Phytochemical profiling of *Hydrocotyle bonariensis* Comm. ex Lam. (Araliaceae)

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Contents

1. INTRODUCTION

Medicinal plants have been used in healthcare since time immemorial. Studies have been carried out globally to verify their efficacy and some of the findings have led to the production of plantbased medicines (Sofowora et al., 2013). The global market value of medicinal plant products exceeds $100 billion per annum. Over 90% of traditional medicine recipes/remedies contain medicinal plants. A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Sofowora et al., 2013). This description makes it possible to distinguish between medicinal plants whose therapeutic properties and constituents have been established scientifically, and plants that are regarded as medicinal but which have not yet been subjected to a thorough scientific study. A number of plants have been used in traditional medicine for many years. The medicinal plants are not only used for the treatment of diseases but also as a potential material for maintaining good health and conditions. Many countries in the world, that is, two third of the world's population depends on herbal medicine for primary health care (Ekor, 2013). The reasons for this is because of their better cultural acceptability, better compatibility and adaptability with the human body and pose lesser side effects. From records, most of the used drugs contain plant extracts. Some contain active ingredients (bioactive components or substances) obtained from plants. Some of the drugs believed to be obtained from plants are aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine and vinblastine (Garg et al., 2021).

Medicinal plants have provided mankind a large variety of potent drugs to alleviate or eradicate infections and suffering from diseases in spite of advancement in synthetic drugs, some of the plant derived drugs still retained their importance and relevance. The use of plant-based drugs all over world is increasing (Ekor, 2013). There have been records

of advances made in the modern (synthetic) medicine there are still a large number of ailments or infection (diseases) for which suitable drugs are yet to be found. This has brought an urgent need to develop safer drugs (both for man and his environment) for the treatment of inflammatory disorders, diabetes, liver diseases, and gastrointestinal disorder.

In this backdrop, the present study envisaged the phytochemical analysis of an underutilized medicinal plant, *Hydrocotyle bonariensis* Comm. ex Lam. (Araliaceae, APG IV). It is well known for its traditional uses and medicinal properties for the treatment of various kinds of diseases such as tuberculosis, relieving the pains of inflammation, rheumatism and arthritis, to increase brain capacity and for longevity (Obaseki et al., 2016).

H. bonariensis is a herbaceous plant with prostate, creeping or floating stems and roots forming at nodes and is closely similar to the well-known medicinal plant C. *asiatica* [Syn. *Hydrocotyle asiatica*] (Apiaceae), in their morphological characteristics. Extensive studies have been conducted on the phytochemical and biological activities of *C. asiatica* (Inamdar et al., 1996; Siddiqui et al., 2007; Hashim et al., 2011; Sabaragamuwa et al., 2022); however, a few studies have been reported on *H. bonariensis* (Haida et al., 2021; Mazumdar et al., 2022). Hence, the present study was undertaken to identify the phytochemical and antioxidant, nutritive analysis and antimicrobial activity of *H. bonariensis*.

2. AIMS AND OBJECTIVES

Aims

The present study aims to study the nutritional content, antioxidant potential and antimicrobial activity of *Hydrocotyle bonariensis*.

Objectives

The specific objectives of the present study are:

- To collect fresh plant materials of *H. bonariensis*.
- To prepare the extract using dried leaves in ethyl acetate, chloroform, distilled water, ethanol and acetone.
- To analyze the phytochemical composition of the plant using the above extracts.
- To perform the proximate analysis of the plant
- To check the antimicrobial activity of the plant using the dried leaf extracts in distilled water and chloroform against the bacterial culture of *Klebsiella pneumoniae*.

3. REVIEW OF LITERATURE

ABOUT THE PLANT: *Hydrocotyle bonariensis* Comm. ex Lam.

Figure 1: Habit of H. *bonariensis*

SYSTEMATIC POSITION AS PER APG IV

Kingdom : Plantae

Clade : Tracheophytes

Clade : Angiosperms

Clade : Eudicots

Clade : Asterids

Order : Apiales

Family : Araliaceae

Genus : *Hydrocotyle*

Species : *H. bonariensis*

Common names: Coastal pennywort, large leaf marsh pennywort etc.

It is a herbaceous plant with prostate, creeping or floating stems and roots forming at nodes. This plant typically forms dense mats along marshes, ponds and wetlands. The leaf shape is quite distinctive and the central location of the stem makes it somewhat unique. This plant has numerous white to creamy-yellow flowers in umbels, and the flower stalks can be up to 30 cm in height.

DESCRIPTION

H. bonariensis is a perennial herb, glabrous; with rhizomes, stems creeping and rooting at the nodes (Figure 1). Leaves peltate with orbicular blades, erect petiole, 2–10 cm diameter. Very small yellowish-white flowers, umbels inflorescence, 1–6 cm diam.; rays many, spreading, with the flowers in whorls in the form of interrupted or sometimes branched spikes and clustered at the base of the rays. Solitary peduncle in each knot of the stem, to 40 cm long, but usually shorter, as long as or usually longer than the leaves; pedicels 2–20 mm long; involucral bracts lanceolate, acute. Orbicular, reniform or elliptical fruits, very laterally compressed, 2 mm in diameter, marinated at the base with rudimentary oil channels and without carpophore; stylopodium depressed.

MEDICINAL AND NUTRITIONAL IMPORTANCE of *H. bonariensis*

Obaseki et al. (2016) reported that the leaves of *H. bonariensis* contain several phytochemical classes, including alkaloids, tannins, flavonoids, phenolics, and saponins. Besides that, *H. bonariensis* extract has also been found to contain medicinal properties to treat inflammation, rheumatism, arthritis, memory improvement, and high antioxidant properties (Yusuf et al., 2010).

