IJPSR (2016), Vol. 7, Issue 11 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 26 May, 2016; received in revised form, 31 July, 2016; accepted, 09 August, 2016; published 01 November, 2016

SYNERGISTIC ANTIBACTERIAL EFFECT OF SOME INDIAN MEDICINAL PLANTS AND COW (*BOS INDICUS*) URINE DISTILLATE AGAINST SELECTIVE PATHOGENIC GRAM-POSITIVE BACTERIA

Vikas Pahal [*1], Arashdeep Kaur [2] and Krishan Swaroop Dadhich [1, 2]

Department of Microbiology [1], Dolphin (P.G) College of Science and Agriculture, Chunni Kalan, F.G. Sahib, Punjab, India.
Department of Microbiology [2], Punjab University, Chandigarh, U.T., India.

Keywords:

Gram-Positive Bacteria, Antimicrobial Agents, Combination Therapy, Cow-Urine Distillate

Correspondence to Author:
Dr. Vikas Pahal

Head, Department of Microbiology Dolphin Post Graduate College of Science and Agriculture, Chunni Kalan, F.G. Sahib, Punjab-140406, India.

Email: vikaspahal@gmail.com

ABSTRACT: Pathogenic bacterial infections are of serious clinical concern around the world. Indiscriminate use of antibiotics makes this situation beyond control. Hence, microbiologists are trying to find out the new and promising therapies against these bacterial infections. In the present research, combination therapy, which comprises extract of different medicinal plants and cow urine distillate, was used against six pathogenic gram positive bacteria. The efficacy of this combination therapy was evaluated by two methods: agar well diffusion method and XTT-colorimetric method. It has been demonstrated that CUD has profound synergistic effect on the efficacy of plants-derived extracts against the pathogenic bacteria at the specified level. The relative activity of different extracts with CUD as solvent medium was found to be increased (R.A.I= 1.61 to 2.13) against every bacteria of present study. MIC & MBC results were greatly improved (upto 2 to 3 folds) by the combination therapy. The results obtained in the present study could be useful for further research to assess the effect of combination therapy against other pathogenic microorganisms like fungi and viruses.

INTRODUCTION: Non-compensated diseases due to various bacterial infections account for the large number of morbidity and mortality cases worldwide. Inconsequential and unsystematic use of antibiotics are fueling the process of evolution of Multiple Drug Resistance (MDR) bacterial strains, making this problem worst at such a level that a common infection could be a life threatening one in today's scenario.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(11).4429-36

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (11).4429-36

This forced the scientific community to look after the alternative remedies, keeping in mind the problem of evolution of resistant strains and undesirable side effects of synthetic antibiotics. Phyto-medicines are the right and the only alternative to this problem [1]. In fact, according to W.H.O reports, more than 80% of the population of developing countries depends directly on plant-derived medicines or remedies for their different ailments as primary health care. Plant-derived drugs (crude extract) are preferred to synthetic drugs for being economical, harmless, and effective [2-4].

In ethano-botanical literature of India there are several hundreds of plants which are known to have great antibacterial activities. Plants defend

themselves from various microbial infections by using products of secondary metabolites like: alkaloids, flavonoids, phenols, volatile essential oils, terpenes etc. These secondary metabolites have also been shown to possess antimicrobial activities against human pathogens. Literature is full of evidences which demonstrate the promising effects of these metabolites against many pathogenic bacteria [3, 4]. These naturally occurring phyto-chemicals have unique built-in chirality due to which they can bind or manipulate the functioning of biologically important enzymes and receptors, which is the reason why they are as effective as bactericidal agents [5]. So, a predominant interest has been observed in evaluating different local plants, used in folk-medicines (mainly from Brazil, China and India), for their antibacterial effects.

Similarly to plants, Indian indigenous white Cow's (*Bos indicus*) Urine Distillate (CUD), known as 'Ark', has been used in various folk-medicines to combat various aliments since time immemorial. Recently, Council of Scientific and Industrial Research (CSIR), India, had obtained U.S patent (no. 6896907/6410059) for various active principles in CUD, which have antimicrobial properties [6]. They also showed the bioenhancer activities of CUD with commonly used antibiotics, antifungal and anti-cancerous drugs. It has been proposed that active principles along with phenols (present in the CUD) may disrupt the flow of electrons in electron transport chain or proton motive force required for oxidative phosphorylation, which directly inhibit the bacterial growth [6, 7]. Not much literature is available to support these findings of CUD as a bactericidal agent; still two studies successfully prove the anti-bacterial effect of CUD [8, 9].

