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Phytochemical Constituents and Antibacterial Activities of Aqueous and Hydromethanolic Leaf Extracts of Chaya (*Cnidoscolus aconitifolius*)

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Abstract

The phytochemical constituents of aqueous and 1:4 (v/v) water: methanol Soxhlet extracts of Cnidoscolus aconitifolius were identified with Perkin-Elmer (Clarus 500 system) Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS). The antibacterial activities of each extract at concentrations (0.25, 0.5, 1 g/10 mL) of Cnidoscolus aconitifolius were tested against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Salmonella typhi, Vibro cholera, Pseudomonas aeroginosa, Shigella flexneri and Klebsiellaoxytoca, using well-in agar diffusion method. Zones of inhibition of extracts were compared with those of a standard antibiotic (Chloramphenicol) to determine the antibacterial activities. Comparing with Chloramphenicol, the zones of inhibition of aqueous extract were 80.76 %, 66.67 %, 62.50 % and 77.20 % against Staphylococcus aureus, Pseudomonas aeroginosa, Vibro cholera and Bacillus subtilis, respectively, whereas hydromethanolic extract had correspondingly, 80.77 %, 38.88 %, 92.59 % and 94.44 %.The extracts showed remarkable inhibition of the bacterial growth compared with Chloramphenicol. The percent mean inhibition of hydromethanolic extract for the eight pathogens was 70.66 % whereas the aqueous extract was 61.13 % of Chloramphenicol. The antimicrobial activity of the Cnidoscolus aconitifolius extracts could be due to the presence of alkaloids and flavonoids identified by GC-MS. Hence, these phytochemicals could be screened to discover the bioactive natural compounds that could serve as leads in the development of new pharmaceuticals.

Key words: Antibacterial, Cnidoscolus aconitifolius, Hydro-methanol, Antibacterial, Phytochemicals

1.0 Introduction

Medicinal plants exist everywhere, especially in Africa which has vast reservoir of plants that had been categorized (Aluyi *et al*., 2003). According to WHO, medicinal plants would be the best source to obtain variety of drugs. About 80 % of individuals from developed countries use traditional medicines, which has compounds derived from medicinal plants. However, such plants should be investigated to better understand their properties, safety and efficiency (Arunkumar & Muthuselvam, 2009).

Plant products have been part of phytomedicines since time immemorial. This can be derived from barks, leaves, flowers, roots, fruits, seed (Craig & David 2001). Knowledge of the chemical constituents of plants is desirable because such information will be of value for synthesis of complex chemical substances (Parekh & Chanda, 2008).

Herbs are widely exploited in the traditional medicine and their curative potentials are well documented (Dubey, *et al*., 2004). About 61 % of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful, especially in the areas of infectious disease and cancer (Cragg & David, 2001). Recent trends, however, show that the discovery rate of active novel chemical entities is declining (Lam, 2007).

Natural products of higher plants may give new source of antimicrobial agents with possibly novel mechanism of action. The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world (Reddy *et al.*, 2001 & Dubey, *et al*. 2004). Plants are rich in wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycoside, etc., which some have been found *in vitro* to have antimicrobial properties (Das et al., 2010).

Ethnobotanical and ubiquitous plants serve as rich resources of natural drugs for research and development (Kong *et al*., 2008). Many of these indigenous medicinal plants are used as spices and food plant (Okwu, 2001). Medicinal plants are of great importance to the health of individuals and communities. World Health Organization (2012) had specified the need to know the composition of biological active botanical substances considered for medicinal purposes.

