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PHARMACOGNOSTIC, TOXICITY, ANTIBACTERIAL AND ANTIHAEMONCHOSIS EVALUATIONS OF *BIDENS PILOSA* L. (ASTERACEAE)

[*1]Cletus A. UKWUBILE, [1] Matthew O. AGU, [1]Salihu Z. ADAMU, [2] ADAMU Abdulrahaman, [3] Daniel C. KAGARU, [4] OTENE J. Matthew

[1]Department of Science Laboratory Technology, [2]Department of Agricultural Technology, Federal Polytechnic, Bali, [3]Department of Pharmacognosy, University of Jos, [4]Applied Biology Unit, Ipaja Lagos, Nigeria.

ABSTRACT

The vast uses of *Bidens pilosa* in traditional medicine for the treatment of various ailments such as cancer, inflammation, diabetes, infections, and haemonchosis in Nigeria, has prompted the research on this plant to evaluate its use in traditional medicine . This study was primarily designed to determine the pharmacognostic parameters, evaluate the safety of the plants in animals, determine its effects on bacteria and nematode parasite which cause haemonchosis in goats and sheep. Pharmacognostic studies as well as toxicity study carried out in two phases using 39 mice of opposite sex . Animals were administered methanol extract (ME) of the crude plant following intraperitoneal (i.p) at 10, 100, 1000, 1600, 2900 and 5000mg/kg body weight(b.w). Antibacterial study was conducted using ME of leaves on *Bacillus subtilis NCTC8239*, *Salmonella typhi ATCC9184*, *Staphylococcus aureus NCTC6571*, *Escherichia coli NCTC10418*, *Klebsiella pneumonia ATCC10031*, and *Staphylococcus aureus ATCC13704*. Effects leaf extracts were tested on *Haemonchus contortus* infective L3 larvae which cause haemonchosis in goats and sheep to determine % embryonation inhibition (%EI), % hatching inhibition (%HI) and % mortality rate of L3 larvae (%MR). Results showed that the plant possess covering trichome, diacytic stomata 22-23.3-25 mm^2 and 31-34.1-38mm^2 , Stomata index 12.5-13.24-14.2% and 22.4-23.80-26%, Vein islet and vein termination numbers 12-14-16 mm^2 and 20-21-22 mm^2 , Palisade ratios 8-9.4-12 mm^2 and 4.2-7.5-10 mm^2 upper and lower epidermis respectively. Phytochemical screening of leaf extract revealed the presence of many metabolites; with alkaloids, saponins, and tannins content more compared to other metabolites in the ME. Physico-chemical parameters showed that water extractive values of leaf were greater than that of stembarks and roots. The plant displayed great antibacterial activities on most of the microbes. Toxicity study showed that the animals survived the intoxication even at dosage of 5000 mg/kg b.w. Antihaemonchosis evaluations showed dose dependent activities of extracts. Methanol extract (ME) showed more potent on L3 larvae in terms of %EI, %HI and %MR from concentration 0.625 to 5.0 mg/mL after 48h. Our study showed that methanol extracts of *Bidens pilosa* were safe at the doses investigated and can serve as an ethnomedicinal prescription as well as treatment for haemonchosis in Nigeria. The data from pharmacognostic studies will serve as a tool for monograph in pharmacy.

Keywords: Pharmacognostic, Toxicity, Antibacterial, Antihaemonchosis, Methanol extracts, *Bidens pilosa*.

INTRODUCTION

Bidens pilosa is an erect perennial herb distributed across temperate and tropical regions of African and South America [1]. It is either glabrous or hairy with green opposite leaves that are serrated, lobed, or dissected. It has white or yellow flowers and long narrow ribbed black seeds, and can grow to an average height of 60 cm and maximum of 150 cm in favourable climates[2]. It is known by various names in Nigeria like: Hausa (Fara-kaya), Igbo (Enyinata), Yoruba (Ekesan) and Igala (Kete-kete).

