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SUSCEPTIBILITY OF MULTI-DRUG RESISTANT STAPHYLOCOCCUS *AUREUS* TO COMBINED EXTRACTS OF *UVARIA CHAMAE, HOLARRHENA FLORIBUNDA* AND *SENNA OCCIDENTALIS*

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ABSTRACT

Multi-Drug Resistant *Staphylococcus aureus* (MRSA) is a serious clinical problem because of its ability to acquire resistance to most antibiotics which consequently reduce treatment successes. In the present study, efficacy of combined leaf extracts of *U. chamae, H. floribunda and S. occidentalis* against five multi-drug resistant *Staphylococcus aureus* isolated from wound swabs was evaluated. The antibiogram was determined by Kirby Bauer technique while in vitro antibacterial efficacy of the plant extracts evaluated using agar well diffusion. Preliminary phytochemical analysis of extracts revealed the presence of tannins, flavonoids, saponins, alkaloids, cardiac glycosides and terpenes. Combined leaf extracts of *U. chamae, H. floribunda and S. occidentalis* exhibited antibacterial activity against multi-drug resistant *Staphylococcus aureus* strains. The result showed that both aqueous and ethanolic extracts exhibited antibacterial activity against all *Staphylococcus aureus* strains. Inhibition was a direct function of concentration of extracts with almost 100% of organisms inhibited at all the concentrations used (400, 200, 100, 50, and 25mg/ml). The methanolic extracts were significantly more active than the aqueous extracts. Finding of this study suggest that the combined plant extracts can be alternative source of potent natural antibacterial agents for management of infections caused by multi-drug resistant *Staphylococcus aureus.*

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INTRODUCTION

Globally, medicinal plants serve as therapeutic resources specifically for primary health care and treatment of diverse illness. In developing nations, majority of population depend chiefly on the use of plant extracts for treatment of both infections and non-infectious diseases (WHO 1993; Chitme *et al*., 2003). Due to this, plants have therefore remain a raw material for over 50% of drugs used clinically and 11% of drugs considered as essential by WHO (Rates, 2001; Kowti *et al.,* 2010). Traditionally, plant such as *Uvaria chamae* commonly called Finger root or "Rukuki" in Hausa is found to promote rapid healing of wounds (Odeja *et al*., 2014).

In traditional settings, *Uvaria chamae* also found wide usage for treatment of yellow fevers, jaundice, breast cancer, tooth ache urinary tract infections, diarrhoea, wounds, coughs, renal and costal pains (Odugbemi, 2008; Chika *et al*., 2007). In Nigeria, *Holarrhena floribunda* is a common medicinal plant called false rubber tree or "Namijinsada" in Hausa, that have been reported to contained both antibacterial and antifungal activities (Yao *et al*., 2017). There is also therapeutic claimed for the treatment of skin infections, dysentery, diarrhoea, malaria, fever, skin infections and as diuretic to treat venereal diseases using *Holarrhena floribunda* (Bogne *et al*., 2007). Similarly, *Senna occidentalis* often referred to as Coffee senna

and "Rairai" in Hausa is reported to possessed antibacterial efficacy against infectious diseases caused by *Staphylococcus aureus* and *Escherichia coli* (Odeja *et al*., 2014). Drug resistance pattern currently presents an ever increasing global health threat that involves all major microbial pathogens and antimicrobial drugs (Olayinka *et al.,* 2009). The appearance of new antibiotic-resistant bacteria requires the development of new alternative treatments (Willer *et al.,* 2013).

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical infections due to increased prevalence of *S. aureus* colonization and its propensity to form biofilms, a difficult-to-treat infections (Chanda and Baravalia, 2010). Over time, with the introduction of methicillin, worldwide epidemic of methicillin-resistant *Staphylococcus aureus* (MRSA) emerged. From all antibiotics resistant microbes, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most persistent and a major cause of infections (Adwan and Mhanna, 2008). This has not only been responsible for the rapid shift in epidemiology, but also propels researches into effective approaches to overcome bacterial resistance. One approach to overcome the bacterial resistance is the evaluation of anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity and synergy of some bioactive plant extracts or synthetic agents (Sumitra and Kalpna, 2013). Therefore, this study determined efficacy of the combined extracts of *Uvaria chamae, Holarrhena floribunda, Senna occidentalis* against multi-drug resistant *Staphylococcus aureus* from wounds.

