Antimicrobial and Antioxidant potential of cow urine

A dissertation for

MIC-651 Discipline specific dissertation

16 credits

Submitted in partial fulfilment of Master/Bachelor's Degree

MSc. In Microbiology

by

DIVIKA ANIL NARVEKAR

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I hereby declare that the data presented in this Dissertation report entitled, "Antimicrobial and Antioxidant potential of cow urine" is based on the results of investigations carried out by me in the MSc. Microbiology at the School Of Biological Sciences and Biotechnology,Goa University under the Supervision of Dr. Sandeep Garg and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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This is to certify that the dissertation report **"Antimicrobial and Antioxidant potential of cow urine"** is a bonafide work carried out by Ms. Divika Anil Narvekar under my supervision in partial fulfilment of the requirements for the award of the degree of **MSc. Microbiology** in the Microbiology programme at the School Of Biological Sciences and Biotechnology, Goa University.

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CONTENTS

PREFACE

The emergence of Multi Drug Resistance in pathogenic organisms has become a global challenge. This is also linked to high annual mortality rates all over the world. Antimicrobial resistance develops the need to identify new drugs against these pathogens. The alternate broad spectrum antimicrobial drug should be non-toxic and can bypass the resistance acquired by the organisms towards the antimicrobial agent. The organisms produce Reactive Oxygen Species during stressed conditions. These reactive oxygen species damage the cellular components. The antioxidants have the ability to reduce the reactive oxygen species. Cow urine is known to have antimicrobial and antioxidant activity.

ACKNOWLEDGEMENT

I would like to express my sincere prayers to God for granting me the strength, perseverance, and good health throughout my academic pursuits. My deepest appreciation goes to my parents, whose unwavering support, encouragement, and sacrifices have been the reason for my success. Their belief in me has been my source of inspiration.

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ABBREVIATION USED

°C	Degree celsius
µg/ml	Microgram per millilitre
µl	Microliter
$1O_2$	Singlet oxygen
AIN	Animal identification number
am	Ante meridiem
AMR	Antimicrobial resistance
BIL	Bilirubin
CAT	Catalase
cm	Centimeter
CU	Coe urine
CUSiN	Cow urine silver nanoparticles
DNA	Deoxyribonucleic acid
DPPH	*Diphenyl-1-picrylhydrazyl*
FTIR	Fourier Transform Infrared spectroscopy
g	Grams
GLU	Glucose
GR	Glutathione reductase
GSH	Glutathione

H_2O_2	Hydrogen peroxide
HIV	Human Immuno Virus
HPLC	High performance liquid chromatography
KET	Ketone
kg	Kilogram
M	Molar
MDR-GNB	Multi-drug resistant gram-negative bacteria
MIC	Minimum inhibitory concentration
ml	Milliliter
mm	Millimetre
MRSA	Methicillin resistance in *Staphylococcus aureus*
O_2^-	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
PhCU	Photoactivated cow urine
PRO	Protein
ROS	Reactive oxygen species
rpm	Revolutions per minute
SG	Specific Gravity
SOD	Superoxide dismutase
URO	Urobilinogen

ABSTRACT

The emergence of multidrug-resistance in pathogenic organisms, such as *Escherichia coli* and *Staphylococcus aureus* have become a growing concern due to extensive antibiotic use. There is a need to find an alternate broad spectrum, non-toxic antimicrobial compound that can bypass the resistance acquired by the organisms towards the antimicrobial agent. In this study the antimicrobial and antioxidant potential of lyophilized cow urine is tested against *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* by Disc Diffusion method. The composition of cow urine is influenced by the health status of the cow and the breed. This may contribute to variations in its antimicrobial and antioxidant properties. Cow urine samples from indigenous breeds, Konkan Kapila and Gir cow, were analysed for antimicrobial and antioxidant activity. Results showed no antimicrobial activity against the three test cultures. The antioxidant activity of the cow urine samples were tested using DPPH. Two cow urine samples collected from Konkan Kapila have shown antioxidant activity.

Keywords: Cow urine, Antimicrobial, Antioxidant.

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1 Antimicrobial compounds

The term antimicrobial refers to the compounds or agents that have the ability to either completely eliminate or reduce the growth of microorganisms like bacteria, viruses, fungus, or protozoa. These may be biological agents such as specific viruses or bacteria. A compound is said to have antimicrobial action if it chemically disrupts the manufacture or function of essential cellular components or if it can bypass the resistance acquired toward the antibacterial agents. An antimicrobial compound can also act by targeting the bacterial ribosomal subunits to decrease protein synthesis and hence fighting against bacterial infection. In bacteria, the enzyme Deoxyribonucleic acid (DNA) gyrase plays a crucial role in winding and unwinding of DNA during replication, DNA repair and transcription. This makes enzyme DNA gyrase a good target for antibacterial substances. Some antimicrobials have the ability to attach to the lipid component of lipopolysaccharide and change its structure by exchanging phospholipids. This may result in an osmotic imbalance and ultimately quick bacterial death (Vaou et al., 2021).

1.1.2 Antimicrobial resistance

The ability of a microorganism to withstand the growth-inhibitory or lethal effects of the antimicrobial compounds beyond the susceptibility of the particular species is known as antimicrobial resistance (Verraes et al., 2013). Antimicrobial resistance is not a recent phenomenon. The term "resistome" refers to the collection of all genes in a microorganism that contribute to antimicrobial resistance. Resistance levels might differ significantly even amongst related bacterial groups.

The susceptibility and resistance of an antimicrobial compound is assessed in relation to minimum inhibitory concentration (MIC), or the lowest amount of drug concentration necessary to stop bacterial growth (Reygaert, 2018).

Factors such as poor or inadequate sanitation system, rigorous use of antibiotics in animal production and clinics, overcrowding, increased worldwide migration and selection pressure that makes the organism evolve are some of the causes contributing to the global resistance (Aslam et al., 2019). Methicillin resistance in *Staphylococcus aureus* (MRSA) is one of the most common instances of antimicrobial resistance (AMR) and has been linked to high annual mortality rates all over the world. The treatment of many infectious diseases like pneumonia and urinary tract infections has become more challenging due to the presence of multi-drug resistant gram-negative bacteria (MDR-GNB) (Dadgostar, 2019). Little is known regarding the epidemiological aspects of antimicrobial resistance in most of Southeast Asian countries (Kumar et al., 2013). In accordance to the meta-analyses of drug susceptibility results from multiple laboratories in India, a growing resistance to commonly used antimicrobials is seen in pathogens such as *Salmonella, Shigella, Vibrio cholerae, Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Klebsiella, Mycobacterium tuberculosis,* Human Immuno Virus (HIV)*, Plasmodium,* and other pathogens (Kumar et al., 2013).

There are four main types of antimicrobial resistance mechanisms: (1) Inhibiting the uptake of the antimicrobial drugs; (2) Modification of the drug targets; (3) Inactivating the drugs; and (4) Efflux of the active drug. Drug target modification, drug inactivation, and drug efflux are examples of acquired resistance mechanisms in the microorganism. Limiting uptake, drug inactivation, and drug efflux are examples of intrinsic resistance mechanisms (Reygaert, 2018). According to Shaw et al. (2004), *Staphylococcus aureus* is the most pathogenic member of the genus *Staphylococcus* in terms of clinical practice. The severity of a disease increases mainly due to the emergence of multiple virulence factors, amplification of antibiotic resistance genes (*VanA, mecA*, staphylococcal exotoxins), and other factors that initiate the disease process such as immune evasion, and host tissue destruction. *Escherichia coli* produces a narrow-spectrum of β-lactamases that are capable of inactivating aminopenicillins and penicillin (Poirel et al.,

2018). There are various mechanisms that confer drug resistance in *Pseudomonas*. Pachori et al., (2019) stated that innate resistance in *Pseudomonas* is due to an overexpressed efflux pump and low outer membrane permeability, while acquired resistance is due to the acquisition of a resistance gene or by mutations in genes that encode penicillin-binding proteins, chromosomal β-lactamase, efflux pumps, and porins. These mechanisms all work together to confer resistance to β-lactams, carbapenems, aminoglycosides, and fluoroquinolones. Since most of these clinical pathogens have acquired resistance to most of the commonly antimicrobial compounds present against them there is a much greater need to come up with novel antimicrobial drugs that have a broad-spectrum activity.

