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Phytochemistry, antibacterial and anticoagulase activities of *Sida acuta* against clinical isolates of *Staphylococcus aureus*

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Abstract : The phytochemical, antibacterial and anticoagulase activities of *Sida acuta* against clinical isolates of *Staphylococcus aureus* were investigated. The phytochemistry of the plant leaves revealed that *S. acuta* is laden with antioxidative compounds with remarkable concentrations of saponins (0.772 mg/100g), flavonoids (0.112 mg/100g), alkaloids (0.076 mg/100g) and tannins (0.0541mg/100g). Their presence conferred a strong bactericidal effect on *Staphylococcus aureus* SS-1VC, *Staph. aureus* SS-2VM, *Staph. aureus* SS-3SW, *Staph aureus* SS-4OM, *Staph. aureus* SS-5BC *Staph. aureus* SS-6AF and *Staph. aureus* SS-7DS isolated from vaginal candidiosis, vaginal mycosis, septic wound, otitis media, buccal cavity, athletes foot and diarrheic stool respectively. Majority of the bacterium strains screened were sensitive to aqueous and methanol extracts of *S. acuta* leaves. All the strains were inhibited by the aqueous extract, but more susceptible were strains SS-2VM isolated from vaginal mycoses and SS-3SW from septic wound, which recorded 25mm and 24mm diameter of inhibition zones respectively, after treatment with 8.0mg/ml of the extract. However, much lower concentration (0.5mg/ml) of the extract was required to halt coagulase activity in both strains. The methanol leaf extract exhibited similar but stronger antibacterial and anticoagulase activities against the clinical isolates of *Staph. aureus*. Marked antibacterial inhibitory effects were observed against most strains tested but SS-6AF and SS-7DS (which exhibited comparatively lower susceptibility), with majority of the strains losing their anticoagulase producing potential at concentrations as low as 0.5mg/ml of alcoholic extract. The strong anticoagulase activity of *S.acuta*, and it's efficacy in inhibiting coagulase elaboration by *Staph aureus* especially *Staph. aureus* SS-3SW isolated from septic wound forms the basis of it's use in folk medicine for wound treatment.

Keywords: Phytochemistry, Antibacterial and Anticoagulase, *Sida acuta*,*Staphylococcus aureus*

INTRODUCTION

The usefulness of ethnobotanical surveys in drug discovery has been reviewed by many researchers (Benjamin, 1980; Sofowara, 1984; Odoemena *et al.*, 1998; Dhawan, 1997; Essien *et al.*, 1999; Ekpendu *et al.*, 2000 and Essien and Akpan, 2004). Naturally occurring plant metabolites are useful sources of new biocidal compounds and some cases have served as leads for new compounds that have been characterized (Emele *et al.*, 1997 and Essien and Akpan, 2004). However the search for new antimicrobial agents is a continuous exercise since target microorganisms often evolve new genetic variants (Agarwal *et al.*, 1980, Locksley *et al.*, 1982, Hughes and Datta, 1983 and Oyagade and Oguntoyinbo, 1997).

Development of resistance to antimicrobial agents by staphylococci is of major concern primarily because they are frequently associated with hospital and community acquired infections (Agarwal e*t al.*, 1980, Iroegbu *et al.*, 1997 and Oyagade and Oguntoyinbo, 1997). *Staphylococcus aureus* is a ubiquitous microorganism. The young and debilitated individual are known to harbor this bacterium in their anterior nare (Ako-Nai *et al.*, 1991) and other parts of the body (Famurewa and Sonntag, 1982). *Staphylococcus aureus* is a normal flora of some parts of the body such as the skin and nasal passage. It produces pustules, carbuncles and boils. It is also a frequent cause of burns and wound sepsis (Bulanda, 1989; and Oyagade and Oguntoyinbo, 1997). The virulence factors produced by human strains of *Staph. aureus* include *a*-toxins, protein A and coagulase. Other factors include the *y*-toxins which damages the tissue cells by its action as a phospholipase and lipase which catalyses the hydrolysis of fats and oils in the sebaceous secretion and blood plasma (Henning *et al.*, 1979 and Oyagade and Oguntoyinbo, 1997). Apart from being associated with wound infections. *Staph. aureus* may contaminate food resulting in food poisoning which is a common cause of vomiting and diarrhea, and staphylococcal enterocolitis (Duguid *et al.*, 1987).

