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In-vitro synergistic antibacterial activity of Punganur cow urine on enrofloxacin

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Abstract
Antimicrobial resistance has emerged as a significant hazard to both livestock and humans. To overcome resistance, the latest trend in antibiotic therapy has been the combination of antibiotics with natural compounds. Indigenous cow urine was believed to have excellent therapeutic importance among numerous natural compounds. As a result, the current investigation was conducted to determine the interaction of Punganur Cow Urine Distillate (PCUD) and Photo Activated Punganur Cow Urine (PAPCU) on enrofloxacin using tube dilution method with MIC as end point. MIC values for enrofloxacin, PCUD, and PAPCU against *E. coli* were 0.223 µg.ml^{-1}, 26.79 µL and 5.12 µL and *S. aureus* were 0.837 µg.ml^{-1}, 35.71 µL and 4.91 µL respectively. The MIC of enrofloxacin reduced from 0.223 µg.ml^{-1} to 0.076 µg.ml^{-1} in the presence of PAPCU and 0.044 µg.ml^{-1} in the presence of PCUD against *E. coli.* The MIC of enrofloxacin against *S. aureus* was reduced from 0.837 µg.ml^{-1} to 0.167 µg.ml^{-1} and 0.153 µg.ml^{-1} and in the presence of PAPCU and PCUD, respectively. Based on findings, it can be concluded that PAPCU has greater antibacterial activity compared to PCUD when used alone. However, PCUD with enrofloxacin provided superior synergistic action compared to the combination of PAPCU with enrofloxacin.

Keywords: Antibacterial activity, Punganur cow urine, PCUD

1. Introduction
India is a sacred land where every live entity and non-living organisms has something to offer. In India, the cow, also known as Kamadhenu, is regarded as a holy animal. The Indian Cow is distinguished by its hump, big ears, and bushy [1]. Cow urine consumption has been practiced widely for several years, and it has been experimentally proven that cow urine is the finest natural medication in the world [2]. Cow urine distillate, also known as cow urine ark, is cow urine that has been completely distilled and is used to cure illness caused by various pathogenic bacteria, opportunistic fungi, and parasitic helminths [3]. Cow urine distillate is patented as an bio-activity enhancer and bio-availability facilitator for anti-infective and anti-cancer bioactive molecules [4].
The Punganur cow is the world's shortest, humped cattle with a long tail and a switch which touches the ground. This breed is well-known for its small stature, efficient milk production and effective reproductive characteristics [5].
Enrofloxacin or 1-Cyclopropyl-6-fluoro-7-(4-ethyl-1-piperazinyl) 1, 4- dihydro-4-oxo-3-quinolinecarboxylic acid, belongs to fluoroquinolone group which is a subgroup of quinolone. Enrofloxacin is the first fluoroquinolone patented in 1984 for veterinary use. Quinolones have an action on bacterial topoisomerase. Enrofloxacin is used as an exclusive veterinary medicine for the treatment of gastrointestinal and respiratory infections caused by Gram-positive and Gram-negative bacteria in several animal species diseases [6, 7]. Although enrofloxacin has been used as an extremely effective treatment for colibacillosis in poultry [8], increasing concern about the consequences of fluoroquinolones resistance led to a ban on its use in poultry production in the USA in 2005 [9]. Over-use of enrofloxacin has resulted in development of resistant bacterial populations leading to reduced clinical efficacy [10].

Development of resistance to different kinds of antibiotics by microbes is an ever-increasing global threat [11]. Indiscriminate use of enrofloxacin has led to development of antimicrobial resistance (AMR) by the bacteria. Therefore, natural products have become alternative adjuncts for antibacterial therapy to revive back the antibacterial potential of the classic antibiotic agents and to reduce the AMR. Keeping the background in view, the present study was designed to determine the *in-vitro* antibacterial activity of enrofloxacin alone and in combination with photo activated punganur cow urine (PAPCU) and punganur cow urine distillate (PCUD) by determining minimum inhibitory concentration (MIC) against *S.aureus* ATCC 25923, *E.coli* ATCC 25922.