It is well known for its traditional uses and medicinal properties for the treatment of various kinds of diseases such as tuberculosis, relieving the pains of inflammation, rheumatism and arthritis, to increase brain capacity and for longevity (Masoumian et al., 2011). This herb has its medicinal uses as emetics, diuretics and laxatives (Evans, 1992). Leaves of this plant have band stems has been reported (Florinsiah et al., 2013). Ajani (2017) reported that chronic administration of *H. bonariensis* aqueous leaf extract may not contribute to liver and renal dysfunctions. The study also indicated that when administered even at an acute dose, the extract may not potentiate any significant toxic effect. It has also been reported that *H. bonariensis* possesses significant antioxidant property that may offer protection from galactose induced oxidative damage in both the lens and the liver (Ajani, 2017).

IMPORTANCE OF PHYTOCHEMICAL ANALYSIS

Phytochemicals are chemical compounds produced by plants, generally to help them resist fungi, bacteria and plant virus infections, and also consumption by insects and other animals. Some phytochemicals have been used as poisons and others as traditional medicine. Phytochemicals are generally regarded as research compounds rather than essential nutrients because proof of their possible health effects has not been established yet. Phytochemicals under research can be classified into major categories, such as carotenoids and polyphenols, which include phenolic acids, flavonoids, stilbenes or lignans. Flavonoids can be further divided into groups based on their similar chemical structure, such as anthocyanins, flavones, flavanones, isoflavones, and flavanols. Flavanols are further classified as catechins, epicatechins, and proanthocyanidins. In total, between 50,000 and 130,000 phytochemicals have been discovered.

Phytochemists study phytochemicals by first extracting and isolating compounds from the origin plant, followed by defining their structure or testing in laboratory model systems, such as in vitro studies using cell lines or in vivo studies using laboratory animals. Challenges in that field include isolating specific compounds and determining their structures, which are often complex and identifying what specific phytochemical is primarily responsible for any given biological activity. Some phytochemicals are known phytotoxins that are toxic to humans; for example, aristolochic acid is carcinogenic at low doses. Some phytochemicals are ant nutrients that interfere with the absorption of nutrients. Others, such as some polyphenols and flavonoids, may be pro-oxidants in high ingested amounts. Non-digestible dietary fibers from plant foods, often considered as a phytochemical are now generally regarded as a nutrient group having approved health claims for reducing the risk of some types of cancer and coronary heart disease.

Phytochemical research of a plant includes several aspects extraction of the compounds to be analysed from a sample or specimen. Separation and isolation of them, identification and/or characterization of the isolated compounds, investigation of the biosynthetic routes of a certain molecule, determination or quantitative assessment.

IMPORTANCE OF ANTIMICROBIAL STUDIES

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties. The understanding of the mechanism of antimicrobial action of medicinal plants extracts is the first step in the optimal utilization of these extracts as natural antimicrobial agents. The antibacterial activity of an agent is mainly attributed to two mechanisms, which include interfering chemically with the synthesis or function of vital components of bacteria, and/or circumventing the conventional mechanisms of antibacterial resistance (Khameneh et al., 2019). Plant-derived compounds have displayed more potential applications in combating bacterial infections. Plantderived chemicals are a wide group of chemical compounds that have been found naturally in plants. The extensive existence of these compounds has demonstrated beneficial advantages in terms of antioxidant, antibacterial, and antifungal activities (Khameneh et al., 2019). They can restore the clinical application of older antibiotics by increasing their potency and as a consequent, avoid the development of resistance.

STUDIES ON *H. bonariensis*

Cytotoxic, acute and sub-chronic oral toxicity profile of the hydroethanol extract of *H. bonariensis* leaves is reported by Kaboua et al., (2021). They have reported the presence of alkaloids, tannins, flavonoids and saponosids in of *H. bonariensis*. The study did not reveal any toxicity and concluded leaves are relatively safe toxicologically when administered orally.

Obaseki et al., (2016) investigated the anti-inflammatory properties of *H. bonariensis.* Phytochemical analysis of the extract revealed the presence of saponin, phenol, flavonoid, tannin, terpenoid and sterol. They have identified a number of anti-inflammatory compounds which were: hexadecanoic acid methyl ester, falcarinol and phytol. They reported high antiinflammatory activity in *H. bonariensis*.

Phytochemical investigation of the under-ground parts of *H. bonariensis* is reported by Tabopda et al., (2012) and isolated of five oleanane-type triterpenoid saponins, 3-*O*-{β dglucopyranosyl-(1 → 2)-[α-l-arabinopyranosyl-(1 → 3)]-β-d-glucuronopyranosyl}-21-*O*- (2methylbutyroyl)-22-*O*-acetyl-R1-barrigenol, 3-*O*-{β-d-glucopyranosyl-(1 → 2)-[α larabinopyranosyl-(1 → 3)]-β-d-glucuronopyranosyl}-21-*O*-(2-methylbutyroyl)-28-*O*-acetyl R1barrigenol, 3-*O*-{β-d-glucopyranosyl-(1 → 2)-[α-l-arabinopyranosyl-(1 → 3)]-β dglucuronopyranosyl}-21-*O*-acetyl-R1-barrigenol, 3-*O*-{β-d-glucopyranosyl-(1 → 2)-[α larabinopyranosyl-(1 → 3)]-β-d-glucuronopyranosyl}-R1-barrigenol, and 3-*O*-{β dglucopyranosyl-(1 → 2)-[α-l-arabinopyranosyl-(1 → 3)]-β-d-glucuronopyranosyl}-22-*O*- (2methylbutyroyl)-A1-barrigenol, together with the known saniculoside-R1. Their structures were established by 2D NMR techniques and mass spectrometry. Six compounds were evaluated against two human colon cancer cell lines, HCT 116 and HT-29. Two compounds showed weak cytotoxicity with IC50 24.1 and 24.0, 83.0 and 83.6 μM against HT-29 and HCT 116, respectively.

Hydroalcoholic extract of *H. bonariensis* is reported as pharmacologically active on the cardiac activity (Kaboua et al., 2022). Leaves's hydroalcoholic extract selectively blocks the potassium current IKs and hence concluded it has a class III anti-arrhythmic effect. The dichloromethane extract of *H. bonariensis* showed broad inhibitory activity over exponenetial growth stage of *Chlamydia trachomatis* and *Chlamydia pneumonia* (Entrocassi *et al,* 2021). The 1H-NMR characterization of dichloromethane extract showed a spectrum with characteristic signals of the fatty acid moiety of lipids or

cerebrosides, volatile phenolics, phytosterols, methyl triterpenes signals, and glucose moiety of the cerebrosides.

