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IN VITRO INHIBITORY EFFECTS OF THE ETHANOL EXTRACT OF *Tetrapleura tetraptera* (Schum and Thonn.) Taub. AGAINST MULTIDRUG RESISTANT *Staphylococcus aureus*

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ABSTRACT. In this study, the antibacterial effect of ethanol extract of *Tetrapleura tetraptera* was investigated *in vitro* against methicillin-resistant *Staphylococcus aureus* by agar diffusion and macrobroth dilution methods. At the lowest concentration of 20 mg/ml of the ethanol extract, 100 µl produced inhibition zones that ranged between 06 and 15 ± 1.0 mm while the inhibition zones ranged between 16 ± 1.0 mm and 22 ± 1.0 mm when the isolates were tested with 100 µl of the highest concentration (100 mg/ml) of ethanol extract. The minimum inhibitory concentrations (MICs) of the ethanol extract were between 0.019 mg/ml and 5.0 mg/ml while its minimum bactericidal concentrations (MBCs) ranged between 0.078 and 10.0 mg/ml. Ten strains had their MICs less than 1.0 mg/ml while the remaining *S. aureus* strains had their MICs at concentrations ranging between 1.25 mg/ml and 5.0 mg/ml. The degree of antibacterial activity exhibited by the extract of *T. tetraptera* demonstrated that its herbal medicine could be as effective as modern medicine in treating diseases associated with the test pathogenic organism and justifying its traditional use in the treatment of bacterial infections.

Keywords: Antistaphylococcal activities, inhibitory concentrations, ethanol extract, *T. tetraptera.*

INTRODUCTION

Although its isolation from urine samples is repeatedly secondary to staphylococcal bacteremia arising in cases like endocarditis in certain patients, *Staphylococcus aureus* is an important pathogen associated with inpatients and community infections (MILLAR *et al.,* 2007). It colonises about 30% of human population (WERTHEIM *et al.,* 2005). It is frequently isolated from abscesses, whitlows, paronychia and infected eczema (ROTTER, 1999) and has been implicated in ascending urinary tract colonization and infection (MUDER *et al.,* 2006). Methicillin-resistant *S. aureus* (MRSA) is the most important healthcare associated infectious agent as a result of its presence in up to 20% of inpatients and 16% of healthcare workers and its ability to survive on surfaces for over 12 days (NIJSSEN *et al.,* 2005; HUANG *et al.,* 2006).

A "search and destroy" method, a difficult but intuitively sensible task to perform (PASTILA *et al.,* 2004; FARIA *et al.,* 2005) when used together in a concerted manner (VOSS, 2004), is a multipronged approach to keeping MRSA infection prevalence low.

To avoid establishment of infection, antibiotic prophylaxis has become the standard of care (DAROUICHE, 2003; BLOCK and STUBBS, 2005). However, the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections (MARCHESE *et al.,* 2001) have forced scientists into having considerable interest in the use of plant materials as an alternative method to control pathogenic microorganisms (AQIL *et al.,* 2005; CHOI *et al.,* 2010). Thus, about 20% of the plants found growing in the world have, therefore, been investigated for their pharmacological importance (SUFFREDINI *et al.,* 2004). Many studies showed that higher plants represent a potential source of potent antimicrobial and new bioactive compounds (NJUME *et al.,* 2011; OLAJUYIGBE and AFOLAYAN, 2012) and accredited the value of plants for medicinal purposes (ARIAS *et al.,* 2004; AYOKA *et al.,* 2008; PRUSTI *et al.,* 2008). In ethnomedicine, the medicinal plants are used in treating and preventing specific ailments and diseases (ANSELEM, 2004). While the advent of bacterial resistance and the occurrence of undesirable side effects of some antibiotics (SOBERON *et al.,* 2007) has become a global challenge, there is an increase in the demand for medicinal plants constituting a reservoir of new antimicrobial substances yet to be discovered (MAKANJUOLA *et al.,* 2010).

