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ANTIBACTERIAL SCREENING OF CRUDE ETHANOLIC LEAF EXTRACTS OF FOUR MEDICINAL PLANTS

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ABSTRACT

Agar well diffusion techniques and macrobroth dilution methods were used to screen the ethanolic leaf extracts of four medicinal plants (Picralima nitida, Chromolaena odorata, Aspilia africana and Hyptis suaveolens) for antibacterial activity against the following bacterial pathogens: Escherichia coli, Pseudomonas aeruginosa, Salmonella spp., Staphylococcus aureus and Bacillus cereus. The minimal inhibitory concentration (MIC) of P. nitida ranged from 1.56 mg/ml to 6.25 mg/ml and that of C. odorata ranged from 2.0 mg/ml to 7.8 mg/ml, while that of A. africana and H. suaveolens varied from 1.56 mg/ml to 4.69 mg/ml and 6.25 mg/ml to 31.25 mg/ml respectively. The extracts appeared to perform best at concentration of 100 mg/ml in agar well diffusion with average inhibition zone diameters ranging from 15 mm to 21 mm (for P. nitida), 16 mm to 22 mm (for C. odorata), 15 mm to 20 mm (for A. africana) and 11 mm to17 mm (for H. suaveolens). P nitida was the most active extract against E. coli and Salmonella spp ($P < 0.05$), while C. odorata and A. africana were the most active against P. aeruginosa and S. aureus. The phytochemical screening of the crude extracts revealed the presence of varied concentrations of bioactive compounds including tannins, flavonoid, alkaloid, etc. The results provide justification for the use of these plants in treatment of various infections in herbal medicine.

INTRODUCTION

The use of medicinal plants for treatment of various infections in traditional communities has been an age-long global practice. It has been estimated that 80% of African population use herbal regimen for treatment and control of diseases (Hugo and Russell, 2003). This provides a

rationalization for studying medicinal plant extracts as a possible source of alternative therapy against infections. Apart from the expensive costs of some antibiotics, most of the clinically important antibiotics have major setbacks. A good number of conventional antibiotics have been found to be neurotoxic, nephrotoxic and hypertensive, and few others cause severe damage to the liver and bone marrow depression (Chong and Pagano, 1997). The primary benefit of using herbal drugs is that they are relatively safer and cheaper than the synthetic alternatives (Aiyegoro and Okoh, 2009). In addition, herbal medicine is a complex mixture of different phytochemicals acting by different mechanisms, which makes it difficult for pathogens to develop resistance (Daferera *et al.*, 2003).

In this study, bacterial isolates of *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa* and *Salmonella* spp., were subjected to an *in vitro* treatment with crude ethanolic leaf extracts of the following medicinal plants. *Picralima nitida, Aspilia africana, Chromolaena odorata* and *Hyptis suaveolens. A. africana* and *C. odorata* are plants used in folklore medicine for topical treatment of wound and skin infections in some parts of Eastern Nigeria, while *P. nitida* and *H. suaveolens* are used for treatment of Malaria and several gastroenteric diseases such as typhoid fever.

The aims of this study were:

i. To evaluate the antibacterial potency of these medicinal plants.
ii. To screen the plant extracts for possible phytochemicals likely to be responsible for any observed antibacterial activity.

MATERIALS AND METHODS

Plant Material

The plants used for this study (*Picralima nitida, Chromolaena odorata, Aspilia africa* and *Hyptis suaveolens),* were harvested from Ozubulu in Anambra state, in Eastern Nigeria. The plants were identified taxonomically by Mr A.O. Ozioko, a former plant taxonomist at the herbarium section of the Department of Botany, University of Nigeria, Nsukka.

Extraction of Plant Material

The leaves of the plants were plucked, rinsed with water and air-dried at room temperature for several days. The dried leaves were pulverized using a milling machine to obtain fine powder. The active ingredients were extracted by percolation using 95% ethanol. Briefly, 100 g of each leaf powder was added to 900 ml of 95% ethanol. The mixture was covered and shaken every 30 min for 6 h, and then allowed to stand for 48 h for extraction. The mixture was then separated by passing through Whatman's No 1 filter paper, after which the filtrate was evaporated to dryness under air pressure. The dried crude extracts were stored in the refrigerator (at 4^{o}C) under aseptic conditions for subsequent use.