Although the resistance of *Staph. aureus* to some antibiotics have been reported (Ako-Nai *et al.*, 1991 and Oyagade *et al.*, 1997), the bacterium is sensitive to many antibiotics (Duguid *et al.*, 1987) and many traditional plant herbs and concoctions have been reported to have some

medicinal value against *Staph. aureus* associated ailments including septic wounds. Certain drug plants *Centella asiatica* and *Aloe vera* have been used for decades, both topically and internally, to enhance wound repair (MacKay and Miller,2003), and scientific studies are now beginning to validate efficacy and explore mechanisms of action for these drug plants. A typical example is the use of *Sida acuta* Burn F. (Malvaceae) as an astringent, cooling tonic and antipyretic herb. It is also a useful antimicrobial agent in nervous and urinary disorders, wound infection and diseases of the blood and bile (Peirce, 2000). However to the best of our knowledge a systematic study on the wound healing activity of *S. acuta* has not been undertaken and little is known about the efficacy or the extent of effectiveness of the plant leaf extracts against pathogenic microorganisms. In the present research we investigate the phytochemistry, antibacterial and anticoagulase activities of *Sida acuta* against clinical isolates of *Staph. aureus*.

MATERIALS AND METHODS

Phytochemistry of *Sida acuta* leaves : Preliminary phytochemical analysis of the petroleum ether extract of the plant leaves was carried out according to the methods outlined by Harborne (1984) and Evans (1998). Precisely, 500g of the fine sample powder was successfully Soxhlet extracted with petroleum ether at room temperature (28 ± 2°C). The extract was concentrated under pressure to yield 40g of petroleum ether-extract. Screening for alkaloids was carried out with Mayer's, Dragendroff's and Picric acid reagents. Cardiac glycosides were detected by the Keller-Killiani and Liberman's tests. The tannins were detected by the Ferric Chloride test, phlobatannins by hydrochloric acid test, saponins by the Frothing and Fehling's tests, flavonoids by Shinoda's test, sterol/triterpenes by sulphuric acid test and the anthraquinones by the Bontruger's test. The incidence of the antioxidants in the plant leaves was graded as being highly present (+++), moderately present (++), present in trace amount (+) and absent (-).

Quantitative measurements of total crude sapogenins were by gravimetric method of Brain *et al* (1968), Alkaloid was quantified by the Harborne (1984), tannin by Van-Burden and Robinson (1981) method and flavonoid by the method of Boham and Kocipai-Abyazan (1974), while the method of Obadoni and Ochuko (2001) was adopted for saponin determination.

Clinical isolates of *staphylococcus aureus* screened : *Staphylococcus aureus* SS-1VC originally isolated from vaginal candidiosis, *Staph. aureus* SS-2VM isolated from vaginal mycosis, *Staph. aureus* SS-3SW isolated from septic wound, *Staph aureus* SS-4OM isolated from otitis media, *Staph. aureus* SS-5BC isolated from buccal cavity, *Staph. aureus* SS-6AF isolated from athletes foot and *Staph. aureus* SS-7DS isolated from diarrheic stool were obtained from the diagnostic laboratory of the University of Uyo Health Centre, Nigeria.

The isolates were streaked on selective media of blood agar, mannitol salt agar and staphylococcus medium 110. The inoculated plates were incubated at 37°C for 24 hours and the emerging colonies were purified by repeated sub-culturing and stocked in nutrient agar slants. Thereafter, the isolates were characterized according to the methods described by Cowan (1985) and Holt *et al* (1994) to ascertain their identities.

