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EFFECT OF COMBINATION THERAPY USING COW (*BOS INDICUS*) URINE DISTILLATE AND SOME INDIAN MEDICINAL PLANTS AGAINST SELECTIVE PATHOGENIC GRAM-NEGATIVE BACTERIA

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ABSTRACT: Inconsequential use of broad-spectrum antibiotics results in the evolution of multiple drug resistant pathogenic gram-negative bacterial strains which is a global health threat and is responsible for high morbidity and mortality rates. This necessitated the search for new effective and safe treatment strategies. One such successful treatment strategy is combination therapy which comprises cow urine distillate and medicinal plants extract. In the present report, this combination therapy was used against five pathogenic gram-negative bacteria- *S. typhimurium*, *K. pneumoniae*, *E. coli*, *E. aerogenes* and *V. cholerae*. It was observed that combination therapy had more profound effect than the individual plant extract against all the bacterial strains at the specified level. Combination therapy with *T. erecta* plant extract was found to be the most effective which improved the antibacterial effect 2.54 times more against *E. coli*. Other plants extract also improved their antibacterial potential when used with CUD (R.A.I=1.28 to 2.54).

INTRODUCTION: Cell wall of gram-negative bacteria is more complex than gram-positive bacteria, due to an extra outer phospholipidic membrane carrying the lipopolysaccharide components, which makes the cell wall less permeable to antimicrobial substances. Evolution of multidrug-resistant gram-negative bacteria due to incongruous use of broad-spectrum antibiotics is responsible for the 30-70% mortality rate due to treatment failure of hospitalized patients.

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Besides being responsible for evolution of antibiotics resistant strains, antibiotics also have adverse effects like hypersensitivity, allergic reactions and immune-suppressions on the health of the host [1].

Plants based folk medicines are considered as safe alternative to synthetic antibiotics since the time immemorial because of their low toxicity, effectiveness and cost-effective. The innate defense mechanism of plants includes the various secondary metabolites like alkaloids, flavonols, phenols, terpenoids, gums, resins etc. which protect them from predation by microorganisms, insects and herbivores. These secondary metabolites of plants have enormous therapeutic potential and hence the best alternative medicinal values for human aliments.

Chemically, these plant-derived biochemicals have unique built-in chirality due to which they act as natural inhibitor or modulator of biologically vital enzymes and receptors, and this is the reason of their being antimicrobial or immunomodulators [2-5]. Efforts have been going on throughout the world to discover the new individual or combination of old/new antibacterial agents from various kinds of sources such as micro-organisms, animals, and plants.

In Indian folk medicine therapy indigenous white Cow's (*Bos indicus*) Urine Distillate (CUD) has been used to combat various infirmities. The active bioenhancer molecules in CUD have been granted U.S patent (no. 6896907/6410059) to Council of Scientific and Industrial Research (CSIR), India [6, 7]. Our earlier report successfully demonstrated the synergistic antibacterial effect of combination therapy (which includes water extract of various plants extract and CUD) against gram-positive bacteria [8]. Enthusiastic results from the previous study motivated us to evaluate the potency of combination therapy against gram-negative bacteria. In the present paper, we are demonstrating our results against five pathogenic gram-negative bacteria, by using water extract of leaves/rhizome from five medicinal plants (*Tagetes erecta, Ocimum sanctum, Azadirachta indica, Curcuma longa, Syzgium aromaticum*) whose antibacterial properties are well documented in literature [9-11]. So, the objective of this research was to find out the improved inhibitory effect of combination therapy against gram-negative bacteria, as we have successfully observed against gram-positive bacteria.

MATERIALS AND METHODS:

Chemicals and Glass wares: Analytical grade chemicals and reagents were used in the present study. Menadione and XTT-salt were purchased from Sigma-Aldrich, India and others chemicals and reagents like nutrient agar, broth etc. were purchased from Hi Media Pvt. Ltd, India. All the glass wares like test tubes, petri plates and beakers used were purchased from borosil, India.

Leaves collection and processing: The leaves of four plants *Tagetes erecta*, *Ocimum sanctum*, *Syzgium aromaticum* and *Azadirachta indica* were collected, first washed under running tap water, then by sterile distilled water and finally air dried at room temperature (35-40^{0}C) for 5-7 days. After that, leaves were homogenized to a fine powder and stored in air tight bottles. Rhizome of *Curcuma longa* was grinded to powder form and UV-irradiated. First of all, as discussed earlier [8], hexane and ethyl acetate (50:50 v/v) extraction was done for 72 hrs to remove the oil and fatty parts of dry matter using Soxhlet assembly. The fatty acid free extract thus obtained was dried and used to extract water-soluble biochemicals in Soxhlet assembly for 72 hrs using Double Distilled Sterile (DDS) water. Each preparation was filtered through a sterilized filter paper (Whatman No. 1) and was finally concentrated to dryness under vacuum at 40-50^{0}C using a rota-evaporator. The dried extracts were then sterilized by UV-irradiation, checked for sterility on nutrient agar plates and stored at 4^{0}C in sterile glass bottles until further use.

