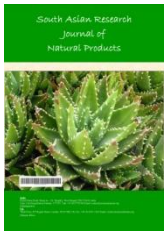

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# Toxicological Evaluation of Different Extracts of *Andrographis paniculata*

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author IED designed the study, wrote the protocol and the first draft of the manuscript. Author ENB managed the literature searches and the experimental process. Author ENB identified the species of plant. Both authors read and approved the final manuscript*.

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## ABSTRACT

**Aim:** The study aimed to evaluate the toxicity of the crude extracts of *Andrographis paniculata* leaves using a Brine Shrimp (*Artemia salina*) lethality assay test to substantiate the ethnopharmacological uses of this plant.
**Methodology:** Ethanol, acetone and ethyl acetate extracts of *Andrographis paniculata* were subjected to qualitative and quantitative phytochemical screening for bioactive compounds using standard methods and protocols. The extracts were further subjected to cytotoxicity screening using Brine Shrimp Lethality Assay.
**Results:** The results of the preliminary screening revealed the presence of alkaloids, saponins, terpenes, anthraquinones, tannin and flavonoids in all the extracts studied, Phlobatannins and cardiac glycosides were not detected in any of the extracts. Total phenols, alkaloids, flavonoids tannins and saponins were determined quantitatively and the results showed that total phenols were the major phytochemical constituent present in highest concentration while the least was tannin in all extracts. The concentrations of these bioactive compounds were highest in ethanol extract. In Brine Shrimp Lethality Bioassay, all the extracts produced dose-dependent cytotoxicity

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effect to Brine Shrimp nauplii with ethanol extract exhibiting highest toxicity having $LC_{50}$ value of 187.5 µg/ml compared to the positive control, potassium dichromate whose $LC_{50}$ value was 130.0 µg/ml.

**Conclusion:** The cytotoxicity exhibited by the crude extracts confirmed the presence of potent bioactive compounds and validates the ethnopharmacological importance of *Andrographis paniculata* in the treatment of different illnesses.

*Keywords: Andrographis paniculat; Brine Shrimp; cytotoxicity; ethnopharmacological; flavonoids.*

## 1. INTRODUCTION

In recent years, the trend has changed towards the utilization of phytochemicals present in like vegetables, fruits, oilseeds, spices and herbs [1]. These plants serve as alternatives for potential antioxidants, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs and functional ingredients [1,2]. These plants are a valuable source of natural phytochemical compounds such as vitamins, phenolic compounds, and flavonoids alkaloids, steroids, tannins, cardiac glycosides, volatile oils, fixed oils and resins [3,4]. It has been reported by many researchers that about 80% of the human population (mostly in underdeveloped countries) globally depend on herbs for their primary health care. The phytochemical compounds of a plant often determine the physiological action on the human body [5]. Screening of the phytochemical components in a given plant extract is aimed at identifying the nature of the compound present which has been reported to be responsible for the observed medicinal action. These compounds exert their effect by destroying the microorganisms responsible for the infection, clearing up residual symptoms, improving immunity and plays a vital role in reducing human's ageing process [6].

Despite the numerous benefits of medicinal plants, some of the plants are known to carry toxicological properties and as such, the effectiveness and potential toxicity of medication used in folk medicine have to be scientifically evaluated [7-9]. Numerous research studies have recently focused on both pharmacology and toxicity of medicinal plants used by humans. This is important to achieve a safe treatment with plant products [10]. The toxicity of plants may originate from different contaminants or from plant chemical compounds that are part of the plant. Various assays are used for the research of potential toxicity of plant extracts based on different biological models, such as *in vivo* assays [11]. However, recent studies employed efforts for alternative biological assays that include species of *Artemia salina, Artemia franciscana, Artemia urmiana* and *Thamnocephalus platyurus* [12]. These toxicity tests are considered a useful tool for preliminary assessment of toxicity [13]. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the Brine Shrimp (*Artemia* sp.) Lethality assay (BSLA) [14]. The selected plants are screened for their cytotoxic effect on *Artemia salina* and correlate results of toxicity with known ethnopharmacological activities. The advantages of Brine Shrimp Lethality Assay include rapid, cost-effective, no need for special equipment and animal serum. The assay can be performed with less than 20 mg sample without any objection from animal right advocates to the use of these invertebrates for the experiment [15].

