

Phytochemistry And Preliminary Toxicity Studies Of The Methanol Extract Of The Stem Bark Of *Garcinia kola* (Heckel)

H Kagbo, D Ejebe

Citation

H Kagbo, D Ejebe. *Phytochemistry And Preliminary Toxicity Studies Of The Methanol Extract Of The Stem Bark Of *Garcinia kola* (Heckel)*. The Internet Journal of Toxicology. 2009 Volume 7 Number 2.

Abstract

Aim of the Study:

Garcinia kola Heckel stem bark is used by traditional medicine practitioners in Ogoni, Nigeria, to treat dysmenorrhea and burns. The decoction and infusion of this stem bark is often taken without any standardized measurement, sometimes in large amounts; hence the need to investigate its phytochemical constituents and acute toxicity profile. The methanolic extract of this part of the plant was therefore investigated for its phytochemical constitution and the Median lethal dose (LD₅₀).

Materials and Methods

: Standard phytochemical screening procedures and internationally acceptable methods for LD₅₀ determination were used. Screening of the effect of the extract on hemoglobin concentration, packed cell volume and red blood cell count was also carried out in rats.

Result

: The result showed a high tannin, phlobatannin and glycoside content; considerable amounts of alkaloid and flavonoid and negligible or no saponin, terpene or anthraquinone. The 24 hour LD₅₀ value, calculated with two arithmetic methods (Arithmetic method of Karber and Arithmetic method of Reed & Muench) was shown to be 358mg/kg.

Conclusion

: The alkaloid and flavonoid content of plant materials has severally been reported to be a major antioxidant, anti-inflammatory and analgesic active principle; while tannins and phlobatannins has been reported to have wound healing properties. The result of this study scientifically proves the rationale behind the traditional use of this part of the plant to treat dysmenorrhea and burns. However, with an LD₅₀ value in the "very toxic" classification range (50 - 500mg/kg), there is need for caution in the "dose" of decoction of this plant part administered in trado-medical care. However, the constituent of the methanol extract of *G. kola* stem bark extract do not seem to impart any significant toxicological effect on the erythrocytes, rather it showed the tendency of increasing the erythrocyte number over time

INTRODUCTION

The use of plants in traditional medical practice has a long drawn history, and remains the mainstay of primary health care in most of the third world (Prescott-Allen and Prescott-Allen, 1982). Traditional medicines are used by about 60% of the world population; in both developing and developed countries (where modern medicines are predominantly used)

(Mythilypriya et al., 2007). Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic benefits (Farnsworth, 1989; Eisner, 1990).

Garcinia kola (Heckel), an angiospermae, belonging to the family Guttiferae, is known in commerce as bitter cola. It is a plant found in the West African sub region, from Sierra Leone to Southern Nigeria, mostly in moist conditions

On chewing, *G. kola* seed has a bitter astringent and resinous taste, somewhat resembling that of raw coffee, followed by a slight sweetness. Bitter cola is a highly valued ingredient in African ethnomedicine because of its varied and numerous uses which are social and medicinal; thus making the plant an essential ingredient in folk medicine.

The relevance of a plant to pharmacy lies in the ability of the plant to elaborate organic compounds that possess pharmacological properties, or compounds that are of use in pharmaceutical formulations as flavouring agents or formulation aids. For example, some plant secondary metabolites such as alkaloids, phenols, tannins, glycosides, terpenoids, saponins, flavonoids and steroids have been implicated in their ability to inhibit the formation of pro-inflammatory signalling molecules such as prostaglandin or leukotrienes (Polya, 2003).

Recently discovered substances that have analgesic properties included those of the alkaloids, flavonoids and terpenoids phytochemical classes (Musa et al, 2008)

Constituents of the seed of *G. kola* include 1-3, 8-11 benzophenones, *Garcinia* biflavonones (GB-1, GB-2) and kolaflavonone (Cotterih et al., 1978). Apigenin based flavonoids represent 60% of the total flavonoids present in the diethyl ether fraction of *G. kola* seeds (Iwu and Igboko, 1982).

