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Brine shrimp lethality and antimicrobial studies on the seeds of *Garcinia kola* (Heckel)

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Abstract

Garcinia kola (Family, Guttiferae) is employed in a variety of therapies ranging from skin, gastrointestinal, chest to tumour problems. Preparations of the stem and roots are used as antitumour in traditional medicine but the potential of the seeds as antitumour had not yet been investigated hence the brine-shrimp lethality and as well as the antimicrobial studies on the seed were carried out. The crude ethanolic extract and methanol fraction elicited good antibacterial activity against *S. aureus, B. subtilis* and, to some extent, *E. coli* but gave minimal activity against *Ps. aeruginosa* and none against fungal isolates (*Aspergillus* spp and *C. albicans*). The brine shrimp lethality assay, analysed using the Finney probit method, showed that the aqueous and crude ethanolic extracts and methanol fraction displayed 'marginal' LD_{50} values at 565ppm, 349ppm and 316ppm respectively compared with literature values below 200ppm which are generally considered "significant". These findings indicate the potential of the seeds as panacea for infectious ailments and therefore scientific justification to some of the folkloric uses of the plant.

Keywords: Brine-shrimp lethality; Antimicrobial; Seeds; *Garcinia kola*.

Introduction

The genus; *Garcinia* which comprises of about 180 species is one of the many genera in the family; Guttiferae. Some of these species include *G. dulcis*, *G. opaca*, *G. spicata* and most especially; *Garcinia kola*. *G. kola* (Heckel) is a tropical tree found in the rain forest zone of West Africa, thriving in moist places near homes. It is widely known and used in Nigerian traditional medicine, especially in the South Eastern Nigeria where it is of high economic value (Keay, 1989).

The stem, roots and branches are used to make chewing sticks and are claimed to inhibit bacterial cultures from oral flora (Fadulu, 1975). The decoctions of the cut and peeled roots are used as analgesic, antidysentry and antitumour preparations and powdered stem bark is used to treat malignant tumours. The root bark is used in tanning and added to palm wine to increase its intoxicating effects (Aplin *et al.*, 1967). The aqueous infusion of the leaves is used to treat malaria by hydrotherapeutic bathing while the

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sap is used to treat parasitic skin infections, gonorrhea and also aid in healing fresh wounds. The masticated nut pulp is used as an antidote to North -- West African arrow poison (Cotteril and Scheinman, 1978). The extract of the seeds is used to treat bronchitis, throat infections, cough, chest cold, relieve diarrhea (Irvine,1961) inflammation of the respiratory tracts, liver cirrhosis (Iwu *et al.* 1990) and to induce aphrodisiac action (Ainslie, 1937). The pulverized seeds together with powdered ginger and garlic are taken with honey to treat tuberculosis and when mixed with palm oil, honey, milk and an un-named leaf is used in treating cases of snake, scorpion and ordinary poison (A.T.H.C., 1988) The seeds have been found to be anti-hepatotoxic (Iwu,1985), inhibit histamine release, platelet adhesion (Adegoke *et al,*1998)and give bittering values which significantly increase to levels comparable to hops on extracting the seeds with organic solvents (Aina and Uko, 1991).

Consequent upon these findings and the uses of *G. kola* in ethnomedicine; this study was designed to determine if the seeds would demonstrate brine shrimp lethality; determinable from the brine-shrimp lethality assay which is a novel, rapid bench top bioassay technique used to predict anti-cancer or anti-tumour ability of bioresources such as medicinal plants and as well as the antimicrobial activity.

Experimental

Materials. The seeds of *G. kola* were bought at the Uyo Main Market in July 2003. Botanical verification and identification were done by the taxonomist; Mr. Abia Williams of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmacy, University of Uyo where a voucher specimen (No 1142) was deposited. Sea water (collected from Kuramo Beach, Lagos), plastic soap case and brine-shrimp eggs (*Artemia salina* Leach) obtained from San Francisco Bay Brand Inc; Newark, CA 94560, U.S.A.

