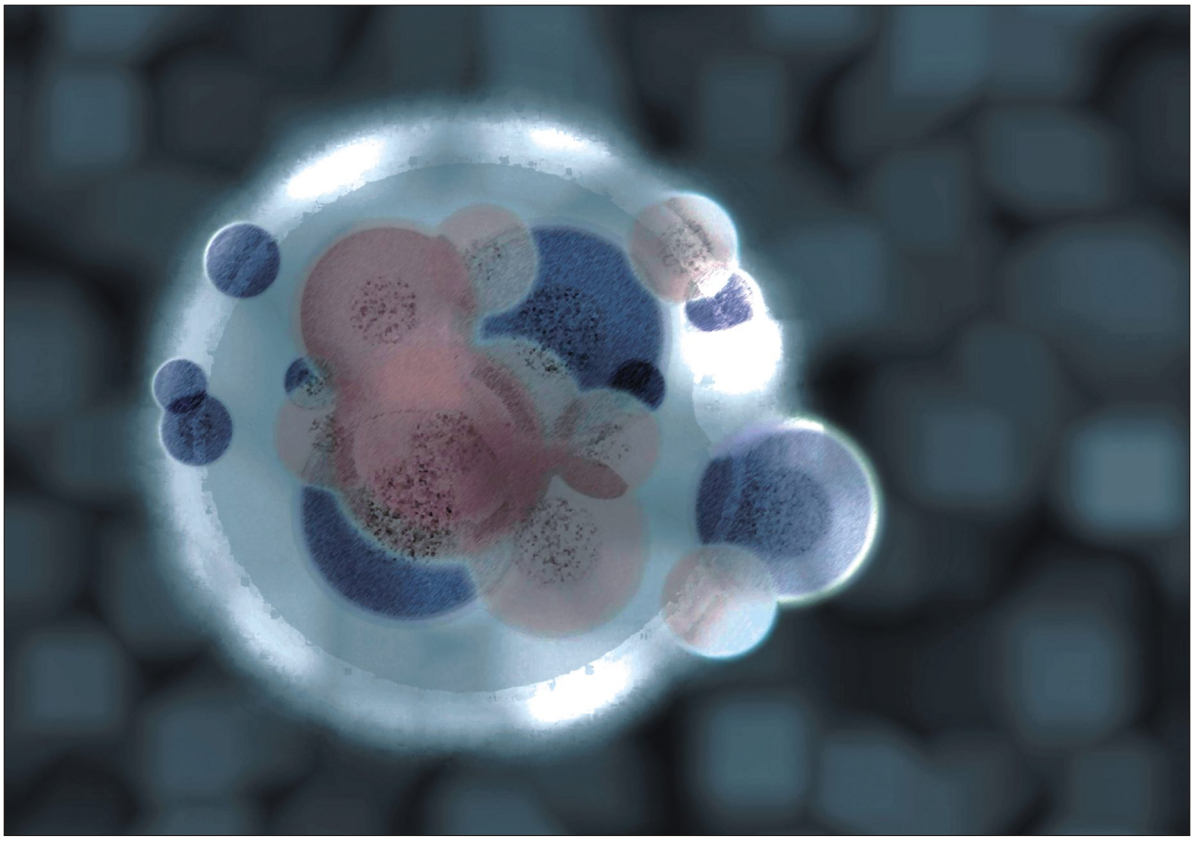

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Research Article
Phytochemical and Toxicological Studies of Methanol and Chloroform Fractions of *Acanthus montanus* Leaves

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Abstract

Background and Objective: *Acanthus montanus* (Nees) T. Anderson (Acanthaceae) is a shrub widespread in Africa, used in African traditional medicine for the treatment of several diseases due to the biologically active compounds present in the plants. Hence, this study evaluated the qualitative and quantitative phytochemical constituents of the methanol and chloroform fractions of *Acanthus montanus* leaves and any possible toxicity effect. **Materials and Methods:** The plant material was macerated in methanol and chloroform in a 1:1 ratio, phytochemical analysis of the methanol and chloroform fractions were done using standard methods. **Results:** Qualitative phytochemical tests revealed that the methanol fraction was rich in alkaloids, reducing sugars, carbohydrates and phenols while the chloroform fraction is rich in alkaloids, flavonoids, carbohydrates and phenols. The quantitative phytochemical tests of the methanol fraction revealed the presence of phenols, alkaloids, reducing sugars, flavonoids, terpenoids, tannins, glycosides and steroids, while chloroform fraction revealed Phenols as carbohydrates, alkaloids, flavonoids, reducing sugars, terpenoids, tannins and steroids. **Conclusion:** The presence of phytochemicals in various proportions in chloroform and methanol fractions also showed that phytochemicals are chemically active compounds that contain polar and non-polar regions thus making it easier to detect the region of the active compounds.