G. kola seeds contains a wide spectrum of organic compounds such as flavonoids which confer on it some anti-microbial and anti fungal actions against gram negative and gram positive microorganisms (Bohn, 1968). The biological activities of flavonoids include action against allergies, inflammation, free radicals, hepatoxins (Terashima et al., 2002).

The anti-inflammatory property of flavonoids is believed to result from inhibition of cyclo-oxygenase enzyme (Liang et al., 1999).

Earlier Harley (1970) had reported the antitusive, antitumour and aphrodisiac activities of *G. kola* while the flavonoids extracted from the seeds have been effective in the treatment of capillary fragility, retinal hemorrhage in hypertension, diabetic retinopathy, purpura, rheumatic arthritis, radiation disease, habitual abortion, frostbite, anaphylactic shock, experimental cancer and in the prevention of chromodacryorrhoea produced by dietary and environmental stress (Elekwa, 1985). Despite these various uses, and most times, over long time periods, little toxicological information is available regarding safety following repeated exposure to *Garcinia kola*.

Furthermore, In Nigeria as in other developing countries, traditional medicines are in widespread use; with the practitioners formulating and dispensing the recipes.

The medicaments are prepared most often from a combination of two or more plant products which many a time may contain active constituents with multiple physiological activities and could be used in treating various disease conditions (Pieme et al., 2006; Ogbonnia et al, 2008). They are administered in most disease conditions over a long period of time without a proper dosage monitoring and consideration of toxic effects that might result from such a prolonged use. The warning regarding the potential toxicity of these therapies means that the practitioners should be kept abreast of the toxicity associated with the ingestion of medicinal herbs (Tédong et al., 2007).

In this study, the stem bark of *G. kola* was assessed for its phytochemical constitution and acute toxicity, in order to establish a baseline data for further research on this very important medicinal plant.

MATERIALS AND METHODS

PREPARATION OF PLANT MATERIAL

The stem bark of *G. kola* used for this work was collected from a single tree (*G. kola* trees from four locations around the Niger Delta area of Nigeria; namely: Bori-Ogoni, Warri, Eket and Port Harcourt city) on the outskirts of Port Harcourt city, Nigeria. It (they were) was reduced to smaller pieces and oven dried for some days at 45°C.

The dried plant material was pulverised with a manual grinder, packaged and labelled G1, G2, G3 and G4 according to the locations from which the samples were collected (G1- Bori-Ogoni; G2- Warri, G3- Eket and G4- Port Harcourt city).

100g of each pulverised plant material was soaked in 300ml of methanol and intermittently shaken. The mixture was kept for 72 hrs after which it was filtered with Whatman No. 1 filter paper and concentrated. The filtrate was stored in a refrigerator until required for use.

PHYTOCHEMICAL SCREENING

Phytochemical screening was carried out on the methanolic extract of the stem bark of *G. kola* according to the method described by (Sofowora, 1993, Edeoga et al, 2005) for testing phytochemical compounds.

Test for tannins: About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration for a positive

test.

Test for phlobatannins: Deposition of a red precipitate when an aqueous extract of the plant sample was boiled with 1% aqueous hydrochloric acid was to be taken as evidence for the presence of phlobatannins.

Test for saponin: About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Test for flavonoids: A few pieces of magnesium metal is added to 5ml of the extract and concentrated Hydrochloric acid. A reddish colouration would indicate a positive test for flavonoids.

Test for Terpenes: 3ml of chloroform was added to 0.5g of extract, shaken and filtered; 10 drops of acetic anhydride followed by 2drops of Conc. Sulphuric acid. A reddish brown colouration at the inter face was looked out for, to show a positive results for the presence of terpenes.

TEST FOR CARDIAC GLYCOSIDES

Lieberman's Test: 0.5g of the extract was dissolved in 2ml of acetic anhydride and cooled in ice. Concentrated sulphuric acid was then carefully added.