Corresponding Author

Cletus A. UKWUBILE
Email id: doccletus@yahoo.com

The plant has variously been used in traditional medicine as: anticancer [3], anti-inflammatory agent, antidiabetic, anti-oxidant, immunomodulator, anti-malarial agent, anti-bacterial agent, anti-fungal agent, anti-helminthics agent[4], wound healing agent and worm eradication. Many compounds has been isolated from the leaves and were evaluated for various biological assays both *in vivo* and *in vitro* [5]. Bacterial infection has remained one of the major problems to humans and their livestock causing an economic loss of up to 56 % annually in developing countries [6]. In most cases, the infection becomes very difficult to be removed from their hosts using both orthodox and herbal remedies [7]. Recent discovering in research have shown that herbal remedies can compete favourably with most first line drug in the treatment of bacterial infections in humans [8].

Haemonchosis is an infection caused by filiariform larvae (L3) of *Haemonchus contortus* in goats and sheep as well as other ruminants. This parasite belongs to the Class; *Secernentea*, Order; *Strongylida*, and Family; *Trichostrongylidae*. The parasites has undergoes four larval form to complete its life cycle and caused heavy losses to meat value as well as market value to goat and sheep [9]. Female worm can lay over 10,000 eggs a day, which pass from the host animal in the faeces. Anti-helminthics drugs are used to prevent and treat these other worm infection, but resistance of the parasite against these drugs is growing Daily [10].This study was designed to determine the pharmacognostic parameters, toxicity of plant parts, anti-bacterial and anti-haemonchosis activities of the crude plant extracts.

MATERIALS AND METHODS

Collection and Preparation of Plant Materials

Three morphological parts of the plant were collected- leaves, stem barks and roots, and identified in the Department of Science Laboratory Technology, Federal Polytechnic Bali, where a voucher code of *FEDPOBAL2013AST001* was deposited for the plant. Freshly collected leaves were kept for determining leaf surface data while others were air-dried alongside other parts. They were grinded using mechanical blender into fine powders, and then stored for further usage.

Chemo Microscopy of Powdered Parts

Chemo microscopy of the powdered parts was carried out using methods described by Evans [11] and Kokate [12].

Examination of epidermal characters of leaves

The fresh leaf of the plant was nicked with razor blade followed by peeling of the epidermis with finger on both upper and lower surfaces. The material was mounted in one drop alcohol and one drop dilute glycerol and observed starting with the scanning objective. Features observed were recorded as well as drawn in laboratory note book, with the aid of camera Lucida and pencil.

Quantitative microscopy of *Bidens pilosa*

The quantitative microscopy of the plant was done using freshly prepared leaves in order to determine the following leaf surface data, and using the methods adopted from Kokate as well as Evans [11, 12]: stomatal number, stomatal index, Vein-islet and vein let termination numbers and palisade ratio. A total of ten readings were taken and values recorded as means of the original values.

Determination of stomatal number and stomatal index

Stomatal number is defined as the average number of stomata per sq mm of epidermis of the leaf while stomatal index is the percentage which the number of stomata form to the total number of epidermal cells, each stoma being counted as one cell. The stomatal index was calculated using the formula:

$$S.I = \frac{S}{E + S} \times 100$$

Where; S.I = stomatal index, E = epidermal cells, and S = number of stomata[11-12].

Determination of vein-islet and vein let termination numbers

Vein-islet number is defined as the number of vein islets per sq mm of the leaf surface midway between the midrib and the margin. A vein termination on the other hand is the ultimate free termination of vein let. This was determined using the methods of Kokate and Evans [11-12].

Determination of palisade ratio

Palisade ratio is defined as the average number of palisade cells beneath each epidermal cell. Scrapings from each of the leaf surface were cleared by boiling with chloral hydrate solution and mounted in glycerine water and then focused under high power (x40) magnification[11-12].

