ORIGINAL ARTICLE

Green extraction using goat urine as menstruum and evaluation for *in vitro* antimycobacterial activity of *Curcuma zedoaria* and *Curcuma caesia* rhizomes collected from Assam

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Abstract

Background: In Indian traditional system of medicine, goat urine is believed to have therapeutic value and is also reported its use in the treatment of tuberculosis (TB). On the basis of reported traditional uses for the treatment of TB and/or leprosy, *Curcuma caesia* and *Curcuma zedoaria* rhizomes were selected. **Aim:** It was aimed to study the antimycobacterial activity of goat urine and extracts of the rhizome of the two plants obtained using goat urine as menstruum. **Materials and Methods:** The rhizomes were amassed from in and around Dibrugarh. The clean sliced rhizomes were dried at room temperature. The dried rhizomes of both the plant species were extracted using raw and photoactivated goat urine as menstruum by maceration process. *In vitro* antimycobacterial activity of the rhizome extracts was carried out by disc diffusion method. **Results and Discussion:** Crude photoactivated goat urine extracts of both the plants *C. caesia* (paGuCc) and *C. zedoaria* were found to have higher antimycobacterial activity against *Mycobacterium smegmatis* than that of raw goat urine extracts of both the plants *C. caesia* and *C. zedoaria*. Among all paGUCc extracts were found to exhibit highest antimycobacterial activity. **Conclusion:** The extracts obtained using photoactivated goat urine showed higher activity than the extracts obtained using raw goat urine. Goat urine also exhibited antimycobacterial activity, but not as much as the extracts. Thus, it is proved that the extracts and goat urine have antimycobacterial activity and extracting with goat urine and thus have improved activity.

Key words: Disc diffusion, goat urine, maceration, mycobacteria, northeast

INTRODUCTION

Tuberculosis (TB) is a life-threatening bacterial disease caused by various *Mycobacterium* species. TB is considered as an old yet emerging disease, which is the leading factor of human mortality and morbidity in developing and underdeveloped countries. The World Health Organization announced this chronic disease to be a "global emergency" and reported that in 2014, about 9.6 million people developed TB and out of those about 1.5 million died.[1,2] The time required to treat TB ranges from 6 to 9 months, which is a long duration that not only increases the risk of infecting the patient but also increases the risk of patient developing drug-induced hepatic damage. Hence, an urge for the development of shorter, simpler therapeutic regiments for the treatment of this deadliest communicable disease is needed.

Incredible advances were made by medical sciences all over the globe during the past centuries. Due to such advancement, the overall mortality rate has decreased and expectancy of life has increased. New advancement in the field of medical sciences and technology has resulted in the discovery of

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new lifesaving drugs which helps us to fight against several infectious diseases. Now, question arises that in spite of such advancements, do the benefits of modern science and technology have found its way to reach every door of the world? Despite so many progresses, modern medical science is finding difficult to reach the ever-increasing problems of every people around the globe mainly in developing and underdeveloped countries. Therefore, the world is awaiting for easily available and cost-effective medicines to avert such problems and provides basic health care to all.

Tralatitious system of medicine is followed in Ayurveda, Unani, and Siddha medicine and considered as a major health-care supportive around the globe, especially in rural areas.[3] Indian society and its traditional medicine are known to play a key role in maintenance of health and cure many diseases since time immemorial. India is considered as a rich repository of medicinal plants and the Indian traditional systems of medicine use these herbal plants and minerals as the vital source for drugs. Around 25,000 effective plant-based formulations and 8000 plant species are used in folk medicine by the rural people of India.[4] In Indian system of medicine like Ayurveda uses 1200–1800 plants, Siddha medicine includes 500–900 plants, Unani uses 400–700 medicinal plants, and Amchi uses nearly 300 plants in different medicines.[5,6] Along with herbs, other ingredients are also used for traditional practices of medicine. In India, traditional practitioners also use animal urine for the treatment of different diseases. In general, cow urine is considered as a mobile medicine house and is a catholicon of all diseases. It is one among the composition of "Panchagavya" and has the ability to treat many minor as well as major diseases and has been used widely in Ayurvedic preparations. According to ancient texts such as "Chakra Samhita," "Ashtanga Samgraha," and "Atharva Veda," cow's urine has an ineradicable place in Ayurveda and is believed to be important animal secretion having significant therapeutic properties. Cow urine therapy has been practiced in different forms and ways by a large population for the treatment of different diseases. Such forms were Panchagavya or Panchakavyam, which is prepared with milk, ghee, cow dung, curd, and urine.[6] Cow urine is pungent, sharp, and hot, alleviates doshas, destroys worms, cures leprosy, and removes itching and if taken internally, it is beneficial in gastrointestinal problems caused by Tridosha.[7] The biochemical estimation of cow urine has shown that it contains Vitamins A, B, C, D, and E and ions such as sodium, nitrogen, and sulfur; and among minerals such as magnesium, manganese, silicon, iron, calcium salts, chlorine, citric, succinic, carbolic acid, phosphate, and lactose; enzymes, creatinine, hormones, urea, and gold acids. There are reports that cow urine contains 95% water, 2.5% urea, minerals, 2.5% enzymes, and 24 types of salts. In addition, it also contains Vitamins A, B, C, D, and E, hormones, sodium, nitrogen, sulfur, manganese, iron, silicon, chlorine, magnesium, citric, succinic, calcium, phosphate, lactose, creatinine, urea, carbolic acid, and gold acids.[8,9]

