



Effect of Extraction Solvents on Bioactive Compounds and Antimicrobial Activities of Two Varieties of *Garcinia kola* (heckel) OBOWO 02 (soft and less bitter) and OBOWO 03 (Hard and Very Bitter)

Okwulehie I. C*., Alozie V. C., Ikechukwu G. C., Nwokeocha O. W.

Michael Okpara University of Agriculture, Umudike, PMB-7267, Umuahia, Abia State, Nigeria

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Corresponding Author:

E-mail : phylyke@yahoo.com

Mob.: xxxxxxxxxxxxxxxxx

Abstract

The aim of this study was to compare the effectiveness of the extracts obtained using the different solvents - water, acetone, methanol and ethanol on the phytochemicals and antimicrobial activity of *Garcinia kola* (Obowo 20 and 03). The following 4 bacterial isolates were used for the investigation of the antimicrobial activities and the minimum inhibitory concentration (MIC) of the extracts. The phytochemical analysis of each of the extract indicated the presence of tannin, saponin, flavonoid, Hydrogen cyanide (HCN), alkaloid, carotenoid, anthocyanin and phenol. The extracts exhibited significant inhibitory action against *S. aureus*, *S. typhi*, *P. aeruginosa* and *E. coli*. The result revealed that methanol extract exerted the highest significant activities ($P > 0.05$) against all the tested organisms at the various treatment regimes with *S. aureus* having a wider zone of inhibition followed by *E. coli*, *P. aeruginosa* and *S. typhi* with the lowest inhibitory zones. The MIC of the methanol extract against the organisms was 12.5, 12.5, 25 and 50 mg/ml respectively. The aqueous extract showed the least significant activity against *S. aureus*, *E. coli*, *P. aeruginosa* and *S. typhi* with MICs of 25, 25, 25 and 50mg/ml respectively.

1 Introduction

Plants are important in our everyday existence. Plants are sources of foods. They are involved in the purification of the air we breathe and in the provision of raw materials for use in various industries, for the manufacture of various finished products such as clothes, rugs, footwear, furniture, biofuels, perfumes, dyes, drugs and pesticides. The implication of plants in both traditional and orthodox medicine cannot be overemphasized¹⁻⁵. According to Mahesh and Satish (2008), the parts of plant used in medicine include the twig, stem, root, flower, fruit. The use of these plant parts in medicine spanned throughout all cultures and civilization⁶. Plant derived substances have recently attracted great research interest owing to their versatile application^{1,7,8}.

Generally, some of these plants especially the edible ones are eaten habitually without any knowledge of their pharmacological effects.

Garcinia kola commonly called bitter kola is one of such medicinal plants. Botanically *G. kola* belongs to the family Guttiferae. It is a dicotyledonous plant found mostly in the moist tropical rain forest zone of Nigeria.

The plant which grows to a height of 12 metres can be cultivated using the seed. When chewed, the seed which is the most valued part of the plant has a resinous astringent bitter taste hence the common name bitter kola⁹. It is the bitter ingredient in the seed that actually constitutes the bioactive compound essential for the cure of diseases. *Garcinia kola* is therefore believed to be an important source of new chemical substances with potential therapeutic benefits¹⁰.

2 Materials and Methods

2.1 Collection of samples

The seeds of the two varieties of *Garcinia kola* Obowo 02 (soft and less bitter) and Obowo 03 (hard and very bitter) were purchased from Ubani market in Umuahia, Abia state.

Photograph of the different varieties were taken before they were further used.

2.2 Preparation of samples

The fresh fruits were peeled to remove the shell covering the pulp which was then chopped to small pieces with a knife and sun dried. Thereafter, the dried seeds were ground using a laboratory blender and powdered samples were stored in polythene bags and placed at room temperature until they were used.