1.1.3 Antioxidant compounds and their mechanism of action

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), and singlet oxygen ($^1O_2$), are the byproducts of cellular metabolism. Under various environmental or internal stress conditions, such as high light intensity, extreme temperatures, drought, pollutants, or toxins, the production of ROS can increase dramatically. ROS can cause damage to cellular components like proteins, lipids, carbohydrates, and DNA through a process called oxidative stress. This damage can lead to disruptions in cell signalling pathways, impairment of enzymatic activities, and even cell death. A substance that reduces the oxidation of proteins, carbohydrates, lipids, and DNA at low concentrations is considered an antioxidant. Antioxidants are grouped based on three primary categories:

1. The antioxidants that act as the primary line of defence, such as glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and minerals like selenium, copper, and zinc.
2. The antioxidants that make up the second line of defence include glutathione (GSH), vitamin C, albumin, vitamin E, carotenoids, and flavonoids.
3. The antioxidants serve as the third line of defence, comprising a complex group of enzymes that repair damaged proteins, oxidised lipids, damaged DNA, and peroxides. Examples include

lipase, protease, transferases, methionine sulfoxide reductase, and enzymes that repair DNA (Sindhi et al., 2013).

Ascorbic acid, commonly known as vitamin C, is a potent antioxidant found in plants. It plays a crucial role in neutralizing ROS, thereby protecting the cells from oxidative damage. The enzyme L-gulonolactone oxidase is required for the biosynthesis of vitamin C. In humans, vitamin C is an essential nutrient that must be obtained from the diet. Since humans lack the enzyme required for the synthesis of vitamin C it cannot be synthesized endogenously. Antioxidants have the ability to prevent further oxidation reactions by themselves being oxidized or by eliminating free radicals, which lowers or stops the chain reactions (Adwas et al., 2019).

1.1.4 Different breeds of cow and the potential of cow urine

According to Indian culture and beliefs, cows are considered sacred. In Sanatan Vedic Hindu Dharma, the cow holds a revered and special status, often referred to as "*Go mata*," meaning mother cow. This reverence for the cow is deeply rooted in ancient scriptures, including the Rigveda, Atharvaveda, and various texts of Ayurveda. गव्यंपदर्त्रंच रसायनंच पथ्यंच हृद्यंबलबुस्मिकृत्स्यात्। आयुीः प्रदं रक्तदर्कारहारी दत्रदोषहृद्रोगदर्षापहंस || Which means Panchagavya is an extremely natural product. It increases life expectancy, strength, and cognitive abilities and is beneficial when incorporated into a diet. It additionally makes the heart healthy. The three *doshas*, or the three faults according to Ayurveda, are destroyed by it. Additionally, it eliminates all blood diseases and pollutants. Heart problems can be cured by it. All issues related to the body, mind, and soul can be resolved by drinking it (Arya, 2023). The cow urine (CU) comprises 95% water, 2.5% urea, minerals, 24 different types of salts, hormones and 2.5% enzymes. It also contains iron, calcium, phosphorus, carbonic acid, manganese, sulphur, amino acids, and cytokines (Godad et al., 2020). Gold has been found in the urine of Gir cows,

along with a number of trace metals like copper, calcium, zinc, and iron. It was found that Gir cow urine distillates enhanced haematological indices and had immunomodulatory effects in rabbits (Sunitha et al., 2023). The hue of the body coat varies greatly in pure Gir cattle. (Fig 1.1.4.1) Gir cattle have unusually curved horns. They emerge from beneath the head, in contrast to other cattle breeds. Once the horns have emerged, they curl backward and downward before inclining slightly upward and forward and making an inner spiral sweep. The Gir breed of cattle has the greatest hump of any native cattle breed. The total milk output of Gir cow varies greatly over each lactation, depending on factors like lactation duration, genetic potential, and environmental conditions (Patbandha et al., 2020).

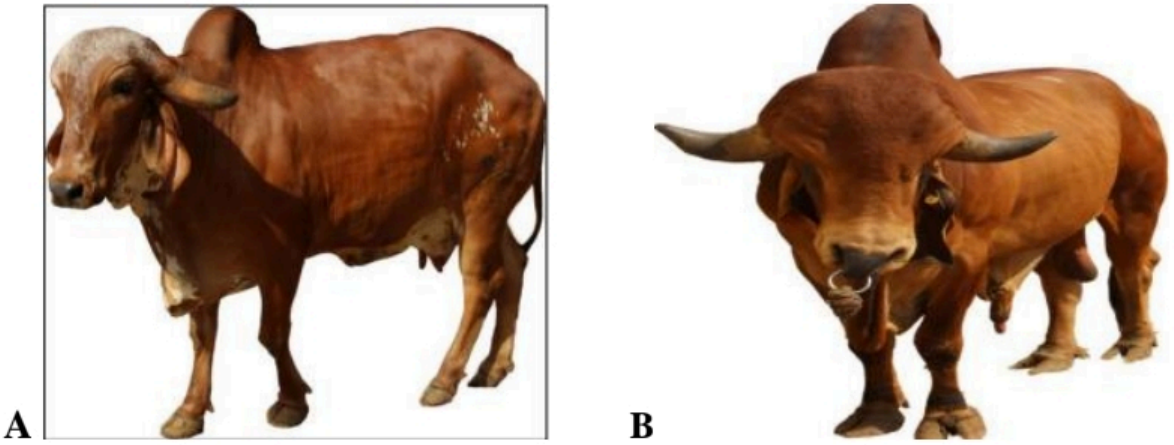

Fig 1.1.4.1: Picture of *Bos indicus* A) Gir cow and B)Gir bull) (Patbandha et al., 2020).

Konkan region of Maharashtra and Goa is known for the rearing of a specific breed of cattle known as Konkan Kapila (Fig.1.1.4.2). Konkan Kapila cattle are indigenous to the Konkan region and are well-adapted to the local environmental conditions. Konkan cattle appear reddish dun, black, white, grey, brown, fawn, or mixed coat hues.Their horns are long, elongated, thick at the base, and pointed at the tips and the horns are straight. The Konkan cattle

were found to have an average daily milk yield of 2.23 kg and a peak milk yield of 3.28 kg (Singh et al., 2019).

A)  B) 

Fig1.1.4.2: Konkan Kapila A) cow B) bull (Bhagat et al., 2022).

The different physical characteristics are due to the difference in genetic makeup of these two breeds. The urine composition may also vary between the two breeds. Therefore, they may have a varying degree of antimicrobial and antioxidant activity.

1.2 AIMS AND OBJECTIVES

1. To analyse the general health status of the cow.
2. Screening of the antimicrobial activity of lyophilized cow urine from healthy cows.
3. Screening of antioxidant activity of lyophilized cow urine.

1.3 HYPOTHESIS

Cow urine is known to have antimicrobial and antioxidant activity. The composition of this cow urine may vary depending on the type of breed and health status of the cow. Therefore, cow urine may have varying antimicrobial and antioxidant potential**.**

1.4 SCOPE

Cow urine samples having antimicrobial and antioxidant activity can be further tested to identify the bioactive compound. This bioactive compound can be purified and tested for its ability to be used as a drug against the antimicrobial resistant organisms.

CHAPTER 2: LITERATURE REVIEW

Godad et al., (2020) carried out the antimicrobial assay of Photoactivated (PhCU). The photoactivation was carried out for 72 hours and then tested against *Escherichia coli* and *Staphylococcus aureus*. Urine samples from two different cow breeds namely Khillari and Gir were taken. Khillari cow urine (CU) showed a zone of 16mm against *E. coli* whereas Gir CU was found to be effective against *S.aureus* showing a zone size of 18mm. Similarly, Kaur et al., (2019) tested the 72 hours PhCU against *S.aureus* which gave a zone size of 21.5mm and against *S.payogenes* a zone size of 20.9mm. They concluded that PhCU becomes highly acidic and therefore has enhanced bactericidal activity. Ghosh and Biswas (2018) tested antimicrobial and antifungal activity of fresh CU. They found that the antimicrobial activity of fresh CU happens to be more than PhCU. This could be due to the presence of various volatile and non-volatile components in fresh CU. The activity seen in PhCU is due to low pH.

