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Pharmacognostic Study and Sedative Activity of *Bryophyllum pinnatum* Stem Methanol Extract and Fractions

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Abstract: Insomnia is known as sleep insufficiency and is considered a capital public health concern. Bryophyllum pinnatum which belongs to the family Grassulaceae is used in folkloric medicine to treat various kind of ailment. The aim of the study was to undertake a pharmacognostic study of the stem and determine the sedative/hypnotic ability of the methanol extract and fraction of the stem. The physicochemical analysis indicated moisture content (12.68%), total ash (3.75%), acid insoluble ash (2.60%), water soluble ash (2.00%), water soluble extractive (6.02%) and alcohol soluble extractive (10.80%). The organoleptic evaluation of the powdered leaves revealed an odorless, green plant with bitter taste. The microscopic analysis of the leaf revealed palisade cell, spongy cell, stoma, collenchyma, xylem tissue, phloem tissue. The transverse section of the stem revealed epidermis, endodermis, xylem and cortex. The acute toxicity result revealed that *Picralima nitida* had no adverse effect in Wistar mice. The result showed that ethyl acetate fraction had sedative/hypnotic ability, affected the level of liver enzymes (ALT, AST, ALP), improved kidney function, and insignificantly increased body weight of mice. This study demonstrated that *B. pinnatum* could be an alternative medicine in pharmacological treatment of insomnia.

Introduction

Insomnia, also known as sleep insufficiency, is considered a capital public health concern reported an estimate of 50–70 million U.S. adults have sleep-related difficulties with its prevalence among general population varying extensively from 10% to 30% (1). Sleep is an essential biological process which plays a crucial role in various bodily functions. These functions include neural development, memory and learning, emotion regulation, cardiovascular and metabolic health, and also, the elimination of cellular toxins (2, 3). Insomnia can be treated pharmacologically, non-pharmacologically or a combination of both (4). People who suffer from insomnia use prescription drugs such as benzodiazepines, zolpidem, zopiclone, zaleplon to sleep (5). Satheesh *et al*. (2020), explained that despite the effectiveness of pharmacologic treatments in treating acute insomnia, some concerns are available regarding the administration of these agents, such as dependency and their side effects (6). Individuals affected by insomnia at any level are concerned about the safety and effectiveness of these pharmaceutical agents and are seeking out novel alternative therapies with lower or no adverse effects (7). Herbal preparations made from leaves, stems, barks and roots of medicinal plants have been used for ages by traditional health practitioners to treat insomnia. More than 75% of most pharmaceutical products currently in use are derived from plants (8). Veeresham stated that natural products discovered so far have played a vital role in improving the human health (9). It was reported that Linalyl acetate isolated from *Lavandula angustifolia* Mill possess sedative activity. Caryphyllone and linalool volatile oils obtained from *Milissa officinalis* L. were shown to be responsible for the plants sedative properties. The resins humulone, 2-methyl-3-butane-2-ol and lupulone which are the main ingredients found in Humulus lupulus

have been shown to possess sedative action (8).

Bryophyllum pinnatum, also known as miracle leaf or wonder of the world (9), belongs to the family Crassulaceae. *B. pinnatum* grows widely and is used in folkloric medicine in Africa, America, India, China and Australia (10). It is used extensively for the treatment of ailments such as conjuctivis, edema, piles, cuts, eczema, constipation, epilepsy, cholera, asthma, chest colds, menstrual disorders, chicken pox, fever (11) and for alleviating abdominal discomforts (12). *B. pinnatum* is enriched with a diverse variety of the pharmacological active chemicals (13) and therefore it necessitates carrying out further scientific research in order to confirm the justification behind its use in folk therapeutics (14).

Figure 1. *Bryophylum pinnatum* (Lam.) Oken plant (Oladejo *et al*., 2021).

This study aims to determine the sedative/hypnotic effect of the methanol extract and fractions of the stem of *B. pinnatum*, and to study the pharmacognostic activity so as to prevent adulteration.

Materials and Methods

Plant Material

B. pinnatum stem was collected from Nsukka, Enugu State Nigeria. The plant material was identified and authenticated in the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Awka, Anambra State Nigeria, with voucher number FPS/PCG/BP-032. The plant material was shredded then air-dried under room temperature for 3 weeks and pulverized with a mechanical grinding machine (GX160 Delmar 5.5HP).