Researchers throughout the world are desperately working to discover the new individual or group of old antimicrobial compounds in different combinations to find solution against the sepathogenic bacteria-related infections. So, it was decided to check whether the new combination therapy of plant-derived phytochemicals with CUD (animal-derived chemical) have any improved effect against the pathogenic bacteria, or not. In the present paper, we are presenting our results against six pathogenic gram-positive bacteria, by using leaf extract of five medicinal plants (*Calendula officinalis*, *Tageteserecta*, *Ocimum sanctum*, *Rosaindica* and *Azadirachta indica*) whose antibacterial properties are well established in literature [2, 3, 10, 11].

MATERIALS AND METHODS:

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

Leaves collection and processing: The leaves of all the five plants *Calendula officinalis*, *Tageteserecta*, *Ocimum sanctum*, *Rosa indica* and *Azadirachta indica* were collected. They were first carefully washed under running tap water, followed by sterile distilled water and then air dried at room temperature (35-40^{0}C) for 5-7 days. After that, leaves were homogenized to a fine powder using a sterilized mixer grinder and stored in air tight bottles. First of all, 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. Then, double distilled Sterilized (DDS) water was used to extract the active principle's using the Soxhlet assembly for 72 hrs. 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: Six gram-positive bacterial strains, *Staphylococcus aureus* (MTCC 3160), *Staphylococcus epidermidis* (MTCC9040), *Streptococcus pneumonia* (MTCC 1935), *Micrococcus luteus* (MTCC106), *Streptococcus mutans* (MTCC 890) and *Streptococcus pyogenes* (MTCC 1927) were obtained 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. The stock cultures were then sub-cultured

at regular intervals for further use during antimicrobial activity testing.

Screening for antimicrobial activity:
Bacteria were grown overnight in broth at 37^0C 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 37^0C. The bacteria were then centrifuged for 900 g for 10 minutes at 4^0C, washed with 10 mM sodium phosphate buffer (pH=7.4), and resuspended in the same cold buffer [12]. The optical density of an aliquot was measured at 620 nm and the concentration of bacteria were standardized (OD_{620}0.20= 5X10^7 CFU/ml).Antimicrobial studies were carried out using cup (well) assay method also known as agar diffusion method. Approximately, 20-25 ml of pre autoclaved agar media cooled at 45^0C 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 Agarplates 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 [13, 14]. Test control was used in each experiment.

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 (5 a.m.). In laboratory, on the same day, urine was 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. The vapours of CU were then collected in distillation process. 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 [6-9].

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^oC for 18-24 hours. DDS water served as the negative control and antibiotic ciprofloxacin 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: Antimicrobial activity with different concentration of plant extracts with DDS and CUD was also evaluated by the XTT-colorimetric method [15]. Using this method, the antimicrobial activity was assessed by measuring the relative reduction informazan production. The XTT assay indirectly measures the microbial activity by assessing the Electron Transport System (ETS) activity using artificial electron acceptors, redox dyes that can successfully compete with oxygen for election's.

The tetrazolium salt 2,3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium - 5 - carboxanilide (XTT)is reduced by the dehydrogenase enzymes present in the ETS system to a water soluble formazen dye, the absorbance of which can be measured colorimetrically at 490 nm. This Assay is valuable in providing a reasonable overall estimate of antimicrobial activity. 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 (**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.30µl 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. On the second day, 100 µl of each well material is transferred into

new 96-wellflat-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 37^0C 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 ciprofloxacin (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:

Percentage reduction (% Rd) =

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

Determination of Activity Index and Relative Activity Index:

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:

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):