2.3 Extraction solvents

Extraction solvents namely: water, acetone, methanol and ethanol were obtained from Hoslab laboratory, Umuahia, Abia state

2.3.1 Preparation of Aqueous, ethanol and methanol extracts.

The extracts were prepared by soaking the sample in each of the solvent at a ratio of 1:10. The mixture was left to stand for 24 hours and then filtered. The filtrate was heated to dryness on a water bath and the dried extract was stored for analysis.

2.4 Phytochemical Analysis

2.4.1 Determination of tannins

The tannin content of the sample was determined by Folin Denms colometric method¹¹. Measured weight of the processed sample (0.2g) was mixed with distilled water in ratio of 1:10 (w/v). The mixture was shaken for 30min at room temperature and filtered.

A standard tannin acid solution was prepared, 2ml of the standard solution and equal volume of distilled water were dispersed into a separate 50ml volumetric flask to serve as standard and reagent blank respectively. Then 2mls of each of the sample extracts was put in their respective flasks and labeled.

The content of each flask was mixed with 35ml distilled water and 1ml of the Folin Denms reagent was added to each. This was followed by 2.5mls of saturated Na₂CO₃ solution. Then each flask was diluted to the 50ml mark with distilled water and incubated for 90min at room temperature. Their absorbance was measured at 760 nm in a colorimeter with the reagent blank at zero.

2.4.2 Determination of saponin

This was done by the double solvent extraction gravimetric method¹¹. 0.2g of the processed sample was mixed with 50mls of 20% aqueous ethanol solution and incubated for 12hours at a temperature of 55^oC with constant agitation. After that, the mixture was filtered through Whatman No 42 grades of filter paper. The residue was re-extracted with 50ml of the ethanol solution.

The combined extract was reduced to about 40mls by evaporation and then transferred to a separating funnel and equal volume (40mls) of diethyl ether was added to it. After mixing well, there was partition and the outer layer was discarded while the aqueous layer was reserved. This aqueous layer was re-extracted with the ether after which its pH was reduced to 4.5 with drop-wise addition of dilute NaOH solution. Saponin in the extract was taken up in successive extraction with 60ml and 30ml portion of named butanol. The combined extract (ppt) was washed with 5% NaCl solution and evaporation dish. The saponin was then dried in the oven (at 600^oc removes any residual solvent); cooling in a desiccators and reweighed.

2.4.3 Determination of alkaloids

The alkaline precipitation gravimetric method of was used¹¹. A measured weight of the processed sample (0.2g) was dispersed in 100ml. of 10% acetic acid in ethanol solution. The mixture was well shaken and allowed to stand for 4hours at room temperature being shaken every 30min. At the end of this period, the mixture was filtered throughout Whatman No 42 grade of filter paper. The filtrate (extract) was concentrated by evaporation; to a quarter if its original volume. The extract was treated with drop-wise addition of concentrated NH₃ solution to precipitate the alkaloid. The dilution was done until the NH₃ was in excess. The alkaloid precipitate was removed by filtration using weighed Whatman No 42 filter paper. After washing with 1% NH₄OH solution, the precipitate in the filter paper was dried at 600^oc and re-weighed after cooling in a desiccator.

2.4.4 Determination of phenols

This was determined by the Folin- ciosptean spectrophotometer (AOAC, 1990). The total phenol was extracted in 200mg of the sample with 10ml concentrated methanol. The mixture was shaken for 30min at room temperature.

The mixture was centrifuged at 500rpm for 15 min and the supernatant (extract) was used for the analysis. 1ml portion of the extract from each sample was treated with equal volume of Folin- Ciosptean reagent followed by the addition of 2ml. of 2% Na₂CO₃. Meanwhile, standard phenol solution was prepared and diluted to a desired concentration. Blue coloration that resulted was measured (absorbance) in a colorimeter at 560nm wavelength.

2.4.5 Determination of flavonoids

Flavonoid was determined using the method described by Harborne (1993)¹¹. A measured weight of the processed sample (0.2g) was boiled in 100mls of 2M HCL solution under reflux for 40 min. It was allowed to cool before being filtered. The filtrate was treated with equal volume of ethyl acetate and the mixture was transferred to a separation funnel.