Sathasivam et al., (2010) tested the distilled CU for potential antibacterial activity against *Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Salmonella typhi.* Maximum antibacterial activity was seen against *Pseudomonas aeruginosa* of a zone size 15.4mm for an amount of 15µl. Similarly, antifungal activity was tested against *Aspergillus niger* and *Aspergillus flavus.* The maximum antifungal activity was seen against *Aspergillus niger* with a zone size of 12mm for an amount of 15µl. Gupta (2020) investigated the antimicrobial activity of undistilled CU and observed that activity was better than the values that have been reported for CU distillate and Ph CU.

Vats and Miglani (2011) tested for the synergistic effect of CU with *Azadirachta indica* (Neem). The extract of *Azadirachta indica* by itself showed antimicrobial activity towards *Candida albicans, Candida glabrata*, *Escherichia coli, Pseudomonas aeruginosa* and *Streptomyces aureofaciens.* Whereas in case of CU strong antimicrobial activity is noted

towards *E.coli, S.aureofaciens* and *C.albicans*. When a formulation was prepared with equal amounts of CU and *Azadirachta indica,* remarkable synergistic effects were observed against *C. tropicalis*, *C. glabrata, P. aeruginosa* and *S. aureofaciens.* Sharma et al., (2023) also tested the synergistic effect of CU with Essential oils. They concluded that essential oils with CU cause significant growth-inhibiting effects on Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Shewale et al., (2016) prepared a gel formulation of propylene glycol, carboxyvinyl polymer (Carbopol 940), glycerin, distilled water and triethanolamine. To this formulation CU distillate and extract of *Azadirachta indica* were added in 2:1 ratio. The prepared gel formulation was tested for antibacterial and antifungal activity wherein a greater zone of inhibition was found against *Bacillus subtilis* as compared to *Staphylococcus aureus.*

The differences in the medicinal potency and effectiveness of cow urine could be attributed to variances in the species' climate as well as differences in the normal diet, water, and grass that they consume. Joshi et al., (2019) evaluated the antibacterial and antioxidant properties of CU that were gathered from Nepal's various climate zones. Compared to low altitude climatic regions, the subalpine region has a larger potential for CU therapy. They concluded that the different activity of the cow urine is due the quality and quantity of active chemical constituents. These vary between cow urine from different climatic zones.

Ahuja et al., (2012) detected the antibacterial activity of Sahiwal breed CU distillate from different cows against pathogenic bacterial strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas fragi, Bacillus subtilis, Streptococcus agalactiae* and *Proteus vulgaris*. Through their studies they concluded that urine samples from different cows within the breed show different degrees of antimicrobial activity which can possibly arise due to different factors. One amongst them is the difference in chemical composition of urine which may arise due to several reasons.

Rai et al., (2022) selected Sahiwal, Tharparkar, Gir and Karan Fries breeds to test for their Panchagavya formulation. The five basic ingredients of panchagavya—cow milk, ghee, curd, urine, and dung from native breeds were combined to create this mixture. The findings of this study revealed that the antifungal efficiency of the panchagavya formulation's is dependent on the ratio of the five main ingredients, the temperature and length of fermentation, and the distillation process. Strong antibacterial activity was shown by the distillate made from the fermented panchagavya formulation. The largest zone of inhibition was seen with fermentation of 37°C after 20 days. The panchagavya formulation that exhibited the strongest antifungal effect was made with the urine and dung of native breeds Sahiwal and Gir.

Kumar et al., (2023) subjected CU to High performance liquid chromatography (HPLC). The 20 fractions obtained were lyophilized and tested for antimicrobial activity against *Escherichia coli, Staphylococcus aureus* and *Streptococcus agalactiae.* Before testing the antimicrobial activity of the fractions, a tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run to detect the presence of peptides. The fractions exhibiting antimicrobial activity were pooled and subjected to nLC-MS/MS for identification of the peptide sequence. The minimum inhibitory concentrations of the aqueous extract of the fractions having antimicrobial activity against *E. coli* was 468µg/mL, for *S. aureus* it was measured to be 3750µg/mL, and for *S. agalactiae* the MIC was 3750µg/mL. After getting the results they concluded that the two possible components behind the antimicrobial activity of cow urine were antimicrobial peptides and plant origin metabolites such as polyphenols. The peptides exhibiting the antimicrobial activity are due to the amino acid sequence and the physicochemical characteristics and the plant origin metabolites arises from its feed.

Nautiyal and Dubey (2021) performed TLC of CU. The fractions obtained were abbreviated as EW1, EW2, CA1 and CA2 from which EW1 exhibited maximum activity against

Staphylococcus aureus with an inhibition zone of 15mm followed by *Klebsiella pneumoniae* with an inhibition zone of 7mm. Fourier Transform Infrared (FTIR) spectroscopy of the EW1 fraction was carried out. The FTIR spectrum was recorded between 4000 and 400 cm^{-1}. The analysis showed the presence of intramolecular bonded alcohol, amide, phosphate ion, strong and medium alkene bending, alkyl halide and polysulfide stretch. Through this study they concluded that these bioactive components can be used for the treatment of various infectious diseases and it also proves the use and significance of CU as mentioned in Vedas.

Santhosh et al., (2021) formulated the cow urine silver nanoparticles (CUSiN) wherein the CU sample was collected from Amrithmahal cattle a breed of Ajjampura, Karnataka, India. The CUSiN were prepared by mixing 3 mL of cow urine with 50 mL of an aqueous solution of 0.01 M silver nitrate solution and stirred the mixture at 500 rpm under the dark condition at room temperature for 12 hours. The CUSiN particle formation was examined by monitoring the UV–visible spectra of the reaction mixture. The CUSiN particles exhibited excellent biocidal effects on cells of *Escherichia coli* and *Staphylococcus aureus.* The cells had severe membrane destruction and DNA damaging activities which were confirmed by microscopy.

CHAPTER 3: METHODOLOGY

3.1 COLLECTION OF COW URINE SAMPLE

The cow urine sample was collected from 2 different breeds. Gir cow urine samples from Krishi Gokul Gaushala Kundai, Ponda, Goa and Konkan Kapila urine samples from Shikeri Gaushala, Mayem. (Fig.3.1.1,3.1.2) The urine sample was collected by the Clean Catch Method (Staerk et al., 2023) in sterile containers. The container was labelled with the Animal Identification Number (AIN) mentioned on the ear of the cow. The sample was collected early in the morning at 4:30am in order to get the First void specimen. The first few volumes of the first void were discarded and the rest were collected in the container. The sample was then transported to the laboratory for urine analysis.

A)

B)

Figure 3.1.1:A) and B) Collection of cow urine sample.

