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In Vitro Antibacterial, Antioxidant And Time-Kill Kinetics Of Cow Ark With *Plectranthus Amboinicus* Extract Against Human Pathogens

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ABSTRACT: The antimicrobial and antioxidant activity of the cow ark with Plectranthus Amboinicus extract on multidrug resistant bacteria and fungi caused by human pathogens. The present study investigated to antibacterial activity of cow ark with Plectranthus Amboinicus (CAM) extract was evaluated by agar well diffusion method against microbial strains. CAM was more susceptible for influential the antimicrobial activity Protein leakage assay and Time Kill assay. The CAM inhibited the growth of bacteria and fungi with high-levels of antibiotic-resistance, such as Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Klebsiella Pneumoniae, Aspergillus niger, Candida albicans. The CAM has antioxidant potentials to reduce the oxidative stress by using DPPH radical Scavenging Assay. The results showed that the protein leakages of bacteria and fungi are correlated to the concentration of CAM. Time-kill kinetics profiles of CAM showed highly significant bactericidal and fungicidal activities. Our results proposed that the CAM bioactive compounds, which may be useful against antibiotic-resistant bacteria and Fungi

Keywords: DPPH, Protein leakage assay, antibiotic-resistance.

1. INTRODUCTION

In Ayurveda, the Indigenous cow urine is the fabulous drug for several diseases such as heart disease, kidney disorder, digestive problems, edema, fever, anaemia, skin irritation and liver diseases etc [1, 2]. The distillate cow urine (DCU) has been patented as a bioactive enhancer for against infection and cancer (US, 2002). DCU components have good therapeutic for drug resistant bacteria [3] antimicrobial and antioxidant. DCU contains few vital compounds such as Pheromones, Urinary protein, N, P, K, Cl, Ca, [4-5]. Their impact is particularly great in developing countries because of the relative unavailability of medicines and the emergence of widespread drug resistance [6]. Helminthes are recognized as a major problem to livestock production throughout the tropics. Parasitic helminthes affect human being and animals by causing considerable hardship and stunted growth. Most diseases caused by helminthes are of a chronic and debilitating in nature [7]. The origin of many effective drugs is found in the traditional medicine practices. Distillated cow and animal urine maintains the equilibrium of volatile compounds and it can be assisted for the treatment of incurable diseases [8-10] It is low cost, eco-friendly with naturally available, and act as a potential therapeutic agent.

Bioenhancer which means that support and enhancement with medicine. Bioenhancers are chemical entities which endorse and supplement with the drugs. Cow urine distillate is a efficient drug and acted as a bioenhancer with medicinal plants, and supplement the efficacy of antimicrobial, antifungal and anticancer drugs. It can also rise the activity of gonadotropin releasing hormone associated with bovine serum albumin (GnRH–BSA) and zinc [11-14].
A numerous peoples have been practised for many disorders by using cowpathy. Previous research has been showed that capability of DCU repress the enlargement of microorganism, fungi, bacteria and helminthes. In our study, the antibacterial activity of Bos indicus, cow urine distillate with *Plectranthus Amboinicus* against the human pathogenic bacterial strains *Staphylococcus aureus, Escherichia coli, Pseudomonas fragi, Bacillus subtilis, Streptococcus agalactiae* and *Proteus vulgaris*.

2. METHODOLOGY

Collection and preparation of cow urine sample

The cow urine (*Bos indicus*) was collected in Karaikudi District. The photoactivated cow urine has maintained at sunlight for 72 h in a transparent sealed glass beaker. Then, DCU was filtered to free it from debris and precipitated other materials.

Preparation of Cow ark with *Plectranthus Amboinicus* (CPA) Plant

The leaves and stems of *Plectranthus Amboinicus* plant was shade dried and powdered material for the preparation of cow ark extract. 10g of powdered plant material was added to 100ml of DCU. Later on, the contents were filtered and stored in refrigerator until use.

