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 *Hhydrocotyle nepalensis* had the highest antiproliferative activity in the human hepatoma Hep3B cells with an IC_{50} 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[8]. Recently, the *in vitro* and *in vivo* antiviral properties of *H. sibthorpioides* against hepatitis B virus and dengue virus replication have been demonstrated[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

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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, anxiolytic 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 poultices 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 mannii* Kook.f. along with *Cynoglossum amplifolium* 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. (Apiaceae)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 ananetis* MTCC 2307 (*P. ananetis*); *Pseudomonas aeruginosa* MTCC 741 (*P. aeruginosa*); *Salmonella typhimurium* MTCC 98 (*S. typhimurium*); *Staphylococcus aureus* MTCC 96 (*S. aureus*); *Staphylococcus epidermidis* MTCC 3086 (*S. epidermidis*); *Streptococcus mutans* MTCC 890 (*S. mutans*) were procured from Microbial Type Culture Collection, IMTECH, Chandigargh, India, and cultured in respective media. The stock culture was maintained in 70% glycerol at -20 °C. The strains

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 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 324.00 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 10^4 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 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.

Active 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 6 000 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 control 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 in 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 re-dissolved in methanol. The active fractions were then air dried and

tested for chromatographic analysis and biological studies.

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 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 8 000 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 cover glass 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.
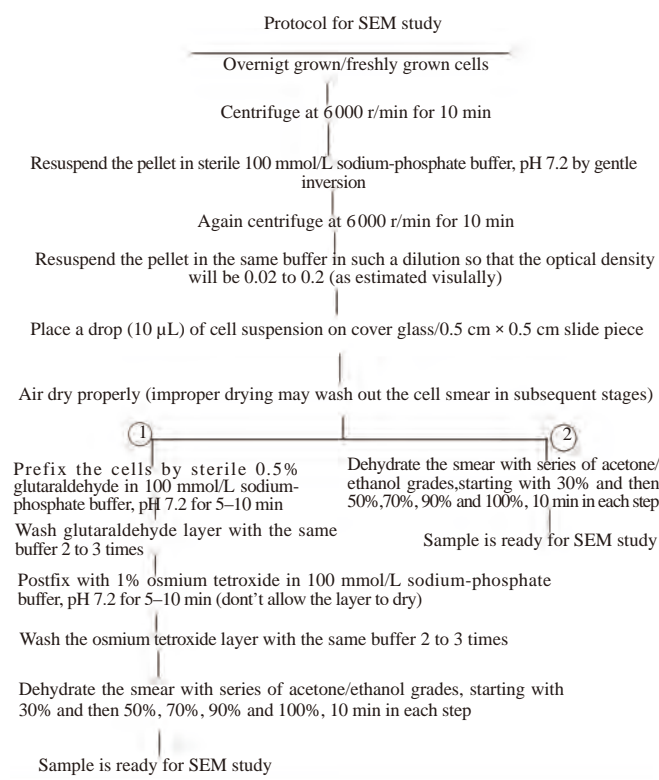
Protocol for SEM study

Overnigt grown/freshly grown cells

Centrifuge at 6 000 r/min for 10 min

Resuspend the pellet in sterile 100 mmol/L sodium-phosphate buffer, pH 7.2 by gentle inversion

Again centrifuge at 6 000 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)

① Prefix the cells by sterile 0.5% glutaraldehyde in 100 mmol/L sodium-phosphate buffer, pH 7.2 for 5–10 min

Wash glutaraldehyde layer with the same buffer 2 to 3 times

Postfix with 1% osmium tetroxide in 100 mmol/L sodium-phosphate buffer, pH 7.2 for 5–10 min (dont't allow the layer to dry)

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%, 90% and 100%, 10 min in each step

Sample is ready for SEM study

② Dehydrate the smear with series of acetone/ethanol grades,starting with 30% and then 50%,70%, 90% and 100%, 10 min in each step

Sample is ready for SEM study

Figure 2. Protocol for SEM study.