The Flavonoid extract (contained in the ethyl acetate portion) was received by filtration using weighed filter paper. The weight was obtained after drying in the oven and cooling in desiccators. The weight was expressed as a percentage of the weight analyzed.

2.4.6 Determination of carotenoids

Measured weight of each sample was homogenized in methanol using a laboratory blender. 1:10(1%) mixture was used. The homogenate was filtered to obtain the initial crude extract. 20ml of ether were added to the filtrate to take up the carotenoid mixed well and then treated with 20ml of distilled water in a separating funnel. The other layer was recovered and evaporated to dryness at low temperature in vacuum desiccators. The dry extract was then saponified with 20ml of ethanolic potassium hydroxide and left over night in a dark cupboard. The next day, the carotenoid were taken up in 20ml of ether and then washed with two portions of 20ml of ether and then treated with a light petroleum (petroleum spurt) and allowed to stand overnight in a freezer (-10°C). The next day, the precipitated steroid was removed by centrifugation and the carotenoid extract was evaporated to dryness in a weighed evaporation dish, cooled in desiccators and weighed.

2.4.7 Determination of Anthocyanin

This was done gravimetrically by the method of Harborne¹¹. 0.2g of each test sample was hydrolyzed by boiling in 100ml or Manganese(II) chloride solution for 30min. The hydrolysate was filtered using Whatman No 42 filter paper. The filtrate was transferred into a separation funnel and equal volume of ethyl acetate was added to it, mixed well and allowed to separate into two layers. The ethyl acetate layer (extract) was retained while the aqueous layer was discarded. The extract was separated to dryness in the crucible over a steam bath. The dried extract was then treated with concentration amyl alcohol to extract the anthocyanins.

After filtration, the alcohol extract and the filtrate was transferred to a weighed evaporating dish and evaporated to dryness. It was then dried in the oven at 30°C for 30min and cooled in desiccators. The weight of anthocyanin was determined and expressed as percentage of the original sample

2.4.8 Determination of Hydrogen cyanide (HCN)

This was determined by alkaline pikrate colorimetric method of Balagopalan *et. al.* (1998)¹².

2.5 Antimicrobial activity

2.5.1 Preparation of stock solution of extract

A stock solution of the extract was prepared by dissolving 0.2g (200mg) of the plant extract in 2ml of Dimethylsulphoxide (DMSO) to give 100 mg/ml of the stock solution. Further dilution

was made from this stock solution to yield concentrations of 50mg/ml and 25mg/ml.

2.5.2 Reactivation of test microorganism (stock cultured)

The strains of microorganism used (*Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhi*) were products purchased from the American Type Culture Collection, USA. The organisms were maintained on nutrient agar slants after reactivation on nutrient agar plates. The nutrient agar used was from Biomarkers laboratories, India. A cell suspension of each microorganism was prepared by transferring colonies from the nutrient agar plates to a sterile bottle containing physiological saline. The turbidity was adjusted to McFarland's turbidity standard tube No 0.5 with sterile physiological saline. Similarly, plates of Mueller Hinton Agar (MHA) were prepared according to manufacturer's instruction. The plates containing MHA were allowed to set and using a sterile swab stick, the cell suspension the agar surface and incubated at 37°C overnight

2.5.3 Antimicrobial testing

Disc diffusion test: This was performed following the standard methods. Filter paper discs (Whatman No1) were sterilized in hot air oven inside glass petri dishes at 160°C for 2 hours. Each disc was impregnated with 20ul of the plant extract solution at various concentrations of 25mg/ml, 50mg/ml and 100mg/ml and labeled accordingly. The discs was placed in an incubator and left for 2hours to dry. They were used immediately and the remaining was stored at 4°C.

