Mediterranean Journal of Medical Research
ISSN: 2789-1895 online
www.mjpe.periodikos.com.br
Mediterr J Med Res

SHORT COMMUNICATION article

Comparative study on the phytochemical composition, amino acid profile, antioxidant, *in vitro* anti-inflammatory, and *in vitro* anti-diabetic activities on the leaf and stem bark of *Acalypha indica*

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Received: 17-04-2025, **Accepted:** 01-06-2025, **Published online:** 03-06-2025

HOW TO CITE THIS

Ogbeide et al. (2025) Comparative study on the phytochemical composition, amino acid profile, antioxidant, *in vitro* anti-inflammatory, and *in vitro* anti-diabetic activities on the leaf and stem bark of *Acalypha indica*. Mediterr J Med Res. 2: 55-64. https://doi.org/10.5281/zenodo.15579785

Keywords: A*calypha indica,* amino acids, anti-diabetic activity, anti-inflammatory, antioxidant

Abstract: Herbal practitioners have long used Indian mercury (*Acalypha indica*) for therapeutic purposes. Research has proven that *A. indica* offers medicinal properties encompassing purgative, antifungal, and antibacterial action. This study aims to evaluate and compare the total phenolic and flavonoid content, amino acid profile, antioxidant, anti-diabetic, and anti-inflammatory activities of the *A. indica* leaf and stem. The quantitative phytochemical screening and amino acid profiling were carried out using standard methods. Antioxidant activities were investigated by the 1,1-diphenyl-2-picrylhydrazyl free radical scavenging method. Anti-inflammatory activities were obtained through the heat-induced hemoglobin denaturation inhibition method and the proteinase inhibition method. Anti-diabetic activities were assessed using an α-amylase inhibition assay. From the quantitative phytochemical screening, the total phenolic and total flavonoid contents were 100.5±0.01 and 93.48±0.40 for the leaf extract, and 92.2±0.26 and 139.0±0.28 for the stem respectively. The amino acid profiling for the stem showed concentration for the essential and non-essential amino acids with isoleucine and proline being the most abundant, and glycine having no concentration. The antioxidant potential at 1.0-200 µg/mL produced an IC_{50} of 4.16±0.04 µg/mL for the leaf extract, 5.57±0.06 µg/mL for the stem extract and 6.37±0.11 µg/mL for standard (ascorbic acid). Analysis for the anti-inflammatory studies revealed that the leaf extract of *A. indica* demonstrated better heat-induced hemoglobin denaturation protection when compared to the stem extract and for the anti-proteinase effects, the stem extract produced a more powerful dose-dependent outcome compared to the leaf extract. The anti-diabetic potential at 0.1-0.5 µg/mL produced an IC_{50} of 0.14±0.06 µg/mL for the leaf extract, 0.18±0.04 µg/mL for the stem extract, and 17.79±0.18 µg/mL for standard (acarbose). The study findings validate *A. indica* as a promising medicinal agent for managing inflammation, diabetes, and oxidative stress.

Introduction

Since ancient times, people have relied on plants for traditional herbal medicine, particularly in rural areas around the world. More than 100 plant species have been used for medicine, and plant products have remained important

in ancient traditional medical systems like Chinese, Ayurvedic, and Egyptian medicine. Many people are now forced to rely on traditional medicine because synthetic drugs are too expensive and healthcare is lacking in poorer countries [1-4]. A report from the World Health Organization in 1990 states that a lot of people still rely on traditional medicines to treat different health problems. These remedies are easy to find, cost less money, and, most importantly, usually have fewer or no side effects than other medicine types [5, 6]. The active phytochemicals in various plant sections give various plants their therapeutic qualities [7, 8]. Due to their excessive pharmacological ability, plants continue to be used in traditional drugs to treat different ailments [9] and despite the fact that many herbal compounds have been utilized extensively in treating many ailments, many of them still require more research and the disclosure of their molecular mechanisms. Finding new, potent therapeutic compounds from medicinal plants with negligible or no adverse effects thus became a top priority for study [10].

