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Mangifera Indica Crude Leaf Extracts Inhibits Efflux Pump System in *Serratia marscencens*

[1]*B.T. Thomas,* [2]*A.A. Olanpeleke,* [3]*O.M. Lakunle and* [4]*A. Davies*

[1]Department of Cell Biology and Genetics, University of Lagos, Akoka, Lagos, Nigeria
[2]Department of Medical Laboratory Science, State Hospital Ijebu-Ode, Ogun State, Nigeria
[3]Department of Pharmacognosy, Faculty of Pharmacy,
Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria
[4]Department of Community Medicine and Primary care, Faculty of Clinical Sciences,
Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria

Abstract: This study evaluated the phytochemical constituents and the anti efflux pump activity of *Mangifera indica* Linn leaves against the strains of efflux positive *Serratia marscencens* isolated from grounded melon and some primary schools pupils in Ijebu North Local government area of Ogun State, Nigeria. The methanolic and aqueous extracts of the tested leaves revealed the presence of tannins, flavonoids, glycosides and saponins while anthraquinones was detected only in the methanolic extract. Fourteen (14) and four (4) multidrug resistant *Serratia marscencens* were isolated from primary school pupils and grounded melon respectively. All the isolates from grounded melon showed hundred percent resistance to the tested antibiotics while the isolates recovered from the primary school pupils displays alarming rate of resistance to all the tested fluoroquinolones and beta lactam antibiotics. The level of resistance observed against gentamicin was relatively very low when compared to other tested antibiotics. The antibiotic resistance patterns were partially transferred from nine of the eighteen donor strains to the transconjugants while the MIC values of the antibiotics were reduced in the presence of the efflux pump inhibitor (CCCP). Also, *Mangifera indica* crude leaf extract caused between 4-fold and 7-fold decrease in the final MIC values relative to the standard drugs when incorporated into the MIC medium. The results of this study confirmed that *Mangifera indica* leaf inhibits antibiotic efflux pump system.

Key words: *Mangifera indica* Linn · Efflux pump · *Serratia marscencens*

INTRODUCTION

Multidrug efflux systems in bacteria are of particular concern for the treatment of patients with infectious diseases, since the substrates of many multidrug transporters include antimicrobials used for therapy [1,2]. Exposure to one substance that is a substrate of the efflux pump can favor its overexpression and the consequence may be cross-resistance to all other substrates which may include clinically relevant antimicrobials [3].

Although genes encoding efflux pumps may be present in plasmids, those found in chromosome are often related to the intrinsic resistance mechanisms and enable the bacteria to survive in hostile environments, as for instance in the presence of antimicrobials [3]. Multidrug transporters can therefore be associated to intrinsic and to acquired antimicrobial resistance [1, 3]. Acquired multidrug resistance can occur via three mechanisms viz; mutation and amplification of genes encoding multidrug transporters which alter their level of expression [4] or their activity level [5], mutation in specific genes or in global regulatory genes, resulting in an increased expression of multidrug transporters, as for example the mutation in *mex R* of *P. aeruginosa* OCR1 [5] and intercellular transfer of resistance genes, such as *qacE*, by plasmids or transposons.

Corresponding Author: Thomas Benjamin Thoha, Department of Cell Biology and Genetics,
University of Lagos, Akoka, Lagos, Nigeria.

The genus *Mangifera* originates from the Asia and is found greatly cultivated in Sumatra, Java, Borneo, Peninsula, Malay, India and Myanmar and practically in every tropical and sub tropical country with India having the largest area for its cultivation [6]. Other common species of this genus include *Mangifera gedebe, Mangifera minor* and *Mangifera mucronulata*. The tree is large and tall (up to 40 m) with a rounded canopy or foliage with leathery leaves and big fleshy edible drupes as fruit [7]. The fruits are eaten and used for juice and wine production. Traditionally, the mango plant has medicinal applications. In Côte d'Ivoire, the leaf-decoction is used as a febrifuge. The bark infusion has been used as gargle to treat mouth infections in children (Tonga). In India and Nigeria, the infusion of the leaves singly or in combination with leaves of *Citrus sinensis* is used in treating diarrhea, dysentery, gastrointestinal tract disorders, typhoid fever, sore throat and scurvy [8-10]. The infusion is also drunk as tea for treating stroke and as a relief from pains and exhaustion (India). The fruits have also been eaten with salt and honey as source of vitamin A and for the treatment of blood disorders. Infusion of the ground seeds have been used as remedy for diabetes. Sap from the leaves and unripe fruits have been eaten as colic and to treat irritations such as scorpion and bee stings. In view of the importance of *M. indica* in ethnobothany as health remedy, this study was aimed at determining the capability of *Mangifera indica* as anti efflux pump inhibitor in *Serratia marcescens*.