The Test Organisms

The test bacterial organisms were clinical and environmental isolates obtained from the Departments of Microbiology and Veterinary Pathology and Microbiology, University of Nigeria, Nsukka. These include at least four isolates of each of the following spices: *E.coli*, *P.aeruginosa*, *B.cereus*, *S.aureus* and *Salmonella* spp.

Screening of the Extracts for Antibacterial Activity

One gram of each crude extract was reconstituted in 20% Dimethyl sulphoxide (DMSO) to obtain extract concentration of 200 mg/ml. This was serially diluted in 2-folds to obtain the following lower extract concentrations: (100, 50, 25, and 12.5) mg/ml. The activities of the plant extracts were determined using agar well diffusion techniques (Perex *et al.*, 1990; Alade and Irobi, 1993) (Nweze and onyishi, 2010). An 18 h old standardized inoculum (10^6 CFU/ml) of each test bacterial isolate was inoculated on dried surface of Mueller-Hinton agar by streaking with a sterile cotton-tipped swab to achieve a confluent growth. The inoculated plates were allowed to dry after which wells were punched on the agar at equidistant positions using a sterile standard 6 mm cork borer. Subsequently, 100 ml of different concentrations of the extract was separately introduced into the different wells that have been labeled accordingly. Equal volume of 20% DMSO was introduced into the well bored in the centre of the plate as a control. This procedure was repeated in duplicate for all the test organisms, and allowed to stay for 30 min on the bench after which they were incubated for 24 h at 37^{o}C. At the end of incubation, observed zones of inhibition were measured and recorded to the nearest millimeter.

Determination of Minimum Inhibitory concentration (MIC) of the Extracts

The MICs of the extracts on the isolates were determined by macrobroth dilutions techniques following the recommendations of the Clinical and Laboratory Standard Institute (CLSI, 2006) (formerly NCCLS). Different concentrations of the extract ranging from 0.78 mg/ml to 50 mg/ml were prepared in tubes of 1 ml Mueller-Hinton broth by serial dilutions.

Then 1ml of an overnight nutrient broth culture of the test isolates (adjusted to 10^6 CFU/ml) was added to each tube of the 1ml Mueller Hinton broth containing the extract. Each tube was mixed and incubated at 37^{o}C for 24 h. The experiment was conducted in duplicate for all the test isolates. Tubes of Mueller-Hinton broth containing only the 1 ml suspension of the test organism without the extract, and the tubes of Mueller-Hinton broth containing different concentrations of the extract without test organisms, were used as controls. The first tube in the series, with no visible growth after incubation period was taken as the MIC.

Phytochemcial Screening

The extracts were subjected to phytochemical tests for plant secondary metabolites such as tannins, saponins, flavonods, alkaloids, terpenoids and glycosides, following the standard laboratory techniques (Harbone, 1994; Trease and Evans, 2004).

Statistical Analysis

All the data were subjected to one way analysis of variance (ANOVA) and the variant means were separated using Ducan multiple range test (DMUT). Significance was accepted at $P \leq 0.05$. The one way ANOVA test was also used to determine if there is any statistically significant difference in the susceptibilities of different test organisms to each extract, and also to determine if there is any significant difference in the activities of the different plant extracts on each test organism.

RESULTS

All the plant extracts exhibited different levels of antibacterial activities and appeared to have the best activity at the extract concentration of 100 mg/ml in the agar well diffusion experiments. Table 1 shows the average minimum inhibitory concentrations (MICs) of different plant extracts on different organisms obtained using macrobroth dilution method.