Similarly, discs of Gentamicin, chloramphenicol and DMSO were also prepared as control. The discs of the extracts and antibiotics were placed on the overnight growth of test organs on MHA with each appropriately labeled at the various levels of concentration. The plates were then incubated at 37°C for 24hours. The diameter of any clear zone of inhibition obtained around the discs was measured manually using a transparent ruler. The experiment was replicated twice for each extract and antibiotics.

2.5.4 Determination of minimum inhibitory concentration (MIC) of plant extract

A set of seven (7) sterile test tubes containing 0.95ml Mueller Hinton Broth (MHB) was arranged in a rack for each of the test organisms. Stock solution of the plant extract (100mg/ml) was diluted by transferring 1ml of the stock solution into the first test tube. To obtain further dilutions, serial dilution technique was employed by transferring 1ml of the mixture in the first tube into the second tube and so on. This was continued till the seventh tube and 1ml was discarded from the seventh tube. This dilution gave a final concentration of 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78mg/ml. Another set of test tubes was labeled organism

control containing MHB and the respective test organisms with no extract.

Similarly, another set of tubes was labeled solvent control containing 1ml of MHB and 1ml of DMSO. This equally was serially diluted as done for the extract. Standard suspension of each of the test organisms were made in Mueller Hinton Broth. Subsequently, each of the test tubes were inoculated with 50ul of the test organism and incubated overnight at 37°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract that prevented visible growth in the test tubes.

3 Results

Table 1: Phytochemical composition of *Garcinia kola* as affected by extraction solvents

Phytochemical	Varieties of <i>G. kola</i>	Water	Methanol	Ethanol	Acetone
Tannin	A	0.36±0.01 ^a	0.94±0.01 ^a	0.87±0.03 ^a	0.97±0.03 ^a
	B	0.35±0.02 ^b	0.92±0.01 ^b	0.86±0.01 ^b	0.96±0.01 ^b
Flavonoid	A	2.27±0.01 ^a	3.16±0.01 ^a	3.34±0.01 ^a	2.85±0.01 ^a
	B	2.20±0.01 ^a	3.16±0.01 ^a	3.26±0.02 ^b	2.78±0.02 ^b
Saponin	A	0.34±0.01 ^a	0.56±0.02 ^a	1.48±0.01 ^a	4.51±0.11 ^a
	B	0.35±0.01 ^b	0.49±0.01 ^b	1.41±0.01 ^b	4.57±0.01 ^a
HCN	A	1.42±0.01 ^a	1.04±0.02 ^a	1.01±0.01 ^a	0.85±0.01 ^a
	B	1.40±0.01 ^b	1.06±0.00 ^a	0.99±0.02 ^a	0.75±0.01 ^b
Alkaloid	A	0.38±0.00 ^b	0.42±0.00 ^b	0.56±0.02 ^b	0.69±0.01 ^b
	B	0.41±0.01 ^a	0.47±0.02 ^a	0.61±0.01 ^a	0.74±0.01 ^a
Phenol	A	0.18±0.01 ^a	0.37±0.01 ^a	0.23±0.01 ^a	0.28±0.00 ^a
	B	0.16±0.01 ^b	0.31±0.01 ^b	0.20±0.01 ^b	0.26±0.00 ^b
Anthocyanin	A	0.48±0.00 ^a	0.53±0.04 ^a	0.68±0.01 ^a	0.53±0.00 ^a
	B	0.47±0.01 ^b	0.43±0.01 ^b	0.60±0.02 ^b	0.51±0.01 ^b
Carotenoid	A	0.04±0.00 ^a	0.09±0.01 ^a	0.07±0.01 ^a	0.97±0.01 ^a
	B	0.04±0.00 ^a	0.07±0.00 ^b	0.05±0.01 ^b	0.90±0.00 ^b

Values are mean ± standard deviation of the three replicates. Means of the two samples with the same superscript letter in each parameter in each column are statistically not different (P>0.05).