The volatile oil obtained from the leaves of *H. bonariensis* was analyzed by GC, ChiralGC and GC-MS (Silva et al., 2009). It was identified 14 compounds and the monoterpene (+) limonene (53.6%) and sesquiterpene γ-muurolene (10.5%) were the main components. The allelopathic effects of the oil were evaluated against two seeds, *Lactuca sativa* and *Allium cepa*. The results showed that the oil exhibited inhibition effects in the germination and seedling growth of plants species relative to the control.

The hexane extract of *H. bonariensis* was subjected to a bioactivity-guided fractionation and identified two chemically related dibenzylbutyrolactone lignans – hinokinin and hibalactone with an aim to discover new drug candidates against the protozoan parasite *Trypanosoma cruzi* (Souza et al., 2021). These compounds showed activity against trypomastigote with EC50 values of 17.0 and 69.4 μM, respectively.

The mutagenic effect of aqueous and methanolic extracts obtained from aerial parts (leaves and stem) and roots of *H. bonariensis* were assayed using the *Salmonella typhimurium* assay or Ames test (Florinsiah et al., 2013). They have concluded that extracts were not mutagenic on *S. typhimurium* strains and has the potential to be used as part of traditional medicine.

Ouviña et al. (2021) reported the phenol and carbohydrate profile in *H. bonariensis*. Principal phenols identified in the methanolic and aqueous extracts (infusion) of the aerial parts are chlorogenic acid, caffeic acid, hyperoside, isoquercitrin, quercitrin, quercetin, apigenin and kaempferol. These metabolites contributed to the anti-inflammatory and antioxidant properties of the species. The main polysaccharides identified in this study are galactorhamnans, galacturonans, rhamnogalacturonans and sulfated galactans; they are related to the diuretic, antiinflammatory and immunomodulatory activity of this species.

4. MATERIALS AND METHODS

1. Collection of plant materials

The fresh leaves of *H. bonariensis* were collected from a home orchard located at [redacted] district (Figure 1). Several collections were made at different time intervals to collect the samples. A voucher specimen was prepared and submitted at [redacted] College Herbarium and identity of the plant is confirmed with the help of teacher in charge of the Herbarium. The collected leaves were repeatedly washed with tap water and followed with distilled water to remove adhering particles and dust. Then the leaves are shade dried and then crushed into fine powder using a mixer grinder. Powdered plant material was stored in an air tight container until further use. Various extracts from this powder is used for investigating the phytochemical, antioxidant, nutritive analysis and antimicrobial activity.

2. Extract preparation of the plant material

a) Solvents used

Organic solvents such as Acetone, Ethyl acetate, Chloroform, Ethanol, and Distilled water were used for the extraction of different bioactive compounds.

b) Extraction

About 8g of the powdered sample was transferred into clean screw cap bottles of 50ml capacity. 40ml of various solvents were added separately and soaked for a week with occasional stirring. The obtained extracts were stored at 4^0C in labeled sterile bottles.

3. Preliminary phytochemical analysis

The different extracts were screened for the qualitative analysis of different classes of natural compounds, using the methodology of Sofowora (1982) and Kepem (1986). The major pharmaceutically valuable compounds analyzed and the method adopted for investigation is listed below.

a) Detection of Alkaloids

Extracts were dissolved individually in dil. HCl and filtered and then subjected to the following tests.

1. **Hager's Test**: Filtrates were treated with Hager's reagent (Saturated picric acid solution). Presence of alkaloid is confirmed by the formation of yellow coloured precipitate.

2. **Dragendoff's Test**: Filtrates were treated with I ml of Dragendoff's reagent. Formation of reddish orange precipitation indicated the presence of alkaloids.

b) Detection of Carboxylic acids

1 ml of each of various extracts was separately treated with a few ml of saturated solutions of Sodium bicarbonate. Observation of effervescence (due to liberation of CO_2) indicated the presence of carboxylic acids.

c) Detection of Coumarins

I ml each of alcoholic extracts was treated with alcoholic 10% NAOH solution. Production of dark yellow colour indicated the presence of coumarins.

d) Detection of flavonoids

1. Alkaline reagent Test: Extracts were treated with a few drops of Sodium hydroxide solution. Formation of intense yellow colour which becomes colourless on addition of dilute acid indicates the presence of flavonoids.

2. Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation yellow colour precipitates indicate the presence of flavonoids.

2.5 ml of each of the various extracts were separately dissolved in 1 ml each of alcohol (stock solution) and subjected to the following test.

3. Ferric chloride Test: I ml each of stock solution was added with a few drops of neutral $FeCl_3$, solution, Formation of blackish red indicated the presence of flavonoid.

e) Detection of Phenols

1. The extract (0.5 ml) was dissolved in 5 ml of distilled water. To this, a few drops of neutral Ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

2. **Ferric chloride Test**: A fraction of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black colour.

f) Detection of protein and amino acids

5 ml each of the various extracts were dissolved in 5 ml of water separately and were subjected to the following tests.

1. Biuret test: 1 ml of each of the various extracts was warmed gently with 10% NaOH solution and a drop of diluted $CuSO_4$ solution. Formation of reddish violet colour indicated the presence of proteins and amino acids.

2. Ninhydrin test: 1 ml each of the various extracts was separately treated with a few drops of Ninhydrin solution. Change in colour showed the presence of proteins and amino acids.

g) Detection of Quinones

1.1 ml of the various extracts was separately treated with alcoholic KOH solution. Quinones give colouration ranging from red to blue

2. A small amount of extracts was treated with conc. HCl and observed for the formation of yellow precipitation (or colouration).

h) Detection of Saponins

1. **Froth Test**: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

2. **Foam Test**: 0.5 gm of extracts was shaken with 2 ml of water. If foam produced persists for few minutes indicates the presence of Saponins.

i) Detection of steroids and phytosterols

5 ml of each of various extracts were dissolved in 5 ml of chloroform separately (stock solution) and was subjected to the following test.