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 F_{254} 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.
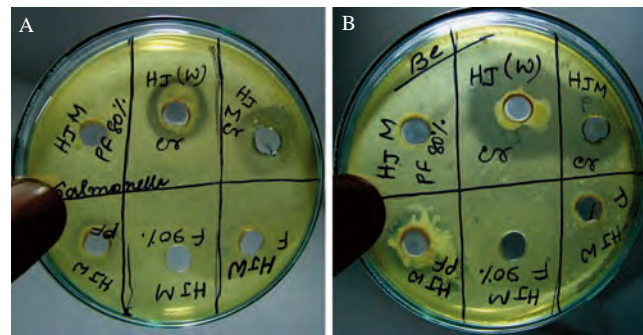

Figure 3. Antibacterial activity of methanol extracts and water extracts against *S. typhimurium* MTCC 98 (A) and *B. cereus* MTCC 1272 (B).
HJ-W and HJ-ME indicate the name of the active fractions harvested from the solvents, water and methanol, respectively.

Table 1

Phytochemical screening of crude methanol and water extracts of *H. javanica*.

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

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)
				Methanol (324 mg/mL)	Water (100 mg/mL)	
Bacterial strains	*B. cereus*	Pathogen of food borne illnesses	TSB, 37 °C	15.000 ± 0.400[a]	19.000 ± 0.240	20.000 ± 0.098
	B. megaterium	Heat resistant strain	TSB, 37 °C	10.000 ± 0.100	13.500 ± 0.360	20.000 ± 0.060
	E. coli	GP, colicin indicator strain	TSB, 37 °C	17.000 ± 0.240	23.050 ± 0.370	22.000 ± 0.141
	L. monocytogenes	HPF borne pathogen causing listerosis	BHI, 37 °C	16.000 ± 0.000[b]	13.000 ± 0.110	17.000 ± 0.144
	P. ananetis	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	Gastroenteritis pathogenic prototrophic strain	TSB, 37 °C	20.000 ± 0.143	26.300 ± 0.260	20.000 ± 0.139
	S. aureus	Pathogenic, antibiotic sensitive strain	TSB, 37 °C	18.000 ± 0.124	12.000 ± 0.160	25.000 ± 0.270
	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	12.000 ± 0.260	15.000 ± 0.167	20.000 ± 0.150
Fungal strains	*Aspergillus parasiticus* MTCC 2796	Aflatoxin producer	ME, 28 °C	0.000 ± 0.000	0.000 ± 0.000	17.300 ± 0.220
	Candida albicans 3018	Pathogenic to human	ME, 28 °C	0.000 ± 0.000	0.000 ± 0.000	13.800 ± 0.150
	Cladosporium herbarum 351	Pathogen of brown spot of rice	ME, 28 °C	0.000 ± 0.000	0.000 ± 0.000	28.000 ± 0.180
	Fusarium oxysporum f. sp. *pisci* MTCC 2480	Pathogen of *Fusarium* wilt of pea	ME, 28 °C	0.000 ± 0.000	0.000 ± 0.000	18.000 ± 0.180

[a]: The figures indicated the average diameter of inhibition zone (mm) of triplicate trials (± SE); [b]: No inhibition; [*]: Bacterial strains used oxytetracyclin and fungal strains used griseofulvin; GP: Gastroenteritis pathogen; HPF: Human pathogenic food; BHI: Brain heart infusion broth; ME: Malt extract.

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

For MIC and MBC study of 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*
324.00	15.00	10.00	14.00	15.00	20.00	9.00	13.00	9.00	8.80	9.00
162.00	13.00	8.00	10.00	9.50	19.00	8.20	11.00	8.50	8.00	8.00
81.00	11.00	7.50	8.00	9.00	18.00	7.00	9.00	7.30	7.50	7.30
40.50	9.00	6.20	5.50	8.00	16.00	6.50	6.50	-	7.00	6.80
20.25	0.70	-	-	-	13.50	-	5.70	-	6.00	6.20
10.12	-	-	-	-	13.00	-	-	-	-	5.80
5.06	-	-	-	-	10.00	-	-	-	-	-
2.53	-	-	-	-	7.00	-	-	-	-	-
1.26	-	-	-	-	-	-	-	-	-	-

The data were the average diameter of inhibition zone of triplicate trials; -: No inhibition zone.

