

Bioassay-Guided Isolation, Purification and Partial Characterization of Antimicrobial Compound from Basic Metabolite of *Garcinia Kola*

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Abstract

Garcinia kola (also called bitter kola) has been identified to possess strong antibacterial and chemotherapeutic activities against several human pathogens such as *Staphylococcus aureus*, *Candida albicans*, *Coliform bacilli*, *E. coli*, *Pseudomonas spp.*, *Salmonella typhi*, and *Streptococcus spp.* although the active principles responsible for these activities are not known with certainty. Therefore the purpose of this study was to purify and characterize the bioactive polyphenolic isolate responsible for the antimicrobial property of the basic metabolite of the plant extract. The bioactive compound, a reddish-brown precipitate, was isolated from the basic metabolite and purified using silica gel packed in a chromatographic column. The various fractions obtained were screened for antimicrobial activity. The fraction that showed the best antimicrobial potential was characterized partially using IR, ¹H- and ¹³C-NMR spectroscopy. Analyses of the combined spectroscopic data suggested that the compound could be *Catechin*, *p-naphtholbenzein*, *α-methyl-dl-tyrosine* or *Naringin*. It was concluded that final identification of this polyphenolic compound would be made from the mass spectrum (MS).

Keywords: isolation, characterization, bioactive compound, basic metabolite, garcinia kola.

INTRODUCTION

Plants are the basis of traditional medicine in Africa and have been used for thousands of years. These plants often exhibit a wide range of biological and pharmacological activities, such as anti-inflammatory, anti-bacterial and anti-fungal properties (Ajayi *et al*, 2011). Due to the need for development of new drugs with better pharmacological activities, dependence on plants grew increasingly as scientists continuously exploited them for isolation of bioactive compounds. *Garcinia kola*, popularly called “bitter kola”, is an indigenous plant used by traditional medicine practitioners as cough suppressant, anti-tumor agent and aphrodisiac. *Garcinia kola* is highly valued in Africa because of its use in traditional medicine (Ghamba *et al*, 2011).

Several workers have investigated the antimicrobial potential of *Garcinia kola* (Dada *et al*, 2009; Nwaokorie *et al*, 2010; Ugbogu *et al*, 2010, Ejele, 2010; Ghamba *et al*, 2011; Penduka and Okoh, 2011; Ejele and Akujobi, 2011). Dada *et al* (2009) investigated the effects of ethanolic extract of *G. kola* seeds on growth and haematology of catfish (*Clarias gariepinus*) broodstock and found that the weight gain increased as amount of extract increased up to 1.0g/kg and decreased thereafter. The authors showed that haematological parameters such as packed cell volume, haemoglobin concentration and red blood cells were not significantly different from those of the control, although there was a significant proliferation

of white blood cells, which probably explains the antimicrobial effect of ethanolic extracts of *G. kola* seeds, in view of the major role white blood cells assume in the immune defense mechanism of man and animals.

Nwaokorie *et al* (2010) evaluated the inhibitory activity of crude methanol and aqueous extracts of *G. kola* on *Fusobacterium nucleatum* isolated from the oral cavity. The results showed that the methanol extract exhibited significant activity against amoxicillin resistant isolates at a concentration of 12.5 mg/ml, suggesting its potential use in oral hygiene. Ugbogu *et al* (2010) studied the susceptibility of aqueous and ethanol extracts of *G. kola* seeds on methicillin resistant *S. aureus* (MRSA) and found that the ethanol extracts were very effective and inhibited the growth of the isolates with zones of inhibition ranging from 11–25mm for different concentrations of the extracts.

Ghamba *et al* (2011) studied the effects of aqueous and diethyl ether extracts of *G. kola* seed on some bacterial isolates, namely; *S. aureus*, *E. coli* and *P. aeruginosa* at concentrations of 50, 100 and 150mg/ml. The results showed that both extracts possessed strong antibacterial and chemotherapeutic activities, although the diethyl ether extract showed a greater activity against the test bacterial isolates. Penduka and Okoh (2011) reported *in-vitro* antimicrobial activities of crude dichloromethane

extract of *G. kola* seed against pathogenic *Vibrio* species. The extract was screened for anti-*Vibrio* activities against a panel of 50 *Vibrio* isolates from five different families. The authors found that the extract inhibited the growth of 16 isolates at 10mg/ml concentration, with zones of inhibition ranging from 9 to 15mm.