*Andrographis paniculata* (Burm. f) Nees., also called as Kalmegh or "King of Bitters" belongs to the family *Acanthaceae* which is a herbaceous plant [16,17].

It is a hardy and erects herb which grows mainly as an under-shrub in the tropical, moist deciduous forest. It has glabrous leaves, about 8.0 cm long and 2.5 cm broad and white flowers with rose-purple spots on the petals [18]. The stem is dark green, about 0.3- 1.0 m in height and 2-6 mm in diameter [19,20]. Some of the chemical constituents that have been found in *A. paniculata* are Diterpenes, flavonoids, terpenoid, lactones, alkanes, alkaloids, cardiac glycoside, tannins, saponins ketones, aldehydes, paniculides, farnesols, polyphenols, arabinogalactan, and several sub-units of andrographolides [20-22]. The biological activities of this plant were attributed to the diterpenoids, flavonoids and quercetin [23]. It is traditionally used to treat gastrointestinal tract and upper respiratory infections, fever, herpes, throat infection hepatitis, tonsillitis, gastroenteritis, pyelonephritis and laryngitis [24,25]. The anti-inflammatory, anti-microbial, anti-malarial, anti-diabetic, anti-diarrhoeal, anti-thrombotic, anti-

venom and hepatoprotective activities of *A. paniculata* has been reported by many researchers [26-33]. *Andrographis paniculata is* commonly used as folk medicine; therefore this research aims to explore the toxicity of different extracts of the leaves on brine shrimp.

## 2. MATERIALS AND METHODS

### 2.1 Collection and Preparation of Sample

Fresh leaves of *Andrographis paniculata* were collected from a bush in Mbiabong in Uyo Local Government Area of Akwa Ibom State. Identification and authentication were carried out in the herbarium section of the Department of Botany, University of Uyo. The fresh leaves were washed with water immediately after collection and then chopped into small pieces, air-dried at room temperature for about 10 days and pulverized into powder form and stored in an airtight container until required for use.

### 2.2 Extraction Procedure

The powdered leaves of *Andrographis paniculata* were extracted by cold maceration method at room temperature with ethanol, ethyl acetate and acetone and left for 72 hours with intermittent shaking. The plant extracts were filtered and concentrated using a rotary evaporator at 40°C. Each extract was transferred into labelled sterile glass vials and stored at 4°C before use.

### 2.3 Qualitative Phytochemical Analysis of Plant Extracts

The leaf extracts (ethanol, ethyl acetate and acetone) were screened for alkaloids, flavonoids, cardiac glycosides, terpenes, deoxy sugars, anthraquinones, saponins, tannins and phlobatannins using procedures described by several authors [34-36].

### 2.4 Test for Alkaloids

Each extract was dissolved individually in 1 ml of 1% hydrochloric acid and filtered. The filtrates were used to test the presence of alkaloids.

**Dragendorff's test:** 1 mL of the filtrate was treated with a few drops Dragendorff's reagent. Formation of orange-brown precipitate indicated the presence of alkaloids.

**Mayers test:** Filtrates were treated with Mayer's reagent. Formation of a yellow cream precipitate indicated the presence of alkaloids.

### 2.5 Test for Tannins

#### 2.5.1 Ferric chloride test

0.5 g of each extract was boiled in 20 ml of distilled water. The mixture was filtered and ferric chloride was added to the filtrate. A dark green color was formed, indicating the presence of tannins.

#### 2.5.2 Test for saponins

**Frothing test:** About 0.5 mg of each extract was shaken with five ml of distilled water. Formation and persistence of frothing showed the presence of saponins.

#### 2.5.3 Test for cardiac glycosides

**Salkowski's test:** 0.5 g of each extract was dissolved in 2 mL of chloroform. Concentrated $H_2SO_4$ (3 mL) was carefully added down the side of the tube to form a layer. An appearance of a reddish-brown colour in the interface indicated the presence of steroidal aglycone portion of cardiac glycosides.

**Keller –Killiani test:** 0.5 g of each plant extract was dissolved in 2 mL of glacial acetic acid containing one drop of ferric chloride solution. 1 mL of concentrated sulphuric acid was subsequently added to the extracts. A brown ring obtained at the interface indicated the presence of a deoxy sugar characteristic of cardenolides

#### 2.5.4 Test for anthraquinones

**Bontrager's test:** About 0.5 g of the extract was transferred into a dry test tube and 5 mL of chloroform was added and shaken for 5 min. The extract was filtered and the filtrate was shaken with an equal volume of 10% ammonia solution. A pink violet or red colour in the lower layer indicated the presence of anthraquinone.