Preparation of leaf extracts for antibacterial and anticoagulase assays : Fresh *Sida acuta* plants were collected from the wild sources in Uyo, Nigeria and authenticated by a plant taxonomist. The leaves were separated, sundried and powdered for easy extraction. Precisely, 75g of leaf powder was extracted with 700 ml of 95% methanol in a Soxhlet apparatus at 60-75°C. Extract was concentrated by evaporation using a rotary evaporator to obtain a pasty oily residue. The traditional extraction process in which 100g of the leaf powder (100g) was refluxed with 400ml of sterile double distilled water for 1 hour at 75°C (MacKay and Miller, 2003) was also adopted. It was cooled and then filtered. This was repeated in three trials. The extracts were pooled and evaporated using a lyophilizer to obtain 250mg/ml extract concentration.

Aliquot of the methanol extract was adjusted using sterile distilled water, to match the concentration derived from the aqueous extract. Serially diluted antibacterial of the extracts were prepared to obtain graded concentrations for the assays.

Antibacterial assay of the leaf extracts : Antibacterial assay of the plant extracts was by the agar-punch-tube method (Stokes,1975), a modification of the agar-diffusion method of Bauer *et al.* (1966) using Oxoid single sensitivity disc and Mueller-Hinton agar as the sensitivity test medium. The inoculum was standardized by streaking isolates of *Staph. aureus* from nutrient agar slants into mannitol salt agar and then incubated at 37°C for 24 hours. Distinct colonies of the emerging isolates were transferred into 5ml of nutrient broth and then incubated for 5 hours until its turbidity matched that of barium sulphate solution (Cheesbrough, 1985).

Using different concentrations of the methanol and aqueous extracts and by the agar-dilution method (Stokes, 1975) the antibacterial effect of graded concentration of extracts were determined for the 7 clinical strains of *Staph. aureus* screened. This procedure described by Cruickshank *et al.* (1982) has previously been used by Emele *et al.* (1997).

Anticoagulase assay of the leaf extracts : Coagulase test is based on the ability of *Staph. aureus* to produce a protein product called coagulase. The test is usually carried out to differentiate the pathogenic *Staph. aureus* from other strains or species of staphylococci. Coagulase causes plasma to clot by converting fibrinogen to fibrin. Bound coagulase (clumping factor) which converts fibrinogen directly to fibrin without requiring a coagulase reacting factor can be detected by the clumping of bacterial cells in the rapid slide techniques.

To determine the anticoagulase potential of the leaf extracts, 0.2ml of broth cultures of each isolate was added to 0.5ml of the extract solution and incubated in a water bath at 37°C. With known control, the test substrate was examined at intervals of 30 minutes for 4 hours (Cheesbrough, 1985 and Cowan, 1985). The effect of the different extracts concentrations on the coagulase activities of the *Staph. aureus* isolates was investigated using inhibited cultures and by the tube test.

For the tube test, free coagulase which converts fibrinogen to fibrin by activating a coagulase reacting factor present in plasma which is indicated by clotting was assayed. To achieve these three clean sterile test tubes were prepared; the first tube containing the inhibited test culture and medium (0.8ml of 24 hour old broth culture), the second tube which served as the positive control contained the uninhibited broth culture while the third tube which served as the negative control had sterile broth. With the aid of sterile pipette 0.2ml of plasma was transferred into each test tube, mixed gently and incubated at 37°C for 1 hour. Observations were made to distinguish positive coagulase which showed clotting of tube content. The time or duration of clotting was determined with the aid of a stop watch (Quartz mechanical stop watch).

RESULTS AND DISCUSSION

Staphylococcus aureus has been one of the most successful of pathogenic bacteria due to its possession of aggressive factors and enzymes that can hydrolyze a wide range of substances (Volk *et al.*, 1986). Among these factors is the production of coagulase, an enzyme-like protein that clots oxalated or citrated plasma in the presence of a factor contained in many sera. The serum factor reacts with coagulase to generate both esterase and clotting activities, in a manner similar to the activation of prothrombin to thrombin (Vassey and Mann, 1978). Coagulase may deposit fibrin on the surface of *Staphylococcus aureus* perhaps altering their ingestion or destruction by phagocytic cells (Volk *et al.*, 1986, Vassey and Mann, 1978). The present study has shown that both the aqueous and methanol leaf extracts of *Sida acuta* are laden with antioxidative compounds with remarkable concentrations of saponins (0.772 mg/100g), flavonoids (0.112 mg/100g), alkaloids (0.076 mg/100g) and tannins (0.0541mg/100g) (Table 1). These antioxidants have strong anticoagulase and antibacterial potentials. Its strong anticoagulase activity forms the basis for its use, and efficacy in inhibiting coagulase elaboration by the bacterium. However, the plant's anticoagulase activity varied with the bacterium strain and extracts concentration.

