In vitro antibacterial potential of Hydrocotyle javanica Thunb.

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ABSTRACT

Objective: To explore the in vitro antimicrobial potential of Hydrocotyle javanica (H. javanica) Thunb. against human pathogenic bacteria and to standardize purification protocol to harvest the active principle in large scale.

Methods: In the present study, methanol and aqueous extracts of H. javanica were examined for in vitro antimicrobial potency against food poisoning, human pathogenic gastrointestinal and topical bacteria.

Results: The methanol and aqueous extracts yielded bioactive crude extracts that have antibacterial potentiality against the human pathogenic bacteria tested. The chemical constituents of the crude extracts showed the presence of alkaloids, flavonoids, phenols, tannins, leucoanthocyanidins and cardiac glycosides in the methanol extract, and alkaloids, saponins and triterpenoids in the water extract. Further solvent fractionation and purification of the crude extracts showed that the active antibacterial compounds were alkaloid in dichloromethane fraction and phenols in methanol fraction with a very strong bactericidal activity as evidenced by SEM and lactate dehydrogenase study.

Conclusions: Based on the findings it could be inferred that H. javanica would be a potential source for developing biotherapeutics against the human pathogenic bacteria. Further research work is going on in our laboratory to elucidate the structure of the active molecules.

1. Introduction

Hydrocotyle javanica Thunb. (H. javanica) (Apiaceae) is a naturally growing perennial herb in Darjeeling hills of Himalayan Range and distributed in several districts of Bhutan, Sikkim, and Darjeeling.[1] Traditionally, the fresh plant parts of H. javanica are used to crushed and ingested orally to cure sores of throats and lungs. Leaf juice is often used as eye drops to cure eye infection and leaf paste was used in dressing of wounds to reduce swelling and juice of shoots can treat gastritis and constipation. Several scientists have evaluated the phytochemical properties, and therapeutic potential of different species of the plant.[2] Matsushita et al.[3] and Yu et al.[4] studied the phytochemical properties and antitumor activity of the methanolic and ethanolic extract respectively of the whole plants of Hydrocotyle sibthorpioides (H. sibthorpioides) and reported that the antitumor activity of the extract is comparable to that of the common antitumor agent 5-fluorouracil while the methanolic extract of Hydrocotyle leucocephala was active in lipopolysaccharide induced cytokine production.[5] The study of in vitro antiproliferative activities of the ethanol and water extracts of four Hydrocotyle species from Taiwan showed that the water extract of Hydrocotyle nepalensis had the highest antiproliferative activity in the human hepatoma Hep3B cells with an IC50 of (435.88 ± 8.64) μg/mL while oleanane-type cytotoxic triterpenoidal saponin and hydrocosisaponins A-F (1–6), were characterized from H. sibthorpioides.[6–8] H. sibthorpioides was found to cure chronic alcohol-induced hepatic injury and fibrosis in rats.[9,10] Quamar and Bera (2014) reported that H. sibthorpioides L. is used as vegetable in Madhya Pradesh, India[11]. The whole plant is used for stomachic disorder and carminative...
and rice beer preparation[11]. The juice of the plant is used in the
treatment of fevers and paste is applied externally on wounds and boils.
The decoction of the whole plant is used in the treatment of abscesses,
boils, cirrhosis, cold, cough, hepatitis, itching, jaundice, sinusitis and
sore throat. While Hydrocotyle umbellata L. extract was reported to
have analgesic and anti-inflammatory, anti-oxidant and sedative effects
in mice[12,13]. Further, Hydrocotyle bonariensis (H. bonariensis)
leaves extracts were reported to have antioxidant and chemopreventive
potentiality effect against oxidative stress and galactose-induced
cataract[14]. The poultces of H. bonariensis Lam. are applied externally
to heal wounds, including freckles. Internally, its decoction is diuretic,
antiphlogistic, hepatic, against dropsy and pectoral. The juice is
emetic, diuretic, and useful against illnesses of lungs, liver, spleen
and bladder[15]. The leaf juice of Hydrocotyle mammii Kook.f. along
with Cynoglossum amphiloium is also used locally in cataract[16].

Hydrocotyle rotundifolia Roxb. leaf juice is taken to cure fever and
sometimes is used as brain tonic in Assam, India[17].

The leaves of H. javanica Thunb. are used in Akha’s traditional
medicine in China and Thailand as liniment on rashes[18]. A high
antioxidant level with genotoxic properties was reported from H. javanica[19] while 95% methanolic extract of H. javanica leaves
had antihemolytic and snake venom neutralizing effect against the
deadly Naja spp.[20]. The extract of H. javanica was also reported to
have larvicidal activity against Culex quinquefasciatus Say (Diptera:
Culicidae)[21]. While Lingaraju et al. (2013) reported that the whole
plant crushed in butter milk is taken orally to cure dysentery and the
leaves of this plant and leaves of Adiantum (a fern) crushed in butter
milk is taken orally to cure menorrhagia. Sometimes the whole plant
pounded with ash is used for fish poisoning[22].