Salkowski Test: 1 ml each of conc. H_2SO_4 was added to the stock solution and allowed to stand for 5minutes after shaking. Turning of golden yellow color in the lower layer indicated the presence of steroids and phytosterols.

j) Detection of Tannins

Gelatin Test: Test solution when treated with gelatin solution would give white precipitate indicating the presence of tannins.

5 ml each of the various extracts was dissolved in minimum amount of water separately filtered add filtrate were then subjected to the following test.

a) Ferric chloride Test: To the above filtrate a few drops of FeCl2 solution were added. The colour change indicates the presence of tannins

b) Basic lead acetate test: To the filtrate a few drops of aqueous basic lead acetate solution are added. Formation of reddish brown precipitate indicated the presence of tannins.

h) Detection of xanthoprotein

1ml of various extracts was treated separately with a few drops of conc. HNO_3 and NH_3 solution. Formation of reddish orange precipitation indicated the presence of xanthoprotein.

i) Detection of Terpenoids

An amount of 0.8 g of selected plant samples was taken in a test tube. Then poured 10 ml of ethanol in it, shaken well and filtered to take 5 ml extract. Then add 2 ml of Chloroform were mixed in exact and 3 ml of Sulphuric acid were added in the extract. Formation of reddish brown colour indicates the presence of terpenoids.

j) Detection of Glycosides

a) **Keller killani Test**: Crude extract was mixed with 2 ml of glacial acetic acid containing 1- 2 drops 26 solution of $FeCl_3$. The mixture was then poured into another test tube containing 2 ml of conc. H_2SO_4 .A brown ring at the interphase indicates the presence of cardiac glycosides.

b) **Bromine water Test**: Test solution was dissolved in bromine water and observed for the formation of yellow precipitates to show a positive result of the presence of Glycosides. **k) Detection of Lignins**

Phosphoglucinol with con. HCl was added with the test solution. Formation of pink colour indicates the presence of lignins.

l. Detection of Carotenoids

1g of each sample was extracted with 10 ml of Chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 85% H_2SO_4 was added. A blue colour at the interphase showed the presence of carotenoids

m) Detection of Diterpenes

Copper acetate test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of Diterpenes.

n). Detection of Sugars

5 ml each of the various extracts was dissolved separately in distilled water filtered and then subjected to the following tests.

a) Molisch's Test: To the filtrate a few drops of alcoholic alpha- naphthol and 2 ml of conc. H_2SO_4 were added slowly through the sides of the test tube. Formation of reddish brown precipitate indicated the presence of sugars.

b) Fehling's Test: A small portion of the various filtrate were treated with 1 ml of Fehling's solution 1 and 2 and then heated gently, Change in colour indicated the presence of sugars.

4. Proximate Analysis

a. Determination of Crude fiber

For the determination of Crude fiber, 2 g of moisture and fat free plant material were treated with 200ml of 1.25% H_2SO_4 with 30 min boiling. After filtration and washing, the residue was treated with 1.25% NaOH with 30 min boiling, then filtered, washed with hot distilled water. The residue was dried overnight at 80-100°C and weighed (W1). It was then ignited and the ash weighed (W2). Loss in the weight gives the weight of crude fiber calculated using the formula. % Crude fibre = 100 x m1- m2 /m0, where m1 is the weight of ashing dish with content before ashing, m2 is the weight of ashing dish with content after ashing, m0 is the weight of sample.

b. Estimation of Protein by Lowry's Method

1g of the sample is ground well in 5-10 ml of the buffer using a pestle and mortar. Supernatant was collected after centrifugation and further used for protein estimation. 0.2-1.0 ml of the working standard was pipetted out into a series of test tubes. 0.5 ml of the sample extracts was pipetted out in another test tube. Made up the volume to I ml in all the test tubes. A tube with 1 ml of water served as blank. 5 ml of Reagent C (Alkaline copper solution) is added to each tube including the blank. Mixed well and allowed to stand for 10 min. Then 0.5 ml of reagent D (Folin- Ciocalteau Reagent) added. Mixed well and incubated at room temperature for 30 min. Blue colour was developed and read at 660nm.

c. Estimation of total Carbohydrate content

The carbohydrate content was detected by Anthrone method. Took 1mg of the sample into a boiling tube, hydrolysed by keeping it in a boiling water bath for three hours with 5.0 ml of 2.5 N HCl and cooled to room temperature. Neutralized it with solid Sodium carbonate until the effervescence ceased. Made the volume to 100 ml and centrifuged, collected the supernatant and took 0.5 ml for analysis. Prepared the standard by taking 0.2 -1.0 ml of the working standards, 1.0 ml of water serves as blank and made up the volume to 1.0 ml in all the tubes with distilled water, then added 4.0 ml of Anthrone reagent, heated for eight

minutes in a boiling water bath, cooled rapidly and read the green to dark green colour at 630nm.

d. Estimation of total Lipid content

Estimation of total lipid content in the sample was carried out using the method of Folch et al. (1957). Weighed 2g of the sample into a wide mouthed boiling tube and added 20ml of ethanol: diethyl ether (3:1) mixture to this and stirred well. Then it was kept in a thermostatic water bath for 2 hours at 55^0C and cooled. The contents were then centrifuged at 3000 rpm for 10 minutes and decanted the clear supernatant to a pre-weighted petri dish. The pellet was collected into the boiling tube, added 20ml of ethanol: diethyl ether mixture and again extracted for 2 hours. Centrifuged the contents and supernatant was decanted to the same Petri dish. Added 20ml of chloroform: methanol (1:1) mixture to the residue and extracted for 1 hour at 50^0 C. Centrifuged and decanted the supernatant to the same petri dish was recorded. The quantification of total lipid content was carried out by reducing the weight of Petri dish before extraction (W1) from weight of petri dish after extraction (W2).