MIC for each test organism was determined by the microdilution broth method. XTT- calorimetric readings were used to determine the effect of different concentrations of various extracts (dissolved in DDS or CUD). 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^oC 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: Different plant extracts in different concentrations (with DDS or CUD as the solvent medium) were found to be effective against every bacterium differentially, as shown in **Table 1** and **2.** *S. aureus* was shown to be inhibited greatly by *T.erecta* extract (14.83±0.28). The combination therapy enhanced the antibacterial activity (24.33±0.55) at a greater level. Relative active index also showed the increased effectiveness (R.A.I=1.63) of combination therapy against *S. aureus*. The rest of the plants extract also showed a great synergistic effect of combination therapy against *S. aureus* (R.A.I ranges from 1.65-1.82), maximum showed by *C. officinalis* (R.A.I=1.82). *R.indica* was found to have highest inhibition activity against *S. epidermidis* (13.66±0.57), which is further improved by combination therapy (22.66±0.57). All the remaining four plants also observed to have high relative activity index which ranges from 1.63 to as high as 2.20. *C. officinalis* showed highest improved activity against the bacteria *S. epidermidis* with CUD as solvent

medium (R.A.I= 2.20). *O. sanctum* extract was found to be more effective against *S. pneumoniae* (11.66±0.57) and also when used in synergism with combination therapy (22.66±0.5). CUD increased the relative activity index to nearly double (R.A.I=1.97). Other plants extract also showed improved activity with CUD (R.A.I= 1.61-1.82), as shown in **Table 1** and **2.**

R.indica extract was again found to be the most effective against *M.luteus* (13.83±0.28), but in case of combination therapy against this bacteria *O. sanctum* (22.5±0.5) was found to have more profound effect in comparison to other plants extract combinations. Both *A.indica* (R.A.I=1.79) and *O. sanctum* (R.A.I=1.78) were found to have improved activity with CUD-mediated combination therapy. *S. mutans* was inhibited by *O. Sanctum* with great effect (14.66±0.57). But, with CUD-mediated therapy *T.erecta* was found to be most effective in comparison to others plants extracts (R.A.I= 1.91) against *S.mutans* (ZOI=22.66±0.57). Other plants extract also showed improved activity with CUD (R.A.I= 1.37-1.86) against *S. mutans*. *T. erecta*water extract showed its profound antimicrobial activity against *S. pyogenes* (13.83+0.28), which was further enhanced (R.A.I= 1.70) by the synergistic effect of CUD (23.33+0.57). The R.A.I was found to be increased by using CUD as a solvent as compared to water with other plants extracts (1.38-1.72) against *S. pyogenes* as shown in **Table 1** and **2.**

XTT-colorimetric analysis also proved the effectiveness of combination therapy against the pathogenic bacteria with respect to the improved MIC and MBC. Six 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**.

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

		ZOI (mm±SD) with respect to concentration of plant extract with DDS or with CUD (I= 1.25 mg/ml; II= 10 mg/ml)											
		S. aureus		*S. epidermidis*		*S. pneumoniae*		*M. luteus*		*S. mutans*		*S. pyogenes*	
Plants	**Solvent**	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
C.officinalis	Water	2.0 ±0.0	10.33 ±0.57	2.83 ±0.28	9.16 ±0.28	2.66 ±0.28	9.83 ±0.28	2.83 ±0.28	11.33 ±0.28	2.0 ±0.0	9.83 ±0.57	2.83 ±0.28	12.66 ±0.57
	CUD	8.66 ±0.58	18.66 ±0.57	6.66 ±0.28	20.16 ±0.28	5.66 ±0.57	17.66 ±0.57	9.16 ±0.28	19.33 ±0.57	6.66 ±0.57	15.66 ±0.28	6.66 ±0.57	21.33 ±0.28
T.erecta	Water	4.33 ±0.57	14.83 ±0.28	4.16 ±0.28	13.66 ±0.28	4.0 ±0.0	12.66 ±0.57	2.33 ±0.28	11.83 ±0.28	4.33 ±0.57	11.83 ±0.28	3.0 ±0.0	13.83 ±0.28
	CUD	9.66 ±0.28	24.33 ±0.55	9.33 ±0.57	22.33 ±0.57	6.66 ±0.28	21.33 ±0.57	8.33 ±0.57	19.66 ±0.28	9.33 ±0.57	22.66 ±0.57	9.66 ±0.57	23.33 ±0.57
A.indica	Water	2.83 ±0.28	11.66 ±0.57	4.0 ±0.0	12.66 ±0.57	4.66 ±0.28	13.83 ±0.28	2.16 ±0.28	10.33 ±0.57	3.0 ±0.0	10.33 ±0.57	2.83 ±0.28	12.66 ±0.57
	CUD	7.33 ±0.57	19.66 ±0.28	9.16 ±0.28	21.16 ±0.28	9.66 ±0.57	22.33 ±0.57	6.83 ±0.28	18.66 ±0.57	6.66 ±0.28	19.33 ±0.57	6.0 ±0.0	18.5 ±0.5
R.indica	Water	4.66 ±0.28	12.83 ±0.28	5.33 ±0.57	13.66 ±0.57	3.0 ±0.0	11.83 ±0.28	3.0 ±0.0	13.83 ±0.28	4.16 ±0.28	13.66 ±0.57	2.0 ±0.0	11.33 ±0.28
	CUD	9.16 ±0.28	21.16 ±0.28	10.83 ±0.28	22.66 ±0.57	7.66 ±0.28	20.16 ±0.28	7.66 ±0.57	19.66 ±0.28	9.16 ±0.28	18.66 ±0.57	5.33 ±0.57	15.66 ±0.28
O. sanctum	Water	2.83 ±0.28	11.83 ±0.28	5.0 ±0.0	12.83 ±0.28	4.0 ±0.0	11.66 ±0.57	4.16 ±0.28	12.66 ±0.57	4.0 ±0.0	14.66 ±0.57	2.66 ±0.28	10.33 ±0.57
	CUD	9.16 ±0.28	19.66 ±0.28	8.66 ±0.57	21.16 ±0.28	10.33 ±0.57	22.66 ±0.5	11.33 ±0.28	22.5 ±0.5	8.66 ±0.57	21.16 ±0.28	5.0 ±0.0	17.66 ±0.57

