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PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITIES OF SIAM WEED (*Chromolaena odorata* L.) ON SELECTED CLINICAL ORGANISMS USING TWO DIFFERENT SOLVENTS

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ABSTRACT

This study evaluated the qualitative and quantitative phytochemical contents and antimicrobial activities of the leaf extracts of *Chromolaena odorata*. Chloroform and ethyl acetate were used in extracting the bioactive components of the leaves, while crude extract was obtained by squeezing fresh leaves. The antimicrobial activities of the extracts were carried out using agar well diffusion method. The result of the phytochemical test showed that tannin, phenol, alkaloids and flavonoids were present with saponins absent in the leaf extracts of *C. odorata* at varying levels. The quantitative analysis showed that tannin contents of the extracts ranged from 5.54 - 8.10%. The phenol, alkaloids and flavonoids content ranged from 2.20 - 5.25%, 3.20 – 7.50% and 4.02 - 7.22% respectively. The antimicrobial study showed that the chloroform extract had the inhibition of 21.00 mm (*Salmonella enterica*), 25.00 mm (*Pseudomonas aeruginosa*), 29.00 mm (*Staphylococcus aureus*), 13.33 mm (*Penicilium digitatum*) and 17.33 mm (*Rhizopus stolonifer*). The zone of inhibition obtained for ethyl acetate extract were 19.00 mm (*Salmonella enterica*), 20.00 mm (*Pseudomonas aeruginosa*), 21.00 mm (*Staphylococcus aureus*), 11.67 mm (*Penicilium digitatum*) and 11.67 mm (*Rhizopus stolonifer*), while the inhibition zone obtained for crude extract were 15.00 mm (*Salmonella enterica*), 10.67 mm (*Pseudomonas aeruginosa*), 8.67 mm (*Staphylococcus aureus*), 14.00 mm (*Penicilium digitatum*) and 11.00 mm (*Rhizopus stolonifer*). The control (gentamicin/ketoconazole) showed the inhibition between 32mm and 44mm for all the test

organisms. The extracts however, showed varying minimum inhibitory concentration against the test organisms, with ethyl acetate extract having the least MIC (3.125 mg/ml) against most of the test organisms.

KEYWORDS: Phytochemical, Antimicrobial, *Chromolaena odorata*, Clinical organisms, Chloroform, Ethyl acetate and crude extract.

INTRODUCTION

From the onset, plants have been recognized to contain natural products which serve as food as well as medicine in the event of human infections (Vital and Rivera, 2009; Kumar *et al*., 2014). The presence of some phytochemicals determines the antimicrobial properties of any plant. They are also responsible for the plants' flavours, odours, colours and majorly their system of defense or resistance against some pathogens (Osman *et al*., 2014).

There has been quest for search of alternative antimicrobial agents from natural plant origin due to increasing microbial drug resistance. When new drug is introduced into clinical practice, micro-organisms particularly bacteria develop resistance to such drug within short period of its introduction. In other words, new chemotherapeutic agents have always been accompanied by corresponding increase in drug resistance (Gislene *et al*., 2000). The problem of drug resistance has prompted researchers to turn their attentions to folk medicines as alternative to conventional chemotherapeutic agents following several reports on the medicinal opportunities derived from higher plants (Santos Pimenta *et al*., 2003).

One of the plants highly utilized for its therapeutic value is *Chromolaena odorata L*. *Chromolaena odorata is a* weed found in tropical Africa, North America, South and Southeast Asia. The common names include Siam weed, Christmas bush, and common floss flower (Chakraborty *et al*., 2011). This weed has been used in several traditional medicines in the treatment of various diseases. Phytochemical components or secondary metabolites derived from this plant such as Alkaloids, Tannins, Flavonoids and other Phenolic compounds are of medicinal value and as well exhibit antimicrobial actions.

