PHYTOCHEMICAL ANALYSIS, CYTOTOXICITY AND MICROBIAL ACTIVITY OF *AGERATUM conyzoides LINN*

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ABSTRACT

Phytochemical screening of Ageratum conyzoides revealed the presence of steroidal glycosides (saponin), cardiac glycosides, aloes and alkaloids. A 100% mortality of the Brine Shrimp was observed at a concentration of 1,000μg/ml for chloroform and methanol extracts. The chloroform extract is more toxic than the methanol extract, at all other concentrations. The LC_{50} for the chloroform and methanol extracts are 165.96±8.91μg/ml and 199.53±19.95μg/ml respectively. All the extracts except the water extract inhibited the growth of Staphylococcus aureus while Escherichia coli growth was inhibited by all the extracts except the methanol and water extracts. Only minimum inhibition was observed on the Escherichia coli for the active extracts even at the highest extract concentration (40,000μg/ml) used for the test. The petroleum ether extract gave the largest zone of inhibition at all concentrations.

INTRODUCTION

Phytochemistry literally means plant chemistry, and it involves the assay of plant extracts by chemical or other means to determine the active constituent(s) of the plant. It gives account of the enormous variety of organic substances accumulated by plants, their biosynthesis, turnover and metabolism; and their natural distribution and biological functions[1].

Plant products, which elicit pharmacological response in animals and man include alkaloids, cardiac glycosides, flavonoids, saponins, tannins, etc. Typical alkaloids derived from plant sources are basic and they contain one or more nitrogen atoms. Two broad divisions are recognised: non-heterocyclic and heterocylic alkaloids.

Glycosides are acetals formed by the combination of one or more sugar units with a non- sugar component through oxide rings. They are characterized by the fact that on hydrolysis by either acid or enzyme they yield one or more sugars together with a non-sugar moiety, the aglycone or genin[2]. Flavonoids form the largest group of naturally occurring phenols. Most flavones are O-glycosides. Flavonoids are known for their anti-inflammatory, anti-allergic, anti-bacterial and anti-fungal properties and inhibition of tumor promotion.

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Saponins are a class of glycosides known for their detergency characterized by their frothing properties. They have haemolytic properties and, when injected into the blood stream, are highly toxic. When taken orally, saponins are comparatively harmless[1]. Two groups are recognized; according to the structure of the aglycone they are steroidal (tetracyclic triterpenoids e.g. cholesterol) and pentacyclic triterpenoid. Tanins are complex chemical substances and consist of mixtures of polyphenols. There are two groups of tannins: hydrolysible tannins, (hydrolysable by acids or enzymes) and condensed tannins (includes all true tannins, which are more resistant to breakage).

The Plant-*Ageratum conyzoides* Linn, is of the order Asterales and the family Compositae, which is one of the largest families of flowering plants with about 1,100 currently accepted genera and 25,000 species and is of world-wide distribution[3]. The genus *Ageratum* is confined to America with the exception of the species *Ageratum conyzoides*, a common weed throughout the warmer regions of the world. Other species of the genus are *A. houstonianum*, *A. strictum*, *A. fastigiatum*, and *A. mexicanum*.

Ageratum conyzoides Linn contains coumarin, and a hydrochloric acid extract of the plant was reported to give positive test for alkaloid. The fresh and dried plant materials of *A. conyzoides* Linn contain a volatile oil and a compound, $C_{12}H_{16}O_2$, identified to be a phenol ester, similar to ethyl eugenol and, on oxidation, gives an oil with an intense odour due to the formation of ethyl vanillin[4].

The plant was reported[5] to yield hydrocyanic acid. Rendao *et al.*[4] described the isolation of the anti-insect hormones, ageratochromene and 6-demethoxyageratochromene from *Ageratum conyzoides*. Tyagi *et al.*[6] revealed that the plant contains vitamin A, proteins and amino acids. In addition, vitamin B and some sugars were obtained from the leaves. Riaz *et al.*[7] confirmed that the main component plant oils were ageratochromene and 6-demethoxyageratochromene. They reported the isolation of another compound, β-caryophyllene.