The aqueous extract (Table 2) delayed coagulase elaboration (plasma clotting within 30 minutes) by *Staph. aureus* strain SS-1VC isolated from vaginal cavity. Coagulase activity was noticed on the 32nd minute, precisely 29 minutes after the activity was noticed (within 3 minutes) in the untreated culture of the test strain which served as control. Similarly, the coagulase activity of *Staph. aureus* strains SS-5BC isolated from the buccal cavity and SS-6AF from athlete's foot were also delayed for 8 minutes and 14 minutes respectively at the highest concentration (8.0mg/ml) of extract applied. The delay in coagulase production by strains SS-1VC, SS-5BC and SS-6AF is an indication of the resistance of the isolates to the anticoagulase properties of the plant extract, and a pointer to the fact that the pathogens may not necessarily loss its virulence when treated with aqueous extract of *Sida acuta* despite their susceptibility to antibacterial properties of the plant. This implies that both attributes have different physiological determinants.

All the bacterial strains were inhibited by the aqueous extract, but more susceptible were strains SS-2VM isolated from vaginal mycoses and SS-3SW from septic wound, which recorded 25mm and 24mm diameter of inhibition zones respectively, after treatment with 8.0mg/ml of the extract. Much lower concentration (0.5mg/ml) of the extract was required to halt coagulase activity in both strains. Other isolates with disrupted coagulase activities were SS-4OM (isolated from otitis media) at 4.0mg/ml and SS-7DS (isolated from diarrheic stool) at 2.0mg/ml of extract concentrations. *Staphylococcus aureus* strains SS-6AF and SS-7DS were the most resistant isolates to the antibacterial components of the aqueous extract.

The methanol leaf extract (Table 3) exhibited similar but stronger anticoagulase and antibacterial activities against the clinical isolates of *Staph. aureus*. Marked antibacterial inhibitory effects were observed against most strains tested but SS-6AF and SS-7DS (which exhibited comparatively lower susceptibility), with majority of the strains losing their anticoagulase producing potential at concentrations as low as 0.5mg/ml of alcoholic extract. The stronger efficacy of the alcoholic extract against coagulase production and bacterial growth may be attributed to the more concentrated level of antioxidants in the methanol extract. This is suggestive that alcohol, as better solvent might have extracted more active components of the plant leaves than water. However in folk medicine *S. acuta* leaf extract are mostly applied in

aqueous extract or free juice forms. Our findings have shown that better results may be achieved if alcoholic extract of the plant leaf is applied or even if used in dried powdery form. Similar observations have previously been reported by Emele *et al* (1997). This stems from the fact that the aqueous extract though with appreciable anticoagulase activity has limited antibacterial activity against many infectious strains of *Staphylococcus aureus*.

Our observation is in agreement with previous report that correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggest their role in a particular disease (Bergdoll, 1970) generally show that different

Table 1. Physicochemistry of *Sida acuta* leaves.

Property	Test method	Observation	Remark	Concentration
Saponins	i. Frothing test	Persistent foam	+++	0.772mg/100g
	ii. Fehlings test	Brown ppt	++	
Tannins	i. Ferric chloride test	Green ppt	++	0.0541mg/100g
Polyphenols	i.Ferric chloride test	No ppt	-	
Alkaloids	i. Dragendroffs test	Red ppt	+++	0.076mg/100g
	ii. Mayer's test			
Flavonoids	i. Shinoda's test		+++	0.112mg/100g
Anthraquinones	i. Bontrugers test			
Sterol/triterpenes	i. Sulphuric acid test	Pink ppt	+	
Cardiac glycosides	i. Keller- Killiani test		+	
	ii. Liberman's test			
	iii. Picric reagent	Redish brown ppt	+	
Phlobatannins	Hydrochloric acid test	No ppt	-	