MATERIALS AND METHODS

Source of Plants: Fresh leaves of *Mangifera indica* was collected from Ilaro, Ogun State, Nigeria. The plant was authenticated by a senior plant taxonomist (Mr T.K. Odewo) at the Department of Botany, University of Lagos, Nigeria.

Preparation of Plant Materials and Extracts: Leaves were air-dried on the herbarium table (25 - 28°C) after which they were shredded and preserved in airtight cellophane bags. The shredded leaves were milled into powder form using a warring commercial blender. Fifty grams of each of the powdered plant materials was soaked in 200 ml of methanol and chloroform separately and stoppered. The flasks were manually agitated at intervals for 5 days. The resulting extracts from each flask was filtered rapidly through layers of gauge and then through Whatman No. 1 filter paper. The resulting filtrates were then concentrated by evaporation in a rotary evaporator. The yield of extracts obtained from methanol was 3.7 and 3.4 g from chloroform.

Phytochemical Studies: Phytochemical tests were carried out to determine the presence of flavonoids, tannins, alkaloids, saponins and anthraquinones using the methods described by Odebiyi and Sofowora [11].

Isolation and Identification of *Serratia marcescens*: One loopfull of suspension of grounded melon samples was plated on blood agar and MacConkeys agar, then incubated at 37°C for 24 h while the urine samples obtained from the primary school pupils in Ijebu North Local government areas were processed following standard procedures [12]. The identification was done using the API 20E kit.

Antimicrobial Susceptibility Profile of the Isolated Organisms: The antimicrobial susceptibility pattern of the isolates was determined using the National Committee for Clinical Laboratory Standard (NCCLS) modified disc diffusion technique as described in Cheesborough [12]. All the strains were tested for their sensitivity to the following antibiotics: Penicillin (5 ug), Ampicillin (25 ug), Gentamycin (10 ug), Ofloxacin, nalidixic acid and ciprofloxacin (2 ug) and Cloxacillin(5 ug) (All from Abtek, U.K.). The zones of inhibition were recorded and classified as "resistant", intermediate" or "sensitive" based on the interpretative chart updated according to the current NCCLS Standards. *S. aureus* NCTC 6571 was used a control for Gram positive organism while *Escherichia coli* ATCC 25922 was used as control for Gram negative organism.

Genetic Transfer: *Serratia marcescens* strains showing resistance to ≥ 1 antibiotic were selected (donor cells) and examined for their ability to transfer the resistance. Conjugation was performed using the strain of *E.coli* J53 (as recipient cell) [13]. Aliquots of overnight cultures of donor and recipient organisms were mixed in a final volume of 4ml Luria-Bertoni medium. The mixture was incubated at 37°C for 2h. Ten-fold serial dilutions of conjugation mixture were made and 0.1ml of each dilution was spread on the agar surface of Mueller Hinton agar supplemented with ampicillin (100µg/ml and sodium azide 200µg/ml, for counting the total number of transconjugants.

Effect of Efflux Pump Inhibitor and *Mangifera indica* Leaf Extract on Minimum Inhibitory Concentration (MIC): To determine the extent of the efflux pump mediated resistance in *Serratia marscencens* isolates, MIC levels for the quinolones were determined using broth dilution method in the presence and in the absence of efflux pump inhibitor [CCCP (Sigma, USA)]. CCCP are proton motive force inhibitors. Stock solution of CCCP was prepared in DMSO to make a final concentrations of (1 mg/l). The concentration of *Mangifera indica* leaf extract used was 50mg/ml.

RESULTS

Results of the phytochemical analysis as summarized in Table 1 showed that both the methanolic and aqueous extracts revealed the presence of tannins, flavonoids, glycosides and saponins while alkaloids were not detected. Anthraquinones was detected only in the methanolic extract.

