



Green extraction and *in vitro* anti-mycobacterial activity of *Hydrocotyle sibthorpioides* Lam. and *Carica papaya* L. leaves collected from Assam, India

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The traditional healers of different parts of India use goat urine for the treatment of tuberculosis. On the basis of ethnomedicinal claims, two plant species namely *Hydrocotyle sibthorpioides* and *Carica papaya* were extracted with raw (fresh) and photo-activated goat urine as a menstruum. The present study reports the *in vitro* antimycobacterial activity of the leaf extracts of *H. sibthorpioides* and *C. papaya* against *Mycobacterium smegmatis* (ATCC 700084 /Mc2155 strain). It was observed that the photo-activated goat urine and raw goat urine leaf extracts could inhibit *M. smegmatis*. Among all the four extracts, the extract of *C. papaya* using photo-activated goat urine showed the highest antimycobacterial activity against *M. smegmatis*.

Keywords: Ajamutra, Anti-tubercular, Ethnomedicine, Goat urine, Maceration, Northeast India.

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Introduction

The Indian Traditional System of Medicine, primarily the *Ayurvedic* system elucidates the use of goat urine in boosting the general health of an individual¹ and is also reported to be used in the treatment of tuberculosis. Several tribes such as the Saharia tribe of Rajasthan, the tribal inhabitants of Attapady hills and other tribes hailing from different parts of India orally administer goat urine as a medication for tuberculosis (TB)². TB which is caused by *Mycobacterium tuberculosis* is considered a highly infectious disease with an annual occurrence rate of more than 9 million and it is reported to have caused about 1.3 million deaths in 2014³. A large number of the world's population is estimated to be infected by it and is regarded to be a major cause of human fatality in the developing world⁴.

With the emergence of efficacious antimycobacterial agents between 1950 and 1970 like ethambutol, isoniazid, rifampicin, and streptomycin there was a substantial reduction in the number of TB cases all over the world, particularly in developing nations⁵. Although these drugs have been developed to treat tuberculosis, they are not always efficient due

to the emergence of drug-resistant strains⁶. It has been reported that there is an increase in cases of Multi-Drug Resistant TB (MDR-TB) and Extensively Drug-Resistant TB (XDR-TB) have deteriorated the situation and constituted a severe health risk. Therefore, potent, novel and cost-effective anti-tubercular drugs with low toxicity are essentially required to encounter the risk of TB⁷.

India is a diverse land with exclusive knowledge about medicinal plants and their uses in treating several diseases⁸. These plants play a crucial role in formulating herbal drugs and provide notable health care aid to a vast section of individuals, especially in developing countries⁹. These medicinal plants have been widely used for healing disease for many centuries¹⁰. Out of the 20,000 medicinal plants that have been recorded to date, the traditional healers use 7000 to 7500 plants to cure various diseases¹¹. A wide range of medicinal plants has been traditionally used to eradicate the symptoms of tuberculosis. Of the 17,500 higher plant species found in India, about 365 species have been estimated to be used for antimycobacterial activities⁵. Researchers have isolated the natural phytochemicals that are essentially required to encounter the risk of MDR-TB. The isoquinoline alkaloid berberine isolated from the roots of *Berberis aristata* and its derivatives [13-

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benzyl (3–6), 13-allyl (7, 8), 8-(2-oxopropyl) (2), and 9-hydroxy (9)] have attributed the anti-tubercular activity against *M. tuberculosis*. Berberine (MIC, 16 µg/mL) anti-tubercular activity is probably due to the 13-benzyl and allyl substitution in the molecule^{12–15}.

