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Preliminary phytochemical screening of *Alternanthera bettzickiana (Regel) G. Nicholson* hydroethanolic extract to evaluate its antioxidant, anti-inflammatory and antimicrobial properties.

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Abstract

This study aimed to assess the phytochemical composition and evaluate the antioxidant, anti-inflammatory, analgesic, and antimicrobial properties of the hydroethanolic extract derived from *Alternanthera bettzickiana (Regel) G. Nicholson*. Initial phytochemical screening revealed a diverse array of compounds in the crude extract, potentially contributing to its observed biological activities. The research employed various methodologies to investigate these properties. Antioxidant capacity was measured using hydrogen peroxide and DPPH radical scavenging assays, utilizing UV-Vis spectrophotometry. Anti-inflammatory activity was examined through an in vitro HRBC membrane stabilization method, comprising hemolytic and membrane stabilization experiments. Hemoglobin release from damaged erythrocyte membranes was quantified at 540 nm. Analgesic effects were assessed using the acetic acid-induced writhing test in mice, while antimicrobial activity was evaluated via the disc diffusion method, comparing plant extracts with standard treatments. The phytochemical analysis identified several key secondary metabolites in the extract, including phenols, tannins, saponins, volatile oils, proteins and amino acids, flavonoids, cholesterols, carboxylic acids, and lignins.While investigating the extract's antioxidant potential through DPPH (2,2-diphenyl-1-picryl-hydrazyl) and hydrogen peroxide scavenging assays, the researchers determined that the concentration required for 50% inhibition (IC_{50}) varied significantly between the two methods, as the DPPH assay yielded an IC_{50} value of 229.6 µg/ml, whereas the hydrogen peroxide scavenging activity demonstrated a considerably lower IC_{50} value of 31.67 µg/ml. In the anti-inflammatory assay, the extract at 400 µg/ml exhibited 82.23% protection against RBC hemolysis, comparable to the 83.16% inhibition shown by diclofenac sodium at the same concentration. Analysis of the analgesic properties demonstrated that, while both diclofenac sodium and *A. bettzickiana* extract exhibited significant writhing inhibition ($p \leq 0.01$) with values spanning from 35.44% to 87.76%, their efficacy differed notably as reflected in their IC_{50} values, where diclofenac sodium showed superior potency at 36.88 µg/ml compared to the *A. bettzickiana* extract's value of 101.39 µg/ml when tested against the control group. Antimicrobial testing showed that the plant extract was effective against *B. megaterium* (9 mm inhibition zone) and *A. flavus* (8 mm inhibition zone) out of six tested microorganisms. These findings suggest promising antioxidant, anti-inflammatory, antimicrobial, and analgesic (Pain-relief) effects of the *A. bettzickiana* hydroethanolic extract, as evidenced by both in vitro and in vivo observations.

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1. Introduction

Throughout human history, as traditional medicine practices have guided the discovery of therapeutic compounds, the natural world has served as an invaluable reservoir of medicinal resources, which has led to the development of numerous contemporary pharmaceutical agents that continue to shape modern medical treatments. (Balick & Cox,2020). According to the World Health Organization's assessment, despite modern medical advancements, indigenous medications continue to serve as the primary healthcare resource for approximately 80% of the world's population, primarily because these traditional remedies remain readily accessible to communities across the globe. (Gul et al., 2022). Compared to synthetic treatments, these natural remedies exhibit a broader range of therapeutic properties, such as antibacterial, antifungal, anti-inflammatory, antioxidant, anticancer, and antidiabetic effects, which make them more versatile in addressing various infections and conditions. (Bari et al., 2021). Traditional medicinal plants are found to contain powerful natural antimicrobial compounds and have historically provided accessible and economical treatment options for communities worldwide. As researchers continue to unlock their therapeutic potential, and with the growing resistance to using synthetic pharmaceuticals, use of natural antimicrobial compounds in future disease management is expected to expand substantially (Bibi et al., 2016).