Figure 1 shows the summary of the antibacterial activity of the extract of *P. nitida* in the agar well diffusion test at different concentration with average inhibition zone diameters that ranged from 15 mm to 21 mm. The mean MICs of the extract varied from 1.56 mg/ml to 6.25 mg/ml as shown in table 1. The susceptibilities of the test organisms to the extract (at 100 mg/ml) can be ranked statistically ($P<0.05$) as follows: *E. coli* > (*B. cereus* = *S. aureus* = *Salmonella* spp) > *P. aeruginosa* The antibacterial activity of the extract of *C. odorata* in the agar well diffusion test is summarized in figure 2. The average inhibition zone diameters of the extract at different concentrations ranged from 16 mm to 22 mm. The average MICs ranged from 2.0 mg/ml to 7.8 mg/ml as shown in table1. The sensitivities of the test organisms to the extract of *C. odorata* at 100 mg/ml are in the following order ($P< 0.05$): *S. aureus* > *B. cereus* > (*Salmonella* spp. = *E. coli* = *P. aeruginosa).* Figure 3 shows the antibacterial activity of *A. africana* extract with average inhibition zone diameters that ranged from 15 mm to 20 mm. The average MICs varied from 1.56 mg/ml to 4.69 mg/ml as shown in tatble1. The sensitivities of the test organisms to the extract at 100 mg/ml were in the following order ($P< 0.05$): (*B. cereus* = *S. aureus*) > (*P. aeruginosa*= *E. coli* = *Salmonella* spp). Figure 4 shows the activity of *H. suaveolens* with average inhibition zone diameters that ranged from 11 mm to s17 mm. The average MICs varied from 6.25 mg/ml to 31.25 mg/ml as shown in table1. The tests organisms were sensitive to the extract (at 100mg/ml) in the following order ($P < 0.05$): (*B cereus*= *Salmonella* spp) > (*E. coli* = *S. aureus)* > P. aeruginosa.
Fig 5.0 compares the activities of different plant extracts on each test organism. In all the test organisms, *H. suaveolens* had the least activity ($P<0.05$). The extract of *P. nitida* was the most active extract against *E. coli* and *Salmonella*, while *C. odorata* and *A. africana* were the most active extracts against *P. aeruginosa* ($P <0.05$). *C. odorata* was also the most active extract against S. *aureus* and *B. cereus.*

Table 2 shows the various concentrations of the phytochemcial constituents of the extracts. The phytochemical screening revealed the presence of active compounds including alkaloids, flavonoids, tannins, etc.

Fig. 1. Antibacterial Activity of Ethanolic leaf Extract of *Picralima nitida*

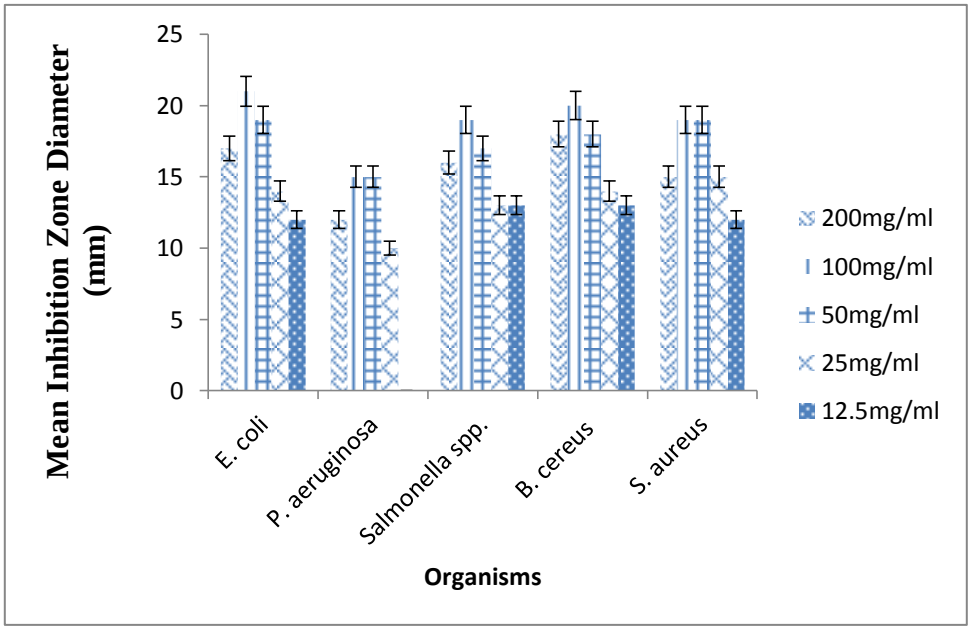

Fig. 2. Antibacterial Activity of Ethanolic leaf Extract of *Chromolaena odorata*

