International Journal of Chemical and Biochemical Sciences

Journal Home page: www.iscientific.org/Journal.html

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Pharmacological investigation of *Asystasia calyciana* for its antibacterial and antifungal properties

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Abstract

The phytochemical investigation of *Asystasia calyciana* (whole plant) extracts revealed the presence of saponins, tannins, steroids, glycosides, flavonoids and anthraquinones. The hexane, ethylacetate and methanol extracts of *Asystasia calyciana* were evaluated *invitro* to determine inhibition of human pathogenic microorganisms made up of six bacteria and six fungi. The plant metabolites inhibited the growth of twelve test organisms at different concentrations between 12.5 and 200mg/ml using agar diffusion plate method. The hexane extract exhibited higher antibacterial properties than both ethylacetate and methanol extracts of the plant. The hexane and ethylacetate extracts revealed higher antifungal properties than the methanol extract of *A. calyciana*, with activity of hexane and ethylacetate extracts comparable to that of the reference drug (tioconazole) against *Candida albicans*, *Rhizopus stolon*, *Pneumonae notatum*, *Tricophyton rubrum* and *Epidermophyton floccosum* and *Candida albicans* and *Epidermophyton floccosum*, respectively.

Keywords: *A. calyciana*, phytochemical screening, agar diffusion method, ethnomedicine, antibacterial and antifungal.

Full length article Received: 22-12-2011 Revised: 05-01-2012 Accepted: 14-01-2012 Available online: 15-01-2012

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1. Introduction

The family Acanthaceae are mostly herbs or shrubs comprising about 250 genera and 2,500 species, including twining forms. The leaves are simple, opposite and decussate; stipulates are lacking. The flowers are bisexual, zygomorphic, and usually are associated with conspicuous, often brightly coloured bracts. One of the species is *Asystasia*. *Asystasia* comprises about 50 species, and distributed in tropics of the old world, with about 30 species in tropical Africa [1]. *Asystasia calycina* is an herb, erect or straggling to 60cm high, recorded from Guinea to South Nigeria and occurring also in East Cameroun. The parts of the plant are medicinal and the leaves are eaten as green in Gabon. The plant has been used as remedy for diverse conditions in the sub-continent. The plant has been used in alternative traditional medicine across West Africa to treat ailments like skin diseases, headache, large craw-craw sores, impotency or weak erection, children's yaw and the twig is said to be aphrodisiac [2-4]. The Therapeutic properties of *A. calyciana* have not been reported. The phytochemistry of the plant has not also been established. In our continuous effort to study the medicinal properties of plants in Nigeria, we report the preliminary phytochemistry, antibacterial and antifungal properties of *A. calyciana* grown in North Central, Nigeria.

2. Material and Methods

2.1. Collection and authentication of the plant material

The whole plant material of *Asystasia calyciana* was collected from Ibadan, Oyo State of Nigeria, October 2010. Botanical identification and authentication was done by Mr. A.W. Ekundayo of the Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher specimen (FHI108471) was deposited.

2.2. Preparation of plant extracts

Fresh whole plant of *Asystasia calyciana* was air-dried, ground and weighed (496g). The dried sample was successively extracted in hexane, ethylacetate and methanol for 10 days, respectively using cold extraction method. The grinded *A. calyciana* was soaked with solvents in an aspirator bottle for ten (10) days each, starting with the least polar solvent n-hexane, to moderately polar solvent ethyl acetate, followed by highly polar solvent methanol. The hexane was used to extract least polar components from the

plant sample, ethylacetate to extract moderately polar components, followed by methanol which was used to extract polar components in the plant sample. Each extract solution was decanted from the plant sample in the aspirator bottle after ten (10) days [5]. The crude extracts were obtained and the weight of each of the crude extracts was weighed. The resultant hexane (whole plant 8g), ethylacetate (whole plant 7g) and methanol (whole plant 10g) extracts were obtained using rotary evaporator. The crude extracts were weighed and stored in the refrigerator for further use.

2.3. Phytochemical studies

The Phytochemical screening of the hexane, ethylacetate and methanol extracts of *Asystasia calyciana* (whole plant) was done using standard procedures [6-10].

1) Saponins: Small quantity of each extract was boiled with 5 ml of distilled water, filtered and cooled. **a). Frothing:** To the filtrate (2.5 ml) about 10 ml of distilled water was added and shaken vigorously for 2 minutes. Frothing observed indicates a positive test. **b). Emulsification:** To the filtrate (2.5 ml) added 3 drops of olive oil and shaken vigorously for 2 minutes. An emulsified layer indicates a positive test.