In Nigeria, the plant is known as *Imi eshu* or *akoyunyun* (Yoruba), *Ibuzolkubu odolo* (Ibo) and *Osu angweri ngwa* (Owerri and Umuahia). In Southern Nigeria, the plant is used in chronic ulcer and intra-vaginally for uterine trouble. A decoction of the leaves is used as a lotion for craw-craw and taken internally for fever. It is also used as a purgative[8].

The chief medicinal uses of the leaves of *Ageratum conyzoides* Linn are as a local remedy for craw-craw, for dressing wounds, treating ulcer and as an eyewash. In Liberia, pneumonia in children is treated by rubbing an extract of the leaves on the chest[9]. In Sierra Leone, the leaves crushed in water are given as emetic while in Portuguese Congo, some tribes use the plant in the treatment of sleeping sickness. In Mexico and India, the plant has been used as a tonic and stimulant and as remedy for fever, colic diarrhoea and rheumatism[8].

EXPERIMENTAL

Sample collection and preparation

The leaves of *Ageratum conyzoides Linn* were collected within the premises of Ahmadu Bello University, Zaria, during the dry season (November and December). The leaves were brushed to remove soils and dust, air-dried in the shade and powdered with a mortar and pestle.

80g of the powdered plant sample was extracted successively using continuous hot Soxhlet extraction method until each solvent was visibly clear of any coloured extract; this normally required about 72hours. The solvents used in the following order are petroleum ether (60°-80°C) → chloroform → ethyl ethanoate → methanol → distilled water.

Phytochemical screening

Standard procedures were used for reagents preparation and phytochemical screening[10,11]

Test for carbohydrates

(i) *Molisch's test (standard test for carbohydrates)*

A small quantity of each extract was added to a test tube containing 1ml of distilled water and the solution mixed with few drops of Molisch's reagent. This was then underlayed with 1ml of tetraoxosulphate (VI) acid. The formation of a red ring at the common surface of the liquid indicated the presence of carbohydrate.

(ii) *Barfoed's test (test for monosaccharides)*

1ml of Barfoed's reagent was added to a small quantity of each extract and boiled for 2 minutes. A red precipitate indicated the presence of monosaccharides.

(iii) Fehling's test (standard test for free reducing sugars)

5ml of a mixture of equal volumes of Fehling's solutions A and B was added to a small quantity of each extract and the solution boiled for 5 minutes. Formation of a red precipitate indicated the presence of free reducing sugars. For the Combined Sugars test the extract was first hydrolysed by boiling with dilute hydrochloric acid and neutralised with 10% sodium hydroxide solution, before the Fehling's solution was added.

(iv) *Resorcinol or Selivanoff's test (standard test for ketoses)*

A crystal of resorcinol and 2ml of hydrochloric acid were added to a small quantity of each extract and the solution was boiled for 5 minutes. A red colour indicated the presence of ketoses.

(v) *Test for pentoses*

To a small quantity of each extract was added 1ml of hydrochloric acid and a little phloroglucinol. The mixture was heated on a low flame and a red colour indicated the presence of pentoses.

(vi) *Osazone test*

A small quantity of each extract was mixed with 0.5g phenylhydrazine hydrochloride, 1g of crystalline sodium ethanoate and 5ml of water. The solution was filtered and boiled for 60 minutes. A yellow precipitate indicated the presence of sucrose.

(vii) *Test for soluble starch*

A small quantity of each extract was boiled with 1ml of 5% potassium hydroxide, cooled and acidified with tetraoxosulphate (VI) acid. A yellow colour indicated the presence of starch.