#### 2.5.5 Test for flavonoids

**Shinoda test:** Few pieces of magnesium turnings and 1-2 drops of concentrated hydrochloric acid were added to 5 mL of each extract. Formation of pink colour was taken as evidence for the presence of flavanoids

**Lead acetate test:** Extracts were treated with a few drops of lead acetate solution. Formation of yellow colour precipitate indicated that the presence of flavonoids.

#### 2.5.6 Test for reducing sugar (De-Oxy sugars)

0.5 g of each extract was macerated with 20 ml of distilled water and filtered. To 1 ml of the filtrates, 1 ml of alkaline copper reagent was added. The mixture was boiled for 5 min and allowed to cool. Then 1 ml of phosphomolybdic acid reagent and 2 ml of distilled water was added and the absorbance read at 420 nm.

### 2.6 Terpenes

0.5 g of the different extracts were dissolved in 4 mL of distilled water and filtered. 2 ml of concentrated tetraoxosulphate VI acid was added to the filtrates. A red colour was observed usually indicates the presence of terpenes.

### 2.7 Phlobatannins

#### 2.7.1 Hydrochloric acid test

About 0.5 g of each plant extract was dissolved in water and filtered. The filtrate was boiled with 1% hydrochloric acid. Deposition of a red color precipitate was taken as a positive test.

#### 2.7.2 Quantitative estimation of phyto-constituents

The phytochemicals which are present in the ethanol, ethyl acetate and acetone extract of *Andrographis paniculata* was determined and quantified.

#### 2.7.3 Determination of polyphenols

The total phenolic content in the extracts was determined by the modified Folin-Ciocalteu method as described by Singleton and Rossi [37] and modified by Ayoola et al. [38]. The sample extract was dissolved in methanol (1 mg/ml). An aliquot of 0.5 ml of each plant extract (1 mg/ml) was mixed with 5 ml of Folin- Ciocalteu reagent which was previously diluted with distilled water (1:10 v/v). The mixture was shaken slightly and allowed to stand at 22°C for 5 mins. Afterwards, 4 ml (75 g/L) of sodium carbonate ($Na_2CO_3$) was added. The tubes containing the mixtures were allowed to stand for 30 min at 40°C to develop colour. Absorbance was read at 765 nm using the spectrophotometer. Results were expressed as gallic acid equivalent in (mgGAE/g) of extracts. All samples were analyzed in triplicate.

#### 2.7.4 Determination of total flavonoids

Total flavonoid contents were determined using the method of Ordon et al. [39]. A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of the sample solution. This reaction mixture was kept four 1 hour at room temperature. The absorbance was measured at 420 nm. The yellow colour indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/mL. Total flavonoid content was calculated as quercetin equivalents (mg/g).

#### 2.7.5 Determination of total alkaloids

Total alkaloids were determined according to the standard method as described by Harbone [36]. 5 g of the sample extract was weighed into a 250 ml glass beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 hours. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and filtered. The residue which contained alkaloids was dried and weighed.

#### 2.7.6 Determination of total tannins

500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and brought to volume. 5 ml of the filtrate was pipetted into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0. I N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 720 nm [40].

#### 2.7.7 Determination of total saponins

20 g of each sample was put into a conical flask and 200 ml of 20% aqueous ethanol was added. The samples were heated in a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation,

the samples were dried in an oven to a constant weight and the saponin content was calculated [41].

## 2.8 Brine Shrimp Lethality Bioassay

The Brine Shrimp Lethality Bioassay was used to predict toxicity activity as described by Meyer et al. [42]. Briefly, 4 mg of each extract was dissolved in 2% dimethyl sulfoxide (DMSO). Solutions of varying concentrations (1000,500, 250,125,62.5,31.25 µg/ml) were obtained by serial dilution technique using seawater. The solutions obtained were then added to pre-marked vials containing 10 live brine shrimp nauplii in 5 ml seawater. The experiments were done in triplicate. About 10 ml of DMSO in seawater and different concentrations of potassium dichromate were taken as negative and positive controls respectively. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. $LC_{50}$ values were determined, based on the percentage mortality, using a linear regression method, plotting % mortality against the correspondent log of concentration. Biological activity was recorded as the concentration when 50% of the larvae were killed within 24 hours. From the data obtained, the percentage lethality of Brine Shrimp nauplii for each concentration and control was calculated.