Recent studies had evaluated the phyto-medicinal, nutritional and electrolyte values of *Cnidoscolus aconitifolius* leaves (CA) consumed in parts of the Niger Delta region of Nigeria (Iwuji *et al.*, 2013a,b). CA is commonly known as Chaya or Tree Spinach. Chaya leaf belongs to the family "*Euphorbiaceae* (Ross-Ibana & Molina-Cruz, 2002). The present study further investigated the phytochemical constituents of aqueous and hydromethanolic leaf extracts of *C. aconitifolius* using Gas Chromatography - Mass Spectrometry (GC-MS) and compared their antibacterial activities

2.0 Materials and Methods

2.1. Collection and Identification of *Cnidoscolus aconitifolius*

Fresh sample leaves of *Cnidoscolus aconitifolius* were collected from residential areas at Ogbeke Obibiezena, Owerri-North, LGA, Imo State. Time of collection was between 6.00 – 8.00 am on March 11, 2015.

The leaves were botanically identified by Prof. Tunde Joseph Ogunkunle, a Taxonomist in the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Oyo State and deposited at the Department of Biomedical Technology, Federal University of Technology, Owerri, Imo state, Nigeria.

2.2. Preparation of the Aqueous Leaf Extract

The preparation of the aqueous leaf extract was carried out as described by Yakubu *et al*. (2008). The leaves were washed with distilled water to remove dust and air-dried completely for some weeks at room temperature before using for study. The leaves were ground into fine powder using a grinder. Approximately 150 g of the powder was extracted with 150 mL of methanol and 600 mL of distilled water (i.e. 1: 4 ratio) using Soxhlet apparatus and concentrated by means of an evaporator 60 ^{0}C. The extract was later evaporated to dryness. The dried mass yielded 12.49 g and the percentage mass extract yielded 8.33 %. The dried extract was stored in a refrigerator until required for use. The extract was dissolved in appropriate volume of distilled water to the desired concentration.

2.3. Preparation of the Hydromethanolic Plant Extract

The method used was described by Yakubu *et al*. (2008). The plants leaf were washed with sterile distilled water to remove dust and air-dried completely for some weeks at room temperature before using for study. The leaves were grinded into fine powder using a grinder. Approximately 150 g of the powder was extracted with 600 mL of methanol and 150 mL of distilled water (i.e. 4:1 ratio) using Soxhlet apparatus and concentrated with an evaporator at 60 ^{0}C. The extract was evaporated to dryness. The dried mass yielded

40.06 g (26.71 % extract yield). The dried extract was stored in a refrigerator until required for use. The extract was dissolved in appropriate volume of distilled water to obtain the desired concentration. The extract was tested for sterility by introducing 2 mL of the supposed sterile extract into 5 ml of sterile nutrient broth. Incubation was done at 37 ^{0}C for 24 hours. A sterile extract was indicated by absence of turbidity or clearance of the broth after the incubation period (Ronald, 1995).

2.4. GC-MS Analysis and Identification of Compounds

The method described by Rajeswari (2012) was employed for GC-MS analysis of *Cnidoscolus aconitifolius* extracts. This was performed using a Perkin-Elmer GC Clarus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-I, fused silica capillary column with agilent 19091S-433HP-5MS (30 mm x 250 µ mm x 0.25 µm), composed of 5 % phenyl methyl silox). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999 %) was used as the carrier gas at constant flow rate of 1.5 mL/min and an injection volume of 1 µl was employed (split ratio of 50:1); Injector temperature 250 °C; Ion-source temperature 300 °C. The oven temperature was programmed from 35 °C (isothermal for 5 min.), with an increase of 4 °C/min, to 150 °C, then 20 °C/min to 250 °C, ending with a 5 minutes isothermal at 300 °C. Mass spectra were taken at 70 eV; a scan interval of 0.25 minutes and fragments from 45 to 450 Da. Total GC running time was 45.75 minutes. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbo mass. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute of Standard and Technology (NIST) library having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The Name, Molecular weight and structure of the components of the test materials were obtained.

2.5. Collection and Maintenance of Test Organisms

The following eight microorganisms were pre-clinical isolates from New Concept Laboratory located at Obinze, FUTO Road Owerri, Imo State: *Bacillus subtillis, Escherichia coli, Staphylococcus aureus, Salmonella typhi, Vibro cholera, Pseudomonas aeruginosa, Shigella flexneri, Klebsiella oxytoca.* Antibacterial *C*hloramphenicol was used as the control. The bacteria were chosen based on their clinical and pharmacological importance. The bacteria were maintained in Nutrient agar plates at 37 ^{0}C and stored in the refrigerator at a temperature 4 ^{0}C.