+++ = highly present, ++ = moderately present, + = present in trace amount, - = absent

Table 2. Anticoagulase (duration of clotting; mins) and antibacterial activity (diameter of zone of inhibition; mm) of aqueous leaf extract of *Sida acuta* against different strains of *Staphylococcus aureus.*

			Concentration					
Strain	**Test**	**Control**	**0.25mg/ml**	**0.5mg/ml**	**1.0mg/ml**	**2.0mg/ml**	**4.0mg/ml**	**8.0mg/ml**
SS-1VC	**CTT**	3mins	9mins	14mins	21mins	28mins	32mins	36mins
	ABT	0mm	5mm	7mm	14mm	16mm	17mm	19mm
SS-2VM	**CTT**	9mins	16mins	NC	NC	NC	NC	NC
	ABT	0mm	4mm	10mm	17mm	20mm	23mm	25mm
SS-3SW	**CTT**	3mins	6mins	NC	NC	NC	NC	NC
	ABT	0mm	7mm	9mm	14mm	18mm	21mm	24mm
SS-4OM	**CTT**	15mins	17mins	17mins	19mins	21mins	NC	NC
	ABT	0mm	3mm	6mm	9mm	10mm	14mm	17mm
SS-5BC	**CTT**	27mins	33mins	33mins	33mins	34mins	34mins	35mins
	ABT	0mm	5mm	6mm	9mm	11mm	16mm	18mm
SS-6AF	**CTT**	30mins	33mins	34mins	36mins	37mins	39mins	44mins
	ABT	0mm	3mm	5mm	8mm	12mm	16mm	17mm
SS-7DS	**CTT**	4mins	8mins	11mins	16mins	NC	NC	NC
	ABT	0mm	4mm	7mm	10mm	11mm	15mm	18mm

CTT= coagulase tube test, NC= no coagulase, ABT= antibacterial test (zone of inhibition)

Table 3. Anticoagulase(duration of clotting; mns) and antibacterial activity (diameter of zone of inhibition; mm) of methanolic leaf extract of *Sida acuta* against different strains of *Staphylococcus aureus*.

			Concentration					
Strain	**Test**	**Control**	**0.25mg/ml**	**0.5mg/ml**	**1mg/ml**	**2.0mg/ml**	**4.0 mg/ml**	**8.0mg/ml**
SS-1VC	**CTT**	3mins	9mins	14mins	40mins	NC	NC	NC
	ABT	0mm	9mm	14mm	19mm	22mm	25mm	27mm
SS-2VM	**CTT**	9mins	26mins	NC	NC	NC	NC	NC
	ABT	0mm	7mm	14mm	19mm	26mm	29mm	28mm
SS-3SW	**CTT**	3mins	6mins	NC	NC	NC	NC	NC
	ABT	0mm	9mm	13mm	19mm	26mm	29mm	29mm
SS-4OM	**CTT**	15mins	27mins	32mins	NC	NC	NC	NC
	ABT	0mm	6mm	9mm	11mm	13mm	16mm	19mm
SS-5BC	**CTT**	31mins	31mins	NC	NC	NC	NC	NC
	ABT	0mm	8mm	11mm	19mm	21mm	26mm	28mm
SS-6AF	**CTT**	33mins	23mins	32mins	NC	NC	NC	NC
	ABT	0mm	6mm	8mm	12mm	17mm	19mm	18mm
SS-7DS	**CTT**	4mins	28mins	NC	NC	NC	NC	NC
	ABT	0mm	7mm	9mm	12mm	14mm	17mm	19mm

CTT= coagulase tube test, NC= no coagulase; ABT= antibacterial test (zone of inhibition)

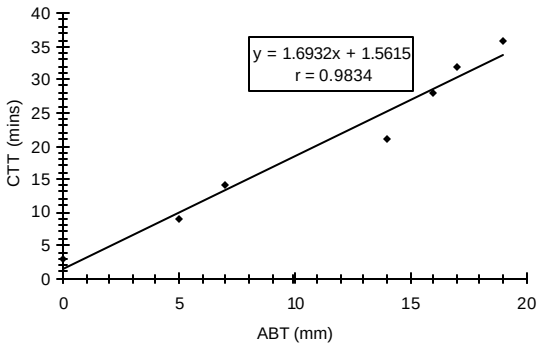

Fig. 1. Relationship between coagulase and antibacterial activities in *Staph. aureus* SS-1VC.