Test microorganisms: Five gram-negative bacterial strains, *Salmonella typhimurium* (MTCC 3224), *Klebsiella pneumoniae* (MTCC 432), *Escherichia coli* (MTCC 443), *Enterobacter aerogenes* (MTCC 2824) and *Vibrio cholerae* (MTCC 3904) were purchased from Institute of Microbial Technology (IMTECH), Sector 39, Chandigarh, U.T, India. Each of the bacterial cultures were freshly cultured before testing by transferring them on to nutrient broth and incubated at 37°C. whenever required, bacterial strains were freshly sub- cultured from the stock culture.

Screening for antimicrobial activity: As described in our earlier study [8], bacteria were grown overnight in broth at 37^{0}C for 18 hrs. Midlogarithmic phase organisms were harvested by inoculating this culture into 50 ml of fresh broth for additional 2.5 to 3.5 hrs at 37^{0}C. The bacteria were then centrifuged, washed and resuspended in 10 mM cold sodium phosphate buffer (pH=7.4) [12]. The optical density of an aliquot was measured at 620 nm and the concentration of bacteria were standardized (OD_{620} 0.20= 5 X 10^7 CFU/ml). Two methods were used to study the antimicrobial effect of combination therapy against gram-negative bacteria. In first method, known as well-agar diffusion method, approximately, 20-25 ml of pre autoclaved agar media cooled at 45^{0}C was poured into petri plates and allowed to solidify at room temperature.

One hundred microliter (100 µL) of the inoculum of each test organism was spread onto the agar plates so as to achieve a confluent growth. The agar plates were allowed to dry and five wells were made with a sterile borer in the inoculated agar plates. The lower portion of each well was sealed with 2-3 µl molten agar medium [8].

CUD (Ark) was prepared as per the standard protocol. Briefly, 2-3 liters of healthy white cow urine was collected in a sterile glass container during morning and filtered first through muslin-cloth and then by Whatman filter no. 1 paper. The urine was yellowish in color and free from solid matter. It was then boiled at 100^0 C to get rid of ammonia gas. The process was repeated thrice and vapours of CU were then collected using distillation assembly. The CUD was checked for any contamination by spreading 100 µl on agar plate and was stored in glass bottles at 4^0C for further use [13, 14].

Initially, stock concentration of 40 mg/ml for all extracts was prepared with DDS water and CUD. From stock concentration, different concentrations of extracts were made (30 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml). The concentration used in agar well diffusion method was 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml. A 100 µL volume of each extract was propelled directly into the wells of the inoculated agar plates for each test organism. The plates were allowed to stand for 1-1.5 hour at room temperature for diffusion of the extract into agar and incubated at 37°C for 18-24 hours. DDS water served as the negative control and antibiotic tetracycline served as the positive controls. The experiments were performed in triplicate and the mean values of the diameter of Zone of Inhibition (ZOI) were calculated in millimeter scale (± standard deviations). This complete procedure was carried out under aseptic conditions in laminar airflow chamber to avoid contamination.

Measurement of antimicrobial activity using XTT-colorimetric method: XTT-calorimetric method was used to evaluate the antibacterial potential of combination therapy. In this method relative reduction in formazan production, which was produced by reduction of XTT salt by dehydrogenase enzymes of Electron Transport System (ETS), was measured colorimetrically at 490 nm [15]. Only those extracts, which showed maximum inhibitory effect against different bacteria in comparison to others as demonstrated by agar well diffusion method, were evaluated for this method. Five combinations were selected as shown in **Fig. 1**.

Briefly, overnight broth cultures of different bacteria were diluted with broth to achieve the standard concentration of bacteria (5 X 10^7 CFU/ml) and 170 µl of the adjusted broth cultures were added to 96-well flat-bottom plate. 30 µl of different plants extract solution (with different concentration: 40 mg/ml, 30 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml) in DDS and CUD were added to the well with gentle mixing, and incubated for 15 hours [8]. On the second day, 100 µl of each well material is transferred into new 96-well flat-bottom plate. Fresh XTT+menadiaone solution was made and 25 µl of that solution was added to each well with gentle mixing. Plates were incubated for 1 hour at 37^0 C and the reading was taken at 490 nm with the help of Plate-reader. Negative control was the media containing respective bacteria without any growth inhibitor and positive control was the media with respective bacteria and antibiotic tetracycline (10 mg/ml). Final readings were adjusted after deducting the reading of blanks. Various blanks included were: individual reading of media, media + XTT/menadione solution, and media + XTT/ menadione solution + antibiotic or different extracts (in DDS or CUD) having different concentrations.

Experiment was performed in triplicate and final reading was taken as mean (± SD) of all readings. Antimicrobial activity was measured as percentage reduction of bacterial growth with the following formula [8].

Percentage reduction (% Rd) =

$$100\% - \frac{[(\text{Experiment well absorbance at 490 nm} - \text{Blank absorbance}) \times 100]}{\text{Negative control absorbance at 490 nm}}$$

Determination of Activity Index and Relative Activity Index: [8] The activity index of the crude plant extract was calculated as follows:

Activity index (A.I) =

$$\frac{\text{Mean of ZOI (or O.D) (individual plant extract effect on bacterial growth)}}{\text{Mean of ZOI (or O.D) (standard antibiotic effect on bacterial growth)}}$$

ZOI readings from agar well diffusion method were analyzed to find out the activity index at highest concentration (10 mg/ml). O.D readings obtained from XTT-calorimetric method were analyzed to find out the activity index at lowest concentration (1.25 mg/ml). These readings were compared with standard antibiotic (10 mg/ml) response to individual bacteria.

