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Preliminary phytochemical standardization and Evaluation of anti-dysentery activity of alcoholic extract of *Hydrocotyle asiatica*

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Abstract

The present study was undertaken to carry out the phytochemical analysis and evaluation of anti-dysentery activity of the leaf extract of *Hydrocotyle asiatica*. The dried leaf powder was extracted with pure alcohol (95%) using soxhlate apparatus. The studies have been evaluated for these extracts. This study was undertaken to ascertain phytochemical & microbial parameters to establish the authenticity of *Hydrocotyle asiatica* leaf.

Keywords: Dysentery,Description of *Hydrocotyle asiatic* plant, Phytochemical evaluation, Thin layer chromatography, Microbiological evaluation.

Introduction

Dysentery [1] is an infectious disease of the large intestine; there are two types: Amoebic dysentery-this type is rare in Britain but can be contracted whilst travelling in tropical & equatorial regions of the world where it is common. Bacterial dysentery-the form most common in Britain, is known as Shigella dysentery & is caused by Shigella, a highly contagious bacteria.

The symptoms may vary but yours may have included:

- Mild or severe diarrhoea often containing blood & or mucous,
- Vomiting,
- Stomach cramps,
- Fever.

Hydrocotyle asiatica, commonly called as thank*uni* in Bengali, *gotu kola* in Sinhala, *mandukaparni* in Sanskrit, it is a small, herbaceous, annual plant of family Apiaceae & is native to India, Indonesia, Iran, Malaysia, Melanesia, Philippine s, Papua New Guinea& other parts of Asia. It is used as a medicinal herb in Ayurvedic medicine, traditional African medicine, & traditional Chinese medicine. Botanical synonyms include *Centella asiatica*[2]. *Hydrocotyle asiatica* grows in tropical swampy areas [2]. The stems are slender, creeping stolons, green to reddish-green in color, connecting plants to each other. It has long-stalked, green, reniform leaves with rounded apices which have smooth texture with palatably netted veins. The leaves are borne on pericardial petioles, around 2 cm. The rootstock consists of rhizomes, growing vertically down. They are creamish in color & covered with root hairs [3]. The flowers are pinkish to red in color, have rounded bunches near the surface of the soil. The hermaphrodite flowers are minute in size. The fruit are densely reticulate, distinguishing it from species of Hydrocotyle which have smooth, ribbed fruit.

The crop matures in three months & the whole plant is harvested manually [4]. Hydrocotyle grows in ditches & in low, wet areas. In Indian & Southeast Asian Hydrocotyle, the plant frequently suffers from high levels of bacterial contamination, because the plant is aquatic & is especially sensitive to pollutants in the water, which are easily incorporated into the plant.

Plant Taxonomy[4]:

Kingdom: Plantae
Subkingdom: Viridaeplantae
Division: Tracheophyta
Subdivision: Spermatophytina
Class: Magnoliopsida
Order: Apiales
Family: Araliacae
Genus: *Hydrocotyle*
Species: *Hydrocotyle asiatica*

Materials and Methods

Materials: Plant material was collected from Durgapur Burdwan district of West Bengal. The collected leaves were claimed to remove dust by using water and then dried under shade. Dried leaves were firstly crushed using sharp knife or blade & then it is pulverized by a mechanical grinder & passed through mesh number 66, then subjected to extraction.

Methodology:

a. Procedure for Soxhlation

1. The leaf powder that is to be extracted is packed in a paper thimble made from a filter paper & it is placed inside the soxhlet extractor.
2. The solvent is placed in the round bottom flask. The apparatus is then fitted.
3. When solvent boiled on heating at 30°C, it gets converted into vapor, this vapor enters into the condenser through the side tube & gets condensed into the hot liquid which falls on the column of the drug.
4. When the extractor gets filled with the solvent, the level of siphon tube was also raised upto its top.
5. The solvent containing active constituents of the drug in the siphon tube, siphon over &run into the flask thus emptying into the body of the extractor.
6. This alteration of filling & emptying of the body of the extractor goes on continuously.
7. The soluble active constituents of the drug remain in flask while the solvent repeatedly volatilized.
8. The process is repeated for total 15 times for complete exhaustion of the drug [5].

Figure 1: Soxhlet apparatusduring soxhelation

b. Preliminary phytochemical screening

It involves testing of different extracts of *Hydrocotyle asiatica* for their contents of different classes of compounds. The methods used for the detection of various phytochemicals were followed by qualitative chemical tests. Various phytoconstituents were carried out for the extracts of *Hydrocotyle asiatica* which are as follows [6],[7],[8].

A. Test for alkaloids:

- Mayer's test: It is used for the detection of alkaloids. It gives cream color precipitate with Mayer's reagent [Potassium mercuric iodide solution].

- Dragendroff's test: Alkaloids give reddish brown precipitate with Dragendroff's reagent [Potassium bismuth iodide solution].
- Wagner's test: Alkaloids gives reddish brown precipitate with Wagner's reagent [Solution of iodine in potassium iodide].
- Hager's test: Alkaloids give yellow color precipitate with Hager's reagent [Saturated solution of Picric acid].