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

	Measurement of Activity index and relative activity index																	
Plants	***S. aureus* (26.0)**			***S. epidermidis* (23.3±0.57)**			***S. pneumoniae* (24.83+0.28)**			***M.luteus* (24.0+0.0)**			***S.mutans* (24.0+0.0)**			***S. pyogenes* (25.33+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**	**A.I (W)**	**A.I (C)**	**R.A.**
C. officinalis	0.39	0.71	1.82	0.39	0.86	2.20	0.39	0.71	1.82	0.47	0.80	1.70	0.40	0.65	1.62	0.49	0.84	1.71
T.erecta	0.57	0.93	1.63	0.58	0.95	1.63	0.50	0.85	1.7	0.49	0.81	1.65	0.49	0.94	1.91	0.54	0.92	1.70
A. indica	0.44	0.75	1.70	0.54	0.90	1.66	0.55	0.89	1.61	0.43	0.77	1.79	0.43	0.80	1.86	0.49	0.73	1.48
R.indica	0.49	0.81	1.65	0.58	0.97	1.67	0.47	0.81	1.72	0.57	0.81	1.42	0.56	0.77	1.37	0.44	0.61	1.38
O. sanctum	0.45	0.75	1.66	0.55	0.90	1.63	0.46	0.91	1.97	0.52	0.93	1.78	0.61	0.88	1.44	0.40	0.69	1.72

TABLE 3: MEASUREMENT OF O.D (MEAN ± STANDARD DEVIATION) OF GROWTH OF DIFFERENT GRAM-POSITIVE BACTERIA AT DIFFERENT CONCENTRATION OF LEAVES 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.