Key words: *Acanthus montanus*, chloroform, methanol, phytochemicals, toxicity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Medicinal plants play a very important role in the health of individuals, especially in many developing countries. Medicinal plants create an effective source of both traditional and modern medicine[1]. Medicinal plants are the main sources of traditional medicine and over the years over 3.3 billion people are living in less developed countries make use of these medicinal plants[2]. These medicinal plants are known to contain certain bioactive molecules that give them these various medical and therapeutic properties. These bioactive molecules produce a type of physiological reaction in the human body and are one of the main reasons for the research in medicinal plants[3].

These bioactive molecules are known as Phytochemicals. Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients[4]. Phytochemicals are classified into primary and secondary metabolites, depending on the role they play in plant metabolism. Primary metabolites include reducing sugars, amino acids, protein, chlorophyll which plays more of a nutritive role etc. Secondary metabolites are the remaining plant chemicals such as alkaloids, terpenoids, tannins, saponins, flavonoids and glycosides which play more defensive roles[5].

Acanthus montanus is a small shrub with sparse branches and soft stems. It belongs to the family *Acanthaceae.* It is commonly known as Mountain Thistle or Bears Breech and is known to have originated from West Africa[6]. Recent pharmacological studies have shown the anti-inflammatory, antipyretic, antimicrobial and immunological properties of this plant[7]. Medicinal plants are the "backbone" of traditional medicine, which means more than half of the less developed countries utilize medicinal plants on a regular basis[4]. Medicinal plants have invariably been a rich source of new drugs and many drugs in use today were either obtained from plants or developed using their chemical structure as starting materials[8].

Toxicology is the study of the adverse effects of chemicals on living organisms. Toxicology testing (safety testing, toxicity testing) is conducted to determine the degree to which a substance can damage living or non-living organisms. It is routinely performed by pharmaceutical manufacturers in the investigation of a new drug.

Acute toxicity (Lethal toxicity) is the ability of a chemical to cause ill effect "relatively soon" after one oral administration or a 4 hrs exposure of to a chemical in the air. It is sometimes also referred to as the "Median Lethal Dose." The LD_{50} for a particular substance is essentially the amount that can be expected to cause death in half (i.e., 50%) of a group of some particular animal species, usually rats or mice when entering the animals' body by a particular route. It is usually expressed as the amount of chemical administered (e.g., mg) per 100 g (for small animals) or kg^{-1} (for bigger subjects) of the body weight of the test animal[9].

Hence, this study evaluated the qualitative and quantitative phytochemical constituents of the methanol and chloroform fractions of *Acanthus montanus* leaves and any possible toxicity effect.

MATERIALS AND METHODS

Study area: This study was carried out in December, 2018.

Collection of plant materials: *Acanthus montanus* plants were obtained from a local market (Ogige Market), Nsukka, Enugu State. The plant leaves were identified and authenticated by Mr. Alfred Ozioko, A plant taxonomist of the International Centre for Ethnomedicine and Drug Development (InterCEDD) Nsukka, Enugu State, Nigeria with Voucher number InterCEDD/035.

Chemicals and equipment: The apparatus, chemicals and equipment used in this research work were gotten from the Department of Biochemistry, University of Nigeria, Nsukka, Nigeria.

Preparation of plant extract: The plant material was shade-dried, powdered and weighed using an electronic weighing balance. A mass of 1168.47 g of the plant material was macerated in 2625 mL of methanol and 2625 mL of Chloroform in a 1:1 ratio for 48 hrs at room temperature. The soaked plant material was filtered with Whatman no. 4 filter paper. Thereafter 20% distilled water equivalent to the volume was added to the filtrate. The mixture was shaken and poured into a separating funnel where it was allowed to stand for some hours. It separated into two layers; the lower layer (chloroform layer) and the upper layer (methanol layer). The chloroform extract was obtained and concentrated using a magnetic stirrer (70°C) to obtain crude extract weighing 95.36 g which was stored in the refrigerator until further use. The methanol layer was also obtained and stored in the refrigerator until further use.