From these earlier studies it is indicated that in comparison to
other species of Hydrocotyle, H. javanica is also a potent source of
different bioactive compounds. To the best of our knowledge, H. javanica
species of the genus Hydrocotyle remains unexplored for its
phytochemical compounds that have therapeutic potential for
antimicrobial activities to treat human pathogens.

In the present paper the major objectives are defined to evaluate the
phytochemical properties and in vitro antimicrobial activity, mode of
action for their therapeutic potential to treat bacterial pathogens of the
crude methanolic extract of H. javanica. Further research works are
going on to purify, characterize, and elucidate the structure of the active
principle.

2. Materials and methods
2.1. Plant collection and identification

The aerial plant parts of H. javanica Thunb. (A. plicata) were collected from Darjeeling hill areas of West Bengal, India (27°3’15.88” N and
88°15’28.10” E. elevation 2034 m) during the month of April-June, 2009 and also in subsequent years when only the vegetative parts
of the plants are available. The prepared herbarium specimen of the plant
was identified by Central National Herbarium, Indian Botanic Garden,
Shibpur, Kolkata, India and deposited there (voucher specimen No.
DGC/SP-02) (Figure 1).

Figure 1. The native plant, H. javanica in its habitat. (inset: the inflorescence
of H. javanica).

2.2. Extraction of active compounds

The fresh leaves along with their petioles (1 kg) of the above two
plants were dried at 60 °C in a hot-air oven for 3–4 days. The dried
leaves (300 g) were crushed into fine powder using mixer grinder.
The 100 g of powder drug was extracted in a Soxhlet extractor in 500
mL of 95% methanol and double distilled water separately at room
temperature (30 °C) for 72–96 h. Then the extracts were filtered using
Whatman No.1 filter paper. The pigments of the filtrate were removed
by running in activated charcoal column. The pigment free samples
were concentrated using rotary vacuum evaporator (Superfit Rotary
Vacuum Evaporator, R-150, Mumbai, India) at 40 °C. From the final
dry weight of 300 g of Hydrocotyle, 30 g of active methanol extract
of H. javanica was recovered. The active extracts of Hydrocotyle
were coded as HJ-ME and HJ-W, for methanol and water extracts,
respectively, and was kept aseptically at 4 °C until use.

2.3. Phytochemical tests to characterize the active constituents

Phytochemical tests for bioactive constituents such as glycosides,
saponins, anthraquinone derivatives, flavonoids, sterols, tannins,
alkaloids, triterpenoids, phenols and leucoanthocyanidins were carried
out on portion of residual material using standard phytochemical
procedures[23].

2.4. Microbial strains and antimicrobial activity

Human pathogenic bacteria, and plant and human pathogenic fungal
strains: Bacillus cereus MTCC 1272 (B. cereus); Bacillus megaterium
MTCC 1684 (B. megaterium); Escherichia coli MTCC 723 (E. coli);
Listeria monocytogenes MTCC 657 (L. monocytogenes); Pantoea
anaritii MTCC 2307 (P. anaritii); Pseudomonas aeruginosa MTCC 741
(P. aeruginosa); Salmonella typhimurium MTCC 98 (S. typhimurium);
Staphylococcus aureus MTCC 96 (S. aureus); Staphylococcus
erpidermidis MTCC 3086 (S. epidermidis); Streptococcus mutans MTCC
890 (S. mutans) were procured from Microbial Type Culture Collection,
IMTECH, Chandigarh, India, and cultured in respective media. The
stock culture was maintained in 70% glycerol at -20 °C. The strains

