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Study of *in-vitro* Antioxidant and Antibacterial Activity of Cow Urine from Different Altitudinal and Climatic Region of Nepal

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Authors' contributions

This work was carried out in collaboration among all authors. Authors DRJ and RB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PA and MKC managed the analyses of the study. Authors BKPK, SPY and NA managed the literature searches, collected the urine samples and involved in overall experimental processes. All authors read and approved the final manuscript.

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ABSTRACT

Background: Cow urine has been widely used as therapeutic or additive agents in different parts of Nepal, mainly to treat infections, cuts, burns, etc. Cow urine has been considered as sacred and holy water and used in many rituals and worshipping purposes. Apart from therapeutic and rituals ceremony cow urines are also used in purification and disinfecting the houses.
Objective: The aim of the study was to investigate the *in-vitro* antioxidant and antibacterial activity of cow urine from different altitudes across Nepal.
Materials and Methods: Antioxidant activity of cow urines was carried out by using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) as free radicals. The antibacterial activity of cow urines was tested by the agar disc diffusion method against *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive).

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Results: The urine sample from Muktinath (Subalpine region) showed the highest 44.8±0.21% inhibition against DPPH free radicals. The urine samples from Jajarkot and Marpha (Temperate zone) showed 39.70±0.43% and 37.30±0.15% inhibition against DPPH free radicals respectively. The urine samples from Palpa and Shyangja (Subtropical region) showed 30.00±0.24 and 34.4±0.01 percent inhibition of DPPH radicals. The samples from Butwal and Dhangadhi (Tropical region) showed the least percentage inhibition of 23.9±0.08 and 21.12±0.1 respectively. Amongst the entire sample, the subalpine region of cow urine (Muktinath 3710m) made known the highest inhibition zone such as 10.56±0.05mm against *S. aureus* and 7.46±0.15 against *E.coli*.

Conclusion: Our study has concluded that cow urines have the antibacterial and antioxidant activities and vary in potency according to altitudinal and climatic differences. Hence cow urine from the subalpine zone has better antibacterial and antioxidant activity than that of lower altitudinal climatic zones.

Keywords: Antibacterial; antioxidant; cow urine; Escherichia coli; subalpine climatic zone; Staphylococcus aureus.

ABBREVIATIONS

DPPH	: 2, 2-Diphenyl-1-Picrylhydrazyl
AIDS	: Acquired Immune Deficiency Syndrome
ROS	: Reactive Oxygen Species
UV	: Ultraviolet
mg	: MilliGram
ml	: Milliliter
mcg	: MicroGram
g	: Gram
µg/ml	: MicroGram per Milliliter
mg/ml	: MilliGram per Milliliter
g/ml	: Gram per Milliliter
mM	: Milli mole
nm	: Nano meter
wt.	: Weight
ZOI	: Zone of inhibition

1. INTRODUCTION

During the search for therapeutic potential effects of plants and animals for human diseases, our ancient people uses various hit and trial methods and came upon with the consumption of poisonous plants to edible natural substances. Evidence of the therapeutic and toxic effects of such materials leads to the documentation of curable and non-curable substances and practiced from generation to generation [1]. Traditional practice makes more challenging and has to develop scientific evidence to explore and to determine sources of a new therapy and promote the use of this traditional and alternative system of medicine in a more sophisticated technique [2,3]. In Nepal, 90% of the population lives in a remote area and 80% of total inhabitants depend upon the indigenous system of medicine.[4,5] The Himalayan region accounts for 39.3 % GDP depends upon farming and livestock [4]. According to most prestigious Hindu scripture "Veda" explains cow (*Bos indicus*) as a sacred animal and considered as a mother of all humankind. One well-known Ayurvedic preparation contains cow urine, milk, dung, curd and ghee which is known as "panchagawya" and is dispense to lactated women. Cow urine has been used to treat various diseases like epilepsy, fever, gastrointestinal disorders, menstruation problem, trauma, burn, cardiac disorders, diabetes, and wounds, etc. Excess production of free radicals and other reactive oxygen species leads to aging, stress, lipid peroxidation, and cardiac disorder, cancer, delayed wound healing and so on. Hence several inorganic and organic compounds present in cow urine are useful in the prevention of oxidative stress and several chronic disorders [6-9].