MATERIALS AND METHODS

Plant material and sample preparation

Fresh leaves of *Uvaria chamae, Holarrhena floribunda* and *Senna occidntalis* were all collected in the month of October 2016 at Mista Ali, Plateau Nigeria. The leaves were identified at the herbarium unit of the Federal College of Forestry, Jos Nigeria. The plants leaves were cleaned with tap water and air dried at room condition under shade for a period of 4 weeks until a constant weight was obtained. Each of the dried leaves was pulverized into fine powder using cleaned laboratory mortar and pestle. The pulverized leaves were then sieved using a mesh of 26µm pore size and stored in air tight plastic container at 4^{o}C until used.

Test Organisms

Five clinical strains of *Staphylococcus aureus* previously isolated from wound swab were kindly provided by Biobank of bacteriological laboratory of National Veterinary Research Institute Vom Nigeria. The isolates collected were sub-cultured and re-identified using conventional bacteriological methods.

Antibiotics

The antibiotic discs (erythromycin (15µg), gentamicin (10µg), clindamycin (10µg), Vancomycin (30µg), Linzolid (30µg), Cloxacillin (5µg), Levofloxacin (5µg), Azithromycin (15µg) (Sigma Chemical Co. St. Louis, M0, USA) were used.

Standardization of Inoculum

A 10ml physiological saline was dispensed in test tubes and a loopful of each isolate was then inoculated and incubated at 37^{0}C for 24h. Concentration of the stock was adjusted using 0.5MacFarland turbidity standard that provides an optical density comparable to bacterial suspension 1.5x 10^{8} (CFU/ml).

Determination of Multi-Drug Resistant *Staphylococcus aureus*

All the confirmed isolates of *Staphylococcus aureus* were tested for their resistance to common antibiotics using the Disc Diffusion Method (Bauer and Tittel, 1996). Standardized cultures of 0.5McFarland obtained were then inoculated on Muller Hinton agar using the spread method. Using a sterile forcet, the antibiotic discs were placed diagonally to each other and incubated at 37^{0}C for 24h. Zones of inhibition formed were then measured to the nearest millimetre (mm) and antibiotic susceptibility determined according to CLSI (2006). Multidrug resistance was taken as resistance to at least 4 first line antibiotics (Salman *et al.,* 2005).

Extraction of Plant Materials

The extraction was done using the maceration method described by Akinyemi *et al* (2005). A total of 200g of pulverized plant leaves were each weighed (using GR Series Semi-Micro analytical balance) and soaked in 1.5L of distilled water for aqueous extract and 1.5L of methanol for methanol extract respectively. The mixture was then left to stand for 48h with intermittent stirring at 12h interval for 48h. The mixture was then filtered through muslin cloth to remove coarse particles and then further filtered with Whatman (No 1) filter paper. The resultant filtrate was evaporated to dryness in a carbonite oven at 45^{0}C and the residue stored in plastic sterile sample bottles at 4^{0}C until used.

Phytochemical Analysis

Qualitative phytochemical analysis of the plant extracts was carried out following the methods of Evans (1989).

Preparation of Stock Concentrations

Stock concentration of plant extracts was prepared by reconstituting 8g of the extracts in10ml of sterile distilled water and 10ml of 20% Dimethyl sulphoxide (DMSO) for aqueous and methanolic extracts respectively. Thus, 800mg/ml of stock was obtained as a standard concentration of for both aqueous and methanolic extracts (Aneja, 2005). The stock concentrations of combined extracts was prepared using ratio of 1:1 by dissolving 5ml each with intermittent manually agitation for 1h. Thereafter, five extract concentrations of 400mg/ml, 200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml were prepared using 2-fold dilution (Aneja, 2005).

Antibacterial Activity Assay of Extracts

The agar well diffusion method of Bauer and Tittel (1996) was adopted. Six wells equidistance to each other were made using a 6mm sterile cork borer on solidified Muller Hinton agar plate already seeded with the standardized resistant strains of *Staphylococcus aureus*. This was then followed with introduction of 0.1 ml aliquot of each concentration of the combined extracts respectively into the wells using 20% DMSO as control. The plates were allowed to stand for one hour for pre-diffusion of the extracts to occur and then incubated at 37^{o}C for 24 hours.