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were subcultured twice before experimentation. Antimicrobial activity of *H. javanica* extract was tested by agar well diffusion assay against a number of human pathogenic and food spoilage bacteria and plant and human pathogenic fungal strains following the method of Mandal et al.[24] published earlier. For antibacterial assay respective bacterial strains were grown overnight and inoculated in melted trypticase soy broth or nutrient broth soft agar (0.7%) at 1% and spread on the pre-poured trypticase soy broth or nutrient broth plates. Agar wells were cut by sterile cork borer of 5 mm diameter and 30 µL of the extract of different concentrations ranging from 32.40 mg/mL to 1.26 mg/mL was loaded into each well. The plates were incubated at 37 °C for 24–48 h and observed for growth inhibition. For antifungal assay the fungal strains were grown in malt extract slants for 7–10 days and incubated at 28 °C till their sporulation. Spore suspension was prepared by scrapping the spores in 0.2% peptone water at a concentration of 10^6 spores/mL and used as fungal inocula. Fungal strain was inoculated in melted malt extract soft agar (0.7%) at 1% and spread on the pre-poured malt extract agar plate. Agar wells were cut and different concentrations of active extracts were loaded into each well as previously described. The plates were incubated at 28 °C for 48 h and observed for growth inhibition. The potency of inhibition was measured as diameter of inhibition zones against the negative control of pure solvent (methanol) and positive control of antibiotic oxytetracycline (100 µg/mL) for bacterial strains and griseofulvin (100 µg/mL) for fungal strains, respectively.

### 2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC is defined as the lowest concentration of the active extract treatment where no visible growth is observed. MIC was determined by agar well diffusion assay as described previously[24,25]. Different concentration of the plant extract was prepared by serially diluting the stock concentrate, with their respective solvent used in the extraction process and in the range of concentration from 324 mg/mL to 1.26 mg/mL. The treated plates were incubated at 37 °C for 24 h and observed for inhibition zone. No inhibition zone was observed at the least concentration noted as the MIC value. The MBC was defined as the minimum concentration of active principle treatment that produced no viable cells. The number of viable cells (CFU/mL) was determined by incubating the respective strains in trypticase soy broth for 12 h in 37 °C. The growth and CFU were measured by optical density 620 nm and by serial dilution, respectively. The active principle was added (at higher concentration of MIC value) and incubated for next 6 h. After that the optical density 620 nm and CFU were measured in both control and treated sets. A total of 100 µL of the diluted sample was spread on trypticase soy broth agar plate and incubated at 37 °C for 24 h. The number of colonies was counted from treated and untreated plates and log of CFU/mL was calculated.

### 2.6. Mode of antimicrobial action on sensitive bacterial cells

#### 2.6.1. Effect of the crude extract on growth and viability of bacterial strains

The effect of bioactive compounds on sensitive pathogenic bacteria was evaluated by using partially purified and crude extract of *HJ-ME* and measuring the growth and viability on cellular integrity of the sensitive bacterial strains.

A crude compound (crude and partially purified, 100 mg/mL each) was mixed with exponentially growing cells of *L. monocytogenes* and *P. aeruginosa* and were incubated at 37 °C for 24 h. The growth was monitored as optical density 620 nm and viability was evaluated by plate count method at every 3 h interval. For plate count method the treated and untreated cells were serially diluted and 100 µL of the dilution (10–4 to 10–6) was plated. The plates were incubated at 37 °C and the viable colonies (CFU) were counted from respective treatment plates and untreated plates and log of CFU/mL was calculated. The culture without treatment was treated as negative control.

#### 2.6.2. Effect of the crude extract on cellular integrity

The effect of active crude constituents on cellular integrity was studied as the amount of lactate dehydrogenase (LDH) activity in the cell-free lysate. For LDH activity study, the active growing cells were harvested by centrifugation at 6000 g for 5 min at 4 °C and washed with sterile water and centrifuged. Cell pellets were suspended in Tris-HCl buffer (50 mmol/L, pH 7.5). The bacterial suspension was divided into three parts. One part was treated with sonication (100% amplitude, at 0.9 cycles for 5 min) (Labsonic-M, Sartorius). Another part was treated with plant extract (partially purified and crude, 100 mg/mL each) and remaining one was treated as untreated and incubated at 37 °C for 5 h. Cell free supernatant, which was treated as enzyme source, was collected at 1 h, 3 h and 5 h of incubation time and the amount of LDH released was measured following the method of Mandal et al.[24] with little modification. The amount of LDH was estimated by measuring the rate of reduced nicotinamide adenine dinucleotide formed. The amount of nicotinamide adenine dinucleotide formed was measured by estimating the samples in a spectrophotometer (UV-vis 1700 Spectrophotometer, Shimadzu, Japan) at 340 nm and plotting a standard curve of absorbance vs. concentration of nicotinamide adenine dinucleotide (standard, mmol/L). The standard assay mixture contained (in a total volume of 3 mL): sodium lactate (1 mL of 10 mmol/L), nicotinamide adenine dinucleotide (150 µL of 20 mmol/L), Tris-HCl (1.75 mL of 50 mmol/L, pH 7.5) and 100 µL enzyme. The reaction was incubated for 5 min and absorbance was measured at 340 nm. One unit of LDH was calculated as 1 µmol of nicotinamide adenine dinucleotide reduction per min.