People and traditional healers of India normally use cow urine as a home remedy and additives in several medicines for the cure of several diseases such as bacterial and viral infection. Cow urine can cure hemorrhoids, reduces antibiotic dosage, increase bioavailability, immunity, and appetizer [10-13]. More importantly, cow urine has been granted as patent no. 6410059 in the field of antimicrobial and bio-enhancer while patent no. 6896907 has been granted for anticancer and anticancer enhancer properties [14-17]. Earlier finding suggest that fresh cow urine contains, sodium, nitrogen, urea, sulphur, Vitamin A, B, C, D, E, minerals, manganese, iron, silicon, chlorine, magnesium, citric, succinic, calcium salts, phosphate, lactose, carbolic acid, aurum hydroxides, H-11 beta- indole acetic acid, anti-neoplastic, phenolic compounds, creatinine, enzymes and hormones such as epithelial growth factor and growth hormones [18-20]. Among them carbolic acid, urea, creatinine, manganese and aurum hydroxide possess

antimicrobial and germicidal effects, while vitamins uric acid, phenol, and potassium shows the antioxidants and anti-inflammatory activity. Substances present in cow urine have immunostimulant, antimicrobial and antiageing activities [21-23].

In our study, we collected cow urine samples from the different climatic zone of Nepal such as tropical (Butwal, Dhangadhi, <500 m), subtropical, (Palpa, Shyangja, 1000-2000 m), temperate (Marfa, Jajarkot, 2000-3000 m) and subalpine (Muktinath, 3710 m) zone. Nepal is a landlocked country and it covers about 147,181 square meters in an area out of which 14% of land consists of Terai/ plain region while remaining 86% of land make up of Hilly and mountain regions. Nepal has immense altitudinal diversity ranging from 60 m above sea level in the Flat Terai region to 8848 m of Mount Everest located in the northeast part. This variation in elevation of land gives a broad variety of climatic form in Nepal. Most of the ethnic groups of Nepal living in these regions are Brahmin, Chhetri, Tamang, Gurung, Magar, Madhesi and Tharu. Among them, Hindus are the most dominant religion. In the Hindu religion, the urine of cow is considered sacred holy water and used in various rituals processes [5,24]. Cow urine is safe for human consumption because it contains 95% water and 2.5% urea, and the rest contains minerals, salts, vitamins, enzymes, and hormones, etc [18]. Also, it has been used as a disinfectant and purification of the houses and human beings [21]. People from rural and hilly areas have become poor because of the low income and consequences of several natural catastrophic events [24].

The modern antibiotic is costly and not easily accessible to remote and rural areas. Cow urines can be used for wound healing purposes and can treat many infectious diseases as described in several sacred books of Hindus and practices the traditional system of medicine [6,25]. Antibacterial activity of human urine was studied by Kaye [26] but in India and Nepal where the majority of the people belong to the Hindu religion and they consider cow as holey so the use of cow urine is very common.

Hence, the present study has been undertaken to investigate and justify the valuable effect like antioxidant and antibacterial activities of cow's urines of four climatic regions of different altitudes of Nepal, where the fresh cow urine is used in many rituals worshipping and other function.

2. MATERIALS AND METHODS

The aim of the study was to evaluate the antioxidant and antibacterial activity of fresh cow urine from the different climatic zone of Nepal.

Urine sample: The Cow urine samples were collected from the different climatic regions of Nepal. In this experiment firstly we selected different regions of Nepal such as Tropical (Butwal, Dhangadhi), Subtropic (Palpa, Shyangja), Temperate (Marfa, Jajarkot) and Subalpine (Muktinath) zone. Our research team went to these areas and collected cow urines after clearing from the veterinary doctor (Birendra Shrestha) to ensure the cows were in healthy condition. Prior to urines collection, all the cows were non- lactating, non-pregnant and free from diseases. The urine samples were collected in the month of August. All the urine samples from cows were collected in the early morning at the time between 6 AM to 7 AM and written permission was taken from all the respective cow's owners from all the areas. Cow urines were collected in sterile collecting bottles and kept in an ice bag. The urine samples were stored at a cool temperature at Pharmacognosy Laboratory of the Crimson College of Technology, Butwal-13, Rupandehi, Nepal.

2.1 Methods

Filtration and proper labeling of urine sample: All of the seven samples are filtered with Whatman filter paper no. 3 and properly labeled.

2.2 Antioxidant Assay by DPPH Radical Scavenging Assay

DPPH free radical scavenging assay was measured using the method described by Bhandari et al., 2015 [25].

Preparation of standard and negative control*:* Ascorbic acid-containing 100 µg/ml solution was prepared. 2 ml of DPPH solution is dissolved in 2 ml of methanol for negative control.

Preparation of DPPH solution: DPPH solution of approximate 60 µM concentration was prepared in standard methanol.

2.3 Measurement of DPPH Free Radical Scavenging Activity

Each urine sample of 2 ml was added to 2 ml of methanolic solution of DPPH. The tubes were shaken vigorously and allowed to stand for 30

min at room temperature in a dark place. Changes in absorbance of samples were measured at 517 nm. Ascorbic acid was used as a standard control. All the tests were performed in triplicates. Free radical scavenging activity was expressed as percentage inhibition and was calculated using the following formula.

