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Synergistic Effect of the Combined Ethanolic and Aqueous extracts of *Garcinia kola* and *Ocimium gratissimum* on Methicillin Resistant *Staphylococcus aureus* and Multi-Drug Resistant *Pseudomonas aeruginosa*

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Abstract: The synergistic antibacterial activity of aqueous and ethanolic, extracts of *Garcinia kola* and *Ocimimum gratissimum* leaves against methicillin resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant *Pseudomonas aeruginosa* (MDRPA) was evaluated. The ethanolic and aqueous extracts of *G. kola* seeds and *O. gratissimum* leaves showed antibacterial activities at varying concentrations (50, 100, 150) against the test organisms (MDRPA and MRSA). The MIC of the aqueous extract of *G. kola* seeds against MDRPA and MRSA were 25mg/ml and MBC of 50mg/ml respectively, 25 and 12.5mg/ml for the ethanolic extract of *Garcinia kola* seed and MBC of 50 and 25mg/ml for *P. aeruginosa* and *S. aureus* respectively. The ethanolic and aqueous extracts of *O. gratissimum* leaves had MIC 50 and 25mg/ml for aqueous extracts, 12.5 and 25mg/ml for the ethanolic extract against the test organisms. The combined aqueous and ethanolic extract of both *G. kola* seeds and *O. gratissimum* leaves showed more antibacterial activities against the test organisms than the single extract with MIC of 12.5mg/ml and MBC of 25mg/ml for *P. aeruginosa* and *S. aureus*. Combined use of both plant parts could find useful application in combating emerging drug resistance caused by methicillin resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa*.

Keywords: Synergism, *Garcinia kola, Ocimium gratissimum*, Methicillin resistant *Staphylococcus aureus,* Multi-drug resistant *Pseudomonas aeruginosa*

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1.Introduction

Plant have long been the principal tools of traditional medicinal systems, although ancient in origin, many traditional medical paradigm and their pharmacopoeias have evolved into quite sophisticated system using the thousands of plants and their natural system (Rates, 2001). Medicinal plants may form a good source of antimicrobial medications or resistance modifying agents to be discovered. Studies on the antimicrobial properties of medicinal plant extracts on resistant strains of microorganismsare scanty and only few antimicrobial agents as isolated compounds have been proven to possess inhibitory properties on multi drug resistance microorganisms (Akinyemi *et al.*, 2005). They present a low risk of resistance development to their action because they are complex mixtures, making microbial adaptability very difficult (Daferera *et al*., 2003).

Plant medicine acts on the body by regulating and balancing its vital processes rather than stopping or combating certain symptoms.

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1.1 Description of plants Used for the Study

Two plants were selected for this work (*G. kola and* O. gratissimum*)* based on ethnobotanical evidence of the plant in the community. *Garcinia kola* popularly known as "bitter kola" is one of the useful indigenous tree in Nigeria and in West and Central Africa (Ogbulie *et al.*, 2007).

It is known as "Orogbo" in Yoruba land, 'Namijin-Ogoro' among the Hausas, 'Akuilu' in Igboland. The fruit is drupe, of the size of an apple and covered with rough hairs. The seed is oval in shape, about 3 cm long with a mass of 8g and a thin leathery testa surrounded by the endosperm.The regions of the plant that are of immense medicinal value are the roots, barks, stems, leaves and seeds (Ogbulie *et al*., 2004). The seeds of *G. kola* have pharmacological uses in the treatment of numerous infections such as bronchitis, hepatitis, liver disorders and stomach upset (Farombi *et al*., 2005; Meserole, 1999). Moreover, *G. kola* is highly recommended in the treatment of opportunistic diseases caused by HIV and AIDS because of its antiviral, detoxification and cleansing properties (Oguntola, 2008). *G. Kola* extracted with 70% ethanol, water and petroleum ether showed the presence of polyisoprenyl benzophenone (kolanone) in petroleum ether extract, hydroxy biflavonols in the ethyl acetone extract. The main component of *G. kola* that has inhibitory activity against Gram-positive and Gram-negative bacteria is hydroxyl flavonol as assessed by Madubunyi *et al.,* (1995).

The seed is used traditionally in treating cough by grinding the seed and mixing with honey (Adebisi, 2007); it is also used as a purgative, antiparasitic and antimicrobial agent (Ghamba *et al*., 2012).