Test for glycosides

(i) *General test for glycosides*

A small quantity of each extract was boiled with 2.5ml of 2.5M tetraoxosulphate (VI) acid, cooled and neutralised with 20% potassium hydroxide and then boiled again with 5ml of a mixture of equal volumes of Fehling's solution A and B. Formation of a brick red precipitate indicated the presence of glycosides.

(ii) *Test for anthracene derivatives (Bornträger's test)*

A small quantity of each extract was shaken in 5ml of chloroform; filtered and the filtrate was shaken with an equal volume of 10% NH_3. A pink, red or violet colour in the ammoniacal lower phase indicated the presence of free hydroxyanthraquinones.

(iii) *Test for cardiac glycosides*

Keller-Kiliani test: To a small quantity of each extract in glacial ethanoic acid containing two drop of iron (III) chloride solution was added 2ml of tetraoxosulphate (VI) acid. A brown ring obtained at the interface indicated the presence of cardiac glycosides.

Salkowski test: To a small quantity of each extract in chloroform (2ml), tetraoxosulphate (VI) acid (1ml) was carefully added to form a lower layer. A reddish-brown ring at the interface showed the presence of a steroidal ring as the aglycone port on of the cardiac glycoside.

(iv) *Test for digitalis glycosides*

A small quantity of each extract was boiled with 10ml of 50% ethanol and 10ml of lead ethanoate. The resulting solution was filtered into a separatory funnel and extracted twice with chloroform. The combined chloroform layer was used for the following tests:

Test for steroidal aglycone:- One portion of the chloroform layer was evaporated to dryness and 1ml of 1% 3,5-dinitrobenzoic acid and 10% sodium hydroxide were added. A red or brown precipitate indicated the presence of steroidal aglycone.

Test for digitoxose sugar:- The second portion of the chloroform layer was also evaporated to dryness and 1ml of xanthydrolhydrochloric acid was added. The resulting solution was boiled, a yellow colouration indicated the presence of digitoxose sugar.

Test for cyanogenetic glycosides: A small quantity of each extract in water was placed in a test tube in the neck of which a moist sodium picrate paper was suspended. The test tube was then placed in an oven for 1 hour at 45°C. Formation of a brick red colour on the paper indicated the presence of cyanogenetic glycosides.

(v) *Test for aloes*

(I) A small quantity of each extract was boiled with 0.1g of borax. 2 drops of this solution was added to a test tube full of water and viewed under UV light. A greenish-yellow fluorescence indicated the presence of aloes.

(II) A small quantity of freshly prepared bromine water was added to 1ml of each extract in a test tube. Formation of a yellow or orange precipitate indicated the presence of aloes.

(vi) *Test for cape aloes*

(I) 1ml of concentrated trioxonitrate (V) acid was added to a small quantity of each extract. Formation of a brown to yellow colour indicated the presence of cape aloes.

(II) To a small quantity of each extract in water, 0.25ml saturated sodium chloride, 0.5ml ethanol and a drop of copper(II) tetraoxosulphate(VI) were added. The mixture was warmed and formation of a brown precipitate indicated the presence of cape aloes.

(vii) *Test for saponin glycosides*

A small quantity of each extract was boiled with [illegible] water, filtered and the filtrate divided into 3 portion.

(I) To the first portion, 2.5ml of a mixture of equal volumes of Fehling's solution A and B was added. A brick red precipitate indicated the presence of [illegible] glycosides.

(II) The second portion was boiled with 2.5ml of tetraoxosulphate (VI) acid, cooled and filtered. The filtrate was made alkaline with sodium hydroxide and [illegible] with 2.5ml of a mixture of equal volumes of Fehling solution A and B. A brick red precipitate indicated the presence of saponin glycosides.

(III) The third portion was thoroughly shaken with [illegible] water and olive oil added. Observation of [illegible] indicated the presence of saponin glycosides.

Test for Tannins

A small quantity of each extract was boiled with 20ml of water, cooled and filtered. The filtrate was used for the following test.

(I) A few drops of lead ethanoate was added to 1ml of the filtrate. Formation of a white precipitate indicated the presence of tannins.