Chemicals, Microorganisms and Media.
The chemicals (reagents) used in this study were: Ethanol , Methanol, Toluene, Chloroform, Acetone and Ammonia; all of AnalaR grade and were obtained from the British Drug House Chemicals Limited, England, Silica gel (254GF), Chloramphenicol and Ampicillin (Healthcare Products Ltd, Lagos , Nigeria) while the micro-organisms used (gram positive bacteria; *Bacillus subtilis* and *Staphylococcus aureus* , gram negative bacteria; *Escherichia coli* and *Pseudomonas aeruginosa* and the fungi; *Aspergillus spp* and *Candida albicans*) were obtained from the Medical Laboratory of the University of Uyo Health Centre and the Pharmaceutical Microbiology and Parasitology Unit of the Faculty of Pharmacy, University of Uyo, Akwa Ibom State, Nigeria. The media used were Mueller Hinton Agar II, Sabouraud Dextrose Agar and Nutrient Broth (Oxoid Ltd, Basingstoke, England).

Extraction and Processing.
The fresh seeds were peeled and pulverized using a wooden mortar and pestle. 200g each of the ground seeds were separately extracted with distilled water and 50% ethanol respectively at 25°C for 48 hours. The filtrates were evaporated to dryness on a rotary evaporator. The dried aqueous and crude ethanolic extracts were then investigated for plants metabolites (alkaloids, saponins, tannins, cardiac glycosides, terpenes, anthraquinones, flavonoids and phlobatannins) according to the laid down phytochemical methods (Akerele, 1984; Harbone, 1984; Brain and Turner, 1975; Stahl *et al.*,1965).

The dried crude ethanolic extract was then chromatographed on silica gel (254GF) column and separated with toluene/acetone/methanol mixture in a gradient elution. The separation was monitored by TLC using toluene: acetone:

water (1:2:1). Eluates showing similar TLC profiles were pooled and bulked separately resulting in toluene, acetone and methanol fractions which were evaporated to dryness and then subjected to antimicrobial screening.

Antimicrobial Sensitivity Test.

The micro-organisms used [*B. subtilis S. aureus E. coli, Ps. aeruginosa, Aspergillus spp* and *C. albicans* were clinical isolates; identified and typed by conventional biochemical tests (Murray *et al.*, 1985). They were maintained by cryopreservation (Gibson and Khoury 1986).

The media (Mueller Hinton Agar II, Sabouraud Dextrose Agar and Nutrient Broth) were prepared and poured into sterile Petri dishes (diameter, 13.5cm) and allowed to set. The hole-in-plate agar diffusion method was used for the antimicrobial screening. The bacteria were cultured in the nutrient agar while the fungi were cultured in the sabouraud dextose agar. The inoculum of each organism (density) was introduced into each Petri dish. Cylindrical plugs were removed from the agar plates by means of a sterile cork borer to produce wells with diameter of approximately 9.0mm. The wells were equidistant from each other and the edge of the plate (Washington, 1995). Concentrations of 20mg/ml, 40mg/ml and 60mg/ml_of the aqueous and crude ethanolic extracts and the fractions (obtained from the silica gel column) at 10mg/ml were separately introduced into wells. Also, a concentration of 10μg/ml of ampicillin, 1mg/ml of chloramphenicol and 1:1 methanol/distilled water were introduced into separate wells as positive and negative controls respectively. The experiments were carried out in triplicates. The plates were left at room temperature for 24hr to allow for diffusion. The plates were then incubated at 37°C for another 24hr. Zones of inhibition were measured in millimeters (mm).

The minimum inhibitory concentration (M.I.C); the lowest concentration of a compound that inhibits the growth of a micro-organism was determined by the standard two-fold dilution technique (Washington, 1995; Hugo and Russel, 1984). Using the Mueller Hinton II agar as medium for the bacteria and the Sabouraud dextrose agar for the fungi.

Brine shrimp Lethality Assay:

Some sea water was placed in a small plastic tank with perforated dividing dam which was fabricated from a plastic soap case. Some shrimp eggs were added to one side of the divided tank. This side was darkened by covering it with a plastic lid while the other compartment was exposed. The set-up was left for 48hr for the shrimp eggs to hatch and mature as nauplii. Mature nauplii usually swim to the exposed compartment.