In Ayurveda, apart from cow's urine which was used more commonly due to the special sanctity attached to the cow in India, the urine of other animals such as goat, sheep, buffalo, elephant, and horse was also suggested for use in the treatment of abdominal enlargements, worms, abdominal tumor, flatulence, colic pain, dropsy, anemia, loss of appetite, poisoning, hemorrhoids, leukoderma, TB, amenorrhea, leprosy, aggravation of Kapha and Vata, and in certain mental diseases.[10] After the urine of cow, the urine of goat "Ajamutra" is referred with great importance in the ancient medical treaties. Goat urine is astringent-sweet, beneficial for channels and alleviates all doshas. Goat urine is pungent and bitter, slightly aggravates and alleviates coughs dyspnea, edema, jaundice, and anemia.[6] Biochemical analysis of normal goat urine constitutes of nitrogenous constituents such as uric acid, nitrogen, urea, creatine, allantoin, creatinine, and ammonia. Non-nitrogenous constituents include bicarbonates and carbonates; phosphates and sulfates; and chlorides, magnesium, and calcium.[11]

In Indian medicine system, goat urine is reported to have antitubercular properties. As reported by many authors, patient suffering from Kshaya/Rajayakshma (pulmonary TB) should live together with goats in the same room; alternatively, the patient's room, in which he stays, should be painted and tiled with goat urine and feces. Goat urine is also taken orally for the treatment of TB.[12,13] According to the previous research done,[14] some plants have shown significant activity against *Mycobacterium* in *in vitro* studies, among which rhizomes of *Curcuma caesia* Roxb. and *Curcuma zedoaria* Roscoe. also show significant activity against *Mycobacterium* species.

Goat urine has also been reported to have antitubercular activity. Considering the traditional therapeutic use of goat urine and potency of herbs, especially *C. caesia* Roxb. and *C. zedoaria* Roscoe., it was thought to be worthy to carry out a study on the efficacy of the extracts of the mentioned plants using goat urine as menstruum.

On the basis of the traditional therapeutic claim of goat urine, it was expected to have improved efficacy (additive/synergistic) of both the plants and the urine. Since this is a novel approach toward the treatment of TB with improved efficacy of urine as well as the plants, so, with the idea in mind, the present work was designed and the results of this study are reported here. The findings of this study would provide a new dimension in establishing, validating traditional claims and also would provide ample opportunities for further research.

MATERIALS AND METHODS

Collection of Plants

Rhizomes of the plant *C. caesia* Roxb. and *C. zedoaria* Roscoe. were collected from in and around Dibrugarh, Assam, in the month of December 2017.

Identification and Authentication of Plant

The plants were identified and authenticated by Botanical Survey of India (BSI), Eastern Regional Centre, Shillong, as follows:

1. *C. caesia* Roxb. vide No.: BSI/ERC/Tech/2017/114 dated: 30.05.2017
2. *C. zedoaria* Roscoe. vide No.: BSI/ERC/Tech./Plant Iden./2018/115 dated: 22.05.2018.

Drying of the Plant Materials

The rhizomes of *C. caesia* Roxb. and *C. zedoaria* Roscoe. were shade dried in room temperature until it was completely dried to be fit for grinding. During drying, the plant materials were kept away from the direct sunlight to avoid the effect of direct sunlight.[15] Compacted sample of rhizome with little air circulation kept for several days also may alter the properties of the sample. Therefore, well-ventilated place and uniform distribution of the plant materials were ensured during drying process. Then, the plant materials were ground to moderate coarse powder form by mechanically using mortar and pastel. The grinding process increases surface area and the penetration of the solvent to the tissues becomes easy, thereby dissolution of the secondary metabolites improves and increases the yield of extraction.

Collection of Solvent

In the present investigation, goat urine "Ajamutra" was used as a menstruum for the extraction process.