Determination of extract yield: The percentage yield of extract of *Acanthus montanus* was calculated by weighing the powdered leaves before extraction and after the concentration of the extract. It was calculated using the formula[10]:

$$\text{Yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of grinded leaves}} \times 100$$

Qualitative phytochemical analysis of the chloroform leaf extract of *Acanthus montanus*: Qualitative phytochemical analysis of the extracts was done to determine the presence of secondary metabolites present according to standard procedure[10,11], while the quantitative phytochemical components of the extract of *Acanthus montanus* were quantified according to the following method[12].

RESULTS AND DISCUSSION

Percentage yield: Results in Table 1 showed that after partitioning the extract with methanol and chloroform, chloroform fraction resulted in the highest yield of both fractions (8.16% w/w), while methanol gave the lesser quantity (4.12% w/w). This might be due to the low polarity associated with chloroform and high molecular weight. The low molecular weight of methanol and the high polarity (used for extraction) could have caused the low percentage yield while the addition of water (used as a solvent for partitioning) which is miscible with methanol increased the polarity of methanol thus giving a better and clear separation of the solvents during partitioning. The lesser yield (4.12% w/w) was obtained using methanol, probably since the plant contains more non-polar components than polar. As expected, the extract yield increased when using solvents of decreasing polarity and increasing molecular weight in partitioning.

Table 1 showed the Percentage yield of the chloroform and methanol fractions of *Acanthus montanus* where chloroform had a higher yield than methanol.

Solubility of chloroform and methanol fractions: As seen in Table 2 below, the greenish colouration and high percentage yield of the chloroform fraction is essentially due to chlorophyll (and/or other pigments) which is present in every green plant in larger quantities. Other pigments and/or oils may also probably have been extracted by the chloroform since they are of poor polarity. Thus the extraction of active principles from the medicinal plants for pharmacological evaluation was to some extent dependent on the polarity of the solvents used in the extraction and partitioning. In this study, *Acanthus montanus* leaf was extracted with methanol and chloroform and partitioned to yield polar and non-polar constituents in two separate fractions. The methanol extract was readily soluble in water as its components consisted of polar Phyto-constituents whose miscibility in water is very high as a result of the polar-polar interaction between molecules, thus allowing dissolution in water. The non-polar Phyto-constituents of the chloroform extract were sparingly soluble in water but were soluble in tween 80 (Polysorbate 80) a non-ionic surfactant and emulsifier of very low polarity facilitating its reaction with molecules of the Phyto-constituents of the chloroform extract.

Table 1: Percentage yield of the chloroform and methanol fractions of *Acanthus montanus*

Weight of leaves (g)	Fractions	Extraction yield (g)	Yield (%)
1168.97	Chloroform	95.36	8.16
	Methanol	48.16	4.12

Table 2: Solubility of chloroform and methanol fractions from *Acanthus montanus* leaf

Extract	Colour	Solubility
MeOH fraction	Light-green	Distilled water
CF fraction	Dark-green	Tween 80 solution

CF: Chloroform, MeOH: Methanol

Table 3: Results of preliminary acute toxicity studies of (phase 1 and 2) methanol fraction of *A. montanus* extract in mice

Groups	Dose (mg kg^{-1})	No. dead/no. alive	Clinical sign(s)
1	10	0/3	No observable signs
2	100	0/3	No observable signs
3	1000	0/3	No observable signs
4	1600	0/3	No observable signs
5	2900	0/3	No observable signs
6	5000	0/3	No observable signs

The above table shows the Solubility of chloroform and methanol fractions of *Acanthus montanus* leaf in which the methanol fraction was soluble in water while the chloroform fraction was soluble in tween 80 solution.