Generally, plant secondary metabolites exhibit their microbial actions by disrupting membrane function and structure, interrupting DNA/RNA synthesis, interfering with intermediary metabolism, inducing coagulation of cytoplasmic constituents and interrupting normal cell communication (Radulovic *et al*., 2013). Several studies have demonstrated

biological activities of this plant as well as its phytopathogenic activity (Sukanya *et al*., 2009), antiviral activity (Pisutthanan *et al*., 2004) as well as antiprotozoal activity to mention a few. This study is focused on determining the bioactive constituents of *chromolaena odorata* using two different solvent as well as its crude form. The antimicrobial activity of these extracts on selected clinical organisms will also be investigated.

MATERIALS AND METHODS

Study Area

The research work was carried out at Emery Research laboratory, located at km 6 Umuahia /Ikot- Ekpene, Expressway, Ahiaeke Ibeku in Umuahia North L. G. A of Abia State.

Collection of plant material and identification

The leaves of *Chromolaena odorata* were collected locally from the premises of Michael Okpara University of Agriculture, Umudike, Abia State and identified at the Herbarium unit of the Department of Plant Science and Biotechnology in Michael Okpara University of Agriculture, Umudike Abia State Nigeria. The fresh and tender leaves of the plant were used for the phytochemical analysis.

Plate 1: Picture showing the leaves of Siam weed (*Chromolaena odorata).*

Preparation of plant material

The leaves of the plant were harvested and washed under running tap to remove dirt. The leaves were divided into two parts; one part was oven-dried at 40^0C for 48 hours and then ground into a fine powder using attrition milling machine. The powdered sample were weighed and stored into two separate sample bottles.

Extraction of plant material

Maceration method of extraction according to Nagappan (2012), was employed for the extraction. Chloroform, ethyl acetate were used for extraction from the powdered leaves, while the remaining fresh leaves were squeezed to obtain the crude extract. Twenty grams of each powdered plant material was macerated in 200 ml of Chloroform and ethyl acetate into a glass bottle and covered, such that the level of the solvent was above that of the plant material. The macerated mixtures were then left to stand over-night at room temperature. The extracts were filtered out from the macerated mixture using a muslin cloth. The Chloroform and ethyl acetate extracts were concentrated, labeled and allowed to stand at room temperature to permit evaporation of residual solvents; the crude extract was used as collected.

Phytochemical analysis

Qualitative analysis of the Leaf Extracts

Alkaloids

The extract from *Chromoleana odorata* (1 ml) was treated with a few drops of Wagner's reagent. The red-brown precipitate indicated the presence of alkaloids (Salehi-Surmaghi *et al*. 1992).

Flavonoids

The presence of flavonoids was estimated by Shinoda test. 1ml of the extracts was treated with a few drops of dilute NaOH solution. The appearance of yellow colour indicated the presence of flavonoids (Somolenski *et al*., 1972).

Tannins

One ml of the extract from each solvent was treated with 1ml of alcoholic $FeCl_3$ reagent. Blue colour indicated the presence of tannins (Segelman *et al*., 1969).

Saponins

The presence of saponins was determined by Frothing test. The extract of each plant (1ml) was vigorously shaken with distilled water (1ml) and was allowed to stand for 10 minutes and classified for saponin content as follows: no froth indicated the absence of saponins and stable froth indicated the presence of saponins (Kapoor *et al*., 1969).

Phenol

The 1 ml of each extract of each plant was treated with 1 ml of dilute HCl and 1 ml of alcoholic $FeCl_3$ reagent. Blue colour indicated the presence of phenol.

QUANTITATIVE PHYTOCHEMICAL ANALYSIS

Determination of alkaloids

The gravimetric method of Harborne (1973) was used in determination of alkaloids. One gram of each extract was dispersed in 50 ml of 10% acetic acid solution in ethanol. The mixture was shaken and allowed to stand for four hours before it was filtered. The filtrate was evaporated to one quarter of its original volume. Concentrated NH_4OH was added drop-wise to precipitate the alkaloids. The precipitate was filtered off and washed with 1% NH_4OH solution, the filtering was done with a weighed filter paper. The precipitate in filter paper was dried in the oven at 60^oC for 30 minutes and reweighed. By weight difference, the weight of alkaloid was determined and expressed as a percentage of the sample weighed analyzed.

