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Bioactivity guided isolation and characterization of antimicrobial principle from *Hydrocotyle javanica* Thunb.

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Hydrocotyle javanica Thunb. (Family Araliaceae) has been used in traditional herbal medicine as a substitute of *Centella asiatica* (L.) Urban. Although, the latter has been studied extensively and various biologically active constituents have been reported, the former has not been studied for its active constituents. The current study followed activity guided isolation and identification of the fraction with antimicrobial activity from *H. javanica*. Plant extracts were prepared by extracting successively with petroleum ether, ethyl acetate and ethanol. Antimicrobial activity was evaluated using gram positive and gram negative bacteria and *Candida albicans*. Bioactivity-guided isolation was carried out by preparative thin layer chromatography and bioautography. Cytotoxicity of the most active antimicrobial principle was evaluated against Human embryonic kidney (HEK293) and Adenocarcinomic Human Alveolar Basal Epithelial (A549) cell lines. The tentative structure of the most active antimicrobial principle was constructed from the data obtained from FTIR, MS, H-NMR and C-NMR.

Keywords: Antimicrobial activity, Bioautography, Cytotoxicity, *Hydrocotyle javanica*, Structure

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Natural products have served as an eternal source to cater to our needs of food, shelter, clothing and healthcare[1]. Plants offer a promising source of biologically active compounds for use as antimicrobial and antioxidant agents, nutraceuticals, food supplements, and pharmaceuticals and as leads for synthetic drugs[2]. Plant derived substances have gained popularity and have become of great importance owing to their versatile applications[3]. It is estimated that approximately three-quarters of pharmacologically active compounds in use today have been discovered from plant derived components by following upon their ethno-medicinal uses[4]. *Hydrocotyle javanica* Thunb. (Family Araliaceae) is an annual, terrestrial freshwater herb widely distributed in tropical Asia and Oceania. It grows as a small herbaceous annual plant with slender stems and creeping stolons. The flowers are white in color and are borne in small, rounded bunches. The species grows wild in areas with warm and humid climatic conditions[5]. It has folkloric use in the treatment of toothache, dysentery, dyspepsia and snake bite and as an alterative, depurative, diuretic, stimulant, tonic and blood purifier[6,7]. The plant has also been used as a piscicide and as a substitute of *Centella asiatica* in herbal preparations[8,9]. The essential oils from the seeds of *H. javanica* have been reported to exhibit antibacterial activity[10]. In our earlier report, we studied the aqueous extract of this plant for antimicrobial effects[11]. In another study by Mandal *et al.*[12], the methanol extract of the plant was reported to exhibit antimicrobial activity. In the current study, we followed the bio-activity guided isolation of the antimicrobial principle from *H. javanica* and elucidated its tentative chemical structure based on various spectroscopic data.

Methodology

Analytical grade solvents and reagents were used for experimentation. These were procured from Merck (Mumbai, India). The distilled water used for the study was generated from Arium Water Purification System (Sartorius Stedim, Germany). The media used for antimicrobial assay were obtained from HiMedia Pvt. Ltd. (Mumbai, India) and the antibiotics- Ciprofloxacin and Clotrimazole were obtained from Sigma-Aldrich (Steinheim, Germany).

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Plant material

Hydrocotyle javanica Thunb. was harvested from the Medicinal Plant Garden of Dibrugarh University, Assam (India). The identity of the plant (Specimen Voucher Number: DULSc 444) was validated by Prof L R Saikia, Department of Life Sciences, Dibrugarh University. The aerial parts of the plant were collected at the flowering stage and cleaned by washing with water. The washed plant material was air-dried at 30±2°C and ground in an electric blender into a fine powder. The powdered plant material was weighed and used for the preparation of extract.

Preparation of organic solvent extracts of plants

The extracts were prepared by successively extracting the powdered plant material (50 g) with petroleum ether, ethyl acetate and ethanol in 1:10 w/v ratio at 28±2°C for 48 h with intermittent shaking. The extracts were filtered through Whatman No. 1 filter paper and the filtrate was concentrated in a rotatory vacuum evaporator (IKA RV-10, Germany) until a constant dry weight of each extract was obtained. The extracts were stored at 4°C.