Ocimium gratissimum linn (Lamiaceae) is a herbaceous shrub notably found in tropical countries including Nigeria where it is commonly called clove basil, sweet basil, te-abush, scent leaf or fever plant, but it is also popularly known with different local names in Nigeria. Nupe: Tan-motsungi-wawagi, Ebira: Ireru; Hausa: Dai doyatagida; Yoruba: Efirin ajase, Ibo: Nchanwu (Mann *et al*., 2003). Several ethanobotanical surveys show that *Ocimium gratissimum* was among the plants reported in Nigerian communities to be used traditionally to treat bacterial infections such as enteric diseases viz: Diarrhoea, dysentery and other gastrointestinal infections; upper respiratory tract infections associated with coughing, pneumonia, asthma and bronchitis; urogenital infections including sexually transmitted diseases, skin infections (Dermatitis, eczema, scabies), wounds and ulcers, headache, ophthalmia, insect bites, nasal bleeding, stroke, measles, paludism and bacterial fevers such as typhoid fever and diabetes including veterinary problems. (Ajibesin *et al*., 2008). It is also used in the treatment of epilepsy, shigellosis, trypanosomiasis, convulsion, pile and anaemia in Nigeria (Idika, 2008). It is also implicated in the oral hygiene and veterinary in Nigeria.

It is used as a tea leaf for fevers often given to children or a as a bath when boiled in water for febrile patients. It is burned in room to drive away spirits and repel mosquitoes (Oparaocha *et al*., 2010). The leaves are used in cooking for flavouring sauces, it is strongly fragrant.

It mixes with alcohol in all proportions and contains thymol varying from 32-65%, it is regarded as an antiseptic and the plant is a drug. Its active agent is Eugenol (Pessoa *et al*., 2002).

Comprehensive biological activities of *O. gratissimum* have been reviewed (Prabhu *et al*., 2009) and it is associated with antibacterial, antifugal, hypoglycaemic, antipyretic, antioxidant, anti-inflammatory, chemopreventive, antidermatophytic and numerous other pharmacological uses (Egesie *et al*., 2006).

O. gratissimum acts as a controlling agent for food spoilage and mycotoxin producing fungi, it is used as a relaxant on isolated ileum from guinea pig (Madeira *et al*, 2002). It's essential oil has insecticidal properties and the main active component is Eugenol reported to be efficient in inhibiting *Haemonchus contortua* (Pessoa *et al*.,2002).

2. Materials and Methods

2.1 Source of Test Organisms

Clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained from Daughters of Mary Mothers of Mercy laboratory, Ahiaeke in Umuahia. The isolates were re-identified and subcultured on nutrient agar slants and stored at 4°C until needed for analysis.

2.2 Confirmation of Test Organisms

The test organisms *S.aureus* and *P. aeruginosa* collected from the laboratory were tested for viability by resuscitating them and re-identifying them by biochemical test. *S.aureus* was re-identified using catalase test, and grams staining while *P. aeruginosa* was re-identify

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by oxidase test. They were then sub-cultured on a nutrient agar slant and stored at 4^0C until needed for analysis as described by Cheesbrough, (2000).

2.3 Identification of Methicilin Resistant *S. aureus* and multi drug resistant *P. aeruginosa.*

Strains of *S.aureus* were subjected to sensitivity test with Oxacillin. Those that were resistant to Oxacilin were selected for the study. For *Pseudomonas aeruginosa* ,they were subjected to sensitivity using Zithromax, Ceftriozone, Cefepin, and Levofloxacin. The strains of *P. aeruginosa* that were resistant to these four major drugs were regarded as multi drug resistant *P. aeruginosa* and were used for the study (Dalitha, 2008).

2.4 Sources of Plant Materials

The *Garcinia kola* seeds and the *Ocimium gratissimum* leaves were purchased at Ndioru market in Umuahia. They were purchased fresh and packed in a clean polythene bags and transported to the Department of Plant Science and Biotechnology for identification. The plants were botanically identified by Dr. Omosun Garuba.

2.5 Extraction of plant Material

2.5.1 Ethanolic Extract

The grounded leaves and seeds were weighed. The method described by Akerele *et al*, (2008) was used to extract the bioactive components from the grounded leaves and seeds. 30g of each of the samples (*Garcinia kola* seed and *Ocimium gratissimum* leaves) were steeped in 200ml of 95% ethanol for 24 hours in a sterile conical flask at laboratory temperature and stirred occasionally. The content was filtered after 24 hours using Whatman filter paper and evaporated to dryness at 48^0C using a rotaevaporator.(Nenaah and Ahmed, 2011; Akerele *et al*, 2008). The residues were collected and stored in a sterile container until required for use.

