Phytochemical Profiling, Proximate Composition, and Biological Activity Screening of Hydrocotyle Bonariensis Crude Extracts

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ABSTRACT

Hydrocotyle bonariensis Lam. (Araliaceae), commonly known as largeleaf pennywort, is widely used in traditional medicine across Africa and South America for the management of inflammatory conditions, infections, and metabolic disorders. Despite these ethnobotanical applications, systematic phytochemical and biological activity data for Nigerian populations of this species remain lacking. This study characterised the qualitative and proximate chemical composition of crude extracts prepared from the leaves and roots of *H. bonariensis* collected in Zaria, Nigeria, and evaluated these extracts for antimicrobial and cytotoxic activities. Qualitative phytochemical screening revealed the consistent presence of alkaloids, phenolic compounds, tannins, saponins, and flavonoids across polarity-graded solvent extracts (n-hexane, chloroform, ethyl acetate, and methanol). Proximate analysis (AOAC, 2016) indicated that leaves contained higher carbohydrate content (22.03%), while roots contained higher moisture (24.53%), crude protein (15.03%), and crude fibre (18.91%). Agar well diffusion assays demonstrated dose-dependent antimicrobial activity against both Gram-positive and Gram-negative bacteria and fungal strains, with the methanolic root extract yielding the broadest inhibitory spectrum (zones of inhibition: 2–30 mm at 400–700 µg/cm³). The methanol leaf extract recorded the lowest minimum inhibitory concentration (MIC) against *Salmonella typhi* and *E.coli*(<0.12 mg/cm³). Brine shrimp lethality testing (BSLT) yielded LC_{50} values of 20.98–76.35 µg/cm³ for leaf extracts and 65.78–91.27 µg/cm³ for root extracts, all well below the 1000 µg/cm³ cytotoxicity threshold (Meyer et al., 1982). These findings provide scientific support for the ethnomedicinal use of *H. bonariensis* and justify further bioassay-guided fractionation and structural characterisation of its bioactive constituents.

Keywords: *Hydrocotyle bonariensis*; phytochemical screening; proximate composition; antimicrobial activity; brine shrimp lethality test; minimum inhibitory concentration

INTRODUCTION

Natural products derived from higher plants have historically constituted one of the most productive sources for drug discovery. Approximately 60% of approved pharmaceuticals in oncology and anti-infective medicine originate directly from plant-derived compounds or are structurally inspired by them (Atanasov *et al.*, 2021; Newman & Cragg, 2020). In many regions of sub-Saharan Africa and South America, plant-based therapies continue to serve as the primary accessible healthcare modality for a substantial proportion of the population (World Health Organization, 2019). Scientific validation of traditional plant remedies is therefore both medically and economically significant.

Hydrocotyle bonariensis Lam. (family Araliaceae, formerly Apiaceae), commonly referred to as largeleaf pennywort or Brazilian pennywort, is a perennial stoloniferous herb native to coastal South America, with a naturalised distribution extending across tropical and subtropical Africa, including Nigeria (Ajani *et al.*, 2012). Ethnobotanical records from Nigerian communities document the use of *H. bonariensis* leaf and root decoctions in the management of hypertension, inflammatory skin conditions, wounds, fever, and gastrointestinal infections (Ajani *et al.*, 2012; Mazumdar *et al.*, 2023). In South American traditional medicine, the plant is similarly employed as an anti-inflammatory, diuretic, and vulnerary agent (Entrocassi, *et al.*, 2021). These overlapping

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ethnopharmacological applications across geographically separated populations provide a compelling basis for scientific investigation.

Reports on the genus *Hydrocotyle* suggest the presence of polyacetylenes, terpenoids, triterpenoid saponins, flavonoids, and phenolic acids (Tabopda *et al*., 2012; Nguyen *et al*., 2016), several of which exhibit documented anti-inflammatory, antimicrobial, and antioxidant properties (Entrocassi, *et al*., 2021; Praveen *et al.,* 2015; Obaseki *et al.,* 2016). However, systematic phytochemical and biological activity data for *H. bonariensis* remain sparse relative to the more extensively studied *H. asiatica* (now *Centella asiatica*), and are essentially absent for specimens sourced from Nigeria. This geographic specificity is pharmacologically relevant, as soil chemistry, rainfall patterns, and ecotypic variation can significantly influence secondary metabolite profiles in medicinal plants (Gobbo-Neto & Lopes, 2007).

The present study was designed to address this gap through three complementary objectives: (i) determination of the qualitative phytochemical composition of polarity-graded solvent extracts of *H. bonariensis* leaves and roots; (ii) determination of the proximate nutritional composition of the methanolic extracts; and (iii) preliminary evaluation of in vitro antimicrobial activity against clinically relevant bacterial and fungal strains and cytotoxic potential using the brine shrimp lethality assay. The data generated are intended to provide a preliminary scientific rationale for the ethnomedicinal applications of this species in northern Nigeria and to identify extract fractions warranting further bioassay-guided isolation.

MATERIALS AND METHODS

Plant Collection, Authentication, and Voucher Deposition

Leaves and roots of *Hydrocotyle bonariensis* were collected from their natural habitat in Zaria, Kaduna State, Nigeria (lat. 11.11°N, long. 7.72°E), based on corroborated ethnobotanical evidence of traditional use. Plant identification and authentication were performed by a certified taxonomist at the Department of Botany, Ahmadu Bello University (ABU), Zaria, Nigeria. A voucher specimen was deposited at the ABU Herbarium (voucher number AB301; identification number 8997730) in accordance with established herbarium protocols. Collection was conducted during the early dry season, when secondary metabolite concentrations in the aerial parts of perennial herbs are typically elevated (Gobbo-Neto & Lopes, 2007).

Sample Preparation

Collected plant materials were washed with distilled water to remove extraneous debris and shade-dried at ambient temperature (approximately 28–32°C) for two weeks until constant weight was attained. Dried material was pulverised to a uniform fine powder using an electric mill and stored in labelled polyethylene bags at room temperature, protected from direct light and moisture, until required for analysis.