Tetrapleura tetraptera is a perennial plant which grows in the lowland forest along the Western Coast of Africa. It is a species of flowering plants in the pea family (Fabaceae) and a deciduous forest plant belonging to the Mimosoideae Sub-family (AKIN-IDOWU *et al.,* 2011). Ethnomedically, the leaves, fruit, bark and pod are used for medicinal purposes (STEENTOFT, 1988). The fruits are used in the management of jaundice, flatulence, fever (BOUQUET, 1971), convulsion, leprosy, inflammation and rheumatism (OJEWOLE and ADESINA, 1983) as well acting as antiparasitic, antidiabetic and anti-inflammatory agents (OJEWOLE and ADEWUNMI, 2004; OJEWOLE, 2005)· The dried fruits have a pleasant aroma (ALADESANMI, 2007) and are popularly used as a seasoning spice in Southern and Eastern Nigeria (OKWU, 2003). Pharmacologically, *T. tetraptera* has anti-inflammatory (ONDA *et al.,* 2017) and antioxidant activities (BADU, 2012) and possess chemical compounds such as triterpenoid glycoside, flavonoids and phenols (MAILLARD *et al.,* 1992). While ADERIBIGBE *et al.* (2007) indicated that the bark has neuropharmacological activity, LEKANA-DOUKI *et al.* (2011) reported that it has antiplasmodial activity. The leaves are used for the treatment of epilepsy (NWAIWU and AKAH, 1986). The aqueous fruit extract possesses hypoglycaemic property (OJEWOLE and ADEWUNMI, 2004). Although there are informations on the ethnopharmacological and nutritional value of *T. tetraptera* (IRONDI *et al.,* 2013; AKINTOLA *et al.,* 2015; ADESINA *et al.,* 2016), there is a dearth of scientific information on the therapeutic significance of this plant. This study, therefore, investigated the inhibitory effects of the ethanol stem bark extract of *T. tetraptera* against multidrug methicillin-resistant *S. aureus* from urine samples to justify its ethnomedicinal importance.

MATERIALS AND METHODS

Collection of plant material

The bark of *T. tetraptera* was collected in May 2016, from the plant growing within a rural settlement in Ogun State, Nigeria. The plant was authenticated ethnobotanically by Prof. O. Adedayo, Kwara State University, Kwara State, Nigeria while voucher specimen was being prepared.

Extract preparation

The bark sample was air-dried at room temperature, pulverized with a milling machine and extracted as described by OLAJUYIGBE and AFOLAYAN (2012). Briefly, 200 g of the pulverized sample was extracted with 1000 ml of ethanol for 72 h with shaking (Stuart Scientific Orbital Shaker, Staffordshire, UK). The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heldolph, city, Germany). The extraction was done for two more consecutive times. The crude extract collected was dried at room temperature to a constant weight. The extract was later dissolved in the extracting solvent to the required concentrations for bioassay analysis. The reconstituted extract solution was filtered through 0.45 µm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract in 10 ml of sterile nutrient broth before being incubated at 37 °C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

Test organisms and inocula preparation

In this study, eighteen clinical strains of *S. aureus* obtained from urine samples and two typed strains *S. aureus* (ATCC 6538 and NCT 6571) used as control were tested. Bacteriologically, each of the clinical strains of *S. aureus* was streaked on mannitol salt agar (MSA) and nutrient agar which were incubated overnight at 37 °C for 24–48 h (FORBES and SAHM, 2007). The bacterial colonies were subjected to Gram staining, microscopic appearance, colony morphology and biochemical tests such as tube coagulase test according to standard protocols (HOLT *et al.,* 1994; CHEESBROUGH, 2002, 2009). The inocula of the test *S. aureus* strains were prepared using the colony suspension method (EUCAST, 2000). Colonies picked from 24 h old cultures grown on nutrient agar were used to make suspensions of the test organisms in saline solution to give an optical density of approximately 0.1 at 600 nm. The suspensions were then diluted 1:100 by inoculating 9.9 ml of sterile nutrient broth with 100 µl of the bacterial suspension and thoroughly agitated before being used.

Determination of methicillin resistant Staphylococcus aureus

The susceptibility of *S. aureus* isolated from urine samples to different antibiotics including cefoxitin (30 µg) (Table 1) was determined by modified Kirby-Bauer disc diffusion method following CLSI guidelines (CLSI, 2013). The strains of *S. aureus* which were found to be resistant to cefoxitin were screened as MRSA. The methicillin susceptibility assay was determined by macrobroth dilution using oxacillin at concentrations ranging between 0.0156 and 128 µg/ml.

Antibacterial assay by agar diffusion method

For the initial determination of the antibacterial activity of the ethanol extract of *T. tetraptera*, the susceptibility screening of the test bacteria to the extract was determined by using the modified Kirby-Bauer diffusion technique (EUCAST, 2000) involving swabbing Mueller-Hinton agar (MHA) (Lab M Ltd, Quest Park, Lancashire, UK) plates with the resultant saline suspension of each adjusted strain of *S. aureus*. Wells were then bored into the agar medium with a heat sterilized 6 mm cork borer. The wells were later filled with 100 µl of 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml, and 100 mg/ml concentrations of the extract taking care not to allow spillage of the solutions onto the agar surface. An antibiotic disk

containing 200 µg of nitrofurantoin was included as a control. The culture plates were allowed to stand on the laboratory bench for 1 h for proper diffusion of the dispensed extract solutions before being incubated at 37 °C for 24 h. Wells in blank Mueller Hinton agar containing 5% ethanol representing the final concentration of the ethanol in the test plates without the extract served as negative control. The determinations were done in duplicates. After 24 h of incubation, the plates were examined for the presence of inhibition zones. While the diameters of the inhibition zones produced by each concentration of the extract was measured in millimetres (WIKLER, 2007), the break point with an inhibition zone of diameter ≥ 11 was chosen for bacterial susceptibility to the plant extracts (NYENJE and NDIP, 2011).