Determination of extractive value

Extractive value was determined by following the standard methods outlined by the World Health Organization[13]. The extractive value was expressed as mg per g of air- dried material.

Quantification of total phenolic content

Folin-Ciocalteu method was followed for the determination of total phenolic content of the plant extracts[14]. Gallic acid was used as standard for preparing the calibration curve and the total phenolic content in extracts was expressed as Gallic Acid Equivalent (mg of GAE/g of extract).

Quantification of total flavonoid content

The Dowd method as described by Meda *et al.* (2005) was followed for quantifying the total flavonoid content of the extracts[15]. A calibration curve was prepared using Rutin and contents were expressed as Rutin Equivalent (mg of RE/ g of extract).

Determination of antimicrobial activity

Test organisms

The plant extracts were screened against 7 bacterial strains, of which three were Gram positive- *Bacillus subtilis* MTCC 441, *Staphylococcus epidermidis* MTCC 435, *Bacillus cereus* MTCC 430, while four were Gram negative- *Escherichia coli* MTCC 739, *Salmonella enterica* MTCC 3219, *Pseudomonas aeruginosa* MTCC 1688 and *Proteus mirabilis* MTCC 1429. Antifungal activity was evaluated using *Candida albicans* MTCC 3017. The microorganisms were procured from MTCC, IMTECH, Chandigarh, India.

Determination of zone of inhibition

The agar well diffusion method was followed for determination of antimicrobial activity of plant extracts[16]. Dimethyl Sulphoxide (DMSO) was used as the solvent for plant extracts. The experiment was performed in triplicate and the results were expressed as the mean of the diameter of zone of inhibition (in mm). Ciprofloxacin (10 µg/mL) and Clotrimazole (30 µg/mL) were used as positive control while DMSO was used as negative control.

Determination of minimum inhibitory concentration

The broth dilution method was followed for the determination of minimum inhibitory concentration (MIC)[17] for the extract that recorded highest antimicrobial activity. Two-fold dilutions of the plant extract were prepared and the minimum concentration of the extract that inhibited microbial growth was considered as the MIC[17].

Isolation and purification of active component(s) by Thin Layer Chromatography (TLC)

The plant extract showing the highest antimicrobial activity was selected for the isolation and purification of active constituent(s) by preparative thin layer chromatography (TLC). TLC was performed on Silica Gel G-60 plates (100 x 200 mm) that were activated at 110°C for 3 h prior to use. The ethanol extract of *H. javanica* was dissolved in ethanol and was applied in the form of bands. The plates were developed in solvent system of petroleum ether, ethyl acetate and ethanol (4:1:1 v/v/v). The chromatograms were visualized under 256 nm, 364 nm and visible light and in iodine chamber. The R_f values for the spots were calculated.

Bioautography of TLC fractions

Antimicrobial activity of TLC fractions was investigated by the method of direct bioautography[18]. After developing, the TLC plates were air-dried and the suspensions of test organisms were diluted and sprayed evenly on them. The plates were dried under air-flow for 2 min and incubated overnight. After incubation, the plates were sprayed with an aqueous solution of *p*- iodonitrotetrazolium violet (INT) and

incubated again till the spots appeared. Finally, the plates were treated with ethanol (70%) before evaluation of bioautograms.

Characterization of structure of antimicrobial principle

The spectral analyses were performed according to standard procedures[19]. The sample was dried and dissolved in chloroform ($CDCl_3$) for recording FTIR spectrum (4000-600 cm^{-1}) (IRAffinity, Shimadzu, Japan). Mass Spectra was recorded on Varian 410 Prostar binary LC with 500 MS IT PDA detectors using electron spray ionization (ESI). Proton (^1H) NMR spectrum was recorded at 400 MHz (Varian, Mercury Plus) and Carbon (^{13}C) NMR spectrum was recorded at 100 MHz (Varian, Mercury Plus).

