ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Evaluation of Antibacterial Activity of *Piliostigma thonningiin* (crude extract) and fractions 1-3, 4, 5, 6 and 7 against Methicillin-resistant *Staphylococcus aureus* (MRSA).

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ABSTRACT

Aim of this study is to investigate the antibacterial activity of traditional Nigeria medicines with special interest in anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity. Hexane, ethylacetate, methanol and aqueous extracts of this plant was prepared. Agar dilution, agar-well diffusion tests and MIC's were used to investigate antibacterial activity. Out of 4 extract that were evaluated for antibacterial activity using agar dilution technique only hexane extract gave the highest inhibition against all the MRSA isolates. Crude extract and fractions showed the highest levels of antibacterial activity overall with MIC's against MRSA in the range of 1.0 and 2.0mg/ml. The presence of antibacterial activity in extracts and fractions of leaves of *P. thonningii* plants traditionally used to treat skin infections, are reported for the first time. Extracts from *P. thonningii*, also used to treat wounds, had the widest spectrum of antibacterial activity.

Keywords: Antibacterial; MRSA; Traditional medicines; Nigeria

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ISSN: 0975-8585

INTRODUCTION

The increasing occurrence, particularly in hospitals, of *S. aureus* resistant not only to methicillin but to a wide range of antimicrobial agents, including all kinds of β- lactams, has made therapy more difficult [1-4]. Although strategies have been proposed in an attempt to control the spread [5]. The search for new ways to treat MRSA infections stimulates the investigation of natural compounds as an alternative treatment of these infections.

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay [6]. Many reports are available on the antifungal, antidiarrhoeal, antiviral, antibacterial, antihelmenth properties of plants [7-11]. Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings. However, not many reports are available on the antimethicillin activity of medicinal plants for developing commercial formulations for applications in health care. The aim of this work therefore was to investigate anti- MRSA activity of the crude extracts and fractions of *P. thonningii* leaves.

MATERIALS AND METHODS

Plant material

The fresh leaves of *P. thoninngii* were collected from Minna metropolis. The taxonomic identities of the plants were confirmed by a botanist of Herbarium Department of National Institute for Pharmaceutical Research and development (NIPRD), Idu, Abuja.

Sources of clinical isolates

MRSA isolates were consecutively isolated from samples submitted to the microbiology laboratories from patients being treated in several hospitals in Niger State including FCT, Abuja. Specimens ranging from blood. Stool, semen, wound, urine were collected.

Preparation of Culture media (Mueller Hinton agar).

Mueller-Hinton agar was prepared from a commercially available dehydrated base according to the manufacturer's instruction. Immediately after autoclaving, it was allowed to cooled at 45-50^{o}C water bath. After cooling it was poured into glass, flat bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4mm. This corresponds to 60-70ml of medium for plate with diameter of 150mm and 25-30ml of medium for plates with diameter 100mm. The agar medium was allowed to cool at room temperature before use.

Trypton soya broth

7.0g of the powder was dissolved in 100mls of distilled water in conical flask, the required volume was dispensed in to bijou bottle and autoclaved for 15min at 121^{o}c.

Detection of MRSA

Methicillin Resistant strains were determined using (Oxoid's PBP2' latex kit) according to manufacturer instructions.

Preparation of Standard inoculums

The inoculum was prepared from broth culture that has been inoculated for 3-4hrs, when growth is considered to be at logarithmic phase. The density of suspension was adjusted to approximately 10^{8}cfu/ml, equivalent to 0.5 Macfarland standards. [12]

Preparation of antibiotic stock solution (Vancomycin)

Vancomycin Hydrochloride commercially prepared (USP Ref STD 70900K). The powder was accurately weighed and dissolved in the appropriate diluents (water). To yield the required concentration, using sterile glass wares. 400µg/ml concentration of the antibiotic vancomycin was prepared by dissolving 10mg vancomycin powder in 10ml of sterile distilled water and 2ml of this stock solution was taken and made up to 5ml with sterile distilled water to get a concentration of 400µg/ml

Preparation of dried filter paper disc

Whatman No. 1 filter paper was used to prepared discs approximately 6mm in diameter, using a paper number. They were placed in a Petri dish and sterilized in hot air oven at 160^{o}C for 30min. After sterilization it was cool at room temperature. The loop used for delivering antibiotic is made of 20 guage wire and diameter of 2mm. This delivers 0.005ml of antibiotic in each discs of 30µg concentration [13].