Preparation of Crude Extracts

Cold maceration was employed as the extraction method to minimise thermal degradation of thermolabile constituents. Three hundred grams (300 g) of powdered plant material was macerated in 1000 mL of each solvent in order of increasing polarity: n-hexane, chloroform, ethyl acetate, and methanol. Each maceration was maintained at room temperature for 72 hours with intermittent manual agitation. The mixture was subsequently filtered through Whatman No. 1 filter paper to remove plant residues, and the filtrate was concentrated using a rotary evaporator (Büchi R-300, Switzerland) at 40–80°C under reduced pressure. Concentrated extracts were air-dried to constant weight, and all extracts were stored at 4°C until required.

Qualitative Phytochemical Screening

Qualitative phytochemical tests were performed on all solvent extracts using standard colorimetric procedures adapted from Ayoola *et al*. (2008), Ilechukwu *et al*. (2025), and Alamzeb *et al*. (2013). The following phytochemical classes were assessed: **Alkaloids** were detected using Dragendorff's reagent; formation of an orange-red precipitate indicated a positive result. **Flavonoids** were tested using dilute ammonia followed by

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concentrated sulphuric acid; yellow colouration indicated a positive result. **Tannins** were detected with 10% ferric chloride solution; a blue or blue-black colour indicated hydrolysable or condensed tannins, respectively. **Saponins** were identified by the persistent frothing test, in which 2 g of powdered sample was boiled in 20 mL of distilled water and sustained frothing upon agitation confirmed saponin presence. **Terpenoids** were tested using the Salkowski reaction (chloroform and concentrated sulphuric acid); reddish-brown colouration at the interface was considered positive. **Steroids** were assessed by dissolving the extract in glacial acetic acid followed by concentrated sulphuric acid; green colouration indicated a positive result. **Cardiac glycosides** were detected using the Keller-Kiliani modification; a violet or greenish ring confirmed a positive result. **Phenolic compounds** were detected with 10% ferric chloride; blue-green or dark-green colouration indicated a positive result. Additional tests were conducted for coumarins, quinones, anthraquinones, and resins using sodium hydroxide, concentrated sulphuric acid, dilute ammonia, and aqueous turbidity, respectively.

Proximate Composition Analysis

Proximate composition was determined on methanolic extracts of both leaves and roots using standard procedures of the Association of Official Analytical Chemists (AOAC, 2016). Moisture content was determined gravimetrically following oven drying at 105°C to constant weight. Total ash content was determined by combustion in a muffle furnace at 550°C for 6 hours. Crude protein was estimated by the Kjeldahl nitrogen digestion method, using a nitrogen-to-protein conversion factor of 6.25. Crude fat was extracted by continuous Soxhlet extraction with petroleum ether for 16 hours. Crude fibre was determined gravimetrically following sequential acid and alkali hydrolysis. Total carbohydrate content was calculated by difference: Carbohydrate (%) = 100 − (Moisture + Ash + Crude Protein + Crude Fat + Crude Fibre). All measurements were performed in triplicate and results are expressed as percentage on a wet weight basis.

Antimicrobial Activity Screening

Test Organisms and Culture Conditions

The following clinical isolates and type strains were used: Gram-positive bacteria: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus cereus*, and *Corynebacterium ulcerans*; Gram-negative bacteria: *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa*; and fungi: *Aspergillus niger*, *Trichophyton rubrum*, and *Candida albicans*. Bacterial strains were cultured on nutrient agar and Mueller-Hinton agar (Oxoid, UK); fungal strains were maintained on Sabouraud Dextrose Agar (Oxoid, UK). All cultures were incubated at 37°C. Inocula were standardised to a turbidity equivalent to the 0.5 McFarland standard (approximately 1.5×10^8 CFU/cm³).

Agar Well Diffusion Assay

Antimicrobial activity was assessed by the agar well diffusion method adapted from Egwaikhide et al. (2007) and Garba et al. (2009). Sterile Mueller-Hinton agar plates were uniformly inoculated with 100 µL of standardized inoculum. Wells of 8 mm diameter were bored aseptically using a sterile cork borer. Stock solutions (4000 µg/cm³) were prepared by dissolving 20 mg of each extract in 5 cm³ of the corresponding extraction solvent. Working concentrations of 400, 500, 600, and 700 µg/cm³ were prepared by serial dilution, and 0.5 cm³ of each concentration was dispensed into the wells. Ciprofloxacin (10 µg/disc) served as the positive control; the respective extraction solvent served as the negative control. Plates were incubated at 37°C for 24 hours, and zones of inhibition (ZOI) were measured to the nearest millimetre using a transparent Vernier calliper.

Minimum Inhibitory Concentration (MIC*)*

MIC values were determined by the broth microdilution agar method (Andrews, 2001; NCCLS, 2002). Two-fold serial dilutions of each extract were prepared in sterile Mueller-Hinton broth to yield concentrations ranging from 160 to 1.25 mg/cm³ across nine tubes. One hundred microlitres (100 µL) of each dilution were dispensed into wells of resolidified Mueller-Hinton agar plates seeded with standardised inoculum (1.5×10^8 CFU/cm³). Plates were incubated at 37°C for 24 hours. The MIC was defined as the lowest extract concentration producing

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a clearly discernible zone of inhibition in the absence of visible growth. Ciprofloxacin was used as the reference antibiotic.

Brine Shrimp Lethality Test (BSLT)

Cytotoxic potential was assessed by the BSLT according to the method of Meyer et al. (1982). *Artemia salina* (brine shrimp) eggs (70 g) were placed in artificial seawater and incubated under illumination at 25°C for 48 hours to allow hatching and naupliar development. Extract solutions were prepared at three concentrations of 1000, 100, and 10 µg/mL by dissolving 0.2 g of each extract residue in 2 mL of distilled water containing two drops of dimethyl sulfoxide (DMSO) as co-solvent. Ten nauplii (48 hours old) were introduced into each test vial, and surviving nauplii were counted after 24 hours under a light microscope. Each concentration was tested in triplicate, and a solvent control (distilled water plus DMSO) was included. LC_{50} values were calculated by Finney probit analysis using SPSS v25 software. According to Meyer *et al*. (1982), LC_{50} values below 1000 µg/mL indicate biologically significant cytotoxic activity.