Cytotoxicity assay

Cytotoxicity of the antimicrobial principle was evaluated using human embryonic kidney (HEK 293) and adenocarcinomic human alveolar basal epithelial (A549) cell lines by following MTT assay[20]. The percentage inhibition of cell proliferation was calculated from the following equation and reported as mean ± standard deviation of triplicates:

Percent inhibition of cell proliferation= $\{(1-A_{Sample}/A_{Control})\} \times 100$

Cytotoxicity was evaluated by comparing with the control well and was expressed as the concentration of the sample required to inhibit 50% of cell proliferation (IC_{50}).

Statistical analysis

Results to all experiments were expressed as mean ± standard deviation which were calculated based on data obtained from three replicates. The *t* Test and ANOVA (α=0.05) were performed to establish the significance of differences among results. IC_{50} values were determined by curve fitting techniques. The various statistical analyses were performed using Sigma Plot (Systat Software, San Jose, CA).

Results

Extracts of *H. javanica* were compared for their extractive values, total phenolic content (TPC) and total flavonoid content (TFC) (Table 1). Highest extractive value was observed for extract prepared with ethyl acetate and the extractive values for the three solvents showed significant differences ($p<0.05$) (Table 1). The TPC of the three extracts showed significant difference (α=0.05) with the ethyl acetate extract recording the highest content. The total flavonoid content (TFC) of the three extracts recorded statistically significant differences (α=0.05) with highest content recorded for petroleum ether extract (Table 1).

The three extracts of *H. javanica* inhibited both Gram positive and Gram negative bacteria and fungi (Table 2). The petroleum ether extract was ineffective against *B. subtilis*, *S. enterica* and *C. albicans.* Among the three extracts, ethanol extract exhibited higher inhibition of most of the test organisms. The MIC of ethanol extract against the test strains was determined and is recorded in Figure 1.

TLC of ethanol extract of *H. javanica* yielded six fractions, which were labeled as HjF1 to HjF6. The results of bioautography of the fractions HjF1-HjF6 are recorded in Table 3. All the 6 fractions inhibited *B. cereus* and *C. albicans* while none of

Table 1 — Extractive value, total phenolic content (TPC) and total flavonoid content (TFC) of different solvent extracts of *H. javanica*

Extract	Extractive Value (in mg/g air-dried plant material)	TPC (in mg GAE/g of extract)	TFC (in mg RE/g of extract)
Petroleum ether	19.67±0.58	1.21±0.02	300.00±2.29
Ethyl acetate	80.00±1.00	7.43±0.02	198.83±5.01
Ethanol	22.33±0.58	7.37±0.05	170.47±1.82

Results expressed as mean ± sd of triplicates

Table 2 — Antimicrobial activity of extracts of *H. javanica* (test concentration= 200 µg/mL)

Test microorganism	Diameter of zone of inhibition (in mm)			
	Petroleum ether Extract	Ethyl acetate Extract	Ethanol Extract	Standard Drug
B. subtilis	NA	10.33±0.58	13.67±0.58	27.67±2.08*
P. mirabilis	10.33±0.58	12.67±0.58	19.67±0.58	26.00±1.00*
B. cereus	12.33±0.58	14.67±0.58	16.00±0.00	19.33±1.15*
E. coli	12.00±1.73	11.67±0.58	13.67±0.58	27.33±0.58*
S. enterica	NA	10.33±0.58	12.67±0.58	27.00±1.00*
P. aeruginosa	10.33±0.58	12.00±1.00	14.33±0.58	21.67±0.58*
S. epidermidis	11.67±0.58	12.67±0.58	13.67±1.15	27.67±0.58*
C. albicans	NA	10.33±0.58	11.67±0.58	16.67±0.58#

NA= No Activity; * = Ciprofloxacin (10 µg/mL); # = Clotrimazole (30 µg/mL)