2.6. Nutrient Agar Media Preparation

This is a basic media mostly used for culturing, sub-culturing and for total viable bacteria count 28 g. Nutrient agar 28 g powder was dissolved in 100 mL of distilled water. It was gently heated to dissolve the medium completely. Then sterilized by autoclaving at 15 psi (121 ^{0}C) for 15 minutes.The medium was dispensed as desired in the Petri dishes and allowed to cool and solidify.

2.7. Antibacterial Activity

In-vitro antibacterial activities of aqueous and hydromethanolic leaf extracts of *Cnidoscolus aconitifolius* were examined. Antibacterial activity testing was carried out using well in agar method. The modified Collins *et al*. (1995) agar well diffusion method was employed to determine the antibacterial activities of the leaf extracts of *Cnidoscolus aconitifolius.* Different concentration of the leaves (1, 0.5, and 0.25 g/10 mL) were prepared. About 0.1 mL of standardized 24 hours old culture of tested organisms in nutrient broth was spread unto sterile prepared Nutrient Agar plates. These were then allowed to set, with the aid of a sterile Cork borer, four (4) wells of about 5 mm in diameter were bored on the solidified medium. About 0.2 mL of each concentration of the extracts were dispersed into the three wells and the control

(Chloramphenicol) was dispersed into the fourth hole, then allowed to stand for about 15 minutes for pre-diffusion of the extracts to occur. These were then incubated for 37 ^{0}C for 24 hours.

2.8. Determination of Zones of Inhibition

At the end of the period, inhibition zone formed on the agar were evaluated in "mm" (Junaid *et al.*, 2006). The diameter of the zones of inhibition in the plates was measured by calculating the difference between the diameter of the cork borer (5 mm) and the diameter of inhibition. The Minimum Inhibition Concentration (MIC) was determined by measuring about 5 mL of nutrient broth into empty sterile tubes. 1 mL of the different concentration of the extracts (0.5 g, 0.25 g, 1 g) were added to 0.5 mL of organisms. This was then incubated for 24 hours at 37 ^{0}C. The tubes were then observed for visible growth with the help of a spectrophotometer. The tubes with the least concentration of the extract showed 'lesser growth' (-ve) was determined as MIC, while the negative tubes were pour plated on Nutrient Agar and incubated for 24 hours at 37 ^{0}C. The tube with the least concentration of the extract that showed "NO" growth at the concentration was reported as the Minimum Bactericidal Concentration (MBC)

3.0 Results

3.1. The results of the Gas Chromatography-Mass Spectrophotometer (GC-MS) analysis of *Cnidoscolus aconitifolius* leaf extracts

The results of the phytochemical analysis of aqueous and hydromethanolic leaf extracts of *Cnidoscolus aconitifolius* were presented in Tables 1 and 2. The Gas Chromatography-Mass Spectrophotometer (GC-MS) study of *Cnidoscolus aconitifolius* leaf extracts identified some chemical constituents which could contribute to the medicinal activity of the plant.

Table 1: Major Phytochemical constituents identified by the GC-MS analysis of aqueous leaf extract of *Cnidoscolus aconitifolius*.

S/N	Major Phytochemical Class	Percent (%) Composition
1.	Alkaloids	67.62
2.	Flavonoids	32.38

Table 2: Major Phytochemical constituents identified by the GC-MS analysis of hydromethanolic leaf extract of *Cnidoscolus aconitifolius*.

S/N	Major Phytochemical Class	Percent (%) Composition
1	Alkaloids	97.86
2	Flavonoids	2.14

3.2. Results of the zones of inhibition of aqueous and hydromethanolic leaf extracts of *Cnidoscolus aconitifolius* and a standard anti-bacterial (Chloramphenicol) against some bacterial test organisms.