It was given thus:

$$\% \, alkaloid = \frac{w_3 - w_2}{w_1} \times \frac{100}{1}$$

Where:

W_1 = weight of sample

W_2 = weight of empty filter paper

W_3 = weight of filter paper + precipitate.

Determination of Tannins

The Follin–Dennis spectrophotometric method was used. The method was described by Pearson (1976). One gram of each extract was dispersed in 10 ml of distilled water and agitated. This was left to stand for 30 mins at room temperature being shaken every 5 min. At the end of the 30mins, it was centrifuged and 2-5 ml of the supernatant was dispersed into a 50 ml volumetric flask. Similarly, 2.5ml of standard tannic acid solution was dispersed into a separate 50 ml flask.

A 1.0 ml Follin-Dennis reagent was measured into each flask, followed by 2.5 ml of saturated to mark in the flask (50 ml) and incubated for 90 minutes at 150 m temperature the absorbance were measured at 250 nm in a Gen-way model 6000 electronic

spectrophotometer, readings were taken with the reagent blank at zero. The tannin content was given as follows;

$$\% \text{ Tannin} = \frac{100}{W} \times \frac{An}{AS} \times C \times \frac{Vf}{Va}$$

Where:

An – absorbance of test sample

As = absorbance of standard solution

C = concentration of standard solution

W = weight of sample used

V_f = total volume of extract

V_a = volume of extract analyzed

Determination of Flavonoid

Flavonoid in the test extracts was determined by the acid hydrolysis gravimetric method of Harborne, (1973). One gram of the plant sample was mixed with diluted 1 ml HCl solution to form a ratio 1:10 w/v. The mixture was boiled for 30 minutes. The boiled extract was allowed to cool and filtered through What-man No 42 filter paper. A portion of the extract (20mls) was measured with a beaker and treated with ethyl acetate to precipitate the flavonoid. The precipitate was measured by filtering with a weighed filter paper and determined by weight difference.

It was given by:

$$\% \text{ flavonoid} = \frac{W_2 - W_1}{\text{Weight of sample}} \times \frac{100}{1}$$

Where:

W_1 = weight of empty filter paper

W_2 = weight of paper + flavonoid precipitate

Determination of Saponin

The method used was described by AOAC (2000). About 5.0 g of dry ground sample was weighed inside extractor thimble and transferred into the soxhlex extractor chamber fitted with a condenser and a round bottomed flask. Some quantity of acetone, enough to cause a reflux was poured into the flask; the sample was exhaustively extracted of its lipid and interfering pigments for 3 hours by heating the flask on a hot plate and the solvent distilled off. This is the first extraction. For the second extraction, a pre-weighed round flat bottomed

flask is fitted unto the soxhlex apparatus (bearing the sample containing thimble) and methanol poured into the flask, the methanol should be enough to cause the reflux, the saponin is then exhaustively extracted for 3 hours by heating the flask on a hot plate after which the difference between the final and initial weights of the flask represents the weight of saponin extracted.

$$\% \text{ Saponin} = \frac{\text{weight of saponin}}{\text{Weight of sample}} \times \frac{100}{1}$$

Determination of Phenol

Phenol was determined by spectrophotometric method described by Pearson, (1976). One gram of the flour sample was added into a test tube and 10 milliliters of methanol was added to it and shaken thoroughly, the mixture was left stand for 15 minutes before being filtered using What-man No 42 filter paper. One milliliter of the extract was placed in a test tube and 1ml Follin Dennis reagent in 5 ml of distilled water was added and colour was allowed to develop for about 1 to 2 hours at room temperature. The absorbance of the developed colour was measured at 760nm wavelength. The phenol content was calculated thus:

$$\% \text{ Phenol} = \frac{100}{W} \times \frac{AU}{AS} \times C \times \frac{Vf}{Va}$$

Where:

W = weight of sample analyzed

Au = absorbance of the test sample

As = absorbance of standard solution

C = concentration of the standard

V_f = total volume of filtrate analyzed

V_a = volume of extract analyzed

Antimicrobial Assay

The antimicrobial assay was done using the agar well diffusion method. One gram for each extracts as dissolved in 2 ml Dimethyl Sulphoxide (DMSO) to get a concentration of 500 mg/ml. Other lower concentrations (200 mg/ml and 100mg/ml) were prepared by diluting serially with DMSO. The various bacterial and fungal isolates were standardized using the 0.5 McFarland turbidity standards. These standardized strains were inoculated onto the surface of sterile plates of Mueller Hinton Broth (MHB). Cork borer (6 mm) was used to make wells on the inoculated sterile plates. Each concentration of the extracts was introduced

into designated wells. These were allowed to be absorbed into the agar, and then incubated at 37^0C for 24h. The antimicrobial activities were determined by the width of the zone of growth inhibition (Bauer, 1996).

Minimum Inhibitory Concentrations

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration or the highest dilution of an antimicrobial agent that prevents visible growth after 18-24 hours of incubation. It was determined by making dilution of various concentrations (200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml) of the chloroform, ethyl acetate and crude extract in test tubes. 1ml stock solution of the plant extract 100 mg/ml was diluted in sterile test tube containing 0.95ml of Mueller Hinton Broth (MHB) to obtain further dilution. Serial dilution techniques were employed by transferring 1 ml from the first test tube to the second test tube and from the second to the third. This was continued to the seventh test tube from where 1ml was discarded to give concentration of 200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml. Another test tube was also prepared with MHB (control) of the test tube which was inoculated with standard suspension (50µl) of the test organisms and incubated at 37°C overnight.

After incubation, the turbidity in each tube was checked. The tube that contains the lowest concentration which showed no turbidity i.e. a clear view, was observed to be the MIC of the antimicrobial agent for the organism tested, the lower the MIC, the more susceptible is the test organism.

3.7 Statistical Analysis

Complete Randomized Design (CRD) was the experimental design used which was replicated three times. The data collected were subjected to Analysis of variance (ANOVA) using SPSS version 20 and the mean separation carried out using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSION

Results

Medicinal plants are known to possess various phytochemical properties, which are responsible for their pharmacological activities and some side effects.

The results of the phytochemical composition of the chloroform, ethyl acetate and crude leaf extracts of *Chromolaena odorata* are presented in Tables 1 and 2 respectively. The qualitative result shows the presence of tannin, phenol, alkaloids and flavonoids and the absence of saponin in the the leaves of *Chromolaena odorata* at varying intensities in the chloroform, ethyl acetate and crude extract used. Tannin was the most abundant phytochemical found in the extracts, followed by alkaloids, while phenol was least.

The result of the quantitative study shows that the tannin content of the extracts ranged from 5.54 – 8.10%. Crude extract had the highest value followed by ethyl acetate extract, while chloroform extract had the least. The phenol content of the extracts was in the range of 2.20% for crude extract to 5.05% for ethyl acetate extract. There was a significant difference ($P<0.05$) in the phenol content of the extract. The alkaloids content obtained ranged from 3.20% for ethyl acetate extract to 7.95% for crude extract. There was a significant difference in the alkaloid content of the extracts. There was a significant difference in the flavonoids content of the extracts. Values obtained ranged from 4.02 – 7.22%. Chloroform extract had the highest value, while crude extract had the least.

Table 1: Qualitative phytochemical analysis of the leaf extract of *Chromolaena odorata* using Chloroform, ethyl acetate and its Crude form.

Parameters	Chloroform extract	Ethyl acetate extract	Crude extract
Tannin	++	++	+++
Phenol	++	++	+
Alkaloid	+	+	++
Flavonoid	++	++	+
Saponin	-	-	-

+ - indicates presence of the phytochemicals; ++ - signifies the intensity of coloration (precipitate) while minus sign (-) indicates absence.

Table 2: Quantitative phytochemical analysis of the leaf extract of *Chromolaena odorata* using Chloroform, ethyl acetate and its Crude form.