Table 4
MBC of crude active methanol extract of *H. javanica* against the pathogenic bacteria strains.

Concentration (mg/mL)	B. cereus	B. megaterium	E. coli	L. monocytogenes	P. ananetis	P. aeruginosa	S. typhimurium	S. aureus	S. epidermidis	S. mutans
324.00	+	+	+	+	+	+	+	+	+	+
162.00	+	+	+	+	+	+	+	+	+	+
81.00	+	MBC	MBC	MBC	+	MBC	+	MBC	+	+
40.50	MBC	-	-	-	+	-	MBC	-	MBC	+
20.25	-	-	-	-	+	-	-	-	-	MBC
10.12	-	-	-	-	+	-	-	-	-	-
5.06	-	-	-	-	MBC	-	-	-	-	-
2.53	-	-	-	-	-	-	-	-	-	-
1.26	-	-	-	-	-	-	-	-	-	-

+: Bactericidal concentration; -: Resistance (growth of bacteria).

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.
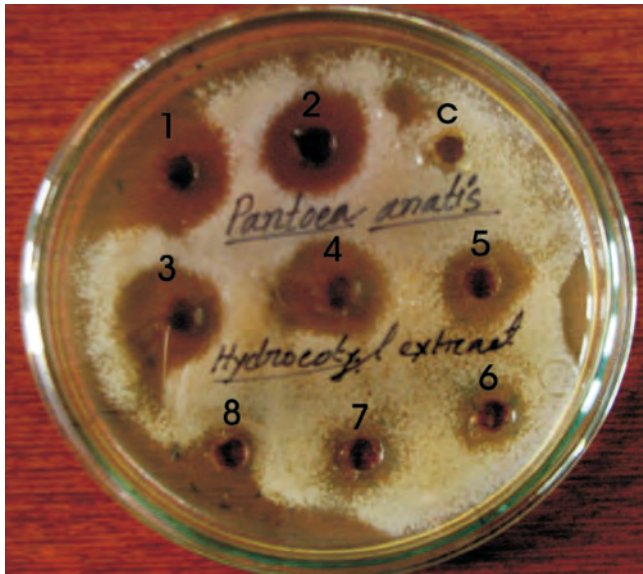

Figure 4. MIC of the crude active extracts against different human pathogenic bacteria by agar well diffusion method against *P. ananetis*.
1: 324.00 mg/mL; 2: 162.00 mg/mL; 3: 81.00 mg/mL; 4: 40.50 mg/mL; 5: 20.25 mg/mL; 6: 10.12 mg/mL; 7: 5.06 mg/mL; 8: 2.53 mg/mL; C: Control (methanol).
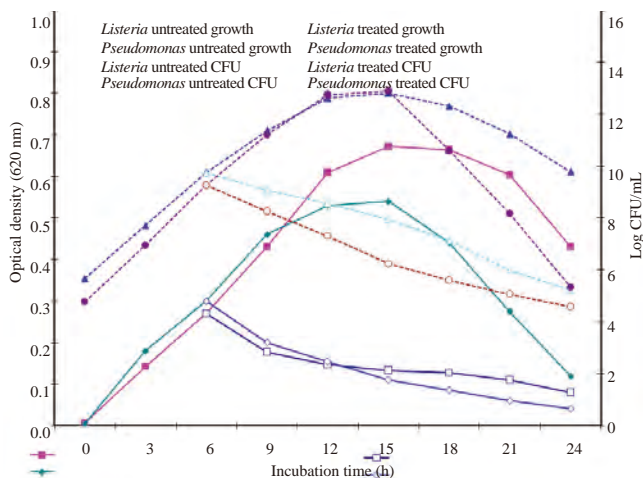