Macrobroth dilution for determining minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) defined as the lowest concentration of the extract which resulted in maintenance or reduction of inoculums' viability was determined by macrobroth tube dilution technique (KHAN *et al.,* 2007) for the *S. aureus* strains. Different concentrations ranging from 0.098 mg/ml to 20 mg/ml of the crude extract prepared by serial dilutions in double strength Mueller Hinton broth medium. A set of tubes was then inoculated with 100 µl of the appropriate strains. Two blank Mueller Hinton broth tubes, with and without bacterial inoculation, were used as the growth and sterility controls. The tubes were incubated at 37 °C for 24 h. After the incubation period, the tubes were observed for the MICs by checking the concentration of the first tube in the series of tubes that showed no visible trace of growth. The lowest concentration in the series with no visible growth after the incubation period was taken as the MICs.

Determination of minimum bactericidal concentrations (MBCs)aliquots

Since the clinical occurrences of tolerance usually necessitate bactericidal testing, the MBC was determined by sampling all the macroscopically clear tubes and the first turbid tube in the MIC series. Before being sampled, the tubes were gently mixed by flushing them with a sterile pipette, and a 100 µl aliquot was removed. Each aliquot was placed on antibiotic-free nutrient agar plate in a single streak down the centre of the plate in accordance with the method of SHANHOLTZER *et al.* (1984). The samples were allowed to be absorbed into the agar until the plate surface appeared dry (after 30 min). The aliquot was then spread over the plate by making a lawn of the bacterial culture with sterile cotton swab. In many studies on microbial susceptibility, this subculturing method has been found satisfactory in eliminating the problem of antimicrobial agent carryover from the 100 µl subcultured volume (MOODY *et al.*, 1987; FASCHING *et al.,* 1990). The growth and sterility controls were sampled in the same manner. The MBC determining plates were incubated for 24 h at 37 °C. After the incubation periods, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as the MBC values for this crude extract. This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation.

RESULTS AND DISCUSSION

In this study, strains of *S. aureus* with inhibition zone ≤21 to cefoxitin disc (30 µg) and having oxacillin MIC ≥4 µg/ml were regarded as methicillin resistant strains as presented in Table 1. The susceptibility of different strains of *S. aureus* using ethanol extract of *T. tetraptera* was determined by agar diffusion assay while the degree of the antibacterial

activity of the extract was determined by macrobroth dilution method. The results were presented in Table 2. The susceptibility of the isolates was concentration dependent. 100 µl of the different concentrations of the extract produced varied degree of inhibition zones. The diameters of inhibition zones decrease with decreases in the concentrations of ethanol extract. At the lowest concentration of 20 mg/ml of the ethanol extract, 100 µl produced inhibition zones that ranged between 6 and 15 ± 1.0 mm while the inhibition zones ranged between 16 ± 1.0 mm and 22 ± 1.0 mm when the isolates were tested with 100 µl of the highest concentration of ethanol extract. On the other hand, the nitrofurantoin antibiotic disk inhibited thirteen of the isolates with inhibition zones ranging between 20 to 37 ± 1.0 mm.

Table 1: Susceptibility of *Staphylococcus aureus* isolated from urine samples to different antibiotic disks.

	Inhibition zones produced by different antibiotic discs against strains of *S. aureus*								
Test *Staphylococcus* strains	Clx (5 µg)	Ery (5 µg)	Gen (10 µg)	Aug (30 µg)	Tet (10 µg)	Chl (10 µg)	Ofl (5 µg)	Cfx (30 µg)	Oxacillin (MIC) µg/ml
SA 1	0	28	0	0	0	0	0	0	8
SA 2	0	30	30	12	0	0	24	14	4
SA 3	0	30	32	0	0	11	25	12	4
SA 4	18	32	20	32	0	36	28	17	8
SA 5	0	32	23	10	0	0	26	17	4
SA 6	0	0	34	12	0	12	26	14	8
SA 7	0	29	26	11	0	0	27	11	16
SA 8	0	18	20	20	19	11	25	14	8
SA 9	8	21	24	0	0	16	31	17	16
SA 10	0	15	36	15	0	14	15	16	4
SA 11	17	28	16	22	15	25	30	20	4
SA 12	0	15	36	15	0	14	15	16	8
SA 13	30	0	20	30	0	20	0	20	4
SA 14	0	28	35	0	0	0	27	15	16
SA 15	0	29	26	11	0	0	27	11	8
SA 16	0	18	20	20	19	11	25	14	4
SA 17	26	38	19	16	0	0	13	18	4
SA 18	0	30	32	0	0	11	25	12	8