2) Alkaloids: Small quantity of each extract was stirred with 5 mL of 1% hydrochloric acid for five minutes on a water bath and then filtered. Of the filtrate of each extract was divided into two portions. Mayer's reagent was added to one portion; occurrence of creamy white precipitate was taken as positive. To the second portion few drops of Dragendorff's reagent was added and appearance of orange red precipitate was regarded as positive for the presence of alkaloids.

3) a.Glycosides (Keller-killiani Test): Small quantity of each extract was diluted in 5 ml of distilled water. Add 2 ml of glacial acetic acid containing one drop of ferric chloride solution (3.5%) to each. This was underlay with 1 ml of concentrated sulfuric acid. A radish brown ring is formed at the interface and upper layer turns bluish green on standing indicates the presence of a deoxy sugar characteristic of cardiac glycosides. **b) Method-2:** Small quantity of each extract moistened with 5 ml in distilled water and filtered. Few drops of chloroform were added to each (to enhance enzymatic activity). A sodium picrate–saturated filter paper strip was hanged at the neck of the flask with the help of the cork and warmed the flask. The filter paper strip turned brick-red or maroon is indicated the presence of cyanogenetic glycosides.

4) Tannins: a) Ferric Chloride Test: Small quantity of each extract was boiled in 10 ml of water in a test tube and then filtered while hot and a few drops of 0.1% ferric chloride solution were added to the filtrate. A brownish green or a blue-black coloration indicates as a positive test. **b) Lead Acetate Test:** Small quantity of each extract was taken in a test tube and diluted with 5 ml of distilled water. Add few drops of a 1% solution of lead acetate to each. A yellow or red precipitate indicates a positive test.

5) Flavonoids: Three methods were used to determine the presence of flavonoids in the extracts. **a) Method-1:** Dilute ammonia solution (5 ml) was added to aqueous filtrate of each extract followed by addition of concentrated H_2SO_4 acid (1 ml). A yellow colouration that disappears on standing indicates the presence of flavonoids. **b) Method 2:** Few drops of 1% aluminium solution were added to aqueous filtrate of the each extract. A yellow coloration indicates the presence of flavonoids. **c) Method 3:** A small portion of the each extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indicates the presence of flavonoids.

6) Steroids: a) Liebermann-burchard's Test: Small amount of each extract was dissolved in 1 ml of chloroform. Added 2ml of acetic anhydride and 1 ml of concentrated H_2SO_4 acid to each portion. A greenish color is produced which turns blue on standing indicates the presence of steroids. **b) Salkowski's Test:** Small amount of the extract was dissolved in 2ml of chloroform. Concentrated sulphuric acid was carefully added to a lower layer. A reddish-brown colour at the interphase indicates the presence of deoxysugar characteristics of cardenolides. A violet ring may form just above the ring and gradually spread throughout the layer.

7) Reducing sugars (Fehling's Test): A small portion of each of the extract was shaken with distilled water and filtered. The filtrate was boiled with drops of Fehling's solution A and B for 2 minutes. An orange-red precipitate on boiling with Fehling's solution indicates the presence of reducing sugars.

8) Anthraquinones: A small portion of each extract was boiled with 10 ml of sulfuric acid, traces of ferric chloride solution was added and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was taken into another test tube and 1 ml of dilute ammonia was added to each portion. Rose-pink color in the aqueous layer indicates the presence of anthraquinones.

2.4. Antimicrobial Assay

Microorganisms: Cultures of six human pathogenic bacteria made up of four gram negative and two gram positive were used for the antibacterial assay. These were; *Salmonella typhii* (UCH 4801), *Escherica coli* (UCH 00260), *Pseudomonas aeruginosa* (UCH 1102) and *Klebsiellae pneumonae* (UCH 2894) belongs to the gram-negative and *Bacillus subtilis* (UCH 74230) while *Staphylococcus aureus* (UCH 2473) belongs to the gram-positive. For the Antifungal assay, six fungi were also utilized. These were; *Candida albicans, Aspergillus niger, Rhizopus stolon, Penicillum notatum, Tricophyton rubrum and Epidermophyton floccosum.* All the microorganisms used were clinical strains from the Medical Microbiology (University College Hospital, Ibadan) and screened in the Laboratory of Pharmaceutical Microbiology Department, University of Ibadan, Ibadan-Nigeria.