Alternanthera bettzickiana, known as the red calico plant or border plant, is a perennial herb from the *Amaranthaceae* family. Native to South America, it is grown worldwide, including in Pakistan. The genus *Alternanthera*, belongs to part of this family and includes about 80 species.(Resources & Us, 2013). It is frequently used as an ornamental bordering plant and possesses both culinary and decorative value. Its leaves can be reddish, green, or a combination of both colors. In Southeast Asian cuisine, the shoots and leaves are consumed similarly to spinach or used in soups, either cooked alone or combined with other vegetables like amaranth or cowpeas (Ali et al., 2012). It is known for its preference for moist habitats such as ditch peripheries, mesh zones, and untamed wastelands. *A. bettzickiana* has been revered in traditional medicinal practices for its diverse therapeutic applications, which encompass blood purification, wound healing, antipyretic properties, lactation enhancement, and gentle laxative effects.(WHO-IARC, 2018). It has also been used to alleviate gastrointestinal discomfort and prevent dementia (Manan et al., 2020). In Thailand, the plant is employed in the treatment of arthritis (Jemal et al., 2011). *A. bettzickiana*, having a diverse array of phytochemical compounds including terpenoids, tannins, steroids, saponins, phenolics, glycosides, flavonoids and alkaloids, has demonstrated its remarkable therapeutic potential through Alzheimer's disease, reduce inflammation, neutralize harmful free radicals, fight against microorganisms, and promote increased urine production, as evidenced by various scientific investigations(Lozano et al., 2012). Research has revealed the presence of fatty acids (linoleic acid) and diterpene alcohol (phytol) in *A. bettzickiana*, which have been associated with therapeutic potential in treating rheumatoid arthritis (RA)(Coleman & Rubinas, 2009). Given the traditional use of *A. bettzickiana* and its reported phytoconstituents, it is valuable to assess its antiarthritic potential, since a comprehensive study has not been conducted to determine its efficacy in treating RA (Gow et al., 2022). Our studies were to validate traditional uses of *A. bettzickiana* and to assess its antibacterial, antifungal, antioxidant, and anti-inflammatory properties through in-vitro tests. Overall, this research aims to advance scientific knowledge and support the development of new, safer treatments from natural sources, aligning with the growing interest in alternative medicine.

2. Materials and Methods

2.1 Gathering and identifying plant species and

In November 2023, researchers gathered the plant specimens from the grounds of the University of Information Technology and Sciences (UITS) in Bangladesh. This particular species is known to grow in areas where the weather is pleasant, with mild temperatures, low humidity, and minimal rainfall during autumn in November. Plant specimens were identified and confirmed using taxonomic keys (Colorful ornamental foliage garden plant), and a sample was subsequently deposited at the National Herbarium of Bangladesh in Dhaka, assigning the accession number DACB 94817.

2.2 Isolation of plant components (Cold extraction)

Approximately 300 grams of dried plant matter were placed in a dark-colored container and immersed in 1 litre of a 75% ethanol solution. The sealed container was stored for roughly a week, during which it was periodically agitated. Subsequently, the mixture underwent filtration, first through cotton and then using Whatman filter paper. A rotary evaporator concentrated the resulting liquid at 50°C under low pressure, yielding 16 grams of raw extract. (Haque et al., 2008).

2.3 Phytochemical analysis

The extracts were screened for carbohydrates, phenols, glycosides, anthraquinone glycosides, cardiac glycosides, steroids, volatile oils, phenols, proteins, amino acids, flavonoids, phytosterols, cholesterol, quinones, carboxylic acid, emodins, lignins using established methods (Harborne, 1998; Sazada et al., 2009). A series of chemical analyses were performed to

detect various compounds within the sample. Carbohydrates were identified using the Molisch test, while a 10% ferric chloride solution was employed to detect phenols. Multiple techniques, including Salkowski's, Keller-Kiliani, and Liebarmann's tests, were used to identify glycosides. A chloroform-based method was applied to analyze terpenoids. Tannins were detected through another application of the ferric chloride test. The presence of saponins was confirmed by observing persistent foam formation upon agitation of the sample. Additional tests were conducted: the hydroxyanthraquinone test for anthraquinone glycosides, ferric chloride for cardiac glycosides, dilute lead acetate and ammonia tests for flavonoids, a specific test for steroids, and a 1% hydrochloric acid test for phlobatannins (Chang et al., 2002, Bloom et al., 2017).