## 3. RESULTS AND DISCUSSION

Phytochemicals can be derived from any part of the plant like bark, leaves, flowers and seeds [43]. Phytochemical screening is one of the techniques to identify new sources of therapeutically and industrially important compounds like alkaloids, flavonoids, phenolics, steroids, tannins and saponins. Knowledge of the chemical compounds of plants is important because such information will be of value for the synthesis of new bioactive compound/s for treating specific disease [44]. The extracts of the leaves of *Andrographis paniculata* were screened qualitatively and quantitatively for the presence of secondary metabolites. The results obtained are presented in Tables 1 and 2. The preliminary qualitative phytochemical screening of ethanol, acetone and ethyl acetate extracts of *Andrographis paniculata* for bioactive compounds indicated that these extracts contained alkaloids, saponins, tannins, flavonoids, terpenes, anthraquinone and cardiac glycosides in varying proportions. However, deoxy- sugars and phlobatannins were absent in all the extracts while cardiac glycosides were not detected in the ethanol extract. These secondary metabolites contribute towards the biological activities of medicinal plants such as hypoglycemic, antidiabetic, antioxidant, anti-microbial, anti-inflammatory, anti-carcinogenic, antimalarial, anticholinergic and antileprosy activities [45]. Preliminary analysis of phytochemicals is essential for the quantitative estimation and separation of pharmacologically active chemical compounds, which subsequently may lead to the drug discovery and development [46].

Quantitative estimation of chemical compounds in the different leaf extracts of *Andrographis paniculata* indicated that all the extracts contained high to moderate levels of bioactive compounds. Generally, it was found that ethanol extract had the highest concentration of bioactive compounds quantitatively estimated. Total phenolic content was highest in ethanol extract (49.24 ± 0.15 mg GAE/g) (Table 2), while the least concentration was recorded for ethyl acetate (11.48 ± 1.43 mg GAE/g). Naturally occurring phenolic compounds, such as phenolic acids, flavonoids, curcuminoids, lignans, tannins, stilbenes and quinones have been reported to be associated with the antioxidant activity of plant and food extracts [47]. This is mainly due to their redox properties, allowing them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, hydroxyl radical quenchers, and metal chelators [48]. The ethanolic extract recorded the highest content for flavonoids (3.30 ± 1.5 mg/g) and the lowest content of flavonoids was noted in ethyl acetate extract (0.78 ± 0.56 mg/g). Flavonoids have been referred to as nature's biological response modifiers because of their inherent ability to modify the body's reaction to allergies, virus and carcinogens [49]. Flavonoids are equally free radical scavengers that prevent oxidative cell damage, have strong anticancer, antiallergic, and anti-inflammatory activities [50,51].

Alkaloids have been reported to possess various pharmacological activities including analgesic, antihypertensive effects, antiarrhythmic effect, antimalarial, bactericidal effects and anticancer activity and they exhibit marked physiological activity when administered to animals [52,53, 50]. The result obtained showed that the extracts contained a moderate concentration of alkaloids.

**Table 1. Phytochemical compounds of different extracts of *Andrographis paniculata***

| Compounds | Ethanol | Acetone | Ethyl acetate |
|---|---|---|---|
| Alkaloids | +++ | ++ | + |
| Saponins | ++ | ++ | + |
| Terpenes | ++ | ++ | + |
| De-oxy sugars | - | - | - |
| Anthraquinone | +++ | ++ | + |
| Tannins | +++ | + | + |
| Cardiac glycosides | - | + | + |
| Flavonoids | ++ | + | + |
| Phlobatannins | - | - | - |

*Keyword: + =low concentration, ++ = moderate concentration, +++ = high concentration, - = absent*

**Table 2. Results of quantification of the phytochemical bioactive compounds in different extracts of *Andrographis paniculata***

| Bioactive compounds (mg/g) | Ethanol extract | Acetone extract | Ethyl acetate extract |
|---|---|---|---|
| Total phenolics | 49.24 ±0.15 | 21.56±1.34 | 11.48±1.43 |
| Total flavonoids | 3.30 ±1.5 | 2.7±1.7 | 0.78±0.56 |
| Total alkaloids | 1.88±0.01 | 0.23±0.04 | 0.22±0.04 |
| Total tannins | 0.23±1.5 | 0.22±1.2 | 0.15±1.4 |
| Total saponins | 10.58±1.7 | 10.2±0.22 | 7.45±1.5 |