Hydrocotyle sibthorpioides Lam. (Araliaceae) and *Carica papaya* L. (Caricaceae) are well-known ethnomedicinal plants. These plants are extensively used in the Indian traditional system to cure ailments. The leaves of *H. sibthorpioides* are traditionally used to treat dysmenorrhoea¹⁶. Freshly crushed leaves are also locally applied to the affected part in the treatment of carbunculus¹⁷. It was also reported to heal rheumatic troubles and skin diseases, including syphilis and liver complaints¹⁸. Studies have also described that the species of *Hydrocotyle* could prohibit the growth of transplanted tumours in mice, such as hepatic carcinoma (Hep), sarcoma (S180) and uterine cervical carcinoma (U14)¹⁹. It was also reported to have antimicrobial and antioxidant activities²⁰. The leaf extracts of *C. papaya* have been studied for a vast range of biological and pharmacological activities like antioxidant, antibacterial, antifungal, antiamebic, wound-healing, antihelminthic, antiulcerogenic, hypolipidemic, antihypertensive, diuretic and antifertility activities²¹. The latex of the plant is utilized as an analgesic in tooth decay. The ripe fruit and seeds of *C. papaya* are used in diarrhoea and as anthelmintics. A decoction of the unripe fruits is taken orally in jaundice^{22,23}. According to *Astanga Samgraha* (an ancient authoritative text on *Ayurveda*) of Acharya Vagbhata^{24,25}, goat urine can be used to treat the ailment of cough, respiratory difficulties, dyspnea, earache, jaundice, oedema and anaemia²⁶. The different tribal communities in the Attappady hills of Western Ghats utilize goat urine for the treatment of tuberculosis²⁷. Goat milk is externally used for the treatment of eye diseases. The pulmonary tuberculosis can be cured by staying in close vicinity in the company of goats and by drinking goat's milk²⁸. The proteins present in goat urine have potential *in vitro* antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*¹. Moreover, it is externally applied to treat skin infections such as itching, ringworms, dermatophytosis or herpes and tinea infections²⁶.

In view of the potential activities of goat urine, *H. sibthorpioides*, and *C. papaya*, the present study was designed and carried out to evaluate the

antimycobacterial activity of raw goat urine extract and photo-activated goat urine extract of *H. sibthorpioides* and *C. papaya*.

Materials and Methods

Raw materials

Rifampicin was procured commercially (Himedia, Mumbai, India), and Berberine (GLR Innovations, New Delhi) was supplied by M/S Vinayak Drugs & Surgicals, Dibrugarh, Assam. *Mycobacterium smegmatis* (*M. smegmatis*) (ATCC 700084 / Mc²155) strain was procured from the Center for Biotechnology & Bioinformatics, Dibrugarh University, Dibrugarh (India).

Plant collection and identification

The leaves of the plant *H. sibthorpioides* and *C. papaya* were collected from Hatiali, Dibrugarh, Assam, India, in December 2017 and February 2018. The plants were identified and authenticated at the Botanical Survey of India (BSI), Eastern Regional Centre, Shillong, Meghalaya, India as *H. sibthorpioides* (BSI/ERC/Tech./Plant Iden./2018/115 dt. 22.05.2018) and *C. papaya* (BSI/ERC/Tech./Plant Iden./2018/114 dt. 22.05.2018). The herbarium specimen was deposited in the Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh.

Drying of the plant materials

The leaves of *H. sibthorpioides* and *C. papaya* were shade dried at room temperature until it was dried entirely to fit for grinding. The plant materials were kept away from direct sunlight during drying to protect the phytoconstituents from degradation²⁹. Then the leaves were grinded to a moderately coarse powder by using a mechanical mixture grinder. The grinding process assists the penetration of the solvent to the cellular structure of the plant tissues, which dissolves the secondary metabolites and increases the yields of extraction.

Collection of menstruum for extraction

In the present experiment, goat urine "*Ajamutra*" was used as a solvent for extraction. Goat urine was collected from two healthy female goats per day at the rate of approximately 600 mL in the morning hours in February 2018. Approximately 5 L of urine was collected and stored in an airtight glass container away from the sunlight.

Photo-activation of the solvent

The goat urine was photo activated by keeping in sunlight for about 72 h in a transparent glass

container. The photo-activation of urine was indicated by colour changes from yellowish to brown^{30,31}. The bactericidal property may be raised in photo-activated urine due to the acidic nature of the photo-activated urine compared to raw urine²⁶. Then, the goat urine was strained out to remove debris.

Extraction

The extraction of the leave extracts was performed by using the maceration technique. The basis of the extraction procedure was to obtain a therapeutically desirable portion and eliminate the inert insoluble material by treating a selective solvent known as the menstruum. The menstruum plays a crucial role in the qualitative and quantitative composition of the extract³². Moderately coarse powdered leaves of *H. sibthorpioides* and *C. papaya* were placed in the maceration chamber and macerated for seven days with raw goat urine (rGUHS and rGUCP) and photoactivated goat urine (paGUHS and paGUCP) with occasional shaking. The menstruum was strained off from the mare. The mare was pressed to recover the filtrate. The strained and expressed liquids were mixed and clarified by filtration. The amount of recovered filtrate was measured. The filtrate was evaporated to concentrate the crude extract. The concentrated crude extracts were stored in an airtight container in the refrigerator for further use.