Chemicals

Chemicals and experimental reagents used include: n-Hexane, butanol, Ethyl acetate, Methanol, Glacial acetic acid and diethyl ether (Guangdong Guanghua Chemical Factory Co., Ltd, China), Tween-80, Ascorbic acid, Fehlings solution (A&B, China), Ammonia solution, Millions reagent, ferric chloride (Griffin & George, England), HCL, Potassium dichromate, Potassium ferricyanide (Hopkin and Williams Ltd, England). All solvent/reagent purchased were of analytical grade. All laboratory reagents were freshly prepared and freshly distilled water was used when required.

Animals

Swiss Albino mice (25 – 30 g) were employed for the study. All the animals were obtained from the animal house of the Department of Pharmacology, Enugu State University of Science and Technology, Agbani. The animals were housed in standard laboratory conditions of 12 h light, room temperature, 40-60% relative humidity and fed with rodent feed (Guinea Feeds Nigeria Ltd). They were allowed free access to food and water. All animal experiments were conducted in compliance with NIH guide for care and use of laboratory animals (National Institute of Health (NIH) (2011) Pub No: 85-23) and animal protocol was approved by Animal care and ethics committee of Enugu State University of Science and Technology with approval number ESUT/AEC/0461/AP 296.

Extraction of the Plant Material

A 6 kg amount of the stem powder of *B. pinnatum* was weighed using a weighing balance (Camry EK5350 Model, China) and extracted using cold maceration in 4.5L of 99% methanol for 72 h with intermittent shaking. The solution was filtered using Whatman filter paper and the filtrate was concentrated to dryness in vacuo using rotary evaporator (RE300 Model, United Kingdom) at 40 °C.

Fractionation of the Plant Extract

A 70 g of the crude methanol extract was dispersed in 10% methanol (200 mL) and was subjected to liquid-liquid partition successively with n-hexane, ethyl acetate and butanol in increasing order of polarity to obtain n-hexane fraction, ethyl acetate fraction, butanol fraction, and water fraction. The extracts and all the fractions were stored in refrigerator between 0-4 °C until they were used. The fractions were filtered and concentrated in vacuo using rotary evaporator at 40 °C.

Organoleptic Evaluation

Sensory organs were used to examine the colour, odour, taste and texture of the plant powder. The organoleptic evaluation was carried out using standard methods as described by Evans (15).

Physicochemical Studies

The physicochemical analysis of the leaf powder was carried out to determine the total ash, acid-insoluble ash, water-soluble ash and extractive value using standard methods as described by Odoh *et al*. (16).

Microscopic Analysis

The qualitative and quantitative microscopy study was carried out on the leaf and stem of *B. pinnatum* according to the method described by Nwafor *et al*. (17). The freehand section of the leaves and stem was

prepared by clearing method. The section was treated with chloral hydrate and stained with safranin for 5 minutes to reveal palisade cell, spongy cell, stoma, collenchyma, xylem and phloem tissue. They were viewed under a compound microscope and photomicrographs were taking through a photographic microscope

Acute Toxicity Studies

Acute toxicity tests were performed in mice according to the method described by Lorke (18). Phase 1: Nine adult albino mice were weighed, marked and randomized into three groups of three mice each. Each group of animals were administered different doses (10, 100 and 1000 mg/kg) of the extracts. The mice were observed for 24 hours for signs of toxicity as well as mortality. Phase 2: Four mice were weighed, marked and randomized into four groups of one mouse each. They received 2000, 3000, 4000 and 5000 mg/kg of the extract. Observation for 24 hours for obvious signs of toxicity and death was recorded accordingly. The LD_{50} was calculated using **Equation 1**.

$$LD_{50} = \sqrt{D_0 \times D_{100}} \quad \textbf{Equation 1}$$

where, D_0 = highest dose that gave no mortality and D100 = lowest dose that produced mortality.

Sedative/Hypnotic Activity

The sedative/hypnotic studies were carried out as described by Vogel and Vogel (19). Eighty-five (85) Acute Swiss mice were divided into seventeen groups. Each group consists of five animals each. One group was administered 40 mg/kg phenobarbital sodium i.p. The fractions (n-hexane fraction, ethyl acetate fraction, butanol fraction and water fraction) each received 50, 100 and 200 mg/kg p.o while chlorpromazine hydrochloride (4 mg/kg i.m) was used as reference drug (positive control). Each animal was injected with 40 mg/kg i.p. phenobarbital sodium thirty minutes after the administration of the test samples. The onset and duration of sleep were noted by recording the time interval elapsed between the loss and regaining of righting reflex.