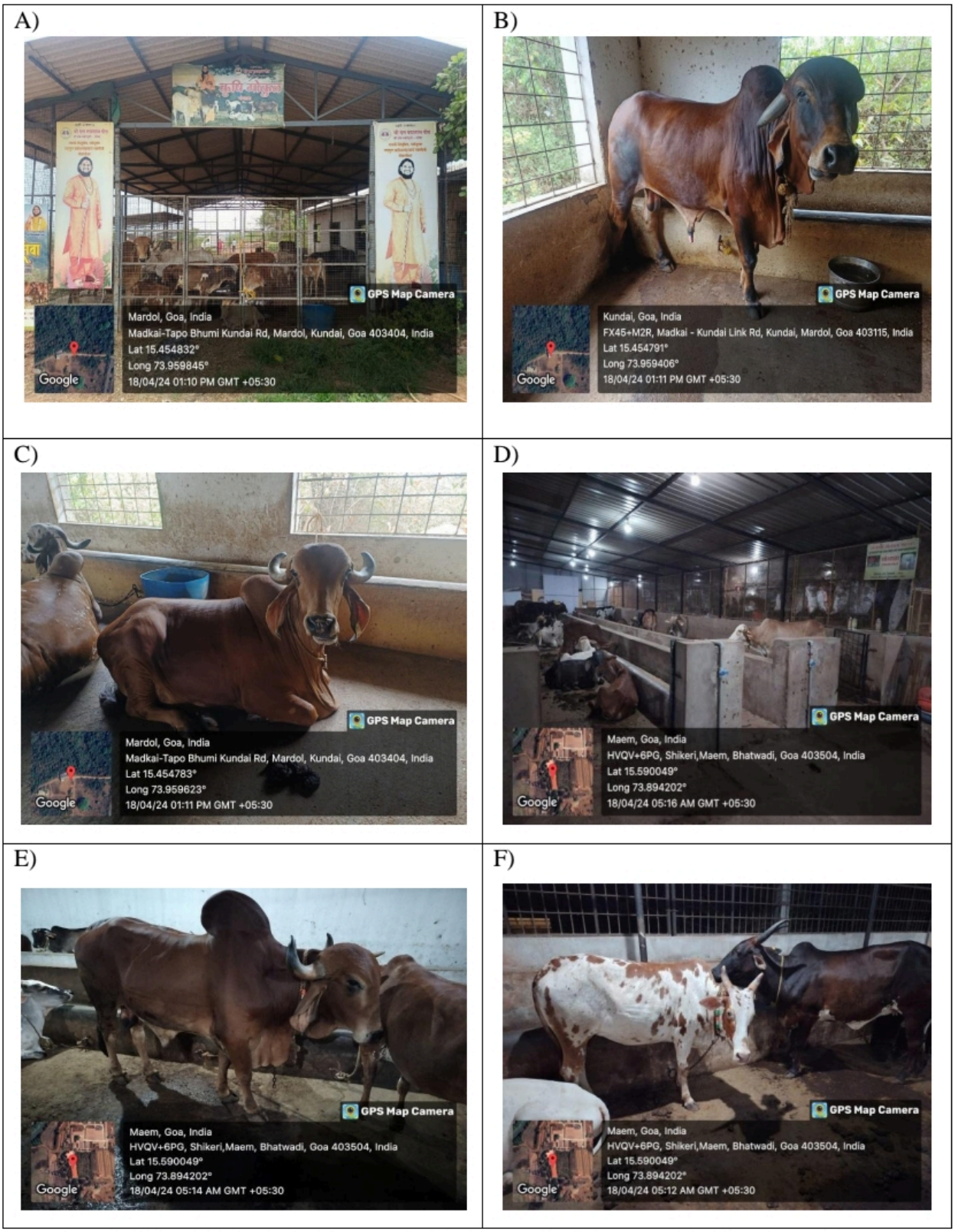

Figure 3.1.2: A) Krishi gokul gaushala, B) Gir bull, C) Gir cow, D) Shikeri gaushala, E) Konkan kapila bull, F) Konkan kapila cow.

3.2 COW URINE ANALYSIS

Cow urine analysis was done by Dipstick Method (Defontis et al., 2013) using the SIEMENS Multiplex® SG IVD Reagent strips. The reagent strip had eight parameters to detect infection in the cow. The parameters tested were Glucose, Bilirubin, Ketone Specific gravity, Blood, pH, Protein and Urobilinogen. The urine sample was taken in a clean test tube. The reagent strip was dipped into the urine sample, then the strip was removed and observed for the colour change. The change of colour on the strip was matched with the reference given on the reagent bottle. The parameters were checked in the following order after a specific time interval after being dipped in the sample as instructed by the manufacturer. Glucose and bilirubin after 30 seconds, ketone bodies after 40 seconds, specific gravity after 45 seconds, blood, pH and urobilinogen after 60 seconds post being dipped into the urine sample.

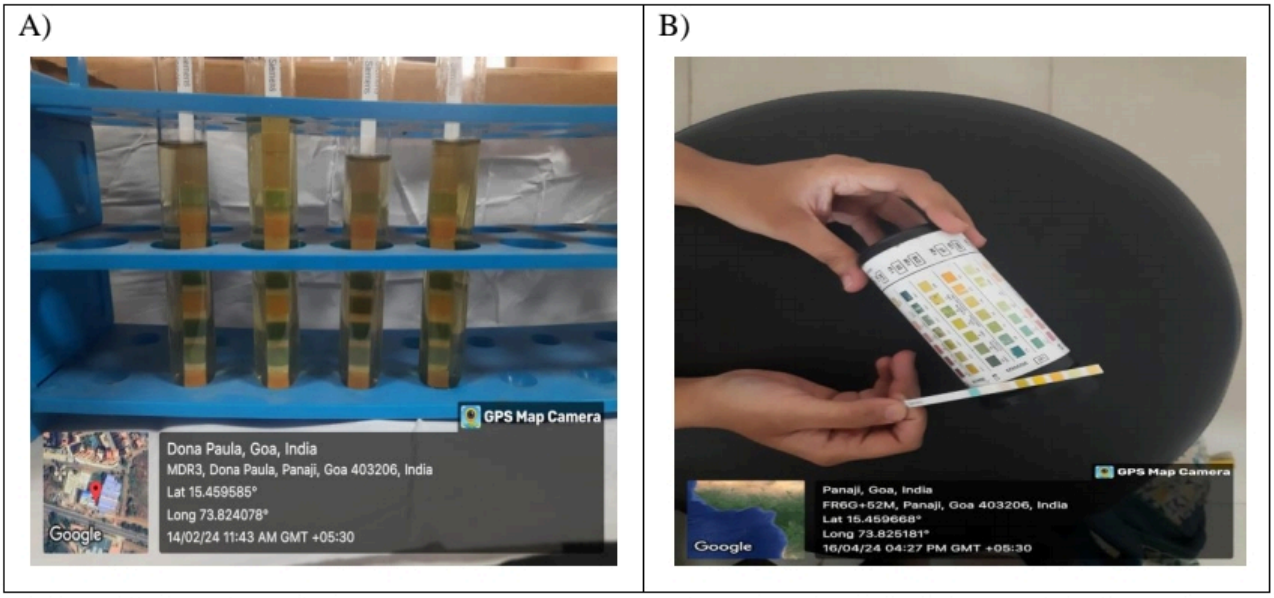

Figure3.2.1 A) Analysing urine sample by dipstick method , B) Checking against standard reference.

3.3 LYOPHILIZATION OF COW URINE

The Cow urine sample of healthy cows was lyophilized using the ilShin Freeze Dry Series system. The CU sample was distributed in three lyophilization flasks of 50ml, 300ml and 600ml adding 10ml, 20ml and 20ml of urine sample respectively. The samples were then

layered on the inner surface of the flask by placing the flask in an Ultra deep freezer at -80°C and rotating periodically for the sample to freeze along the sides of the flask. The layered sample flasks were kept overnight in the freezer at -80°C. The ilShin Freeze Dry Series system was put on and allowed to reach a temperature of -77°C. The vacuum pump was then put on and allowed the pressure to drop to 200mTor. Layered sample flasks were placed on to the nuzzle and the valve was turned to vent mode. As the pressure dropped to 10mTor the sample began to lyophilize. Lyophilization took 8 hours. The lyophilized sample was then collected in sterile empty vial (Fig.3.3.1).

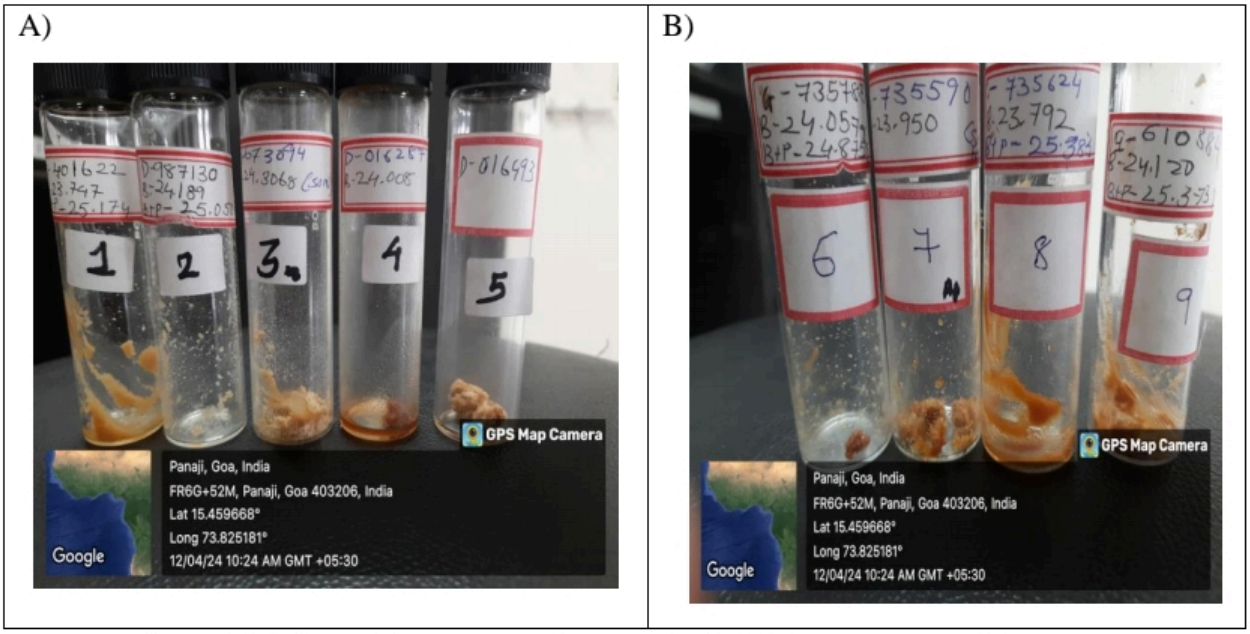

Figure 3.3.1: Lyophilized cow urine sample A) Konkan Kapila, B) Gir cow.