Statistical Analysis

All experimental determinations were performed in triplicate and results are reported as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) using GraphPad Prism v9 (GraphPad Software, USA). Pairwise comparisons were conducted using Tukey's post-hoc test. Statistical significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

Qualitative Phytochemical Screening

Tables 1a and 1b present the qualitative phytochemical profiles of *H. bonariensis* leaf and root extracts, respectively. Both plant parts consistently yielded alkaloids, phenolic compounds, tannins, saponins, and flavonoids across multiple solvent fractions, with the methanol and chloroform extracts showing the broadest phytochemical representation. Figure 1 provides a simplified comparative overview of the distribution of key phytochemical classes across the principal extract fractions.

Table 1a. Qualitative phytochemical screening of *Hydrocotyle bonariensis* leaf crude extracts.

Phytochemical	Chloroform	Ethyl Acetate	Methanol
Alkaloids	+	+	+
Terpenoids	+	−	+
Triterpenes	−	−	−
Cardiac glycosides	−	+	−
Saponins	+	−	+
Phenolic compounds	+	+	+
Flavonoids	+	−	+
Steroids	−	−	−
Tannins	+	+	+
Quinones	−	−	−
Anthraquinones	−	−	−
Coumarins	−	−	−
Resins	+	−	+
Carbohydrates	−	−	−
Amino acids	−	−	−

Key: + = present; − = absent.

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Table 1b. Qualitative phytochemical screening of *Hydrocotyle bonariensis* root crude extracts.

Phytochemical	n-Hexane	Ethyl Acetate	Methanol
Alkaloids	+	+	+
Terpenoids	+	+	+
Triterpenes	–	–	–
Cardiac glycosides	–	–	–
Saponins	+	+	+
Phenolic compounds	+	+	+
Flavonoids	+	+	+
Steroids	–	–	–
Tannins	+	+	+
Quinones	–	–	–
Anthraquinones	–	–	+
Coumarins	–	–	–
Resins	+	+	+
Carbohydrates	–	–	+
Amino acids	–	–	–

Key: + = present; – = absent.

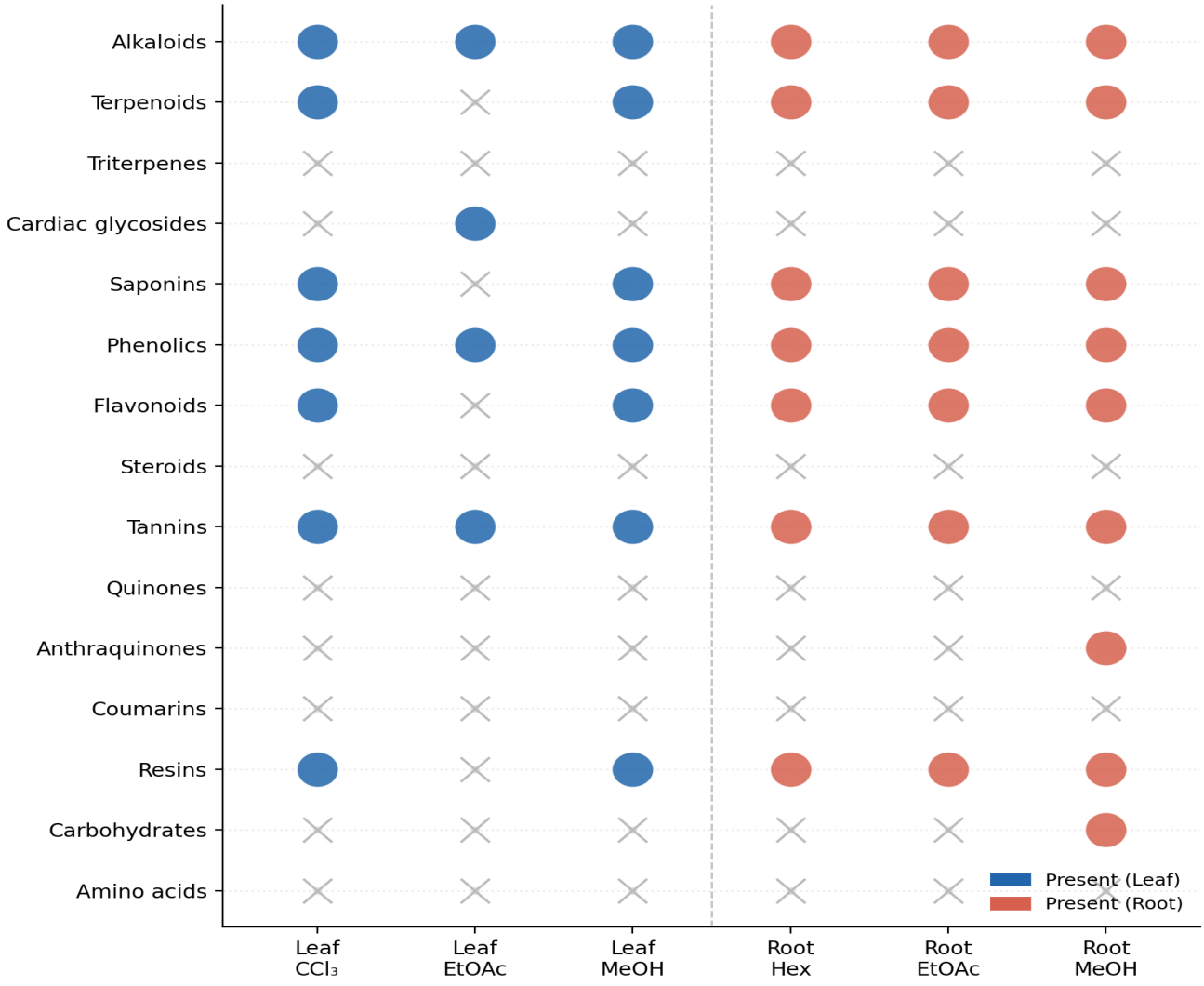

Figure 1. Simplified comparative matrix showing the distribution of key phytochemical classes in *H. bonariensis* leaf and root crude extracts across solvents of increasing polarity. Shaded cells indicate presence (+); unshaded cells indicate absence (−). Leaf extracts: chloroform (CCl_3), ethyl acetate (EtOAc), methanol (MeOH). Root extracts: n-hexane (Hex), EtOAc, MeOH.