Results expressed as mean ± sd of triplicates

Table 3 — Bioautography of TLC Fractions of Ethanol Extract of *H. javanica* (R_f values shown for bands observed under visible light)

Fraction	Rf value	Test microorganism							
		Bs	Pm	Bc	Ec	Se	Pa	Sep	Ca
HjF1	0.19	+	+	+	-	-	-	+	+
HjF2	0.28	+	+	+	-	-	+	+	+
HjF3	0.40	+	-	+	-	-	+	+	+
HjF4	0.49	-	+	+	-	-	-	+	+
HjF5	0.67	+	-	+	-	-	-	+	+
HjF6	0.96	+	+	+	-	-	-	-	+

Bs = *B. subtilis* Pm = *P. mirabilis* Bc = *B. cereus*
Ec = *E. coli* Se = *S. enterica* Pa = *P. aeruginosa*
Sep = *S. epidermidis* Ca= *C. albicans*
+ = Inhibitory Activity Present -= No Activity

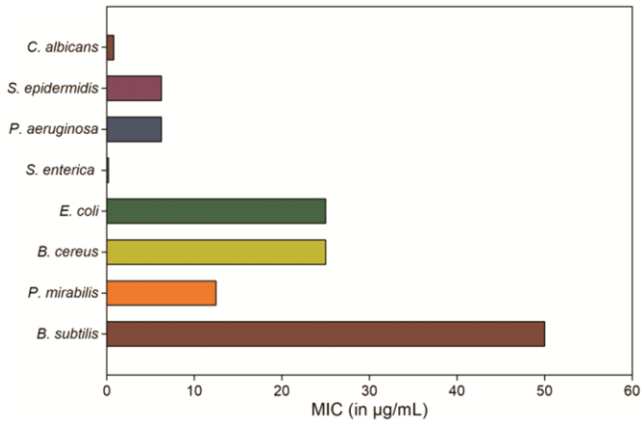

Fig. 1 — Minimum Inhibitory Concentration (MIC) of the ethanol extract of *H. javanica*.

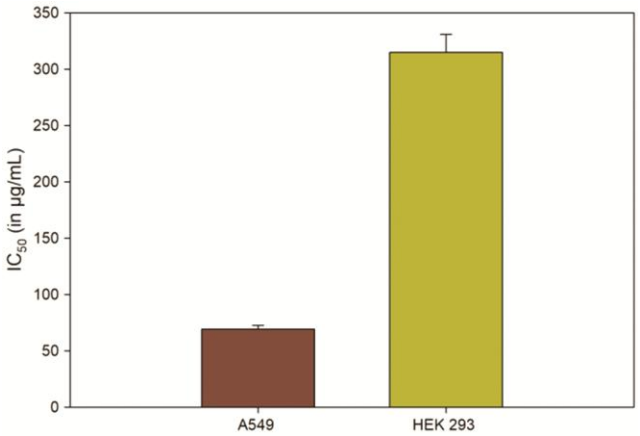

Fig. 2 — Toxicity of antimicrobial principle HjF2 of *H. javanica* towards A549 (lung cancer cell line) and HEK 293 (normal human embryonic kidney cell line).

them inhibited *E. coli* and *S. enterica.* HjF2 inhibited most of the test organisms as compared to the other fractions.