2.4 Antioxidant Assay

2.4.1 DPPH radical scavenging assay

The test sample or standard (1 ml), with concentrations ranging from 8.125 to 400 µg/ml, was combined with a 0.004% 1,1-diphenyl-2-picrylhydrazil (DPPH) solution (3 ml). The mixture was incubated at ambient temperature for 30 minutes. The absorbance was then measured at 517 nm using a UV-visible spectrophotometer, and a blank was used as the reference. The calculation of the inhibition percentage was performed using the formula: [(AC-AS)/AC] × 100. In this equation, the absorbance of the control was represented by AC, while the absorbance of the tested sample or standard was denoted by AS. (Gulcin & Alwasel, 2023).

2.4.2 Hydrogen peroxide scavenging assay

A hydrogen peroxide solution (40 mM) was prepared using a phosphate buffer (pH 7.4). The extract was diluted in distilled water to achieve concentrations between 25 and 1000 µg/mL, and these dilutions were combined with the hydrogen peroxide solution (0.6 mL). Following a 15-minute incubation period, spectrophotometric measurements were taken at 230 nm. A phosphate buffer lacking hydrogen peroxide was utilized as the reference blank. The following equation was employed to determine the hydrogen peroxide scavenging activity of both the extract and standard compounds:

Scavenging Percentage = [(AC-AS)/AC] × 100

Linear equation, $Y = ax + b$, $IC_{50} = (0.5 - b)/a$.

Where AC indicated the control absorbance, and the absorbance of extract- or standard-containing samples were represented by AS. (Keser et al., 2012).

2.5 Anti-inflammatory Assay

2.5.1 Acetic acid-induced writhing test in mice

The experimental setup involved five groups of Swiss albino mice, with each group containing six animals. All procedures were carried out in accordance with institutional guidelines for the care and use of laboratory animals and the study protocol received prior approval from the Department of Pharmacy Ethical Committee (PEC) under the approved reference number (UITS/Pharm/PEC/24/18).

For the negative control, Group I mice received an intraperitoneal (i.p.) injection of normal saline (10 ml/kg). The experimental groups were administered different concentrations of the extract via i.p. route: 50 mg/kg was given to Group II, 100 mg/kg to Group III, and 200 mg/kg to Group IV (based on body weight). Group V, serving as the positive control, was treated with diclofenac sodium (20 mg/kg body weight) through i.p. injection. After a 30-minute interval, acetic acid solution (0.06%) was administered i.p. to all mice at a dose of 1 ml per 100 g body weight. Five minutes after the acetic acid administration, the number of abdominal constrictions exhibited by each mouse was observed and recorded over 10 minutes. A predetermined formula was used to calculate the percentage reduction in writhing movements, which indicated the analgesic effectiveness of the treatments (Thangjam et al., 2020).

$$\text{Inhibition \%} = \frac{\text{Mean no of writhes (control)} - \text{mean no of writhes (test)}}{\text{Mean no of writhes (control)}} x100$$

2.5.2 In vitro anti-inflammatory activity by red blood cell membrane stabilization

A 10 mL sample of fresh whole human blood, collected in a heparin-treated centrifuge tube, was processed in accordance with institutional ethical guidelines for human biological material, with approval from the Pharmacy Ethical Committee (PEC) under the approved reference number (UITS/Pharm/PEC/24/18) and informed consent obtained from the donor. The sample was centrifuged at 3000 rpm for 10 minutes, after which the supernatant was discarded. The remaining packed cells were washed three times with equal volumes of normal saline, and, following the final wash, a 10% v/v red blood cell (RBC) suspension was prepared using normal saline.