Test microorganism

Saprophytic, rapidly growing, non-pathogenic mycobacteria; *M. smegmatis* strain was used as test organisms in the primary screening process.

Antimycobacterial susceptibility test

The antimycobacterial susceptibility test is performed by the determination of the zone of inhibition by the disc-diffusion method. It is the prescribed procedure applied in microbiology laboratories for regular antimicrobial susceptibility testing. In this method, the test microorganism's standardized 1×10^8 bacterial/mL (equivalent to 0.5 McFarland) inoculum is inoculated at agar plates. Then, about 6 mm (in diameter) filter paper discs are kept on the agar surface containing the test substance. The dishes are incubated in an incubator at a temperature of 37 ± 1 °C. The antimicrobial substance diffuses into the agar medium and subsequently inhibits the process of germination as well as the growth of the microorganism. Finally, the diameters of the zones of inhibition are measured. The antibiogram qualitatively separates the

microorganism as intermediate, susceptible, or resistant. The disc-diffusion method provides many advantages over other methods such as low cost, simplicity and the ability to test enormous antimicrobial agents^{33,34}.

In vitro antimycobacterial susceptibility assays

Muller-Hinton agar medium

About 38 g of Muller-Hinton agar was suspended in 1 L of distilled water and then boiled to dissolve the media completely. It was sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes and then allowed to cool to 50 °C. Then, 25 mL of freshly prepared medium was poured into flat bottomed sterilized Petri-plates, to give a uniform depth of approximately 4 mm. The mycobacterial strain was inoculated into the Petri-plates containing the media with the help of a sterilized inoculating loop. The plates were then incubated overnight at 37 ± 1 °C.

Preparation of working solutions

A set of three working solutions were prepared by dissolving each of the crude extracts in sterilized distilled water in a volumetric flask to obtain concentrations of 1000, 2000, and 3000 µg/mL. The solutions were filtered through a membrane filter to remove the impurities before the test.

Preparation of inoculums

The turbidity of the inoculums equivalent to 0.5 McFarland standard was used for the susceptibility test. The standard of 0.5 McFarland was prepared by adding 0.05 mL of Barium chloride dehydrate (1.175% w/v $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution in 9.95 mL of Sulphuric acid (1% v/v H_2SO_4) with constant stirring to obtain a suspension containing a Barium sulphate precipitate. The suspension of bacteria was compared visually with the prepared standard³⁵.

Determination of the zone of inhibition

The antimycobacterial sensitivity test was carried out by the paper disc-diffusion method²⁷. For determination of antimycobacterial activity, 25 mL of Muller-Hinton agar medium was poured into petri-plates and allowed to dry for 15 min. Three concentrations (1000, 2000, 3000 µg/mL) of each plant extract were prepared in 10% DMSO. About 100 µL of the inoculum was placed on the molten Muller-Hinton agar medium in petri-plates and spread throughout the plate by spread plate technique to

disperse the microorganisms homogeneously. The paper discs (6 mm diameter) were cut from Whatman filter paper No. 1 and were sterilized by autoclaving at 15 lbs (121 °C) for 15 minutes. The discs were impregnated in 10 µL above three concentrations of the rGUHS, paGUHS, rGUCP, paGUCP plant extract and kept aside for 15 minutes to dry. The extract-soaked filter paper disks were then placed on the surface uniformly seeded with test microorganisms of the Mueller-Hinton agar plates. Drug impregnated discs (Rifampicin of concentration 10 µg/mL and Berberine of concentration 25 µg/mL) were prepared by the same method. 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 measuring the diameter of the zone of inhibition using a transparent ruler under the colony counter. The test was performed in triplicate to minimize the errors and the average zones of inhibition (mm) were recorded³⁶.

Determination of MIC

Plant extracts (rGUHS, paGUHS, rGUCP, paGUCP) were used to determine Minimum Inhibitory Concentration (MIC) using the broth microdilution method³⁰. In this method, serial 5-fold dilutions of the plant extracts were prepared in the

10% DMSO. Bacterial inoculum was prepared in Mueller–Hinton broth, and the turbidity was adjusted to approximately 0.5 McFarland turbidity standards to prepare 1×10^8 bacterial/mL. the 96-well microtitre plates were used for the MIC assay. Exactly 150 µL of each plant extract was added to each well of the microplates. Exactly 50 µL of bacterial suspension was added to each well except the negative controls. Rifampicin and Berberine were used as the positive control. The 10% DMSO and plant extracts without bacterial suspension were used as the negative controls. The 96-well microtitre plates were incubated at 37 ± 1 °C for 24 h. This procedure was repeated twice for the *M. smegmatis* strain. The MIC endpoint for the *M. smegmatis* strain was assessed by measuring absorbance at 630 nm using a microplate reader as the lowest concentration of each plant extract at which there is no absorbance was regarded as MIC³⁶.