Total lipid = W2 – W1

e. Determination of Nutritive value

Nutritive value was calculated as per the formula used by Nile and Khobragade (2009). Nutritive value (4 x percentage of protein) + (9 x percentage of fat) + (4 x percentage of carbohydrate).

5. Quantitative Estimation

a. Determination of total phenolic content

The total phenolic content of extracts was determined using the Folin-Ciocalteau's Phenol reagent. Pipetted out 0.5ml of sample into test tubes. Made up volume in each test tube to 3ml with distilled water. Add 0.5ml of Folin- Ciocaltueau reagent. After 3 minutes, added 2ml of 20% sodium carbonate solution to each test tube. Mixed thoroughly. Place the tubes in boiling water for exactly 1 minute. Cool and measure the absorbance at 650 nm against a reagent blank. The total phenol content was determined using the regression equation x= 0.576y + 0.057 (y is the OD of the sample) based on catechol and expressed the content in mg/g.

b. Determination of Flavonoid content

2.5gm of the sample was mixed with 80% of aqueous methanol and let it kept for 24 hrs. Discarded the supernatant, the residue re-extracted three times with same volume of methanol with Whatman filter paper. Sample filtrate was transferred to a crucible and evaporated to dryness over a water bath. The content in the petri dish is cooled.

c) DPPH Free Radical Scavenging Assay:

The DPPH free radical scavenging assay was determined by the method of Shimada et al. (1992). 0.1mM DPPH (2, 2-diphenyl -1-picrylhydarzil) was prepared in methanol solution. 0.5g of sample was homogenized using 5ml of methanol and centrifuged the contents. The supernatant was collected, different aliquots (0.5 and 1 ml) were prepared and final volume was made up to 1 ml using methanol. To this mixture added 2ml of 0.1mM DPPH solution (control) and reaction mixtures were measured at 517 nm against methanol as blank. The assay was carried out in triplicates. Lesser values of absorbance of the reaction mixture indicate higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the formula.

DPPH Scavenged (%) = ((Absorbance of control – absorbance of test) ÷ (Absorbance of control)) × 100

d) Reducing Power Assay:

Antioxidant capacity as per reducing assay was measured according to a method reported by Oyaizu (1986). 1g of sample was extracted in 10ml phosphate buffer, contents were centrifuged and supernatant was collected for the assay. Volumes of 2.5ml of different concentrations of the extract were mixed with 2.5ml phosphate buffer solution (0.2M, pH = 6.6) and 2.5ml of 1% Potassium ferric cyanide in test tubes. The mixtures were placed in water bath for 20 minutes at 50^0C. After that 2.5 ml of 10% Trichloro acetic acid (TCA) was added and mixed thoroughly. Then 2.5ml was taken from the mixture and added 2.5 ml of distilled water and 0.5ml of 0.1% $FeCl_2$ solution. The reaction mixture was allowed to stand for 10 minutes. Then the absorbance of the mixture was read at 700 nm against phosphate buffer as blank. The assay was carried out in triplicates. Higher values of absorbance of the reaction mixture indicate greater reducing power. Ascorbic acid standard was used as a positive control.

Testing for antimicrobial activity

Bacterial culture used

Klebsiella pneumoniae bacterial cultures are used to investigate the antimicrobial activity of the plant *H. bonariensis*

Extract used for the culture

Chloroform and distilled water

Culture media used

Nutrient agar was used for the culturing of bacteria and the disc diffusion method is used for this culture.

Preparation of discs

Sterile filter paper discs of 6mm diameter (Whatman No.1) were used for the present study. In each sterile disc, 200µl of the plant extracts were added using a 10 µl pipette. Precautions were taken to prevent the flow of the plant extract from the disc's upper surface. This was obtained by applying the condensed extract in small quantities and the discs were allowed for air drying. In the culture also an antibiotics disc (Chloramphenicol, for checking antibiotic susceptibility) is used.

Assay of antimicrobial activity

The antimicrobial activity was assessed using the disc's diffusion assay. For the culture nutritive agar is used as the culturing medium. Chloroform and distilled water extract of the sample was used for this study. The nutrient agar was made up into 100ml and it is melted in the oven. The melted nutrient agar was poured in to the sterilized Petri dish in the laminar air flow. Bacterial culture was spread on air dried nutrient agar plates using sterile cotton swab using sterile forceps, sterile disc loaded with the plant extract was placed on the surface of nutrient agar plates swabbed with bacterial culture. Control was

also maintained by incorporating the respective solvents on sterile discs. Then the plates were incubated at 37± 0.5ºc for 12 to 14 hours. The zone of inhibition was observed.

5. RESULTS AND DISCUSSION

1. Preliminary Phytochemical analysis

The results of preliminary phytochemical screening of various extracts from dried leaves of *H. bonariensis* indicated the presence of selected primary and secondary metabolites (Figure 2 and 3). The result is summarized below in Table 1.

Table 1: Qualitative phytochemical analysis of various extracts of *H. bonariensis.*

Phytochemical constituents	**Ethyl acetate**	**Chloroform**	**Distilled water**	**Ethanol**	**Acetone**
Flavonoids	+	+	+	+	+
Xantho protein	-	+	+	+	+
Phenols	+	-	+	+	+
Tannins	+	-	+	+	+
Steroids & Phytosterols	+	+	-	-	-
Proteins	+	+	+	+	+
Glycosides	+	-	+	-	+
Saponins	+	-	+	-	+
Carboxylic acids	-	+	+	-	-
Terpenoids	+	+	-	+	+
Quinones	-	+	+	+	-

Coumarin	-	+	-	+	-
Carotenoids	+	+	-	+	-
Resins	-	-	+	-	+
Sugars	+	+	+	-	+
Diterpene	+	-	+	+	+

Ethyl acetate extract showed the presence of flavonoid, phenol, tannins, steroids and polysterols, proteins, glycoside, saponines, alkaloids, terpenoids, carotenoids, sugars and diterpenes. Analysis of Chloroform extract displayed the presence of flavonoids, xantho protein, steroids and polysterols, proteins, alkaloids, carboxylic acids, terpenoids,quinones, coumarins, carotenoids and sugars. Distilled water extract revealed the presence of flavonoids, xantho protein, phenols, tannins, proteins, glycosides, saponins, carboxylic acids, quinones, coumarins, resins, sugars and Diterpenes. Ethanol extract showed the presence of flavonoids, phenols, xanthoproteins, phenols, tannins, steroids and polysterols, carboxylic acids, terpenoids, quinones, carotenoids and diterpenes. Acetone extract indicated the presence of flavonoids, phenols, xanthoproteins, tannins, proteins, glycosides, saponins, terpenoids, coumarins, sugars and diterpenes.