#### 2.7. Partial purification and characterization of the active fraction

As the water extract of the plant is very much prone to fungal growth, even in the refrigerated condition, therefore, purification study was conducted only from the methanol extract, which is stable at room temperature (30 °C) for more than six months. For purification of active ingredients, the pigment free active extract was concentrated to dryness in a rotary vacuum evaporator (Superfit Rotary Vacuum Evaporator, R-150, Mumbai, India) and fractionated by dichloromethane, repeatedly, to harvest the alkaloid fraction and the residue was redissolved in methanol. The active fractions were then air dried and
2.8. Effect of the active purified extract on cell morphology

The effect of the active fractions of the extract was studied by SEM. To study the effect of the active dichloromethane and methanol fractions of the methanol extract, 500 µL of each of the active fractions (which contained approximately 0.06 µg of dichloromethane fraction and 15.0 µg of methanol fraction) was taken in a sterile Eppendorf tubes and evaporated to dryness under laminar air flow, to exclude the solvent effect on microbial morphology. Then 100 µL of the exponentially growing bacterial culture of _L. monocytogenes_ was added to Eppendorf tubes containing active compound, and incubated at 37 °C for 1 h. The untreated and treated cells were harvested by centrifugation at 8000 r/min for 10 min at 4 °C and washed twice with sterile sodium-phosphate buffer (100 mmol/L, pH 7.0). The pellet was then re-suspended in the same buffer, a drop of the cell suspension was placed on a coverslip and air dried. The samples were processed for SEM (EVO 18, Zeiss, Inca Penta FETX3, Oxford, England with Q150R ES, Quorum gold sputter) following our standardized protocol as represented schematically in Figure 2. This study was conducted from the Centre for Research in Nanoscience and Nanotechnology, University of Calcutta, Kolkata, West Bengal, India.

Protocol for SEM study

| Protocol for SEM study | Description |
|------------------------|-------------|
| Overnight grown/freshly grown cells | Resuspend the pellet in sterile 100 mmol/L sodium-phosphate buffer, pH 7.2 by gentle inversion |
| Centrifuge at 6000 r/min for 10 min | A gain centrifuge at 6000 r/min for 10 min |
| Resuspend the pellet in the same buffer in such a dilution so that the optical density will be 0.02 to 0.2 (as estimated visually) | Place a drop (10 µL) of cell suspension on cover glass/0.5 cm × 0.5 cm slide piece |
| Air dry properly (improper drying may wash out the cell smear in subsequent stages) | Dehydrate the smear with series of acetone/ethanol grades, starting with 50%, 70%, 80%, and 100%, 10 min in each step |
| Prefix the cells by sterile 0.5% glutaraldehyde in 100 mmol/L sodium-phosphate buffer, pH 7.2 for 5–10 min | Postfix with 1% osmium tetroxide in 100 mmol/L sodium-phosphate buffer, pH 7.2 for 5–10 min (don’t allow the layer to dry) |
| Wash glutaraldehyde layer with the same buffer 2 to 3 times | Wash the osmium tetroxide layer with the same buffer 2 to 3 times |
| Dehydrate the smear with series of acetone/ethanol grades, starting with 30% and then 50%, 70%, 80%, and 100%, 10 min in each step | Dehydrate the smear with series of acetone/ethanol grades, starting with 30% and then 50%, 70%, 80%, and 100%, 10 min in each step |
| Sample is ready for SEM study | Place a drop (10 µL) of the each of the active fractions (which contained approximately 0.06 µg of dichloromethane fraction and 15.0 µg of methanol fraction) was taken in a sterile Eppendorf tubes and evaporated to dryness under laminar air flow, to exclude the solvent effect on microbial morphology. Then 100 µL of the exponentially growing bacterial culture of _L. monocytogenes_ was added to Eppendorf tubes containing active compound, and incubated at 37 °C for 1 h. The untreated and treated cells were harvested by centrifugation at 8000 r/min for 10 min at 4 °C and washed twice with sterile sodium-phosphate buffer (100 mmol/L, pH 7.0). The pellet was then re-suspended in the same buffer, a drop of the cell suspension was placed on a coverslip and air dried. The samples were processed for SEM (EVO 18, Zeiss, Inca Penta FETX3, Oxford, England with Q150R ES, Quorum gold sputter) following our standardized protocol as represented schematically in Figure 2. This study was conducted from the Centre for Research in Nanoscience and Nanotechnology, University of Calcutta, Kolkata, West Bengal, India. |

2.9. Analysis of the active purified extract by high performance liquid chromatography (HPLC), thin-layer chromatography and UV-vis spectroscopy

The crude pigment free methanol extract of the plant was further analyzed in HPLC system from Central Drug Research Institute, Lucknow, India. The study conditions were as follows: run time: 30 min; inject volume: 5.0 µL; column: photodiode array 281.1 nm and analyzed in Empower 2 software. The solvent fractionated purified compounds were run on pre-coated thin-layer chromatography silica gel 60 F254 with solvent system absolute methanol and observed under UV-254 nm chamber to check the purity of the factions. UV-vis spectrum study of the purified compounds was done with 95% ethanol in a spectrophotometer (LI-295, UV-vis spectrophotometer, Lassany, Haryana, India) and observed for absorbance peak.