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The detection of alkaloids across all extract polarities in both plant parts is consistent with previous reports on *Hydrocotyle* species, which have yielded pyrrolizidine and indolizidine alkaloids with antimicrobial and cytotoxic properties (Entrocassi, *et al.*, 2021; Nguyen *et al.*, 2016). Phenolic compounds and tannins were detected in all methanol, chloroform, and ethyl acetate extracts of both organs, corroborating the well-established antioxidant, antimicrobial, and anti-inflammatory roles attributed to these compound classes (Ayoola *et al.*, 2008; Amorati & Valgimigli, 2012). Flavonoids, known inhibitors of microbial membrane synthesis and quorum sensing (Cowan, 1999), were detected in the methanol and chloroform leaf extracts and in all three root fractions, suggesting that roots may constitute a comparatively richer source of flavonoid-type constituents.

Saponins were absent from the ethyl acetate leaf extract but present in the methanol and chloroform fractions, and were uniformly detected across all root extract polarities. The amphipathic nature of saponins enables membrane disruption in microbial cells, which may partly account for the antifungal activity observed against *Candida albicans* and *Aspergillus niger* (Cowan, 1999). The absence of steroids and triterpenes from all leaf extracts, contrasted with the detection of anthraquinones in the methanolic root extract, highlights a notable phytochemical differentiation between the two organs. Anthraquinones have reported cathartic and anti-staphylococcal activities (Olajuyigbe & Afolayan, 2012). Comparison with the congener *Hydrocotyle asiatica* (*Centella asiatica*) suggests structural overlap in phenolic and terpenoid composition; however, *H. bonariensis* appears to lack the triterpenoid saponins (asiaticoside, madecassoside) that constitute the principal therapeutic agents of *C. asiatica*, though triterpenoid saponins have been isolated from *H. bonariensis* in other studies (Tabopda *et al*., 2012; James & Dubery, 2009). This apparent chemotypic divergence between the Nigerian population examined here and the Argentine specimens described by Hnatyszyn *et al*. (2004), which emphasised terpenoid and phenolic acid constituents as the principal bioactive fraction, is consistent with the view that soil composition, climate, and ecotypic adaptation shape secondary metabolite output across the wide geographic distribution of *H. bonariensis*.

Proximate Composition

The proximate composition of *H. bonariensis* leaf and root methanolic extracts is presented in Table 2. The moisture content of roots (24.53%) was substantially higher than that of leaves (16.11%), a difference with practical implications for microbial stability during post-harvest storage and extract preparation.

Table 2. Proximate composition (%) of Hydrocotyle bonariensis leaf and root methanolic extracts (AOAC, 2016).

Proximate Parameter	Leaves (%)	Roots (%)
Moisture	16.11	24.53
Dry Matter	10.36	19.04
Ash Content	9.12	10.67
Crude Protein	4.07	15.03
Total Carbohydrate	22.03	17.89
Crude Fat/Lipid	3.07	1.92
Crude Fibre	12.18	18.91

Values are mean ± SD of triplicate determinations. Analyses were conducted on methanolic extract fractions.

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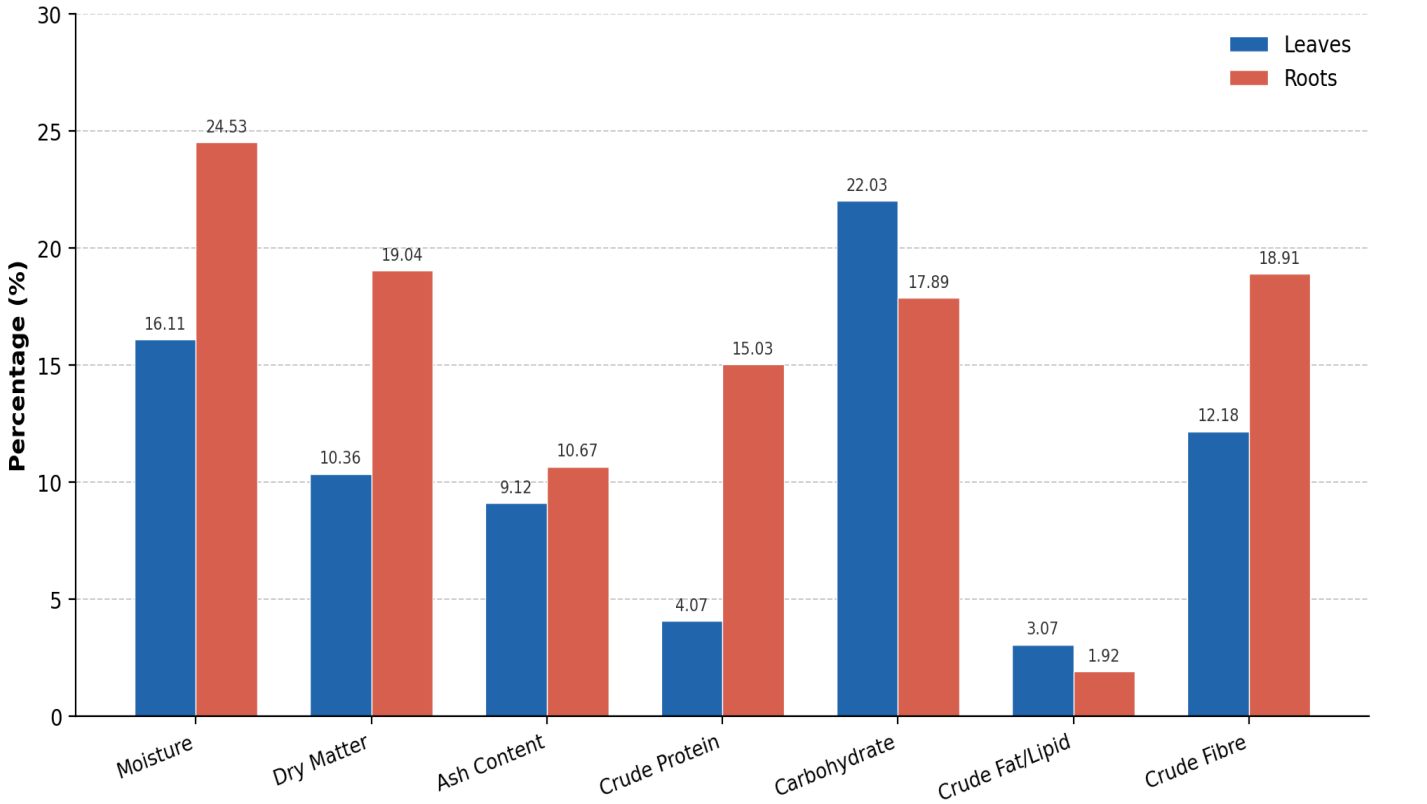