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} * 100$$

Where,

Abs control is the absorbance of DPPH radical + methanol and
Abs sample is the absorbance of DPPH radical + urine sample

2.4 Antibacterial Activity

Antibacterial activities of cow urine samples were screened by the disc diffusion method according to the method described by Benbelaid et al., 2013 [27]. The sub-cultured microorganisms were swabbed into Petri plates containing Mueller Hilton agar. Test discs (6 mm in diameter) were prepared by punching the filter paper by punching machine. The sterile test discs were made by infusing with 10 µl of the urine sample. Ciprofloxacin (10 mcg/disc) and Ampicillin (10mcg/disc) were used as standard drugs. The test and reference disc were placed over at equidistance on top of the agar layer. The discs were allowed to diffuse for an hour and then incubated at 37ºC for 24 h in an incubator. Before selecting the appropriate antibiotic as positive control antimicrobial susceptibility tests to different antibiotics (Ofloxacin, Ciprofloxacin, Ampicillin, and Streptomycin) were determined. Only Ciprofloxacin and Ampicillin were susceptible to bacteria supplied. Other antibiotics were resistant to respective strains of bacteria.

2.5 Statistics

All the data expressed as mean ±SD (*n*=3).

3. RESULTS

Our study measured the antioxidant and antibacterial activities of cow urine collected from the different climatic zone of Nepal such as Muktinath (Subalpine), Jajarkot, Marfa (Temperate), Shyangja, Palpa (Subtropical), and Butwal, Dhangadhi (Tropical Region).

3.1 DPPH Radical Scavenging Assay

In this experiment, the antioxidant activities of collected different cow urine samples were carried out using DPPH as a free radical. The result of the antioxidant activity is summarized in Table 1. The urine sample from Muktinath (Subalpine region) shows the highest percent inhibition while a sample from Butwal and Dhandgadi (Tropical region) shows the least percentage inhibition. The antioxidant activity of cow urine from the temperate region (Marfa and Jajarkot) is greater than the subtropical region (Palpa and Shyangja). The radical scavenging activity was found to be in decreasing order from the subalpine region to the tropical climatic region. Among the entire sample tested the highest percent inhibition was found by a reference sample such as ascorbic acid whose concentration was taken as 100 µg/ml.

Table 1. Free radical scavenging activity of fresh cow urine by DPPH method (*in vitro*)

Sample	% inhibition
Ascorbic acid	92.33± 0.57
Muktinath	44.8±0.21
Jajarkot	39.7± 0.43
Marfa	37.3± 0.15
Palpa	30.0± 0.24
Shyangja	34.4±0.01
Butwal	29.9± 0.08
Dhangadhi	28.12± 0.1

All the value expressed as mean ±SD (n=3)

3.2 Antibacterial Activity of Cow Urine

Antibacterial activity of all the climatic regions of urine samples was evaluated against two virulent strains of bacteria such as *E. coli* and *S. aureus*. The antibacterial effect of urine samples was assessed by the presence or absence of inhibition zone using the disc diffusion method. The standard reference drugs used were ampicillin and ciprofloxacin for Gram-positive and Gram-negative bacteria respectively. The zone of inhibition of urine samples from the different regions was shown in Table 2. Our data summarized in the table indicate that cow urines have fascinating antibacterial activity and also greatly vary in their antibacterial activity according to their altitudinal and climatic differences. Amongst the entire sample tested, subalpine regions of cow urine from Muktinath revealed the highest inhibition zone. Our preliminary data showed that the antibacterial activity of cow urine from temperate (Jajarkot and

Table 2. Zone of inhibition (mm) of cow urines, ciprofloxacin (10 mcg) and ampicillin (10 mcg)

SN	Sample	*S. aureus*	*E. coli*
1	Muktinath	10.56±0.05	7.46±0.15
2	Jajarkot	8.36±0.15	6.43±0.40
3	Marpha	7.40±0.17	5.56±0.05
4	Shyangja	6.83±0.15	5.50±0.20
5	Palpa	6.13±0.11	5.30±0.10
6	Butwal	5.16±0.20	3.00±0.26
7	Dhangadhi	4.76±0.15	2.10±0.10
8	Ciprofloxacin		25.83±0.76
9	Ampicillin	22.50±0.04	

All the value expressed as mean ±SD (n=3)

Marpha) region has slightly less than that of the subalpine region and greater zone of inhibition than that of subtropical (Palpa and Shyangja) and tropical region (Dhangadi and Butwal) of cow urines. According to results obtained from our study showed that cow urines were more susceptible to Gram-positive bacteria than Gram-negative bacteria.