Phytochemical profile of *H. bonariensis* observed in the present study is a clear indication of the value of this plant as a medicinal plant. These compounds may be responsible for the pharmacological and physiological potential reported elsewhere (Obaseki et al., 2016). This is the first report on the extensive phytochemical profiling of this plant. A few studies have reported earlier in this regard but mainly focusing on anti-inflammatory activity of hexane extract (Obaseki et al., 2016), a comparative account between *H. bonariensis* and *Centella asiatica,* two taxonomically unrelated generas in the light of recent treatments in APG IV.

Figures

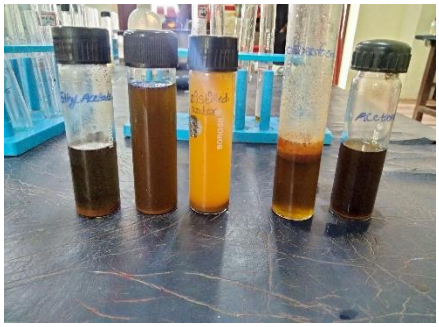

Fig 2: Plant Extract

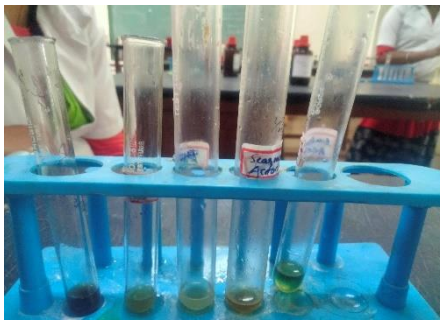

Fig 3: Saponins

Fig 4: Diterpenes

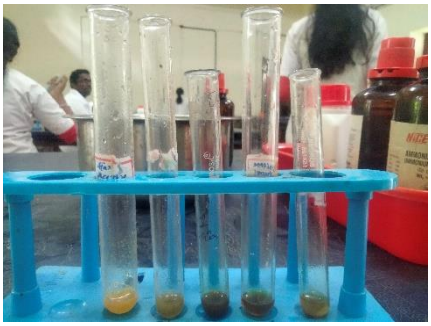

Fig 5: Coumarins

Fig 6: Carboxylic acids

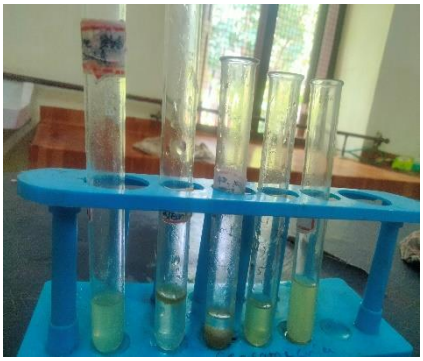

Fig 7: Resin

Fig 8: Xanthophyll

Fig 9: Terpenoids

2. Analysis of the Proximate Composition

Determination of the Crude fiber

Crude fiber is the amount of cellulose and lignin present in plants. The crude fibre content in *H. bonariensis* observed in this study is 6.05%. It is reported that crude fibre helps in the prevention of colon cancer by nourishing the beneficial *Bifidobacterium* in the colon, which enables the bacteria to prevent the growth of harmful bacteria (Slavin, 2013). Crude fibre content reported in *H. bonariensis* is comparable to previous reports in the leaves of other plants like, 6.49% in *Malva sylvestris* (Busuttil-Griffin et al., 2015).

3. Analysis of Nutritional quality.

Protein content

Protein is one of three primary macronutrients (the others being carbohydrate and fat). Macronutrients are the chemical compounds that humans ingest the most of, and which provide us with most of our energy. Proteins consist of amino acids, and are the most commonly found molecules in cells. A large number of proteins isolated from the medicinal plants have been shown to exhibit anti-microbial, anti-oxidant, anti-HIV, anticancerous, ribosome-inactivating and neuro
modulatory activities (Wani et al., 2020). The protein content determined in *H. bonariensis* by Lowry's method is 4.5 mg/g.

Carbohydrate Content

Carbohydrates are the main source of energy for the body. They are the sugars, starches, and dietary fiber that occur in plant parts. The carbohydrates content in *H. bonariensis* is 9.2 mg/g.

Lipid content

Plant lipids are diverse and essential for cells. They are essential for the integrity of cells and organelles by acting as a hydrophobic barrier for the membrane. The lipid content in the leaves of *H. bonariensis* is 2.1%.

Phenolic content

Plant phenolics are generally involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as contributing to plants' colors. They are ubiquitous in all plant organs. The phenol content of *H. bonariensis* estimated in this study is 0.47 mg/g.

Determination of Flavonoid content

Flavonoids possess a number of medicinal benefits, including anticancer, antioxidant, antiinflammatory, and antiviral properties. They also have neuroprotective and cardio-protective effects. The total flavonoid content detected in *H. bonariensis* is 6%.

Determination of Antioxidant activity

Antioxidants are substances that can prevent or slow damage to cells caused by free radicals, unstable molecules that the body produces as a reaction to environmental and other pressures. The total antioxidant capacity was measured as the cumulative capacity of the compounds present in the sample to scavenge the free radicals by using DPPH. The antioxidant activity of *H. bonariensis* was determined using methanol solution of DPPH and is 6%.

Determination of Reducing Power Assay

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm. The reducing power assay of *H. bonariensis* is find out as 1.167mg/g.