The relative activity index of the same plant extract in two different solvent (DDS and CUD) was calculated as follows:

$$\text{Relative activity index (R.A.I)} = \frac{\text{Activity index of plant extract (CU as solvent)}}{\text{Activity index of same plant extract (DDS as solvent)}}$$

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): Microdilution broth method was used to find out the MIC and MBC values. XTT-calorimetric readings were used to determine the MIC and MBC values as described earlier [8]. The lowest concentration that did not permit any growth, as confirmed by the O.D of the plate was considered as MIC. MBC is the lowest concentration of antimicrobial agent that will not allow the growth of an organism after sub-culturing on antibiotic free media. MBC was determined by sub-culturing the preparations that did not show any bacterial growth (by the method of MIC determination). A 100 µL aliquot from the selected tube (showing MIC) was spread over the nutrient agar plate and incubated at 37^{o}C for 24 hours and examined for bacterial growth. The MBC, lowest concentration of the extract (DDS and CUD) giving 99.9% reduction of the bacterial growth, was determined [16, 17].

RESULTS: As a solvent medium CUD improved the inhibitory effect of different plants extract against all gram-negative pathogenic bacteria as shown in **Table 1** and **2**. *T. erecta* extract (with water as solvent) was found to be the most effective against *S. typhimurium* (ZOI: 15.66±0.57) and *K. pneumoniae* (ZOI: 12.33±0.28). The inhibitory effect was found to be increased in *S. typhimurium* (ZOI: 23.33± 0.57) and *K. pneumoniae* (ZOI: 22.66± 0.57), when CUD was used as solvent medium. Inhibitory effect of *T. erecta* extract was improved by the CUD solvent medium against all bacteria (R.A.I= 1.50 to 1.89) as shown in **Table 2**. Water extract of *O. sanctum* was found to possess good inhibitory effect against *K. pneumoniae* (ZOI: 13.16± 0.28) and *E. aerogenes* (ZOI: 13.66± 0.57). The combination therapy increased the effectiveness to a greater level against both the bacteria, *K. pneumoniae* (ZOI: 23.33± 0.57) and *E. aerogenes* (ZOI: 21.33± 0.28). When CUD was used as solvent medium for *O. sanctum* extract, the relative activity index increased (1.52 to 1.84) in each case, maximum shown by *E.coli* (R.A.I=1.84).

A. indica was found to have highest inhibition activity against *K. pneumoniae* (ZOI: 12.66± 0.57) and *E. aerogenes* (ZOI: 14.33± 0.57), which is further improved by combination therapy in both *K. pneumoniae* (ZOI: 17.33± 0.28) and *E. aerogenes* (ZOI: 19.66± 0.28). Zone of inhibition was again observed to be improved in all other bacteria with relative activity index ranges from 1.37 to 1.76, where *E. coli* showed highest improved inhibitory effect with CUD as solvent medium (R.A.I=1.76). *C. longa* showed highest inhibitory effect against *K. pneumoniae* (ZOI: 15.66± 0.28) and *E. coli* (ZOI: 15.66±0.28), which further enhanced by combination therapy in both bacteria *K. pneumoniae* (ZOI: 20.16±0.28) and *E. coli* (ZOI: 21.16±0.28). The synergistic effect of combination therapy had improved the inhibitory effect against all other bacteria with R.A.I ranges from 1.37 to 1.80. In case of *E. coli* the synergistic effect was observed maximum (R.A.I=1.80). Water extract of *S. aromaticum* showed potential inhibitory effect against *E. aerogenes* (ZOI: 13.83±0.28) followed by *S. typhimurium* (ZOI: 13.66±0.57) and *K. pneumoniae* (ZOI: 12.83±0.28). The effect was further enhanced by the combination therapy against *E. aerogenes* (ZOI: 20.16±0.28), *S. typhimurium* (ZOI: 17.5± 0.5) and *K. pneumoniae* (ZOI: 18.66± 0.57), as shown in **Table 1** and **2**.

The improved efficacy of plants extract against all bacteria with CUD as solvent medium was tested again by using XTT-colorimetric analysis. The analysis proved the effect of combination therapy when MIC and MBC results were used as criteria of effectiveness. Five combination of bacteria and