2.5.2 Aqueous Extraction (Hot and Cold Water)

30g each of the plants (*Garcinia kola* seed and *Ocimium gratissimum* leaves) were weighed and steeped in 200ml of hot water and cold water respectively for 24 hours, after which both were filtered using Whatman filter paper and evaporated to dryness at 48^oC using a rota-evaporator. The residues were collected and stored until required for analysis.

2.6 Extraction of Combined Plant Materials

15g of *G. kola* seeds and *O. gratissimum* leaves were combined making it 30g. The 30g sample was steeped in 200ml of ethanol for 24 hours in a sterile conical flask and was stirred occasionally. The content was filtered using Whatman filter paper and the extract was evaporated to dryness using a rotaevaporator. (Akereke *et al*, 2008). The residues were collected into a sterile container until required for use.

The aqueous extracts of the combined *G. kola* seeds and *O. gratissimum* leaves was carried out using hot and cold water respectively. 15g of each sample were weighed and mixed thoroughly after which they were steeped in hot and cold water for 24 hours. The content was filtered using whatman filter paper and the extracts were evaporated to dryness using a rotaevaporator at 48^0C. The residues were collected into a sterile container until required for use (Nennah and Ahmed, 2011; Akerele *et al.*, 2008).

2.7 Screening of Extracts for Antibacterial Activity

The antibacterial activity of the different extracts (both combined and single) were evaluated using methicillin resistant *S. aureus* and multi drug resistant *P. aeruginosa*. The ability of the various extracts to inhibit growth of the clinically significant bacteria was determined using 6mm diameter hole in agar well diffusion technique (Ogueke *et al*, 2006). The bacterial isolates were first grown in nutrient broth for 18 hours at 37^oC, the broth was collected aseptically using a swab stick and smeared evenly on Muller-Hinton agar. 0.2ml of the extracts was inoculated into the holes bored on the Muller-Hinton agar. The extracts were tested at 50,100 and 150mg/ml concentrations. The plates were incubated at 37^oC for 24 hours; the zones of inhibition were measured in millimeter diameter using a meter rule (Adegboye *et al.*, 2008).

2.8 Determination of Minimum Inhibitory Concentration (MIC) of Extracts

Determination of Minimum Inhibitory Concentration (MIC) was carried out using the broth dilution method as described by Dalitha (2008). The MIC of the extracts were determined for the test organisms (MRSA and MDRPA) at varying concentrations. A stock solution of 20mg/10ml was prepared for each extract. One millimeter (1ml) of nutrient broth was dispensed into test tubes and sterilized by autoclaving at 121^0C at 15Psi for 15 minutes. The different extracts were serially diluted from the stock solutions to obtain varying concentrations. The concentrations were 100, 50, 25, 12.5, 6.25mg/ml. 0.1ml of each test isolate was inoculated into the various test tubes containing varying concentrations and then incubated at 37^0C for 24 hours.

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After incubation, the presence or absence of growth on each tube was rated. The minimum inhibitory concentration was taken as the lowest concentration of the plant extract that inhibited the growth.

2.9 Phytochemical Screening test for the Plant Extracts

A portion of the crude plant extracts of *Ocimium gratissimum* leaves and *Garcinia kola* seeds were subjected to phytochemical screening for identification of various classes of active chemicals constituents such as Tannins, saponins, flavonoids, alkaloids, using methods described by (Amadi *et al*., 2004).

3. Results and Discussion

The evaluation of the antibacterial properties of the single plant extracts and the synergistic effect of *Garcinia kola* seeds and *Ocimium gratissimum* leaves has shown antibacterial activity against the test organisms (methicillin resistant *Staphylococcus aureus* and multi drug resistant *Pseudomonas aeruginosa*). The antibacterial activity in this study was expressed as a measure of the diameter of the inhibition of growth in millimeters. Table 1 to 3 show the mean zone diameter of inhibition at different concentrations of 50,100 and 150mg/ml for the single plant extracts and combined extracts of *Garcinia kola* seeds and *Ocimium gratissimum* leaves.

A high inhibitory activity for the single plant extracts was obtained from the ethanolic extracts; *P. aeruginosa* had a high zone of inhibition for ethanolic extract at 13.50mm for *O. gratissimum* leaves extract while *S.aureus* gave 13.25mm for ethanolic extract of *G. kola*. The combined extracts gave the highest zones of inhibition both the ethanolic and aqueous extracts (hot and cold water) at different concentrations (50, 100, 150mg/ml). The ethanolic extract of the combined plants had a high zone of inhibition of 18.05mm and 16.45mm for *S.aureus* and *P. aeruginosa* respectively (Table -3).

