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Full Length Research Paper

In vitro activity of certain antimicrobial agents in combination with Augouardia letestii hexane extracts against methicillin-resistant Staphylococcus aureus

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The ability of methicillin-resistant Staphycoccus aureus to acquire resistance to most antibiotics is a worldwide concern that necessitated the study of the antimicrobial activity of the medicinal plant Augouardia lestestii alone and in combination with existing antibiotics. The minimum inhibitory concentration was determined by broth microdilution method and the evaluation of synergy was determined by the checkerboard dilution test. In this study, the antimicrobial activity of *A.* lestestii leaves, stem barks and roots were investigated against methicillin resistant *S.* aureus strains. The A. lestestii hexane fraction showed good antibacterial activity against all strains with minimum inhibitory concentration ranging from 125 to 250 µg/ml. Further, synergistic effects were observed for all antibiotics (ampicillin, norfloxacin, ciprofloxacin and erythromycin) except for nisin. The study also validated the traditional use of *A. l*estestii against infectious diseases.

Key words: Augouardia lestestii, antimicrobial activity, synergistic combinations, drug-resistance.

INTRODUCTION

Methicillin-resistant Staphycoccus aureus (MRSA) is a commensal organism that represents a worldwide threat by its ability to acquire resistance to most antibiotics (Aqil et al., 2006; Gibbons, 2004). This pathogen is associated with a variety of infectious diseases that bring the average mortality rate from 36 to over 50% (Baltch et al., 2007; Choi et al., 2010; Dancer, 2008). The use of natural products from plants alone or in combination with antimicrobial agents could be useful, particularly in developing countries where the availability of drugs is limited (Aqil et al., 2006; Miranda-Novales et al., 2006; Kastoris et al., 2010). Furthermore, MRSA strains are not only resistant to betalactam antibiotics but also to Flouroquinolones and other families of antibiotics (Aqil et al., 2006). Thus, the different antibiotics were chosen based on the ability of this Gram-positive organism to be resistant to them.

On the other hand, the medicinal plant *Augouardia letestii Pellegr* (Caesalpiniaceae) is an endemic tree, 20 m high growing in Ogooué Invindo, in Gabon. The leaves and barks are used by traditional healers for different ailments such as back pain or infectious diseases. This knowledge of traditional healers is to be taken into consideration as it has led to effective anti-malaria, anti cancer, antioxidative or antimicrobial activities (Adjuik et al., 2004; Mills et al., 2005; Hemaiswarya et al., 2008; Amoo et al., 2009). The success compelled the WHO to promote the development of traditional medicine (Akinyemi et al., 2005; Elujoba et al., 2005). The CH_3OH extract of *A. letestii* stem bars have been previously tested for

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Table 1. Minimum inhibition concentration (MIC) values of bacterial growth in the presence of MeOH extracts from *A. letestui*.

***S. aureus* strain**	**MIC (mg/ml)**			
	Leaf	**Stem**	**Root**	**Ampicillin**
ATCC 33591*	>0.5	0.5	0.25	0.5
ATCC 25923*	>0.5	0.5	0.25	0.00006

*American Type Culture Collection.

cytotoxicity and antileishmanial activity (Lamidi et al., 2005). However, to our knowledge, this is the first report investigating its leaves, stem barks and roots antimicrobial activities against MRSA.

In this in vitro study, we attempted to evaluate the possible antimicrobial interaction between hexane extract of *A. letestui* roots and certain antimicrobial drugs (ampicillin, norfloxacin, ciprofloxacin, erythromycin and nisin) against MRSA strains.

MATERIALS AND METHODS

Plant materials and extraction

The plant samples (leaves, barks and roots) were collected in January 2010 in the Lopé reserve at 250 m from the Pygmy village of Masseguelani Ramba (Ogooé Invindo, Gabon). The identification of the species was carried out at the National Herbarium of Gabon, where a voucher specimen is kept.