B. Test for cardiac glycosides:

- Keller-killiani test: Extract the drug with chloroform & evaporate it to dryness. Add 0.4 ml of glacial acetic acid containing a trace amount of ferric chloride. Transfer to a small test tube, add carefully 0.5 ml of concentrated sulfuric acid by the side of the test tube, blue color appears in the acetic acid layer.

C. Test for tannins and phenolic compounds:

- Gelatin test: Test solution with 1% gelatin solution containing 10% sodium chloride gives white precipitate.
- Ferric chloride test: Test solution gives blue green color with ferric chloride.

D. Test for flavonoids:

- Alkaline reagent test: To the test solution add few drops of sodium hydroxide solution which leads to the formation of yellow color which then turns into colorless on addition of few drops of diluted acid, indicates the presence of Flavonoids.

E. Test for proteins and amino acids:

- Ninhydrin test: Amino acids & Proteins were boiled with 0.2% of Ninhydrin, gives violet color.

F. Test for steroids and terpenoids:

- Salkowski test: Treat extract with few drops of chloroform along with concentrated sulfuric acid, shake well and allow standing for some time, red color appears at the lower layer which indicates the presence of steroids & formation of yellow color at the lower layer indicates the presence of terpenoids.

G. Test for carbohydrates:

- Molisch's test: Treat the test solution with few drops of alcoholic α-napthol. Add 0.2 ml of concentrated sulfuric acid slowly through the sides of the test tube, a purple to violet color ring appears at the junction.
- Fehling's test: Equal volume of Fehling's A (Copper sulfate in distilled water) and Fehling's B (Potassium tartarate and Sodium hydroxide in distilled water) reagents are mixed and few drops of sample is added and boiled, a brick red precipitate of cuprous oxide forms if reducing sugars are present.

H. Test for saponin glycoside:

- The extract is transferred into a test tube & shaken vigorously then is left to stand for 10 minutes. A thick persistent froth indicates the presence of saponins.

I. Test for fats and fixed oils:

- Copper sulphate test: Treat 5 drops of sample with 1 ml of 1% copper sulphate solution, then add 10% NaOH solution. A clear blue solution will obtain which shows the presence of glycerin in the sample.

c. Thin layer chromatography

Thin Layer Chromatography (TLC) is a chromatography technique used to separate mixtures. Thin Layer Chromatography is performed on a sheet of glass which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide or cellulose. This layer of adsorbent is known as Stationary phase. After the sample is applied on the plate, a solvent or solvent mixture known as the Mobile phase is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates & the separation is achieved. The extracts of *Hydrocotyle asiatica* were subjected to thin layer chromatographic analysis to find the presence of number of chemical constituents to support the chemical test.

The details of the method are as follows:

Analytical TLC plates were prepared by pouring the silica gel G slurry on the glass plates. Prepared chromatographic plates were there by dried for 30 minutes in air and then in dryer at 60°C for another

30 minutes. Now the alcohol extract of *Hydrocotyle asiatica* was loaded on silica plate. The separation was carried out by using Toluene: Acetone: Formic acid (of ratio 9: 6: 1) [9] as a solvent system. Then TLC plate is kept at iodine chamber for 5 minutes. Now the spotted TLC plate was placed at *45*°C in the development chamber covering the bottom of the plate by solvent up to nearly 1cm.The solvent front was marked & the plate was finally allowed to dry. The qualitative evaluation of the plate was done by determining the migrating behavior of the separated substances in the form of R_f value.

Figure 2: Preparation of TLC plates

d. Anti-Bacterial Assay

i. Principle of Disc diffusion method – Dried & sterilized filter paper discs of about 6 mm diameter containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. These plates are kept at low temperature for 24 hours to allow maximum diffusion of the test materials to the surrounding media. Then the plates are incubated at 37°C for 24 hours to inhibit microbial growth in the media surrounding the disc yield a clear distinct area defined as 'zone of inhibition' [9].

ii. List of test bacterias – 1. *Salmonella typhi*
2. *Staphylococcus aureus*
3. *Escherichia coli*

iii. Culture media & composition – Following media is used to determine the anti-microbial activity of the test organisms, (Nutrient agar media for 200 ml):

Table 1: Culture media & its composition

Ingredients	Amounts (gms)
Agar	4
Beef extract	2.4
Peptone	2.4
Sodium chloride	1
Dextrose	2.4
pH	7.5

Figure 3: Preparation of culture media

iv. Sterilization procedure – In order to avoid any type of contamination by microorganisms, the microbial screening was done in Laminar hood and all types of precautions were maintained. UV light was made on 1 hour before working in the laminar hood. Petridishes were sterilized by autoclaving at a temperature of 121°C & pressure of 20 lbs/sq inch for 10 minutes. Micropipette tips, cotton, forceps, blank discs were also sterilized [10].

v. Preparation of subculture – Bacterial subculture was done by preparing fresh lactose broth. In an aseptic condition under laminar air cabinet, the test organisms were transferred from pure cultures to the lactose slants to have fresh pure cultures. The inoculated strains are then incubated for 24 hours at 37°C for optimum growth [10].