The antibacterial activities of the extracts of *Cnidoscolus aconitifolius* and standard drug control (Chloramphenicol) were studied at different concentrations (0.25, 0.5, and 1 g/10 ml) against eight pathogenic bacteria (*Staphylococcus auerus, Escherichia coli, Salmonella typhi, Klebsiella oxytoca, Pseudomonas aeruginosa, Shigella flexneri, Vibro cholera, Bacillus subtilis)*. The antibacterial potential of an extract was determined in terms of zone of inhibition of bacterial growth. The results of the antibacterial activities were presented in Tables 3 and 4.

Table 3: Inhibition zones of different aqueous extract concentrations of *Cnidoscolus aconitifolius* leaves and a Control (Chloramphenicol) against bacterial test organisms.

Micro-organism	a (mm)	B (mm)	c (mm)	Control (mm)
Staphylococcus auerus	8	12	21	26
Escherichia coli	10	15	17	35
Salmonella typhi	11	12	16	32
Klebsiella oxytoca	12	15	15	30
Pseudomonas aeruginosa	9	12	20	30
Shigella flexneri	10	14	16	30
Vibro cholera	12	18	20	32
Bacillus subtilis	12	15	17	22

(Keys: a, b, and c are concentrations of the extract: a = 0.25 g/10 mL; b = 0.5 g/10 mL; c = 1 g/10 mL; zones of inhibition in millimetres (mm))

Table 4: Inhibition zones of different hydromethanolic extract concentrations of *Cnidoscolus aconitifolius* leaves and a Control (Chloramphenicol) against bacterial test organisms.

Micro-organism isolate	a (mm)	B (mm)	c (mm)	Control (mm)
Staphylococcus auerus	11	12	21	26
Escherichia coli	12	14	17	35
Salmonella typhi	7	12	17	22
Klebsiella oxytoca	8	11	18	32
Pseudomonas aeruginosa	9	12	14	36
Shigella flexneri	12	17	26	34
Vibro cholera	11	15	25	27
Bacillus subtilis	12	15	17	18

(Keys: a, b, and c are concentrations of the extract: a = 0.25 g/10 mL; b = 0.5 g/10 mL; c = 1 g/10 mL; zones of inhibition in millimetres (mm))

3.3. Results of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of aqueous and hydromethanolic extracts of *Cnidoscolus aconitifolius leaves.*

The results of MIC and MBC of each extract concentration and the standard antibacterial agent, Chloramphenicol, are presented in Tables 5 and 6.

Table 5: Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of aqueous extract of *Cnidoscolus aconitifolius* leaves.

Micro-organism	a	b	c	MIC%	MBC%
		Turbidity			
Staphylococcus auerus	++	+	-	1 g/10 ml	>1g/10 ml
Escherichia coli	+	-	-	0.5 g/10 ml	> 0.5 g/10 ml
Salmonella typhi	+	+	-	1 g/10 ml	>1 g/10 ml
Klebsiellaoxytoca	+	-	-	0.5 g/10 ml	>0.5 g/10 ml
Pseudomonasaeruginosa	+	+	-	1 g/10 ml	>1 g/10 ml
Shigellaflexneri	+	-	-	0.5 g/10 ml	>0.5 g/10 ml
Vibro cholera	+	-	-	0.5 g/10 ml	>0.5 g/10 ml
Bacillus subtilis	+	-	-	0.5 g/10 ml	>0.5 g/10 ml

(KEY: : a, b, and c are concentrations of the extract: a= 0.25 g/10 mL; b = 0.5 g/10 mL; c = 1 g/10 mL; Turbidity of ++ =Very turbid with bacterial growth; + = Slightly turbid; -= No bacterial growth)

The lesser the turbidity, the higher the zone of inhibition / the higher the antibacterial effect but the higher the turbidity the lower the zone of inhibition.)

Table 6: Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of hydromethanolic extract of *Cnidoscolus aconitifolius* leaves.