Zones of inhibition formed were measured in millimetre, halos equal to or greater than 7 mm were considered susceptible to tested extract (Nascimento *et al.,* 2000).

RESULTS

Trend in the resistance pattern of *Staphylococcus aureus* strains to Gentamicin (20%), Linezolid (40%), Levofloxacin (40%), Azithromycin (60%), Erythromycin (60%), Vancomycin (80%) Clindamycin (80%) and Cloxacilin (100%) a representative methicillin group is presented in Table 1.

The results revealed the presence of alkaloids, tannins, saponin, resins and anthraquinones as predominant secondary metabolites of the plants. Finding of this study revealed that cardiac glycoside, terpenes and steroids were absent in both aqueous and methanolic extracts of *U. Chamae.* Similarly, flavonoid was not detected in both aqueous and methonolic leaf extracts of *Uveria chamae* and *S. Occidalis.* Antibacterial efficacy of the combined leaf extracts of *Uveria chamae, Senna occidentalis* and *Holarrhena floribunda* both for aqueous and methonolic extraction is presented in Table 3. The mean inhibition zone diameter ranged from 10 to 22mm for ethanolic extracts, while lower mean values of 0.0 to 16mm in the aqueous extract.

Table 1: Antibiotic Resistance Profile of *Staphylococcus aureus* isolated from Wounds

Antibiotic	SA1	SAII	SAIII	SAIV	SAV	Total %Resistance	CLSI Interpretation
Gentamicin (10µg,)	14(I)	0(R)	20(S)	15(S)	18(S)	20	S; ≥ 15, R;≤ 12, I; 13-14
Linezolid(30µg)	20(I)	0(R)	22(S)	22(S)	12(R)	40	S; ≥ 21, R; ≤ 20,
Cloxacilin(5µg)	12(R)	0(R)	18(R)	24(R)	14(R)	100	S; ≥ 18, R; ≤ 18
Azithromycin(15µg)	10(R)	0(R)	14(I)	18(S)	10(R)	60	S; ≥ 18, R; ≤ 13, I; 14-17
Clindamycin(2µg)	18(I)	0(R)	12(R)	12(R)	8(R)	80	S; ≥ 21, R; ≤ 14, I; 15-20
Erythromycin(15µg)	20(I)	0(R)	10(R)	10(R)	20(S)	60	S; ≥ 23, R; ≤ 13, I; 14-22
Levofloxacin(5µg)	22(S)	0(R)	8(R)	20(S)	22(S)	40	S; ≥ 19, R; ≤ 15, I; 16-18
Vancomycin(30µg)	12(R)	0(R)	18(S)	12(R)	18(S)	60	S; ≥ 17, R; ≤ 14, I; 15-16
Total %resistance	37.5	100	50	50	50		

Key: S = Susceptible, R= Resistant, I = Intermediate, SAI = *S. aureus* isolate I, SAII = *S. aureus* isolate II, SAIII = *S. aureus* isolate III, SAIV = *S. aureus* isolate IV, SAV = *S. aureus* Isolate V.

Table 2: Phytochemical Constituents of *Uvariachamae, Holarrhena floribunda,* and *Senna occidentalis* Leaf Extracts

Constituents	*U. chamae*		*S. occidalis*		*H. floribunda*	
	ME	AE	ME	AE	ME	AE
Saponin	+	+	+	+	+	+
Tannin	+	+	+	+	+	+
Flavonoid	-	-	-	-	-	+
Resin	+	+	+	+	+	+
Alkakloids	+	+	+	+	+	+
Glycoside	+	+	+	+	+	+
Anthraquinones	+	+	+	+	+	+
Cardiacglycosides	-	-	+	-	+	+
Terpenes	-	-	+	-	+	+
Steroids	-	-	+	-	+	+

Key:+ = Present, - = Absent, ME = Methanol, AE = aqueous.