**Table 3. $LC_{50}$ result of Brine shrimp lethality bioassay of different extracts of *Andrographis paniculata***

| Extracts | $LC_{50}$ in µg/mL |
|---|---|
| Ethanol | 187.5 |
| Acetone | 375 |
| Ethyl acetate | 750 |
| Potassium dichromate | 130 |

The different extracts of *Andrographis paniculata* contained varying concentrations of tannins, with ethanol extract recording the highest concentration (0.23 ± 1.5 mg/g). The defensive properties of tannins are generally attributed to their ability to bind proteins [54]. It exerts anti-inflammatory effects probably by inhibiting the release, synthesis and /or production of inflammatory cytokines and mediators, including prostaglandins, histamine and polypeptide [55]. Tannins have astringent properties, hasten the healing of wounds and inflamed mucous membranes. They are also known to have various physiological effects like anti-irritant, anti-scretolytic anti-parasitic and anti-microbial activities. Plants containing tannin are used to treat non-specific diarrhoea and inflammation of the mouth [56].

The concentration of saponins in this study ranged from 7.45 ± 1.5 mg/g to 10.58 ± 1.7 mg/g. Ethanol contained the highest concentration of saponins, while ethyl acetate recorded the least concentration. Saponins are known to exhibit antimicrobial activities and protect plants from microbial pathogens [57]. It is also reported that saponins are capable of precipitating and coagulating red blood cells [58]. Saponins are known to possess antioxidant, anti-cancer and anti-inflammatory properties [59,60]. These classes of compounds present from phyto-chemical screening are responsible for the medicinal activity of *Andrographis paniculata* against several pathogens and therefore justify the ethnopharmacological uses of this plant in the treatment of different illnesses.

## 3.1 Brine Shrimp Lethality Test

The brine shrimp test represents a rapid, inexpensive and simple bioassay for testing plant extract lethality which in most cases corroborates reasonably well with cytotoxic and anti-tumour properties. It has been proven to be a convenient system for monitoring biological activities of natural products [61]. Brine shrimp lethality assay after 24 hours of exposure to the crude extracts of *Andrographis paniculata* and positive control, potassium dichromate were investigated and the $LC_{50}$ values are presented in Table 3. Different measures of lethality were observed with exposure to different concentrations of the test samples. The results obtained showed that the brine shrimp lethality assay of the three plant extracts was concentration-dependent, with maximum mortalities at a concentration of 1000 µg/mL whereas least mortalities were at 10 µg/ml

concentration. According to Meyer's toxicity index, herbal extracts with $LC_{50}$ < 1000 µg/mL are considered toxic, while extracts with $LC_{50}$ > 1000 µg/mL are considered non-toxic [42]. Equally, according to Rieser et al. [62], crude extracts resulting in $LC_{50}$ values less than 250 µg/ml were considered active. The leaf extracts of *Andrographis paniculata* exhibited positive brine shrimp larvicidal activity concerning the toxicity indexes proposed by Meyer et al. [42] and Rieser et al. [62]. Among the extracts studied, ethanol had the highest toxicity ($LC_{50}$ = 187.5 µg/mL) which is comparable to the positive control ($LC_{50}$ 130 µg/ml), confirming that ethanol may be a more suitable medium for obtaining specific bioactive compounds responsible for the toxicity. It is possible that a broad range of structurally diverse compounds contribute to the overall pharma-cological activity of the crude extract and synergistic effects between active principles may exist [63].

## 4. CONCLUSION

Qualitative and quantitative evaluation of the different leaf extracts of *Andrographis paniculata* revealed the presence of bioactive compounds such as phenols, flavonoids, alkaloids, tannins and saponins in varying concentrations. The extracts were further subjected to toxicity assay using Brine shrimps. The results indicated that the leaf extracts of *Andrographis paniculata* exhibited cytotoxic activity against brine shrimp since their $LC_{50}$ values were less than 1000 µg/mL. The cytotoxicity exhibited by the crude extract further confirmed the presence of bioactive compounds and gave credence to the ethnopharmacological uses of the plant in the treatment of various diseases. However, Brine Shrimp Lethality Assay is inadequate in determining the mechanism of action of the bioactive substances in the plant, further investigations such as isolation and structural elucidation of these compounds are necessary.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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