Direct Colony Suspension Method

The inoculum was prepared by making a direct broth suspension of isolated colonies from 18-24hrs, this was further sub-culture to 3-4hours in fresh broth. The suspension was adjusted to 0.5 MacFarland turbidity standard using saline and a vortex mixer. This approach is the recommended method for testing staphylococci for potential Methicillin or Oxacillin Resistance [14].

Inoculation of Test Plate

Optimally within 15min after adjusting the turbidity of the inoculums suspension, a sterile cotton swab (No 03-048) was dipped into the adjusted suspension, the swab was rotated several times and pressed finally on the inside wall of the bijou bottles above the fluid level. This was to remove the excess inoculums from the swab. The dried surface of the MHA plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plates approaching 60^{o} each to ensure an even distribution of inoculums. The lid was left ajar 3-5min, to allow any excess surface mixture to be absorbed before applying the drug impregnated disc.

Application of Disc to Inoculated agar plates

The predetermined battery of antimicrobial disc was dispensed unto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface; they were distributed evenly so that they were not closer than 24mm from center to center.

The plates were inverted and placed in an incubator at 35^{o}C within 15min after the disc was applied.

Reading plate and Interpreting Result

After 24hours of incubation each plate were examined. The resulting zones of inhibition were uniformly circular and there were confluent lawns of growth. The diameter of the zones of complete inhibitory (as fridges by the unaided eye) were measured; including the diameter of the disc.[15]

Zones are measured to the nearest whole number, using a ruler which was held on the back of the inverted petri plate. The Petri plate was held a few inches above a transmitted light (plate held up to light) is used to examine the Vancomycin zone for light growth of Methicillin or Vancomycin Resistant colonies, respectively, within apparent zones of inhibition. Any discernable growth within zone of inhibition was indicative of Methicillin or Vancomycin Resistant. The size of zones of inhibition are interpreted by (zone diameter interpretive standard and equivalent MIC break point)[16]

Fractionation of *P. thonningii* extract using Column and Thin layer chromatography

A glass column (460 mm x 26 mm, Büchi, AG, Flavil, Switzerland) was used for the fractionation of the antimicrobially most potent extracts of ***P. thonningii***. Twenty grams of leaf powder of *Piliostigma thonningii* was extracted in 500 ml of n-hexane This extract was applied to the column and eluted with n-hexane and ethyl acetate using a step gradient, flow rate 3.0 ml/min. The fractions were collected with a fraction collector (LKB, Bromma, 2111 Multirac, Bromma, Sweden). The fractions obtained were investigated with reversed phase thin layer

chromatography (RP-TLC) in order to be able to combine the fractions containing the same pattern of molecules. TLC was performed on (Merck, Darmstadt, Germany) TLC plates. The plates were developed with hexane : ethyl acetate 4 : 1 (v/v). Fractions were combined according to the pattern information obtained by RP-TLC.

Fractionation procedure

The crude hexane extract was obtained by soxhlet extraction.The crude extract was then subjected to vacuum liquid chromatography (VLC) to fractionate them. Twenty grams of extract was dissolved in hexane suitable solvent and preabsorbed on TLC- grade silica gel (Merck Kieselgel 60G). The preabsorbed sample was allowed to dry and made into fine powder. The sintered glass funnel (porosity 3) used for the VLC was loaded with silica gel under vacuum to ensure that it was well compacted and uniformily spread. A non-polar solvent (hexane) was run through the column under vacuum. Then, the preabsorbed sample was evenly spread on the silica gel. Suction was applied to compress the sample to the silica gel and cotton wool was used to cover the surface to prevent disturbance during the course of the experiment. The column was eluted with 100ml of different solvent systems (hexane : ethyl acetate) of increasing polarity starting with hexane and then ethyl acetate. The concentrated fractions were subjected to TLC (hexane : ethyl acetate, 4 : 1) and similar fractions were pooled together. The fractions were also subjected to antibacterial assay.