Table 4 to 9 shows the minimum inhibitory concentrations (MIC) and the minimum bacteriocidal concentrations (MBC) for the aqueous and ethanolic extracts at various concentrations against the test organisms. The minimum inhibitory concentration (MIC) of the aqueous extract of *G. kola* seeds against the isolates were 25mg/ml and MBC value of 50mg/ml for both *P. aeruginosa* and *S.aureus* (Table -4). *P. aeruginosa* and *S.aureus* were killed by the ethanolic extracts at the concentration of 50mg/ml and 25mg/ml respectively (Table -7).

The aqueous extract of *O. gratissimum* leaves had an MIC value of 50 and 25mg/ml for *P. aeruginosa* and *S. aureus* respectively (Table- 5) while the ethanolic extract inhibited the growth of *P. aeruginosa* and *S. aureus* at concentration of 12.5 and 25mg/ml respectively (Table 8).

The combined aqueous and ethanolic extract of *G. kola* seeds and *O. gratissimum* leaves had an MIC value of 12.5mg/ml and MBC value of 25mg/ml for *P. aeruginosa* and *S.aureus* (Table 6 and 9). The phytochemical screening test revealed the presence of Tannins, Saponins, Flavonoids and Alkaloids for *O. gratissimum* leaves and *G. kola* seed (Table 10).

4.Discussion

This study reveals that the combined and single plant extracts of *O. gratissimum* leaves and *G. kola* seeds possess antibacterial activities at varying concentrations against the clinical isolates (methicillin resistant *S.aureus* and multi-drug resistant *P. aeruginosa)* indicating their broad spectrum activity (Bankole, 1995). The ethanolic and aqueous extracts of *G. kola* seeds was active against *S.aureus* and *P. aeruginosa*. This is similar to the work of Ogueke *et al*., (2006) which showed that ethanolic and aqueous extracts of *G. kola* seeds exhibited antibacterial activities against both Gram-positive and Gram-negative organisms. Muanya (2008) also identified *G. kola* to have strong antibiotic activities and found the plant to be very effective against disease-causing microorganisms such as *E.coli, S.aureus, P. aeruginosa*, *Salmonella specie,* etc. *P. aeruginosa* and S.*aureus* was found to be sensitive to the ethanolic and aqueous extracts of *O. gratissimum* leaves. This finding justifies the ethnomedical use of this plant in the treatment of wounds of which *P. aeruginosa* and *S. aureus* have been highly incriminated.

The combined extracts of *G. kola* seeds and *O. gratissimum* leaves produce greater zones of inhibition against the test organisms (MRSA and MDRPA) than *G. kola* seeds and *O. gratissimum* leaves separately as shown in table 3. This could be attributed to the additive effect of the active components present in these plants which is in agreement with Onwuakor and Ukaegbu-Obi (2014).

The phytochemical screening tests of the plant extracts were also determined in this study. The *G. kola* seeds and *O. gratissimum* leaves revealed the presence of Tannins, Saponins, Flavonoids and Alkaloids (Table 10). The significant roles these phytochemical compounds play in the antibacterial activity lies on their

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Table -1: Antibacterial activity of *Garcinia kola* seeds extract on clinical isolates

Isolates	Concentration(mg/ml)	Mean zone diameter of inhibition (mm)		
		Hw	Cw	Eth
P. aeruginosa	50	10.33	9.33	10.36
	100	11.40	10.40	11.50
	150	12.02	12.32	13.38
S. aureus	50	11.40	10.50	10.59
	100	12.00	12.30	11.50
	150	13.14	12.50	13.25

Key: HW – How water extract; CW – Cold water extract, Eth – Ethanolic extract

Table -2: Antibacterial activity of *Ocimium gratissimum* leaves extract on clinical isolates

Isolates	Concentration(mg/ml)	Mean zone diameter of inhibition (mm)		
		Hw	Cw	Eth
P. aeruginosa	50	10.50	9.50	10.60
	100	12.32	10.90	12.70
	150	13.01	12.45	13.50
S.aureus	50	10.23	9.20	10.45
	100	11.50	10.20	11.50
	150	12.00	12.05	13.15

Key: HW – How water extract; CW – Cold water extract, Eth – Ethanolic extract

Table - 3: Antibacterial activity of combined extracts of *Ocimium gratissimum* leaves and *Garcinia kola* seeds on clinical isolates

Isolates	Concentration(mg/ml)	Mean zone diameter of inhibition (mm)		
		Hw	Cw	Eth
P. aeruginosa	50	13.50	12.25	13.50
	100	14.40	13.30	14.03
	150	16.40	15.20	16.45
S.aureus	50	13.19	13.80	13.70
	100	13.56	14.30	14.25
	150	15.50	15.04	18.05

Key: HW – Combined (Hot water extract), CW– Combined (Cold water extract), Eth-(Combined Ethanolic extract).

