MBX2319, a novel pyranopyridine efflux pump inhibitor with potent activity against RND efflux pumps which binds with the -12.5 kcal/ mol binding energy. Another well studied efflux pump PAbN also binds with acr B protein with -7.95 binding energy. Results of the present study also in agreement with these researches indicating that piperine bind with acr B, a receptor protein in the complex structure of AcrAB-Tol C, hence has the ability to overcome the action of this efflux pump.

Conclusion

Piperine down regulated the expression of *acr A*, *acr B* and *tol C* genes of the efflux pump in *E. coli* isolates resistant to enrofloxacin, ciprofloxacin. Down regulation of *acr B* gene in *E. coli* isolates resistant to enrofloxacin, ciprofloxacin was more compared to *acr A* and *tol C* genes. Efflux pump inhibition for all the three genes on co treatment with piperine was high in *E. coli* isolates resistant to ciprofloxacin compared to enrofloxacin. To conclude, piperine, a phytochemical can act as potentiator with enrofloxacin and ciprofloxacin in resistant *E. coli* isolates from mastitis. Thus the efflux pump inhibitory activity of piperine can be used against resistant *E. coli* isolates to combat efflux pump

mediated drug resistance. Combination therapy of piperine is a promising approach to overcome tetracycline and fluoroquinolones resistance. This synergistic action of piperine can be exploited in other bacteria too in different antibacterial combinations to overcome multiple drug resistance.

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