Determination of Physico-chemical Parameters of Powdered Plant Parts

Using the methods as described by Brain and Turner [13], the following physical constants were determined: moisture content, Ash value, acid insoluble ash value, water soluble ash value, alcohol and water extractive values.

Extraction of Plant Materials

2000g (2Kg) of each of the powdered parts was weighed using electronic scale balance into three separating funnels respectively. 1.5mL of methanol was poured into 500mL capacity separating funnel using cold maceration techniques. The extracts were weighed and a percentage yield for each extract was calculated as:

$$\% \ yield = \frac{Final\ weight\ of\ extract}{Total\ Weight\ of\ ground\ plant} \times 100$$

Preliminary Phytochemical Screening of Methanol Extract of *Bidens pilosa* Leaves

The methods described by Odebiyi and Sofowora[14] as well as Evans were used to test for the presence of saponins, flavonoids, alkaloids, cardiac glycosides, tannins, anthraquinones, carbohydrates, triterpenes, and unsaturated steroids.

Toxicity Investigations of *Bidens pilosa* Crude Methanol Extracts in Mice

0.5 g of the crude extracts were weighed and prepared into appropriate mL in order to determined the lethal dose (LD_{50}) of the extracts in Swiss albino mice of both sexes numbering 39 and average weight 20 g. The animals were weighed using an electronic scale balance. The experiment was divided into two phases. In phase one, 9 animals of 3 animals per group were used and doses of 10mg/kg, 100mg/kg and 1000mg/kg b.w methanol extract administration i.p were given to the animals. Animals were monitored for any deaths and changes in behaviour for one week during the working days. In second phase, the remaining 4 animals were divided into 3 groups of one animal per group and administered doses of 1600mg/kg, 2900mg/kg and 5000mg/kg b.w methanol extract (i.p) and observed for changes and death for 24h. LD_{50} was calculated as the geometric mean of the dosage that resulted in 100% mortality and that which cause no mortality at all. The experiment was terminated after two weeks [15].

Biological Evaluation of Methanol Extract of leaves of *Bidens pilosa*

This was carried out in order to assess the potency of the crude extract of the leaf on selected bacteria based on the ethnomedicinal use of the leaf. Bacteria tested include: *Bacillus subtilis NCTC8239*, *Salmonella typhi ATCC9184*, *Staphylococcus aureus NCTC6571*, *Escherichia coli NCTC10418*, *Klebsiella pneumonia ATCC10031*, and *Staphylococcus aureus ATCC13704.*

Antibacterial Screening of *Bidens pilosa* Methanol Extract

Using well diffusion method, solution concentration of 20mg/mL was used as the initial concentration, and blood agar was used as the growth medium. 40g of the medium was dissolved in a litre of distilled water contained in a flask capped with cotton wool. The medium was boiled to dissolve on a Bunsen burner [16].

Determination of Minimum Inhibitory Concentration (MIC) And Minimum Bactericidal Concentration (MBC) of *B. pilosa* Leaf Methanol Extract

MIC and MBC determination were carried out on the microorganisms that showed sensitivity to the extract. This was done using broth dilution methods [16-17]. The aim was to know the least concentration of the extract that will inhibit or prevent the growth of the bacteria or kill the microbes.

Anti-haemonchosis Evaluation Various Leaf Extracts of *Bidens pilosa* on Filiariform Larvae of *Haemonchus contortus*

The effects of *Bidens pilosa* leaf extract on L3 larvae of *Haemonchus contortus* was evaluated using the methods described by Wabo *et al*. [18] with slight modifications.

Statistical Analysis

All statistical analysis was based on the student's t-test and analysis of variance (ANOVA). Statistical difference was determined at $p \leq 0.05$ for triplicate values analysed by Graph pad prism version 6.0 2013.