Parameters	Chloroform extract	Ethyl acetate extract	Crude extract
Tannin	5.54^{c}±0.57	6.34^{b}±0.00	8.10^{a}±0.14
Phenol	4.06^{b}±0.23	5.25^{a}±0.14	2.20^{c}±0..00
Alkaloid	4.16^{b}±0.20	3.20^{c}±0.00	7.95^{a}±0.11
Flavonoid	7.22^{a}±0.00	6.71^{b}±0.00	4.02^{c}±0.00

Values are mean ± SD. Values on the same row with different superscripts are significantly different ($P<0.05$).

Table 3 presents the result of the antimicrobial activities of *Chromolaena odorata*. The result shows that all the extract showed activity against all the test organisms. Chloroform extract had the inhibition of 21.00 mm (*Salmonella enterica*), 25.00 mm (*Pseudomonas aeruginosa*), 29.00 mm (*Staphylococcus aureus*), 13.33 mm (*Penicilium digitatum*) and 17.33 mm (*Rhizopus stolonifer*). The zone of inhibition obtained for ethyl acetate extract were 19.00 mm (*Salmonella enterica*), 20.00 mm (*Pseudomonas aeruginosa*), 21.00 mm (*Staphylococcus aureus*), 11.67 mm (*Penicilium digitatum*) and 11.67 mm (*Rhizopus stolonifer*), while the inhibition zone obtained for crude extract were 15.00 mm (*Salmonella enterica*), 10.67 mm (*Pseudomonas aeruginosa*), 8.67 mm (*Staphylococcus aureus*), 14.00 mm (*Penicilium digitatum*) and 11.00 mm (*Rhizopus stolonifer*). The control (gentamicin/ketoconazole) showed the inhibition between 32mm and 44mm for all the test organisms. The control gave inhibition that is significantly higher than that of all the test extract against all the test organisms. There was also a significant difference ($P<0.05$) in the inhibition of the test extracts against S. aureus. There was no significant difference ($P>0.05$) in the inhibition obtained for the test extracts against *Penicillium digitata* and *Rhizopus stolonifer*. There was also no significant difference ($P>0.05$) in the inhibition obtained for chloroform and ethyl acetate extract against *S. enterica* and *P. aeruginosa.*

Table 3: Antimicrobial activities of the leaf extracts of *Chromolaena odorata.*

	Zone of inhibition (mm)				
Plant extracts mg/ml	***S. enterica***	***P. aeruginosa***	***S. aureus***	***P. digitata***	***R. stolonifer***
Chloroform extract	21.00b±2.00	25.00b±5.00	29.00b±3.61	13.33b±2.52	17.33b±3.52
Ethyl acetate extract	19.00bc±1.00	20.00b±2.00	21.00c±3.61	11.67b±2.52	11.67b±4.04
Crude extract	15.00c±5.00	10.67c±4.04	8.67c±1.53	14.00b±5.29	11.00b±4.00
Control (Gentamicin) Ketoconazole	32.00a±0.00	38.00a±0.00	45.00a±0.00	44.00a±0.00	44.00a±0.00

The result of the minimum inhibitor concentration (MIC) (Table 4(a-c) showed that chloroform extract had an MIC of 3.125mg/ml for *Salmonella enterica, Pseudomonas aeruginosa* and *Staphylococcus aureus* and an MIC of 6.25mg/ml for *Penicillium digitata and Rhizopus stolonifer.* Ethyl acetate extract had an MIC of 6.25mg/ml *Salmonella enterica, Pseudomonas aeruginosa* and *Staphylococcus aureus* and an MIC of 25mg/ml for *Penicillium digitata and Rhizopus stolonifer,* while the crude extract had an MIC of 25mg/ml for all the test organisms.

Table 4(a-c): Minimum inhibitory concentration of the extracts of *Chromolaena odorata* (mg/ml).

Table 4a Chloroform extract.

Test organisms	25	12.5	6.25	3.125	1.76
S. enterica	-	-	-	-	+
P. aeruginosa	-	-	-	-	+
S. aureus	-	-	-	-	+
P. digitatum	-	-	-	+	+
R. stolonifer	-	-	-	+	+

+ = growth, - = no growth

Table 4b: Ethyl acetate extract.