4. DISCUSSION

Cow urine contains many organic and inorganic substances and helps to maintain bodily homeostatic [23]. Traditionally cow urine has been used as a therapeutic remedy for several human diseases and hypothetically used to treat several illnesses and also included in Ayurvedic formulation as mentioned in a sacred ancient book like Veda and Charaka Samhita etc.

The biochemical investigation revealed that the presence of potent antimicrobial agents such as creatinine, calcium, phenols, aurum hydroxide carbolic acid, manganese, and creatinine, etc [16,17,22,28]. Phenol compounds are phytoconstituents and contain many hydroxyl groups. This hydroxyl group is toxic to bacteria and can kill Gram-positive as well as Gram-negative bacteria [29,30]. Hence the significance role of cow urines in human and veterinary medicine from scientific literature and ancient holy books evoked us to carry out this experiment to determine the antibacterial and antioxidant effects of cow urine collected from the different climatic and altitudinal region of Nepal.

Our study has revealed that cow urine is more active against *S. aureus* (Gram-positive) bacteria than Gram-negative bacteria such as *E. coli*. Phenolic compounds are responsible to kill the bacteria and more specifically effective against Gram-positive bacteria [17,29]. Therefore, the zone of inhibition against *S. aureus* may be comparatively higher than that of *E. coli*. The cow urine from the subalpine and temperate zone exhibit good antimicrobial activity to ampicillin against *S.aureus*. The urine samples from Muktinath (subalpine region) has the highest inhibition zone against Gram-negative bacteria such as *E.coli*, while urine sample from the tropical region showed the lowest inhibition zone against *E.coli*. Apart from the antimicrobial properties cow urine also exhibit antioxidant activity. According to Table 2, cow urine from the subalpine zone (Muktinath) showed higher percentage inhibition and urine from the lowest altitude (Tropical zone, Dhangadi) exhibits the lowest free radical scavenging activity. This variation in bioactivity may be due to the variation in diets they usually consume [31].

According to our study the diets for Dhangadi/Butwal (Tropical region): Dana, Cereals, Grains, Wheat hay etc, for Palpa/Syangja (Sub-tropical region): *Ficus lacor*, *Bambusa arudinacea*, *Cyanodon dactylon* in addition to Dana and cereals etc, for Marfa/Jajarkot (Temperate region): *Bauhinia variegata*, *Oxalis corniculata*, *Cyanodon dactylon* in addition to Dana and cereals etc. and for Muktinath (Sub-alpine region): Buckwheat, Millet, *Xanthoxylum armatum*, *Euphrobia hirta*, *Cyanodon dactylon* in addition to cereals etc. were found. Hence, these naturally available plants such as ficus species, *Bambusa arundinacea*, *Bauhinia variegata*, *Oxalis corniculata*, *Cyanodon dactylon* have flavonoids, phenolic compounds, carbohydrates, proteins, triterpenoids, alkaloids, glycosides, sterols, tannins, etc [32-35].

The several free radicals are the consequence of cellular and other metabolism and produce oxidative stress and are believed to cause several harmful chronic disorders such as aging, cardiovascular, diabetes, kidney disease, and cancer, etc [17,36]. According to ancient records, cow urine has been supplied to many diseases like kidney, cardiac and edema [31]. Experiment on cow urine exhibit protective effect against genotoxic agents, this antioxidant and the protective effect is due to the presence of volatile fatty acid, uric acid, vitamin A, B, C, D and E presence in cow urine [8,22]. Hydroxyl groups presence in phenolic compounds accountable for free radical scavenging activity [30].

5. CONCLUSION

The investigation of our experiment is highly supportive and evidence of the antibacterial and antioxidant effect of cow urine carried out by previous scientific researchers. From our study's data and results, we have concluded that antioxidant and antibacterial effects of cow urine significantly increase with an increase in altitude. The therapeutic potentiality of cow urine is greater in the subalpine region than low altitude climatic region. This variation of activity may be due to the variation of quality and quantity of active chemical constituent present in the different climatic regions of cow urines. The dissimilarity in cow urine therapeutic potency and efficacy may be due to the climatic changes within the species and maybe the variation in food, water and grass they regularly take. Further qualitative research should be carried out to investigate the active chemical constituents and their metabolites present in different climatic and altitudinal cow's urines due to their habitats, plants, and the food they use.

DECLARATIONS

The results presented in this manuscript entitled "Study of *in-vitro* Antioxidant and Antibacterial Activity of Cow Urine from Different Altitudinal and Climatic region of Nepal" has been entirely carried out by all the authors from Department of Pharmaceutical Sciences, Crimson College of Technology, Pokhara University, Nepal. We hereby declare that this work is original and has not been submitted elsewhere.

CONSENT

Written permission was taken from all the respective cow's owners from all the areas.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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