3.4 TESTING THE ANTIMICROBIAL ACTIVITY OF LYOPHILIZED COW URINE USING DISC DIFFUSION METHOD

In a sterile 2 ml Eppendorf tube 0.25g of lyophilized cow urine sample was taken. To this 1 ml of sterile distilled water was added. The sample was allowed to dissolve completely. The lyophilized cow urine sample was tested for antibacterial activity against three test cultures. One gram positive organism *Staphylococcus aureus* and two gram negative organism *Escherichia coli* and *Pseudomonas aeruginosa.* The three test cultures were inoculated in 50 ml Muller Hinton's Broth. The flasks were incubated overnight on the shaker at 37°C to obtain

24 hours old culture. The antimicrobial activity was tested using sterile Muller Hinton's Agar plates by Disc Diffusion Method (Nishanth et al., 2010). The sterile discs were placed on a clean petri dish with the help of a forceps and 20µl of the sample was impregnated onto the disc. Another 20 µl of sample was then added on the disc and the sample was allowed to absorb on the surface of the disc for 20 minutes. With the help of a sterile swab the culture broth was swabbed onto the surface of the sterile Muller Hinton's Agar plates and kept aside for 10 minutes. The discs impregnated with the samples were then placed on the Muller Hinton's Agar plates swabbed with the test culture. These plates were kept in the refrigerator at 4°C for 10 minutes. Finally, plates were transferred to the incubator at 37°C. The results were observed after 24 hours of incubation.

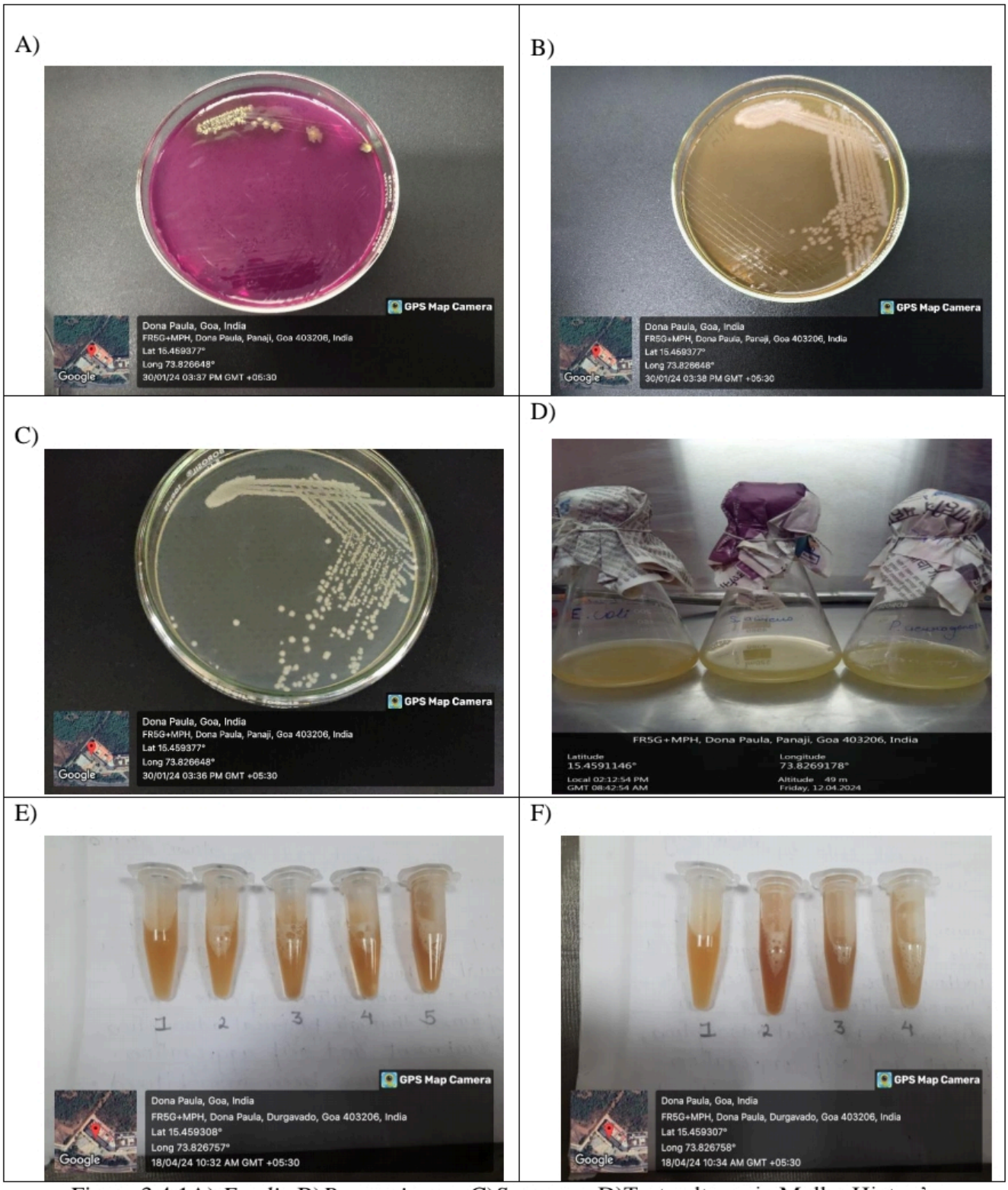

Figure 3.4.1A) *E.coli* , B)*P.aeruginosa* , C)*S.aureus* , D)Test cultures in Muller Hinton's broth, E) Konkan Kapila samples, F) Gir cow samples

3.5 TESTING THE ANTIOXIDANT ACTIVITY OF LYOPHILIZED COW URINE

3.5.1Preparation of lyophilized cow urine sample to test antioxidant activity:

In a clean 15ml centrifuge tube 0.1g of the lyophilized cow urine sample and 1ml sterile distilled water was added and was allowed to dissolve while maintaining the tube at 4°C in the ice bath. Methanol was added slowly till the volume reached 10ml.

3.5.2Preparation of Ascorbic acid (positive control) for antioxidant test:

The 0.1g of Ascorbic acid was weighed in a 15ml centrifuge tube and dissolved in 10ml of methanol.

3.5.3Preparation of 2,2 Diphenyl-1-picrylhydrazyl (DPPH) *solution*:

A DPPH solution was prepared to test the antioxidant activity of the cow urine sample. To obtain a 254mM DPPH solution, 0.1g of DPPH was dissolved in 1ml 95% methanol. The 0.1ml of 254mM DPPH solution was put into 0.9ml 95% methanol to obtain 25.4 mM DPPH which was used as a stock to prepare the working solution of DPPH.