A*calypha Indica* (*A. indica*) holds different common names such as Indian Nettle while also being known as Indian Mercury and Three-Seeded Mercury [11], and it combines traditional medical knowledge with contemporary scientific research. Scientific studies of *A. indica* were scarce when the choice to focus on it was made despite its widespread medical uses and traditional North Karnataka medicine practitioners utilized different parts of the plant for treatment [12]. The phytochemicals separated from *A. indica* consist mainly of three classes: alkaloids, glycosides, and polyphenols. Alkaloids and glycosides: Ambelline, lupinine, pergolide sulfone, rescinnamine, and cyanogenic glycosides [10]. The polyphenolic compounds of Acalypha include flavonoids together with tannins and coumarins and phenolic acids that include gallic acid, syringic acid, and caffeic acid. Flavonoids: Quercetin [13] and kaempferol glycosides [14]. Tannins and coumarins: Antioxidant and anti-inflammatory compounds. Sitosterol anti-inflammatory and cholesterol-lowering [10]. Other compounds: Acalyphin, acalyphamide, aurantiamide, succinimide, and flindersine [10]. Iridoids include the compound Isodihydronepetalactone which draws feline species [14]. Aldehydes alongside ketones and terpenoids, steroids, and fatty acids belong to the group of volatile and fatty compounds. Additional phytochemicals: The plant contains organic compounds, peptides, phenols, and alkaloids.

The presence of bioactive compounds like flavonoids and polyphenols, makes *A. indica* a natural source for treating inflammatory ailments, enhancing insulin sensitivity, cancer, mutagens, microbial infections, and oxidative stress [15-18]. Depending on where they come from, antioxidants can be further divided into two categories: natural and synthetic. These days, it is well accepted that naturally occurring antioxidant molecules are safe and they can help raise the nutritional standards of our diets, thereby promoting good health. They aid in stopping free radicals and the oxidation reactions they trigger, preventing the human system's cells from being destroyed as a result of the oxidation reactions [17]. Inflammation can be viewed as the composite genetic reaction of vascular tissues to destructive stimuli like irritants or pathogens, through a protecting effort by the organism to get rid of the damaging stimuli and also commence the restorative development of the tissue. Inflammation has become the focus of international scientific exploration due to its impact on both animal and human illnesses [17]. Inflammation has been increasingly linked to prostate enlargement, specifically benign prostatic hyperplasia. Benign prostatic hyperplasia is characterized by the increase in the epithelial and stromal cells within the prostate, which leads to the formation of large lumps that compress the urethra [19]. This compression results in lower urinary tract symptoms, such as increased urinary frequency, nocturia, and difficulty initiating urination. Factors such as Infection, oxidative stress, and autoimmune reactions contribute to inflammatory processes within the prostrates [21]. Therefore, this study aims to compare the total phenolic and flavonoid content, amino acid profile, antioxidant, antidiabetic, and anti-inflammatory activities of the leaf and stem of the ethyl acetate extract of *A. indica.*

Materials and methods

Sample collection and preparation: Fresh *A. indica* leaves and stems were collected on September 26, 2024, from Ekhewan, Benin City, Nigeria, and identified by Henry A. Akinnibosun. A voucher specimen number (UBH-A658) was deposited at the University of Benin Herbarium. Samples were air-dried (20-25°C) and ground to powder, yielding 474.52 g (leaves) and 361.12 g (stems), following Wokocha and Okereke [22].

Extraction process: For extraction, 250 g of each powdered sample was macerated in 2.5 L of ethyl acetate for three days with occasional shaking, and then filtered. The filtrates were concentrated at 40°C using a rotary evaporator, and the extracts were stored at 4°C for further analysis.