The results showed that the plant extracts possessed strong antibacterial activities against bacterial isolates at the various treatment regimes 100, 50, 25mg/ml as indicated in table 2. The results revealed that the methanol extract exerted significant activities (P>0.05) against all the tested bacteria at the various treatment regimes with *Staphylococcus aureus* having a wider zones of inhibition followed by *E. coli*, *P. aeruginosa* and *S. typhi* with the lowest inhibitory zones. The minimum inhibitory concentration of methanol extract against the organisms was 12.5, 25 and 50mg/ml for *E. coli*, *P. aeruginosa* and *S. typhi* respectively as shown in table 3. The *in vitro* activity of ethanol

The results of phytochemical composition of the two varieties of *Garcinia kola* are presented in table 1. The result shows that tannin, flavonoid, saponin, HCN, alkaloid, phenol, anthocyanin and carotenoid were present in *G. kola*. Tannin was most extracted with acetone in varieties A and B (0.97±0.03 and 0.96±0.01), respectively and least with water (0.36±0.01 and 0.35±0.02). Flavonoid was most extracted with ethanol and least with water. Saponin was most present in acetone extract and least with water. Alkaloid, phenol, anthocyanin and carotenoid were least present in the water extract and most present in acetone, methanol, ethanol and acetone extracts respectively. HCN was most extracted with water and least extracted with acetone.

extract was also showed to exhibit inhibitory effects (P>0.05) at the various treatment regime against the *S. aureus*, *E. coli*, *P. aeruginosa* and *S. typhi* with MICs 12.5, 25, 25, and 100 respectively as shown in table 3. The acetone extract also indicated a significant (P>0.05) activity against the bacteria at the various treatment regime with *S. aureus* yielding the highest zones of inhibition followed by *E. coli*, *P. aeruginosa* and *S. typhi* with MICs 12.5, 25, 25 and 50mg/ml respectively.

The aqueous extract also showed activity against *S. aureus*, *E. coli*, *P. aeruginosa* and *S. typhi* with MICs of 25, 25, 25, and 50

respectively as shown in table 3. From the result, Gentamicin was used as a control and it showed zones of inhibition against

S. aureus, *E. coli*, *P. aeruginosa* and *S. typhi* (32, 29, 27 and 23) respectively.

Table 2: Diameter of zone of inhibition (mm) produced by *G. kola* against the test organisms

Organism	Samples	Methanol			Acetone			Ethanol			Water			Gentamicin (control)
		100	50	25	100	50	25	100	50	25	100	50	25	
<i>E. coli</i>	A	11.35 ^a	10.35 ^a	8.5 ^a	6.0 ^b	5.0 ^b	3.00 ^b	9.70 ^a	6.0 ^a	3.0 ^b	7.10 ^a	4.50 ^a	3.0 ^a	29
	B	10.50 ^b	8.0 ^b	6.5 ^b	9.45 ^a	6.25 ^a	4.05 ^a	9.25 ^b	5.65 ^b	3.75 ^b	6.80 ^a	4.10 ^b	2.90 ^a	
<i>S. aureus</i>	A	13.70 ^b	10.30 ^a	8.40 ^a	10.0 ^a	6.50 ^b	4.00 ^b	12.60 ^a	9.50 ^b	6.10 ^b	6.70 ^a	4.35 ^a	3.10 ^b	32
	B	11.88 ^a	9.90 ^b	8.10 ^a	9.45 ^b	7.35 ^a	5.45 ^a	11.60 ^b	8.50 ^b	5.90 ^b	6.70 ^a	4.40 ^a	3.50 ^a	
<i>P. aeruginosa</i>	A	10.10 ^a	7.90 ^a	5.0 ^a	8.10 ^a	5.10 ^b	3.50 ^b	8.50 ^a	6.10 ^a	4.60 ^a	6.0 ^a	4.65 ^b	3.60 ^a	27
	B	19.45 ^b	7.30 ^b	4.50 ^b	7.70 ^b	6.65 ^a	4.10 ^a	7.75 ^b	6.0 ^a	4.10 ^b	7.30 ^b	5.10 ^a	3.90 ^a	
<i>S. typhi</i>	A	6.00 ^a	3.60 ^a	0.00 ^a	5.85 ^a	3.05 ^a	0.00 ^a	4.0 ^a	0.00 ^a	0.00 ^a	4.10 ^a	3.10 ^a	0.00 ^a	23
	B	5.60 ^b	3.10 ^b	0.00 ^a	3.75 ^b	0.00 ^b	0.00 ^a	3.60 ^b	0.00 ^a	0.00 ^a	4.0 ^a	3.0 ^a	0.00 ^a	