(II) To the filtrate was added few drops of 1% Iron (III) chloride solution, a blue-black, green or blue-green precipitate was taken as evidence for the presence of tannins.

(III) 2ml of 10% ethanoic acid and 10% lead ethanoate was added to 1ml of each filtrate. Formation of a white precipitate indicated the presence of tannins.

(IV) The filtrate was boiled with 3 drops of 10% HCl and 1 drop of methanal, a red precipitate was taken as evidence for the presence of tannins.

Extraction and test for alkaloid

(i) *General test*:- The extracts were cleaned (to remove non-alkaloidal compounds capable of giving 'false positive' results)[10]; and then divided into equal portions and each was treated with few drops of Mayer's, Dragendorff's reagent, 10% tannic acid solution and 1% picric acid solution. Observation of a white precipitate with Mayer's reagent, orange-red colour with Dragendorff's reagent, yellowish-brown

precipitate with tannic acid and yellow precipitate with picric acid, indicated the presence of alkaloids.

(ii) *Vitali-Morin Test*:- Another portion of the 'cleaned' alkaloid extract was evaporated to dryness and 1ml of glacial ethanoic acid was added and the mixture evaporated again. 3 drops of trioxonitrate (V) acid was added and the mixture digested, cooled and dissolved in a few drop of propan-2-one. 2 drops of 3% methanolic potassium hydroxide solution was quickly added; an immediate purple colour indicated the presence of tropane alkaloids.

Cytotoxicity test

The method used for the cytotoxicity test was the Brine Shrimp (*Artemia salina*). This test was carried out on 30mg each of the chloroform and methanol extracts. A stock solution of each of the extracts was prepared to a concentration of 10mg/ml in Dimethyl Sulphoxide (DMSO). For each extract, concentrations of 1000, 500, 250, 125, and 62.5 µg/ml were made by serial dilution from the stock. A test tube containing only DMSO was used as the control. Triplicate determinations were made.

Ten shrimps, initially hatched from its eggs in sea water, were collected with teat pipettes and placed in each test tube and made up to 5.0ml with sea water immediately after adding the shrimps, giving a total of thirty shrimps per dilution. The test tubes were maintained under illumination for twenty-four hours and the survivor shrimp larvae were counted macroscopically in the tubes against a lighted background. From the survivors the number of deaths at each dose and control were recorded. The LC_{50} at 95% confidence interval, were determined for the extracts based on the recorded percentage death using the method of linear regression analysis[12].

Antimicrobial test using the Agar Diffusion method

The microorganisms (concentration is ½ of Macfanland 1. Macfanland 1 is $3x10^6$ million organisms per ml): *Staphylococcus aureus* and *Escherichia coli*, were sub-cultured to nutrient agar slants, and incubated for 24hours at 37°C. The agar medium was prepared by dissolving 23g of the nutrient agar in 1000ml of water, boiled, autoclaved and cooled (slightly above room temperature). This was then dispensed into sterilized medium sized petri dishes and the plate allowed to set). Before inoculating the plates, the broth culture was further diluted (one drop of broth culture to four drops of normal saline- 9% NaCl solution). A control containing only normal saline was also prepared.

Solutions of the extract were prepared using 20% propan-2-one. The concentrations prepared were 50, 100, 1,000, 10,000, 20,000 and 40,000 µg/ml. 0.1ml of the bacterium (*Staphylococcus aureus, Escherichia coli*) was spread on a plate using a bent glass rod and 10mm holes were bored on each plate using 10mm cork borer. 0.1ml of the prepared concentrations of each extract was poured into the holes in duplicate. A control experiment using the solvent only was also set up.

The plates were incubated for 24hours at a temperature of 37°C, after which the zones of inhibition of the bacterium for each extract was measured using a calibrated ruler.