2. Materials and Methods

2.1 Chemicals

Enrofloxacin (CAS No: 85721-33-1) was procured from Sigma-Aldrich Chemicals, Pvt. Ltd, Bengaluru, Mueller-Hinton Broth, Magnesium Chloride Hexahydrate, Calcium chloride and Indonitrotetrazolium chloride (INT) was obtained from M/s Hi Media Laboratories Pvt. Ltd. Mumbai, India.

2.2 Microbial cultures

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 cultures were procured from Department of Veterinary Public Health and Epidemiology, CVSc, Tirupati, SVVU.

2.3 Punganur cattle urine Sample collection

Urine was collected from the healthy Punganur cows at Livestock Research Station, SVVU, Palamaner, Chittoor (Dist.), Andhra Pradesh. All the adult cows were taken into the consideration; randomly urine was collected and pooled from cows, without taking estrus cycle into the consideration. All the animals are in the semi-intensive care. Early morning first voided and mid-stream urine samples were collected in sterile amber colored glass containers.

2.4 Preparation of Cow urine distillate (CUD)

Punganur cattle urine was collected and filtered by muslin cloth followed Whatman filter paper No.1. Cow urine distillate (CUD) was prepared by using CUD apparatus at 100 ºC for 4hours. After distillation, CUD is stored in amber colored airtight glass bottles at 4-8 °C.

2.5 Preparation Photo Activated cow urine

Punganur cattle urine was exposed to sunlight from 10 AM to 5 PM (7 h/day) for 140 h during the month of March (early summer). After photo-activation, cow urine was filtered through muslin cloth and followed by Whatman filter paper No.1 and stored at 4 -8ºC in amber colored airtight glass bottles [12].

2.6 Preparation of 0.5 McFarland turbidity standards

Stock solutions of 0.18 M (0.36 N) H_2SO_4 (1% v/v) and 0.048 M $BaCl_2$ (1.175% w/v $BaCl_2 \bullet 2H_2O$) were prepared. 0.5 mL of the $BaCl_2$ solution was added to 99.5 mL of the H_2SO_4 stock solution while constantly swirling to maintain a suspension. The turbidity standard's density was validated by measuring absorbance with a spectrophotometer with a 1 cm light path and matched cuvettes. The absorbance at 625 nm for the 0.5 McFarland standard ranged from 0.08 to 0.13. According to CLSI, 2012 guidelines, 5 mL aliquots of $BaSO_4$ were placed into screw cap tubes of the same size as those used for standardizing the bacterial inoculum. (13).

2.7 Preparation of cation stock solutions

A stock solution containing 10 mg of magnesium ions (Mg2+) per milliliter was made by dissolving 8.36 g of $MgCl_2.6H_2O$ in 100 ml of deionized distilled water, while a stock solution containing 10 mg of Ca^{2+} per milliliter was made by dissolving 3.68 g of $CaCl_2.2H_2O$ in 100 ml of deionized distilled water. They were sterilized through membrane filtration and stored at temperatures ranging from 2 to 8 °C.

2.8 Preparation of cation-adjusted Muller-Hinton broth (CAMHB)

Muller-hinton broth (100ml) was prepared as directed by the manufacturer, autoclaved, and cooled overnight at 2 to 8°C. With steady stirring, 0.2 mL of $MgCl_2$ stock solution was added to this chilled broth, followed by 0.4 mL of $CaCl_2$ stock solution, resulting in a final concentration of Mg^{2+} and Ca^{2+} ions in the broth of 10 and 20 mg/L, respectively. The pH of the broth following cation addition ranged from 7.2 to 7.4.

2.9 Test sample preparation (Enrofloxacin)

Stock solution was prepared by adding 10mg of enrofloxacin in10ml 0.1N NaOH (1 $mg.ml^{-1}$). To calculate the MIC of enrofloxacin alone, a working standard of 100 g/ml enrofloxacin was generated by diluting the stock solution with distilled water. Working standards of (100 g/ml) were established for the interaction investigation by diluting stock solution (1mg/ml) separately with PCUD and PAPCU.