IN VITRO ANTIBACTERIAL ASSESSMENT

Culture collection

Pure bacterial and fungal culture were collected from the Doctor's Diagnostic Centre, A Unit of Multispecialty Lab Service Pvt Ltd, (DDC) in Trichy, the following Bacteria and Fungi were collected from liver Abscess and keratitis patients. The pure culture, *Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Klebsiella Pneumoniae, Aspergillus niger, Candida albicans* were streaked on Nutrient broth and Potato Dextrose broth slant and stored at 4°C.

Agar well diffusion assay

For antimicrobial activity, 100µl of bacterial and fungal culture were taken by using Agar well diffusion method. The culture was swabbed uniformly on Muller Hinton Agar Plates and using sterile cotton swabs. Wells of 10 mm diameter were made with the help of a sterilized stainless steel cork borer. Different concentrations of (25, 50, 75, 100µg/ml) cow ark with *Plectranthus Amboinicus* (CPA) solution was poured onto each well. Control experiments were carried out under similar condition by using gentamycin for antibacterial activity and nystatin for antifungal activity as standard drugs [15]. The zone of inhibition was measured after incubation at 37°C for 24 hrs.

Time kill assay

Bactericidal activity of CAI was examined using the time kill assay. 100 µl of more susceptible Gram positive and Gram negative (*Staphylococcus aureus, Escherchia coli*) and fungi *(Candida albicans)* were added to suitable medium and exposed to 1X, 2X and 4X the MIC of CPA were taken for colony counts at the interval of 0, 30 min, 2, 4, 6, 8, 10, 12 and 24 h. Chlorhexidine diacetate monohydrate (CHX, 0.1 %) and extract free medium were used as the positive and negative controls, respectively. The feasible counts were determined after appropriate incubation and each experiment was performed in triplicate.

Protein leakage assay

Protein leakages from bacterial cells were estimated by following the method described by Lowry *et al.*, (1951) [16]. 100µl of selected bacterial strain (*Stephylococcus aureus, Escherchia coli)* and fungal strain (*Candida albicans*) treated with different concentrations (25, 50, 75, and 100 µg/ml) of cow urine solution and incubated in a shaker for 6 hrs. BSA (Bovine Serum Albumin) was used as a standard protein Then, the incubated samples were centrifuged for 10 min at 3000 rpm, from these supernatant was collected separately and mixed with 800µl of Lowry's reagent. Subsequently, the optical density was measured for each sample at 595 nm.

DPPH Radical Scavenging Activity

The 2,2-diphenyl-1-1picrylhydrazyl (DPPH) radical scavenging activity of the CAI was determined [17]. Different concentrations of 25 ml of the CPA were mixed with 60 ml of a 0.04 mg/ml methanolic solution of DPPH. The mixture was vortexed and then incubated in the dark at room temperature for 20 min. Absorbance of the mixture was measured at 515 nm. Trolox was used as the standard and was analysed in the same manner as the sample. Ascorbic acid, butylated hydroxytoluene (BHT) and quercetin were used as positive controls. The percentage inhibition of the DPPH radicals by the CAI was calculated.

Statistical analysis

All the experiments were performed three times. SPSS 20 and GraphPad Prism 5 were used for data analysis.

3. RESULTS AND DISCUSSIONS

Due to the presence of phytoconstituents, numerous medicinal plants have been high therapeutic potentials against many diseases including heredity disease [18]. The use of plants with cow ark has drawn the attention of researchers as it is rapid, cheap and simple step method and much faster as compared to medicinal plant extracts.

Table.4 Antimicrobial activity of CPA against human pathogenic bacteria and fungi

Human Pathogen	**Antibiotics Gentamycin/ Nystatine**	**Zone of inhibition (mm)**				
		Control (100 µl)	**CAI (25µl)**	**CAI (50µl)**	**CAI (75µl)**	**CAI (100µl)**
Gram positive bacteria						
Staphylococcus aureus	08.00±1.53	7.70±0.10	12.00±1.0	15.00±2.0	17.00±3.2	21.00±2.4
Enterococcus faecalis	08.00±1.53	8.830±0.10	11.00±1.0	15.00±2.2	16.00±3.1	20.00±3.0
Gram negative bacteria						
Escherichia coli	05.00±1.20	9.77±0.30	11.33±1.3	13.67±1.6	15.67±2.1	17.67±1.5
Klebsiella pnemoniae	06.00±0.11	8.63±0.15	09.33±2.5	10.67±1.5	12.00±2.0	14.00±1.0
Fungi						
Aspergillus niger	5.00±0.09	5.40±0.10	9.30±1.53	10.30±0.5	11.00±1.0	13.70±1.3