Media: Nutrient agar, Sabouraud dextrose agar, nutrient broth and tryptone soya agar were used in this study. Hexane, ethylacetate and methanol were also used in solubilizing the extracts and as negative controls in the assays.

Table 1: Phytochemical constituents of the hexane, ethylacetate and methanol extracts of *Asystasia calyciana* (whole plant)

	Extracts		
Secondary metabolites	**Hexane**	**Ethylacetate**	**Methanol**
Alkaloids	-	-	++
Saponins	-	++	++
Tannins	++	++	++
Reducing sugars	-	-	++
Steroids	++	-	++
Glycosides	++	++	-
Flavonoids	++	++	-
Anthraquinones	-	++	++

- Absent ++ Present

Table 2: Antibacterial activities of the hexane, ethylacetate and methanol extracts of *Asystasia calyciana* (whole plant)

Extracts	**Extract conc/Ref./ Control (mg/ml)**	**Diameter of well = 8 mm**					
		Diameter of zone of inhibition of bacteria(mm)					
		S.a	**E.coli**	**B.sub**	**Ps.a**	**Kleb**	**Sal**
Hexane	6.25	-	-	-	-	-	-
	12.5	10	-	10	-	10	10
	25	14	10	14	10	12	12
	50	18	12	18	12	14	14
	100	20	14	20	14	18	16
	200	24	18	26	16	20	18
	Hexane	-	-	-	-	-	-
	Gentamycin	38	36	34	34	36	38
Ethylacetate	6.25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
	25	10	-	10	-	-	10
	50	12	10	14	10	10	12
	100	14	12	16	12	14	14
	200	18	16	20	14	18	16
	Ethylacetate	-	-	-	-	-	-
	Gentamycin	38	37	35	34	36	38
Methanol	6.25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
	25	-	-	10	-	-	-
	50	10	10	12	-	10	10
	100	12	12	14	10	12	12
	200	14	14	16	12	14	14
	Methanol	-	-	-	-	-	-
	Gentamycin	37	36	34	34	36	38

Table 3: Antifungal activities of the hexane, ethylacetate and methanol extracts of *Asystasia calyciana* (whole plant).

Extracts	Extract conc/Ref./ Control (mg/ml)	Diameter of well = 8 mm					
		Diameter of zone of inhibition of fungi(mm)					
		C.a	A.n	Rhiz	Pen	T.r	E.f
Hexane	6.25	-	-	-	-	-	-
	12.5	10	-	-	10	10	10
	25	12	-	10	12	12	12
	50	16	10	12	14	14	14
	100	18	12	14	16	18	16
	200	20	14	16	20	20	20
	Hexane	-	-	-	-	-	-
	Tioconazole	26	24	20	24	26	26
Ethylacetate	6.25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
	25	10	-	-	-	-	10
	50	12	10	10	10	10	12
	100	14	12	12	12	12	10
	200	18	14	14	14	14	16
	Ethylacetate	-	-	-	-	-	-
	Tioconazole	26	25	20	24	26	26
Methanol	6.25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
	25	-	-	-	-	-	-
	50	10	-	-	-	-	-
	100	12	10	10	10	10	-
	200	14	12	14	12	12	12
	Methanol	-	-	-	-	-	-
	Tioconazole	26	24	20	25	26	26

Key

S.a *Staphylococcus aureus*
E.coli *Escherichia coli*
B.sub *Bacillus subtilis*
Ps.a *Pseudomonas aeruginosa*
Kleb *Klebsiellae pneumonae*
Sal *Salmonellae typhii*
C.a *Candidas albicans*
A.n *Aspergillus niger*
Rhiz *Rhizopus stolon*
Pen *Penicillum notatum*
T.r *Tricophyton rubrum*
E.f. *Epidermophyton floccosum*

Antimicrobial agents: Gentamycin (10 µg/mL) and Tioconazole (0.7 mg/mL) were included as standard reference drugs in the study.

2.4.1. Antimicrobial activity determination

Agar diffusion-pour plate method (bacteria): An overnight culture of each organism was prepared by taken two wireloop of the organism from the stock and inoculated each into the sterile nutrient broth of 5ml, each incubated for 18-24hr at 37^{o}C. From overnight culture, 0.1 mL of each organism was taken and put into the 9.9mL of sterile distilled water to obtained 10^{-2} inoculum concentration of the organism.