Total phenolic content: Total phenol content was determined using Kim et al. [23]. Extract (0.5 mL, 1000 µg/mL) was mixed with Folin-Ciocalteu's reagent, then sodium carbonate. After incubation, absorbance was measured at 750 nm. Results were expressed as µg GAE/g extract, using a gallic acid standard curve (12.5-150 µg/ml).

Total flavonoid content: Total flavonoid content was estimated using Ebrahimzadeh et al. [21] method. Extract (0.5 mL, 1.0 mg/mL) was mixed with methanol, aluminum chloride, potassium acetate, and distilled water. After 30 min at room temperature, absorbance was measured at 415 nm. Total flavonoid content was expressed as mg quercetin equivalents per gram of extract, using a quercetin standard curve (12.5-150 µg/L).

Amino acid profile determination: Amino acid profiling was performed using a modified Henderson et al. method [24]; Samples (0.1 g) were hydrolyzed in 15 mL 6 M HCl (N2, 110°C, 24 hr) and filtered. Hydrolysates were then derivatized by vacuum-drying, treatment with methanol-water-phenylisothiocyanate, drying, addition of derivatizing reagent, incubation, evaporation (N_2), storage (4°C), and dilution. Separation was achieved via gradient elution chromatography at 30°C with Eluant A (triethylamine, sodium acetate, pH 6.2) and Eluant B (acetonitrile/distilled water).

Determination of antioxidant potential: Following Jain et al. [25], the DPPH assay used 3.0 mL of plant extract (5-200 µg/mL) combined with 1.0 mL of 0.1 mM DPPH methanol solution. After 30 min, absorbance was read at 517 nm. DPPH radical scavenging potential was calculated. IC_{50} represents the concentration required for 50.0% free radical inhibition.

Heat-induced hemoglobin assay: Adapted from Sakat et al. [26], samples (01.0 mL, 100-500 µg/mL) or saline (control)+1.0 mL 10.0% RBCs were incubated (56°C, 30 min), centrifuged, and absorbance (supernatant, 560 nm) was read (triplicate, aspirin standard).

Anti-proteinase activity assay: Following a modified Sakat et al. method [26], trypsin, Tris-HCl buffer+samples (1.0 mL, 100-500 µg/mL) were incubated (37°C, 5 min). Casein was added, incubated (20 min), stopped (perchloric acid), centrifuged, and absorbance (supernatant, 210 nm) was read (triplicate).

Anti-diabetic study: Following the Miller method [27]; the α-amylase solution was mixed with extract, buffer, and NaCl, incubated (10 min), then 01.0% starch was added (10 min incubation). The reaction was stopped with DNSA. After heating and cooling, the mixture was diluted, and absorbance was read at 540 nm.

Statistical analysis: Data analysis was performed using Statistical Package for the Social Sciences (SPSS version 20.0) for Windows. The results are expressed as the mean ± standard deviation (SD). One-way analysis of variance (one-way ANOVA) was conducted, followed by either the Student-Newman-Keuls post hoc test or the Bonferroni t-test. The statistical significance difference was set at $P<0.05$.

Results and discussion

Quantitative phytochemical screening: The therapeutic benefits of *A. indica* leaf and stem bark were evaluated by determining the quantitative phytochemical screening of the ethyl acetate leaf and stem extract of *A. indica*, and was presented in **Table 1** which shows the results for the Total Phenolic and Total Flavonoid content (TPC and TFC). Phenolic compounds are important for plant defense and antioxidant activity. This result aligns with studies on varying phenolic content due to metabolism and environment [28-32]. Flavonoids, known for antioxidant and anti-inflammatory effects [33], found in stems displayed a higher concentration level of 139±0.28 µg/ml compared to leaf sampling which achieved 93.48±0.40 µg/ml [33]. The flavonoid content exhibited higher variability in leaves which is similar to what other research established through studies of gene expression and enzyme activity levels [34].