Micro-organism	a	b	c	MIC%	MBC%
		Turbidity			
Staphylococcus auerus	+	+	-	1 g/10 gml	>1 g/10 ml
Escherichie coli	+	-	-	0.5 g/10 ml	>0.5 g/10 ml
Salmonella typhi	++	+	-	1 g/10 ml	>1 g/10 ml
Klebsiellaoxytoca	+	+	-	1 g/10 ml	>1 g/10 ml
Pseudomonasaeruginosa	+	+	-	1 g/10 ml	>1 g/10 ml
Shigellaflexneri	+	-	-	0.5 g/10 ml	>0.5 g/10 ml
Vibro cholera	+	-	-	0.5 g/10 ml	>0.5 g/10 ml
Bacillus subtilis	+	-	-	0.5 g/10 ml	>0.5 g/10 ml

(KEY: : a, b, and c are concentrations of the extract: a= 0.25 g/10 mL; b = 0.5 g/10 mL; c = 1 g/10 mL; Turbidity of ++ =Very turbid with bacterial growth; + = Slightly turbid; - = No bacterial growth)

The lesser the turbidity, the higher the zone of inhibition / the higher the antibacterial effect but the higher the turbidity the lower the zone of inhibition.)

3.4. Results of the comparisons of the zones of inhibition of aqueous and hydromethanolic extracts of *Cnidoscolus aconitifolius* leaves with the standard drug against bacterial test organisms

The results of the comparisons of the zones of inhibition of aqueous and hydromethanolic extracts of *Cnidoscolus aconitifolius* and the standard control drug (Chloramphenicol) against bacterial test organisms were presented in Tables 7 and 8.

Table 7: Comparisons of the zones of inhibition of aqueous extract of *Cnidoscolus aconitifolius* and standard control drug against bacterial test organisms

Bacteria	**A (mm)**	**B (mm)**	**C (%)**	**D (%)**
Staphylococcus auerus	21	26	80.76	14.79
Escherichie coli	17	35	48.57	11.97
Salmonella typhi	16	32	50	11.27
Klebsiella oxytoca	15	30	50	10.56
Pseudomonas aeruginosa	20	30	66.67	14.08
Shigella flexneri	16	30	53.33	11.27
Vibro cholera	20	32	62.50	14.08
Bacillus subtilis	17	22	77.20	11.97

[KEY: A and B were the zones of inhibition (in millimetres) by 1 g/10 mL *aqueous leaf extract and standard drug (Control), respectively. C = [A/B X 100] = the percentage (%) inhibition of the aqueous extract compared with Chloramphenicol (Control); and D = [A/∑A X 100] = individual sensitivity of isolate to* 1 g/10 mL *aqueous extract;*

Mean percent inhibition = *[∑C]/ No, of isolates* =489.03/8 = 61.13 %]

Table 8: Comparisons of the zones of inhibition of hydromethanolic extract of *Cnidoscolus aconitifolius* and standard control drug against bacterial test organisms

Bacteria isolate	**A (mm)**	**B (mm)**	**C (%)**	**D (%)**
Staphylococcus auerus	21	26	80.77	13.55
Escherichie coli	17	35	48.57	10.97
Salmonella typhi	17	22	77.27	10.97
Klebsiella oxytoca	18	32	56.25	11.61
Pseudomonas aeruginosa	14	36	38.88	9.03
Shigella flexneri	26	34	76.47	16.77
Vibro cholera	25	27	92.59	16.13
Bacillus subtilis	17	18	94.44	10.97

*[KEY: A andB were the zones of inhibition (in millimetres) by*1 g/10 mL *aqueous leaf extract and standard drug (Control), respectively. C = [A/B X 100] = the percentage (%) inhibition of the aqueous extract compared with Chloramphenicol (Control); and*

D = [A/∑A X 100]= individual sensitivity of Isolate to 1 g/10 mL *aqueous extract.*

Mean percent inhibition = *[∑C]/ No, of isolates* =565.24/8 =70.66 %]

4.0. Discussion

The presence of various bioactive ingredients called secondary plant metabolites revealed by the Gas Chromatography and Mass Spectrophotometer (GC-MS) phytochemical analysis supported the medicinal potential of *Cnidoscolus aconitifolius leaf* extracts. This is in agreement with previous reports (Sofowora, 1993).