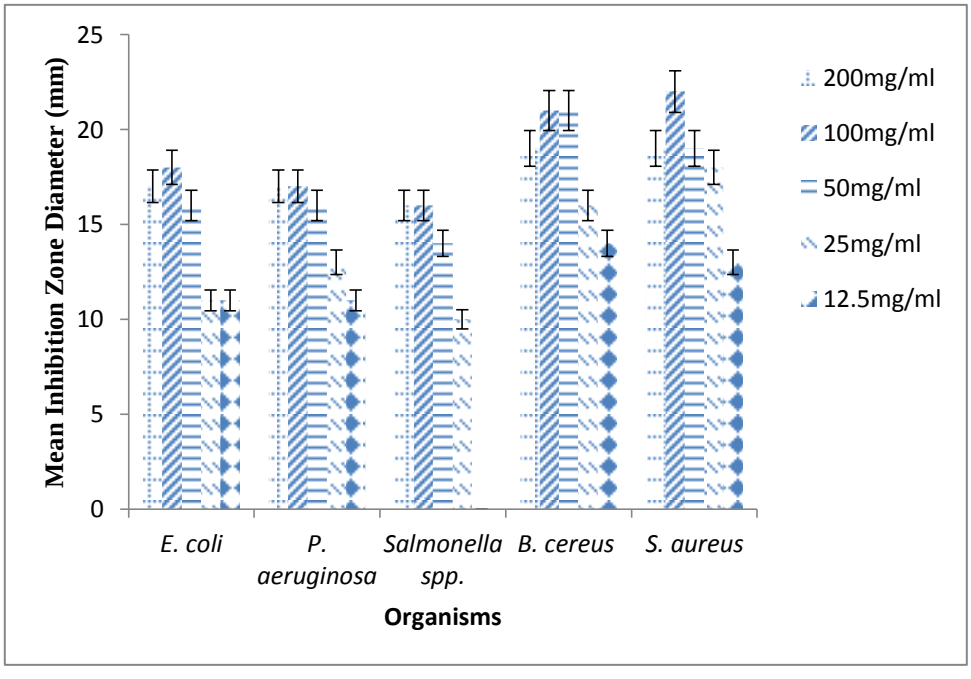

Fig. 3. Antibacterial Activity of Ethanolic leaf Extract of *Aspilia africana*

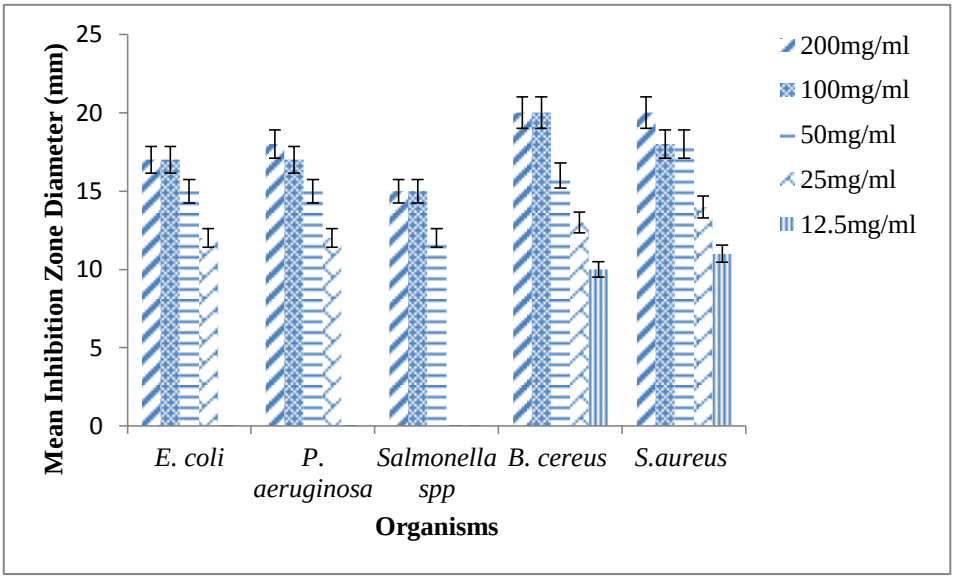

Fig. 4. Antibacterial activity of Ethanolic leaf Extract of *Hyptis Suaveolens*

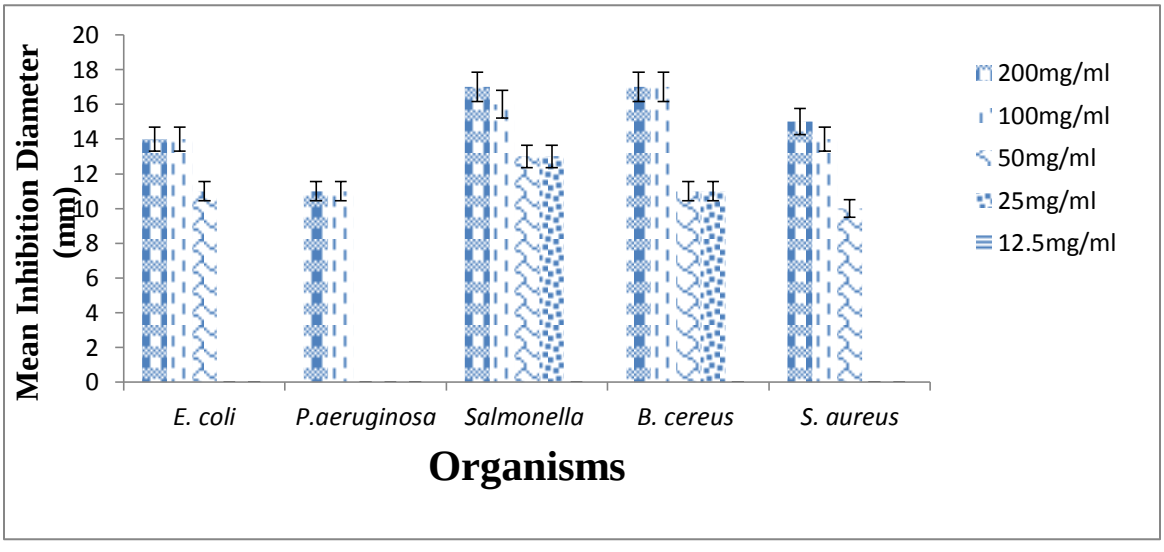

Table-1. The Mean MIC Values of Crude Extracts of Different Plants

Organisms	Mean MIC Values of Different Extracts (mg/ml)			
	P. nitida	*C. odorata*	*A. africana*	*H. suaveolens*