Values are means of triplicate determination. Value in the sample column of sample A and B with same superscript letter are not significantly different (P>0.05)

Table 3: Minimum Inhibitory Concentrations (MIC) of the extracts against test organisms

Organisms	Sample	Methanol	Acetone	Ethanol	Water
<i>E. coli</i>	A	12.5	25	25	25
	B	12.5	25	25	25
<i>S. aureus</i>	A	12.5	25	12.5	25
	B	12.5	25	25	25
<i>P. aeruginosa</i>	A	25.0	25	25	25
	B	25.0	25	25	25
<i>S. typhi</i>	A	50	50	100	50
	B	50	50	100	50

Values are means of triplicate determination

4 Discussions

This study shows that *Garcinia kola* contains tannin, flavonoid, saponin, HCN, alkaloid, phenol, anthocyanin and carotenoid. Flavonoid and saponin were the most abundant. Similar results were also obtained by Nyananyo *et al.*, 2010 on some medicinal plants who observed the presence of flavonoids, tannins, alkaloids and saponins in large quantities¹³. Flavonoid which are hydroxylated phenolic substances are known to be synthesized by many plants in response to infection by some pathogens. Their influence stems from the fact that they are able to complex with bacterial cells wall as reported by Marjorie, (1996)¹⁴.

Flavonoids are also effective antioxidant and show strong anticancer activities. The medical potential of the seeds of *G. kola* is further emphasized by the presence in large quantity of saponins. The characteristics of saponins include hemolytic activity, cholesterol-binding properties and bitterness¹⁵. This reveals the reason why variety B had a more bitter taste than variety A in this study.

Phytochemical screening using water, methanol, ethanol and acetone on the seeds of *G. kola* have been used as extraction solvents by many authors, however alcohol solvents such as methanol, and ethanol have been found to be more suitable than the other solvent of medicinal plants¹⁶⁻¹⁸. One rational explanation to this observation is that the solubility of phytochemicals differs depending on the solvent of extract. As a result, some metabolites which are sparingly soluble in water but completely soluble in other solvents would be extracted lesser in water¹⁹. The result of the present investigation which shows methanol as the best solvent for extraction of the active ingredients of the seeds of *G. kola*. Similarly this study reveals that the extract obtained with methanol, ethanol, acetone and water as solvents possess in vitro antibacterial activities at varying concentrations, as also reported by Esimone *et al.*, (2007)²⁰, but the extracts obtained using, methanol is more active, while aqueous extract showed least activity. This is in conformity with the findings of Muna *et al.* (2011)²¹.

5 Conclusion

One could rationally attribute the antimicrobial properties of *Garcinia kola* to its bioactive content. Moreover, this study suggested that methanol is a better solvent for the full exploitation of the therapeutic potentials of *Garcinia kola* since it exhibited higher antimicrobial properties than the other extracts.

Hence, *Garcinia kola* is a potential antimicrobial candidate that helps in the management of degenerative diseases.

6 Recommendations

Based on the results of this study, I recommend that: Methanol should be used as the best extraction solvent for an effective antimicrobial activity of *Garcinia kola*.

7 Conflicts of interest

None

8 Author's contribution

OIC and AVC, carried out literature review, carried out the experiment, did the analyses IGC drafted the manuscript while NOW participated in reading and approval of the final work.

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