plants extract were chosen, which showed very good R.A.I in agar-well diffusion method, for XTT-colorimetric analysis as shown in **Fig. 1**, **Table 3** and **4**. The analysis was done on the results shown by lowest concentration (1.25 mg/ml) of combination therapy. *T. erecta* extract showed very profound effect on inhibition of *E. coli* with R.A.I as high as 2.54. This means CUD distillate increase the efficacy of plant mediated inhibitory effect upto 2.5 fold (33.5% vs. 85.1%). MIC & MBC improved from 20 & 30 mg/ml to 5 & 10 mg/ml, respectively. Similarly, *S. aromaticum* also showed great inhibitory effect against *V. cholerae*. In this case, R.A.I at lowest concentration was observed to be 2.13. CUD improved the percentage reduction of the bacterial population when compared with the water as solvent medium (20.9% vs. 44.6%). In this case, MIC & MBC improved from 30 & 40 mg/ml to 20 & 30 mg/ml, respectively. *C. longa* extract inhibited the growth of *E. aerogenes* more profoundly when used with CUD as solvent medium. CUD improves the reduction of *E. aerogenes* from 25.9% (water as solvent medium) to 49.7% (R.A.I=1.91). In this case, MIC & MBC improved from 30 & 40 mg/ml to 20 & 30 mg/ml, respectively. Combination therapy by using *O. sanctum* extract also showed the profound effect against *K. pneumoniae* when compared with water as solvent medium. Combination therapy increased the reduction percentage of *K. pneumoniae* from 40.8% to 73.6% (R.A.I=1.80). The MIC and MBC values were also improved from 20 & 30 mg/ml to 5 & 10 mg/ml respectively. Similarly, extract of *A. indica* with CUD also showed a great inhibitory effect against bacteria *S. typhimurium*, where the percentage reduction in bacterial population was from 34.9% to 60.2% (R.A.I=1.72). It also improved the MIC and MBC values when CUD was used as solvent medium (30 & 40 mg/ml to 20 & 30 mg/ml, respectively). So, it was observed that plants extract in combination with CUD improved the percentage inhibition of bacterial growth.

TABLE 1: MEASUREMENTS OF ZOI AS OBSERVED AGAINST VARIOUS GRAM-NEGATIVE BACTERIA WITH EXTRACT OF VARIOUS MEDICINAL PLANTS PREPARED USING DDS OR WITH CUD AS SOLVENT MEDIUM. ZOI READINGS WITH CONCENTRATIONS 1.25 mg/ml (I) AND 10 mg/ml (II) ARE SHOWN

Plants	Solvent	ZOI (mm±SD) with respect to concentration of plant extract with DDS or with CUD (I= 1.25 mg/ml; II= 10 mg/ml)									
		V. cholerae		*S. typhimurium*		*K. pneumoniae*		*E. coli*		*E. aerogenes*	
		I	II	I	II	I	II	I	II	I	II
T. erecta	water	2.83±0.28	11.66±0.57	4.66±0.28	15.66±0.57	4.33±0.57	12.33±0.28	2.0±0.0	9.33±0.57	2.66±0.28	11.5±0.5
	CUD	7.66±0.57	17.66±0.57	9.33±0.57	23.33±0.57	6.83±0.28	22.66±0.57	4.16±0.28	17.66±0.57	5.0±0.0	17.33±0.28
O. sanctum	water	2.0±0.0	9.16±0.28	4.66±0.28	11.66±0.57	4.66±0.28	13.16±0.28	2.0±0.0	10.0±0.00	4.0±0.0	13.66±0.57
	CUD	6.83±0.28	13.66±0.28	8.66±0.57	20.33±0.28	8.66±0.57	23.33±0.57	5.0±0.0	18.16±0.28	9.16±0.28	21.33±0.28
A. indica	water	3.33±0.28	12.83±0.28	3.0±0.0	10.83±0.28	3.0±0.0	12.66±0.57	4.0±0.0	10.83±0.28	4.0±0.0	14.33±0.57
	CUD	8.66±0.57	20.16±0.28	6.66±0.28	17.66±0.57	5.66±0.57	17.33±0.28	9.16±0.28	19.33±0.57	8.66±0.57	19.66±0.28
C. longa	water	4.16±0.28	13.33±0.57	4.0±0.0	14.33±0.57	4.66±0.28	15.66±0.28	4.66±0.28	15.66±0.28	2.0±0.0	9.33±0.57
	CUD	9.16±0.28	22.33±0.57	7.66±0.57	19.66±0.28	7.33±0.57	20.16±0.28	10.83±0.28	21.16±0.28	5.66±0.28	16.66±0.57
S. aromaticum	water	3.0±0.0	11.83±0.28	3.0±0.0	13.66±0.57	4.16±0.28	12.83±0.28	2.0±0.0	10.33±0.57	2.66±0.28	13.83±0.28
	CUD	7.33±0.57	20.66±0.57	5.0±0.0	17.5±0.5	8.66±0.57	18.66±0.57	6.83±0.28	17.33±0.28	6.66±0.28	20.16±0.28

TABLE 2: MEASUREMENT OF ACTIVITY INDEX AND RELATIVE ACTIVITY INDEX OF DIFFERENT PLANTS EXTRACT AGAINST PATHOGENIC GRAM-NEGATIVE BACTERIA. DATA IN BRACKETS WITH EACH BACTERIUM ARE THE ZONE OF INHIBITION PRODUCED BY STANDARD ANTIBIOTIC AS DESCRIBED IN TEXT. (W: DOUBLE DISTILLED STERILE WATER; C: CUD AS SOLVENT)