Goat urine was collected from two healthy female goats in the morning hours in the month of February 2018. The volume of goat urine collected from two goats per day was about 600 ml. The total volume of urine collected for the investigation was 5 L. Urine collected was stored in an airtight glass container away from the sunlight.

Photoactivation of Goat Urine

The half of the goat urine used in this present investigation was photoactivated by maintaining it in sunlight for 72 h in a transparent glass container.[8] Then, the goat urine was strained out to free it from debris and precipitated materials.

Extraction

Plant samples were extracted by maceration technique. The purpose of following this basic extraction procedure for the samples was to obtain the therapeutically active constituents without exposing to heat. Such type of extraction procedure plays a decisive role for the qualitative and quantitative properties of the extract.[2]

The extracts of the rhizomes of *C. caesia* Roxb. with raw goat urine (rGUCc) and with photoactivated goat urine (paGUCc) and also the extracts of *C. zedoaria* Roscoe. with raw goat urine (rGUCz) and with photoactivated goat urine (paGUCz) were prepared. The plant extracts were evaporated by heating by maintaining the temperature at 50–60°C until the concentrated crude extracts were obtained. The concentrated crude extracts were stored in airtight container in the refrigerator for further use.

Test Microorganism

A species of saprophytic, rapidly growing, non-pathogenic mycobacteria, namely, *Mycobacterium smegmatis* MC^2155 was used as the test model organism in the initial screening process, while *Mycobacterium tuberculosis* is usually used at a later stage for further studies.

Antimycobacterial Susceptibility Testing

Antimycobacterial susceptibility testing was performed by the determination of the zone of inhibition by disc diffusion method.[16]

Preparation of Medium

The mediums used for the growth of bacteria were as follows:

1. Mueller-Hinton agar medium
   About 38.00 g of Mueller-Hinton agar was suspended in 1000 ml of distilled water. Then, it was heated to dissolve the media. It was then sterilized by autoclaving at 15 lb pressure (121°C) for 15 min and then, it was allowed to cool to 50°C. Then, 25 ml of medium was poured into flat-bottomed sterilized Petri dishes to produce a layer of approximately 4 mm thick. The mycobacterial strain was inoculated into the Petri dishes containing the media with the help of a sterile inoculating loop. The plates were incubated at 36°C overnight.
2. Mueller-Hinton broth medium
   About 21 g of Mueller-Hinton broth was suspended in 1000 ml distilled water and was dissolved by heating. Sterilization was carried out by autoclaving at 15 lb pressure (121°C) for 15 min. On the basis of the morphological similarity of the colonies, five such well-isolated colonies were selected. The inoculum was added to flask containing sterilized Mueller-Hinton broth medium with a sterile loop and the broth culture was incubated at 35°C overnight.

Turbidity Standard for Inoculums Preparation

For a susceptibility test, 0.5 McFarland standard was prepared by adding 0.05 ml of barium chloride dihydrate (1.175% w/v $BaCl_2.2H_2O$) and 9.95 ml of sulfuric acid (1% v/v H_2SO_4) with constant stirring to maintain a suspension. Mixing

these two, barium sulfate precipitate was obtained resulting turbidity in the mixture. The standard was compared visually to a suspension of bacteria. If the bacterial suspension was too turbid, it was diluted with more diluent. If the suspension was not turbid enough, more bacteria were added.

Preparation of Working Solutions

Working solutions were prepared by dissolving each of the crude extracts in sterile distilled water. A set of three dilutions were prepared from each extract. The concentration of the three dilutions was 1000, 2000, and 3000 µg/ml. Each of the working solutions was filtered using syringe filter (22 µ) to remove the minute impurities before the test.

Preparation of Standard Drug Solution

Rifampicin and berberine were used as standard drugs for this study. Standard drug solution was prepared by dissolving the drugs in distilled water to make 10 µg/ml and 25 µg/ml, respectively.

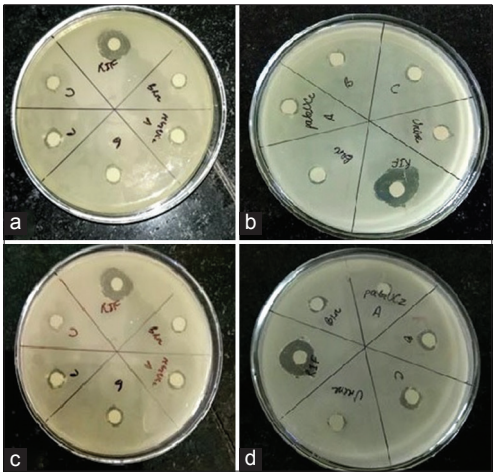

Figure 1: Zone of inhibitions of different concentration of extracts against *Mycobacterium smegmatis*; (a) rGUCc, raw urine, rifampicin, and berberine; (b) paGUCc extract, photoactivated urine, rifampicin, and berberine; (c) rGUCz extract, raw urine, rifampicin, and berberine; (d) paGUCz extract, photoactivated urine, rifampicin, and berberine