2.10. Statistical analysis

Antimicrobial activities was calculated by mean = SE. The least significant data were calculated to justify the observed values. Differences at _P_ < 0.05 were considered to be significant.

3. Results

3.1. Preliminary screening for secondary metabolites in the plant extracts

Phytochemical screening showed the presence of alkaloids, cardiac glycosides, flavonoids, leucoanthocyanidins and phenols in the methanol extract while the water extract showed the presence of alkaloids, cardiac glycosides, phenols, saponins, sterols, and triterpenoids as shown in Table 1.

3.2. Antimicrobial activity of the crude extract

_In vitro_ antibacterial and antifungal assay of the methanol extract of _H. javanica_ (324 mg/mL) was carried out by agar well diffusion method as shown in Table 2 and Figure 3. It was observed that the methanol and water extracts had wide antibacterial activity against both Gram-positive and Gram-negative bacterial strains under study. No activity was detected against the fungal strains. The antimicrobial activity (the diameter of inhibition zones) of the plant extracts was comparable to standard antibacterial antibiotic, oxytetracycline at a concentration of 100 µg/mL. The above spectrum of antibacterial activity showed that the methanol and water extracts contained some active constituents that could be used against food spoilage, gastroenteritis and topical bacterial pathogens.
Table 1
Phytochemical screening of crude methanol and water extracts of *H. javanica*.

| Chemical tests | Observations | Inferences |
|----------------|--------------|------------|
| Fehling’s test for reducing sugars (in glycosides) | Presence of brick-red precipitate | Present |
| Frothing test for saponins | Formation of thick persistent froth | Absent |
| Test for phenol | Formation of a red, blue, green, or purple coloration | Present |
| Borntrager’s test for anthraquinone derivatives | Formation of pink-red colour in the ammoniacal (lower) layer | Absent |
| Test for flavonoids | Formation of yellow precipitate | Present |
| Test for sterol | Formation of brownish red color | Absent |
| Test for glucosides (Molisch’s test) | Formation of red precipitate | Present |
| Ferric chloride solution test for tannins | Formation of green colour was formed in the extract | Absent |
| Colour tests for alkaloids (Wagner’s test) | Formation of reddish brown precipitate | Present |
| Test for leucoanthocyanins | A reddish color was formed in the extract | Absent |
| Test for cardiac glycosides (Keller-Kiliani test) | A brown ring at inter-phase was present | Present |
| Test for triterpenoids (Liebermann-Burchard test) | Presence of brown ring at the junction | Absent |

Table 2
Spectrum of antimicrobial activity of crude active extracts of *H. javanica* against the bacterial and fungal strains.

| Strains | Strain properties | Growth conditions | *H. javanica* extracts | Antibiotics* (100 µg/mL) |
|---------|------------------|------------------|-----------------------|-------------------------|
| Bacterial strains | Pathogen of food borne illnesses | TSB, 37 °C | 15.000 ± 0.400* | 19.000 ± 0.240 | 20.000 ± 0.098 |
| *B. cereus* | Heat resistant strain | TSB, 37 °C | 10.000 ± 0.100 | 13.500 ± 0.360 | 20.000 ± 0.060 |
| *B. megaterium* | GP, colicin indicator strain | TSB, 37 °C | 17.000 ± 0.240 | 23.050 ± 0.370 | 22.000 ± 0.141 |
| *E. coli* | HPF borne pathogen causing listeriosis | BHI, 37 °C | 16.000 ± 0.000* | 13.000 ± 0.110 | 17.000 ± 0.144 |
| *L. monocytogenes* | Food borne pathogen | TSB, 37 °C | 10.000 ± 0.950 | 15.200 ± 0.370 | 20.000 ± 0.080 |
| *P. aeruginosa* | Skin and lung infection pathogen | TSB, 37 °C | 15.000 ± 0.138 | 20.000 ± 0.250 | 20.000 ± 0.130 |
| *S. typhimurium* | Gastrointestinal pathogenic protothecosis strain | TSB, 37 °C | 20.000 ± 0.143 | 26.300 ± 0.260 | 20.000 ± 0.193 |
| *S. aureus* | Pathogenic, antibiotic sensitive strain | TSB, 37 °C | 18.000 ± 0.124 | 25.000 ± 0.270 | 20.000 ± 0.130 |
| *S. epidermidis* | Air borne skin infecting pathogen | TSB, 37 °C | 10.000 ± 0.220 | 19.000 ± 0.133 | 22.000 ± 0.240 |
| *S. mutans* | Dental caries pathogen | BHI, 37 °C | 15.000 ± 0.167 | 20.000 ± 0.150 | 20.000 ± 0.150 |
| Fungal strains | A flavotxin producer | ME, 28 °C | 0.000 ± 0.000 | 0.000 ± 0.000 | 17.300 ± 0.220 |
| *A. parasitica* | Pathogenic to human | ME, 28 °C | 0.000 ± 0.000 | 0.000 ± 0.000 | 15.800 ± 0.150 |
| *C. albicans* 3018 | Pathogen of brown spot of rice | ME, 28 °C | 0.000 ± 0.000 | 0.000 ± 0.000 | 28.000 ± 0.180 |
| *F. oxysporum f. sp. pisi* MTCC 2480 | Pathogen of *Fusarium* wilt of pea | ME, 28 °C | 0.000 ± 0.000 | 0.000 ± 0.000 | 18.000 ± 0.180 |