The FTIR spectra of HjF2 recorded peaks at 3413 cm^{-1} (ArO-H bonded), 2923 cm^{-1} and 2853 cm^{-1} (-CH_2-), 1537 and 1560 cm^{-1} (R-CO-NH-R'). In addition, peaks were also recorded at 1414 cm^{-1} (Ar C-C stretch), 1092 cm^{-1}(RCOOH, C-O stretch) and 1022 cm^{-1}(RCOOR', C-O stretch). The ESI-MS spectra of HjF2 recorded molecular ion [$M+H^+$] at m/z 302 that suggests a molecular mass of 301 Da. The ^{1}H- NMR spectrum of fraction recorded the following chemical shifts - ^{1}H NMR (Chloroform-*d*, 400 MHz) δ 1.08 (3H, t, *J*=6.7 Hz), 2.00 (2H, qd, *J*=6.7, 1.0 Hz), 2.28 – 2.33 (6H, m), 4.51 (1H, s), 5.39 (2H, d, *J*=6.5 Hz), 5.48 (1H, s), 5.97 (1H, t, *J*=1.0 Hz), 6.18 (2H, s), 7.33 – 7.40 (2H, m), 7.85 – 7.92 (2H, m). The spectra recorded one three-proton triplet (δ 1.08) and a two proton quartet (δ 2.00) which were assigned to –CH_2CH_3. The ^{13}C- NMR spectra of the fraction showed peaks which were recorded as follows- ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 28.6, 29.8, 95.6, 107.3, 122.7, 127.5, 127.8, 136.2, 136.6, 157.7, 168.6, 168.9, 182.9, 196.1. HjF2 exhibited dose-dependent toxicity and showed higher inhibition of A549 cell line (lung cancer cell line) (IC_{50}= 69.11 ± 3.46 µg/mL) as compared to HEK 293 cell line (normal human embryonic kidney cell line) (IC_{50}=315.07 ± 15.75 µg/mL) (Fig. 2).

Discussion

The plant constituents responsible for various biological activities, exhibit solubility in solvents of different polarities; hence, selection of an ideal solvent for extraction is a prerequisite before proceeding with the evaluation of plant extracts. Extractive value refers to the total soluble constituents obtained from the dried plant material in any particular solvent or mixture of solvents[21]. In the present study, extracts prepared with solvents of different polarities exhibited significant differences in extractive values, which may be due to the differences in the quantity and quality of phytoconstituents

obtained with these solvents. Significant differences were also observed in the contents of phenolics and flavonoids in the extracts. Low polarity solvents like petroleum ether, yield more lipophillic components, while alcoholic and aqueous solvents yield a broad spectrum of polar components[22]. In addition, solvents with low-polarity are known to only rinse or leach the sample, while solvents with higher polarity, like alcoholic solvents can rupture the cell membranes and extract a greater amount of endo-cellular materials[23]. Petroleum ether, which has the least polarity among the three solvents used in the experiment, extracted more of flavonoids than phenolics, thus suggesting the non-polar nature of flavonoids available in the plant, while, ethyl acetate and ethanol extracted higher quantities of phenolics (Table 1). Phenolics and flavonoids are secondary metabolites that are known to exert different biological effects like, inhibition of reactive oxygen species, inhibition of alpha-amylase and alpha- glucosidase enzymes and cytotoxic effects[24-26]. The three extracts of *H. javanica* inhibited both gram positive and gram negative bacteria with different efficacy (Table 2). The plant extracts have been reported to exhibit variations in their inhibitory activity against Gram positive and Gram negative bacteria[27,28]. Gram positive bacteria have been reported to be more susceptible to plant extracts than the Gram negative ones[29-34]. This difference in susceptibility to plant extracts is attributed to the structure of the bacterial cell membrane. The presence of structural lipo-polysaccharide components in the cell membrane of Gram negative bacteria make the cell membrane impermeable to lipophilic molecules and neutralize the effects of most of the drug molecules[35,36]. However, in the present study it was observed that the Gram negative strains were more susceptible to the ethanol extract than the Gram positive ones (Table 2). This may be regarded as an important finding as most of the pathogenic strains of bacteria are Gram negative and are responsible for various infections in humans. *S. enterica* is known to cause different diseases like gastroenteritis, bacteremia and enteric fever[37,38]. *P. mirabilis*, a rod-shaped Gram negative bacterium, is responsible for the infections of the urinary tract[39]. Similarly, *P. aeruginosa*, a Gram negative cocco-bacillus, is an opportunistic pathogen[40]. *E. coli*, a Gram negative rod, is responsible for food poisoning in humans[41,42]. In addition to antibacterial activity, the ethanol extract of *H. javanica* was active against *C. albicans* and recorded low inhibitory and fungicidal concentrations. *C. albicans* is a fungus that grows both as yeast and as filamentous cells. It is a commensal and a constituent of the normal flora of human mouth and gastrointestinal tract[37,43]. The ethanol extract was selected for isolation of the active principle(s) with antimicrobial activity by thin layer chromatography followed by direct bioautography. TLC-bio-autography is a standard method for visualizing the antimicrobial activity of separated components on TLC plates and is based on detecting the dehydrogenase activity of test organism. The living microorganisms metabolize the tetrazolium salt (*p*- iodonitrotetrazolium violet) into corresponding formazan which is intensely colored. Thus, components with antimicrobial activity develop as pale or clear spots on a colored tlc plate background. It was interesting to observe that though the ethanol extract of *H. javanica* inhibited *E. coli* and *S. enterica* (Table 2), but none of the isolated TLC fractions inhibited these strains (Table 3). These components failed to exert antimicrobial activity in isolation but exhibited inhibition when present together in the crude extract. This suggests synergism among the components of the crude extract. Similar observations have also been reported by other workers, but there are limited reports that mention of such interactions[44,45]. Such interactions among the components of plant extracts are an interesting avenue for further research. Fraction HjF2 (R_f=0.28) showed inhibitory activity against the highest number of test organisms in comparison to the other fractions. Therefore, it was selected for further characterization. The assignments of the various signals in the ^{1}H and ^{13}C NMR spectra were made in consultation to the published literature[19]. Based on spectroscopic data, a tentative structure was derived with the chemical formula- $C_{17}H_{19}NO_4$ and chemical name 4-(3-Ethyl-1,5-dihydroxy-7-oxo-octa-1,3,5-trienyl)-benzamide (Fig. 3). HjF2 exhibited peaks for the presence of aromatic ring along with –OH (hydroxyl), -$CONH_2$ (amide), -COOH (carboxylic acid) and –COO (ester) as the functional groups in the FTIR spectrum. The hydroxyl (OH) group is reported to affect the antimicrobial activity of extracts against Gram positive and Gram negative bacteria. The present work is the first to report this antimicrobial fraction from *H. javanica.* A dose-dependent toxicity was observed towards HEK and A549 cell lines, hence