Toxicity of the different fractions: The fractions were tested for their acute toxicity effects (LD_{50}) using Lorke's method in a two-phase study which produced no mortality as can be seen in Table 3 and 4. Table 3 showed the toxicity of the methanol fraction while Table 4 showed that of chloroform. In the acute toxicity studies, all the treated mice were closely examined for signs of toxicity from the time of administration of extracts to more than 48 hrs. None exhibited any obvious signs of toxicity or change of behaviour at various doses ranging from 10-5,000 mg kg^{-1}. All the treated mice remained alive even long after a week of observation. This suggests an oral LD_{50} greater than 5,000 mg kg^{-1}. The high LD_{50} value implies a remote risk of acute intoxication and a high degree of relative safety[13] when the extract is administered orally. It is therefore considered practically non-toxic. Earlier[14], established an LD_{50} greater than 5,000 mg kg^{-1} when the aqueous root extract of *A. montanus* was administered orally and intraperitoneally. In

Table 4: Results of preliminary acute toxicity studies of (phase 1 and 2) chloroform fraction of *A. montanus* extract in mice

Groups	Dose (mg kg^{-1})	No. dead/no. alive	Clinical sign(s)
Phase 1			
1	10	0/3	No observable signs
2	100	0/3	No observable signs
3	1000	0/3	No observable signs
4	1600	0/3	No observable signs
5	2900	0/3	No observable signs
6	5000	0/3	No observable signs

Table 5: Qualitative phytochemical components of the chloroform and methanol fraction of the leaves of *Acanthus montanus*

	Inference	
Phytochemicals	Chloroform	Methanol
Alkaloids	+	+++
Flavonoids	++	++
Tannins	+	+++
Phenols	+	++
Saponins	ND	ND
Glycosides	+	+++
Reducing sugars	+++	+++
Carbohydrates	+	+++
Terpenoids	++	+++
Steroids	++	+++

+: Slightly present, ++: Moderately present, +++: Highly present, ND: Not detected

an earlier study[15] demonstrated LD_{50} of the same plant to be greater than 8,000 mg kg^{-1} in rats. This probably explains why the undetermined amount of aqueous leaf extract is taken locally in folk medicine without side effects.

The above table showed the results of preliminary acute toxicity studies of methanol fraction of *A. montanus* extraction mice where there was no mortality or any observable clinical sign.

The above table showed the results of preliminary acute toxicity studies of (phase 1 and 2) chloroform fraction of *A. montanus* extract in mice where there was no mortality or any observable clinical sign.

Qualitative phytochemical constituents: The presence of these phytochemicals in the *Acanthus montanus* can be seen in Table 5. Findings suggested that these bioactive compounds are actively responsible for the pharmacological properties of medicinal plants[16] and this explains why this plant is used in the treatment of various diseases. However, some phytochemicals were present in smaller concentrations while some are present moderately and some in abundance.

Flavonoids are hydroxylated polyphenolic compounds and have been reported to possess significant pharmacological properties which include protection against allergies, inflammation, free radicals, platelet aggregation, microbes, ulcers, hepato-toxins, viruses and tumor[17]. Besides, flavonoids also exhibit anti-fungal, anti-viral and anti-cancer properties[18]. Flavonoids also have significant activities when ingested and there is great interest in their potential health benefits, particularly for compounds such as isoflavonoids, which have been linked to the anticancer benefits of soy-based foods and the stilbenes in red wine that are believed to contribute to reduced heart disease[19]. It has been known for several years that plant polyphenols such as steroids, terpenoids, flavonoids etc. are antioxidants *in vitro*[20]. Therefore, flavonoids protect against oxidative cell breakdown and damage has anti-inflammatory properties. This may be the reason *A. montanus* is used in the treatment of furuncles as reported previously[14]. Flavonoid also lowers the risk of heart diseases and cancer. Quercetin which is used to treat atherosclerosis, CVDs and prevent cancer is a flavonoid.

Tannins have high potency for intestinal disorders such as diarrhoea and dysentery[8]. Tannins bind to produce rich proteins in the body and interfere with protein synthesis. They are known to exert anti-microbial activities by iron deprivation, hydrogen bonding or specific interactions with vital proteins such as enzymes in microbial cells[17]. Besides, the high concentration of tannins also shows antimicrobial and antifungal activities by coagulating the protoplasm of microorganisms[21]. Tannins can also be effective in protecting the kidneys[22] and as anti-inflammatory, antiseptic; antioxidant and haemostatic pharmaceuticals[23]. Thus the presence of tannins in *A. montanus* fractions could suggest its activity against microbial related diseases.