From the diluted organism (10^{-2}), 0.2mL was taken into the prepared sterile nutrient agar cooled to about 40-45^{o}C, then poured into sterile Petri dishes and allowed to solidify for about 45-60min. Using a sterile cork-borer of 8mm diameter, the wells were made according to the number of the test tubes for the experiment. For this work 8 wells were made. The graded concentrations of the extracts were put into the wells accordingly including the controls. The studies were done in duplicates to ascertain the results obtained. The plates were left on the bench for about 2hrs to allow the extract diffuse properly into the nutrient agar i.e. pre-diffusion. The plates were incubated for 18-24hr at 37^{o}C.

Agar diffusion-surface plate method (fungi): A sterile sabouraud dextrose agar was prepared accordingly and aseptically poured into the sterile plates in triplicates and solidified properly. From the prepared diluted organism, 0.2mL of the 10^{-2} inoculum concentration of the organism was spread on the surface of the agar using a sterile Petri-dish cover to cover all the surface of the agar. Eight wells were bored using a sterile cork-borer of 8mm diameter. The graded concentrations of the extracts were put into the including the controls. All the plates were left on the bench for 2hr to allow the extract diffuse properly into the agar i.e. pre-diffusion. The plates were incubated at 25^{o}C for 72hr [11-13].

3. Results and discussion

The metabolite screening of *A. calyciana* (Table 1) revealed the presence of tannins in hexane, ethylacetate and methanol extracts of the plant; saponins and anthraquinones in only ethylacetate and methanol extracts; glycosides and flavonoids in only hexane and ethylacetate extracts of *A. calyciana*; while steroids were found in hexane and methanol extracts of the plant. Meanwhile alkaloids and reducing sugar were established in only methanol extract of *A. calyciana* (whole plant) but absent in both hexane and ethylacetate extracts of the plant.

The result of antibacterial activities of hexane, ethylacetate and methanol extracts of *A. calyciana* is presented in table 2. The six tests bacteria were sensitive to the extracts at concentrations between 12.5 and 200 mg/ml. The hexane extract of the plant effectively inhibited the growth of *Saphylococcus aureus and Bacillus subtilis* (gram positive), *Klebsiellae pneumonae* and *Salmonellae typhii* (gram negative) at concentrations between 12.5 and 200 mg/ml, but showed inhibition on *Escherichia coli* and *Pseudomonas aeruginosa* (gram negative) at concentrations between 25 and 200 mg/ml of the extract. Further, *Staphylococcus aureus, Bacillus subtilis* and *Salmonellae typhii* were sensitive to ethylacetate extract at concentrations ranging from 25 to 200 mg/ml, while *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiellae pneumonae* showed lower sensitivities on ethylacetate metabolites at concentrations between 50 and 200 mg/ml. Methanol extract of *A. calyciana* inhibited the growth of tests bacteria only at higher concentrations between 50 and 200 mg/ml, except in *Bacillus subtilis* where its sensitivity to the extract was established at concentrations between 25 and 200 mg/ml.

Hexane extract exhibited higher antifungal properties than both ethylacetate and methanol extracts of the plant. The activity of the extract (hexane extract) was compared to that of the reference drug tioconazole against *Candida albicans, Penicillum notatum, Tricophyton rubrum* and *Epidermophyton floccosum*. In addition, ethylacetate extract revealed appreciable antifungal properties on the test organisms at higher concentrations between 50 and 200 mg/ml, while methanol metabolites of *A. calyciana* showed lower inhibition on the fungi only at higher concentrations between 100 and 200 mg/ml. However, the antibacterial and antifungal activities of hexane, ethylacetate and methanol metabolites on the organisms were concentration dependent, activity being higher at higher concentrations of the extracts.

4. Conclusion

The higher antibacterial and antifungal properties of hexane extract of *A. calyciana* (whole plant) suggest the use of the plant in alternative traditional medicine for the treatment of bacteria infectious diseases like gonorrhea, syphilis, typhoid; and treatment of fungi diseases such as skin and mouth sores, craw-craw, aphrodisiac etc. This study proves the efficacy of *A. calyciana* in ethnomedicine while also recommends it as a potential usefulness in the invention of new antibacterial and antifungal drugs.

Acknowledgements

The authors acknowledged the Department of Chemistry of both University of Ilorin and University of Ibadan, Nigeria for the assistance rendered during this research.

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