Results and Discussion

In ancient India, *H. sibthorpioides* and *C. papaya* have been traditionally utilized (*Rig-Veda*) and by Chinese people to cure several infections, including bacterial and viral infections^{37,38}. In the present study, we examined the *in vitro* antimycobacterial activity of the *H. sibthorpioides* and *C. papaya* leaves against the *M. smegmatis* strain. The zone of inhibition of both raw and photoactivated goat urine extracts are shown in Table 1 and Fig. 1 and 2.

Table 1 — Zone of inhibition of plant extracts against *Mycobacterium*

Name of the extracts	Zones of inhibition (mm)					
	Concentration of plant extract (µg/mL)			Goat urine	Berberine (25 µg/mL)	Rifampicin (10 µg/mL)
	1000	2000	3000			
1. rGUHS	8.33±0.47	9.66±0.43	10.66±0.47	8.41±0.47	14.74±0.47	16.41±0.47
2. phGUHS	9±0.43	10.33 ±0.57	12± 0.81			
3. rGUCP	10±1.73	11.33±0.48	12.67±0.94			
4. phGUCP	10.67±0.48	11.67±0.48	12.67±0.48			

*Values represented as mean±S.D. of replicates

Fig. 1 — a) Zone of inhibition of Goat urine, Standard drugs against *M. smegmatis*, b) Zone of inhibition of different concentration of plant extracts against *H. smegmatis*.

From the experiment, it was found that the zone of inhibition of raw goat urine extract of *H. sibthorpioides* in the concentrations of 1000, 2000, and 3000 µg/mL were 8.33 ± 0.47 , 9.66 ± 0.43 , and 10.66 ± 0.47 mm respectively. The zone of inhibition of photo activated goat urine extract of *H. sibthorpioides* in concentrations of 1000, 2000, and 3000 µg/mL were 9 ± 0.81 , 10.33 ± 0.57 , and 12 ± 0.81 mm respectively. The zone of inhibition of raw goat urine extract of *C. papaya* in concentrations of 1000, 2000, and 3000 µg/mL were found to be 10 ± 1.73 , 11.33 ± 0.48 , and 12.67 ± 0.94 mm respectively. The zone of inhibition of photo-activated goat urine extract of *C. papaya* in concentrations of 1000, 2000, and 3000 µg/mL were found to be 10.67 ± 0.48 , 11.67 ± 0.48 , and 12.67 ± 0.48 mm respectively. The raw goat urine inhibition zone was 8.41 ± 0.47 mm against *M. smegmatis* strain.

The MIC values obtained from plants that exhibited antibacterial activity ranged between 400 ± 1.6 to 660 ± 2 µg/mL against *M. smegmatis*

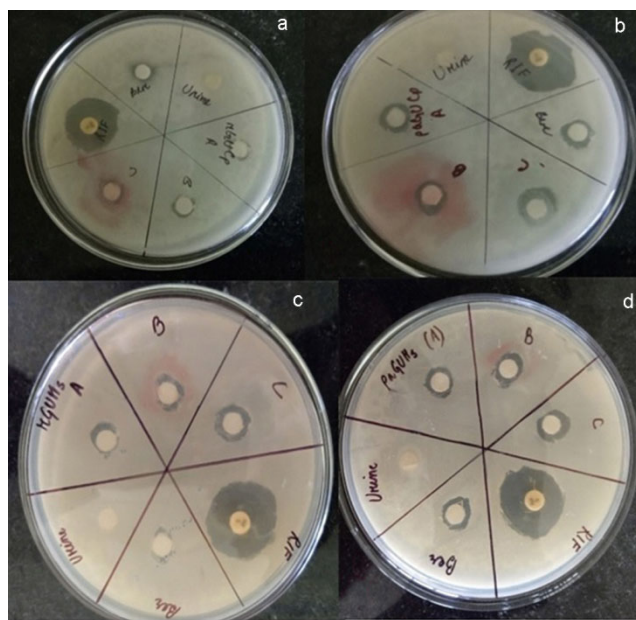


Fig. 2 — a) Antimycobacterial activity of raw goat urine extract of CP, b) Antimycobacterial activity of photoactivated goat urine extract of CP, c) Antimycobacterial activity of raw goat urine extract of HS, d) Antimycobacterial activity of photoactivated goat urine extract of HS.