Figure 5. Effects of crude active extract (methanol, 100 mg/mL) on growth and viability of *L. monocytogenes* and *P. aeruginosa*.
Here solid lines indicated untreated control and dotted lines indicated treated with active extract. Square and star for growth and triangle and circle for CFU of *Listeria*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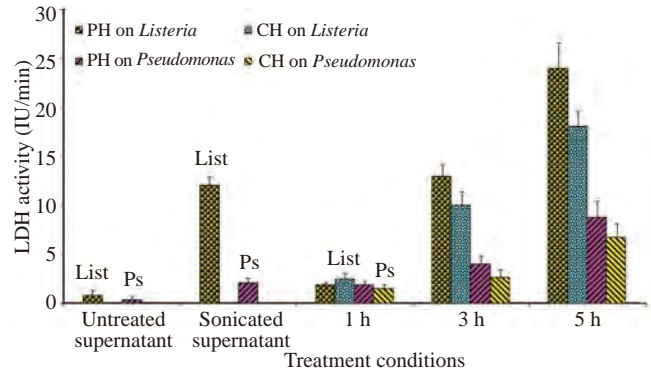

Figure 6. LDH activity of *L. monocytogenes* and *P. aeruginosa* induced by the crude active constituent (methanol, 100 mg/mL).
Results shown here were the average of triplicate trials (± SE). Each point represented the mean of triplicate trials and error bars represented standard errors; PH: Purified 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 UV_{254} with R_f values 0.68 and 0.307, respectively. UV-vis spectra showed a prominent peak (λ_{max}) at 285.7 nm and 238.5 nm for dichloromethane fraction and 218.0 nm for methanol fraction (Figure 9).
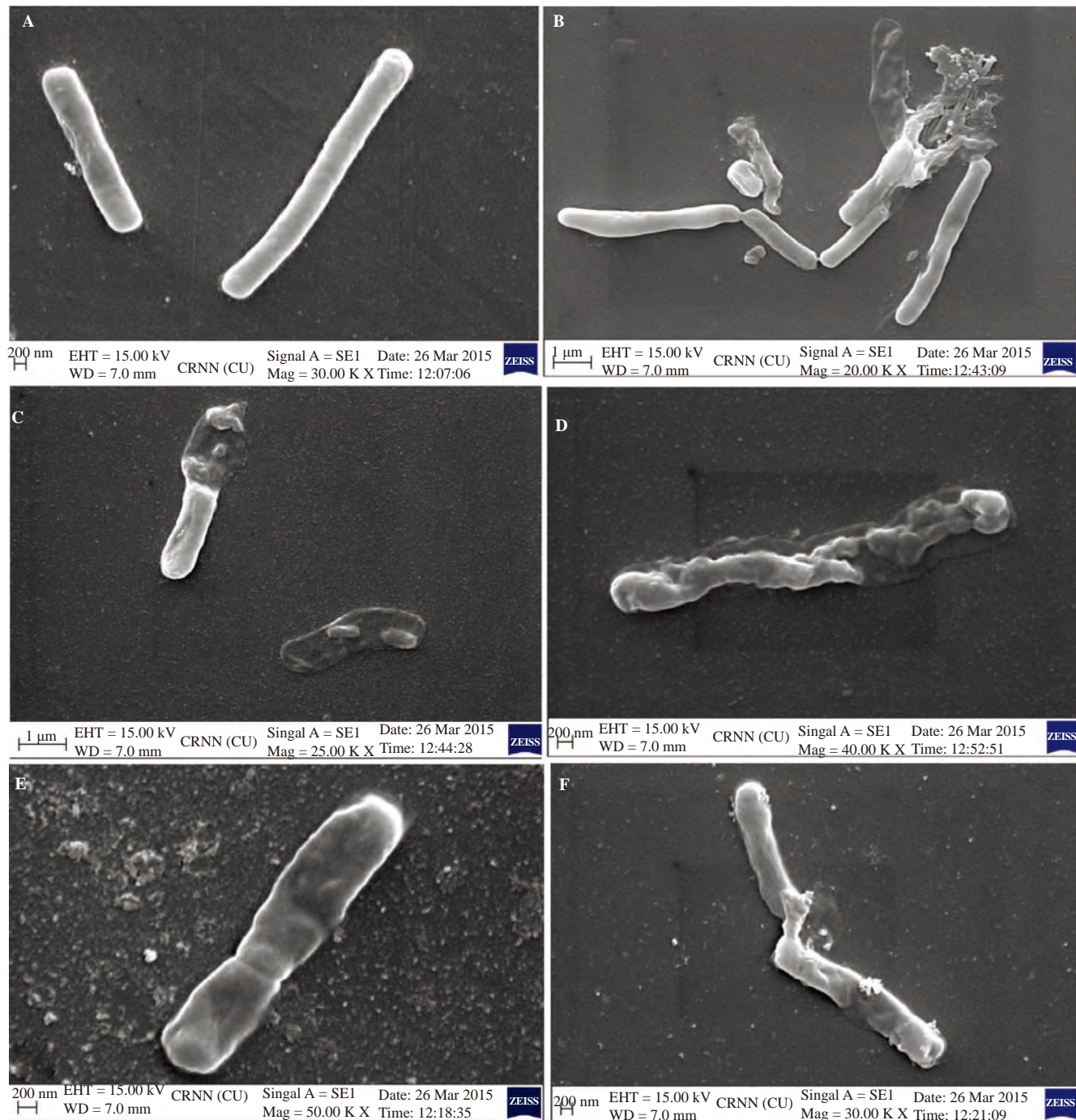
Figure 7. SEM of the active fractions (methanol and dichloromethane) of methanolic extract of *H. javanica* Thunb. against *L. monocytogenes*. Schematic representation of the SEM protocol, as standardized by our laboratory.
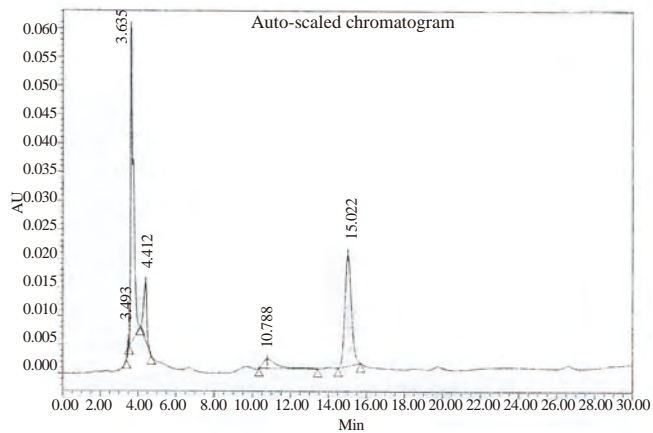