Candida albicans	6.00±0.10	6.87±0.15	10.00±1.7	12.00±1.0	14.33±2.5	16.67±2.0

According to earlier researcher reported that plant extracts have been used widely in the health industry, medicine, wound dressing, antiseptic creams and a number of environmental applications due to their antimicrobial properties [19-20]. In the present study, the antibacterial activity of cow ark with CPA at different concentrations (25, 50, 75, and 100 µg/ml) was tested against human pathogenic bacteria (*Escherichia coli, Enterococcus faecalis, staphylococcuc aureus, Klebsiella pnemonae, Aspargillus niger,Candida albicans*) by using agar well diffusion assay (Table 1). The results showed that highest zone of inhibition was observed in Gram positive bacteria *Staphylococcus aureus* (21 nm) at 100µg/ml concentration whereas in Gram negative bacteria *Escherichia coli* (17 mm). The highest zone of inhibition in fungi was observed in *Candida albicans* (16 mm) at 100µg/ml.

Time kill Assay

Time kill curves were performed for human pathogenic bacteria and fungi representative the killing activity depended on time and concentrations of cow urine extracts. Generally, 1× MIC could reduce the number of the CFU by approximately 50%, although complete sterility was not achieved. At 4× MIC and 2× MIC, selected human pathogenic bacteria and fungi was killed after 12 and 24 h, while respectively (Fig.1-3). The killing of the positive control (CHX) was observed within 30 min. Time–kill studies have been used to investigate numerous antimicrobial agents and also frequently used for pharmacodynamic drug interactions. The relationship between bactericidal and fungicidal activity and concentration of antimicrobial agents is not quantitatively characterized. From the results, it is recommended to researchers for development of drug concentrations.