E .coli	2.0	6.25	3.125	12.5
P. aeruginosa	6.25	5.47	3.125	31.25
Salmonella	3.125	7.8	4.69	6.25
B. cereus	2.0	3.125	1.56	7.8
S. aureus	1.56	2.0	2.0	12.5

Key: MIC = minimum inhibitory concentration

Fig. 5. Antibacterial Activities of Different Plant Extracts on Each Test Organism (at extract concentration of 100mg/ml)

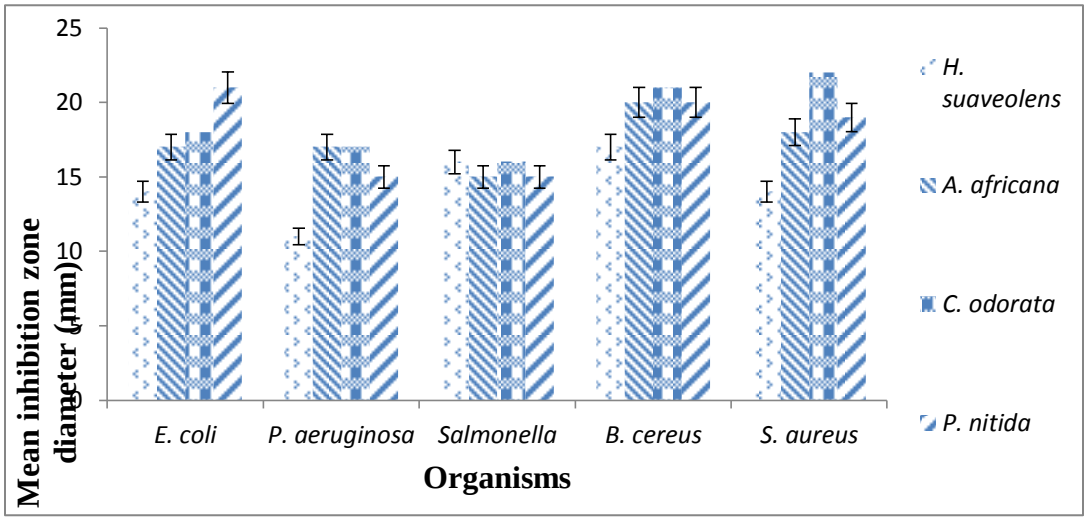

Table-2. The Photochemical Screening of Ethanolic leaf Extracts of the four Test plants