Table - 4: Minimum Inhibitory concentration and Bacteriocidal concentration of aqueous extracts of *Garcinia kola* seeds on *S.aureus* and *P. aeruginosa*

Isolates	Concentration (mg/ml)	Turbidity	Growth on plate	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	100	-	-		
	50	-	-		50
	25	-	+	25	
	12.5	+			
	6.25	+			
S.aureus	100	-	-		
	50	-	-		50
	25	-	+	25	
	12.5	+			
	6.25	+			

Key: - No growth + Growth

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Table 5: Minimum Inhibitory concentration and Bacteriocidal concentration of aqueous extracts of *Ocimium gratissimum* leaves on *S. aureus* and *P. aerugionsa*

Isolates	Concentration (mg/ml)	Turbidity	Growth on plate	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	100	-	-		
	50	-	-	50	50
	25	+			
	12.5	+			
	6.25	+			
S. aureus	100	-	-		
	50	-	-		50
	25	+	+	25	
	12.5	+			
	6.25	+			

Key: - No growth + Growth

Table 6: Minimum Inhibitory concentration and Bacteriocidal concentration of combined aqueous extracts of *Garcinia kola* seeds and *Ocimium gratissimum* leaves on *P. aeruginosa* and *S. aureus*

Isolates	Concentration (mg/ml)	Turbidity	Growth on plate	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	100	-	-		
	50	-	-		
	25	-	-		25
	12.5	-	+	12.5	
	6.25	+			
S. aureus	100	-	-		
	50	-	-		
	25	-	-		25
	12.5	-	+	12.5	
	6.25	+			

Key: - No growth + Growth

Table -7: Minimum Inhibitory concentration and Bacteriocidal concentration of ethanolic extracts of *Garcinia kola* seeds on *S. aureus* and *P. aeruginosa*

Isolates	Concentration (mg/ml)	Turbidity	Growth on Plate	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	100	-	-		
	50	-	-		50
	25	-	+	25	
	12.5	+			
	6.25	+			
S. aureus	100	-	-		
	50	-	-		
	25	-	-		25
	12.5	-	+	12.5	
	6.25	+			

Key: -No growth + Growth

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Table -8: Minimum Inhibitory concentration and Bacteriocidal concentration of Ethanolic extracts of *Ocimium gratissimum* leaves on *P. aeruginosa* and *S. aureus*

Isolates	Concentration (mg/ml)	Turbidity	Growth on Plate	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	100	-	-		
	50	-	-		
	25	-	-		25
	12.5	-	+	12.5	
	6.25	+			
S. aureus	100	-	-		
	50	-	-		50
	25	-	+	25	
	12.5	+			
	6.25	+			

Key: -No growth + Growth

Table -9:Minimum Inhibitory concentration and Bacteriocidal concentration of combined ethanolic extracts of *Garcinia kola* seeds and *Ocimium gratissimum* leaves on *P. aeruginosa* and *S.aureus*

Isolates	Concentration (mg/ml)	Turbidity	Growth on Plate	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	100	-	-		
	50	-	-		
	25	-	-		25
	12.5	-	+	12.5	
	6.25	+			
S. aureus	100	-	-		
	50	-	-		
	25	-	-		25
	12.5	-	+	12.5	
	6.25	+			

Key: - No growth + Growth

Table -10: Phytochemical Screening Test of *Ocimium gratissimum* and *Garcinia kola*

Bioactive Components	**Ocimium gratissimum**	**Garcinia kola**
Tannins	+	+
Saponins	+	+
Flavonoids	+	+
Alkaloids	+	+

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ability to produce a definite and specific action on the human body (Adegboye *et al*., 2008). The significant activities of both plants observed in this study could thus be attributed to the interaction of one or more of the identified phytochemical compounds against the test organisms.

Conclusion

This research work have shown that the single extracts of *Garcinia kola* seeds and *Ocimium gratissium* leaves have antibacterial activity against methicillin resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa* but their activities were more better when combined than when used singly. This therefore suggest for the possible exploration of these plants as sources of natural product for future use in the management of multi-drug resistant pathogens such as *P.aerugionosa* and *S.aureus* that cause wide range of infections.

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