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SYNERGISTIC EFFECTS OF METHANOLIC EXTRACTS OF *Allium sativum Lin.* BULBS AND *Garcinia kola* (HECKEL) SEEDS ON SELECTED BACTERIAL ISOLATES

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Abstract

This study investigated the individual and combined antimicrobial activities of the methanolic extracts of *Allium sativum* bulbs and *Garcinia kola* seeds on selected bacterial isolates, assessed the minimum inhibitory concentrations of the crude extracts with a view to determine if the crude extracts have synergistic effect. The seeds of *Garcinia kola* and bulbs of *Allium sativum* were purchased from Central Market, Ile– Ife Southwest Nigeria, dried, and ground into fine powder separately. The extract of ground plant materials were obtained by soaking in 60% methanol separately, filtered, concentrated *in vacuo*, lyophilized and screened for phytochemical components and antibacterial activities individually and in combined form against some selected bacterial isolates. Minimum inhibitory concentrations (MIC) assay of plant extracts and their combinations were performed. Phytochemical screening revealed the presence of Saponins, Tannins, Flavonoids, reducing sugars, Alkaloids and Steroids in *G. kola* while Saponins, Triterpenes and Steroids were present in *A. sativum*. The plant extracts exhibited different degrees of antimicrobial activities against the selected bacteria and synergistic effect against some tested pathogens. The MIC of the methanolic extract of *G. kola* ranges from 0.313 mg/ml to 2.5 mg/ml, MIC exhibited by *A. sativum* was 2.5 mg/ml while MIC that ranged from 0.313 mg/ml and 1.25 mg/ml was observed when the extracts were combined at equal proportion. The study concluded that crude extracts of *G. kola* and *A. sativum* exhibited potentials of synergy against pathogenic bacteria which can be explored in providing solutions to multidrug resistance.

Keywords: Antimicrobial, Phytochemical components, Synergistic, *Garcinia kola, Allium Sativum.*

Introduction

Increasing emergence and spread of bacteria resistant to first-line antibiotics are now serious global problems. The problem is more pronounced in bacterial infections which contribute most to the global disease burden which include gastrointestinal tract infection and sexually transmitted infectious diseases (Straif-Bourgeois *et al.*, 2014). This incidence has necessitated alternative therapy with the aid of medicinal plants due to their ability to produce metabolites with antimicrobial properties and this had instigated finding solutions to microorganism resistances to the existing antibiotics (Akinpelu *et al.*, 2008). Widespread emergence of multidrug resistant organisms (MDR) which is characterized by

high mortality rate coupled with abnormal use of antibiotics had necessitated the need for combination therapy with plant based therapeutic agents so as to reduce such incidence. *Syzygium aromaticum* (cloves), *Mikania glomerata* (guaco), *Psidium guajava* (guava), *Allium sativum* (garlic), *Cymbopogon citratus* (lemon grass), *Zingiber officinale* (ginger), *Baccharis trimera* (carqueja), and *Mentha Pieria* (mint) extracts and when combined with some antibiotics, were shown to exhibit synergistic effect against *Staphylococcus aureus* (Betoni, 2006).

A study (Natchimutha *et al.*, 2012) discovered synergistic antibacterial activities of aqueous extracts of *Cassia auriculata* and *Cissus quadrangularis* against *Escherichia coli*. Synergistic activity was observed with methanol and n-hexane extracts of leaves and roots of *Withania somnifera* against *Salmonella typhimarium* and *E. coli* (Arora *et al.*, 2004). Another study also reported that ginger and honey showed combined antimicrobial effect against *Salmonella typhi, Shigella dysenteriae, Escherichia coli and Candida albicans* (Omoya and Akharaiyi, 2012). *Allium sativum* cloves and *Zingiber officinale* rhizomes were found to synergistically inhibit multiple-drug resistant clinical pathogens such as *P. aeruginosa*, *E. coli* and *S. aureus* in the study carried out (Natchimutha *et al.*, 2012). Omoya and Akharaiyi (2012) discovered *in-vitro* antifungal effects of *G. kola* and *A. sativum* against *Candida* isolates obtained from vagina. Therefore, *G. kola* and *A. sativum* are of great importance due to their significant inhibitory effect on both fungi and bacteria (Akinpelu *et al.*, 2008; Omoya and Akharaiyi, 2012).