Toxicological Evaluation of the Most Active Fraction

Twenty healthy albino mice were randomized into 4 groups of 5 animals each. Group A was administered 100 mg/kg of the most active fraction (ethyl acetate). Group B and C were administered 200 mg/kg and 400 mg/kg respectively. Group D served as control and received only 10 mL/kg of 5% Tween 80 for 28 days. The animals were weighed before treatment and at the end of the 28 days treatment. On the 29th day, 5 mice from each group were anaesthetized using diethyl ether and blood samples were collected through the retro-orbital plexus. The blood samples were used as estimate of biochemical toxicity markers. For the estimation of the serum enzyme levels, the blood samples were allowed to coagulate for 30 minutes. The clear serum was centrifuged at 2500 rpm for 10 minutes and used for the analysis of biochemical hepatic markers - Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), and kidney function markers - Blood Urea Nitrogen (BUN) and Creatinine using standard methods.

Biochemical Assay of Serum Liver Marker Enzymes

The quantitative determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Alkaline phosphate (ALP) were estimated by the method described by Colville (20) using the test kit (Span Diagnostics Ltd., India.

Kidney Function Markers

Serum creatinine and blood urea nitrogen (BUN) were estimated by the method described by Tietz, (21) and Heinegard and Tiderstrom (22) respectively using BUN and creatinine test kits (Teco Diagnostics, USA).

Quantitative Determination of Creatinine

Creatinine working reagent was prepared by combining equal volumes of 10 mM picric acid and Creatinine buffer reagent (10 mM sodium borate, 240 nM sodium hydroxide). A 3.0 mL of the reagent was added to labelled tubes (test, blank and standard) and 100 µL of serum (test), 5 mg/dl of Creatinine (Standard), and distilled water (blank) were added. These were mixed in their designated test tubes. The tubes were incubated at 37 OC for 15 minutes and the absorbance measured spectrophotometrically at 520 nm against test blank. The concentration of Creatinine (mg/dl) were calculated using **Equation 2**.

$$Marker\,(\%) = \frac{A_{test}}{A_{standard}} \times C_{standard} \quad \textbf{Equation 2}$$

where, A = Absorbance and C = concentration.

Quantitative Determination of Blood Urea Nitrogen

A 1.5 mL of BUN Enzyme reagent (containing 10,000 µ/l Urease, 6.0 mmol/l sodium salicylate, 3.2 mmol/l sodium nitroprusside) was added to 10 µL of Test (serum), Standard (20 mg/dL) and Blank (distilled water) and were incubated for 5 minutes at 37 °C. At timed interval, 1.5 mL of BUN colour developer (6 mmol/L of sodium Hypochlorite and 130 mmol/l sodium hydroxide) was added to each of the labelled tubes and were incubated for another 5 minutes at 37 °C. The absorbance of the tests and standard were measured spectrophotometrically at 630 nm against blank. Urea nitrogen concentration (mg/dl) were calculated using **Equation 2**.

Statistical Analysis

The results were analysed using SPSS version 16 and presented as mean ± standard error of mean (SEM). Significance between control and extract-treated groups were determined using one-way analysis of variance (ANOVA). Differences between means were considered statistically significant at $p < 0.05$ using turkey HSD post hoc test.

Results

Yield of *B. pinnatum* Methanol Extract and Fractions

The methanol extract yielded 1.83% calculated from 6000 g of air-dried stems. The butanol, ethyl acetate, n-hexane and water fractions calculated from 70 g of the methanol extract yielded (butanol 17.43%), (ethyl acetate 26.86%), (n-hexane 22.00%) and (water 33.71%) respectively. The yield in gram and percentage of the methanol extract and fractions of *B. pinnatum* stem are presented in **Table 1**.

Table 1. Yield of *B. pinnatum* methanol extract and fractions.

Extracts/Fractions	Yield (g)	Yield (%w/w)
Methanol extract	110	1.83[a]
Butanol fraction	12.2	17.43[b]
Ethyl acetate fraction	18.8	26.86[b]
n-hexane fraction	15.4	22.00[b]
Water fraction	23.6	33.71[b]

Note: [a]Yield calculated from 6000 g of powdered stem, [b]Yield calculated from 70 g of methanol extract.