Fractions of 100 g dried plant material (leaves, stem barks and roots) were refluxed with MeOH for 3 h, three times. The MeOH extract (4.22 g) was then partitioned with organic solvents of different polarities to yield n-hexane (71 mg), EtOAc (1.28g), *n*-BuOH (1.82 g) and H_2O(0.32g) fractions, in sequence.

Bacterial strains and culture medium

For the S. aureus strains used in this study, clinical isolates (MRSA) were obtained from different patients at the Wonkwang University Hospital (Iksan, South Korea). S. aureus ATCC 33591 which is methicillin-resistant strain (American Type Culture Collection, Manassas, VA) was commercially purchased. Before use, all bacteria were stored in 30% glycerol and frozen at -70°C. The bacteria were cultured in Mueller–Hinton broth (MHB) and Mueller-Hinton agar (MHA) (Difco Laboratories, Baltimore, MD) and incubated at 37°C for 20 h.

Determination of the minimum inhibitory concentration (MIC)

Preparation of the microorganism inocula was done on 12-h broth cultures, and the suspensions were adjusted to a 0.5 McFarland standard turbidity. Susceptibility tests were carried out by the standard broth microdilution method as described by Clinical Laboratory Standards Institute (formerly the National Committee for Clinical laboratory Standards)with an inoculum of approximately 5 x 10^4 CFU/ml in MHB. The MHB was supplemented with serial ampicillin concentrations ranging from 0.00006 to 1 mg/ml, and *A. letestii* MeOH extract and fractions concentrations from 0.031 to 1 mg/ml. The other antimicrobials tested included norfloxacin, ciprofloxacin, erythromycin and nisin. The data were reported as MICs, the lowest concentration of commercially purchased antibiotics and *A. letestii* MeOH extract and fractions inhibiting visible growth after 24 h of incubation at 37°C. All antibiotics were purchased from Sigma chemical Co (St. Louis, Mo. USA).

Colorimetric assay using MTT test

As a result of the coloration obtained from plant extracts and fractions, a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of the presence of bacteria was used as previously described (Abate et al.,1998; Scheuber et al., 1983; Shi et al., 2008). Basically, a stock solution of MTT (Sigma) concentration of 5 mg/ml was prepared in phosphate buffer saline (PBS), and was kept at -70°C. A final concentration of 1 mg/ml of MTT was used in the assay. After 24 h of incubation, a 37°C, 20 µl of the yellow MTT was added to the 96 well microtitre plate (Nunc, 0.3 ml volume) and incubated for an additional 20 min. The presence of a blue color indicates the presence of bacteria.

The checkerboard dilution test

Evaluation of interactions between antimicrobial agents and plant extracts were assessed by the checkerboard test. The serial dilutions of the two different agents were mixed in cation-supplemented MHB. The inocula were prepared from colonies that had been grown on the MHA overnight. The final bacterial concentration after inoculation was 5 x 10^4 CFU/ml. The MIC was determined after 24 h of incubation at 37°C. Each experiment was repeated three times. The fractional inhibitory concentration (FIC) index was determined by the following formula:

FIC index = FIC A + FIC B

FIC index= [A] /MIC A+ [B]/MIC B

Where, [A] is the concentration of drug A, MIC A is its MIC, and FIC A is the FIC of drug A for the organism, while [B], MIC B and FIC B are defined in the same fashion for drug B. The FIC index thus obtained was interpreted as follows: <0.5, synergy; 0.5 to 0.75, partial synergy; 0.76 to 1.0, additive effect; >1.0 to 4.0, indifference; and >4.0, antagonism (Lim et al., 2007).

RESULTS

The preliminary screening of *A. letestii* (Table 1) showed that its MeOH extracts of roots as compared to the leaves and barks have a better antimicrobial activity with a MIC of 0.25 mg/ml. The barks also have a potential to be therapeutic with a MIC of 0.5 mg/ml.