Ejele (2010) studied the effect of some plant extracts on the microbial spoilage of *Cajanus cajan* and found that several plants extract (including *G. kola*) were effective against the microbial spoilage. In another study, Ejele and Akujobi (2011) evaluated the effects of secondary metabolites of *G. kola* extract on the microbial spoilage of *C. cajan* extract and found that the acidic metabolite totally inhibited the microbial growth. In a more recent study, Ejele *et al* (2012) isolated and compared the antimicrobial potential of two crude phenolic compounds obtained from *G. kola* extract and showed that the product isolated from the basic metabolite possessed a greater antimicrobial activity and inhibited the growth of several human pathogens, including *Candida albicans*, *Coliform bacilli*, *E. coli*, *Pseudomoniaspp.*, *Salmonella typhi*, *Staphylococcus aureus* and *Streptococcus spp.* with inhibition zone diameters ranging between 10 and 30 mm.

These studies show that *G. kola* seed extract could be an important source of useful bioactive compounds, which may be employed for the treatment of infections caused by several human pathogens. In this paper we report the isolation, purification and partial characterization of a bioactive antibiotic compound from the basic metabolite of *G. kola* seed with a view to identify the active principle responsible for the observed antimicrobial properties of *G. kola*.

MATERIALS AND METHODS

Plant Material: Fresh *Garcinia kola* seeds were obtained from the open market in Owerri, Imo state of Nigeria. The seeds were sun-dried for a period of one week after which they were ground to semi-powder. 50g of the ground seeds was put in a Soxhlet extractor, fitted with reflux condenser and extracted with 250 ml of ethanol for 12 h. The solvent was evaporated at reduced pressure using Rotary evaporator. The solid residue obtained was dissolved in ethanol/water mixture (4:1) and filtered. The filtrate was used without further purification for the preparation of the basic metabolite as described by Ejele and Alinnor (2010).

Preparation of Basic Metabolite: The filtrate obtained above was treated with dilute HCl in a separatory funnel and extracted with chloroform. The lower chloroform layer was removed and the HCl layer was treated with Na_2CO_3 powder until the mixture became basic, allowed to stand overnight and filtered (Ejele and Alinnor, 2010).

Isolation of Bioactive Principle: The bioactive compound was isolated from the basic metabolite as described by Ejele *et al* (2012). The filtrate obtained above was acidified with conc H_2SO_4 . A reddish-brown precipitate was obtained, allowed to stand overnight and filtered. The crude mixture of phenolic compounds (called **Tannin-1**) was washed with distilled water and allowed to dry in air (Ejele *et al*, 2012).

Chromatographic Purification: The crude mixture obtained was purified using silica gel packed in a chromatographic column. A slurry of finely powdered silica gel (in chloroform) was packed in glass column to a height of about 12" and loaded with 10ml of the isolated phenolic mixture dissolved in ethanol and separated by gradient elution with chloroform/ethanol mixture (ratio 1:2) and finally eluted with 95% ethanol. Different fractions were collected at intervals of 1h, although differently coloured compounds were collected in different conical flasks. The solvent in each flask was allowed to evaporate at room temperature and the different fractions obtained were screened for antimicrobial activities. The results are presented in Table 1.

Antimicrobial Screening of Various Fractions:

This experiment was carried out at the Department of Microbiology, Federal Medical Centre, Owerri, Imo State Nigeria, using the disk diffusion method as described by Garred and O. Graddy (1983). An inoculating loop is touched to four or five isolated colonies of the pathogen growing on agar and then used to inoculate a tube of culture broth. The culture was incubated for a few hours at 35-37°C until it became slightly turbid and was diluted to match a turbidity standard. A sterile cotton swab was dipped into the standardized bacterial test suspension and used to evenly inoculate the entire surface of an agar plate. After the agar surface has dried for about 5 minutes, the appropriate antibiotic test disk of each fraction was placed on it and the agar plate was immediately placed in incubator maintained at 35-37°C. After 16-18h of incubation, the diameters of zones of inhibition were measured and recorded. The results are shown (Table 1). The fraction with highest inhibition zone diameter was selected for spectroscopic identification using IR and NMR analysis.

Spectroscopic Analysis: IR spectroscopic analysis of the selected antimicrobial active fractions was performed at the Central Research laboratory, University of Ibadan, using the Perkin-Elmer IR equipment while the NMR analysis was carried out at the Obafemi Awolowo University, Ile-Ife, Nigeria using the VNMR-1 Mercury-20088 machine. The solvent used was deuteriated-methanol or DMSO.