Physicochemical Studies of *B. pinnatum* Powdered Stem

The result of the physicochemical analysis of the powder revealed that *B. pinnatum* stem methanol extract contains moisture content 12.68%, total ash 3.75%, acid insoluble ash 2.60%, water soluble ash 2.00%, water soluble extractive value 6.02%, alcohol extractive value 10.08% as shown below in **Table 2**.

Table 2. Physicochemical Analysis of *B. pinnatum* stem.

Parameters	Yields (% w/w)
Moisture content	12.68 ± 0.25
Total ash	3.75 ± 0.07
Acid insoluble ash	2.60 ± 0.05
Water soluble ash	2.00 ± 0.02
Water soluble extractive	6.02 ± 0.03
Alcohol soluble extractive	10.80 ± 0.02

Microscopic Analysis of the Leaf and Stem of *B. pinnatum*

The fresh leaf microscopic features presented in **Figure 2** revealed a thin adaxial and abaxial epidermal layer of the leaf with less prominent cells. The cross section of the mid rib of the leaf revealed palisade cell, spongy cell, stoma, collenchyma, xylem and phloem tissue as presented in **Table 3**. The transverse section of the stem revealed the epidermis, endodermis, cortex and xylem as shown in **Figure 5**. The quantitative microscopy analysis is presented in **Table 3**.

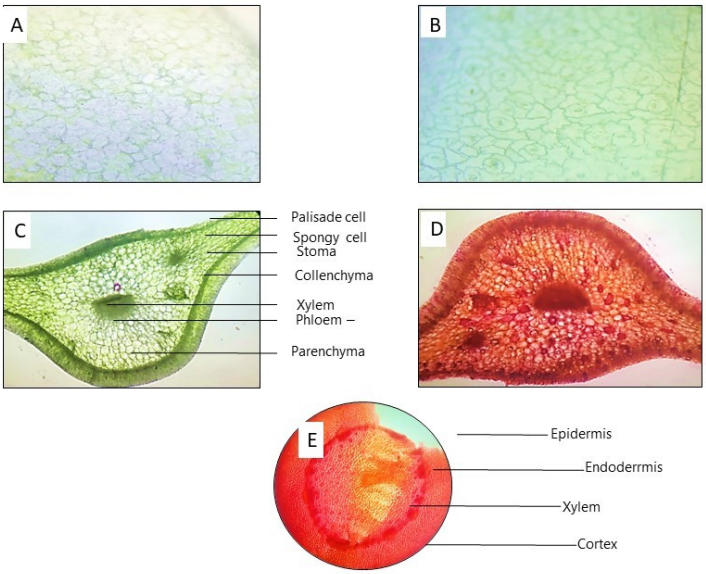

Figure 2. Microscopic analysis of *B. pinnatum*; A= Adaxial (upper) surface of the leaf; B= Abaxial (lower) surface of the leaf; C & D= Cross section of the mid rib; E= Transverse section of the stem.

Table 3. Quantitative microscopic analysis of *B. pinnatum* stem.

Parameters	Range
Stomata density	37.10 ± 1.73 mm^2
Stomata length	35.12 ± 2.13 µm
Stomata width	26.81 ± 1.52 µm
Stomata size	1101.04 ± 41.11 $µm^2$
Vein islet number	3.24 ± 0.09 mm^2
Veinlet termination number	6.84 ± 1.33 mm^2
Palisade ratio	09.25 ± 0.21

Acute Toxicity Result

The result of the acute toxicity (LD50) is presented in **Table 4**. No mortalilty was observed in both phases of the acute toxicity test. Reduced animal activity and sedation were observed in all groups especially at second phase which received higher doses of the extract. The LD50 was estimated to be greater than 5000 mg/kg.

Table 4. Acute toxicity of methanolic extract of *B. pinnatum* stem.

Group	Dose (mg/kg)	Number of death	% Death
A	2000	0	0.00 ± 0.00
B	3000	0	0.00 ± 0.00
C	4000	0	0.00 ± 0.00
D	5000	0	0.00± 0.00

Phenobarbital-Induced Sleep Activity of the Methanol Extract and Fractions

The results presented in **Table 5** demonstrate a significant increase in both onset and duration of sleep at 200 mg/kg for the crude extract and ethyl acetate fraction in the Phenobarbital-induced sleep activity. Notably, the n-hexane fraction, administered at the same dosage, exhibited no significant changes compared to the vehicle and positive control, prompting further exploration into its specific components and potential pharmacological actions.