Table 1: Total phenolic content and total flavonoid content of *A. indica* leaf and stem bark

Parameters	**Value:** concentration ± standard deviation	
	Leaf extract	**Stem extract**
Total phenolic content (µg GAE/100 g)	100.5±0.01	92.2±0.26
Total flavonoid content (µg QE/100 g)	93.48±0.40	139.0±0.28

Amino acid profile analysis: Stems from *A. indica* exclusively contain amino acids and do not share this trait with leaves according to Brosnan and Brosnan [35]. Secondary metabolite production proves more significant for Leaves according to Kumar et al. [36] and Shah et al. [37]. Further testing of stem extracts was carried out to test their antioxidant and anti-inflammatory properties due to the presence of these amino acids. According to **Table 2**, *A. indica* analysis showed a complete amino acid profile: essential amino acids (Threonine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Histidine, Tryptophan, Valine) and non-essential amino acids (alanine, serine, proline, aspartate, glutamate, arginine, tyrosine, cystine, glycine) (mg/100g). Isoleucine and proline were the most abundant which contributes to muscle metabolism immunity [38] and protein stability stress resistance [39], respectively. Significant levels of leucine, lysine, and methionine (protein synthesis, collagen formation, metabolism) [36, 38], along with glutamate, aspartate, and glycine were also found. This suggests that A. indica is a potential dietary amino acid source [40], thereby meriting further in vivo nutraceutical research. Metabolite allocation showed that amino acids in the stem, and secondary metabolites in the leaves [39], enhance its phytochemical pharmacological potential.

Antioxidant potential: The DPPH assay is a method used to assess the antioxidant activity in a sample. It measures the ability of a substance to scavenge free radicals by causing the discoloration of the DPPH solution [41]. The antioxidant capacity measurement aligns directly with the degree of solution discoloration according to Baliyan et al. [42], and **Table 3** displays these results. Ascorbic acid (standard) reached the highest antioxidant inhibition through its DPPH tests when compared against stem and leaf extracts. The leaf extract showed higher antioxidant effects when used at reduced concentrations which implies it contains powerful antioxidant components.

The IC_{50} method stands as the accepted measurement to determine the blocking effectiveness of substances on biological or metabolic operations. The antioxidant capacity of a substance becomes higher when its IC_{50} value decreases because it allows smaller extract quantities to achieve equal inhibition levels according to Ogbeide et al. [43]. According to the data shown in **Table 4** the antioxidant capacity of the leaf extract proved stronger than stem extract and ascorbic acid while being linked to its higher content of phenolics and flavonoids. The extraction method using ethyl acetate succeeded in obtaining flavonoids together with phenolic acids. The leaf extract exhibits a greater antioxidant activity than the other samples because it contains higher levels of compounds.

Ascorbic acid demonstrated a higher IC_{50} value compared to the extracts since multiple antioxidants work synergistically as stated by Rice-Evans et al. [44], and extract polyphenols and flavonoids perform various antioxidant functions reported by Gülçin [45]. *A. indica* leaf extracts show antioxidant properties that exceed those of ascorbic acid. The leaf extract's lower IC_{50} suggests its potential as a natural antioxidant source for nutraceutical and pharmaceutical applications against oxidative stress-related disorders.

Table 2: Amino acid concentration of *A. indica* stem

	Amino acid	Value (mg/100 g)
Essential amino acids	Threonine	15.37
	Isoleucine	32.08
	Leucine	2.59
	Lysine	4.42
	Methionine	9.08
	Phenylalanine	2.51
	Histidine	2.16
	Tryptophan	1.76
	Valine	4.33
Non-essential amino acids	Alanine	6.74
	Serine	7.60
	Proline	30.87
	Aspartate	9.74
	Glutamate	3.74
	Arginine	9.16
	Tyrosine	3.81
	Cystine	1.32
	Glycine	-