RESULTS AND DISCUSSION

From the successive extraction of *A. conyzoides*, the weight of the extracts obtained based on the initial 80g plant sample are 5.662g for petroleum ether, 2.412g for chloroform, 0.247g for ethyl ethanoate, 9.870g for methanol and 10.350g for water.

Table 1: Results of phytochemical screening

Table 1.1: Test for Carbohydrates

Extract	Carbohydrates						
	Molisch	Barfoed	Fehlings Fr	Fehlings Comb	Ketoses	Pentoses	Osazone	Soluble starch
Pet. ether	+	-	+	+	-	+	-	+
$CHCl_3$	+	-	+	+	-	+	+	+
Ethyl acetate	+	-	+	+	-	+	-	+
Methanol	+	+	+	+	+	+	+	+
Water	+	+	+	+	-	+	-	+
Crude	+	+	+	+	+	+	+	+

Fr = Free sugar Comb = Combined sugar

Table 1.2: Test for glycosides

Extract	Glycosides									
	General	Anthracene	Cardiac		Digitalis			Aloe	Cape aloe	Saponin	Frothing
			KK	S	SA	DS	CG				
Pet. ether	+	-	+	+	-	-	-	+	-	-	-
$CHCl_3$	+	-	+	+	-	-	-	+	-	+	+
Ethyl acetate	+	-	+	+	-	-	-	+	-	-	-
Methanol	+	-	+	+	-	-	-	+	+	+	+
Water	+	-	+	+	-	-	-	+	+	+	+
Crude	+	-	+	+	-	-	-	+	+	+	+

KK =Keller Kiliani S = Salkowski SA = Steroidal aglycone DS = Digitoxose sugar
CG = Cyanogenetic glycoside

Table 1.3: Test for tannins and alkaloids

Extract	Tannin						Alkaloid		
	I	II	III	IV	Mayer	Tannic	Dragendoff	Picric	Tropane
Pet. ether	-	-	-	-	+	+	+	+	-
$CHCl_3$	-	-	-	-	+	+	+	+	-
Ethyl acetate	-	-	-	-	+	+	+	+	-
Methanol	-	-	-	-	+	+	+	+	-
Water	-	-	-	-	+	+	+	+	-
Crude	-	-	-	-	+	+	+	+	+

Results of the phytochemical screening, on each of the extracts and the crude sample of *A. conyzoides*, are shown in Tables 1.1-1.3. As is common to all plants, the test for carbohydrates was positive in the extracts and crude sample. Free reducing sugar, combined reducing sugar, pentoses and starch were found in all the extracts, either as a primary constituent or secondary products of chemical reactions occurring during the test. Monosaccharides test was positive in the crude sample and the methanol and water extracts. Only the crude sample and methanol extract gave positive test for ketoses while osazone was present only in the crude sample, and methanol and chloroform extracts.

Since sugars are confirmed component of this plant extracts, the presence of glycosides are as expected positive. The glycosides in *A. conyzoides* are cardiac glycosides and aloes in all the plant extracts and crude sample; and saponin glycoside in the chloroform, methanol and water extracts as well as in the crude sample. However, the tests for steroidal aglycone, digitoxose sugar, cyanogenetic glycoside and free anthraquinone derivatives were negative. Cape aloes was found only in the polar (methanol and water) extracts and crude sample.

Cardiac glycosides are known to have the ability to stimulate heart muscles and are used in the treatment of congestive heart failure. Saponins are effective in the treatment of syphilis, rheumatism and certain skin diseases[1]; and on abscess or other swellings, ulcers and septic wounds.