2.10 MIC by broth microdilution method

Enrofloxacin's MIC against *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC25922 was determined using the broth microdilution method. In a 96-well microtiter plate, enrofloxacin was serially diluted two times in CAMHB until the final volume in all the wells was 100µl. After incubating the bacterial culture in CAMHB at 37±1 °C for 6 to 8 hours, the turbidity was modified to 0.5 McFarland turbidity standards (1 X 10^8 CFU/ml), which was subsequently diluted 1:20 in CAMHB. When 0.01 ml of this suspension was added to the broth, the final bacteria concentration was around 5 X 10^5 The CFU per mill (ranging from 2 - 8X 10^5 CFU/ml or 5 X 10^4 CFU/well). Each plate was properly sealed to avoid drying during incubation. The inoculated microdilution trays were then incubated in an ambient BOD incubator at 35±2 °C for 16 to 20 hours.

2.11 MIC End Point

The MIC is the lowest antimicrobial agent concentration that totally suppresses organism growth in microdilution wells as observed by the unassisted eye. Bacterial growth and inhibition were measured by adding 25µL of p-Iodonitrotetrazolium (INT) dye to each well and incubating for 30 minutes at 35±2 °C. Biologically active organisms, in this example dividing bacteria, convert INT to a red formazan molecule. When the solution in the well stayed clear, bacterial growth was inhibited. This concentration was designated as the MIC. Each experiment included growth controls [13].

3. Results and Discussion

The minimum inhibitory concentration (MIC) of enrofloxacin, punganur cow urine distillate (PCUD), Photo Activated Punganur Cow Urine (PAPCU) alone and

enrofloxacin in combination with PCUD and PAPCU was carried out *in vitro* on *Escherichia coli* and *Staphylococcus aureus.* Results were expressed as mean MIC and percentage reduction of MIC of enrofloxacin and enrofloxacin in combination with PCUD and PAPCU which have presented in Table No 1 & 2 and Fig No. 1, 2, 3 & 4.

The *in vitro* bioenhancing property of enrofloxacin in the presence of PCUD and PAPCU was explored by determining MIC against *E. coli* and *S. aureus*. The MIC of enrofloxacin alone was 0.223µg.ml^{-1} and 0.837µg.ml^{-1} against *E. coli* and *S. aureus*, respectively (Table No 1). The above obtained values of MIC of enrofloxacin against *E. coli* and *S. aureus* agreed with the findings of Grobel *et al*., 2007 [14]. The MIC values of PAPCU alone against *E. coli* and *S. aureus* were 5.12 µL and 4.91 µL respectively (Table No 1). The PCUD alone showed the MIC end point against *E. coli* and *S. aureus* as 26.79µL and 35.71 µL respectively (Table No 1). Reduced MIC values of PAPCU in comparison to PCUD might be due to hydrolytic state and increased hydrophobicity of cow urine. Further enhanced antibacterial activity of PAPCU might be due to acidic pH, presence of a greater number of cations and biogenic volatile and non-volatile compounds like sulffinol, ketones, urinary peptides, phenols, and nitrosamines [12, 15, 16].

Enrofloxacin's minimal inhibitory concentration (MIC) alone was 0.223 µg.ml^{-1} against *E. coli* and which was reduced to 0.076 µg.ml^{-1} in the presence of PAPCU and even more decreased to 0.044 µg.ml^{-1} in the presence of PCUD. The MIC of enrofloxacin was 0.837µg.ml^{-1} against *S. aureus* that was decreased to 0.167 µg.ml^{-1} in the presence of PAPCU and further changed to 0.151µg.ml^{-1} in the presence of PCUD. The MIC of enrofloxacin was reduced to 81% in the presence of PCUD against *E. coli* and *S. aureus* whereas MIC of enrofloxacin was reduced 68% against *E. coli* and 80% against *S. aureus* in the presence of PAPCU, which indicates positive drug interaction between enrofloxacin and Punganur cow urine. PCUD and PAPCU showed effective *in vitro* bioenhancing property on enrofloxacin. In accordance with Mohanvel *et al.*, Cow urine distillate (CUD) increases the bio-activity and bio-availability of bioactive compounds [17]. The combination of enrofloxacin with PAPCU and PCUD increased antibacterial activity of enrofloxacin as has been evidenced by the reduction of MIC values.