Assay for antibacterial activity of a crude extract and fractions RF(1-3) - RF7 of *P. thonningii* extract

An agar diffusion method [17-19] was used to screen the plants for antibacterial activity of crude extract and fractions. The bacterial strains to be used were inoculated on Mueller Hinton agar (MHA), and grown for 24 h. Different concentrations of the extract, 10 mg, 20mg, 30mg, 40mg, extract were used. The petri dishes were kept in room temperature for one hour prior to incubation at 35^0C for 24 h. The diameter of the inhibition zones were measured after incubation. Vancomycin (30µg) (Oxoid) was used as positive controls.

Minimum Inhibitory Concentration(MIC) of Vancomycin (the positive control drug)

One millilitre of sterile nutrient broth was pipetted into each of 12 sterile test- tube using an automated pipette. One millilitre of the 400µg/ml vancomycin was introduced into the first test tube and mixed thoroughly. One millilitre of the mixture was then pipetted and transferred into test-tube 2 thus reducing the concentration downwards serially until the 11^{th} test tube after which the excess 1ml was discarded. One millilitre of the test organism of approximately 10^8 CFU/ml was then inoculated into each test tube –including tube 12. One tube was left with just the broth and everything was incubated at 37^0C for 24 hours and the plates were read. The work was done in triplicates and under aseptic conditions.

ISSN: 0975-8585

Minimum Inhibitory Concentration (MIC) of the fractions (1-3,4,5,6,7)

One millilitre of sterile nutrient broth was pipetted into each of 8 sterile test- tube using an automated pipette. One millilitre of the 4mg/ml plant extract was introduced into the first test tube and mixed thoroughly. One millilitre of the mixture was then pipetted and transferred into test-tube 2 thus reducing the concentration downwards serially until the 7^{th} test tube after which the excess 1ml was discarded. One millilitre of the test organism approximately 1x 10^6CFU/ml was then pipetted into each test tube –including tube 8. One tube was Left with just the broth and another with 1ml of the broth and 1ml of the plant extract, all the tubes were was incubated at 37^0C for 24 hours and the turbidity was read. This was done in duplicates and under aseptic conditions.

RESULTS AND DISCUSSION

The methicillin resistance rate in S. aureus isolates varies extensively amongst different sources between 5% to 80% [20]. Infection control Management requires a suitable treatment strategy and a rapid resistance identification procedure. PBP2' was chosen due to its high specificity and sensitivity that has been successfully used around the world for several years. Total test time is 15 minutes, while traditional agar methods take 1-2 days. 100% sensitive and 99% specific (from TSA with blood); more accurate than agar and MIC methods. Saves time and money by eliminating the need for expensive PCR or time consuming agar methods. Uses common laboratory equipment.. The overuse of antibiotic drugs has led to the extensive antibiotic resistance in human pathogenic bacteria, which highlights the research need on new antimicrobial agents. In this study, *P. thonningii* crude extract and fractions were tested for their putative antibacterial activity against 20 clinical MRSA isolates. *P. thonningii* showed antibacterial activity at different degrees. Antibaterial effects of a crude extract and fractions RF(1-3)-RF7 isolated from a leaf hexane extract of *P. thonningii* is presented in Table 1. The crude extract of *P. thonningii* gave the highest susceptibility against all the MRSA isolates tested with zones of inhibition ranging from 24mm to 34mm. Fractions RF(1-3)-RF7 was also active against all the MRSA isolate with zone of inhibition of 13mm to 25mm. Zone of inhibition recorded for positive control antibiotic(Vancomycin) ranged from 9mm to 30mm (Table 1).The MIC results of the fractions and the positive control drug (Vancomycin) are presented in Table 2. The Minimum Inhibitory Concentration of fraction1-3 and 567, on all the MRSA was 100µg/ml and 200µg/ml, that of the control was 31.25mg/ml – 1mg/ml. Greater susceptibility was observed with the crude extract than the individual fractions. This is not suprising because the crude extract tested positive for tannins which had been implicated in previous studies to be antimicrobial. It is probable that these bioactive compounds might have played a significant similar role in the observed activity. The activity recorded by these extract against both MRSA and MSSA in our previous study then necessitated their purification by chromatography into fractions. Unexpectedly, the fractions afforded less antibacterial activity against MRSA isolates than the activity of individual fractions. This could be attributed to loss of purity inherent in the fractions. The use of solvents and fractionation is expected to result in greater concentrations of active compounds and stronger activity. But this is not always the case, sometimes total activity decrease with fractionation as obtained in our result. The classical method gives undue

importance to the isolation of pure active compounds from medicinal plants, while isolaton and identification of single active compound is interesting for studies of structure-activity relationships and may be stimulating for the scientist, it will not contribute to any significant extent to the solution of health problems of developing countries. It is therefore imperative that research methodologies be more respondent to the principles of traditional medicine has, in many countries, there is greater prevalence and accessibility than modern medicine.