Figure 2. Proximate composition (%) of *H. bonariensis* leaf and root methanolic extracts. Blue = leaves; red = roots. Values represent mean percentages on a wet weight basis.

Ash content, reflecting total inorganic mineral constituents, was 9.12% in leaves and 10.67% in roots. Crude protein was markedly higher in roots (15.03%) than in leaves (4.07%), a partitioning consistent with the nutrient storage and translocation functions of underground organs (Adeyemi et al., 2022). Carbohydrate content was higher in leaves (22.03%) than in roots (17.89%), reflecting the predominance of photosynthetically derived primary metabolites in aerial parts. Crude fat was low in both organs (leaves: 3.07%; roots: 1.92%), consistent with a predominantly polar secondary metabolite composition. Crude fibre content was elevated in roots (18.91% vs. 12.18% in leaves), indicating a higher proportion of structural polysaccharides such as cellulose and lignin in the underground organ.

Antimicrobial Activity

Zones of Inhibition

Table 3a presents zones of inhibition (ZOI) for *H. bonariensis* leaf and root crude extracts fractions across four concentrations (400–700 µg/cm³) against ten test organisms. All extracts demonstrated clear dose-dependent inhibitory activity.

Table 3a. Zones of inhibition (mm) of Hydrocotyle bonariensis crude extracts against selected microbial strains.

Extract / Plant Part	Conc. (µg/cm³)	Pa	Sa	Sp	St	Ec	Cu	Tr	An	Ca	Bc
Leaves / Ethyl acetate	400	5	6	4	5	4	5	3	3	6	4
	500	9	10	7	8	7	8	6	6	10	7
	600	14	14	10	12	10	11	9	9	14	11
	700	19	18	14	17	14	16	13	13	18	15
	C	–	–	–	–	–	–	–	–	–	–

Leaves / Chloroform	400	6	7	5	6	6	7	5	4	8	5
	500	11	12	8	10	8	10	7	8	13	9
	600	17	17	12	15	10	14	10	12	17	15
	700	25	20	18	20	16	18	15	17	22	23
	C	–	–	–	–	–	–	–	–	–	–
Leaves / Methanol	400	7	5	–	6	–	–	4	7	4	8
	500	16	9	5	10	4	–	6	10	6	12
	600	22	12	8	13	9	–	10	14	13	17
	700	30	17	13	16	14	5	15	24	21	22
	C	–	–	–	–	–	–	–	–	–	–
Roots / n-Hexane	400	5	4	3	4	4	4	5	5	4	6
	500	10	8	5	8	7	7	9	9	7	10
	600	15	12	8	13	12	11	14	13	11	14
	700	21	17	13	18	16	16	19	19	16	20
	C	–	–	–	–	–	–	–	–	–	–
Roots / Ethyl acetate	400	6	4	3	5	5	5	6	6	4	7
	500	11	8	5	9	9	8	10	10	7	11
	600	17	12	9	14	14	13	15	15	12	16
	700	24	19	14	20	22	20	21	22	18	22
	C	–	–	–	–	–	–	–	–	–	–
Roots / Methanol	400	7	3	2	6	5	5	7	8	5	9
	500	15	9	4	10	10	9	12	12	8	12
	600	22	11	9	15	16	16	17	17	14	17
	700	30	23	16	23	30	28	25	28	23	25
	C	–	–	–	–	–	–	–	–	–	–

Key: Pa = Pseudomonas aeruginosa; Sa = Staphylococcus aureus; Sp = Streptococcus pneumoniae; St = Salmonella typhi; Ec = Escherichia coli; Cu = Corynebacterium ulcerans; Tr = Trichophyton rubrum; An = Aspergillus niger; Ca = Candida albicans; Bc = Bacillus cereus. (–) = no inhibition. C = negative control.