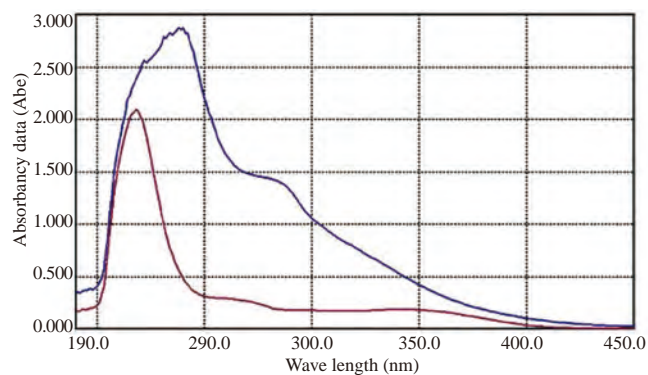**Figure 8.** HPLC analysis of the crude methanol extract of *H. javanica*.

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 alkaloids, cardiac glycosides, flavonoids, leucoanthocyanidins, phenols, saponins, sterols and triterpenoids (Table 1) many of which have been reported to have health protective efficacy. Saponins, anthraquinone and terpenoids were reported to be present in the methanol fraction in other species of *Hydrocotyle*[3-7].

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.*[15] 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, Ranuncoside 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 IR, 1-D and 2-D nuclear magnetic resonance (^{1}H and ^{13}C), 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* had also 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.

Acknowledgments

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