RESULT AND DISCUSSION

Chromatographic Purification / Antimicrobial Results:

Six different fractions were obtained from the chromatographic separation of the polyphenolic mixture and identified as: F₁, F₂, F₃, F₄, F₅ and F₆. Each fraction was considered as a pure natural product. The results of antimicrobial activities of these fractions and the control against the test microbes; *Candida albicans*, *Coliform bacilli*, *E. coli*, *Salmonella typhi*, *Staphylococcus aureus* and

Streptococcus spp. were presented in Table 1 from which it was observed that the control (Amoxil) had no inhibitory activity against *Salmonella typhi* and *Candida albicans* (at the concentration of 0.3mg/ml), but showed bioactivity against *E. coli*, *Staphylococcus aureus*, *Streptococcus spp.* and *Coliform bacilli*. All the fractions (F₁, F₂, F₃, F₄, F₅ and F₆) showed antimicrobial activity against *Salmonella typhi* although F₅ exhibited highest inhibition zone of 32mm.

Table 1: Results Of Antimicrobial Screening Of Various Fractions

Microbes	Zone of inhibition of the different fraction (mm)					
	F ₁ , F ₂ 0.3mg/ml	F ₃ 0.3mg/ml	F ₄ 0.3mg/ml	F ₅ 0.3mg/ml	F ₆ 0.3mg/ml	Control (amoxil) 0.3mg/ml
<i>E. coli</i>	15mm	12mm	20mm	10mm	28mm	15mm
<i>S. aureus</i>	14mm	12mm	15mm	10mm	18mm	10mm
<i>Strepto. spp</i>	10mm	15mm	20mm	17mm	30mm	20mm
<i>C. bacilli</i>	10mm	12mm	18mm	10mm	12mm	20mm
<i>S. typhi</i>	16mm	10mm	15mm	32mm	27mm	0mm
<i>C. albicans</i>	0mm	0mm	30mm	0mm	27mm	0mm

Candida albicans was not susceptible to F₁, F₂, F₃ and F₅ as only F₄ and F₆ showed activity against this microbe. F₄ exhibited the highest inhibition zone of 30mm. The activity of Amoxil (control) was equal to that of F₁, F₂ against *E. coli*, F₄ against *Streptococcus spp.* and F₅ against *Staphylococcus aureus*, as shown by their respective zones of inhibition. Also the activities of F₁, F₂ and F₃ against *Streptococcus spp.*, *Salmonella typhi* and *Coliform bacilli* as well as F₂ against *E. coli* and F₆ against *Coliform bacilli* were smaller than that of Amoxil (control). In general, it may be said that F₁, F₂ and F₃ had lower antimicrobial potential while F₄ and F₆ showed greater antimicrobial potential compared to Amoxil, a conventional antibiotic drug used as the control in this study. However, fraction F₆ was chosen for IR and NMR analysis because it showed a better antimicrobial activity against the microorganisms.

Spectroscopic Results

Results of the IR and NMR analysis are presented in Table 2, which shows the absorption peaks (cm⁻¹) of different functional groups of the F₆ fraction and the chemical shifts of different protons present in the molecule. The IR spectrum showed a strong broad absorption peak at $\delta = 3360\text{cm}^{-1}$ indicating presence of hydrogen-bonded -OH group probably, of a phenol or alcohol. The sharp peak at $\delta = 3072\text{cm}^{-1}$ probably indicates the presence of C-H group of benzene while the peak at $\delta = 2902\text{cm}^{-1}$ corresponds to C-H absorption of aliphatic group. The weak wave-like absorption peaks around $\delta = 2728$ and 2528cm^{-1} probably suggests presence of carboxylic acid (-COOH) while the peak at $\delta = 2355\text{cm}^{-1}$ suggests presence of triple bond of alkyne or cyanide. The peak at $\delta = 2135\text{cm}^{-1}$ probably shows substitution pattern of the benzene ring in the

molecule. The peak at $\delta = 1638\text{cm}^{-1}$ probably indicates presence of amide, carboxylic acid, α,β -unsaturated ketone (-C=C-C=O) or quinone. The twin peaks at $\delta = 1451$ and 1389cm^{-1} are due probably to presence of -CH₃ or -CH₂- of methylene groups, suggesting the presence of aliphatic groups in the molecule. The strong sharp peak at $\delta = 1050\text{cm}^{-1}$ probably indicates presence of C-O bond stretch of alcohol, carboxylic acid, amide or phenol while the peak at 880cm^{-1} probably shows para-substitution in benzene ring.

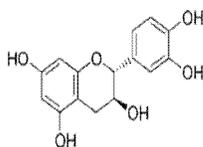
Based on absorption peaks in the IR spectrum of F₆, the Fluka library of Perkin-Elmer, manufacturers of the equipment, suggested following compounds as possible structures for F₆: *α -Methyl-dl-tyrosine*, *5-Aminotetran*, *L-Alanine*, *α,β -dichloroisobutyric acid (sodium salt)*, *Catechin*, *2,6-dichlorophenolindophenol (sodium salt)*, *Pyridine hydrobromide perbromide*, *p-naphtholbenzein*, *Naringin*. *Thiourea*.