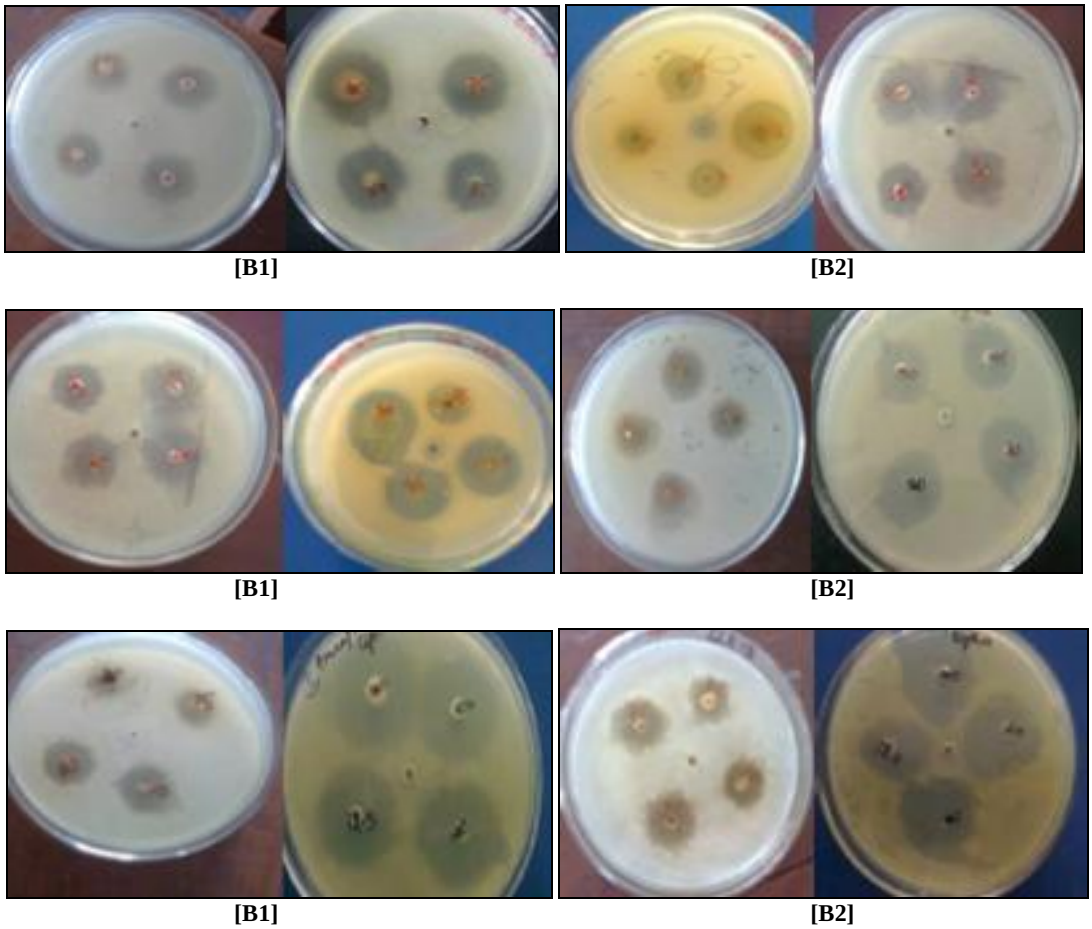
BC	PC	C3 (20 mg/ml)		C4 (10 mg/ml)		C5 (5 mg/ml)		C6 (2.5 mg/ml)		C7 (1.25 mg/ml)	
		W	CUD	W	CUD	W	CUD	W	CUD	W	CUD
B1	0.074±0.016	0.00	0.00	0.008±0.006	0.00	0.105±0.015	0.00	0.305±0.033	0.094±0.016	0.594±0.031	0.175±0.025
B2	0.099±0.022	0.009±0.006	0.00	0.127±0.045	0.00	0.366±0.037	0.008±0.002	0.536±0.047	0.128±0.036	0.680±0.038	0.324±0.055
B3	0.144±0.031	0.006±0.002	0.00	0.101±0.014	0.008±0.002	0.274±0.035	0.110±0.030	0.435±0.039	0.267±0.057	0.667±0.058	0.418±0.033
B4	0.065±0.020	0.00	0.00	0.007±0.002	0.00	0.151±0.030	0.00	0.358±0.047	0.010±0.001	0.503±0.041	0.195±0.022
B5	0.089±0.008	0.00	0.00	0.005±0.003	0.00	0.232±0.026	0.00	0.408±0.043	0.038±0.017	0.589±0.026	0.204±0.030
B6	0.120±0.024	0.00	0.00	0.050±0.019	0.00	0.318±0.065	0.069±0.018	0.552±0.045	0.154±0.026	0.718±0.037	0.397±0.055

[PC: Positive control; **B1:** *C. officinalis* **&** *S. epidermidis***; B2:** *T. Erecta* **&** *S. mutans***; B3:** *T.erecta* **&** *S. aureus***; B4:** *R. Indica* **&** *M. luteus***; B5:** *O. Sanctum* **&** *S. pneumoniae***; B6:** *T.erecta* **&** *S. pyogenes***]**

TABLE 4: MEASUREMENT OF PERCENTAGE REDUCTION IN BACTERIAL POPULATION USING DIFFERENT LEAF 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 THE SOLVENT MEDIUM.