RESULTS

Table 1. Cell wall material s of *Bidens pilosa* powdered parts

Cell wall materials	Test	Observation	Conclusion/location
Cellulose/Hemicelluloses	N/50 iodine	Blue colour	Cell wall layer of (R,S)
	N/50 iodine + 80% w/w H_2SO4	Blue colour	-
	Ammoniacal CuO	Cellulose ppt.	Cellulose is stained(L,R,S)
Cutin	Chlor-zinc-iodine	Yellow to Brown colour	Cuticle (L,R,S), cork cell (SR)
Suberin	Chlor-zinc-iodine	Yellow to Brown	Cuticle (L,R,S)
Gums/Mucilage	Ruthenium red	No colour change	-
Lignin	Pgnl + HCl	No colour change	-

*L (Leaf), S (Stem) and R (Root), Pgnl (Phloroglucinol), CuO (Copper II oxide).

Table 2. Examination of cell inclusions of *B. pilosa* powdered parts

Description	Starch	COX	$CaCO_3$	Fats/oils	Tannins
Shape :	Spherical	prism	-	-	-
Size :	28 µm (Large)	18 µm (Large)	-	-	-
	4 µm (small)	4 µm (Small)	-	-	-
Hilium :	Eccentric	-	-	-	-
Striation :	None	-	-	-	-
Frequency:	Numerous	Rare	-	-	-
Aggregation:	Simple grains	-	-	-	-
Location :	Cortex, Parenchyma (LRS)	Parenchyma (LS),		-	Vacuole

* - = not applicable or not detected, L (Leaves), S (Stem barks) and R (Roots), COX (Calcium oxalate), $CaCO_3$ (Calcium carbonates).

Table 3. Quantitative microscopical features of the Leaf of *B. pilosa Linn*.

Parameter (mm^2)	Upper Surface	Lower Surface
	Range	Range
SN	22-**23.3**-25	31-**34.1**-38
SI	12. 5 -**13.24**- 14.2 %	22.4-**23.80**-26 %
VIN	12-**14**-16	–
VTN	20 -**21**-22	–
PR	8 -**9.4**-12	4.2-**7.5**-10

***** SN (stomatal number), SI (stomatal index), VIN (vein islet number), VTN (vein termination number), PR (palisade ratio), numbers in bold are means of original values at n = 5, - = not applicable.

Table 4. Physico-chemical constants of *Bidens pilosa*

Parameters Mean + SEM	Leaves	Stembarks	Roots
Moisture content	4.10 ± 0.08	3.10 ± 0.06	2.60 ± 0.05
Ash value	12.47 ± 0.20	6.22 ± 0.14	3.04 ± 0.09
Acid insoluble ash	1.34 ± 0.17	6.60 ± 0.25	3.50 ± 0.22
Water soluble ash	5.20 ± 0.46	6.90 ± 0.49	4.40 ± 0.44
Alcohol extractive Value	2.50 ± 0.15	1.70 ± 0.06	0.50 ± 0.12
Water extractive Value	6.32 ± 0.50	2.91 ± 0.08	1.51 ± 0.06

*Note results are means of ± SE of duplicate estimations at n = 5.

Table 5. Phytochemical screening of methanol extracts of *B. pilosa leaf*

Constituent	Test	Observation	Inference		
			L	S	R
Saponins	Frothing test	Frothing occur more than 30 min	+	+	-
	Haemolysis test	Haemolysis in tube	+	+	-
Flavonoids	NaOH test	Yellow colour	+	+	-
	Shinoda's test	Orange colour	+	+	-
Tannins	Ferric chloride test	Green colour	+	+	+
	Lead acetate test	Brown ppt.	+	+	+
	Goldbeater's skin	Black colour	+	+	+
Alkaloids	Dragendorff's reagent	Rose-red ppt.	+	-	-
	Wagner's reagent	White ppt.	+	-	-
	Mayer's reagent	Cloudy ppt.	+	-	-
	Picric acid test	Yellow colour	+	-	-
	Tannic acid	Black cloudy ppt.	+	-	-
Anthraquinones	Borntrager's test	Violet colour	+	-	-
	Modified Borntrager's test	Cherry-red colour	+	-	-

●Key: +=present, - = absent, L = leaf extract, S= stembark extract, R= root extract.