Hence, this study aims at examining the antibacterial activities of methanolic extract of *A. sativum* bulbs and *G. kola* seeds individually and in combination against some selected bacterial isolates with a view of determining synergistic effects or otherwise.

Materials and Methods

Plant Extract Preparation

The seeds of *Garcinia kola* and bulbs of *Allium sativum* were purchased from Central Market Ile – Ife. Seeds of *Garcinia kola* were air dried while the bulbs of *Allium sativum* were peeled, cut and oven dried at 45 °C. Both the dried seeds and bulbs were then ground into a fine powder. About 550 g of powdered plant materials each were soaked using 2 L of 60% methanol (analytical grade) for four days with regular agitation after which they were filtered using whatman filter paper No. 1. The extracts obtained were concentrated *in-vacuo* using rotary evaporator (Buchi Rotavapor R-100, Switzerland) at 40 °C and lyophilized (Christ Beta 1-8 LD plus, Germany) at -55 °C. The yields were kept in refrigerator for future use.

Phytochemical screening

The methanolic plant extracts were screened for phytochemical parameters individually and in combination as described previously (Trease and Evans, 2002). The phytochemical qualitative tests were done in duplicates to assess the presence of reducing sugars, Saponins, Anthraquinone, Flavonoids, Triterpenes, Tannins and Alkaloids.

Test for reducing sugars

Two milliliters of plant extracts were measured into a test tube. One milliliter of each of Fehling solution I and II were added, shaken and heated in a heating block for 5 min. Production of brick red precipitate indicates positive for reducing sugar.

Test for saponins

Two millilitres (2 ml) of each methanolic extract were dispensed into separate test tubes and shaken continuously and then left for 10 min. A thick persistent froth indicates positive test for saponins.

Anthraquinone derivatives test

Two millilitres (2 ml) of each plant extracts were dispensed into separate test tubes with the addition of one milliliter of 10 % ammonia followed by vigorous shaken of the mixture. Pink-red coloration in the ammoniacal layer means presence of anthracene derivatives.

Test for flavonoids

Two millilitres (2 ml) of each plant extracts were dispensed into test tubes followed by addition of dilute NaOH and dilute HCl. Solubility and change in color was assessed. Yellow solution with NaOH that changed to colorless when dilute HCl was added confirmed the presence of flavonoids.

Test for triterpenes

Twenty milligrams of each extract were dissolved in 10 ml chloroform. The solution was heated slightly and filtered, after which sulphuric acid was added to the filtrate. Red coloration indicated the presence of triterpenes.

Test for tannins

One gram of each extract was dissolved in 20 ml of distilled water and filtered**.** In 2 ml of the filtrate then about three drops of 10% $FeCl_3$ added. One milliliter of bromine water was added to another 2 ml of the filtrate. The color change was recorded. A precipitate was taken as an indication positive for tannins.

Test for Alkaloids

Half gram (0.5 g) of each methanolic extracts was dissolved in 5 ml of 1% HCl on a heating block and filtered. Dragendorff's precipitating reagent was used to treat 1 ml of the filtrate. Formation of precipitate indicates the presence of alkaloids.

Test for Steroids

Half gram (0.5 g) of each plant extract was dissolved in 3 ml of $CHCl_3$ and made to pass through filter paper followed by addition of concentrated H_2SO_4 to the filtrate to form a lower layer. Red brown coloration indicates presence of steroids.

Preparation of Inoculum

Bacteria utilized in this research were obtained from the Department of Microbiology, Obafemi Awolowo University, Ile Ife, and includes both typed and locally isolated organisms consisting of both Gram positive and Gram-negative strains. Organisms were subcultured by picking one or more discrete colonies of each bacterial isolates and inoculating into test tubes containing sterile nutrient broth, incubated at 37 °C for 18 h. The suspensions were adjusted to 0.5 McFarland standards.