	Test Plants			
Photochemical	*P nitida*	*A. africana*	*C. odorata*	*H. suaveolens*
Alkaloids	+++	++	+++	+++
Flavonoids	+++	++	+++	+++
Glycosides	+	+	+++	+
Saponins	+	++	++	+
Tannins/Phenols	+++	+++	+++	+++
Terpenoids	+++	+++	+++	+
Steroids	+	+	++	+++
Fats and oil	+	+	+	+++

Key: + = low concentration

++ = moderate concentration

+++ = high concentration

DISCUSSION

The phytochemical screening of the plant extracts revealed the presence of high concentrations of bioactive compounds including tannin, terpenoids, alkaloids, etc. These phytochemicals have been proved to possess biocidal and inhibitory activities against a wide range of microorganisms (Cowan, 1999; Iwu *et al.*, 1999; Aiyegoro and Okoh, 2009). The presence of these phytochemicals in the extracts could therefore explain their antibacterial activities as observed in this study. The extract of *P. nitida* appeared to be the most active extract against *E. coli* and *Salmonella* spp ($P<0.05$). This is quite significant as *Salmonella* and *E. coli* are among the bacterial pathogens responsible for gastroenteric diseases against which herbal preparations of *P. nitida* is highly recommended and often used in folklore medicine in some parts of Eastern Nigeria.

There was no significant difference in the activities of the extracts of *A. africana and C. odorata (*P < 0.05) except on *S. aureus*, were *C. odorata* had the highest antibacterial activity followed by *A. africana* and *P. nitida*. This could be due to the fact that *A. africana* and *C. odorata* belong to the same family of *Asteraceae* (Schmidt and Schilling, 2000; Oluyemi *et al.*, 2007). In some parts of Eastern Nigeria, the extracts of the two medicinal plants (*A. africana* and *C. odorata)* are used for treatment of wound and skin infections. The phytochemical screening of the extract of *H. suaveolens* revealed a high concentration of some bioactive compounds in amounts similar to those of other extracts. However, the extract of *H. suaveolens* was far less active than other plant extracts against all the test organisms. The reason for this is not obvious. Although the activities of the extracts declines as their concentration decreases in the agar well diffusion experiment, the plant extracts generally appeared to have the best activity at extract concentration of 100 mg/ml and not at the highest concentration of 200 mg/ml. The reason for this observation could be attributed to the limitation of high concentration of plant extracts in diffusing through agar medium due to their high viscosity. Some researchers have noted that this impairment in drug diffusion is a major limitation in the evaluation of the antimicrobial activities of plant extracts by agar well diffusion (Esimone *et al.*, 2006; Adwan and Mhanna, 2008). This will constitute another important research frontier in this multidimensional search for a role for plants in the search for a solution to multidrug resistance among infectious agents.

The Gram positive bacterial organisms (*B. cereus* and *S. aureus)* were more sensitive to the extracts of *C. odorata* and *A. africana* than the Gram negative organisms (*E. coli, P.aeruginosa* and *Salmonella)* (P<0.05) .This agrees with the observation made by some researchers that plant extracts show considerable activity against Gram positive bacteria than Gram negative bacteria due to the double membrane barrier of Gram negative bacteria (Nostro *et al.*, 2000). However, the activities of the extracts of *P. nitida* and *H. suaveolens* did not correlate with this trend suggesting that these plant extracts may be inhibiting microorganisms through different mechanisms thus exhibiting selective action against separate genera. There was no significant difference in the sensitivities of the tested Gram negative bacteria and the Gram positive bacteria. These results provide a rationalization for the use of these medicinal plants for treatment and control of various infections in traditional medicine. Acute toxicity study needs to be conducted on the extracts of these plants to evaluate the safe limit of their dosage and consumption.

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