Fig.1 Time Kill Assay of *Staphylococcus aureus*

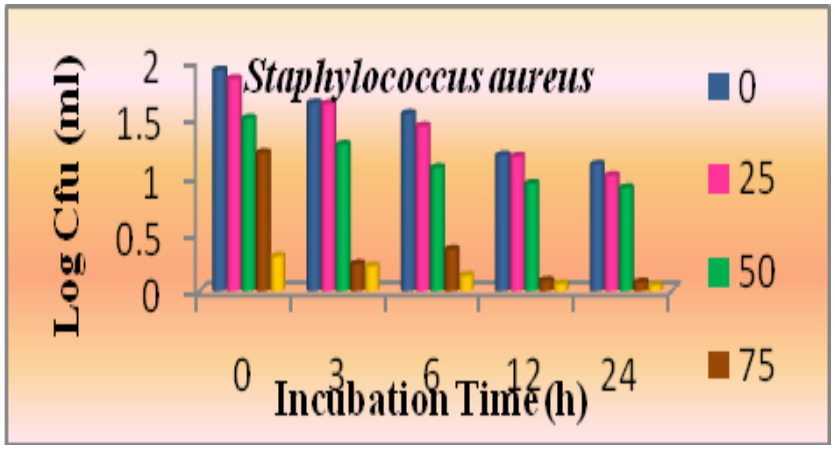


Fig. 2 Time Kill Assay of *Escherichia coli*

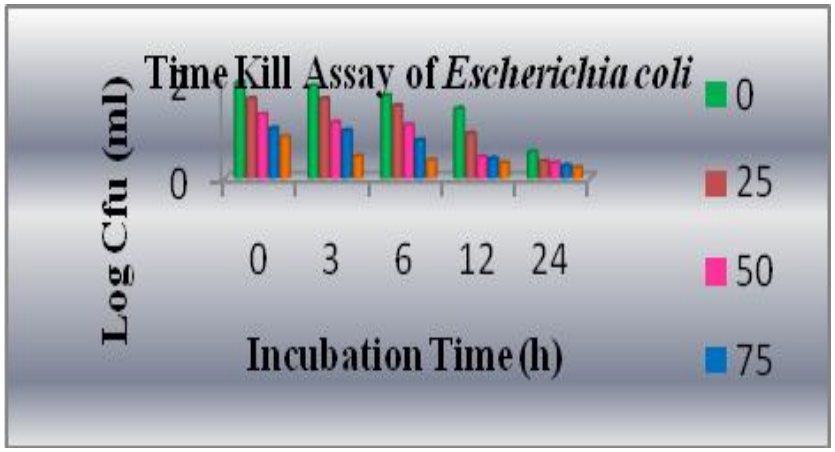
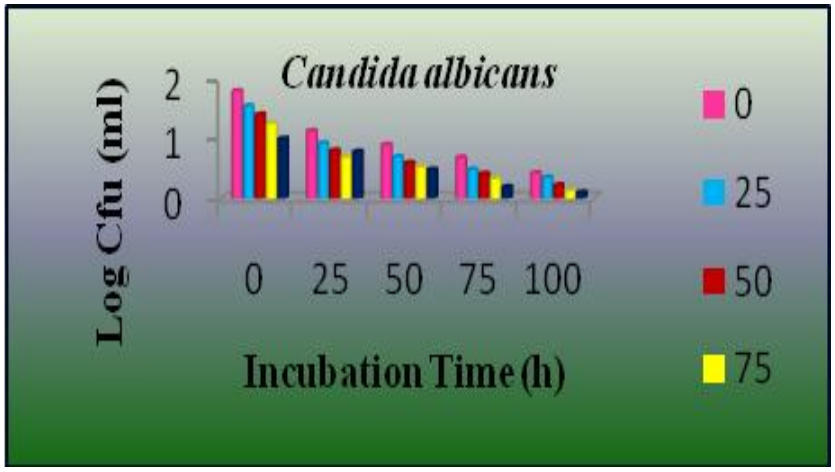


Fig.3 Time Kill Assay of *Candida albicans*



Protein leakage assay

The results of Protein leakage assay are shown in Figure.(4-6). The cow urine extracts enhanced with for *Plectranthus Amboinicus* the protein leakage by increasing the membrane permeability of the tested bacterium and fungi at 100µg/ml. After six hour incubation, the amount of protein leakages from the treated CAI was considerably increased (at 100 µg/ml) when compared to the control group. Protein leakage studies have been used to analyse the accurate expression regarding bacterial growth. The results showed that the protein leakages of bacteria and fungi are correlated to the concentration of cow ark with *Acalypa indica*. The *Bos indicus*, cow urine act as a bioenhancer and antibiotics against various disease [21-23].