The results of the antimicrobial susceptibility tests were expressed in terms of minimum inhibitory concentration (MIC). The MIC of the crude extract was also determined and this ranged between 100µg/ml and 200µg/ml while that of the vancomycin control ranged between 31.25mg/ml to 1.0 mg/ml (Table 2). NCCLS guidelines define *Staphylococcus aureus* for which the MIC of Vancomycin is ≤4 µg/ml to be susceptible, while isolates for which the MIC is 4 to 16 µg/ml are intermediate and those for which the MIC is ≥32 µg/ml are resistant. Japan, however, considers some isolates for which the MIC is 8 µg/ml to be resistant; as a result, some isolates reported as resistant in Japan have been reclassified as intermediate in the United States. Although the MICs of *P. thonningii* extracts were higher than those of the control (vancomycin) [21] , one has to remember that the *P. thonningii* extract was a crude agent compared to these control. Ebi [22] reported the inhibition of bacterial growth by leaf extracts of *Alchornea cordifolia* against MRSA. Ebi found chloroform soluble extracts to be active against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* but demonstrated greater anti-staphylococcal and anti-pseudomonal activity in the fractions that were insoluble in chloroform. Okeke *et al*[23] (1999) also reported that leaf extracts from *Alchornea cordifolia* were active against Gram positives. The activity they reported was similar but slightly higher than ours with MICs ranging from 5 to 20mgml−1 but lower than that of George *et al* [24] with an MIC of >50mg/ml from *Cassia alata*. In contrast MICs as low as 0.5mg/ml against MRSA was recorded with purified components from *Vernonia amygdaline; vernolide and vernodalol* [25]. Iwalokun *et al* [26] found that aqeous extract of *Vernonia amygdalina* produce low activity against MRSA with MICs of 22-26mg/ml. Abu-Shanab *et al* [27] recorded lower MICs of 0.395-0.780mg/ml against MRSA, a much lower value than that of [28] who recorded 18.2-24mg/ml

CONCLUSION

This preliminary evaluation indicated that certain medicinal plants, especially *P. thonningii*, possessed significant activity against MRSA isolates. This activity may be indicative of the presence of metabolic toxins or broad spectrum antimicrobial compounds. *P. thonningii* is known to possess tannins, and the antimicrobial property of this substance is well known. The results presented in this report suggest that this plant extract should be analysed further, it might provide a new compound effective against multi-resistant *S. aureus* infections. Such simple and inexpensive alternatives to conventional treatment of bacterial infections may be worth of further rigorous investigation. It has already been reported that tea tree leaf extract gives results equivalent to conventional antibiotics in the therapy of impetigo contagiosa [29], while Bassett[30] have reported favourable results with tea-tree oil in the treatment of acne. It can be speculated that *P. thonningii* extract may be effective in the form of a nasal ointment or body wash, but much more experimental work is needed. Considering that the extract is in the

crude form, this observation has promise as a veritable of source of active pure antimicrobial compounds. Thus improvement on such extract by pharmaceutical industry to produce antimicrobial drug of natural source will go a long way in healthcare delivery. The minimun inhibitory concentration (MIC) of the plants yielded promising results that are worthy of note. *P. thonningii* crude and fractions had low MICs of 1.0-2mg/ml respectively for MRSA isolates. This suggests that they can be gainfully employed in the production of antibiotics, as low MICs mean that only a small quantity of the extract will be required to impair bacterial growth[31].

ACKNOWLEDGEMENT

The authors thank medical laboratory personnel from General Hospital, Minna in Niger State and National Hospital for providing specimens. Mal.Yusuf and Mal. Abbas of National institute for Pharmaceutical Research and Development, Idu, Abuja for confirming the identity of the isolates. Thanks to Mr Andrew for assisting me in the area of fractionation of the extract and Mrs G.E. Ugbabe ,a botanist of Herbarium Department of National Institute for Pharmaceutical Research and development (NIPRD), Idu, Abuja for identifying the plant species.