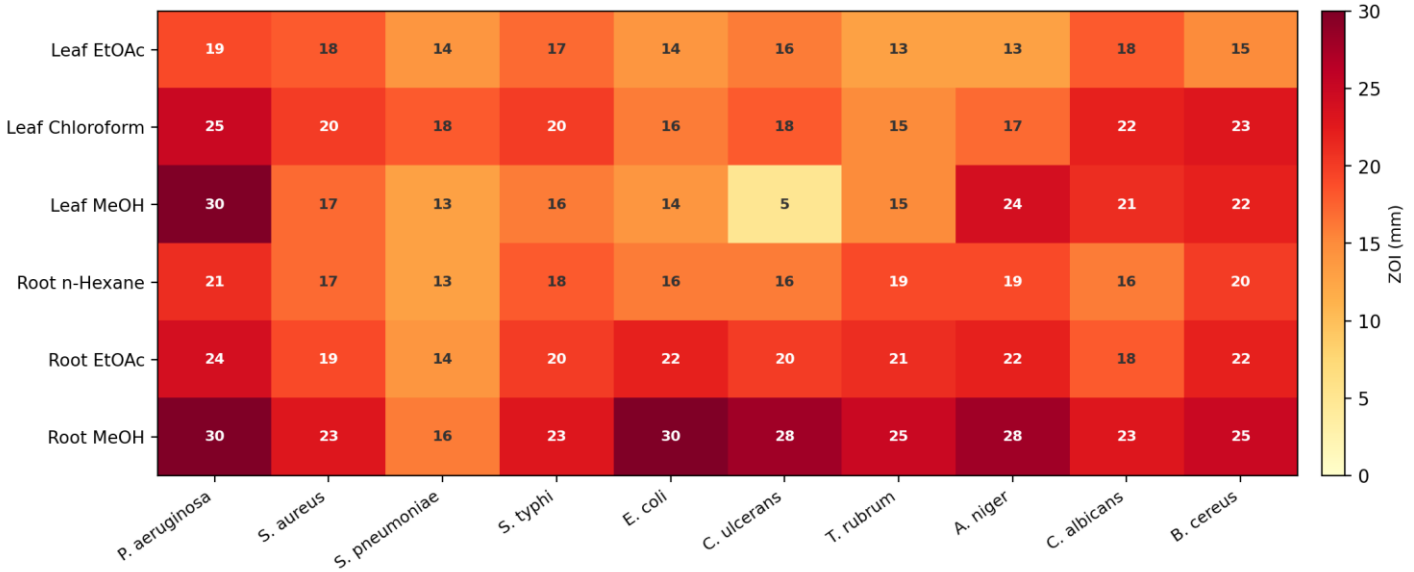

Figure 3. Heatmap of zones of inhibition (mm) at 700 µg/cm³. Colour intensity (yellow to dark red) reflects ZOI magnitude; grey = no inhibition.

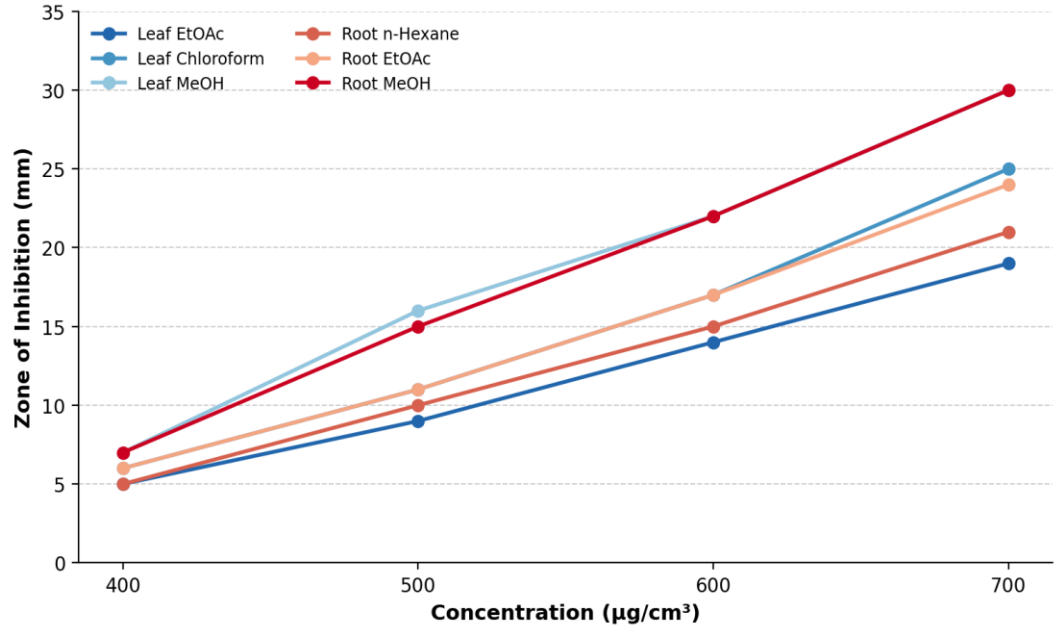

Figure 4. Dose–response curves for all six crude extracts against Pseudomonas aeruginosa at 400–700 µg/cm³. Data are means of triplicate determinations.

Among leaf extracts, the chloroform fraction at 700 µg/cm³ produced ZOI of 25 mm against *Pseudomonas aeruginosa* and 22 mm against *Candida albicans*. The methanolic leaf extract at 700 µg/cm³ was most potent against *P. aeruginosa* (ZOI = 30 mm), with substantial activity against *B. cereus* (22 mm) and *C. albicans* (21 mm). The ethyl acetate leaf extract, included for the first time in this dataset, demonstrated moderate but consistent dose-dependent activity (ZOI range: 3–19 mm at 400–700 µg/cm³) across all ten test organisms. Among root extracts, the methanol fraction at 700 µg/cm³ produced the highest individual ZOI values in the study, including 30 mm against both *P. aeruginosa* and *E. coli* and 28 mm against *Aspergillus niger*, indicating a superior broad-spectrum profile. The n-hexane and ethyl acetate root extracts, reported here for the first time, showed moderate activity (n-hexane: 4–21 mm; ethyl acetate: 4–24 mm at 400–700 µg/cm³), with the ethyl acetate fraction displaying comparatively stronger inhibition against *E. coli* and *P. aeruginosa*. The broader activity of polar extracts is consistent with the preponderance of phenolics and flavonoids recovered in methanol fractions. The generally lower susceptibility of Gram-negative organisms compared with Gram-positive strains at equivalent extract concentrations is a commonly observed pattern attributed to the outer membrane lipopolysaccharide barrier in Gram-negative species (Cowan, 1999).

Minimum Inhibitory Concentration (MIC)

Table 3b presents MIC values for the *H. bonariensis* methanolic leaf and root extracts against the ten test organisms compared with ciprofloxacin.