Antibacterial screening and Minimum inhibitory concentration (MIC)

The susceptibility of the bacterial isolates to the plant extracts was determined using agar-well diffusion method (Irobi *et al.*, 1994). Mueller-Hinton agar were uniformly seeded with standardized (0.5 McFarland) 18 h inoculum suspensions. Three wells were made in the agar plate using pre-sterilized cork borer (6 mm diameter), filled with *G. kola* extract (50 mg/ml), *A. sativum* extract (50 mg/ml), and streptomycin (1mg/ml) as positive control, incubated at 37 °C for 24h, examined for zones of inhibition and measured in millimeter (using ruler). The experiment was performed in replicates.

The MIC of each plant extract was determined using the method described by (Akinpelu and Kolawole, 2004). Two-fold dilutions of the plant extracts were done to vary the concentrations of the extracts, producing solutions ranging between 0.078 mg/ml and 10 mg/ml. About 2 ml of each solution was added to 18 ml of sterile molten agar at 40 °C, transferred into Petri dish, allowed to solidify then standardized inoculum were streaked on the plate and incubated at 37 °C for 48 h. The plates were examined for growth and MIC was taken as the lowest concentration that inhibited visible bacterial growth.

Combination studies of methanolic

extracts of *Garcinia kola* and *Allium sativum*

The extracts were mixed at equal concentrations (25mg/ml each) to make up the active concentration (50 mg/ml) and subjected to antimicrobial screening as described above, followed by determination of MIC. The fractional inhibitory concentrations (FIC) were obtained through the lowest concentration of extracts in combination that permit no physical growth of the bacterium (Mandal *et al.*, 2004). The FIC value for each agent was calculated using the formula;

$$\text{FIC (extract A)} = \frac{\text{MIC of extracts in combination}}{\text{MIC of extract A alone}}$$

$$\text{FIC (extract B)} = \frac{\text{MIC of extracts in combination}}{\text{MIC of extract B alone}}$$

The interaction between the two extracts was determined using Fractional Inhibitory Concentration indices calculated with the formulae;

$$\text{FIC Index} = \Sigma\text{FIC} = \text{FIC (extract A)} + \text{FIC (extract B)}$$

Combination with FIC indices < 1 is regarded as synergy, FIC indices =1 is additive, FIC indices between 1 and 2 were indifferent, and FIC indices > 2 were considered antagonistic (Deresse, 2010).

Results and Discussion

Phytochemical examination revealed the presence of Saponins, Tannins, Flavonoids, reducing Sugar, Alkaloids as well as Steroids in *G. kola* while Saponins, Triterpenes and Steroids were present in *A. sativum* (Table 1). Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants and are synthesized by phenylpropanoid pathway (Kumar and Pandey, 2013). Reports showed that secondary metabolites of phenolic nature including flavonoids are responsible for the variety of pharmacological activities (Mahomoodally *et al.*, 2005, Kumar and Pandey, 2013).

The extracts of the plant materials individually and in combined form exhibited different degrees of antimicrobial activities as shown in Table 2. *Garcinia kola* at 50 mg/ml exhibited broad spectrum antimicrobial activities against all 15 test organisms, with zones of inhibition ranging between 12 ± 0.00 and 22±0.00 which agrees with results of other researchers (Adegboye *et al.*, 2008; Indabaw and Arzai, 2011) thus validating local use of the *G. kola* seeds in treating oral and respiratory tract infections. Broad spectrum activities noticed in methanolic extract of *G. kola* can also be due to the presence of phytochemical compounds in *G. kola* which had been reported by several researchers to possess antibacterial and antifungal properties (Akinpelu *et al.*, 2008; Omoya and Akharaiyi, 2012). Methanolic extract of *A. sativum* at 50 mg/ml exhibited antimicrobial activities against only four Gram-positive bacteria isolates out of fifteen used in this study. Absence of flavonoids, exposure to heat (Deresse, 2010), geographical location and variation in the strain of *A. sativum* species may be responsible for limited antibacterial activity observed with *A. sativum* used in this study. When the plant extracts were combined at equal concentration to make 50 mg/ml, broad spectrum activity was obtained with the exception of *M. luteus* and this compared very well with the antibiotics (Streptomycin) utilized in this research because their zones of inhibition fell within the same range (10-30 mm). The resistance to the combined form of the extracts seen with *M. luteus* could probably be that the strain of *M. luteus* used in this study was a plasmid-bearing *M. luteus* strain (Eady *et al.*, 2000).