Plants	Measurement of Activity index and relative activity index														
	V. cholerae (24.83±0.28)			*S. typhimurium* (25.33±0.57)			*K. pneumoniae* (23.66±0.57)			*E. coli* (25.16±0.28)			*E. aerogenes* (26.33±0.57)		
	A.I (W)	A.I (C)	R.A	A.I (W)	A.I (C)	R.A	A.I (W)	A.I (C)	R.A	A.I (W)	A.I (C)	R.A	A.I (W)	A.I (C)	R.A
T. erecta	0.46	0.71	1.54	0.61	0.92	1.50	0.52	0.95	1.82	0.37	0.70	1.89	0.43	0.65	1.51
O. sanctum	0.36	0.55	1.52	0.46	0.80	1.73	0.55	0.98	1.78	0.39	0.72	1.84	0.51	0.81	1.58
A. indica	0.51	0.81	1.58	0.42	0.69	1.64	0.53	0.73	1.37	0.43	0.76	1.76	0.54	0.74	1.37
C. longa	0.53	0.89	1.67	0.56	0.77	1.37	0.66	0.85	1.28	0.62	0.84	1.35	0.35	0.63	1.8
S. aromaticum	0.47	0.83	1.76	0.53	0.69	1.30	0.54	0.78	1.44	0.41	0.68	1.65	0.52	0.76	1.46

TABLE 3: MEASUREMENT OF O.D (MEAN ± STANDARD DEVIATION) OF GROWTH OF DIFFERENT GRAM-NEGATIVE BACTERIA AT DIFFERENT CONCENTRATION OF DIFFERENT PLANTS EXTRACT (WITH DDS OR CUD AS SOLVENT). WITH CONCENTRATION C1 (40 mg/ml) AND C2 (30 mg/ml) BOTH THE SOLVENT MEDIUM SHOWED COMPLETE INHIBITORY EFFECT AGAINST ALL BACTERIA. HENCE, ONLY THE RESULTS OF CONCENTRATION C3 (20 mg/ml) TO C7 (1.25 mg/ml) ARE SHOWN. (W: DOUBLE DISTILLED STERILE WATER)

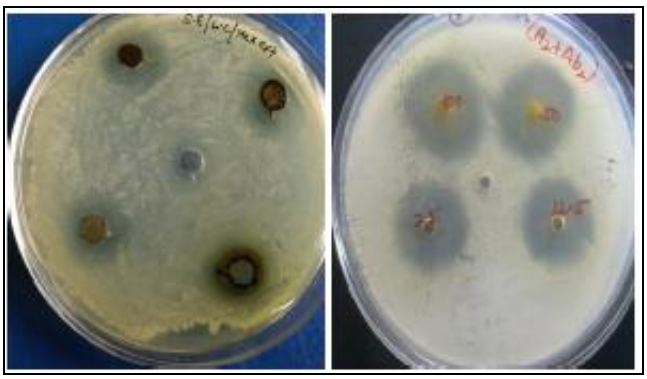
BC	PC	C3 (20 mg/ml)		C4 (10 mg/ml)		C5 (5 mg/ml)		C6 (2.5 mg/ml)		C7 (1.25 mg/ml)	
		W	CUD	W	CUD	W	CUD	W	CUD	W	CUD
U1	0.064± 0.010	0.00	0.00	0.152± 0.029	0.00	0.258± 0.044	0.00	0.436± 0.037	0.089± 0.027	0.665± 0.052	0.149± 0.039
U2	0.088± 0.008	0.00	0.00	0.122± 0.018	0.00	0.215± 0.031	0.00	0.407± 0.056	0.158± 0.017	0.592± 0.022	0.264± 0.018
U3	0.110± 0.009	0.067± 0.010	0.00	0.176± 0.017	0.032± 0.008	0.373± 0.017	0.116± 0.011	0.484± 0.020	0.344± 0.025	0.651± 0.032	0.398± 0.008
U4	0.152± 0.016	0.095± 0.016	0.00	0.191± 0.014	0.034± 0.011	0.421± 0.037	0.155± 0.015	0.579± 0.022	0.341± 0.018	0.741± 0.021	0.503± 0.014
U5	0.174± 0.017	0.124± 0.024	0.00	0.281± 0.045	0.039± 0.018	0.445± 0.033	0.166± 0.024	0.637± 0.024	0.353± 0.039	0.791± 0.083	0.554± 0.051

[PC: Positive control; U1: *T. erecta* & *E. coli*; U2: *O. sanctum* & *K. pneumoniae*; U3: *A. indica* & *S.typhimurium* ; U4: *C. longa* & *E.aerogenes*; U5: *S. aromaticum* & *V. cholerae*]

TABLE 4: MEASUREMENT OF PERCENTAGE REDUCTION IN BACTERIAL POPULATION USING DIFFERENT PLANTS EXTRACTS WITH DIFFERENT SOLVENT MEDIUM-DDS & CUD. RELATIVE ACTIVITY INDEX AT 1.25 MG/ML CONCENTRATION WERE MEASURED FOR RESPECTIVE BACTERIAL AND PLANT COMBINATION IN BOTH SOLVENT MEDIUM. (W: DOUBLE DISTILLED STERILE WATER; C: CUD AS SOLVENT; CR: COMPLETE REDUCTION).