The ¹H NMR spectrum of F₆ showed peaks at $\delta = 12$, probably indicating the presence of carboxylic acid (RCOOH), peaks around $\delta = 9-10$, which suggest presence of aldehydic proton (H-C=O). The peaks around $\delta = 6.5-8$ show the presence of benzene ring (Ar-H), the nature of which indicates that the benzene ring may be para-disubstituted. The peak at $\delta = 5.8$ probably indicates presence of vinyl hydrogen atom (-C=C-C-H) or α,β -unsaturated ketone (C=CH-C=O) or quinone. The strong and sharp peak around $\delta = 2-3$ is probably indicative of presence of benzylic hydrogen atoms (Ar-CH₂-) or acetyl hydrogen atoms (CH₃C=O). The peaks around $\delta = 0.9-1.7$ are probably due to methyl/methylene hydrogen atoms. Therefore, it may be concluded that the IR and NMR spectra of F₆ suggests that the compound could be carboxylic acid or aldehyde / ketone or phenol (that is

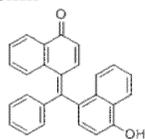
capable of enolization to give quinone) and contains at least one benzene ring with α,β -unsaturated ketone or quinone groups.

The ^{13}C NMR shows peaks around 160ppm, probably due to $-\text{C}=\text{O}$ group of aldehyde / ketone or carboxylic acid, etc; peaks at 130 and 110ppm, probably due to presence of benzene ring; peaks around 95–100ppm due to presence of quinone or vinyl carbon atoms. When the combined spectroscopic data (from IR, ^1H - and ^{13}C -NMR) are considered, it was concluded that the bioactive antimicrobial compound from the basic metabolite of *Garcinia kola* (F_6) could be one of the following:

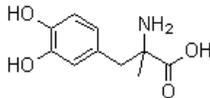
Catechin, p-naphtholbenzein, α -methyl-dl-tyrosine, Naringin



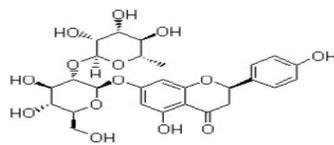
Catechin



p-naphtholbenzein



α -methyl-dl-tyrosine



Naringin

This conclusion was made in view of the fact that all the spectra (IR, ^1H - and ^{13}C -NMR) showed the presence of benzene ring, thereby eliminating from the possible structures those compounds which do not possess the benzene ring.

Several researchers have studied the phytochemical composition of *G. kola* and shown that it contains large proportions of phenolic compounds (Etkin, 1981; Ebana *et al*, 1991; Okunji and Iwu, 1991; Narcissi and Sacor, 1996; Onayade *et al*, 1998; Otor *et al*, 2001; Terashima *et al*, 2002; Ibikunle and Ogbadoyi, 2011). The compounds isolated from *G. kola* include: *alkaloids, cardiac glycosides, saponins, tannins* (Ebana *et al*, 1991; Onayade *et al*, 1998), biflavonoids such as *kolaflavone* and *2-hydroxybiflavonols* (Okunji and Iwu, 1991), *xanthenes, kolanone, amekoflavone, 2,4-methylene-*

cyclartenol, coumarin and phenylatebenzophenones (Narcissi and Sacor, 1996). Two new chromanols, *garcioic acid* and *garcinal*, together with *tocotrienol* have also been isolated (Otor *et al*, 2001; Terashima *et al*, 2002; Ibikunle and Ogbadoyi, 2011). These compounds are potent antioxidants, and have ability to reduce oxidative damage associated with cardiovascular diseases, cancer, diabetes, asthma, hepatitis, liver injury, arthritis and ageing (Field and Lettinga, 1992; Karou *et al*, 2005). The presence of these phenolic compounds indicates that *G. kola* could be useful as anti-inflammatory, anti-clotting, antioxidant agent, an immune enhancer and hormone modulator because phenols are known to modify prostaglandin pathway and protect platelets from clumping (Gislene *et al*, 2000; Ghamba *et al*, 2011)..

SUMMARY AND CONCLUSION

An antimicrobial compound was isolated from the basic metabolite of *Garcinia kola* extract and partially characterized using IR, ^1H and ^{13}C NMR spectroscopy. Analyses of the combined spectroscopic data suggested that the compound (F_6) could be *Catechin, p-naphtholbenzein, α -methyl-dl-tyrosine* or *Naringin*. It was concluded that final identification of this compound would be made more certain after the mass spectrum was obtained. Hence more work would be done and the mass spectrum of on the product obtained to enable final decision of the structure.

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