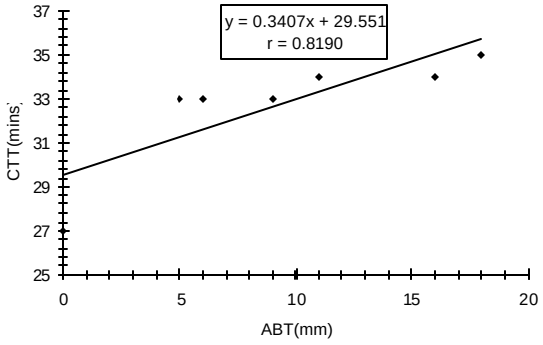

Fig. 2. Relationship between coagulase and antibacterial activities in *Staph. aureus* SS-5BC.

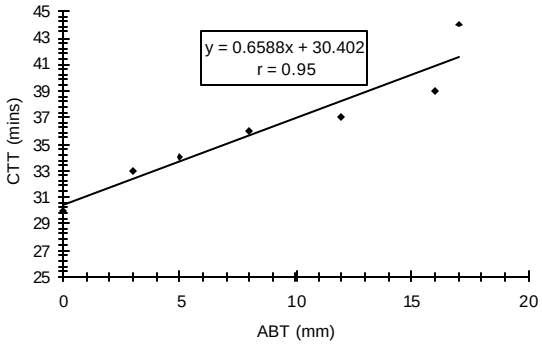

Fig. 3. Relationship between coagulase and antibacterial activities in *Staph. aureus* SS-6AF.

strains exhibit different levels of virulence. As a confirmation, analysis of the relationships between duration of clotting (coagulase activity) and zones of inhibition (antibacterial activity) in resistant strains SS-1VC (Fig. 1), SS-5BC (Fig. 2) and SS-6AF (Fig. 3) and *Staph. aureus* treated with the aqueous extract revealed significant positive relations between the two attributes. This implies that coagulase production is necessary for the growth of the etiological agent and that resistant strains of *Staph aureus* can effectively elaborate coagulase under inhibitory conditions. Therefore controlling the rate of either using *S. acuta* leaf extract may retard the pathogenicity of *Staph aureus*. However, halting both the coagulase and cells multiplication would enhance healing of wound sepsis caused by *Staph. aureus*.

Conclusion

Wound contraction is the process of mobilizing healthy skin surrounding the wound to cover the denuded area (Shanbhag *et al.*, 2006). The centripetal movement of the wound margin is believed to be due to activity of myofibroblast (Gabbaiani *et al.*, 1976). Like other wound healing botanicals such as *Kaempferia galanga*, the anticoagulase and antibacterial activities of *Sida acuta* against wound sepsis inducing strain of *Staph. aureus* would enhanced the contractile property of myofibroblast or increased the number of myofibroblasts recruited into the wound area. Our research have shown that its potential to enhance the property of myofibroblast via reduced anticoagulase and enhanced antibacterial activities of wound sepsis causing *Staph. aureus* varies with etiological strain, extract concentrations, nature of wound and the method in which the extracts were derived. Alcoholic extract exhibited stronger efficiency than aqueous extract usually used by traditional wound healers in folk medicine. These findings may reflect the different modes of action of the unrefined antioxidative components of the plant leaf.

In recent years oxidative stress has been implicated in a variety of degenerative process and diseases. These include acute and chronic inflammatory condition such as wound healing (Maiere and Chan, 2002). Wound healing plants are known to possess antioxidants especially flavonoids which are responsible for free radical scavenging activity required for wound healing (Devipriya and Shyamaladevi, 1999). Phytochemical screening revealed the presence of remarkable concentration of flavonoids in *Sida acuta*. This plus the presence of other antioxidants, such as saponins, tannins and alkaloids could be the reason for the strong anticoagulase and antibacterial potentials, and pro-healing activity of *S. acuta.*

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