ANTIMICROBIAL ACTIVITY

Antimicrobial activity of *H. bonariensis* was tested against the bacterial culture *Klebsiella pneumoniae*. *K. pneumoniae* is a gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar. The plant extracts was made up of distilled water and Chloroform and the method obtained is disc diffusion method. The disc are prepared using Whatman filter nutrient agar is used as the culture medium. As shown in Figure. , antibacterial activity is not onserved in this study. But a detailed antibacterial activity studies using different microorganisms is warranted for a conclusion.

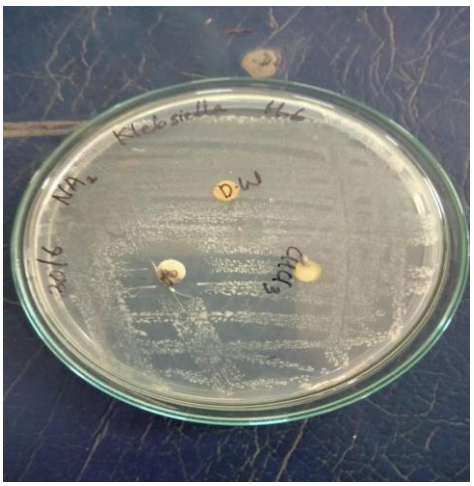

Figure 10: Plate showing the result of antibacterial activity of *H.bonariensis* against *Klebsiella pneumoniae*.

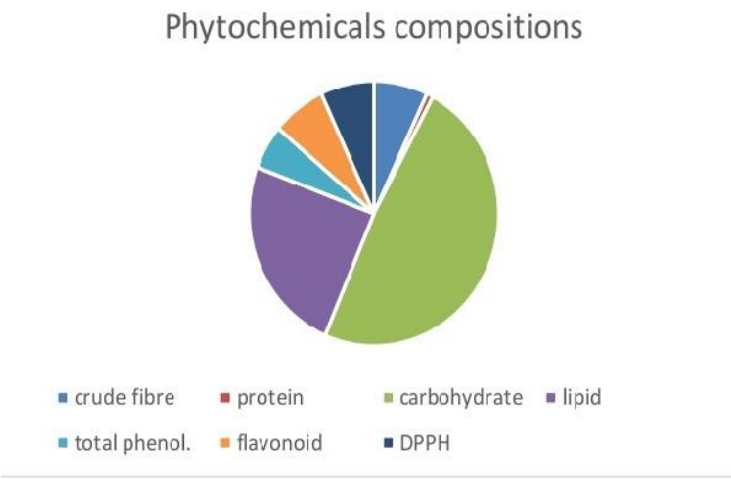

Figure 11: Pie chart showing the phytochemical profile of *H. bonariensis*

The world is rich with natural and unique medicinal plants. Medicinal plants are now getting more attention than ever because they have potential of myriad benefits to society or indeed to all mankind, especially in the line of medicine and pharmacological. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladun et al., 2007). Some of the most important bioactive Phytochemical constituents are alkaloids, essential oils, Flavonoids, tannins, terpenoid, saponins, phenolic compounds and many more (Edeoga et al., 2005). These natural compounds formed the foundations of modern prescription drugs as we know today (Goh et al., 1995). Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers, leaves and roots that work with nutrients and fibers to act as an defense system against disease or more accurately, to protect against disease. Phytochemicals are divided into two groups, which are primary and secondary constituents; according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids and phenolic compounds (Krishnaiah et al., 2007) and many more such as lavonoids, tannins and so on.

Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine (Ang-Lee et al., 2001; Goldman, 2001). In India and China, herbal medicines are still widely used, and developed countries have rediscovered many of these

Traditional medicines as cheap sources of complex bioactive compounds (Phillipson, 1994). Modern pharmaceuticals are typically oral dosage Forms containing single synthetic chemicals that have potent clinical activity. However, natural products from higher plants continue to be used in pharmaceutical preparations either pure or as extracts (Gogtay et al., 2002). Despite the availability of different approaches for the Discovery of therapeutic agents, natural products still remain as one of the best reservoirs of new structural types (Hostettmann, 1999). To this effect, in the constant effort to improve the efficacy and ethics of modern medicinal practice, researchers are increasingly turning their Attention to folk medicine as a source of new drugs (Wayne, 1998; Hoareau & Dasilva, 1999). Phytochemicals are a powerful group of compounds, belonging to secondary metabolites of plants and including a diverse range of chemical entities such as polyphenols, flavonoids, steroidal saponins, Organosulphur compounds, and vitamins.

They have important roles in plant development, being part of relevant physiological process, i.e., Reproduction, symbiotic association, and interactions with other organisms and the environment. Though most of these compounds occur constitutively, their synthesis can be enhanced under stress conditions, in a manner dependent on the growth conditions and on stress. In plants, following the exposure to stressful conditions, an oxidative burst may cause an imbalance between ROS production and scavenging, leading to the activation of reactive antioxidant enzymatic and non-enzymatic responses. The first one includes changes in the activity of antioxidant enzymes, such as superoxide dismutase, peroxidases, and catalase, while the non-enzymatic response is related to the synthesis of low molecular (ascorbic acid, glutathione, carotenoids, phenolic acids, flavonoids, and others) and high molecular weight antioxidants (tannins).Many of these plant metabolites have been tested on animal and human cells, showing very interesting biological activities. They have been shown to be useful in pharmaceutical applications and in cosmetics, nutrition, and dietary supplements. Plants have been always considered as source of food and medical compounds actually, up to 200 species are considered as medicinal plants and about 25% of the medicines have plants origins. The first antioxidant molecule discovered is ascorbic acid, i.e., vitamin C, that is, produced during aerobic metabolism, and reacts rapidly with , singlet oxygen and ozone (chemically), and H2O2 (enzymatically) through ascorbate peroxidase to neutralize their toxic effects. Besides this, in plants, such acid is also involved in the regeneration of

carotenoids and vitamin E (tocopherol).activity of antioxidant enzymes, such as superoxide dismutase, peroxidases, and catalase, while the non-enzymatic response is related to the synthesis of low molecular (ascorbic acid, glutathione, carotenoids, phenolic acids, flavonoids, and others) and high molecular weight antioxidants (tannins).Many of these plant metabolites have been tested on animal and human cells, showing very interesting biological activities .