vi. Preparation of test plates – The test organisms was then transferred from subculture to the petridishes containing sterilized and solidified agar medium with the help of a transfer loop in an aseptic area. The test tubes were manually rotated clockwise & anticlockwise to assure uniform distribution of the test organisms in the media.

vii. Preparation of sample discs with test samples of *Hydrocotyle asiatica* – The crude ethanolic extract of the plant sample were dissolved in carbon tetrachloride solution at different concentrations of 1 mg/ml, 0.5 mg/ml & 0.25 mg/ml respectively were taken in test tubes & the sterilized filter paper discs were soaked with the sample test solutions & embedded on the sterile solid agar media. Then the embedded media was allowed to incubate at 37°C for 24 hours.

viii. Minimum Inhibition Concentration– After incubation the bacterial susceptibility of the drug *Hydrocotyle asiatica* was observed by measuring the diameter of the distinct circular areas surrounding the discs.

Results and Discussion

a. Preliminary phytochemical screening

Table 2: Fluorescence analysis of leaf extract of plant extract

Extract	Day light	UV light at 366nm	UV light at 254nm
Ethanol extract	Brownish color	Greenish black	Fluorescent green

Table 3: Phytochemical analysis of plant extract

Phytochemicals	Tests	Ethanol extract
Alkaloid	1. Mayer's test 2. Wagner test 3. Hager's test	+ + +
Cardiac glycoside	Kellar killiani test	-
Tannin	1. Gelatin test 2. Ferric chloride test	+ +
Flavonoid	Alkaline reagent test	+
Proteins and amino acids	Ninhydrin test	-
Steroids and terpenoids	Salkowski test	+
Carbohydrate	1. Molish test 2. Fehlings test	- +
Fats and fixed oil	Copper sulphate test	-
Saponin glycoside	Froth test	+

Figure 3: Test for detection of Tannin

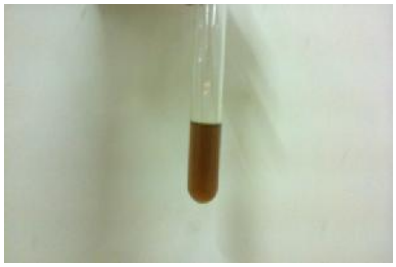

Figure 4: Test for detection of Steroid

Figure 5: Test for detection of Flavonoid

Figure 6: Test for detection of Alkaloid

b. Thin layer chromatography

$$R_f \text{ (retention factor) value} = \frac{\text{Distance travelled by solute from the base line}}{\text{Distance travelled by solvent from the base line}}$$

For alcohol extract = R_{f1} =4.8cm/5cm = 0.96
R_{f2}= 3cm/5cm = 0.6
R_{f3}=0.8cm/5cm = 0.16

c. Anti-Bacterial Assay

Table 4: Zone of inhibition (cm) & minimum inhibition concentration (gm/ml) after 24 hours incubation

Bacterial strains	Conc. 1gm/ml	Conc. 0.5gm/ml	Conc. 0.25gm/ml	MIC_{50} (gm/ml)
Escherichia coli	1.6 cm	1 cm	0.5 cm	0.25
Staphylococcus aureus	1.2 cm	0.8 cm	0.5 cm	0.25
Salmonella typhi	1.3 cm	0.2 cm	0.5 cm	0.5

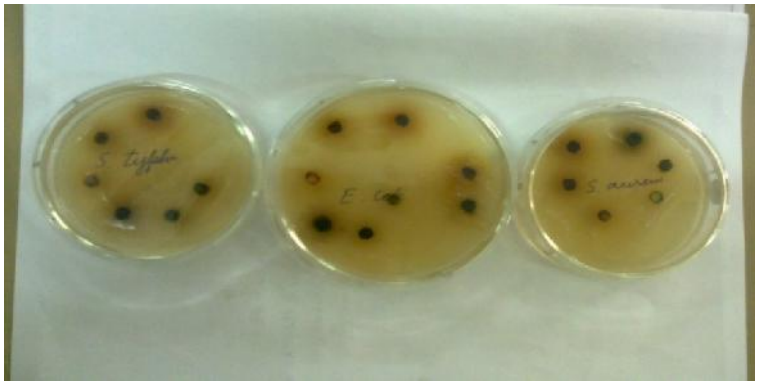

Figure 7: Anti-Bacterial assay by using Disc diffusion method

Conclusion

Preliminary phytochemical screening shown that the leaf of *Hydrocotyle asiatica* had phytoconstituents like alkaloids, tannins, flavonoids, steroids, terpenoids, carbohydrates &saponin glycosides.

Thin layer chromatography (TLC) results of the alcoholic extract shown that at least three different constituents were present in the leaf extract of *Hydrocotyle asiatica*.

The bacterial strain *Escherichia coli* was found to be more susceptible towards the extract of *Hydrocotyle asiatica*and the MIC_{50} (Minimum inhibitory concentration) value was found to be 0.25 gm/ml which can effectively act as an anti-dysentery drug of therapy.

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