The evaluation of membrane stabilization against heat-induced hemolysis was conducted using a modified version of an existing protocol (Mahmuda et al., 2020). The researchers prepared test tubes by adding 5 mL of isotonic buffer solution containing the test substances at different concentrations (50, 100, 200, and 400 µg/mL). Each concentration was tested in six replicates. The vehicle (5 mL) was placed in negative control tubes, while Diclofenac was used as the reference standard. To each tube, 0.05 mL of the RBC suspension was added and mixed carefully. One set of tubes underwent incubation at 54 °C, while the remaining sets were maintained at 0-4 °C, both for 20 minutes. Following the heating period, the tubes were cooled and subjected to

centrifugation at 3,000 rpm for 3 minutes. The absorbance measurements of the supernatants were taken at 540 nm, using isotonic buffer solution as the blank. The researchers calculated the percentage inhibition of hemolysis by comparing the absorbance values of heated and unheated test samples against a heated control sample using a specific formula.

% Inhibition of hemolysis= {1- (OD2- OD1 / OD3- OD1)} × 100

Linear equation, Y = ax + b, IC_{50} = (0.5 - b)/a.

Where OD1 represents the test sample's absorbance when it is not heated, OD2 is the test sample's absorbance when it is heated, and OD3 is the absorbance of the heated control sample.

2.6 Antimicrobial assay

The antimicrobial properties of various extracts were evaluated using the disc diffusion technique, which involved testing both Gram-positive and Gram-negative bacterial strains, as well as fungi, with the specific bacterial and fungal strains listed in Table: 6 to prepare the test samples, measured quantities of the extracts were dissolved in appropriate solvents to achieve known concentrations (mg/ml), and sterilized 6 mm diameter filter paper discs were infused with precise amounts of these solutions using a micropipette. Thereafter, they were placed on nutrient agar medium inoculated with the microbial strains. For comparison, a positive control disc, containing 30 µg of kanamycin, considered as a standard due to its broad-spectrum antimicrobial activity and well-established efficacy in susceptibility testing, and a negative control disc, treated with the extraction solvent, were also included in the assay. The plates were kept at 4°C for 24 hours to allow for optimal diffusion, creating a concentration gradient around each disc. Subsequently, the plates were incubated at 37°C for an additional 24 hours to promote optimal microbial growth. Extracts with antimicrobial activity showed inhibited microbial growth, resulting in clear, distinct zones of inhibition surrounding the discs. These zones were used to quantify the antimicrobial efficacy of the extracts (Bauer et al., 1966).

3. Results and Discussion

3.1 Phytochemical Screening

Most of the biochemicals shown in Table 1 were found in the hydroethanolic extract of *Alternanthera Bettzickiana*, according to phytochemical tests.

Table 1: Results of phytochemical screening

S/L	Test name	Result
1	Carbohydrate	++
2	Phenol	++
3	Glycoside	-
4	Terpenoids	-
5	Tanins	++
6	Saponins	++
7	Anthraquinon glycoside	-
8	Cardiac glycoides	-
9	Steroids test	-
10	Volatile oils test	+
11	Phlobatannins	-
12	Proteins and Amino acid	++
13	Flavonoids	++
14	Phytosterols	-
15	Cholesterol	++
16	Quinones	-
17	Carboxylic acid	+
18	Emodins	-
19	Lignins	++

***The presence of phytochemical compounds is denoted by a plus sign (+), while their absence is indicated by a minus sign (-).**

3.2 Antioxidant

3.2.1 DPPH Free Radical Scavenging Activity

The antioxidant properties of the *A. bettzickiana* extract were assessed using the highly regarded DPPH radical scavenging method and Ascorbic Acid as a standard. This technique evaluates how effectively the extract can neutralize the stable DPPH radical, which contains an unpaired electron. The test measures changes in absorbance as the DPPH is reduced, indicating the extract's capacity to neutralize free radicals. Analysis of the hydroethanolic extract yielded an IC_{50} value of 229.66 µg/ml (Table 2), with the equation y = 11.867x + 9.9429 and an R^2 value of 0.9815 (Figure 1).