3.5.4Preparation of the microtiter plate:

The microtiter plate was prepared to check the antioxidant activity of the cow urine sample. In the 1st well and the subsequent 2 wells below it (1A, 1B, 1C) 200µl of Methanol was added as Blank. In the 2nd well and the well below it (2A, 2B) 200µl of DPPH and 100µl of DPPH was added respectively. In the 3rd well (3A) 200µl of Ascorbic acid was added. Ascorbic acid was used as positive control. In the 4th well 200µl of distilled: methanol was added in 1:9 ratio. In the wells 5A, 6A, 7A, 8A, 9A, 10A, 11A, and 12A 200 µl of test samples was added. To the remaining wells 100µl of methanol was added to perform dilutions of the positive control (ascorbic acid) and the test sample. To perform dilutions 100µl of the contents from well (3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A and 12A) were transferred to the wells below it (3B, 4B, 5B, 6B, 7B, 8B, 9B, 10B, 11B and 12B) and there by further diluting the sample by transferring 100µl of the content to the subsequent wells below it. Finally 100 µl of the content was

discarded from the last wells (3H, 4H, 5H, 6H, 7H, 8H, 9H, 10H, 11H and 12H). Add 100µl of the prepared DPPH solution to all the wells in which dilution was carried out. Keep the microtiter plate for incubation in a dark room for 30 minutes. Observe the colour change in the sample and compare it with the ascorbic acid standard. The final reaction mixture in each well is given in the table below.

Table 3.5.1.: Microtiter plate preparation.

	1 (blank)	2 (DPPH)	3 (+ve control)	4 (d/w:methanol)	5 (S2)	6 (S3)	7 (S4)	8 (S5)	9 (S6)	10 (S7)	11 (S8)	12 (S9)
A	200µl methanol	200µl DPPH	100µl ascorbic acid +100µl DPPH	100µl d/w:methanol +100µl DPPH	100µl S2 +100µl DPPH	100µl S3 +100µl DPPH	100µl S4 +100µl DPPH	100µl S5 +100µl DPPH	100µl S6+100µl DPPH	100µl S7 00µl PPH	100µl S8 +100 µl DPPH	100µlS9 +100µl DPPH
B	200µl methanol	100µl DPPH	100µL (3A)+100µl DPPH	100µL (4A)+100 µl DPPH	100µL (5A)+100µl DPPH	100µL (6A)+100µl DPPH	100µL (7A)+100µl DPPH	100µL (8A)+100µl DPPH	100µL (9A)+ 100µl DPPH	100µL (10A) +100µl DPPH	100µL (11A) +100µl DPPH	100µL (12A)+100µl DPPH
C	200µl methanol		100µL (3B)+100µLDPPH	100µL (4B)+100 µl DPPH	100µL (5B)+100µl DPPH	100µL (6B)+100µl DPPH	100µL (7B)+100µl DPPH	100µL (8B)+100µl DPPH	100µL (9B)+ 100µl DPPH	100µL (10B) +100µl DPPH	100µL (11B) +100µl DPPH	100µL (12B)+100µl DPPH
D			100µL (3C)+100µLDPPH	100µL (4C)+100 µl DPPH	100µL (5C)+100µl DPPH	100µL (6C)+100µl DPPH	100µL (7C)+100µl DPPH	100µL (8C)+100µl DPPH	100µL (9C)+ 100µl DPPH	100µL (10C) +100µl DPPH	100µL (11C) +100µl DPPH	100µL (12C)+100µl DPPH
E			100µL (3D)+100µLDPPH	100µL (4D)+100 µl DPPH	100µL (5D)+100µl DPPH	100µL (6D)+100µl DPPH	100µL (7D)+100µl DPPH	100µL (8D)+100µl DPPH	100µL (9D)+ 100µl DPPH	100µL (10D) +100µl DPPH	100µL (11D) +100µl DPPH	100µL (12D)+100µl DPPH
F			100µL (3E)+100µLDPPH	100µL (4E)+100 µl DPPH	100µL (5E)+100µl DPPH	100µL (6E)+100µl DPPH	100µL (7E)+100µl DPPH	100µL (8E)+100µl DPPH	100µL (9E)+ 100µl DPPH	100µL (10E) +100µl DPPH	100µL (11E) +100µl DPPH	100µL (12E)+100µl DPPH
G			100µL (3F)+100µLDPPH	100µL (4F)+100 µl DPPH	100µL (5F)+100µl DPPH	100µL (6F)+100µl DPPH	100µL (7F)+100µl DPPH	100µL (8F)+100µl DPPH	100µL (9F)+100µl DPPH	100µL (10F)+ 100µl DPPH	100µL (11F)+ 100µl DPPH	100µL (12F)+100µl DPPH
H			100µL (3G)+100µLDPPH	100µL (4G)+100 µl DPPH	100µL (5G)+100µl DPPH	100µL (6G)+100µl DPPH	100µL (7G)+100µl DPPH	100µL (8G)+100µl DPPH	100µL (9G)+ 100µl DPPH	100µL (10G) +100µl DPPH	100µL (11G) +100µl DPPH	100µL (12G)+100µl DPPH

Key

+ve controle- Ascorbic acid

The respective samples in the wells are as follows:

S2-AIN-987130, S6-AIN-735783

S3-AIN-673094, S7-AIN-735590

S4-AIN-061287, S8-AIN-735624

S5-AIN-016493, S9-AIN-610884

CHAPTER 4: ANALYSIS AND CONCLUSION

4.1ANALYSIS OF COW URINE SAMPLE

All the urine samples of the breed Konkan Kapila had normal levels of Urobilinogen ranging from 0.1 to 0.8 mg/dL (Table 4.1.1). The specific gravity of all the samples was 1.010. The urine samples had trace amounts of protein that is equivalent to 10mg/ml. Blood, Ketone, Bilirubin and Glucose was absent in all the urine samples. The pH of the urine sample with AIN 401622 and AIN 987130 was 8.5. Urine samples with AIN 670394, AIN 016287 and AIN 016493 had a pH of 8.0.

Table 4.1.1: Analysis of cow urine sample of the breed Konkan Kapila.

Animal Identification Number (ANI)	**Urobilinogen (URO)**	**Protein (PRO)**	**Blood (BLO)**	**Specific gravity (SG)**	**Ketone (KET)**	**Bilirubin (BIL)**	**Glucose (GLU)**	**pH**
401622	Normal	Trace	-ve	1.010	-ve	-ve	-ve	8.5
987130	Normal	Trace	-ve	1.010	-ve	-ve	-ve	8.5
670394	Normal	Trace	-ve	1.010	-ve	-ve	-ve	8.0
016287	Normal	Trace	-ve	1.010	-ve	-ve	-ve	8.0
016493	Normal	Trace	-ve	1.010	-ve	-ve	-ve	8.0

Key

Absent- -ve

Figure 4.1.1: Urine analysis of Konkan Kapila sample A) AIN-401622, B) AIN-987130, C) AIN-670394, D) AIN-016287, E) AIN-016493

The urine samples of Gir cow had normal levels of Urobilinogen ranging from 0.1 to 0.8 mg/dL (Table 4.1.2). The protein levels in these cow urine samples were trace which is equivalent to 10mg/ml. Blood, Ketone, Bilirubin and glucose were absent in the cow urine sample. The specific gravity of the cow urine samples was 1.005. The pH of all the cow urine samples was 8.0.

Table 4.1.2: Analysis of cow urine sample of Gir cow.

Animal Identification Number (ANI)	**Urobilinogen (URO)**	**Protein (PRO)**	**Blood (BLO)**	**Specific gravity (SG)**	**Ketone (KET)**	**Bilirubin (BIL)**	**Glucose (GLU)**	**pH**
735783	Normal	Trace	-ve	1.005	-ve	-ve	-ve	8.0
735590	Normal	Trace	-ve	1.005	-ve	-ve	-ve	8.0
735624	Normal	Trace	-ve	1.005	-ve	-ve	-ve	8.0
610884	Normal	Trace	-ve	1.005	-ve	-ve	-ve	8.0