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Indian system of medicine such as ayurveda and siddha uses majority of the crude drugs that are of plant origin .it is necessary that standards have to be laid down to control and check the Identity of the plant and as certain it's quality before use.A detailed pharmacognostic evaluation therefore it's highly essential prerequisite (Ramana,2007).Moreover,the antibiotic usage for the prevention and treatment of microbial infections resulting in the emergence and spread Antibiotic usage for the prevention and treatment of microbial infections resulting in the emergence and spread of resistant organisms.Many organisms acquire several resistance mechanism making them multi drug resistant (MDR) (Kenneth ,2009).

The presence of antifungal and antimicrobial in the higher plant is well establishes as they have provided a source of inspiration for novel drug compounds as plants derived medicines have made system, providing protection against lipid peroxidation . alI Indian system of medicine such as ayurveda and siddha uses majority of the crude drugs that are of plant origin .it is necessary that standards have to be laid down to control and check the identity of the plant and as certain it's quality before use. A detailed

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The presence of antifungal and antimicrobial in the higher plant itis well establishes as they have provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health. Phytomedicines have significant contribution towards human health. Phytomedicines have been used for the treatment of diseases as in done in cases of Unani and Ayurvedic system of medicines, natural blueprint for the development of new drugs. The potential for developing antimicrobial from higher plants appears rewarding, as it will lead to the development of a phytomedicine to act against microbes .plant based antimicrobials have enormous therapeutic potential as they can survive the purpose without any side effects that are often associated with synthetic antimicrobials, continued further research and exploration of plant derived antimicrobial test form the basis.

Many of the studies were useful in identifying the active principle responsible for such potentials and develop clinically important therapeutic drugs for mankind .The ethnobotany can provide new effective pharmaceutical alternatives to existing drugs because of the emergence of drugs, resistant strains of many infections microorganisms. Medicinal plants does many potentially valuable therapeutic agents .our traditional folklore medications revels the been used for the treatment of diseases as in done in cases of Unani and Ayurvedic system of medicines, natural blueprint for the development of new drugs.

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provide new effective pharmaceutical alternatives to existing drugs because of the emergence of drugs, resistant strains of many infections microorganisms. Medicinal plants are potentially valuable therapeutic agents and our traditional folklore medications revels the identification of some novel treatment modalities (Locher et al., 1995).Moreover, many organisms acquire several resistance mechanisms against microorganisms. Pathogens that have become resistance to currently available agents are one tactics for compacting resistant organisms (Kenneth, 2009).

The studies of phytochemical and antimicrobial activity of *Hydrocotyle bonariensis* revealed the presence of the phytochemical consistent in order Ethyl acetate reveals the presence of Flavonoid, phenol, tannins, sterols and polysterols, proteins, Glycoside, saponines, slkaloids, terpenoids, carotenoids, sugar and diterpene. Chloroform reveals the presence of flavonoid, xanthoprotein, sterolss and polysterols, protein, alkaloids, carboxylic acid, terpenoids, quinones, coumarins, carotenoids and sugar. Distilled water reveals the presence of flavonoid, xantho protein, phenol, Tannins, Protein, Glycoside, saponine, carboxylic acids, quinones, coumarins, resin, sugar and Diterpene. Ethanol reveals the presence of flavonoid, phenol, xanthoproteins, phenol, tannins, sterols and polysterols, carboxylic acids, terpenoids, quinones, carotenoids, diterpene and acetone reveals the presence of flavonoid, phenol, xanthoproteins, tannins, protein, glycoside, saponine, terpenoids, coumarins, sugar and diterpene. *H. bonariensis* showed no antimicrobial property against *Klebsiella pneumoniae*. Anti-inflammatory property of *H. bonariensis* is detected in this study and the phytochemicals identified could serve as lead compounds for designing anti-inflammatory drugs.

6. SUMMARY AND CONCLUSION

The leaves of the *Hydrocotyle bonariensis* Comm ex Lam were collected and shade dried. Dried plant materials were powdered and used for extraction by percolation method employing various solvents like Ethyl acetate, Chloroform, Distilled water, Ethanol and Acetone. These extracts were subjected to qualitative phytochemical screening and proximate analysis. The antimicrobial activity of dried leaves of *H. bonariensis* was also evaluated using the bacterial culture *Klebsiella pneumoniae*.

The preliminary phytochemical analysis of *H. bonariensis* was elucidated the chemical profile of the plant. The Ethyl acetate extract of the dried leaves contained phytochemicals such flavonoid, phenol, tannins, sterols and polysterols, proteins, glycosides, saponines, alkaloids, terpenoids, carotenoids, sugar and diterpene. The chloroform extract showed the presence of flavonoids, xanthoproteins, sterols and polysterols, proteins, glycosides, alkaloids, carboxylic acids, terpenoids, quinones, coumarins, carotenoids and sugar. Distilled water extract contained flavonoids, xanthoprotein, phenols, tannins, proteins, glycoside, saponines, carboxylic acids, quinones, coumarins, resin, sugars and diterpenes. Analysis on ethanol extract displayed the presence of flavonoids, phenols, xanthoproteins, phenols, tannins, sterols and polysterols, carboxylic acids, terpenoids, quinones, carotenoids and diterpenes. Acetone extract showed the presence of flavonoids, phenols, xanthoproteins, tannins, proteins, glycosides, Saponines, terpenoids, coumarins, sugars and diterpenes. Interestingly the flavonoids and proteins are found in all the five extract used in this study. The blue print of phytochemical profile revealed in this study will be an excellent resource for the future exploitation of this plant.

DPPH assay and reducing power assay indicated a significant level of antioxidant property in *H. bonariensis*. The Antimicrobial activity was also performed in the shade dried leaves of *H. bonariensis* using disc diffusion method and the result showed no antimicrobial activity against *Klebsiella pneumonia*. Further study using more microorganisms is warranted for a conclusion.

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