Table 3: Antibacterial efficacy of Combined *Uvariachamae, Holarrhena floribunda,* and *Senna occidentalis* Leaf Extracts

Isolates	Methanolic extract(mg/ml)						Aqueous extract (mg/ml)					
	Zone of Inhibition(mm)											
	400	200	100	50	25	C	400	200	100	50	25	C
SAI	22	20	18	14	12	14	16	14	13	10	8.0	14
SAII	22	18	14	12	10	0.0	16	14	12	11	10	0.0
SAIII	20	16	14	12	10	20	14	12	10	7.0	0.0	20
SAIV	22	18	16	14	13	15	16	14	12	10	8.0	15
SAV	20	18	16	14	12	18	14	13	11	8.0	0.0	18

Key: Control (C) = Gentamicin (10µg), S = Susceptible, R= Resistant, I = Intermediate, SAI = *S. aureus* isolate I, SAII = *S. aureus* isolate II, SAIII = *S. aureus* isolate III, SAIV = *S. aureus* isolate IV, SAV = *S. aureus* Isolate V.

In this study, strains of *Staphylococcus aureus* exhibited *variation in the* level of *resistance* to the antibiotics use. Among the *Staphylococcus aureus* isolates, the Cloxacilin (100%) resistance was found highest in all the isolates (88%) followed by Clindamycin (80%) and Gentamicin (20%) least. All the 5 isolates exhibited Multi Drug Resistant (MDR) to three or more antibiotics belonging to different classes. Preliminary phytochemical constituents of the crude methanolic and aqueous extracts of *Uveria chamae, Senna occidentalis* and *Holarrhena floribunda* is presented in Table 2.

Generally, finding of this study showed that the combined extracts exhibited activity against the *S. aureus* that increased with concentrations gradient. Comparatively, strains of *S. aureus* exhibited higher susceptibility with methanolic extracts than the aqueous extracts.

DISCUSSION

The phytochemical analysis of *U. Chamae, H. Floribunda* and *S. occidentalis* revealed the presence of flavonoids, saponins, alkaloids, tannins and terpenes, cardiac glycosides as major active secondary metabolites.

In this study, the extraction agents used serves as a main factor that influences variation and availability of phytochemical constituents. This result explained the fact that phytochemical compounds of the plant were more soluble in moderate polar organic solvent such as methanol. This is in agrees with previous established reports that these active compounds could be effectively extracted with aqueous methanol (Syahidah *et al*., 2017). These phytochemicals have been established to be frequently responsible for antimicrobial properties of most medicinal plants (Cowan, 1999; Aspidi *et al*., 2008). Phenolic compounds are although most diverse groups of secondary metabolites found in plants. This study revealed that flavonoids was least detected although it is widely occurring among the classes of polyphenols follow by tannins and lignins (D'Archivio *et al.,* 2007). In this study, high activity for the combined extracts of *U. chamae, H. floribunda and S. occidentalis* affirms synergistic interaction and additive effect of the individual extracts as previously reported (Chanda *et al.* 2010**).** Comparatively, methanolic extracts exhibited higher activity compared to aqueous extracts. This could be due to the reason that methanol is a better extraction solvent of plant active constituents. These results support the findings of Ogbulie *et al*. (2007) that the ethanol extracts of *Uvaria chamae* was more active against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Salmonella* Typhi. Sujan Ganapathy *et al*. (2008) similarly showed that ethanolic extracts of *Hollarrhena floribunda* have significant antibacterial effect against *Staphylococcus aureus* (ATCC-29737) strains. The activity of ethanolic extracts of leaves of *Holarrhena floribunda* against *Staphylococcus aureus* ATCC 29213 and clinical strain of *Staphylococcus aureus* (Hoekou *et al.,* 2017) also suggest its therapeutic potentials. An important finding of this study is the fact that combined extracts of *U. chamae, H. floribunda and S. occidentalis* have affirms consistently demonstrated activity against strains of *Staphylococcus aureus.* However, high activity of combined extracts of *U. chamae, H. floribunda and S. occidentalis* even against high Multi-Drug Resistant strains of *Staphylococcus aureus* expresses reasons for their used as traditional medicinal plants commonly in West Africa (Iwu, 1993; Anderson, 1996).

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