Table 2. Antimicrobial activity of MeOH, hexane, EtOAc, n-BuOH and water extracts of *A. lestestii* roots against 12 MRSA isolates.

S. *aureus* strain	Class	Mec A gene	MIC (mg/ml)				
			MeOH	Hexane	EtOAc	n-But	H_2O
ATCC33591[a]	MRSA	+	0.25	0.125	0.25	0.25	0.5
ATCC25923[a]	MSSA	-	0.25	0.25	0.125	0.125	0.5
Clinical isolates							
DPS-1[b]	MRSA	+	0.25	0.25	0.25	0.25	0.5
DPS-2	MRSA	+	0.25	0.25	0.25	0.25	>0.5
DPS-3	MRSA	+	0.25	0.25	0.25	0.25	0.5
DPS-4	MRSA	+	0.25	0.25	0.25	0.25	0.5
DPS-5	MRSA	+	0.25	0.125	0.25	0.25	0.5
DPS-6	MRSA	+	0.25	0.125	0.25	0.25	0.5
DPS-7	MRSA	+	0.25	0.125	0.25	0.25	0.5
DPS-8	MRSA	+	0.25	0.25	0.25	0.25	0.5
DPS-9	MRSA	+	0.25	0.125	0.25	0.25	>500
DPS-10	MRSA	+	0.25	0.125	0.25	0.25	>0.5
DPS-11	MRSA	+	0.25	0.125	0.25	0.25	0.5
DPS-12	MRSA	+	0.25	0.25	0.25	0.25	0.5

[a] American Type Culture Collection; [b]DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.MeOH, methanol; EtOAc, ethanol; n- But, n, butane.

A. letestii roots were then fractioned into MeOH, *n*-hexane, EtOAc and H_2O. The extracts were used for MIC determination using the microdilution broth method. The results were recorded as MIC in Table 2. All the extracts showed good antimicrobial activity with MICs ranging from 0.125 to 0.5 mg/ml; the hexane fraction showed the best activity against some strains of MRSA. The MeOH, EtOAc and *n*-BuOH extract also showed good activity (0.25 mg/ml) against all strains of MRSA.

In order to determine if the hexane fraction of *A. letestii* roots was synergistic in combination with other existing antibiotics, four strains of MRSA (DPS) selected from four different patients together with the clinical isolate ATCC 33591 were tested against the selected antibiotics as shown in Table 3. The values obtained in terms of MICs confirmed the resistance of MRSA strains to these existing antibiotics with MICs ranging from 0.062 to 0.5 mg/ml for ampicillin, 0.25 to 1 mg/ml for norfloxacine and ciprofloxacine, 1 mg/ml and more for erythromycin and a constant value of 0.25 mg/ml for nisin.

All antibiotics showed either synergistic or partial synergistic effect in combination with hexane fraction of *A. lestestii* roots (Table 4). Nisin is synergistic only with one strain of *S. aureus* (DPS-1). Among the test antibiotics, norfloxacin combination is the most promising with a synergistic effect obtained with three of the five strains with FICI of 0.19, 0.13, 0.56, 0.31 and 0.75, respectively.

DISCUSSION

A. letestii as previously mentioned is used in Gabon by traditional healers for back pain and infectious diseases. Its antimicrobial activity (leaves, stem barks and roots) is mostly found in roots. These roots, still in their impure form present a good prospect even though natural products from plants are not very amenable to rapid high-throughput screening for desirable activity as drugs (Li and Vederas, 2009). Because of the good antimicrobial activity of *A. letestii*, we may suggest that it contains many different antimicrobial compounds. However, it does not mean that a single compound has a wide antimicrobial spectrum (Kastoris et al., 2010).

The water extract has a MIC value of 0.5 mg/ml for most strains and it can be considered relevant in clinical settings. The use of extracts in traditional medicine in Gabon is mostly done with water, however, solvents such as MeOH, hexane, EtOAC or BuOH are extracting more active compounds, increasing the ability of the plant to inhibit the activity of MRSA.