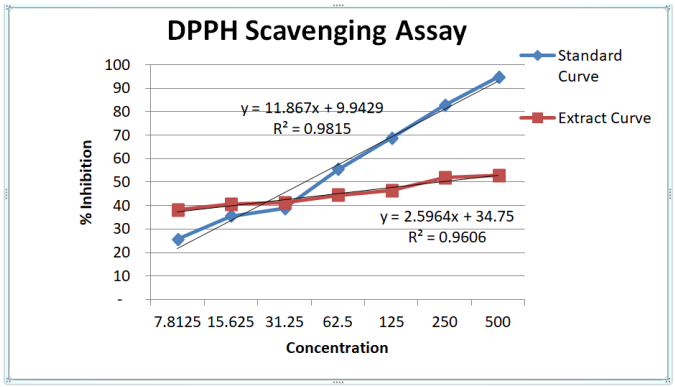

Figure 1: Calibration curve of Ascorbic acid (Standard) and plant extract (*A. bettzickiana*)

Table 2: Percentage inhibition of DPPH radicals at different concentrations of A. bettzickiana extract

Sample	Concentration(µg/ml)	Absorbance of Ascorbic Acid	% inhibition	IC_{50}
Standard (Ascorbic Acid)	7.8125	0.592	26	40.53
	15.625	0.513	36	
	31.25	0.487	39	
	62.5	0.355	55	
	125	0.249	69	
	250	0.136	83	
	500	0.041	95	
Plant Extract (A. Bettzickiana	7.8125	0.493	38.07	229.66
	15.625	0.472	40.70	
	31.25	0.468	41.21	
	62.5	0.442	44.47	
	125	0.426	46.48	
	250	0.382	52.01	
	500	0.374	53.02	
Control	0	0.796	0	

Table 3: Percentage inhibition of H_2O_2 at different concentrations of A. bettzickiana extract

Sample	Concentration n(µg/ml)	Mean Absorbance	% inhibition	IC_{50}
Standard (Ascorbic Acid)	7.8125	1.605	32.020	11.73
	15.625	0.904	61.711	
	31.25	0.762	67.726	
	62.5	0.512	78.314	
	125	0.306	87.039	
	250	0.198	91.614	
	500	0.072	96.950	
Plant Extract (A. Bettzickiana)	7.8125	1.912	19.017	31.67
	15.625	1.402	40.618	
	31.25	1.067	54.807	
	62.5	0.769	67.429	
	125	0.575	75.646	
	250	0.473	79.966	
	500	0.314	86.701	
Control	0	2.361	0.000	

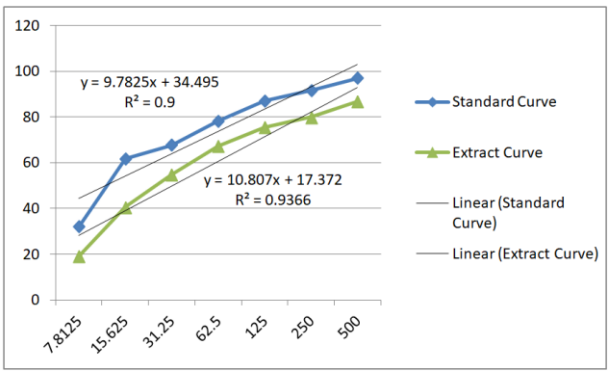

Figure 2: Calibration curve of ascorbic acid (Standard) and plant extract (*A. bettzickiana*)

3.3 Anti-inflammatory Assay

3.3.1 In-vivo acetic acid-induced abdominal constriction assay

A statistically significant reduction ($p \leq 0.01$) in writhing movements was observed when animals received different doses of *A. Bettzickiana*extract or diclofenac sodium, compared to those treated with placebo. The writhing inhibition analysis revealed that Groups II, III, IV, and Group V (treated with diclofenac sodium) showed inhibition rates of 19.23%, 34.61%, 61.53%, and 84.61% respectively, when compared against the control group (as depicted in Table 4).

