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Anti-Bacterial Effect of Different Fractionations of Acetic Acid Leaf Extract of *Parinari curatellifolia*

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Authors' contributions

This work was carried out in collaboration between both authors. Author EHT designed the study, performed the statistical analysis, and managed the literature searches. Author WAU managed the analyses of the study, and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

Research Article

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ABSTRACT

The research was aimed at determining the anti-bacterial effect of different fractions of acetic acid leaf extract of *Parinari curatellifolia.* The research was conducted in laboratory unit of peace specialist hospital, Yola, Nigeria between May and November, 2011. Different solvent extracts of the leaf were prepared using the soxhlet apparatus. Disc diffusion method was used to test for the anti-bacterial activities of the different solvent extracts. The Minimum Inhibitory Concentration (MIC) of the acetic acid extract which gave the highest zones of inhibition against the microbial isolates used revealed that the MIC for *Streptococcus pyogenes* and *Pseudomonas sp* is 5mg/ml while that for *Klebsiella sp* and *Staphylococcus aureus* is 50mg/ml The acetic acid extract was fractionated with different solvents using the column chromatography. Four fractions (TiA, TiB, TiC, and TiD) were obtained from the column. Fraction TiC gave the highest zone of inhibition ranging from 10.0±0.6 to 17.3±0.9 against the test organisms. This is significantly (*P=0.5*) different from the crude acetic acid extract with inhibition zones ranging from 20.0±0.6 to 28.3±0.3. Inhibition zones were measured using a metre rule. Fraction TiB did not have any anti-bacterial activity against all the test organisms. Fraction TiC gave three bands on thin layer chromatography with Rf values of 0.38, 0.36, and 0.23. The activities of the separated fractions were lower compared to the crude extract. This may be due to

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synergistic effect of various secondary metabolites present in the crude extract. Results were discussed in respect to the anti-bacterial effect of the different fractions.

Keywords: Parinari curatellifolia; leaf extract; antibacterial activity; thin layer chromatography; disc diffusion.

1. INTRODUCTION

The use of herbal medicine by the traditional practitioners for the treatment of diseases remains the main stay of health care system and is gaining increasing popularity especially among the rural populace in developing countries. Many of the herbal remedies used by herbal practitioners are also employed therapeutically in orthodox medicine after the crude extract has been greatly improved upon. Research programs have been going on to assay and improve the medicinal principles found in drugs for use in the development of new pharmacotherapeutic agents in the management and cure of diseases [1].

In Eha-Amufu of Enugu State, Nigeria, *Parinari curatellifolia* is a valuable and cherished medicinal plant in which different parts of the plant are widely used by the traditional herbalist in the treatment of Epiglottitis, diabetes and other diseased conditions. However, there is very little scientific information documented on the efficacy of this plant. The anti-diabetic potentials of the plant have been evaluated and reported. Recently, we presented the curative effect of the crude leaf extract on epiglottitis [3].

Here we present the probable synergistic anti-bacterial effect of the different fractions of the acetic acid leaf extract of *Parinari curatellifolia.*

2. MATERIALS AND METHODS

2.1 Identification of the Plant Material

The plant was collected from Isu village in Eha-Amufu of Enugu State, Nigeria. It was identified and authenticated by a staff of Botany Department, University of Nigeria Nsukka. The voucher specimen was deposited at the biological science department of Modibbo Adama University of Technology, Yola.

2.2 Source of the Test Organism

The clinical isolates of *Streptococcus sp, Staphylococcus aureus, Pseudomonas sp,* and *Klebsiella sp* were obtained from Federal Medical Centre Yola, Nigeria. Each test bacterial strain was re-identified using standard bacteriological and biochemical methods [4]. Stock cultures were maintained in nutrient agar slants at 4ºC.

2.3 Preparation of the Plant Extract

The acetic acid, ethyl acetate and methanolic extracts of the leaf of the plant were prepared. The plant sample collected was air dried and ground using a milling machine. The powdered material (50g) was transferred into a Soxhlet apparatus and extracted in the Soxhlet extractor using, ethyl acetate, methanol, acetic acid (glacial), and water separately for 24hrs each [5,6]. The extracting solvents used were concentrated ones. The extracts were

concentrated to dryness. The residues were transferred into pre-weighed sample containers, and stored at 4ºC until when required for use.

2.4 Determination of Viable Cell

The viable cells were determined as described by Kanika, 2009 [7]. Serial dilutions of hour broth cultures were prepared. 1ml of suspension was inoculated into nutrient agar plates labeled according to the dilutions used. They were incubated in an inverted position at 37ºC for 24hrs. After the incubation, plates with number of colonies ranging from 30-300 were counted. Plates with spreaders were discarded.