Table 1: Minimum inhibitory concentration of PAPCU, PCUD, enrofloxacin alone and in combination of enrofloxacin with PAPCU and PCUD

	Organisms	1	2	3	4	5	6	7	MEAN±SEM
PAPCU (µL)	*E. coli*	6.25	6.25	6.25	3.125	1.5625	6.25	6.125	5.12±0.736
	S. aureus	6.25	6.25	6.25	6.25	3.125	3.125	3.125	4.91±0.631
PCUD(µL)	*E. coli*	12.5	25	25	25	25	50	25	26.79±4.25
	S. aureus	25	50	50	25	25	25	50	35.71±5.05
ENR(µg.ml^{-1})	*E. coli*	0.1953	0.1953	0.1953	0.1953	0.3908	0.1953	0.1953	0.223±0.02
	S. aureus	0.3906	0.78125	0.78125	0.78125	1.5625	0.78125	0.78125	0.837±0.13
ENR+PAPCU(µg.ml^{-1})	*E. coli*	0.048	0.048	0.048	0.097	0.097	0.097	0.097	0.076±0.009
	S. aureus	0.1953	0.1953	0.1953	0.1953	0.097	0.1953	0.097	0.167±0.018
ENR+PCUD(µg.ml^{-1})	*E. coli*	0.024	0.024	0.048	0.048	0.097	0.024	0.048	0.044±0.009
	S. aureus	0.1953	0.097	0.1953	0.1953	0.097	0.1953	0.097	0.153±0.019

ENR-Enrofloxacin; PCUD-Punganur Cow Urine Distillate; PAPCU-Photo Activated Punganur Cow Urine

Table 2: Effect of PCUD and PAPCU on MIC of enrofloxacin

	ENR µg.ml^{-1}	PCUD+ENR		PAPCU+ENR	
		µg.ml^{-1}	% reduction	µg.ml^{-1}	% reduction
E. coli	0.223	0.044	81%	0.076	68%
S. aureus	0.837	0.153	81%	0.167	80%

Note: ENR-Enrofloxacin; PCUD-Punganur Cow Urine Distillate; PAPCU-Photo Activated Punganur Cow Urine.