The MIC of *G. kola* extract ranges from 0.313 mg/ml and 2.5 mg/ml, MIC exhibited by *A. sativum* was 2.5 mg/ml while MIC ranging between 0.313 mg/ml and 1.25 mg/ml was observed when the extracts were combined at

equal proportion. The MIC exhibited by streptomycin which was the control antibiotics ranged between 0.06 mg/ml and 0.5 mg/ml (Table 3).

The combined study using FIC Index, revealed synergistic effect on two Gram positive (*B. cereus* and *B. subtilis*) and three Gram negative (*E. coli*, *Salmonella* sp. and *Shigella* sp.) bacteria (Table 4). The synergistic effect obtained with this research could be attributed to the presence of flavonoids and polyphenols which had been reported to possess resistance modifying potentials (Trease and Evans 2002; Cushnie and Lamb, 2005; Kumar and Pandey, 2013). The synergistic effect observed against Gram-positive bacteria may be due to inhibition of DNA synthesis and damage to cell membrane as in *Bacillus subtilis* (Ponmurugan and Shyamkumar, 2012). High level of antibiotics resistances in pathogenic bacteria are as a result of efflux pumps (Leon *et al.*, 2005). Efflux pumps could be the mechanism of resistance adopted by some of the organisms used in this study in response to the antibacterial activity of combined methanolic extract of *G. kola* and *A. sativum*.

Table 1: Results of phytochemical analysis of *G. cola* and *A. sativum* extracts

Chemical Test	*Garcinia kola*	*Allium sativum*
Alkaloids	++	--
Tannins	++	--
Saponins	++	++
Flavonoids	++	--
Triterpenes	--	++
Reducing sugar	++	--
Antraquinone	--	--
Steroids	++	++

Key: ++ denotes Positive, -- denotes Negative

Table 2: The sensitivity pattern exhibited by Methanolic Extracts of *G. kola*, *A. sativum*, combined G. *kola* and *A. sativum*, and streptomycin against the bacterial isolates

	Zones of Inhibition (mm**) Control			
Bacterial isolates	***G. kola* (50 mg/ml)**	***A. sativum* (50 mg/ml)**	***G. kola*& *A. sativum* (50 mg/ml)**	**Streptomycin (1 mg/ml)**
Bacillus anthracis (LIO)	14 ±1.52	0 ±0.00	13 ±0.00	28±0.76
B. cereus (NCIB 6349)	13±0.71	15±0.71	23±0.71	26±0.50
B. stearothermophilus (NCIB 8222)	17±1.41	0±0.00	19±0.72	30±0.58
B. subtilis (NCIB 3610)	15±0.71	0±0.00	20±0.00	27±1.00
Clostridium sporogenes (NCIB 532)	14±2.12	0±0.00	14±0.35	30±0.50
Streptococcus pyogenes (LIO)	12±0.00	13±1.41	20±0.00	24±0.00
E. coli (NCIB 86)	16±2.12	0±0.00	22±1.00	26±0.00
Klebsiella pneumoniae (NCIB 418)	22±0.00	0±0.00	28±0.71	24±1.00
Micrococcus luteus (NCIB 196)	16±0.71	20±0.00	0±0.00	25±0.50
Proteus vulgaris (LIO)	15±1.41	0±0.00	18±1.72	26±1.00
Pseudomonas aeruginosa (NCIB 418)	15±0.71	0±0.00	16±0.71	20±0.00
Salmonella sp. (LIO)	14±0.71	0±0.00	17±0.14	0±0.00
Shigella sp. *(LIO)*	14±0.00	0±0.00	20±0.0	20±1.00
Staphylococcus aureus (NCIB 8588)	16±0.42	0±0.00	16±0.35	29±1.50
Enterococcus faecalis (LIO)	14±0.71	13±0.71	18±0.71	20±2.00

Key: LIO=Locally Isolated Organisms, NCIB=National Collection of Industrial Bacteria, mm** = mean of two replicates, 0= Not sensitive *G.k* & *A.s* = Combination of *G. kola* and *A. sativum.*

Table 3: Minimum Inhibitory Concentrations exhibited by the methanolic extracts individually and in combined form as well as streptomycin.