Table 3: DPPH-scavenging potential of crude extract of *A. indica* leaf and stem

Concentration (µg/mL)	Inhibition (%)		
	Ascorbic acid	Leaf extract	Stem extract
1	18.07	41.43	34.58
2	20.25	41.61	40.81
5	38.32	52.33	47.66
10	62.93	58.88	53.58
20	89.41	60.44	65.52
50	94.08	70.09	69.16
100	94.39	70.4	77.26
200	98.44	80.06	81.30

Table 4: IC_{50} Value of leaf and stem extract of *A. indica* and standard (ascorbic acid)

Sample	**IC_{50} values expressed in µg/mL** Concentration ± standard deviation
Leaf extract	4.16±0.04
Stem extract	5.57±0.06
Standard	6.37±0.11

Heat-induced hemoglobin assay: This study assessed *A. indica's* anti-inflammatory properties by subjecting leaf and stem extracts from 100 to 300 mg/ml to a heat-induced hemoglobin assay for denaturation inhibition testing using aspirin as the control substance. The inhibition rate of heat-induced hemoglobin denaturation increased with concentration for both *A. indica* leaf and stem extracts as demonstrated in **Table 5**. The inhibition rates from the stem extract were slightly superior at 100 mg/ml yet the leaf extract displayed superior results in general. Shinde et al. [46] reported aspirin demonstrated maximum inhibitive effects across all measurement points (Aspirin100:

75.900%, Aspirin200: 79.501%, and Aspirin300: 83.865%). At 300 mg/ml, the inhibitory effect of leaf extract exceeded stem extract and recorded a 57.618% result while stem extract achieved 49.654%. Studies have verified that plant polyphenols and flavonoids defend proteins from denaturation thus indicating their potential use as anti-inflammatory agents [9]. The leaf extract of *A. indica* demonstrated better heat-induced hemoglobin denaturation protection when compared to stem extract as research indicates it contains superior anti-inflammatory compounds ([45]; warranting additional study for therapeutic purposes).

Table 5: Heat-induced hemoglobin denaturation inhibition of *A. indica* leaf and stem extracts.

Concentration (mg/mL)	Inhibition (%)		
	Aspirin	Leaf Extract	Stem extract
100	75.900	36.427	40.789
200	79.501	48.199	44.529
300	83.865	57.618	49.654

Table 6: Anti-proteinase activity of *A. indica* leaf and stem extract

Concentration (mg/mL)	Inhibition (%)		
	Aspirin	Leaf extract	Stem extract
100	65.138	43.303	39.725
200	80.000	51.652	59.545
300	82.661	55.230	77.982

Tissue breakdown and inflammatory promotion during inflammatory processes happen through the action of proteinases [26]. The administration of anti-inflammatory drugs succeeds in blocking enzymatic activity and the therapeutic control of inflammatory conditions depends heavily on enzyme inhibition [43]. The results show that *A. indica* extract solutions from leaves and stems prevent proteinase activity at volumes 100, 200, and 300 mg/mL relative to aspirin standards as demonstrated in **Table 6**. The anti-proteinase effects of the *A. indica* stem extract surpassed those of the leaf extract at the 300 mg/ml concentration level. The anti-inflammatory properties of aspirin proved stronger than any other substance tested because it delivered the highest level of inhibition. The stem extract produced a more powerful dose-dependent outcome compared to the leaf extract but both substances demonstrated enhanced inhibitory activity based on concentration rises. A superior inhibitory effect of the stem extract matches its higher content of amino acids, phenolics, and flavonoids. Flavonoids along with phenolics stabilize proteins [47] but amino acids could change proteinase structure [46]. The results indicate that *A. indica* shows promise as a natural anti-inflammatory compound because its properties align with earlier studies about plant-based polyphenols and flavonoids performing effectively as proteinase inhibitors [26]. Research about bioactive compounds within stem extract shows it has superior activity thus validating studies that define plant-based compounds while validating traditional medicinal applications.