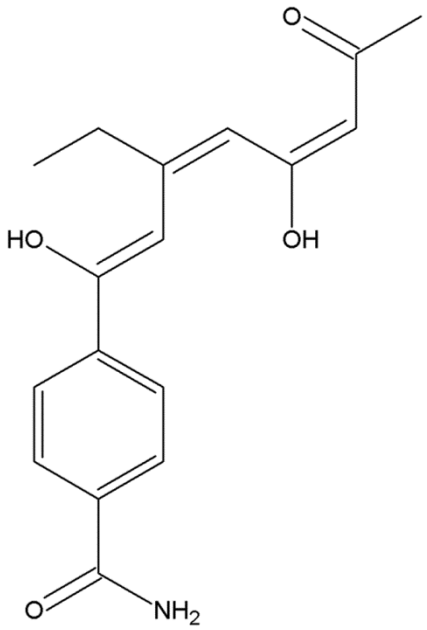

Fig. 3 — Structure of the antimicrobial principle HjF2 of *H. javanica.*

implying a probable therapeutic utility of the antimicrobial principle. The findings indicate that HjF2 is more toxic to cancer cells than to normal cells.

Conclusions

The present work has reported the isolation and characterization of antimicrobial principal from *H. javanica* and provided a scientific foundation to the ethnomedicinal uses of the plant. The antimicrobial principal has shown broad spectrum antimicrobial activity and toxicity to cancer cells. Further studies may be conducted using *in vivo* models to evaluate the efficacy of the isolated fraction and also to study the synergism among the components of plant extracts.

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Conflict of Interest

None

Authors' Contributions

KS contributed to experimental design, conducted experiments, compiled and analysed results and drafted the manuscript. RNSY contributed to experimental design, interpretation of results, refining of manuscript draft and supervised the entire study.

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