Our findings confirm the synergism found between plant extract and antibiotics (Aqil et al., 2010; Liu et al., 2000; Rosata et al., 2007; Wagner and Merzenich, 2009). Ampicillin, ciprofloxacin and erythrocycin in combination also showed good synergy as they are able to partly or completely suppress bacterial resistance mechanisms. The practice of multi-drug therapy worldwide and some *in*

Table 3. Antimicrobial activity of hexane fraction *of A. lestestii* roots, ampicillin, norfloxacin, ciprofloxacin, erythromycin and nisin against 4 MRSA isolates.

S. *aureus* strain	MIC (mg/ml)					
	HF[a]	AC[b]	NOR[c]	CIP[d]	ERY[e]	NI[f]
ATCC33591	0.125	0.5	0.5	0.5	1	0.25
Clinical isolates						
DPS-1[g]	0.25	0.25	0.25	0.25	1	0.25
DPS-2	0.25	0.062	1	1	>1	0.25
DPS-3	0.25	0.25	1	1	>1	0.25
DPS-4	0.25	0.062	0.25	0.25	>1	0.25

a = hexane fraction, b = ampicillin, c = norfloxacin, d = ciprofloxacin, e = erythromycin and f = nisin, [g]DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University.

Table 4. MICs (mg/ml) and FIC indexes of antibiotics in combination with hexane fraction of *A. letestii* roots against 5 MRSA isolates.

Parameter	ATC33591	DPS-1	DPS-2	DPS-3	DPS-4
HF/AC[a]	0.031/0.062	0.5/0.5	0.062/0.015	0.015/0.015	0.125/0.015
FIC	0.38	4	0.5	0.13	0.75
Outcome	Synergy	Indifferent	Synergy	Synergy	Partial synergy
HF/NOR[b]	0.015/0.031	0.015/0.031	0.125/0.062	0.015/0.062	0.062/0.125
FIC	0.19	0.13	0.56	0.31	0.75
Outcome	Synergy	Synergy	Partial synergy	Synergy	Partial synergy
HF/CIP[c]	15.63/31.25	15.63/31.25	15.63/15.63	31.25/62.5	125/250
FIC	0.19	0.13	0.75	0.38	1.5
Outcome	Synergy	Synergy	Partial Synergy	Synergy	Indifferent
HF/ERY[d]	0.062/0.062	0.015/0.062	0.25/0.062	0.062/0.031	0.25/0.062
FIC	0.56	0.13	1.06	0.28	1.06
Outcome	Partial synergy	Synergy	Indifferent	Synergy	Indifferent
HF/NI[e]	0.125/0.015	0.015/0.015	0.25/0.062	0.125/0.015	0.25/0.015
FIC	1.06	0.13	1.25	0.56	1.06
Outcome	Indifferent	Synergy	Indifferent	Partial Synergy	Indifferent

MIC for a = ampicillin plus hexane fraction, b = norfloxacin plus hexane fraction, c = ciproflocaxin plus hexane fraction, d = erythromycin plus hexane fraction and e = nisin plus hexane fraction.

vitro antimicrobial combinations studies have been undertaken to validate the role of synergism in phytotherapy (van Vuuren and Viljoen, 2008; Wagner and Merzenich, 2009). *A. letestii* has the potential to be used to minimize the spread of drug resistance. However, synergy of herbal drug combination does not represent 100% evidence for use in humans (Wagner and Merzenich, 2009). Some adverse effects in combined use of synthetic drugs have been reported (Wagner and Merzenich, 2009; Bailey, 1998; Strandell et al., 2004). While no antagonism was observed for any of the combinations, it cannot yet be confirmed if MICs in combination will be achieved therapeutically. From this present study, *A. letestii* can be seen as a new source of novel therapeutics; confirming its traditional use. One of the options to tackle the issue of drug resistance is the use of secondary metabolites from plants sources for combination therapy (Shi et al., 2008).

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