Table 1. Antibaterial effects of a crude extract and fractions RF(1-3)-RF7 isolated from a leaf hexane extract of *Piliostigma thonningii* . Average diameter of zones of inhibition in mm. Vancomycin (30µg) was used as positive control.

	Mean diameter(mm) of inhibition zones for MRSA isolates[a]																			
CRD/FR	SAY	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	B_1	B_2	B_3	C_1	C_2	D	E	F	G	H	I
CE	33	28	26	26	24	28	29	34	28	26.1	32.3	29	30	32	26.1	31.1	26	26.2	36.1	29
*RF(1-3)	16	16	22	18	16.6	15	18	19	16	16	15	18.2	16.2	14	15	18	20	16	19	16
RF4	17.2	13	18	18	15	17	17	25	18	13.6	19	22	17.2	16	16.4	16	18.9	15	17	19
RF5	16	16	16	19	15	16.6	20	22	19.4	15	24	24	19.1	14	16	15.8	22.2	17	20	18
RF6	17	16	16	18.8	15.8	15	18	25	15.7	16	15	15	16.4	15	16	16	20.5	17	17	19
RF7	17	16.2	16	18	18	16	18	21	17	16	15	15	17	14	16	16	19.9	17	16	19
Van	17.8	18.2	17	19	17.8	17	15.6	19	28	10.4	16	15.8	29.2	16.9	11.5	30	18.4	18.7	9.0	16.2

Key: [a] MRSA isolates and their origins A:1,2,3,4,5,6,7,8- Urine; B: 1,2,3- Ear swab; C: 1,2 -Sputum; D-Urethra swab; E- Endocervical swab; F- Stool; G- Eye swab; H-Wound swab; I- Semen, SAY: ATCC 12365-Reference strain, Van-Vancomycin(30µg) positive control, CE-Crude extract.

ISSN: 0975-8585

Table 2. Minimum Inhibitory Concentration of fractions on MRSA.

Tub No	Con/ml	80u	208	15	304	78	14u	46s	85	299	23	36
1	200ug/2mg	+	+	+	+	+	+	+	+	+	+	+
2	100ug/1mg	+	+	**	++	+	+	+	+	+	+	+
3	50ug/0.5mg	**	**	**	**	**	**	**	**	**	**	**
4	25ug/250ug	**	**	**	**	**	**	**	**	**	**	**
5	12.5ug/125ug	**	**	**	**	**	**	**	**	**	**	**
6	6.25ug/31.25ug	**	**+	**+	**	**	**	**	**	**	**	**
7	3.125ug/31.25ug	**+	**	**	**	**+	**+	**+	**+	**+	**+	**+
8	1.563ug/--	**	**	**	**	**	**	**	**	**	**	**
9	0.7813ug	**	**	**	**	**	**	**	**	**	**	**
10	0.3906ug	**	**	**	**	**	**	**	**	**	**	**
11	0.1953ug	**	**	**	**	**	**	**	**	**	**	**

Table 2 Cont'd

Tub No	Con/ml	36	377	138	131	98	49	6	C	53	FRACTION 1-3
1	200ug/2mg	+	+	+	+	+	+	+	+	+	+
2	100ug/1mg	+	++	**	+	+	+	+	+	++	+
3	50ug/0.5mg	**	**	**	**	**	**	**	**	**	**
4	25ug/250ug	**	**	**	**	**	**	**	**	**	**
5	12.5ug/125ug	**	**	**	**+	**	**	**	**	**	**
6	6.25ug/31.25ug	**	**	**	**	**	**	**	**+	**	**
7	3.125ug/31.25ug	**+	**	**+	**	**+	**+	**+	**	**	**
8	1.563ug/--	**	**	**	**	**	**	**	**	**	**
9	0.7813ug	**	**	**	**	**	**	**	**	**	**
10	0.3906ug	**	**	**	**	**	**	**	**	**	**
11	0.1953ug	**	**	**	**	**	**	**	**	**	**
12	= =	**C	O	N	T	R	O	L	S	**	

KEY

\+ There was activity against the microorganism.

** Very turbid

\+ Vancomycin Control

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