The phenolic compounds play important roles in health in addition to enhancing antimicrobial activity in this plant[24]. Phenols also contain wound healing potential by providing materials for the lignifications of cells[25]. These phenolic compounds include tannins, flavonoids and glycosides among others. They are known to be biologically active and their presence has been reported for several activities like anti-bacterial, molluscicidal[26], antidirrhoeal[27], anti-vibrio[28], analgesic[29], anti-hepatotoxic and anti-ulcer[30], anti-microbial and antioxidant. Phenolic compounds usually contribute to antioxidant activity due to the presence of hydroxyl functional groups in their chemical structures[31]. Besides, it has been hypothesized to inhibit carcinogenesis through electrophile trapping mechanism[32].

Steroids are known mainly for their effect on lipid metabolism[33]. Phytosterol, a class of steroids also helps to inhibit the intestinal absorption of cholesterol and anti-inflammatory role[34,35]. Steroids exhibit anti-mutagenic (e.g., stigmasterol), anxiolytic, analgesic, anticonvulsant, sedative, hypnotic and anaesthetic properties by enhancing GABA receptor function in a non-genomic manner and some steroid

Table 6: Quantitative phytochemical composition of the chloroform and methanol leaf extract of *Acanthus montanus* (mg/100 g)

	(mg/100 g)	
Phytochemicals	Chloroform	Methanol
Alkaloids	372.50±7.26	1393.35±54.45
Flavonoids	362.56±73.43	180.84±4.13
Tannins	11.78±1.04	12.05±0.13
Phenols	4067.21±115.91	5883.06±302.50
Glycosides	5.79±0.93	4.06±0.12
Reducing sugars	235.51±23.26	728.55±27.41
Carbohydrates	428.99±21.45	1028.55±27
Terpenoids	181.22±14.35	80.19±11.73
Steroids	14.74±0.62	1.12±0.03

Values were expressed as Mean±SD of n = 3

derivatives have been synthesized as GABA receptor antagonist for the treatment of CNS abnormalities e.g., stress, anxiety, seizures caused by epilepsy and to prevent muscle tension, depression and to cause anesthesia[36]. Steroids are also used in the synthesis of drugs and several sex hormones. This perhaps justifies the use of the plant with *Costus* spp. in the treatment of urogenital infections and urethral discharge in[14] and in the treatment of false labour and threatened abortion[37]. Furthermore, steroids are known for their antibacterial activity specifically associated with membrane lipids and cause leakage from liposomes[38]. It is probable that the presence of steroids in *Acanthus montanus* also validates its use as an anti-inflammatory[15] and anti-bacterial agent.

Terpenoids have medicinal properties such as anti-carcinogenic (e.g., perilla alcohol), anti-malarial (e.g., artemisinin), anti-ulcer, hepaticidal, anti-microbial or diuretic (e.g., glycyrrhizin) activity and the diterpenoid anticancer drug-taxol[39]. Terpenes have unique antioxidant activity in their interaction with free radicals. They react with free radicals by partitioning themselves into fatty membranes under their long carbon side chain[40]. The anti-inflammatory and radical scavenging ability of terpenoids is also probably responsible for the use of *Acanthus montanus* in treating hepatitis (a disease that results in liver inflammation), boils, various inflammatory conditions[15] as well as other anti-oxidant functions.

Alkaloids are very important in phytomedicine and constitute most of the valuable drugs, for example, anti-malarial. Alkaloids are well known for their anti-microbial activity. Some studies have been reported on the anti-microbial activity of alkaloids from several medicinal plants such as *Jatropha curcas*, *Carica papaya*, *Mangifera indica* and *Psidium guajava*[41]. Pure, isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents all over the world for their analgesic, antispasmodic and bactericidal effects. These are used for the help of mankind and found beneficial for certain life-threatening disease[42]. Morphine, Codeine and berberine are among the isoquinoline alkaloids that have been used in the treatments of neurodegenerative diseases (NDDs) such as Alzheimer's Disease (AD), Parkinson's Disease (PD) and Epilepsy. Also, caffeine is a stimulant for the Central Nervous System (CNS) promoting CNS coordination and fighting NDDs[43]. Thus the presence of alkaloids alludes to the ability of the fraction of A. montanus to exhibit numerous pharmacological activities.