Test organisms	25	12.5	6.25	3.125	1.76
S. enterica	-	-	-	+	+
P. aeruginosa	-	-	-	+	+
S. aureus	-	-	-	+	+
P. digitatum	-	+	+	+	+
R. stolonifer	-	+	+	+	+

+ = growth, - = no growth

Table 4c: Crude extract.

Test organisms	25	12.5	6.25	3.125	1.76
S. enterica	-	+	+	+	+
P. aeruginosa	-	+	+	+	+
S. aureus	-	+	+	+	+
P. digitatum	-	+	+	+	+
R. stolonifer	-	+	+	+	+

+ = growth, - = no growth

DISCUSSION

Tannin was the most abundant phytochemical found in the extracts, followed by alkaloids, while saponin was least. The presence of this phytochemical have been reported in *C. odorata* by Ndukwe and Ikpeama (2013). Tannins exhibited antimicrobial activities by iron deprivation, hydrogen bonding or specific interactions with vital protein interaction. Leaves that have tannins are used for the treatment of intestinal disorders such as diarrhoea and dysentery (Akindahunsi and Salawu, 2005). Tannins have astringent properties, hasten the healing of wounds and inflamed mucous membranes. Tannins are reported to have various physiological effects like anti-irritant, antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects. Phytotherapeutically, tannins are used to treat non-specific diarrhea,

inflammations of mouth, throat and slightly injured skins (Iniaghe *et al.*, 2009**;** Prasad *et al.*, 2008). Phenols protect plants from predators and pathogens. They produce poisons that protect the plants. The presence of phenolic compounds in the plant indicates that this plant may contain anti-microbial agents. Phenols are used to eliminate bacteria and also used as poisons to burn up parasites (Sofowora, 1993). Flavonoids have been shown to have antibacterial, anti-inflammatory, anti-allergic and anti-tumour and protect organisms from free radicals attack. Flavonoids, are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anticancer activity. Flavonoids in intestinal tract lower the risk of heart disease (Okwu, 2004). This may be the reason for the use of *C. odorata species* in the treatment of intestinal trouble when consumed in soup. Alkaloids are mainly blended from amino acids and also protect the plant from herbivorous animals and may also be useful pharmacologically.

Saponins are a special class of glycosides which have soapy characteristics. It has also been shown that saponins are active antifungal agents (Prasad *et al*., 2008).

The results of the antimicrobial study showed that all the extract showed inhibition against all the test organisms. However, the results showed that the activities of the extract against the test organisms were concentration dependent, as higher concentrations showed higher zone of inhibition. Herbal medicine has been considered as alternative to treating both infectious and non-infectious diseases because of their proven efficacy in traditional medicine practice. Several studies have reported the antimicrobial ability of *C. odorata* leaf extract to inhibit most clinical organisms. Angus reported the inhibition of *S. aureus, S. typhi* and *E. coli* by ethyl acetate extract of *C. odorata*. Lifongo *et al*. (2014) reported the use of *C. odorata* in the treatment of diarrhoea of bacterial origin. Extracts of *C. odorata* are used conventionally in treating headache, haemorrhage, skin diseases, boils, fevers, etc. Its stem bark extracts have antibacterial, antidiabetic, antihyperglycaemic and hepatoprotective activities (Jain *et al*., 2013).

The sensitivity of *S. aureus* and *P. aeruginosa* is very important since the two organisms reportedly show resistance to antibiotics. They both show resistant to vancomycin and ampicillin and first generation cephalosporin (Bernardo-Mazariegos *et al*., 2018).

The lower MIC observed with chloroform extract against the test organisms could suggest that the bioactive component need for the inhibition of the growth of organisms were better

extracted with chloroform than the others. The lower MIC of the extracts obtained compared to the result of the antimicrobial activity in the agar well diffusion study could be attributed to limitation of the extract in diffusing through the agar (Prasad *et al*., 2008).

Aeruginosa and *Staphylococcus aureus* and an MIC of 6.25mg/ml for *Penicillium digitatum and Rhizopus stolonifer* at 200, 100 and 50mg/ml concentrations

The extracts however, showed varying minimum inhibitory concentration against the test organisms, with ethyl acetate extract having the least MIC (3.125mg/ml) against all most of the test organisms.

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