The GC-MS analysis of aqueous extract of *Cnidoscolus aconitifolius* leaves identified two major phytochemicals. The percent compositions of these phytochemicals from aqueous extract were different from those of hydromethanolic extract. These showed that the type of extraction solvent could affect the phytochemicals extracted: hydromethanolic extract yielded more alkaloids than aqueous in this study. The percentage of flavonoid was lower in hydromethanolic leaf extract compared with aqueous extract. This indicated that hydromethanolic extract of *C. aconitifolius* could be more active with alkaloids than aqueous extract.

The results of the study on the antibacterial activities of *Cnidoscolus aconitifolius* against S*taphylococcus auerus, Salmonella typhi, Klebsiella oxytoca, Pseudomonas aeroginosa, Escherichia coli, Shigella flexneri, Vibro cholera, and Bacillius subtilis* shows that the aqueous and hydromethanolic leaf extractsinhibited the growth of majority of the test isolates. The inhibition zones of both extracts on microbes were less than that of the Control (Chloramphenicol). This indicated that Chloramphenicol is more effective than the extract concentrations used.

It is implied that the extracts possess compounds that inhibit the growth of some bacteria. This is in agreement with the reports of Sarmiento-Franco *et al*. [2002] and Awoyinka *et al*. [2007]. More so, the observed inhibitory effects increased with extract concentrations (0.25 g/10 mL, 0.5 g/10 mL, and 1 g/10 mL) and therefore they are concentration dependent. 0.25 g/10 mL had no inhibitory effect on any bacterial isolate; 0.5 g/10 mL had selective effects while 1 g/10 mL had possible bactericidal effect on all the isolates.

Comparatively, hydromethanolic extract gave higher zone of inhibition on some microbes (such as *Salmonella typhi, Klebsiella oxytoca, Pseudomonas aeroginosa, Shigella flexneri and Vibro cholera*) than aqueous extract. *Staphylococcus auerus, Escherichia coli* and *Bacillus subtilis* had the same zone of inhibition with both aqueous and hydromethanolic extracts.

Comparisons of the zones of inhibition of aqueous extract of *Cnidoscolus aconitifolius* and Chloramphenicol against bacterial test organisms showed that the sensitivity of the microbes was in the following decreasing order: *Staphylococcus auerus* > *Bacillus subtilis* > *Pseudomonas aeroginosa* > *Vibro cholera* > *Shigella flexneri* > *Salmonella typhi* > *Klebsiella oxytoca* > *Escherichie coli* and that of hydromethanolic extracts was also in the following decreasing order: *Bacillus subtilis* > *Vibro cholera* > *Staphylococcus auerus* > *Shigella flexneri* > *Salmonella typhi* > *Klebsiella oxytoca* > *Escherichie coli* > *Pseudomonas aeroginosa*. The increase in inhibitory effects or inhibition zone could depend on the concentration of active constituents in the plant extracts (Obi & Onuoha, 2000).

5.0. Conclusion

The (1:4 v/v) hydro-methanol leaf extract of *Cnidoscolus aconitifolius* gave higher yield of chemical constituents (alkaloids) compared with aqueous extract. The hydro-methanol leaf extracts of *Cnidoscolus aconitifolius* were found to be more active on most of the pre-clinically isolated bacteria when compared with aqueous extract.

The study justified the use of the leaf extracts in the traditional medicine to treat various infectious diseases. It also formed the basis for selection of plant extraction solvents in the discovery of new natural bioactive compounds.

Further studies are needed to identify and evaluate the effectiveness of the phyto-compounds as antibacterial agents. More studies should also be carried out on inhibition of other pathogenic bacteria by different identified compounds in *Cnidoscolus aconitifolius* leaves.

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