Key: Clx = Cloxacillin; Ery = Erythromycin; Gen = Gentamicin; Aug = Augmentin; Tet = Tetracycline; Chl = Chloromphenicol; Ofl = Ofloxacin (5 µg); Cfx = Cefoxitin.

The degree of the antibacterial activity of *T. tetrapleura* against the isolated *S. aureus* as determined by the macrobroth dilution assay is presented in Table 3. The MICs of the ethanol extract were between 0.019 mg/ml and 5.0 mg/ml while its MBCs ranged between 0.039 and 10.0 mg/ml. Of the ten isolates that had their MICs at concentrations less than 1.0 mg/ml, seven had their MICs at concentrations 0.019 mg/ml, one strain had a MIC of 0.039 mg/ml, two strains had MIC of 0.325 mg/ml while the remaining *S. aureus* strains had their MICs at concentrations ranging between 1.25 mg/ml and 5.0 mg/ml.

In this study, the degree of the antibacterial activity of the ethanol stem bark extract of *T. tetraptera* was very high. The extract was able to effectively inhibit 50% of the test organisms at concentrations less than 1000 µg/ml while the isolates inhibited at

concentrations less than 1000 µg/ml had their MICs at concentrations ranging between 0.019 and 0.3125 mg/ml. ELOFF (2008) and KUETE (2010) classified the antimicrobial activity of plant extracts and fractions to be significant if the MIC is 0.1 mg/ml or lower, moderate if 0.1 < MIC ≤ 0.625 mg/ml and weak if MIC > 0.625 mg/ml. Based on these criteria, the ethanol extract had significant antibacterial activity against eight strains of *S. aureus* with MICs of 0.019 mg/ml, moderate activity against two strains with MICs of 0.3125 mg/ml while it was weak against the rest of the strains having MICs ranging from 1.25 and 5 mg/ml. Although previous studies have focused on the antibacterial activities of the fruit extracts of this plant (ACHI, 2006; AWOFISAYO *et al.,* 2010), this study indicated the antibacterial activity of its stem bark extract against human bacterial pathogen.

Table 2: Antistaphylococcal effects of ethanol extract of *Tetrapleura tetraptera* by agar diffusion assay.

	Inhibition zones produced by 100 µl of different concentrations of ethanol extract (± 1.0 mm)					**Nitrofurantoin**
Test *Staphylococcus* strains	**100**	**80**	**60**	**40**	**20**	**30**
	mg/ml					**µg/ml**
SA 1	22 ± 1.0	20 ± 1.0	18 ± 1.0	16 ± 1.0	15 ± 1.0	20 ± 1.0
SA 2	20 ± 1.0	18 ± 1.0	16 ± 1.0	15 ± 1.0	13 ± 1.0	06 ± 1.0
SA 3	17 ± 1.0	16 ± 1.0	14 ± 1.0	14 ± 1.0	06 ± 1.0	20 ± 1.0
SA 4	19 ± 1.0	15 ± 1.0	16 ± 1.0	15 ± 1.0	15 ± 1.0	06 ± 1.0
SA 5	20 ± 1.0	18 ± 1.0	16 ± 1.0	15 ± 1.0	15 ± 1.0	28 ± 1.0
SA 6	17 ± 1.0	15 ± 1.0	13 ± 1.0	14 ± 1.0	13 ± 1.0	06 ± 1.0
SA 7	18 ± 1.0	15 ± 1.0	14 ± 1.0	14 ± 1.0	12 ± 1.0	06 ± 1.0
SA 8	16 ± 1.0	15 ± 1.0	15 ± 1.0	14 ± 1.0	14 ± 1.0	06 ± 1.0
SA 9	18 ± 1.0	16 ± 1.0	15 ± 1.0	15 ± 1.0	14 ± 1.0	06 ± 1.0
SA 10	20 ± 1.0	17 ± 1.0	14 ± 1.0	15 ± 1.0	15 ± 1.0	33 ± 1.0
SA 11	17 ± 1.0	16 ± 1.0	15 ± 1.0	15 ± 1.0	12 ± 1.0	25 ± 1.0
SA 12	17 ± 1.0	16 ± 1.0	15 ± 1.0	15 ± 1.0	15 ± 1.0	30 ± 1.0
SA 13	19 ± 1.0	16 ± 1.0	14 ± 1.0	14 ± 1.0	13 ± 1.0	26 ± 1.0
SA 14	19 ± 1.0	17 ± 1.0	15 ± 1.0	12 ± 1.0	12 ± 1.0	23 ± 1.0
SA 15	20 ± 1.0	17 ± 1.0	14 ± 1.0	15 ± 1.0	14 ± 1.0	06 ± 1.0
SA 16	22 ± 1.0	19 ± 1.0	17 ± 1.0	16 ± 1.0	15 ± 1.0	35 ± 1.0
SA 17	19 ± 1.0	18 ± 1.0	15 ± 1.0	15 ± 1.0	13 ± 1.0	37 ± 1.0
SA 18	20 ± 1.0	18 ± 1.0	17 ± 1.0	17 ± 1.0	15 ± 1.0	30 ± 1.0
SA 19 ATCC 6538	20 ± 1.0	18 ± 1.0	16 ± 1.0	14 ± 1.0	14 ± 1.0	25 ± 1.0
SA 20 NCT 6571	21 ± 1.0	19 ± 1.0	18 ± 1.0	16 ± 1.0	13 ± 1.0	30 ± 1.0