Table 2 depicts the distribution of *Serratia marscencens* isolated from primary school pupils in Ijebu North Local government area of Ogun State, Nigeria, as well those obtained from grounded melon. As shown in the table, fourteen (14) and four (4) multidrug resistant *Serratia marscencens* were isolated from primary school pupils and grounded melon respectively. All the isolates obtained from grounded melon showed hundred percent resistance to the tested antibiotics while the isolates recovered from the primary school pupils displays alarming rate of resistance to all the tested fluoroquinolones and Beta lactam antibiotics. The level of resistance observed against gentamicin was relatively very low when compared to other tested antibiotics. The antibiotic resistance pattern of the studied isolates are recorded in Table 3. Results obtained reveals that some of the resistance patterns were transferred genetically through conjugation (Table 4). On the determination of the efflux positive organisms, the minimum inhibitory concentration of the most resisted antibiotics (fluoroquinolones) against the *Serratia marscencens* isolates were determined both in the presence and in the absence of carbonyl cyanide m chloro phenyl hydrazone (CCCP) and it was found that the MIC values were decreased in the presence of CCCP (Table 5). The activity of *Mangifera indica* as shown by the MIC values disclosed between 4-fold and 7-fold decrease in the MIC values relative to the standard antibiotics.

Table 1: Phytochemical analysis of extracts of *Mangifera indica*

	Phytochemical constituents					
Extract	Tannins	Alkaloids	Saponin	Anthraquinones	Flavonoids	Glycosides
WATER	+	-	+	-	+	+
METHANOL	+	-	+	+	+	+

Table 2: Distribution of resistant *Serratia marscencens*

Sources	N	n	%
PSP	100	14	14
GM	83	4	4

PSP = Primary school pupils, GM = Grounded melon, N= Sample size

Table 3: Antibiotic resistance pattern of *Serratia marscencens* isolates

Antibiotic	PSP (14)	GM(4)
Penicillin	7(50)	4(100)
Ampicillin	5(35.7)	4(100)
Gentamicin	4(28.6)	4(100)
Ofloxacin	10(71.4)	4(100)
Nalidixic acid	10(100)	4(100)
Ciprofloxacin	14(100)	4(100)
Cloxacillin	11(78.6)	4(100)

Table 4: Antimicrobial resistance patterns of the studied *Serrratia marscencens*

	Antimicrobial Resistance Pattern	
Isolates designation	Original	Transferred
A21 P,	AMP,GN,OFL,NAL,CIP	P, OFL, NAL, CIP
L6	P, GN,OFL, NAL,CIP	-
B13	P,AMP, CX,OFL,NAL,CIP	-
A23	P, AMP, CX,OFL, NAL,CIP	-
A24	P, CX, OFL,NAL,CIP	-
B12	P, CX,OFL,NAL,CIP	P, OFL,NAL,CIP
C16	P,OFL,CIP,NAL	P, OFL,CIP,NAL
L13	P,OFL, NAL, CIP	P,OFL, NAL, CIP
C18	P, NAL, CIP, OFL	-
D12	P, CIP, OFL, NAL	-
E6	P, CIP, NAL, OFL	-
R8	P, CIP, NAL, OFL	P, CIP, NAL, OFL
AA1	P, CIP, OFL, NAL	P, CIP, OFL, NAL
AA2	P, CIP, GN, NAL,OFL	P, CIP, GN, NAL,OFL
BC3	P, OFL, CX,NAL,OFL	OFL, NAL
AD9	P,CIP,CX,NAL,OFL	NAL,CIP,OFL
AD4	P, CIP, GN, NAL, OFL	-
L63	P,GN, CX, NAL,OFL, CIP	-

Table 5. Antibacterial activity of *Mangifera indica* against Efflux positive *Serratia marscencens*

	MIC (mg/L) with/without CCCP						
	CIP		OFL		NAL		
Lab code	WITHOUT	WITH	WITHOUT	WITH	WITHOUT	WITH	MGI
A21	32	0.5	32	0.25	64	1	3.3
L6	64	1	32	1	64	0.25	1.65
B13	64	1	64	0.25	128	2	3.3
A23	16	0.06	32	2	128	1	0.8
A24	32	0.03	32	0.125	128	1	6.25
B12	32	0.25	32	0.03	128	1	3.3
C16	32	0.016	64	0.5	64	1	3.3
L13	32	0.016	64	0.5	64	1	3.3
C18	32	0.008	64	0.25	64	1	1.65
D12	32	0.008	16	0.5	64	0.5	1.65
E6	32	0.008	16	0.03	64	0.5	3.3
R8	8	0.06	64	0.016	64	1	1.65
AA1	16	0.06	64	0.008	128	1	0.8
AA2	16	0.03	32	0.06	128	4	1.65
BC3	32	1	32	0.03	64	0.25	1.65
AD9	32	1	32	0.03	64	0.25	1.65
AD4	16	0.008	32	0.016	64	1	0.8
L63	16	0.06	32	0.016	64	2	0.8