Numbers of viable cells were calculated as follows;

$$\text{No of cells/ml} = {}^{\text{No of colonies}}/_{\text{Volume of sample}} \times \text{dilution factor}$$

2.5 Determination of Antimicrobial Activity of the Extract

The test for sensitivity of each organism was done by the disc diffusion technique [8]. A number of sterile paper discs (diameter 5mm) were mixed completely with a reconstituted extract (500mg/ml) in a sterile 10ml glass beaker. The discs were allowed to remain in contact with the extract for at least an hour to enable them absorbs the extracts which became embedded in the discs. The discs were brought out and allowed to air dry. As a control, a number of sterile paper discs (diameter 5mm) were mixed in the solvents without the extract. Each organism was cultured by the spread plate technique [9]. The test organisms were adjusted to 11×10^7 cells/ml. They were spread evenly on the surface of the agar medium with a sterile glass hockey. Then using a flame needle, the prepared sensitivity test discs were carefully picked and placed on top of the inoculated plate at some distances from one another. The plates were allowed to stand for about 5 minutes and incubated at 37ºC in an electronic incubator. They were observed for 24hours for growth and possible clear zone around the disc as a mark of sensitivity to the test extract. The antimicrobial activity of the partially fractionated extract was also determined using disc diffusion method. The experiment was done in triplicates.

2.6 Determination of the Minimum Inhibitory Concentration (MIC) of the Extract

The minimum inhibitory concentration was determined as the least concentration of the extract which inhibits each test organism [8]. 500mg of the extract was separately reconstituted in sterile distilled water and diluted to concentrations of 50, 5, 0.5, 0.05, 0.005 mg/ml. The regenerated concentrations were used for the sensitivity test. After incubation, the plates were observed for inhibition zones. The list concentration which caused inhibition was taken to be the minimum inhibition concentration.

2.7 Fractionation of the Extract of the Leaf of *Parinari curatellifolia*

The extract of the leaf of *Parinari curatellifolia* was fractionated using the Column and the Thin-Layer Chromatographic Techniques for possible identification of the active component responsible for the observed activity. For column chromatography, a piece of cotton wool was inserted into a column. Silica gel (grit size 200–300 mesh) was used to fill the glass column. The packed column was clamped onto a retort stand. Enough solvent (ethyl

acetate) was added into the column and gently tapped with a cork ring to ensure proper packing of the column. Dry sample of the extract was slowly poured on top of the column and continuously eluted with different solvents starting from the least polar to the most polar, in the following order; ethyl acetate, methanol, acetic acid, and water. The fractions obtained (TiA, TiB, TiC, TiD) were evaporated using a rotary vapor system. The residue obtained was investigated for its antimicrobial activity. Analytic TLC was done with silica gel glass plates (5 × 15cm) as stationary phase and a mixture of acetic acid, ethyl acetate, and hexane (5:3:2) as solvents for the mobile phase. The glass plates were coated with the silica gel and allowed to air dry. The coated glass plates were activated by heating in an oven at 110^{o}C for 30 minutes. A thin pencil line mark was made on the plate at about 1cm from one end of the plates known as point of application. A small amount of the extract dissolved in the extracting solvent was spotted on the line using a spotter made from micro-capillary tube. The spot was allowed to air dry. The plate was put into a thin-layer chromatographic tank saturated with the solvent vapor. The plates were allowed to remain in the tank until the solvent is few centimeters away from the top of the plates. The distance moved by the samples and the solvents were noted. Pigments visible at daylight were documented by photographing.

2.8 Statistical Analysis

The results were subjected to a statistical software SPSS (15.0) for analysis. Results were expressed as Mean ± SEM. Significant differences were determined using the student's t-test. Differences were considered significant if $p<0.05$.

3. RESULTS AND DISCUSSION

Table 1 shows the results of anti-bacterial activities of the various crude leaf extracts *Parinari curatellifolia*. Acetic acid extract had the highest zone of inhibition ranging from 20.0-28.7mm. The difference in the zones of inhibition (Table 1) is an indication of the differences in the solubility of the active components of the leaf of this plant in different solvents. The result implies that the active components are more soluble in acetic acid (glacial) compared to ethyl acetate, methanol, and water, thus making acetic acid to extract more of the active components compared to the other solvents used in this research. The antimicrobial activities observed in the leaf of this plant may be attributed to the presence of some secondary metabolites in the leaf extract [3].

Table 1. Results of antibacterial activity of different leaf extracts of *P. curatellifolia*

Microorganisms	Extracting solvent and inhibition zones (mm)			
	Water (mm)	Methanol (mm)	Ethyl acetate (mm)	Acetic acid (mm)
Streptococcus pyogenes	11.7±1.2	09.0±0.6	14.7±0.9	20.0±0.6
Staphylococcus aureus	13.7±1.3	10.0±0.6	16.3±0.9	28.3±0.3
Klebsiella sp	12.3±1.5	12.0±0.6	14.7±2.3	22.3±0.3
Pseudomonas sp	13.0±1.2	13.0±0.6	15.3±1.8	28.3±0.3

Results are express as mean ± SEM

The efficacy of this extract against both gram positive and gram negative organisms is an indication of broad spectrum activity.