Toxicological Evaluation of the Most Active Fraction

Effect of Ethyl Acetate Fraction on Liver Function Enzymes

There was a significant reduction in serum ALT ($p < 0.05$) at 200 and 400 mg/kg doses of the ethyl acetate fraction treated groups while significant ($p < 0.05$) reduction in serum AST occurred only at higher dose of 400 mg/kg. For serum alkaline phosphatase (ALP) enzyme activity, treatment with the ethyl acetate fraction produced slight alteration in enzyme concentration. At 100 mg/kg, there was an increased enzyme concentration while at 200 and 400 mg/kg, reduction in concentration was recorded. These changes in ALP enzyme concentrations were found not to be significantly ($p > 0.05$) different from the concentration recorded for the vehicle control group. The result is presented in **Figure 3**.

Table 5. Effect of methanol extract and fractions on phenobarbital-induced sleep in mice.

Treatment	Dose (mg/kg)	Onset of sleep	Duration of sleep
Pentobarbitone	40	8.9 ± 0.6	56.3 ± 1.5
Extract	50	11.6 ± 0.4	64.9 ± 1.6
	100	18.3 ± 0.7*	105.4 ± 1.9*
	200	26.9 ± 0.9*	135.8 ± 2.2*
Hexane Fraction	50	8.9 ± 0.2	56.6 ± 1.2
	100	9.4 ± 0.6	52.3 ± 1.5
	200	9.4 ± 0.8	54.9 ± 1.4
Ethyl acetate Fraction	50	20.1 ± 0.6*	76.4 ± 1.2*
	100	28.4 ± 0.9*	126.0 ± 1.5*
	200	32.7 ± 0.5*	195.8 ± 1.8*
Butanol Fraction	50	9.1 ± 0.7	60.3 ± 1.1
	100	9.6 ± 0.4	62.5 ± 1.4
	200	8.9 ± 0.6	78.3 ± 1.6*
Water fraction	50	20.5 ± 0.6*	55.3 ± 1.1
	100	24.9 ± 0.4*	50.9 ± 1.5
	200	27.3 ± 0.8*	50.1 ± 1.3
Chlopromazine	4	8.5 ± 0.5	88.5 ± 1.7*

Note: *$p < 0.05$ compared to pentobarbitone

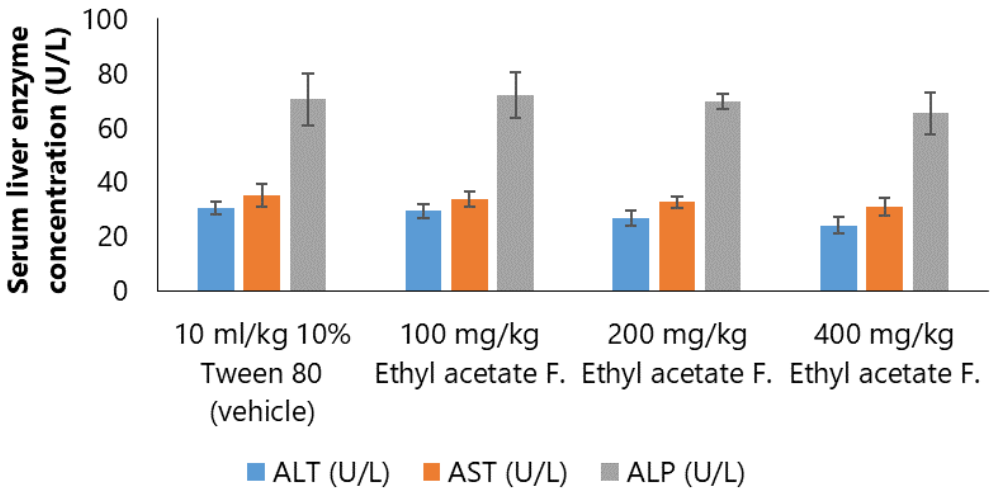

Figure 3. Graphical representation of the effect of ethyl acetate fraction on liver function enzymes (*, $p < 0.05$ compared to vehicle control group).