BC	PC	C3		C4		C5		C6		C7		R.A.I.
		W	C	W	C	W	C	W	C	W	C	C7 (W) Vs. C7(C)
U1	93.6	CR	CR	84.8	CR	74.2	CR	56.4	91.1	33.5	85.1	2.54
U2	91.2	CR	CR	87.8	CR	78.5	CR	59.3	84.2	40.8	73.6	1.80
U3	89.0	93.3	CR	82.4	96.8	62.7	88.4	51.6	65.6	34.9	60.2	1.72
U4	84.8	90.5	CR	80.9	96.6	57.9	84.5	42.1	65.9	25.9	49.7	1.91
U5	82.6	87.6	CR	71.9	96.1	55.5	83.4	36.3	64.7	20.9	44.6	2.13

[PC: Positive control; **U1:** *T. erecta* & *E. coli***; U2:** *O. sanctum* & *K. pneumoniae***; U3:** *A. indica* & *S.typhimurium***; U4:** *C. longa* & *E.aerogenes***; U5:** *S. aromaticum* & *V. cholerae***]**



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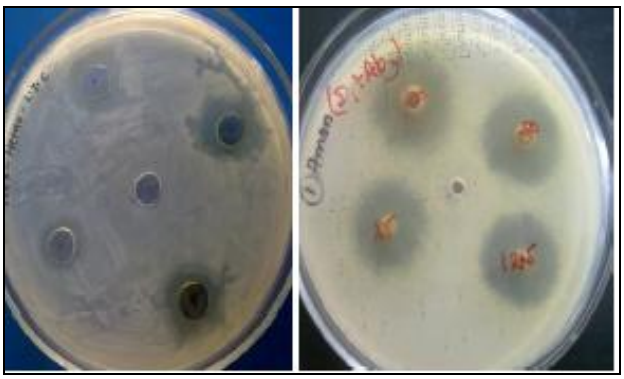

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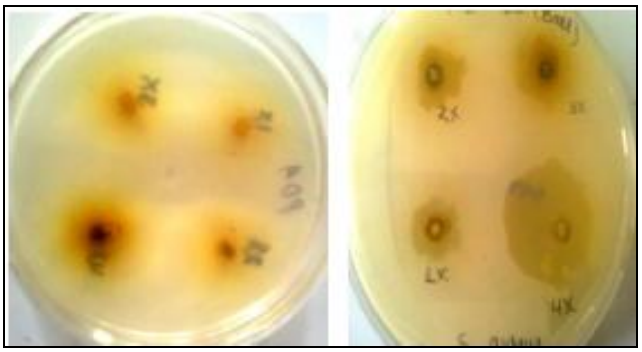
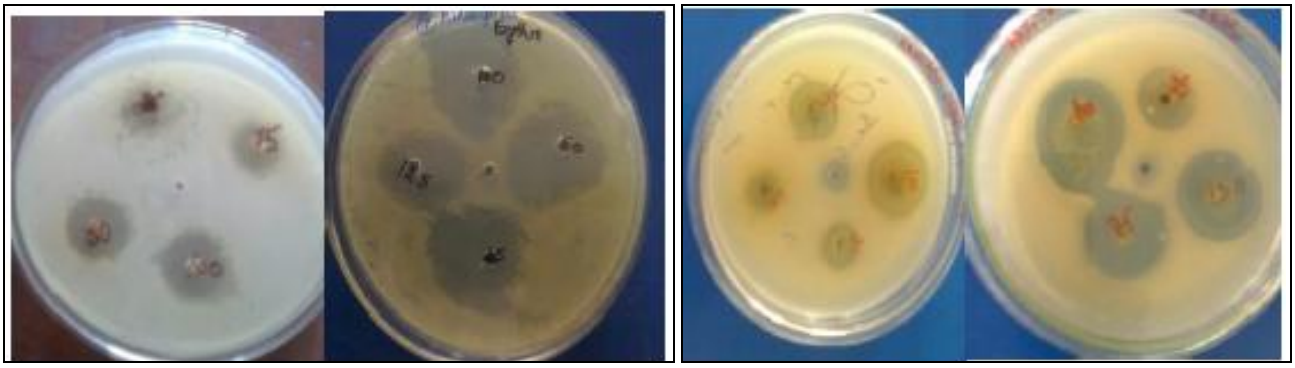


[B3]



[B4] [B5]

FIG. 1: ZOI OBSERVED AGAINST DIFFERENT BACTERIA USING DDS AND CUD AS SOLVENT MEDIUM FOR DIFFERENT CONCENTRATIONS OF LEAF EXTRACTS OF DIFFERENT PLANTS. LEFT PICTURE IN EVERY GROUP SHOWED THE INHIBITORY EFFECT WITH DDS AS SOLVENT, AND RIGHT PICTURE SHOWED THE IMPROVED INHIBITORY EFFECT AGAINST THE SAME BACTERIA WITH CUD AS SOLVENT MEDIUM. [**B1:** *T. erecta* & *E. coli*; **B2:** *O. sanctum* & *K. pneumoniae*; **B3:** *C. longa* & *E. aerogenes*; **B4:** *A .indica* & *S. typhimurium*; **B5:** *S. aromaticum* & *V. cholerae*].