Anti-diabetic activity: A. indica leaf and stem ethyl acetate extracts were tested for their anti-diabetic potential using an α-amylase inhibition assay and acarbose as a standard. The percentage inhibition results are presented in **Table 7**. Notably, the stem extract showed the greatest inhibition at 0.5 µg/mL (80.142%), surpassing both the leaf extract (58.682%) and the acarbose standard (31.611%).

Table 7: Anti-diabetic activity of the Acarbose (standard) and *A. indica* leaf and stem extract

Concentration (µg/mL)	Inhibition (%)		
	Acarbose	Leaf extract	Stem extract
0.1	18.522	49.668	35.440
0.2	20.837	50.311	56.100
0.3	21.282	53.963	60.520
0.4	23.152	54.764	61.086
0.5	31.611	58.682	80.142

To better understand the effectiveness of the extract, the IC_{50} values which represent the concentration needed to inhibit 50.0% of α-amylase activity were calculated. A lower IC_{50} values indicate greater potency.

Table 8: IC_{50} values of anti-diabetic activity for the Leaf, stem, and acarbose (standard)

Sample	**IC_{50} values in µg/mL, ±standard deviation**
Leaf extract	0.14±0.06
Stem extract	0.18±0.04
Standard	17.79±0.18

The leaf extract demonstrated the strongest inhibitory effect on α-amylase since its IC_{50} value reached 0.14±0.06 µg/mL in the results shown in **Table 8**. Acarbose showed less effectiveness as the standard anti-diabetic reference with a high IC_{50} value of 17.79±0.18 µg/ml while the stem extract followed by 0.18±0.04 µg/ml and the leaf extract exhibited the lowest value at 0.14±0.06 µg/ml. This implies that both the leaf and stem extract of *A. indica* are stronger α-amylase inhibitors than acarbose which indicates a significant potential for managing diabetes and this high effectiveness can be attributed to the phenolics and flavonoids present in the extracts that inhibit α-amylase activity as reported in Sales et al. [48]. The complex structure of acarbose demands greater concentrations for matching inhibition strength according to Kwon et al. [49]. Ethyl acetate used as the extraction solvent may be related to the high degree of α-amylase inhibition in the extracts. Peak α-amylase inhibition results occur while using ethyl acetate because it successfully extracts both the flavonoids and phenolic acids that show enzyme-blocking effects [50]. The superior activity of the extracts may, therefore, be due to ethyl acetate's efficient extraction of these key bioactive compounds. *A. indica* extracts, especially from leaves, showed better α-amylase inhibition than acarbose due to lower IC_{50} values, suggesting potential as a natural diabetes treatment.

Conclusion: This study documented different medicinal properties in leaf and stem extracts of *Acalypha indica*. Antioxidant effects with anti-diabetic potential were more abundant in the leaf extract. Notably, the leaf extract surpassed the stem extract when it came to inhibiting hemoglobin denaturation although both showed anti-inflammatory outcomes. When tested at a higher concentration the stem extract displayed precise anti-proteinase properties despite its enrichment with flavonoids and amino acids.

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Acknowledgments: This research was supported by TETFUND through the IBR Grant of 2024.
Authors' Contribution: OKO conceived and designed the study, and SO & FIA collected the data. SO, FIA, OJO, MOI, MOI, DO, & PI performed data analysis and interpretation. SO & FIA drafted and reviewed the manuscript. All authors contributed to the data analysis approved the final version of the manuscript and agreed to be accountable for its contents.
Conflict of interest: The authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Ethical issues: The authors completely observed ethical issues including plagiarism, informed consent, data fabrication or falsification, and double publication or submission.
Data availability statement: The raw data that support the findings of this article are available from the corresponding author upon reasonable request.
Author declarations: The authors confirm that they have followed all relevant ethical guidelines and obtained any necessary IRB and/or ethics committee approvals.