Bacterial isolates	*G. kola* (mg/ml)	*A. sativum* (mg/ml)	C-PE (mg/ml)	Streptomycin (mg/ml)
Bacillus anthracis (LIO)	0.31	2.5	0.63	0.25
B. cereus (NCIB 6349)	5.0	2.5	0.31	0.06
B. stearothermophilus (NCIB 8222)	5.0	2.5	5.0	0.06
B. subtilis (NCIB 3610)	1.25	5.0	0.63	0.25
C. sporogenes (NCIB 532)	0.31	5.0	0.63	0.06
S. pyogenes (LIO)	1.25	5.0	5.0	0.13
E. coli (NCIB 86)	5.0	5.0	1.25	0.50
K. pneumoniae (NCIB 418)	0.31	2.5	0.31	0.50
M. luteus (NCIB 196)	5.0	5.0	5.0	0.25
P. vulgaris (LIO)	0.31	2.5	0.63	0.50
P. aeruginosa (NCIB 950)	5.0	2.5	5.0	0.50
Salmonella sp. (LIO)	5.0	5.0	0.63	0
Shigella sp. *(LIO)*	2.5	5.0	1.25	0.25
S. aureus (NCIB 8588)	0.31	5.0	0.63	0.13
E. faecalis (LIO)	5.0	2.5	5.0	0.25

Key: LIO = Locally Isolated Organisms, NCIB = National Collection of Industrial Bacteria, ND = Not determined, C-PE = Combination of *G. Kola* and *A. sativum*

Table 4: Fractional inhibitory Concentration (FIC) values and indices for the combinations of the methanolic extract of *G. kola* and *A. sativum*.

Bacterial isolates	Mean FIC (*G. kola*)	Mean FIC (*A. sativum*)	FIC Index	Interaction
B. anthracis (LIO)	2.00	0.25	2.25	Indifference
B. cereus (NCIB 6349)	0.06	0.13	0.19	Synergy
B. stearothermophilus (NCIB)	1.0	2.0	3.0	Antagonisms
B. subtilis (NCIB 3610)	0.50	0.13	0.63	Synergy
C. sporogenes (NCIB 532)	2.00	0.13	2.13	Indifference
S. pyogenes (LIO)	4.17	1.00	5.17	Antagonisms
E. coli (NCIB 86)	0.25	0.25	0.50	Synergism
K. pneumoniae (NCIB 418)	1.00	0.13	1.13	Additivity
M. luteus (NCIB 196)	1.00	1.00	2.00	Indifference
P. vulgaris (LIO)	2.00	0.25	2.25	Indifference
P aeruginosa	1.00	2.00	3.00	Antagonisms
Salmonella sp. (LIO)	0.13	0.13	0.26	Synergy
Shigella sp. (LIO)	0.50	0.25	0.75	Synergisms
S. aureus (NCIB 8588)	1.00	2.00	3.00	Antagonisms
E. feacalis (LIO)	1.00	2.00	3.00	Antagonisms

Conclusions

In this study, *G. kola* extract exhibited antimicrobial activities against both Gram positive and Gram-negative bacteria, indicating a broadspectrum antibacterial potential. The synergy obtained between the methanolic extracts of *Garcinia kola* and *Allium sativum* is an important finding that demonstrated the therapeutic potentials of these plants especially against pathogenic bacteria (*Salmonella* sp. and *Shigella* sp.) often involved in antibiotic resistance. This could be an indication for the potential of these plant materials as a source of novel antimicrobial agents for antibiotic resistance pathogens.

With these important results, this study concludes by recommending the necessity to establish the molecular basis of the interaction between *G. kola* and *A sativum*.

Conflict of Interest

Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of Interest.

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