Fig.4 Protein Leakage Assay of *Staphylococcus aureus*

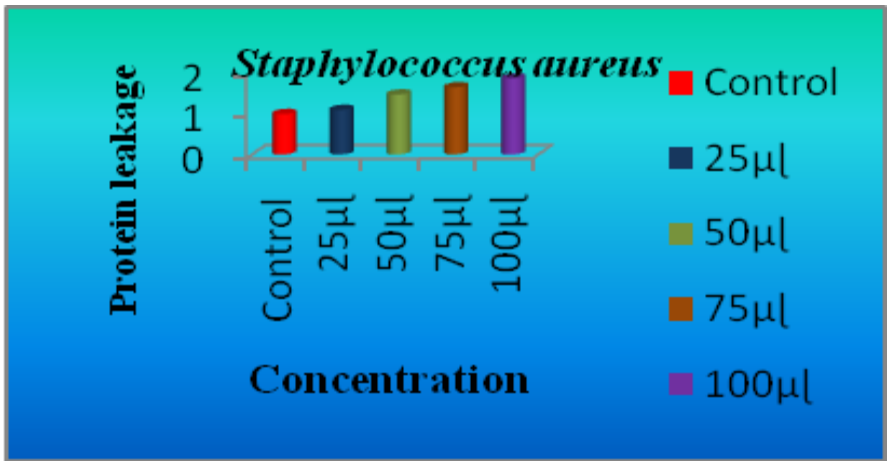


Fig.5 Protein Leakage Assay of *Escherichia coli*

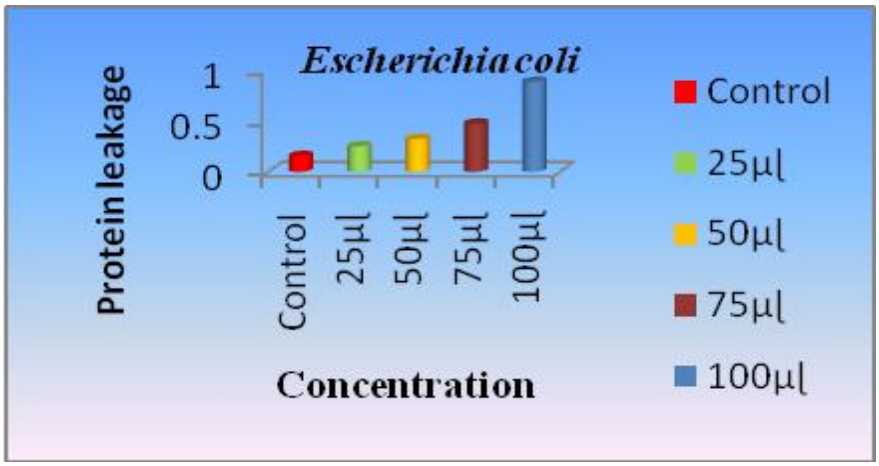


Fig.6 Protein Leakage Assay of *Candida albicans*

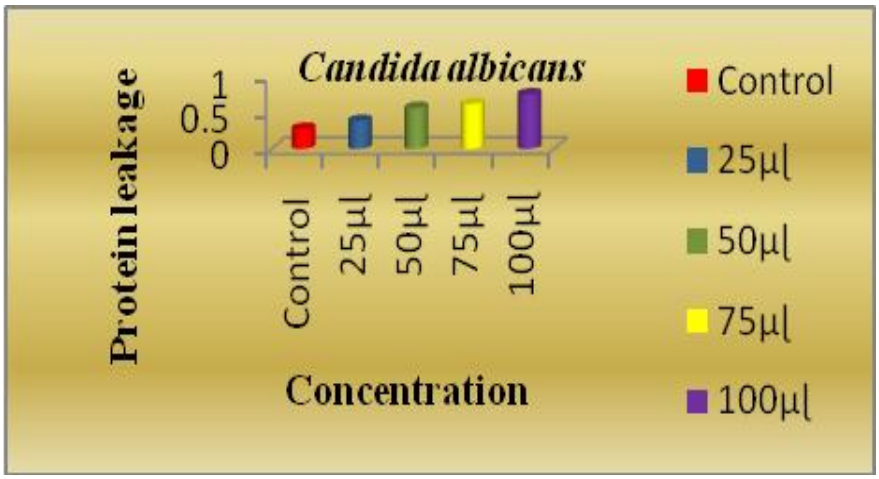


Table – 2 Antioxidant Activity by using DPPH Radical scavenging Assay

Con.µg/ml	Ascorbic acid	CPA extracts
20	8.85	7.88
40	31.34	32.45
60	54.78	53.67
80	57.04	60.08
100	65.98	68.72

The present study investigated that the antioxidant activity of Cow ark with *Plectranthus Amboinicus* (68.72) has the high oxidative potential when compared with Ascorbic acid. Commonly, the antioxidants have radical scavenging activities, potential complexes and reducing components etc.

4. CONCLUSION

In this study the cow urine with *Plectranthus Amboinicus* plant extract were demonstrated to have tremendous potential of antimicrobial, antioxidant activities. Time kill assay, protein leakage assay were performance against the human pathogenic bacteria and fungi. From the above, it is concluded that the cow ark with *Plectranthus Amboinicus* plant extract has traditional therapeutic activities due to their biological components.

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