BC	PC	C3		C4		C5		C6		C7		R.A.I. C7 (W) Vs. C7(C)
		W	C	W	C	W	C	W	C	W	C	
B1	92.6	C.R	C.R	99.2	C.R	89.5	C.R	69.5	90.6	40.6	82.5	2.03
B2	90.1	99.1	C.R	87.3	C.R	63.4	99.2	46.4	87.2	32.0	67.6	2.11
B3	85.6	99.4	C.R	89.9	99.2	72.6	89.0	56.5	73.3	33.3	58.2	1.74
B4	93.5	C.R	C.R	99.3	C.R	84.9	C.R	64.2	99.0	49.7	80.5	1.61
B5	91.1	C.R	C.R	99.5	C.R	76.8	C.R	59.2	96.2	41.1	79.6	1.93
B6	88.0	C.R	C.R	95.0	C.R	68.2	93.1	44.8	84.6	28.2	60.3	2.13

[PC: Positive control; **B1:** *C. officinalis* **&** *S. epidermidis***; B2:** *T. Erecta* **&** *S. mutans***; B3:** *T.erecta* **&** *S. aureus***; B4:** *R. Indica* **&** *M. luteus***; B5:** *O. Sanctum* **&** *S. pneumoniae***; B6:** *T.erecta* **&** *S. pyogenes***]**



[B1] **[B2]**

[B1] **[B2]**

[B1] **[B2]**

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:** *C. officinalis* **&** *S. epidermidis***; B2:** *T. Erecta* **&** *S. mutans***; B3:** *T.erecta* **&** *S. aureus***; B4:** *R. Indica* **&** *M. luteus***; B5:** *O. Sanctum* **&** *S. pneumoniae***; B6:** *T.erecta* **&** *S. pyogenes*].

At lowest concentration (1.25 mg/ml), *C. officinalis* extract showed R.A.I with CUD as high as 2.03. It means, CUD increases the efficacy of the antimicrobial effect significantly upto two folds with CUD as solvent medium. MIC & MBC improved from 10 & 20 mg/ml to 5 & 10 mg/ml respectively. *T. erecta* extract was found to be most effective against three bacteria- *S. mutans*, *S. aureus* and *S. pyogenes* with CUD as solvent medium. The *T.erecta* extract at lowest concentration (1.25 mg/ml) showed excellent improved efficacy (with CUD as solvent medium) against *S.mutans* (R.A.I=2.11), *S. aureus* (R.A.I=1.74)) and *S. pyogenes* (R.A.I=2.13).

Similarly, MIC and MBC values were also improved with combination therapy against *S.mutans* (20 mg/ml & 30 mg/ml to 5 mg/ml & 10 mg/ml), *S. aureus* (20 mg/ml & 30 mg/ml to 10 mg/ml & 20 mg/ml) and *S. pyogenes* (20 mg/ml & 30 mg/ml to 10 mg/ml & 20 mg/ml). *R. indica* extract inhibited *M. luteus* with great potential (1.25 mg/ml) with CUD as solvent medium (R.A.I=1.61). It also improved the MIC and MBC values (10 mg/ml & 20 mg/ml to 2.5 mg/ml & 5 mg/ml). Similarly, *O. sanctum* was found to be most effective against *S. Pneumoniae* (R.A.I=1.93 at 1.25 mg/ml concentration). It also improved the MIC and MBC values when CUD was used as solvent medium (20 mg/ml & 30 mg/ml to 10 mg/ml & 20 mg/ml).

Conclusively, the combination of CUD and plant extracts at different concentration gradients have given very convincing results to derive a concept of combination therapy against pathogenic gram-positive bacteria.

DISCUSSION: Multiple Drug resistance is of widespread feature against nearly 70% pathogenic bacteria, which is a matter of high clinical concern. Hence, microbiologists are diverting their focus on new life saving drugs [1]. One of the alternatives suggested recently was use of animal derived preparations against many pathogens and disease, e.g. snake, toad, scorpion and spider's venom as curative agents used successfully against cancerous cells, nerve disease and various viral infections [18, 19]. Nevertheless, CUD is being used in rural population as an antiseptic agent for wound infection and dermatitis since time immemorial.