DISCUSSION AND CONCLUSION

The presence of *S. marcescens* has been linked to several episodes of bacteremia traced to infusion pumps [14-19] and a wide spectrum of infectious diseases, including urinary, respiratory, biliary tract infections, wound infections, intravenous catheter-related infections, septic arthritis, osteomyelitis, infective endocarditis and peritonitis [20-21]. The susceptibility rate of *S. marcescens* isolates to fluoroquinolone in this study was lower than expected. For instance, nalidixic acid and ofloxacin shows 28.6% of resistance while 100% resistance was observed to ciprofloxacin. This observation suggest a progression in the resistant rate of ciprofloxacin since the reporting of the Taiwan Surveillance of Antimicrobial Resistance study in 2000 [22, 23]. Sheng *et al*. [24] reported that the susceptibility rate of *S. marcescens* to ciprofloxacin decreased from 100% in 1985-1986 to 80% in 1996-1997.

The continuous increase in fluoroquinolone resistance among clinically important Gram-negative bacilli poses a serious problem because of the widespread use of fluoroquinolones to treat both community-acquired and nosocomial infection. The resistance observed against the beta lactam antibiotics in this study may be suggesting that these isolates were producing ESBLs. The most common enzymes associated with resistance to third-generation cephalosporins in certain Gram-negative bacilli, including *S. marcescens,* are chromosomally encoded, inducible AmpC beta- lactamases. However, ESBLs were increasingly found among clinical *S. marcescens* isolates in the past decade. CTX-M-3, TEM-47 and SHV-5 were discovered in 19% of 347 *S. marcescens* isolates in Poland from 1996 to 2000 [25]. In Taiwan, Wu *et al.*[26] reported that 21 (62%) of 34 *S. marcescens* isolates non-susceptible to cefotaxime exhibited an ESBL-resistant phenotype and all possessed CTX-M-3, suggestive of the wide spread of such a beta-lactamase, at least among *S.marcescens*. The presence of ESBL will further limit the choice of appropriate antimicrobial therapy for cefotaxime-resistant *S. marcescens* bacteremia. The transfer of antibiotic resistance pattern to the transconjugants is an indication that the isolates may be harbouring conjugative plasmid. Resistance to high levels of antibiotics has been ascribed in most instances to the presence of plasmids [27-30]. The fact that the MIC value of the fluoroquilonones were reduced in the presence of carbonyl cyanide m chloro phenyl hydrazone disclosed that the level of resistance engineered to the antibiotics was caused by the presence of efflux pump system in the studied isolates. Exposure to one substance that is a substrate of the efflux pump can favor its overexpression and the consequence may be cross-resistance to all other substrates which may include clinically relevant antimicrobials [3]. Majority of the phytochemical constituents present have been known to possess antibacterial activities [31-33]. Some of this phytochemicals especially tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention and are thought to be responsible for coagulating the wall proteins of pathogenic organisms. Thus, *M. indica* containing this compound may serve as a potential source of bioactive compounds in the treatment of infectious diseases such as pneumonia. Flavonoids have been shown to exhibit their actions through effects on membrane permeability and by inhibition of membrane bound enzymes such as the ATPase and phospholipase [34]. They also serve as health promoting compounds as a result of their anion radicals [35]. These observations support the usefulness of this plant in folklore remedies in the treatment of stress-related ailments and as dressings for wounds. Alkaloids were also detected and their common biological property is cytotoxicity [36]. In this study, the presence of *Mangifera indica* in the MIC medium lowered the MIC values of the multidrug resistant isolates to between 4-fold and 7-fold relative to the standard antibiotics. This observation is not surprising as many other authors have documented the antibacterial activity of this plant [37-39] and this findings suggest the possible use of this plant as an antibiotic efflux pump inhibitors in *Serratia marscencens.*

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