A stock solution of sample was prepared by dissolving 20mg of the material in 2ml of 1:1 methanol/distilled water. To obtain the desired final concentrations such as 1000 μg/ml 100μg/ml and 10μg/ml; 0.5ml, 0.05ml and 0.005ml of the stock solution were transferred into the three vials respectively. The solvent was then evaporated by leaving the vials in a vacuum desiccator for 24hr. 10 shrimp nauplii were counted into each vial (i.e. 30 nauplii per dilution). The total volume of solution in each vial was adjusted to 5ml by adding the sea water (5ml/vial). The control (1:1 methanol/distilled water) was prepared in the same way except that the sample of the extract was omitted. The vials were maintained in the laboratory with normal fluorescent illumination and the set-up left for 24hr. The number of survivors, usually swimming was counted with the aid of a magnifying lens for each of the vials at the end of 24hr. Thus, the number of the dead was computed; hence the LD_{50} in ppm (parts per million) was determined using the Finney probit analysis software (Mclaughlin 1988).

Results and Discussion

The phytochemical screening revealed the presence of saponins, tannins, cardiac glycosides, terpenes, flavonoids and phlobatannis in both the aqueous and crude ethanolic extracts. However, alkaloids and anthraquinones were absent in the extracts. It was noted that there was a higher degree of qualitative positive results given by the crude ethanolic extract to the phytochemical tests than those furnished by the aqueous extract (Table 1), thereby suggesting that ethanol was a better extractive solvent than water. This observation then necessitated the chromatographic purification of the crude on silica gel 254GF to give the fractions.

The extracts and the fractions (obtained from the chromatographic purification of the crude ethanolic extract) were screened for antibacterial and antifungal activities using *B. subtilis* and *S. aureus* (gram positive) *E. coli* and *Ps aeruginosa* (gram negative) and the fungi, *Aspergillus spp* and *C. albicans* to represent a desirable spectrum of microbes. The extracts were tested for the antimicrobial activities at 20mg/ml, 40mg/ml and 60mg/ml while the fractions were tested at 10mg/ml. From the results presented on Table 2, it could be inferred that the activities elicited were concentration – dependent. The crude ethanolic extract gave higher antibacterial activity against B. *subtilis, S. aureus* and *E. coli* than the aqueous extract but recorded minimal activity against *Ps. Aeruginosa* but demonstrated poor antifungal activity against *Aspergillus spp* and *C. albicans*. This is not surprising because gram negative bacteria like *Pseudomonas spp* posses sophisticated three – layered envelope which does not allow the permeation of external agents (Brown,1975). Also the antifungal activity given by the extracts and fractions was insignificant probably because of the pleomorphic and facultative nature of these organisms, their integral structures differing from the cell walls of bacteria and resembling those of higher plants and hence limiting the permeation of the substance into them.

It must however be stated that, among the fractions, the methanol fraction at 10mg/ml gave comparable activities as those given by the crude ethanolic extract at 60mg/ml. This could be attributed to its level of purity. The results of the M.I.C determinations as presented on Table 3 generally show that higher concentrations of the extracts were required to inhibit the microbes than those required of the fractions. The same reason of level of purity applies.

The brine-shrimp assay determines the lethalities of materials toward brine-shrimp larvae (nauplii) and in doing so predicts the ability to kill cancer cells in cell cultures, kill various pests and exert a wide range of pharmacologic effects. The shrimp nauplii have been used for a number of bioassay systems in which natural products extracts, fractions or pure isolates are tested a concentrations of 1000μg/ml, 100μg/ml and 10μg/ml in vials containing 5ml of brine and ten nauplii in each of the three replicates (Meyer *et al.,* 1982). The LD_{50} values in p.p.m. are estimated with 95% confidence using the appropriate mathematical estimates; the Finney probit analysis programme being the model routinely employed. The LD_{50} values of the aqueous and crude ethanolic extracts and methanol fraction of the seeds of *G. kola* obtained in this present study with the other plants in literature (Murray *et al.*, 1985; Stahl *et al.*, 1965; Washington 1995) are presented on Table 4. The results show that the aqueous and crude ethanolic extracts and methanol fraction displayed "marginal" LD_{50} values at 565ppm, 349ppm and 316 ppm respectively in comparison to *Myrsine africana* (114 ppm) and *Pogonopus speciosus* (50ppm) whose LD_{50} are considered "significant" (Murray *et al.*, 1985; Stahl *et al.*, 1965; Washington 1995).