Key

Absent- -ve

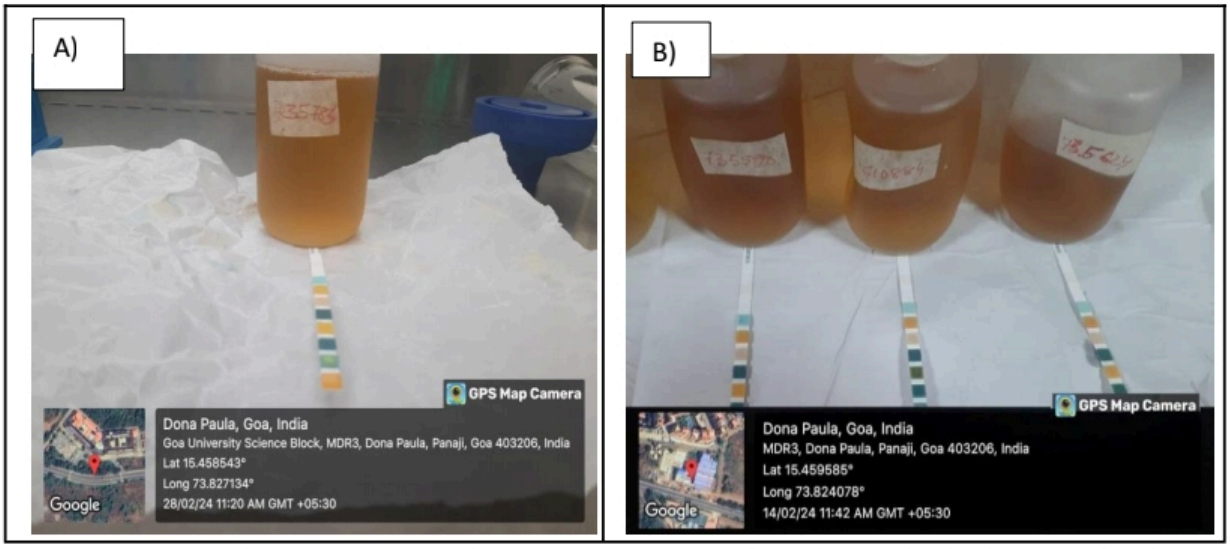

Figure 4.1.2: Urine analysis of Gir cow sample A) AIN-735783, B) AIN-735590, AIN-735624, AIN-610884.

4.2ANALYSIS OF COW URINE SAMPLE FOR ANTIMICROBIAL ACTIVITY

The antimicrobial activity of the lyophilized cow urine sample was tested against three pathogenic cultures of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The antimicrobial activity was tested using the disc diffusion method. An amount of 40µL of sample was used. The samples did not exhibit antimicrobial activity against the three test cultures (Fig.4.2.1, 4.2.2, 4.2.3)

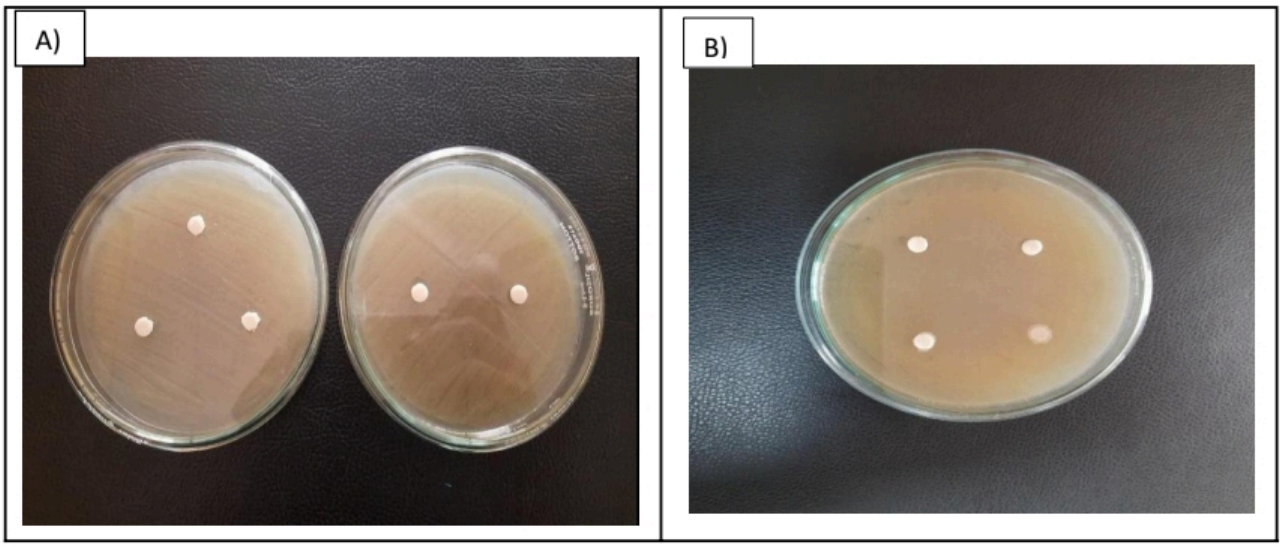

Figure 4.2.1 Antimicrobial test against *Escherichia coli* A) Konkan Kapila samples, B) Gir cow samples.

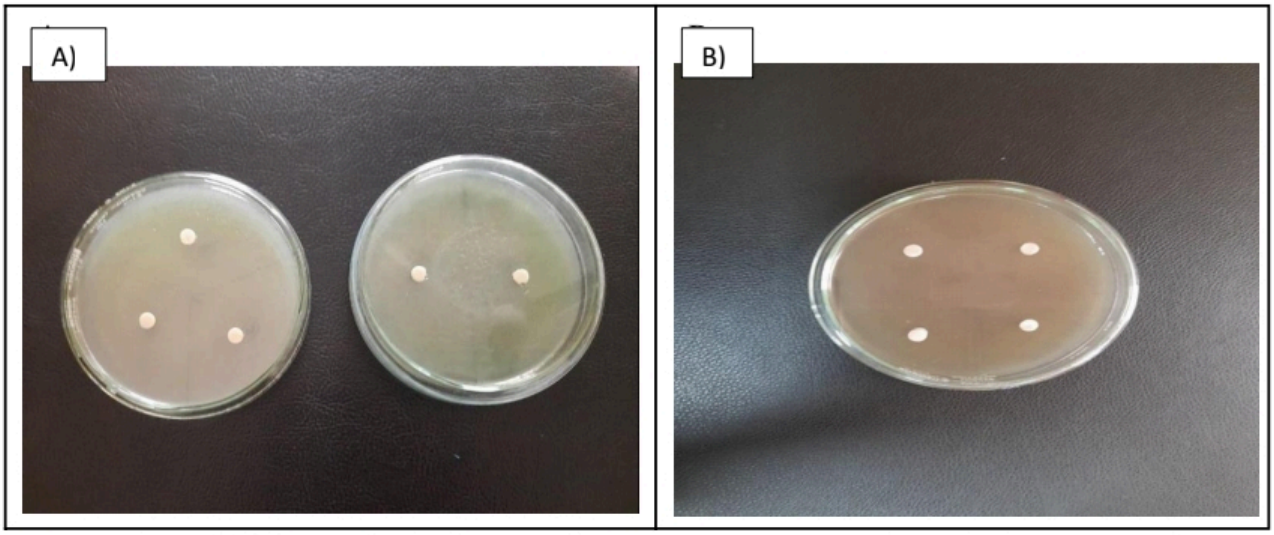

Figure 4.2.2 Antimicrobial test against *Pseudomonas aeruginosa* A) Konkan Kapila samples, B) Gir cow samples.

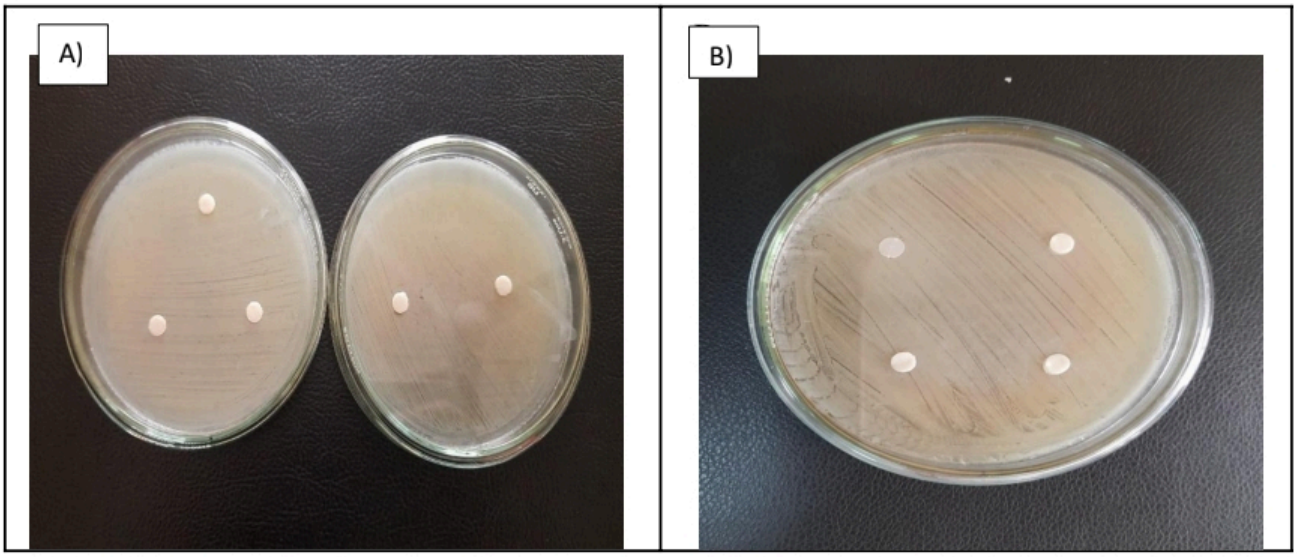

Figure 4.2.3 Antimicrobial test against *Staphylococcus aureus* A) Konkan Kapila samples, B) Gir cow sample