3.3. Determination of MIC, MBC and mode of action of the active crude compound

For MIC and MBC study in the *H. javanica* extracts, the methanol extract was used as shown in Table 3, Figure 4 and Table 4, respectively. The results showed that MIC and MBC were in the range from 2.53 to 81.00 mg/mL and 5.06 to 81.00 mg/mL, respectively. The results of this study were indicative of possible pure active principle of natural origin from that extract with possible high potency which could serve as chemotherapeutic agents against the bacterial pathogens. The effect of bioactive compound of methanol extract on growth and viability was determined against human pathogenic bacteria *L. monocytogenes* and *P. aeruginosa* as shown in Figure 5. From this study it was observed that after 6 h of incubation, when methanol extract was administered in actively growing *Listeria* and *Pseudomonas* cells, the growth and viability decreased simultaneously with incubation period. It was also observed that the rapid decrease in growth and viability of treated bacterial cells indicated that the active compounds had bactericidal mode of action[24]. The results obtained were also statistically very significant at $P = 0.005$ ($df = 6$) for the methanol extract effect on the *L. monocytogenes* and *P. aeruginosa* growth and viability. The effect of active constituents of methanol extracts on cellular integrity was measured by LDH assay (Figure 6). LDH is an important cytoplasmic enzyme of living cells. So the presence of LDH in extracellular

Table 3
Diameter of inhibition zone of crude active methanol extracts of *H. javanica* at different concentrations against the pathogenic bacteria strains, mm.

| Concentration (mg/mL) | B. cereus | B. megaterium | E. coli | L. monocytogenes | P. ananetis | P. aeruginosa | S. typhimurium | S. aureus | S. epidermidis | S. mutans |
|-----------------------|-----------|--------------|--------|-----------------|------------|--------------|----------------|---------|---------------|---------|
| 32.40                 | 15.00     | 10.00        | 14.00  | 15.00           | 20.00      | 9.00          | 13.00          | 9.00    | 8.80          | 9.00    |
| 16.00                 | 13.00     | 8.00         | 10.00  | 9.50            | 19.00      | 8.20          | 11.00          | 8.50    | 8.00          | 8.00    |
| 8.00                  | 11.00     | 7.50         | 8.00   | 9.00            | 18.00      | 7.00          | 9.00           | 7.30    | 7.50          | 7.30    |
| 4.05                  | 9.00      | 6.20         | 5.50   | 8.00            | 16.00      | 6.50          | 6.50           | -       | 7.00          | 6.80    |
| 2.05                  | 0.70      | -            | -      | -               | 13.50      | -             | -              | 5.70    | -             | 6.00    |
| 1.00                  | -         | -            | -      | -               | 13.00      | -             | -              | -       | -             | 5.80    |
| 0.60                  | -         | -            | -      | -               | 10.00      | -             | -              | -       | -             | -       |
| 0.53                  | -         | -            | -      | -               | 7.00       | -             | -              | -       | -             | -       |
| 0.26                  | -         | -            | -      | -               | -          | -             | -              | -       | -             | -       |

The data were the average diameter of inhibition zone of triplicate trials; -: No inhibition zone.
supernatant was an indicative of loss of cellular integrity. From this experiment it was found that addition of active constituents into bacterial cells did not increase the LDH activity upto 1 h treatment compared to sonicated supernatant but after 3 h and at 5 h of treatment its activity had increased sharply indicating their drastic lytic activity. The growth and viability experiment also support this observation.