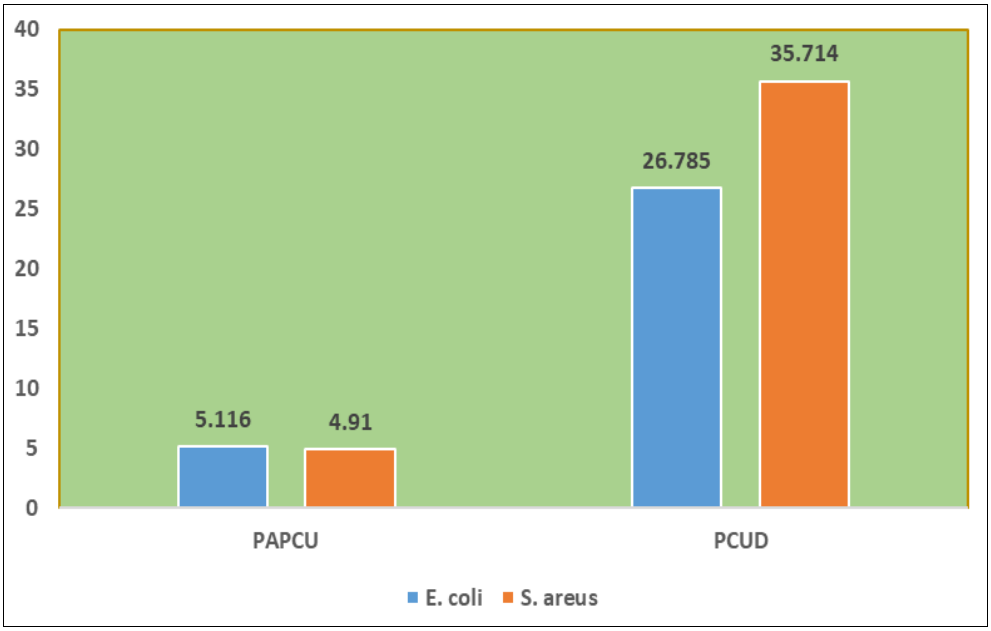


Fig 1: MIC (µL) of Photoactivated Punganur Cow Urine (PAPCU) and Punganur Cow Urine Distillate (PCUD) on *E. coli and S. aureus.*

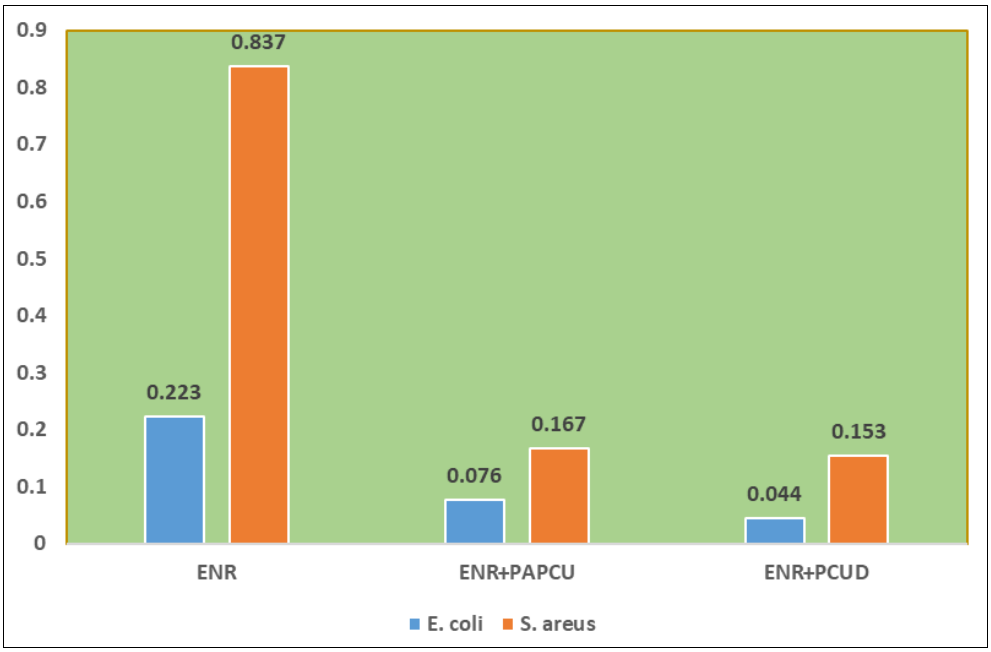
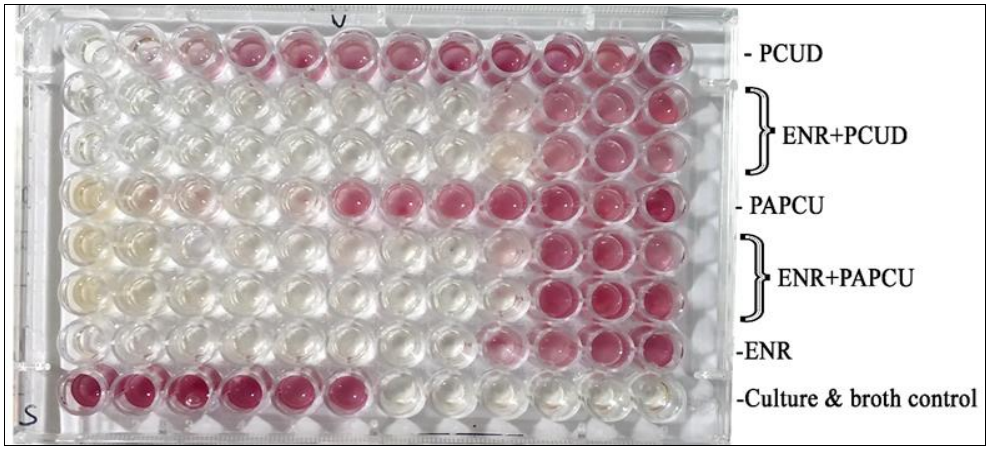
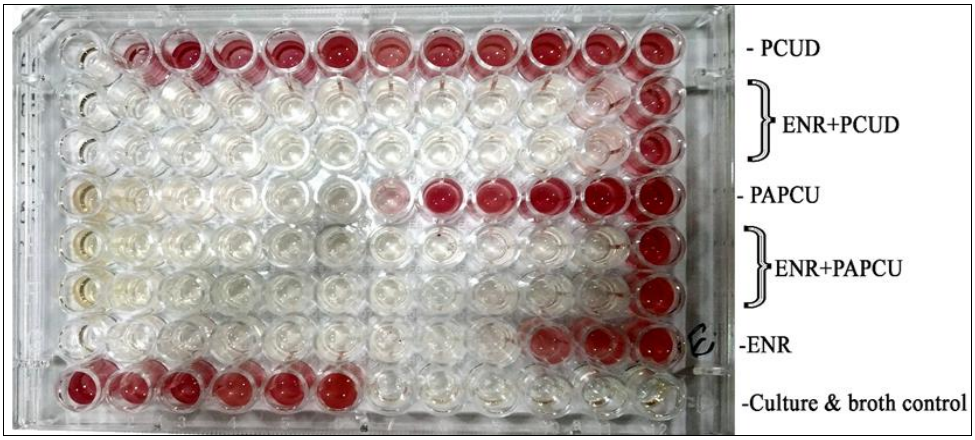