Preparations of the stem bark and roots of *G. kola* tree are used as antitumour in ethnomedicine. The results obtained with the seeds in this study have revealed a possible correlation between the traditional ethnomedicinal use as an antitumour and the brine shrimp lethality.

Table 1: Phytochemical screening of the aqueous and crude ethanolic extracts of the seeds of *G. kola*

Plant metabolite	Test	AE	CEE
Alkaloids	Dragendorff's	-	-
	Mayer's	-	-
Saponins	Froth	++	+++
	Emulsion	++	+++
Tannins	Ferric chloride	++	+++
Cardiac glycosides	Salkowski's	+	++
	Lierbermann- Burchard's	+	++
Terpenes	Sulphuric Acid	+	++
Anthraquinones	Borntrager's	-	-
Phlobatannins	Hydrochloric Acid	+	++
Flavonoids	Shinoda's	++	+++

Key: AE = Aqueous extract; CEE = Crude ethanolic extract; + = Trace; ++ = Moderate; +++ = Abundant; - = Absent

Table 2: Antimicrobial sensitivity of extracts and fractions of the seeds of *G. kola* at different concentrations in methonol/ water.(1:1)

Test organism	*Zone of inhibition in millimeters (mm)											
	AE (mg/ml)			CEE (mg/ml)			TF	AF	MF	AMP	CPL	NC
	20	40	60	20	40	60	10mg/ml	10mg/ml	10mg/ml	10μg/ml	1mg/ml	
B. subtilis	4	6	7	5	7.5	9	4	6	10	-	4	-
S. aureus	5	6	8	7	8	10	6	6	10	20	2	-
E. coli	4	5	5.5	5	6.5	9	6	7	10	22	4	-
Ps. aeruginosa	-	2	2	-	2	2	3	3	5	-	1	-
Aspergillus spp.	-	-	1	1	1	1.5	1	1.5	1.5	-	-	-
C. albicans	-	-	1	1	2	2	1.5	3	3.5	-	5	-

Key: AE = Aqueous extract; CEE = Crude ethanolic extract ; TF = Toluene fraction; AF = Acetone fraction; MF = Methanol fraction; AMP = Ampicillin; = CPL = Chloramphenicol = NC = Negative control (methanol/ water - 1:1); *Zone recorded is zone of inhibition less diameter of cup (9mm).

Table 3: The Minimum Inhibition Concentration (M.I.C.) of extracts and fractions of seeds of *G. kola*

Test organisms	M.I.C (μg/ml)*						
	AE	CEE	TF	AF	MF	AMP	CPL
B. subtilis	> 400	>400	ND	ND	200	0.6	ND
S. aureus	> 200	>200	ND	ND	ND	1.2	ND
E. coli	> 400	>400	ND	>200	250	5.2	ND
Ps. aeruginosa	> 400	>400	ND	>400	300	>100	ND
Aspergillus spp.	1000	1000	ND	ND	ND	ND	92
C. albicans	1000	1000	ND	ND	ND	ND	72

Key: Refer to Table 2; * mean of three determinations; ND = Not done

Table 4: Brine shrimp lethality assay of extracts and fraction of seeds of *G. kola*.

Sample	Concentration			LD_{50} (ppm)
	1000μg/ml	100μg/ml	10μg/ml	
AE	6.7	1.7	1.3	565
CEE	7.3	2.3	1	349
MF	9.7	7.7	2.3	316
Myrsine africana (ethanolic extract of root)				114
Pogonopus speciosus (dry sap)				50

Key: Refer to Table 2.

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