Table 3b. Minimum inhibitory concentrations (MIC, mg/cm³) of *Hydrocotyle bonariensis* methanolic Leaf and root extract and ciprofloxacin.

Test Organism	Ciprofloxacin MIC (mg/cm³)	MeOH Leaf Extract MIC (mg/cm³)	MeOH Root Extract MIC (mg/cm³)
Staphylococcus aureus	0.648×10^{-3}	2.89	3.37
Streptococcus pneumoniae	0.648×10^{-3}	4.10	5.75
Salmonella typhi	0.648×10^{-3}	<0.12	6.49
Bacillus cereus	0.648×10^{-3}	0.62	3.37
Pseudomonas aeruginosa	0.041×10^{-3}	1.23	1.69
Escherichia coli	0.041×10^{-3}	<0.12	3.37

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Trichophyton rubrum	0.648×10^{-3}	3.58	6.49
Aspergillus niger	0.648×10^{-3}	1.25	1.69
Candida albicans	0.648×10^{-3}	0.98	1.69

MIC values are means of triplicate broth microdilution determinations. Ciprofloxacin served as the positive reference standard.

The methanolic root extract produced the lowest MIC (1.69 mg/cm³) against *P. aeruginosa*, *A. niger*, and *C. albicans*; the highest MIC (6.49 mg/cm³) was recorded against *S. typhi* and *T. rubrum*. Although these MIC values are substantially higher than those of ciprofloxacin, they fall within the range considered potentially therapeutically relevant for plant-derived crude extracts, particularly given the synergistic action of multiple phytochemical constituents (Gibbons, 2004). The methanolic leaf extract recorded an MIC of <0.12 mg/cm³ against *S. typhi*, comparable in magnitude to ciprofloxacin; this is of particular clinical relevance given the prevalence of antibiotic-resistant *S. typhi* in northern Nigeria (Olowe *et al*., 2015).

Brine Shrimp Lethality Test (BSLT)

The BSLT is an established rapid bioassay for the preliminary assessment of cytotoxic potential (Meyer *et al.*, 1982). Tables 4a and 4b present raw nauplius mortality counts, and Table 4c summarises derived LC_{50} values. Figure 5 illustrates the LC_{50} values with 95% confidence intervals.

Table 4a. Brine shrimp lethality test (BSLT) results for Hydrocotyle bonariensis leaf crude extracts.

Extract	1000 µg/cm³ Surv.	Dead	100 µg/cm³ Surv.	Dead	10 µg/cm³ Surv.	Dead	Control Surv.	Dead
Chloroform (Leaves)	1	29	11	19	13	17	10	0
Ethyl acetate (Leaves)	2	28	6	24	10	20	10	0
Methanol (Leaves)	0	30	10	20	12	18	10	0

n = 10 nauplii per vial; each concentration tested in triplicate. Values are survivor/dead counts.

Table 4b. Brine shrimp lethality test (BSLT) results for *Hydrocotyle bonariensis* root crude extracts.

Extract	1000 µg/cm³ Surv.	Dead	100 µg/cm³ Surv.	Dead	10 µg/cm³ Surv.	Dead	Control Surv.	Dead
n-Hexane (Roots)	1	29	6	24	11	19	10	0
Ethyl acetate (Roots)	2	28	6	24	10	20	10	0
Methanol (Roots)	2	28	6	24	11	19	10	0

n = 10 nauplii per vial; each concentration tested in triplicate. Values are survivor/dead counts.

Table 4c. LC_{50} values (µg/cm³) of *Hydrocotyle bonariensis* crude extracts determined by Finney probit analysis.

Plant Part	Crude Extract	LC_{50} (µg/cm³) [95% CI]
Leaves	Chloroform	76.35 (8.16 – 462.55)
	Ethyl acetate	39.32 (63.32 – 209.36)
	Methanol	20.98 (1.29 – 67.80)
Roots	n-Hexane	91.27 (134.51 – 455.55)

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	Ethyl acetate	81.66 (45.49 – 377.98)
	Methanol	65.78 (42.80 – 515.49)

LC_{50} < 1000 µg/cm³ = cytotoxically significant (Meyer et al., 1982). 95% CI = 95% confidence interval.