Table 6. LD_{50} determination of crude methanol extracts of *Bidens pilosa*

Doses (mg/kg) 1.p	Animal died/Animal survived		
	Leaves	Stembarks	Roots
10	0/3	0/3	0/3
100	0/3	0/3	0/3
1000	0/3	0/3	0/3
1600	0/1	0/1	0/1
2900	0/1	0/1	0/1
5000	0/1	0/1	0/1

● LD_{50} > 5000mg/kg, n=3 in phase I, n=1 in phase II.

Table 7. Zones of inhibition of leaf extract of *B. pilosa* against the microbes

Test Organism	Mean zone of inhibition + SE (mm)		
	Extract	Ceftriaxone	Water
Staph aureusATCC13704	33± 0.22	13±0.05	0
Staph aureusNCTC6571	32±0.22	14±0.06	0
Salmonella typhiATCC9184	16 ± 0.31	28+0.13	0
K. pnemoniaATCC10031	18 ± 0.10	22±0.14	0
B. subtilisNCTC8239	27±0.14	20±0.12	0

●Note zero (0) denotes organism that showed resistant to the drugs, $p<0.05$ (t- test), n = 3.

Table 8. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Bidens pilosa* leaf extract against the microbes

Test organism	MIC (mg/mL)					MBC (mg/mL)				
	20	10	5	2.5	1.25	20	10	5	2.5	1.25
Staph aureus ATCC13704	-	-	0*	+	+ +	-	0^a	+	++	+++
Staph aureus NCTC6571	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Salmonella typhi ATCC9184	-	-	-	0*	+	-	-	0^a	+	++
K. pnemoniaATCC10031	-	-	0*	+	++	-	0^a	+	++	+++
B. subtilisNCTC8239	-	-	-	-	-	-	-	-	-	-
	-	-	0*	+	++	-	0^a	+	++	+++

Key:- (clear), + (light growth), ++ (dense growth), 0* (MIC),0^a (MBC), +++ (highly growth), nd (not determined).

Table 9. Effects of methanol extract of *Bidens pilosa* leaf extracts on embryonation and egg hatching against *H. contortus* L3 larvae

% Anti-helminthic activity of *B. pilosa* extracts						
Embryonation inhibition				Egg hatching inhibition		
Conc.(mg/mL)	ME±SE	IAE±SE	MAE±SE	ME±SE	IAE±SE	MAE±SE
0.625	10±0.12	10±0.12	10±0.12	20±0.14	10±0.12	10±0.12
0.725	-	20±0.14	-	-	20±0.14	-
1.200	20±0.14	-	-	30±0.16	-	-
1.250	30±0.16	30±0.16	20±0.14	40±0.18	30±0.16	20±0.14
2.500	40±0.18	40±0.18	30±0.16	60±0.32	-	30±0.16
3.750	60±0.32	-	-	70±0.34	-	-
5.000	80±0.36	80±0.36	40±0.18	80±0.36	40±0.18	-
TW80(1.5%)	-	-	-	-	-	-

*ME(methanol extract), IAE (infused aqueous extract), MAE (macerated aqueous extract), TW (tween solution), - (no visible activity), n = 3 for each determinant, SE(standard error of mean).