Determination of the Zone of Inhibition

The antimycobacterial sensitivity test was performed by agar disc diffusion method. Agar disc diffusion testing developed in 1940, is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing.[16] To determine the antimycobacterial activity, Mueller-Hinton agar medium (30 ml) was poured in Petri plates and allowed to set. Within 15 min after adjusting the turbidity of the inoculums suspension, 100 µl of the inoculums was placed to the molten Mueller-Hinton agar medium on Petri plates and spreads throughout the plate by spread plate technique to disperse the microorganisms homogeneously. Sterilized filter paper discs (Whatman filter paper No. 1, 6 mm in diameter) impregnated with different concentrations (1000, 2000, and 3000 µg/ml of the test solutions (rGUCc, paGUCc, rGUCz, and paGUCz) were prepared. Standard drug impregnated disc (rifampicin of concentration 10 µg/ml and berberine of concentration 25 µg/ml) was also prepared. Discs were placed in such a way that they were not closer than 24 mm center to center. Each test plate consisted of three dilutions of the plant extract, two standards (rifampicin and berberine), and a disc impregnated with urine. The test plates were then incubated at 37°C for 24 h. After 24 h of incubation, antimycobacterial activity was recorded by taking the measurement of the diameter of zone of inhibition using a transparent ruler under colony counter. The test was done in triplicate and the average zone of inhibition (in mm) was recorded.

RESULTS

The zones of inhibition of rGUCc and rGUCz; paGUCc and paGUCz against *M. smegmatis* are shown in Table 1 and Figure 1.

From the results, it was found that plant extracts exhibited susceptibility to antimycobacterial activity test. In both the cases, it was observed that the extracts with photoactivated urine showed more susceptibility to antimycobacterial activity than the extracts with raw urine. Therefore, we can say that photoactivated goat urine has higher activity against *Mycobacterium* than the raw goat urine.

The antimycobacterial activity of the photoactivated goat urine extracts of both the plants was higher than that of the

Table 1: Zone of inhibitions of different concentration of extracts against *Mycobacterium smegmatis*

Name of the extract	Zone of inhibition (diameter in mm)					
	Concentration of plant extract (µg/ml)			Urine (raw)	Rifampicin (10 µg/ml)	Berberine (25 µg/ml)
	1000	2000	3000			
rGUCc	8.33±0.48	8.67±0.48	9.66±0.48	7.33±0.48	17.33±0.48	10.67±0.48
paGUCc	8.67±0.48	9.33±0.48	11.67±0.94	8.33±0.48	17.33±0.48	11.67±0.48
rGUCz	7.66±0.47	8.33±0.59	9.66±0.47	7.66±0.47	9.66±0.47	17.66±0.43
paGUCz	8.33±0.47	9.66±0.43	10.66±0.47	8.33±0.47	9±0.81	17.66±0.47

*Values presented are mean±SD; *n*=3

raw goat urine extracts. It was due to the fact that during photoactivation, biogenic volatile organic and inorganic compounds such as acetone, methane, methanol, propanol, carbon dioxide, ammonia, and some metabolic secondary nitrogenous products are formed.[17] Photoactivated urine is highly acidic in comparison to fresh urine for which there was an increase in the antimycobacterial activity.[18] It was observed that both the urine and the plant materials exhibit a zone of inhibition in *in vitro* antimycobacterial test. However, the zone of inhibition exhibited by the plants using goat urine exhibits much higher susceptibility than that of the urine. Thus, it can be said that additive/synergistic effect of both the plants and urine has improved efficacy.

CONCLUSION

It was found that both the plant materials, the rhizome of *C. caesia* and the rhizome of *C. zedoaria* using both raw and photoactivated goat urine as menstruum showed significant activity against *M. smegmatis*. Among all the four different extracts, the extract of the plant material obtained using photoactivated goat urine shows higher activity than the extracts obtained using raw goat urine. Photoactivated goat urine itself also showed higher antimycobacterial activity than the raw goat urine. Thus, it can be concluded that photoactivated urine has high antimycobacterial activity than the raw goat urine. This also validates the traditional use of goat urine in the treatment/management of TB. Among all the four extracts, the extract of *C. caesia* Roxb. using photoactivated urine showed highest antimycobacterial activity against *M. smegmatis*.

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