**Figure 4.** MIC of the crude active extracts against different human pathogenic bacteria by agar well diffusion method against *P. ananetis*.

1: 324.00 mg/mL; 2: 162.00 mg/mL; 3: 81.00 mg/mL; 4: 40.50 mg/mL; 5: 20.25 mg/mL; 6: 10.12 mg/mL; 7: 5.06 mg/mL; 8: 2.53 mg/mL; C: Control (methanol).

**Figure 5.** Effects of crude active extract (methanol, 100 mg/mL) on growth and viability of *L. monocytogenes* and *P. aeruginosa*. Here solid lines indicated untreated control and dotted lines indicated treated with active extract. Square and star for growth and triangle and circle for CFU of *Listeria* and *Pseudomonas*, respectively.

**Figure 6.** LDH activity of *L. monocytogenes* and *P. aeruginosa* induced by the crude active constituent (methanol, 100 mg/mL).

Results shown here were the average of triplicate trials (± SE). Each point represented the mean of triplicate trials and error bars represented standard errors; PH: Purified extract; CH: Crude extract; Ps: *Pseudomonas*; List: *Listeria*.

### 3.4. Purification and effect of the active purified fractions on the cell morphology

The solvent fractionation and subsequent phytochemical test of the active methanol extract showed the presence of alkaloid and phenol compounds in the dichloromethane and the methanol fractions, respectively. The effect of the active fractions (dichloromethane and methanol) on the morphology of *L. monocytogenes* showed in the Figure 7. A drastic change in the morphology, like formation of blebbing, notch and rupture of entire cell walls, entire dissolution of cell integrity were observed in the treated cells of both fractions (Figures 7B to 7F) than the untreated one (Figure 7A). Further investigation is going on in our laboratory to pin-point the exact mode of action of the active compounds.

### 3.5. Chromatographic and spectrophotometric analysis of the active fractions

The HPLC analysis of the crude pigment free active plant extract (methanol) showed the presence of three major components with retention time 3.635 (peak area 45.49%), 4.412 peak area 9.66%) and 15.022 peak area 38.06%) as shown in Figure 8. This observation was in accordance with the chemical tests performed for chemical characterization of the active extract. Thin-layer chromatography of the active fraction of dichloromethane and methanol showed one prominent band under UV254 with *Rf* values 0.68 and 0.307, respectively. UV-vis spectra showed a prominent peak (λ<sub>max</sub>) at 285.7 nm and 238.5 nm for dichloromethane fraction and 218.0 nm for methanol fraction (Figure 9).
Figure 7. SEM of the active fractions (methanol and dichloromethane) of methanolic extract of *H. javanica* Thunb. against *L. monocytogenes*. Schematic representation of the SEM protocol, as standardized by our laboratory.

Figure 8. HPLC analysis of the crude methanol extract of *H. javanica*.

Figure 9. UV-vis study of the active fractions (methanol and dichloromethane) of *H. javanica*. Blue line: The dichloromethane fraction; Pink line: The methanol fraction of the methanol extract of *H. javanica*.
4. Discussion

4.1. Phytochemical screening

The phytochemical screening of the methanol and water extracts of *H. javanica* showed the presence of the alkaloid, cardiac glycosides, flavonoids, leucoanthocyanidins, phenols, saponins, sterols and triterpenoids (Table 1) many of which have been reported to have health protective efficacy. Saponins, antheraquione and terpenoids were reported to be present in the methanol fraction in other species of *Hydrocotyle*.[13-17]