(Table 2). Therefore, the highest antimicrobial activity was observed for the pHGUCP extract.

It was observed that leaf extracts of *H. sibthorpioides* and *C. papaya* were found to be effective in the antimycobacterial susceptibility tests. The experiment demonstrated that the extracts with photo-activated urine showed better antimycobacterial activity against *H. smegmatis* in comparison to the extracts with raw urine. Thus, both plants have a prominent antimycobacterial properties compared to the standard drugs Berberine and Rifampicin. The study also found that the antimycobacterial activity shown by both the plant materials containing goat urine as menstruum was higher than the activity demonstrated by raw goat urine. During the photo-activation process, volatile biogenic organic as well as inorganic compounds for instance acetone, methane, methanol, propanol, carbon dioxide, ammonia and secondary nitrogenous metabolic products are formed³⁹. Thus, urine becomes highly acidic in comparison to fresh urine and enhances the antimycobacterial efficacy of photo-activated urine⁴⁰.

C. papaya leaves are reported to show good antibacterial activity. Romasi and co-workers reported that ethyl-acetate extract have a significant zone of inhibition in comparison with ethanol extract of leaves of *C. papaya* against *Listeria monocytogenes* and *Bacillus stearothermophilus*⁴¹. The researchers found that the maximum zone of inhibitions was observed for the *C. papaya* leaves at acidic pH of 4. Francis and co-workers reported that aqueous and organic (petroleum benzene) extracts of leaves and seeds of *C. papaya* demonstrated antibacterial action against the most common disease-causing bacteria namely *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*³⁸. Kiran Peter and co-workers demonstrated that aqueous extract of *C. papaya* seeds exhibited a zone of inhibition against *S. aureus*, *P. aeruginosa*, and *E. coli* on increasing the concentration of seed extract⁴².

Handique *et al.* reported that methanolic extract of the whole plant of *H. sibthorpioides* showed significant antibacterial activity against both gram-negative and gram-positive bacteria [*Klebsiella*

Table 2 — MIC values of plant extracts against *M. smegmatis*

Name of bacterial strain	MIC (µg/mL) of the plant extracts			
	rGUHS	pHGUHS	rGUCP	pHGUCP
<i>M. smegmatis</i>	660±2	575±1.2	540±4	400±1.6

*Values represented as mean ± S.D of replicates

pneumonia (MTCC 432) and *Staphylococcus aureus* (MTCC-96)]¹⁸. Similarly, Mandal *et al.* showed that both methanol and aqueous extracts of *Hydrocotyle javanica* Thunb have a potential antibacterial activity against *Bacillus cereus*, *P. aeruginosa*, *L. monocytogenes*, and *S. aureus*⁴³.

In light of the different available literatures of *C. papaya* and *H. sibthorpioides* and the results of the present study, it can be concluded that these plants have effective antibacterial and antimycobacterial constituents. These phytoconstituents need to be identified and isolated to understand their activity better. Furthermore, from the study, it can be concluded that the photoactivated goat urine resulted in a synergistic effect of the extracts of *H. sibthorpioides* and *C. papaya* with the photoactivated urine and therefore improved the antimycobacterial efficacy of the plants.

Conclusion

In the present study, the antimycobacterial activity of the leaves of *H. sibthorpioides* and *C. papaya* was evaluated based on the ethnomedicinal claims of these plants as good antibacterial agents. The results showed that goat urine extract of *H. sibthorpioides* and *C. papaya* leaves have prominent activity against the *H. smegmatis* strain. The photo-activated goat urine showed significant activity in comparison to the extracts obtained using raw goat urine. The leaf extract of photo-activated goat urine extract of *C. papaya* at a 3000 µg/mL concentration showed the highest antimycobacterial activity compared to the other three extracts. In future, compounds having antimycobacterial activity can be isolated from the goat urine extracts of *H. sibthorpioides* and *C. papaya* using different analytical techniques. Furthermore, these compounds can also be studied for their effects on multidrug-resistant mycobacteria. Also, exhaustive experimentations should be carried out to understand the mechanism of antimycobacterial inhibition by *H. sibthorpioides* and *C. papaya* as well as goat urine.

Conflict of interest

The authors have no conflict of interest.

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