DISCUSSION: Two main reasons- evolution of multiple drug resistant strains and the strains with reduced susceptibility to present antibiotics- necessitated the search for novel antibacterial compounds and effective treatment strategies like combination therapies. Since the last decade, an alternative which was suggested was to use animal derived preparations (venom of snake, toad, scorpion, spider etc.) to combat many diseases like cancer, nerve disorder, viral diseases etc [18, 19]. In our medicinal system CUD, an animal derived preparation, has been used as an antiseptic agent since time immemorial. Scientifically, the effect of CUD as antibacterial agent, was proved by two studies which demonstrated the inhibitory potential of CUD against *P. aeruginosa* (15.4±1.23), *S. typhi* (13.6±0.17), *K. pneumonia* (11.0±0.14) [20], *E. Coli* (30.0 mm), *B. subtilis* (32.0 mm), *S. aureus* (25.0 mm), *K. pneumonia* (28.0 mm) and *P. vulgaris* (28.0 mm) [21].

Literature has ample evidences that all the plants, which are used in present report, have good inhibitory potential against various pathogenic gram-negative bacteria, as *A. indica* was found to be a good inhibitor of *E. coli*, *K. pneumonia* & *V. cholera* [22-24], whereas *S. aromaticum* possesses good antibacterial potential against *S. typhimurium* and *K. pneumonia* [23, 25]. *C. longa* is well known for its antibacterial effect against many pathogenic gram-positive and gram-negative bacteria [26, 27]. Till date, only in one study MDR strains of *E. Coli* (12.68 mm), *S. aureus* (>8.66 mm) and *P. aeruginosa* (>8.66 mm) were examined for their response to combination therapy comprising CUD and *A. indica* extract [28].

In our previous report, we demonstrated the successful and effective antibacterial activity of combination therapy against six gram-positive bacteria [8]. We observed superior antibacterial potential of combination therapy when compared with individual plants extract. Here, in the present report, at the highest concentration (10 mg/ml) with CUD as solvent medium, it has been observed that all the plants extract showed great synergistic anti-bacterial effect. Combination therapy with *T. erecta* extract has been observed to be the most effective against two gram-negative bacteria *K. pneumonia* and *E. coli*, where R.A.I was improved nearly two times, 1.82 & 1.89, respectively. These results were reproducible with XTT-calorimetric assay also, where lowest concentration (1.25 mg/ml) of combination therapy was used. Again, combination therapy with *T. erecta* extract showed 2.5 times improved antibacterial effect against *E. coli*.

Similarly, *S. aromaticum* extract with CUD as solvent medium also improved the antibacterial effect more than two times (2.13) against *V. cholerae*. Further, as shown in **Table 3**, CUD also improved the results of MIC and MBC activities from 2 to 3 times against all gram-negative bacteria.

CUD owes its clinical significance due to the presence of phenols. The bioactive compounds present in plant and animal source may have synergistic effect towards abolition of gram-negative bacterial infection. The appraisal of obtained results shows that combination therapy has highly effective antibacterial potential as compared to water extract of individual plants, and

secondly, the therapy has some potential advantages like, avoidance of evolution of multiple drug resistant strains and less toxic effect on host health.

CONCLUSION: The objective of this research was to authenticate the use of combination therapy against gram-negative bacteria and the results were in favor of combination therapy. This new trend of drug development, which includes the biochemicals from both plant and animal origin, has great potential as antimicrobial therapy along with minimizing the menace of emergence of MDR strains. Hence this study holds importance in using combination therapy as alternative source for treating various diseases caused by gram-negative bacteria. So, to conclude, the present study has shown that the combination therapy has a wide spectrum antibacterial activity and can serve as a substantial potent antibacterial agent.

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CONFLICT OF INTEREST STATEMENT: We declare that we have no conflict of interest.

REFERENCES:

1. Tamma PD, Cosgrove SE, Maragakis LL: Combination therapy for treatment of infections with gram-negative bacteria. Clin Microbiol Rev. 2012; 25(3):450-70.
2. Chitravadivu C, Manian S, Kalaichelvi K: Qualitative analysis of selected Medicinal plants, Tamilnadu, India. Middle-East Journal of Scientific Research 2009; 4(3):144-146.
3. Kaur S, Mandal P: Study of total phenolic and flavonoid content, antioxidant activity and antimicrobial properties of medicinal plants. Journal of Microbiology and Experimentation 2014; 1(1):1-6.
4. Chattopadhyay RR, Bhattacharyya SK, Medda C, Chanda S and Bag A: A Comparative Evaluation of Antibacterial Potential of Some Plants Used in Indian Traditional Medicine for the Treatment of Microbial Infections. Brazilian archives of Biology and Technology 2009; Vol.52, 5: 1123-1128.
5. Sinha S, Mishra P, Amin H, Rah B, Nayak D, Goswami A, Kumar N, Vishwakarma R and Ghosal S: A new cytotoxic quinolone alkaloid and a pentacyclic steroidal glycoside from the stem bark of Crataeva nurvala: Study of antiproliferative and apoptosis inducing property. European Journal of Medicinal Chemistry 2013; 60: 490-496.
6. Dhama K, Chauhan RS, Singhal L: Anti-cancer activity of Cow urine: current status and future directions. International Journal of Cow science 2005; 1(2):1-25.
7. Kishore VS, Rao LR, Ramesh B, Aditya AK: Indian cow urine distillation and therapeutic uses. Mintage journal of pharmaceutical and medical sciences 2015; Vol 4:1-5.
8. Pahal V, Kaur A and Dadhich KS: Synergistic antibacterial effect of some Indian medicinal plants and cow (*Bos indicus*) urine distillate against selective pathogenic gram-positive bacteria. Int J Pharm Sci Res 2016; 7(11): 1000-09.
9. Mehrotra S, Srivastava AK, Nandi SP: Comparative antimicrobial activities of neem, amla, aloe, assam tea and clove extracts against *Vibrio cholera*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Journal of Medicinal Plants research 2010; Vol.4 (22):2393-2398.
10. Dasgupta N, Ranjan S, Saha P, Jain R, Malhotra S and Mohamed SMA: Antibacterial activity of leaf extract of Mexican marigold (*Tagetes erecta*) against different gram positive and gram negative bacterial strains. Journal of pharmacy research 2012; 5(8): 4201-4203.
11. Mistry KS, Sanghvi Z, Parmar G, Shah S: The antimicrobial activity of *Azadirachata indica*, *Tinospora cardifolia*, *Ocimum sanctum* and 2% chlorhexidine gluconate on common endodontic pathogens: An *in vitro* study. Eur J Dent 2014; 8:172-7.
12. Lehrer RI, Rosenman M: Ultrasensitive assays for endogenous antimicrobial polypeptides. Journal of microbiological methods 1991; 137: 167-173.
13. Sathasivam A, Muthuselvam M, Rajendran R: Antimicrobial activities of cow urine distillate against some clinical pathogens. Global journal of pharmacology 2010; 4(1):41-44.
14. Jarald E, Edwin S, Tiwari V, Garg R, Toppo E: Antioxidant and antimicrobial activities of Cow urine. Global journal of pharmacology 2008; 2(2):20-22.
15. Al-Bakri GA**,** Afifi FU: Evaluation of antimicrobial activity of selected plant extracts by rapid XTT colorimetry and bacterial enumeration. Journal of Microbiological Methods 2007; 68:19-25.
16. Ncube NS, Afolayan AJ, Okoh AI: Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. Afr J Biotechnol 2008; 7:1797-1806.
17. Das K, Tiwari RKS, Shrivastava DK: Techniques for evalu-ation of medicinal plant products as antimicrobial agent: Current methods and future trends. J Med Plants Res 2010; 4:104-111.
18. Gomes A, Bhattacharjee P, Mishra R, Biswas AK, Dasgupta SC and Giri B: Anticancer potential of animal venoms and toxins. Indian Journal of Experimental Biology 2010; 48:93-103.
19. Shanbhag VKL: Applications of snake venoms in treatment of cancer. Asian Pacific Journal of Tropical Biomedicine 2015; 5(4):275-76.
20. Sathasivam A, Muthuselvam M, Rajendran R: Antimicrobial activities of cow urine distillate against some clinical pathogens. Global journal of pharmacology 2010; 4(1):41-44.
21. Jarald E, Edwin S, Tiwari V, Garg R, Toppo E: Antioxidant and antimicrobial activities of Cow urine. Global journal of pharmacology 2008; 2(2):20-22.
22. El-Kamali HH and EL-Karim EMA: Evaluation of Antibacterial Activity of Some Medicinal Plants Used in Sudanese Traditional Medicine for Treatment of Wound Infections. Academic Journal of Plant Sciences 2009; 2 (4): 246-251.

23. Yamasaki S, Asakura M, Neogi SB, Hinenoya A, Iwaoka E, Aoki S: Inhibition of virulence potential of *Vibrio cholerae* by natural compounds. Indian J Med Res 2011; 133:232-239.
24. Shaik G, Sujatha N, Mehar SK. Medicinal plants as source of antibacterial agents to counter *Klebsiella pneumoniae*. Journal of Applied Pharmaceutical Science 2014; 4 (01):135-147.
25. Atteia HG, Hussein EAM. *In vitro* antibacterial and synergistic effects of some plant extracts against *Staphylococcus aureus* and *Klebsiella pneumoniae*. Journal of Antimicrobials 2014; 129: 338-346.
26. Marasini BP, Baral P, Aryal P, Ghimire KR, Neupane S, Dahal N, Singh A, Ghimire L and Shrestha K. Evaluation of Antibacterial Activity of Some Traditionally Used Medicinal Plants against Human Pathogenic Bacteria. BioMed Research International 2015; 1-7.
27. Chakraborty S**.** Plant derived Extracts and Respective Compounds against major Life-Threatening Water contaminant Bacteria *Vibrio cholerae*: A comprehensive Mini Review. Int. Res. J. Environment Sci 2015; 4(7): 96-105.
28. Rajapandiyan K, Shanti S, Murugan AM, Muthu GA, Singh AR: *Azadirachta indica*-cow urine extract, a novel controlling agent towards clinically significant Multi drug resistant pathogens. Journal of Applied Pharmaceutical Sciences 2011; 01(10):107-113.

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