4.3ANALYSIS OF COW URINE SAMPLE FOR ANTIOXIDANT ACTIVITY

Antioxidant activity of the lyophilized cow urine samples were carried out. DPPH was used to detect the antioxidant activity of the sample Ascorbic acid antioxidant was used as positive control. Samples from well number 6A,6B and 6C (S3- AIN-673094)and well number 8A,8B,8C,8D,8E and 8F (S5- AIN-016493) had shown a change in color from purple to yellow indicating the presence of possible antioxidant activity(Fig.4.3.1)

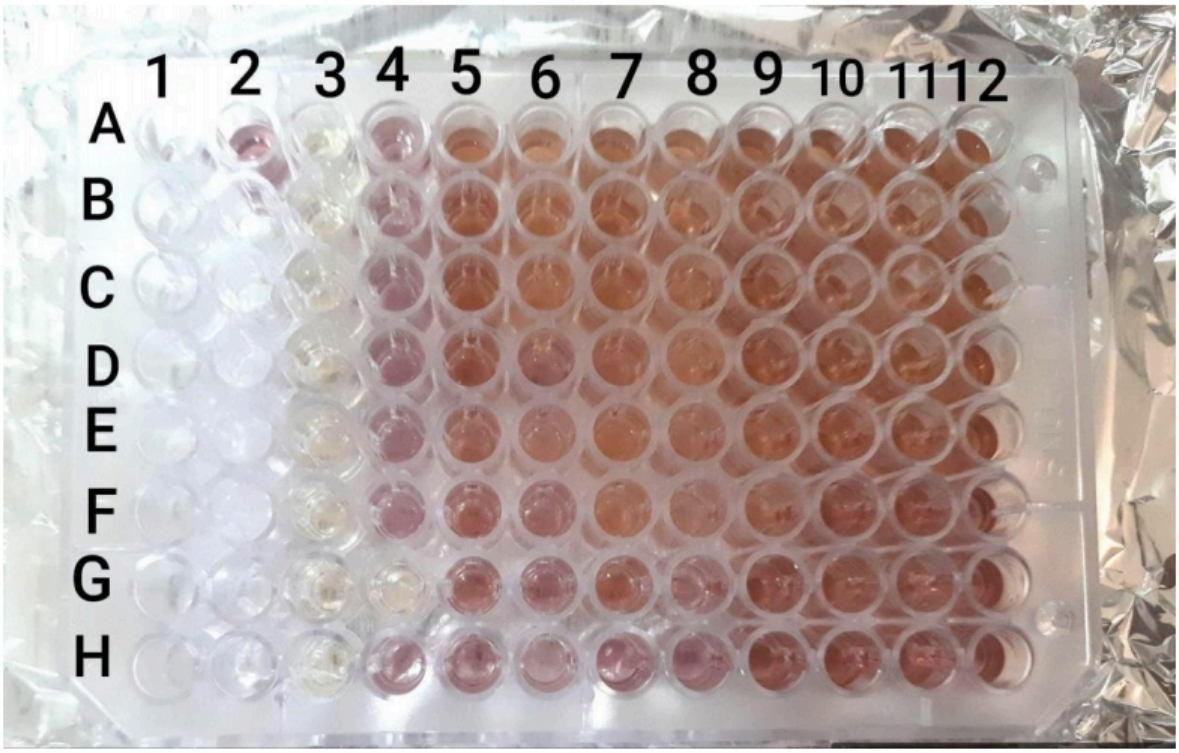

Figure 4.3.1: Antioxidant activity of Cow urine sample

The emerging antimicrobial resistance amongst the pathogenic bacteria has surged the need to identify new antimicrobial compounds. Cow urine is known to have antimicrobial and antioxidant potential. Cow urine has been used in India in various forms. For this study cow urine samples were taken from the breeds Konkan Kapila and Gir cow. The lyophilized powder of the cow urine samples were tested for their antimicrobial and antioxidant activity. The urine samples were analysed for the health status of the cow by the Dipstick Test Method. All the urine samples of the breed Konkan Kapila had normal levels of Urobilinogen ranging from 0.1 to 0.8 mg/dL. The specific gravity of all the samples was 1.010. The urine samples had trace amounts of protein that is equivalent to 10mg/ml. Blood, Ketone, Bilirubin and Glucose was absent in all the urine samples. The pH of the urine sample was in the range 8.0-8.5. All the urine samples analysed of the breed Konkan Kapila indicated that the cows were healthy. The urine samples of Gir cow had normal levels of Urobilinogen ranging from 0.1 to 0.8 mg/dL. The protein levels in these cow urine samples were trace which is equivalent to 10mg/ml. Blood, Ketone, Bilirubin and glucose were absent in the cow urine sample. The specific gravity of the cow urine samples was 1.005. The pH of all the cow urine samples was 8.0. All the urine samples analysed of the breed Gir cow indicated that all cows were healthy.

The lyophilized powder of cow urine sample was tested for antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The urine samples of Konkan Kapila and Gir Cow did not exhibit antimicrobial activity against these pathogens for an amount of 40µl. Sharma *et al*., (2019) tested the antimicrobial activity of lyophilized cow urine samples of the indigenous Hariana breed and found a zone of inhibition of 18.67 mm against. DPPH was used to test the antioxidant activity of the lyophilized cow urine samples. It is a stable free radical. DPPH changes its colour from violet to colourless or pale yellow upon reaction with an antioxidant. Sample with AIN 673094 and AIN 016493 had possible antioxidant activity. These two samples were from the breed Konkan Kapila. Nautiyal and Dubey (2020) tested the antioxidant activity of the Badri cow urine sample. At a concentration of 80mg/ml the sample showed a maximum reduction of 54%.

CONCLUSION

The lyophilized cow urine samples of the breed Konkan Kapila and Gir cow were tested for the antimicrobial activity. The cow urine samples did not exhibit antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Cow urine from the breed Konkan Kapila may have possible antioxidant activity.

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APPENDIX I

Nutrient Agar

Beef extract	3g
Peptone	5g
Sodium Chloride	5g
Agar	15g
Distilled water	1000ml

Endo Agar

Peptic digest of animal tissue	10g
Lactose	10g
Dipotassium phosphate	3.5g
Sodium sulphate	2.5g
Basic fuchsin	0.5g
Agar	15g
Distilled water	1000ml
Final pH	7.5±0.2

Citrimide Agar

Pancreatic digest of gelatin	20g
Agar	15g
Potassium sulphate	10g
Magnesium chloride	1.4g
Cetrimide	0.3g
Distilled water	1000ml

Mueller Hinton's Agar

Beef infusion	300ml
Casein hydrolysate	16g
Starch	1.5g
Agar	1.5g
Distilled water	1000ml
pH	7.2±0.2

Mueller Hinton's Broth

Beef infusion	300ml
Casein hydrolysate	16g
Starch	1.5g
Distilled water	1000ml
pH	7.2±0.2

APPENDIX II

(REAGENTS)

95% Methanol

Composition	Milliliters
Methanol	95ml
Distilled water	5ml

0.1g/10 ml Ascorbic acid

Composition	Grams
Ascorbic acid	0.1g
Distilled water	10ml

254mM DPPH solution

Composition	Grams
DPPH	0.1g
95% Methanol	1ml

25.4 mM DPPH solution

Composition	Grams
254mM DPPH solution	0.1ml
95% Methanol	0.9ml