The spectra of efficient results of CUD was demonstrated by two studies against *Pseudomonas aeruginosa* (15.4±1.23), *S. typhi* (13.6±0.17), *K. pneumonia* (11.0±0.14) [8], *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) [9]. 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 of CUD with *A. indica* extract [20].

These successful treatments galvanized the enthusiasm of microbiologists to use combination therapy to get rid of the problems of resistant bacterial strains. In the present study, we investigated the effect of combination therapy (CUD + Plants extracts) against six pathogenic gram-positive bacteria. This combination therapy had convincing results. We have seen a strong improved efficacy *in vitro*. The combination of CUD and *T. erecta* was found to be the most effective in present study, which improved the MIC and MBC results upto 3 folds. The present study demonstrated the significant synergistic effect of combination therapy on every gram-positive bacterium. Therefore, the results obtained in this study suggest that the combination could lead to the development of new curative agent against these pathogenic gram-positive bacteria.

The use of combination therapy can broaden the continuum of anti-bacterial activity, as described successfully in this paper. First, the synergistic effect of potential antibacterial compounds in both sources, results in to significant anti-microbial activity greater than what would be expected from individual antimicrobial agents. Secondly, and quite important too, it reduces the chances of emergence of resistant microorganisms. The main aim of this study is to validate and authenticate the antibacterial potential of combination therapy and hence to justify their use to fight against bacterial diseases. Furthermore, the data presented in this study are indicative of the fact that use of combination therapy against various pathogenic fungi and viruses could be of practical use.

CONCLUSION: In an endeavour to astound the serious threats of widespread multidrug and antibiotic resistant bacterial strains, it is now

absolutely imperative to devise alternative methods like combination therapies against pathogenic bacteria. This innovative study which comprises of plants and animal derived compounds is promising and results oriented. To the best of our knowledge, this is the first detailed work on the combined effect of plants & animal derived compounds to substantiate the synergistic anti-microbial activity against the pathogenic gram-positive bacteria. Pharmacokinetic and pharmacodynamic properties of this combination therapy must be further evaluated by *in vivo* experiments to check the safety, and the molecular basis of this synergistic interaction. Hence future research should be reoriented in this direction.

ACKNOWLEDGEMENT: Authors are grateful to Dr. Vinod Mittal (M.D), Dolphin (P.G) College of Science and Agriculture for providing financial supports to this study. We are highly indebted to the Principal, Dr. S.P. Jindal, for his encouragement to present research and giving final shape to this paper.

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

REFERENCES:

1. 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.
2. 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.
3. Dasgupta N, Ranjan S, Saha P, Jain R, Malhotra S and Mohamed SMA: Antibacterial activity of leaf extract of Mexican marigold (*Tageteserecta*) against different gram positive and gram negative bacterial strains. Journal of pharmacy research 2012; 5(8):4201-4203.
4. 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.
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 Crataevanurvala: 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. 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.
9. 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.
10. Ahmad I, Mehmood Z, Mohammad F: Screening of some Indian medicinal plants for their antimicrobial properties. J Ethnopharmacol2008; 62: 183-193
11. Kiranmai M and Ibrahim M: Antibacterial potential of different extracts of *Tageteserecta* linn. International journal of pharmacy 2012; 2(1):90-96.
12. Lehrer RI, Rosenman M: Ultrasensitive assays for endogenous antimicrobial polypeptides. Journal of microbiological methods 1991; 137: 167-173.
13. Aneja KR, Sharma C: Antimicrobial potential of fruit extracts of Elettaria Cardamomum Maton (Chhotielaichi) against the pathogens causing ear infection. Pharmacologyonl 2010; 3:750-756.
14. Aneja KR, Sharma C, Joshi R: *In vitro* efficacy of amaltas (*Cassia fistula* L.) against the pathogens causing otitis externa. Jundishapur J Microbiology 2011; 4(3): 175-183.
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 Biotechnol2008; 7:1797-1806.
17. Das K, Tiwari RKS, Shrivastava DK: Techniques for evaluation 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. 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.

How to cite this article:
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): 4429-36.doi: 10.13040/IJPSR.0975-8232.7(11).4429-36.