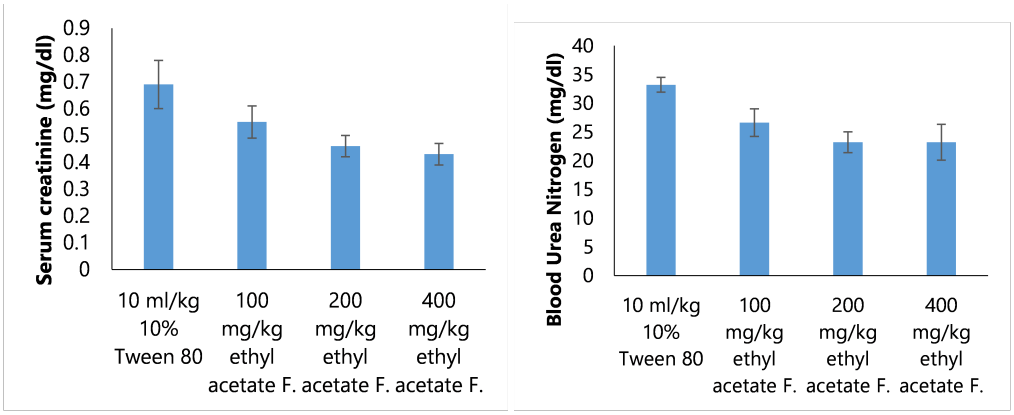

Figure 4. Graphical representation of the effect of ethyl acetate fraction on kidney function parameters (*, $p < 0.05$, compared to vehicle control group).

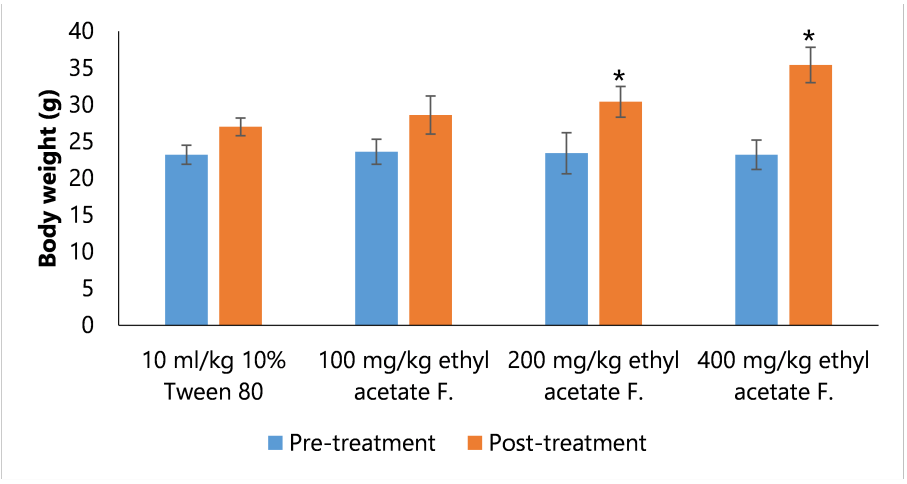

Figure 5. Graphical representation on the effect of ethyl acetate fraction on body weight (*, p ˂ 0.05, compared to vehicle control group).

Effect of ethyl acetate fraction on kidney function parameters

Reduction in blood urea nitrogen and serum creatinine were observed after 28 days administration of the ethyl acetate fraction. Progressive significant ($p<0.05$) reductions were recorded at all tested doses compared to the vehicle control group. The serum creatinine and BUN can be seen in **Figure 4**.

Effect of the Ethyl Acetate Fraction on Body Weight

The body weights of various groups of animals for the sub-acute study were closely related prior to treatment with no significant ($p > 0.05$) differences across groups. However, after 28 days treatment, progressive increase in body weight was observed with increasing dose of the ethyl acetate fraction (see **Figure 5**). These increase in body weights became significantly ($p < 0.05$) different from the weights of vehicle control group at 200 and 400 mg/kg doses of the ethyl acetate treated groups.

Discussion

This paper reports the yield of methanol extract and different fractions of Bryophyllum pinnatum stem, the pharmacognostic parameters which comprises the physicochemical and microscopic study of fresh and powdered stem as well as the sedative/hypnotic activity of the extract and fraction on mice.

The efficiency of extraction solvents varies based on the nature of the extracted compound, solubility, and polarity of the solvent used (23). A polar solvent may be more effective at extracting polar compounds while a non-polar solvent may be more effective at extracting nonpolar compounds. The result in table 1 showed that water had the highest extraction yield (33.71%) while butanol had the lowest (17.43%). Water is a polar solvent that is commonly used for the extraction of polar compounds like sugars, amino acids, and organic acids (24).