Glycosides are the well-known and well-established class of phytochemicals that have cardiotonic, anti-bacterial, anti-viral, anti-cancer, anti-oxidant, anti-inflammatory, neuroprotective, hepatoprotective and immunomodulatory actions[44]. They can also inhibit the oxidase enzymes like tyrosine's and are useful in dermatological treatments. Cardiac glycosides, a class of glycosides are usually used to treat heart diseases by strengthening heart tissues and allowing them to function more efficiently[45]. They can inhibit sodium ions, Na^+ pump and decrease the concentration of Na^+ ions in the myocytes. This will lead to an increasing in the number of calcium ions, Ca^{2+} available for the heart muscle. Then, it will increase the cardiac output and decrease the distension of the heart. In addition to their role in cardiac health, cardiac glycosides have anticancer properties as well[36]. These glycosides, therefore, play significant roles in the use of *Acanthus montanus* in ethnomedicine to treat hypertension, cardiac dysfunctions and various heart diseases[44].

Reducing sugars and carbohydrate are also present. Carbohydrates are nutrients that are required in adequate amounts in the diet for the supply of energy in the body[16]. The incorporation of the leaves of *A. montanus* into diet provides an adequate source of carbohydrate for the diabetic and obese patient. The plant has been also reported to have hypoglycemic properties[6]. Plant polysaccharides have also shown diverse biological activities such as wound healing, enhancement of the reticuloendothelial system, stimulation of the immune system, treatment of tumours and effects on the hematopoietic system[46]. The effectiveness of *Acanthus montanus* in treating wounds, boils, inflammatory conditions[15] as well as other diseases in local medicine could also be attributed to the presence of these reducing sugars.

Quantitative phytochemical constituents: The presence of these phytochemicals in the *Acanthus montanus* can be seen in Table 6 suggested that these bioactive compounds are actively responsible for the pharmacological properties of medicinal plants[16] and this explains why this plant is used in the treatment of various diseases. However, some phytochemicals were present in smaller concentrations while some are present moderately and some in abundance and these can be as a result of the different polarity index of the

solvents used in partitioning the extract. From Table 6 alkaloids were present in both fractions, however, the concentration was more in the methanol fraction than the chloroform fraction and this could be because most alkaloids are basic and comparatively methanol is more basic than chloroform. A similar result was also expressed in carbohydrates and reducing sugars. Carbohydrates are the main source of energy for the system and they are mainly found in plant foods little wonder why *Acanthus montanus* is included in diets. In folk medicine, plants containing polysaccharides have been used as hypoglycemic[47] and anti-inflammatory[48]. Tannins and glycosides were found to be present in a non-significant ratio in both fractions. Terpenoids, steroids and flavonoid are found to be present in more concentration in the chloroform fraction than the methanol fraction. Phenols are aromatic organic compounds consisting of one or more hydroxyl groups. Phenols are known to exert so many pharmacological effects such as antimicrobial[24], analgesic[29] and anti-ulcer[30]. In the present study, phenolics were found to be present in higher concentrations in the methanol fraction than the chloroform fraction.

CONCLUSION

The findings of this study have provided toxicity, qualitative and quantitative estimation of the phytochemicals of the methanol and chloroform fractions of *Acanthus montanus* leaves. The presence of these phytochemicals at various concentrations suggests potential medicinal value and possibility of use in pharmaceutical research. Individual isolation of these phytochemicals should be done to determine their potency against various diseases. More so further research is indispensable to reveal the detailed molecular mechanism(s) behind the biological activities of the phytochemicals present in the plant.

SIGNIFICANCE STATEMENTS

This study discovered that both the chloroform and methanol fraction are safe for laboratory practices and they contain a bioactive compound that could be potent against some diseases. This study will help the researchers uncover the solubility, toxicity and phytochemical contents of the different fractions of *Acanthus montanus* that many researchers were not able to explore. Thus the new theory on solubility, toxicity and the potency of *Acanthus montanus* may be arrived at.

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