4.2. Spectrum and mode of antimicrobial activity of the extract

In vitro antibacterial study showed that the methanol and water extracts of *H. javanica* had wide antibacterial activity against both Gram-positive and Gram-negative bacterial strains under study. No antifungal activity was detected. The above spectrum of antibacterial activity showed that the methanol and water extracts contained some potent antibacterial compounds that could be used against bacterial pathogens associated with gastroenteritis, topical and also food spoilage. Sood and Yadav[26] also reported that *H. javanica* showed the highest inhibitory activity against *B. cereus* MTCC 441 (activity index = 0.63), *E. coli* MTCC 739 (activity index = 0.45), Salmonella enterica MTCC 3219 (activity index = 0.44), and *P. aeruginosa* MTCC 1688 (activity index = 0.53), *S. epidermidis* MTCC 435 (activity index = 0.37) than *Hydrocotyle rotundifolia* Roxb. In a study by Eduardo et al[18] showed that the dichloromethane extract of *H. bonariensis* had antibacterial activity against *Bacillus subtilis* ATCC 6633 while it was negative for *P. aeruginosa* ATCC 27853, *S. aureus* (MRSA) (clinical strain), *S. aureus* ATCC 25923 and *E. coli* ATCC 25422. Hugo and Rusell[25] reported that “with most bactericidal antimicrobials, the MIC and MBC are often near or equal in value” as has typically been observed in this study. The effect of bioactive compound of methanol extract of *H. javanica* on growth and viability was determined against human pathogenic bacteria *L. monocytogenes* and *P. aeruginosa* MTCC 741 showed that the growth and viability decreased simultaneously with incubation period. The rapid decrease in growth and viability of treated bacterial cells indicated that the active compounds had a very strong bactericidal mode of action.[24] The LDH assay and the SEM study proved the loss of cellular integrity due to active compounds which are alkaloid and phenol in nature, in the dichloromethane and methanol fractions, respectively. Among these active constituents, the alkaloid is more potent than the phenol constituent, as the dose of treatment alkaloid compound was much lower than the phenol compound. Though our study did not show any inhibitory activity against the fungal pathogens (molds), in a recent study by Sood and Yadav[26] showed that the water extract of *H. javanica* is inhibitory to *Candida albicans* MTCC 3017, which also justifies its applicability in fungal diseases of human.

4.3. Spectroscopic and chromatographic characterization of the active compound

The HPLC analysis of the active plant extract (methanol extract of *H. javanica*) correlates with the observation of the chemical tests performed for chemical characterization. Thin-layer chromatography and the UV-vis spectra correlate the degree of purity in the purification strategy and the compounds are also assumed to be alkaloid and phenol in dichloromethane and methanol fractions, respectively. Though different species of *Hydrocotyle* have been evaluated for their potential phytochemical biotherapeutics, but there is a scarcity in vivid phytochemical characterization of metabolites from different species of *Hydrocotyle*. Recently Maulidiani et al[27] reported that two species of *Hydrocotyle*, viz. *H. bonariensis* and *H. sibthorpioides* contained oleanane-type triterpenes (like *Hydrocotyloside* I-III, *Ranuncuside* I-Ⅴ, *Barrigenol* derivative), phenolics (like chlorogenic acid, dehydrotriferulic acid) and flavonoids (like quercetin retinoside, quercetin 3-O-glucoside, and other similar compounds). But there were no report of the presence of alkaloids in the species of *Hydrocotyle*. The active dichloromethane fraction, which is an alkaloid in nature, might be the new compound to be reported from *H. javanica*. Further research works like 1R, 1D and 2-D nuclear magnetic resonance (1H and 13C), gas chromatography–mass spectrometry and tandem mass spectrometry are going on in our laboratory to characterize the active principles.

In conclusion it can be stated that *H. javanica* Thunb. contained some bioactive active principles mainly alkaloid and phenols in the methanol extract that have strong antibacterial efficiencies that can be exploited to treat against gastrointestinal bacterial pathogens and also some skin infecting bacterial pathogens. *S. aureus* is a polygenic invasive skin infecting bacterium responsible for superficial and deep follicular lesson. The prevalence of *S. aureus* strains resistant to conventional antibiotics has increased to high levels of some hospitals[28,29]. The methanol extract of *H. javanica* could serve as a remedy to such resistance folklorically. The methanol and water extracts of *H. javanica* also had showed high level of activity against *B. cereus* which is responsible for food borne illnesses (2%-5%), causing severe nausea, vomiting and diarrhea. It also causes keratitis, a condition in which the eye’s cornea becomes inflamed[30]. The methanol and water extracts of *H. javanica* also could be used as a possible drug for such diseases. The methanol and water extracts of *H. javanica* showed high level of activity against *L. monocytogenes* and *P. aeruginosa*. *L. monocytogenes* which is a common contaminant in raw meat, dairy products, vegetables, and sea foods, is well-known human pathogenic food borne pathogen causing listeriosis. *P. aeruginosa* is skin and lung infecting opportunistic pathogen in immune-compromised individuals and typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections. *Pseudomonas* can also cause community-acquired pneumonias, as well as ventilator-associated pneumonias. The methanol and water extracts of *H. javanica* also could serve as a potential drug for these two human pathogenic bacteria. Thus, the active principles of the plant, which had been used as folk medicine for years, had tremendous potential to solve many deadly pathogenic bacterial invasions and infections in man and could substitute conventional antibiotic use. The elucidation of the structures of the active compounds, chemical synthesis and vivid mode of action would explore better application strategies in human endeavor.
Conflict of interest statement

We declare that we have no conflict of interest.

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