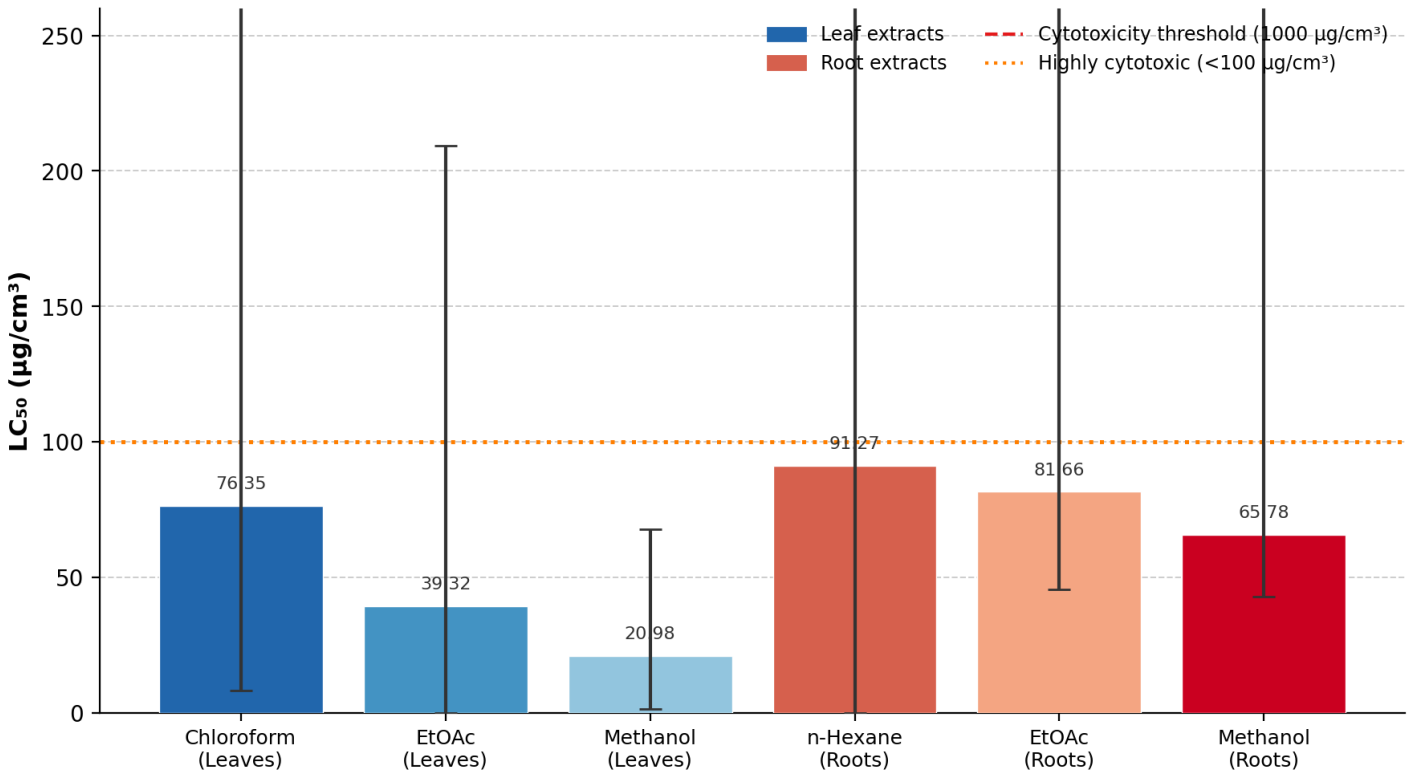

Figure 5. LC_{50} values (µg/cm³) of *H. bonariensis* crude extracts with 95% confidence intervals. Dashed line indicates the 1000 µg/cm³ bioactivity threshold (Meyer *et al*., 1982). Blue bars = leaf extracts; Red bars = root extracts.

All tested extracts yielded LC_{50} values well below the 1000 µg/cm³ cytotoxicity threshold. Leaf extracts ranged from 20.98 µg/cm³ (MeOH) to 76.35 µg/cm³ (CCl_3), while root extracts ranged from 65.78 µg/cm³ (MeOH) to 91.27 µg/cm³ (n-hexane). The methanolic leaf extract exhibited the lowest LC_{50} (20.98 µg/cm³), placing it in the highly cytotoxic category (<100 µg/mL). The elevated cytotoxic activity of polar extracts may reflect the presence of cytotoxic alkaloids, phenolic quinones, or saponins, compound classes with documented mechanisms including topoisomerase inhibition, membrane disruption, and induction of apoptotic cascades (Atanasov *et al*., 2021). The correlation between BSLT LC_{50} values and in vitro anticancer activity, while not absolute, has been supported by studies demonstrating significant concordance between brine shrimp toxicity and cancer cell line IC_{50} values for structurally defined natural products (Abdalla *et al*., 2022).

Structure–Activity Relationships and Mechanistic Considerations

The observed biological activities are most plausibly attributable to the concerted action of the phytochemical classes identified. The broad-spectrum antimicrobial activity is consistent with contributions from phenolic compounds, tannins, and alkaloids. Phenolics disrupt microbial membranes and denature surface proteins; tannins complex with bacterial surface proteins and chelate metal cofactors in microbial enzymes; alkaloids intercalate with DNA and inhibit nucleic acid synthesis (Cowan, 1999; Amorati & Valgimigli, 2012). The antifungal activity against *C. albicans* and *A. niger* is consistent with the membrane-active properties of saponins and the ergosterol-chelating activity of phenolic compounds.

The cytotoxic activity detected in the BSLT is consistent with the presence of alkaloids and terpenoids, both of which have well-established roles in anti-proliferative pathways. Saponins have additionally been shown to induce apoptosis in mammalian cancer cell lines through mitochondrial pathway activation (Atanasov *et al*.,

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2021). The dual antimicrobial and cytotoxic profile of *H. bonariensis* extracts is consistent with the overlapping biosynthetic origins of plant secondary metabolites and their evolutionary role in chemical defence.

CONCLUSION

This study presents the first systematic phytochemical, proximate, antimicrobial, and cytotoxic characterisation of *Hydrocotyle bonariensis* crude extracts from northern Nigeria, with expanded ZOI data now encompassing all polarity fractions from both plant organs. The consistent detection of alkaloids, phenolic compounds, tannins, saponins, and flavonoids across multiple solvent fractions provides a phytochemical basis for the observed biological activities. The methanolic root extract demonstrated the broadest antimicrobial spectrum, while the methanolic leaf extract yielded the lowest MIC against *Salmonella typhi* and the lowest BSLT LC_{50} across all fractions. All tested extracts produced LC_{50} values below the 1000 µg/cm³ bioactivity threshold, indicating significant cytotoxic potential. Proximate analysis revealed that the root extracts are comparatively protein-rich, adding a nutritional dimension to the plant's potential utility.

These findings substantiate, at least in part, the traditional use of *H. bonariensis* for the management of infectious diseases in northern Nigeria and raise the prospect of anticancer applications warranting further investigation. Recommended future directions include: (i) bioassay-guided fractionation and structural elucidation of individual bioactive constituents by mass spectrometry and NMR spectroscopy; (ii) in vitro cytotoxicity assessment against human cancer cell lines; (iii) in vivo antimicrobial and anti-inflammatory studies to establish safety and efficacy thresholds; and (iv) quantitative phytochemical profiling by HPLC to standardise extracts for reproducible biological testing.

Declarations

Ethical Approval: Not applicable (plant study only).

Competing Interests: The authors declare no competing interests.

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Author Contributions: A. Umar and A. B. Adedoyin both supervised the study, and V. O. Adole performed laboratory analyses, data interpretation and manuscript preparation.

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