The Minimum Inhibitory Concentration(MIC) of the acetic acid extract which gave the highest zones of inhibition against the microbial isolates used revealed that the MIC for *Streptococcus pyogenes* and *Pseudomonas sp* is 5mg/ml while that for *Klebsiella sp* and *Staphylococcus aureus* is 50mg/ml (Table 2). The low Minimum Inhibitory Concentration (MIC) of the acetic acid extract observed against the isolates used in this research is an indication of the efficacy of the plant leaf extract against these bacteria.

Table 2. Minimum inhibitory concentration of the acetic acid extract

Microorganism	Extract concentration (mg/ml)				
	50mg/ml	5mg/ml	0.5mg/ml	0.05mg/ml	0.005mg/ml
Streptococcus pyogenes	-	-	+	+	+
Staphylococcus aureus	-	+	+	+	+
Klebsiella sp	-	+	+	+	+
Pseudomonas sp	-	-	+	+	+

Note: – indicates no growth, + indicates growth

Table 3. Results of antibacterial activities of the different fractions of acetic acid extract of the leaf of *P.curatellifolia*.

Microorganisms	Different Fractions and inhibition Zones (mm)			
	TiA (mm)	TiB (mm)	TiC (mm)	TiD (mm)
Streptococcus pyogenes	06±0.6	0	10±0.6	10±0.6
Staphylococcus aureus	06±0.6	0	15±0.6	15±0.5
Pseudomonas sp	08±0.6	0	10.7±0.9	0
Klebsiella sp.	07±0.6	0	17.3±0.9	10.7±0.3

Results are express as mean ± SEM

Fractionation of plant extracts may result in improved or loss of activity [10]. Four fractions (TiA, TiB, TiC, and TiD) were obtained on fractionation of the acetic acid extract which gave the highest inhibition zone. With reference to the susceptibility of the test strains, TiC recorded the highest zone of inhibition of 17.0mm against *Klebsiella sp*. However, the inhibition zone was significantly (P=0.05) less than that observed with the crude extract. Fraction TiB did not show any activity against all the test organisms. The remaining two fractions (TiA and TiD showed varying levels of activity against the test organisms (Table 3). Fraction TiC gave three distinct bands on thin layer chromatography with Rf values of 0.38, 0.36, and 0.23 (Fig 1).

Combination of secondary metabolites enhances the activity of combined agents and susceptible organisms [11]. In the present study it was observed that the activity of the crude extract was higher compared to the separated fractions. This could be partly due to the ability of the active principle to dissolve in different solvents at different rates or it could be due to synergistic effect of various secondary metabolites present in the crude extract. Fraction TiC with the highest anti-bacterial activity yielded three bands on thin layer chromatography.

TiA = first fraction (ethyl acetate fraction), TiB = second fraction (methanol fraction), TiC = third fraction (acetic acid fraction), TiD = fourth fraction (water fraction).

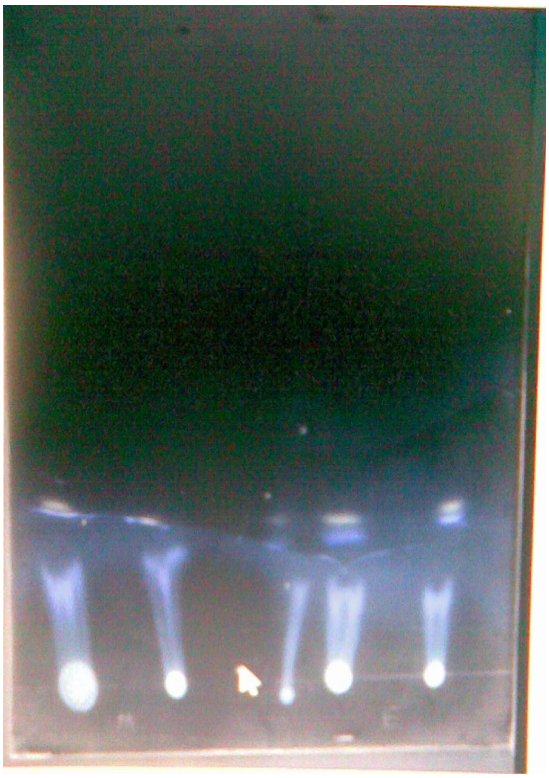

Fig. 1. TLC of the Acetic Acid Extract of the Leaf of *Parinari curatellifolia*

4. CONCLUSIONS

Parinari curatellifolia leaf extract has antimicrobial activity. This activity may be due to the presence of secondary metabolites present in the plant. These agents may have acted synergistically to produce the observed effect in the crude extract. The efficacy of this extract against both gram positive and gram negative organisms is an indication of broad spectrum activity. There may be three different compounds which may be responsible for the observed activity of the separated fractions. Further work is going on to identify, characterized and elucidate the structure of the anti-microbial agent(s) present in the plant.

CONSENT

Not applicable.

ETHICAL APPROVAL

The authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

The authors declare that no competing interest exists.

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