Phytochemical screening results are shown in Table 1. *A. conyzoides* also contains alkaloids, which are known to be useful as respiratory stimulants,

Table 2: Result of the Brine Shrimp lethality bioassay

Concentration (µg/ml)	1000		500		250		125		62.5		C*
	CH	ME	CH	ME	CH	ME	CH	ME	CH	ME	
Total shrimp per test sample	30	30	30	30	30	30	30	30	30	30	30
Number of survivors	0	0	7	14	16	19	19	20	24	25	30
Number of death	30	30	23	16	14	11	11	10	6	5	0
% mortality	100	100	77	53	47	37	37	33	20	17	0

C* = Control CH = Chloroform ME = Methanol

insecticides, skeletal muscle relaxant and analgesic. For the alkaloid test reagents, only the Mayer's and Dragendroff's reagents gave positive test while the picric acid and tannic acid solutions were not sensitive enough to detect the alkaloids. The tests for tannins on each of the extracts and the crude sample of *A. conyzoides* were negative.

The Brine Shrimp cytotoxicity test has been used as a simple bioassay in the detection and isolation of plant constituents with pharmacologic activities. It has the advantages of being rapid, inexpensive and simple. The activity of known compounds or extracts is manifested as toxicity to the shrimps[13].

Only the methanol and chloroform extracts of *A. conyzoides* were used for this test because of inadequate quantity of the Brine Shrimp available during the course of this work. As shown in Table 2, 100% mortality of the Brine Shrimp was observed at a concentration of 1,000µg/ml for both extracts. The mortality rate of the Brine Shrimp decreases with decrease in sample concentration. The percentage mortality at lower concentrations is higher in the chloroform plant extract than in the methanol plant extract.

Another method used for estimating the potency of the extracts, was to determine the median lethal concentration, LC_{50} (the concentration of the extract that kills 50% of the brine shrimp), at 95% confidence interval using the method of linear regression analysis[12]. The probits of the percentage death responses give a quantitative measure of the toxicity of the extract. The calculated LC_{50} for the chloroform extract is 165.96±8.9µg/ml and methanol extract 199.53±19.9µg/ml. The cytotoxicity of the extracts are significant in terms of activity, since the LC_{50} values are <1000µg/ml. Extracts are said to display toxicity in the Shrimp assay when LC_{50} is <1000µg/ml, and values >1000µg/ml are said to be too toxic for medicinal use without probable side effects.

All the extracts except the water extract showed inhibitory ability to the growth of *Staphylococcus aureus* while *Escherichia coli* growth was inhibited by all the extracts except the methanol and water extracts. The ether extract gave the largest zone of inhibition at all concentrations, which implies that the components of this extract can suppress the growth of *Staphylococcus aureus* more effectively than the components of the other extracts. However, only the minimum inhibition was observed for the *Escherichia coli* for active extracts even at the highest extract concentration (40,000µg/ml) used for the test. Any extract with zone of inhibition, 6mm or more is considered active[14].

This finding lends credence to the traditional use of the plant as a remedy for wounds, ulcer, abdominal upsets and pains, diarrhoea, infections, e.t.c. which are caused by *Staph. aureus* and *Escherichia coli*.

CONCLUSION

Detailed phytochemical screening of *A. conyzoides* Linn for chemical constituent is reported. The median lethal concentration, LC_{50}, on the brine shrimp

Table 3: Results of antimicrobial sensitivity test

Concentration (µg/ml)	Average Zone of Inhibition (mm) of Extracts									
	Petroleum ether		Trichloro-methane		Ethyl ethanoate		Methanol		Water	
	SA	EC	SA	EC	SA	EC	SA	EC	SA	EC
40,000	14.3	6.0	8.0	6.0	7.3	7.0	9.0	0.0	0.0	0.0
20,000	11.7	0.0	7.0	0.0	7.0	0.0	7.3	0.0	0.0	0.0
10,000	8.7	0.0	6.0	0.0	6.0	0.0	6.5	0.0	0.0	0.0
1,000	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

SA= Staphylococcus aureus EC=Escherichia coli

cytotoxicity assay, of the chloroform extract is 165.96±8.9μg/ml and methanol extract 199.53± 19.9μg/ml. The plant extracts showed some level of inhibition on the growth of *E. coli* and *Stap. aureus*.

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Accepted 22 / 8 / 99
Received 12 / 8 / 99