Key: SA = Different strains of *S. aureus.*

The antibacterial activity of the ethanol extract may be attributed to the presence of many pharmacologically bioactive compounds such as alkaloids, flavonoids, tannins, anthraquinones and phenolic compounds which have been previously associated with the antibacterial activity of many plants (EDEOGA *et al.,* 2005; NAWROT *et al.,* 2007). To have attained such degree of antibacterial activity, the extract may have possessed certain membrane active compounds capable of disrupting the function and permeability of biological membranes (ABEL *et al.,* 2002; RAMACHANDRAN *et al.,* 2004). While the extent of adsorption onto the membranes due to surface activity has been correlated with the damaging effects likely to be produced (ATTWOOD and FLORENCE, 1983), the degree of the susceptibility of strains of *S. aureus,* used in this study, is not surprising. Being Gram positive cocci, the

cytoplasmic membrane with plenty of pores allowed inflow of foreign molecules without any difficulty (LIN *et al.,* 2003; GOUDA, 2008). Although, with the exception of SA15 that had MIC of 0.019 mg/ml, all the isolates resistant to nitrofurantoin had MICs equal to 5.0 mg/ml of the extract. The difference in the susceptibility of these isolates to the extract and antibiotic used as control may be due to the incomparable differences in the crudeness of the extract and the degree of purity of the nitrofurantoin as well as stearic hindrance preventing the extract from diffusing easily through the agar gel.

Table 3: Minimum inhibitory and minimum bactericidal concentrations of ethanol extract of *Tetrapleura tetraptera* against different strains of *Staphylococcus aureus.*

ORGANISMS	**Ethanol extract**	
Test *Staphylococcus* strains	**MIC**	**MBC**
	mg/ml	
SA 1	0.3125	1.25
SA 2	2.5	5.0
SA 3	0.039	0.078
SA 4	5.0	10.0
SA 5	0.019	0.078
SA 6	5.0	10.0
SA 7	2.5	5.0
SA 8	5.0	5.0
SA 9	5.0	5.0
SA 10	2.5	2.5
SA 11	0.019	0.078
SA 12	1.25	5.0
SA 13	0.019	0.039
SA 14	0.019	0.156
SA 15	0.019	0.039
SA 16	0.3125	0.625
SA 17	0.019	0.156
SA 18	0.019	0.078
SA 19 ATCC 6538	5.0	5.0
SA 20 NCT 6571	5.0	5.0

Key: SA = Different strains of *S. aureus*

CONCLUSION

In conclusion, the degree of antibacterial activity exhibited by the extract of *Tetrapleura tetraptera* demonstrated that its herbal medicine could be as effective as modern medicine in treating diseases associated with the test pathogenic organism and justifying its traditional use in the treatment of bacterial infections. Since all herbal medicines and botanical drugs will have to fulfil the international requirements on quality, safety and efficacy, further study, which is ongoing in our laboratory, should be carried out on its toxicity in order to know its safety and toxicity profile and establish a safe dosage regimen since the concoction is taken orally by local people for treating gastrointestinal infections as well as isolate and purify novel, effective and inexpensive drugs of great importance.

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