Fig 2: MIC (µg.ml^{-1}) of enrofloxacin alone and in combination with Photoactivated Punganur Cow urine (PAPCU) and Punganur Cow Urine Distillate (PCUD) on *E. coli* and *S. aureus.*



Note: Concentration of test sample in first well (PCUD and PAPCU 50µL; ENR, ENR+PAPCU and ENR+PCUD 50 µg.ml^{-1})

Fig 3: MIC of different test samples on *S. aureus.*



Note: Concentration of test sample in first well (PCUD and PAPCU 50µL; ENR, ENR+PAPCU and ENR+PCUD 50µg.ml^{-1});
ENR-Enrofloxacin; PCUD- Punganur Cow Urine Distillate; PAPCU-Photo Activated Punganur Cow Urine

Fig 4: MIC of different test samples on *E. coli.*

IAEC Approval details
The experimental protocol was approved by the IAEC vide reference number 281/ GO/ ReBi/ S/2000 /CPCSEA /CVSc/ TPTY/006/Pharmacology/2020dated 30.01.2020.

4. Conclusion

Based on the findings, it is concluded that, while PAPCU demonstrated superior antibacterial activity when compared to PCUD when used alone, PCUD demonstrated superior *In vitro* bioenhancing effect on enrofloxacin than PAPCU, which enhanced the antibacterial effect of enrofloxacin against selected bacteria.

It has been concluded that the punganur cow urine distillate and photoactivated punganur cow urine both have antibacterial activity and bioenhancing property on enrofloxacin, which activity can be used to control the bacteria of various origins. The synergistic activity of enrofloxacin with natural compounds like PCUD and PAPCU can be made use of in reducing the effective doses of actual antibiotic, thereby reducing the development of antimicrobial resistance.

Further studies are required to analyze which components of cow urine were responsible for the antimicrobial and bioenhancing property. Future suitable animal model studies could reveal antibacterial and bioenhancing property of punganur cow urine distillate and photoactivated punganur cow urine *in vivo*.

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