According to the WHO, the organoleptic and microscopic description of a medicinal plant is the first step in establishing the identity, degree of purity (25), authenticity and identifying the adulterants in medicinal plant raw drugs. The powdered stem of *B. pinnatum* is green, odorless, astringent and soft. The microscopic study of the fresh leaf showed the adaxial and abaxial surfaces. The adaxial epidermal layer is thin, small and less prominent while the abaxial is less distinct. This agrees with the result of Kamboj *et al*. (26). The ground tissue of the midrib is parenchymatous and homogenous and the cells are circular or angular and compact. Stomata are abundant and are anisocytic. Xylem elements are narrow, angular, thin-walled and diffused while the phloem appears as thick hemispherical arc (Figure 3). The microscopically diagnostic features of the transverse section of the stem include single layered epidermis consisting of thin-walled rectangular cells and covered by moderately thick and striated cuticle.

Evaluation of physicochemical parameters is important in preparing herbal monograph (27). Determination of ash values, extractive values and moisture content signifies standard parameters to ensure the quality and purity of the crude drug. The imminent composition of *B. pinnatum* stem extract contains moisture content 12.68%, total ash 3.75%, acid insoluble ash 2.60%, water soluble ash 2.00%, water soluble extractive value 6.02% and alcohol extractive value 10.08%. Less value of moisture content of drugs could prevent content bacterial, fungal or yeast growth through storage and according to the report of Pandey (28), it is recommended that the moisture content in crude drugs should not be more than 14%. Since the value is below the maximum limit, it implies that the powder of this plant material can be stored for a longer period with lower chances of microbial attack. The total ash indicates the presence of inorganic matter present in a plant material (26). The extractive values are indicative of approximate measures of their chemical constituents extracted with specific solvents (27).

Butanol fraction gave a fast onset of sleep and a short duration of sleep. When compared to vehicle induced control there is no significant difference in onset of sleep at dose 200 mg/kg and not much significant increase in duration of sleep at dose 200 mg/kg. N-hexane fraction treated group showed no significant difference both in onset of sleep and duration of sleep when compared to vehicle induced control as presented in **Table 5**. Ethyl acetate fraction treated group and crude extract treated group gave a significant difference in onset of sleep and duration of sleep, this means that the crude extract and ethyl acetate fraction took a long time for onset of sleep and a long duration of sleep at a high dose of 100 mg/kg and 200 mg/kg when compared to the vehicle induced control and positive control as shown in **Table 5**. Ethyl acetate fraction dose of 50 mg/kg showed a significant increase in time for onset of sleep and no much increase in duration of sleep when compared to vehicle induced control and positive control. Water fraction gave a significant difference in onset of sleep, this shows that water fraction treated group took a long time to sleep when compared to the vehicle induced control and positive control as indicated in table 9 below. Following the results presented in the **Table 8** below it showed a dose dependent increase onset and duration of pentobarbitone-induced sleep. *B. pinnatum* has showed a marked effect on the CNS and it has been proven that the methanolic extract produced a significant change in behaviour pattern, a study results have demonstrated that the herb caused the CNS

depression and dose-dependent stimulation of pentobarbitone sleeping time (27). The administration of ethyl acetate fraction also increases the body weights of the mice. This could be an indication of some compounds present in the fraction.

Conclusion

Insomnia is among the most prevalent health complaints. To maintain good health and high quality of life, it is crucial to get quality sleep. The present study revealed that *B. pinnatum* stem is more polar in water than other extractive solvent. This study also established the pharmacognostic profiling of *B. pinnatum* will help in proper identification and authentication of the plant thereby preventing adulteration. The crude methanol extract and ethyl acetate fraction produced a significant increase in sleep quality and duration at a high dose of 100 mg/kg and 200 mg/kg. The crude methanol extract and ethyl acetate fraction produced a significant increase in sleep quality and duration at a high dose of 100 mg/kg and 200 mg/kg. The administration of the methanolic extract produced a significant change in behaviour pattern and the administration of ethyl acetate fraction also increases the body weights of the mice. This study demonstrated that *B. pinnatum* could be an alternative medicine in pharmacological treatment of insomnia.

Declarations

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Conflict of Interest

The authors declare no conflicting interest.

Data Availability

The unpublished data is available upon request to the corresponding author.

Ethics Statement

The study was approved by Animal care and ethics committee of Enugu State University of Science and Technology with approval number ESUT/AEC/0461/AP 296.

Funding Information

Not applicable.

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