Table 10. Effects of methanol extract of *Bidens pilosa* leaf extracts on larval mortality rate in *H. contortus*

% Anti-helminthic activity of *B. pilosa* extracts			
Mortality rate of L3 Larva			
Conc.(mg/mL)	ME±SE	IAE±SE	MAE±SE
0.625	10±0.12	10±0.12	-
0.725	-	20±0.14	-
1.200	-	-	-
1.250	20±0.14	30±0.16	10±0.12
2.500	60±0.32	-	30±0.16
3.750	80±0.36	-	-
5.000	100±0.38	40±0.18	40±0.18
TW80(1.5%)	-	-	-

*ME(methanol extract), IAE (infused aqueous extract), MAE (macerated aqueous extract), TW (tween solution), - (no visible activity), n = 3 for each determinant, SE(standard error of mean), larval mortality increased with concentration.

Plate 1. Pictorial view of *Bidens pilosa* L. (Asteraceae) in its natural habitat.(Source: Gashaka forest, Nigeria)

DISCUSION

The study on the leaf revealed some important diagnostic features that would help in the identification of the plant, since there are close resemblances between *B. pilosa* and other species in Asteraceae. The starch contains hilium at eccentric position and does not have striations. The walls of the granules are thick and isodiametric in all or part of the plant. Suberin and cutin are water proof in the organ where they occur, and it is possible that these materials prevent the loss of water to the surrounding, ensuring that plant survive adverse conditions [18,19]. This is an adaptation to reduce water lose from the stomata [tables 1,2,3]. Thus, the presence of calcium oxalate and $CaCO_3$ in the plant can serve as an indicator of the presence of limestone in the soil where they grow. Diacytic stomata of *B. pilosa* are deeply sunken and this is an adaptation to survive drought. This helps the plant to minimize the rate of transpiration through the leaves.

Excess moisture in drug suggests not only that the buyer could be paying a high price for unwanted water but also that the drug has been prepared incorrectly or subsequent to Excess moisture in drug suggests not only that the buyer could be paying a high price for unwanted preparation, has been wrongly stored. This can also lead to break down of important constituents due to enzyme activity and the other microbial attack. % moisture was found to be 4.10±0.08 w/w (for leaf), 3.10±0.06 w/w (for stem) and 2.60±0.05 w/w (for root). These values are within the range by Edward and Tyler [20], who suggested that the value should not exceed 10%w/w[table 4].

The presence of secondary metabolites such as alkaloids, saponins, tannins, flavonoids and others in the methanol extracts of Bidens pilosa is undoubtedly responsible for the observed biological activities in tables 7,8, 9,10 above. Acute intoxication of the plant parts in mice showed that the plant is safe as an ethnomedicinal prescription in traditional medicine (table 6). Although, there were reduced metabolic activities in the animals at dosage of 5000 mg/Kg b.w, yet this is biologically unimportant. Antibacterial evaluation showed that methanol extract of leaf exhibited greater zones of inhibition, minimum inhibition as well as bactericidal effects on most of the microbes. Resistance strains of *Staph aureus* were mostly affected by *Bidens pilosa* extract. This discovering has been evaluated on most Gram negative strains in the family Enterobacteriaceae [21] [tables 7,8]. In tables 9 and 10, percentages of embryonation inhibition, egg hatching

inhibition and mortality rate of L3 larvae were concentration dependent. This is because as the concentration of extracts increased from 0.625 to 5.000 mg/mL of extracts, developmental as well life cycle activities decreased progressively. But the methanol extract proved to be more effective in slowing down these activities in *Haemonchus contortus* life cycle than other extracts investigated at these concentrations. This result was in agreement with that obtained by Wabo *et al.* [22].

CONCLUSION

This present study revealed that leaf methanol extract of *Bidens pilosa* has antibacterial as well as anti-haemonchosis activities. The study also showed that the plant parts were safe to be used as an ethnomedicinal plant since no death or necrosis as well as liver congestion was seen *in vivo* mice model at different dosages.

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CONFLICT OF INTEREST

Anthelminthic activities of the *Bidens pilosa* Linn. has been previously evaluated by Wabo *et al.* and only methods used were adopted from the study. No any other conflicting interest existed as at the time of this research.

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