3.3.2 In vitro anti-inflammatory activity by HRBC membrane stabilization

The study demonstrated that when methanol-based extracts were used in concentrations between 50 and 400 µg/ml, they effectively prevented red blood cell membrane breakdown in hypotonic conditions, as shown by the experimental data. For comparison, diclofenac sodium was tested at similar concentrations (50-400 µg/ml) and also exhibited significant protective effects against hypotonic damage. The highest protection rate of 82.23% (Table 5) was achieved with hydroethanolic extracts at 400 µg/ml concentration, which was comparable with diclofenac sodium's maximum protection rate of 83.16% at the same concentration when measured against the control group.

Table 4: The acetic acid-induced writhing test of plant extracts and diclofenac standard in mice

Groups	Treatment	Dose (mg/kg)	(Mean ± SEM)	% inhibition
Group I	Saline	Solvent	26.33±1.20	-
Group II	Extract	50	21.33±1.20	19.23
Group III	Extract	100	17.33±0.88	34.23
Group IV	Extract	200	10.66±0.88	61.53
Group V	Extract	400	6.7±0.88	74.72
Group VI	Diclofenac Na	20	4.66±0.88	84.61

Table 5: The anti-inflammatory effects of the hydroethanolic extract, in comparison to the standard drug Diclofenac, were investigated.

Sample	Concentration (µg/ml)	OD1	OD2	OD3 (Control)	% of Hemolysis	% Inhibition of Hemolysis	IC_{50}
Standard Diclofenac Sodium	50	0.249	0.153	0.027	43.24	56.76	28.82
	100	0.231	0.162		33.82	66.18	
	200	0.221	0.169		26.80	73.20	
	400	0.217	0.185		16.84	83.16	
Plant Extract (A. bettzickiana	50	0.204	0.112		52.07	47.93	60.34
	100	0.227	0.145		40.90	59.10	
	200	0.231	0.157		36.38	63.62	
	400	0.203	0.172		17.77	82.23	

3.4 Antimicrobial activity

Antimicrobial tests conducted on the ethanol-based extracts of *A. Bettzickiana* against six different microbial strains (Table 6). Revealed that the extract slightly restricted microbial growth, producing inhibition zones of 7.5mm against *A. flavus and* 8.5mm against *B. megasterium*.

Table 6: The anti-microbial effects of the hydroethanolic extract, in comparison to the standard drug kanamycin

Entry	Organism classes	Organism	Zone of inhibition (mm)	
			Extract	Kanamycin
1	Gram Positive	*Bacillus megaterium*	8.5	21
2		*Staphylococcus aureus*	Nil	23
3	Gram Negative	*Escherichia coli*	Nil	21
4		*Salmonella typhi*	Nil	24
5	Fungi	*Aspergillus niger*	Nil	23
6		*Aspergillus flavus*	7.5	22
*400 µg per disc was applied; *5 µg per disc was used as reference (Kanamycin)				

4. Conclusion

Research findings demonstrate the promising therapeutic potential of *A. Bettzickiana*ethanol extract. The experimental results revealed that this extract exhibited remarkable properties in a concentration-dependent manner, specifically showing potent pain-relieving capabilities (analgesic effect), strong antioxidant activity, and significant anti-inflammatory effects. The multi-faceted therapeutic properties displayed by the ethanol extract of *A. Bettzickiana*in our investigations suggest its valuable potential as a medicinal agent. These results add to the expanding body of research that highlights the therapeutic potential of this plant extract, especially when ethanol is utilized as the solvent for extraction. The consistency and significance of our results across multiple therapeutic parameters highlight the extract's versatility as a potential pharmaceutical resource.

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