Keller-Killani Test: 0.5g of the extract was dissolved in 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid.

A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Salkowski Test: 0.5g of the plant extract was dissolved in 2ml of chloroform.

Concentrated Sulphuric acid was carefully added to form a lower layer.

TEST FOR ANTHRAQUINONES

FREE HYDROXY ANTHRAQUINONES

The Borntrager's test for anthraquinones was used. 5mg of the plant extract was shaken with 10ml of benzene, filtered and 5ml of 10% ammonia solution added to the filtrate and

the mixture shaken.

COMBINED ANTHRAQUINONES

5mg of plant extract was boiled with 10ml aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5ml of benzene, the benzene layer separated and half its own volume of 10% ammonia solution added.

LD DETERMINATION

ANIMALS AND EXPERIMENTAL DESIGN

Preliminary test, with 4 mice per pilot dose level, was conducted to establish the range of toxicity so that the proper dose levels could be established for LD₅₀ determinations. With the pilot tests it was possible to establish the highest dose of the extract that killed none of the exposed animals (200mg/kg) and the lowest dose that killed all the animals (500mg/kg). The dose levels used in the acute toxicity study ranged between these two dose extremes.

30 mice weighing between 20 - 25 g were kept in 5 cages (6 per cage) and handled according to standard guidelines for the use and care of laboratory animals.

The animals were maintained on standard animal diet and water. However food was withdrawn 18 h before the start of the experiment according to the method of Amresh et al. (2008).

The LD₅₀ value was calculated with Arithmetic Method of Karber (Turner, 1965; Aliu & Nwude, 1982) and Arithmetic Method of Reed & Muench (Reed & Muench, 1938)

The five groups of mice were administered intraperitoneally with 250mg/kg, 300mg/kg, 350mg/kg, 400mg/kg & 450mg/kg of the extract respectively and the groups were observed for mortality for 24 hours; a sixth group (the control) was administered with serially diluted 5% Na₂CO₃, the solvent in which the extract showed relative solubility.

ASSESSMENT OF HAEMATOLOGICAL PARAMETERS

ANIMALS

50 male Wister albino rats (weighing 200-250g) used for this study were obtained from the Faculty of Pharmacy Animal house, University of Uyo. They were housed in plastic cages, with food and tap water available ad libitum. The rats were randomly assigned to two sets of twenty five animals each. Each set was further classed into five groups of five animals each. They were allowed an acclimatization period

of 5 days, prior to commencement of the tests.

ADMINISTRATION OF GARCINIA KOLA STEM BARK EXTRACT.

Animals in all groups except the controls, were given by gavage, different concentrations of the methanol extract of *G.kola* stem bark powder daily for 7 and 14 days respectively. The control group was given 5% Na_2CO_3 serially diluted with distilled water. Four doses were administered to four different groups of experimental animals (200mg/kg, 400mg/kg, 600mg/kg and 800mg/kg).

COLLECTION AND HANDLING OF BLOOD SAMPLES

All animals were sacrificed at the end of 7 and 14 days of treatment with *G.kola* stem bark extract, and whole blood was obtained via cardiac puncture. The blood obtained was conditioned for subsequent analysis with 0.2ml of the blood placed in vials containing 0.1mg Na_2EDTA anticoagulant for Hb assay and Blood cell (RBC and packed cell volume) estimation.

ESTIMATION OF PACKED CELL VOLUME (PCV)

Blood sample anticoagulated with EDTA was made to enter a plain glass capillary tube, one end of which was later sealed with non absorbent sealer clay. The tube was then spun at 11,000 rpm for 5 min in a microhaematocrit centrifuge.

The PCV value was then read, using a microhaematocrit reader.

ESTIMATION OF BLOOD CELL PARAMETERS

Haemoglobin (Hb) concentration and red blood cell (RBC) were estimated using a semiautomatic haematological analyzer (SWELAB IEO Model). The auto counter utilized 20 μl of blood in 16ml of a commercially prepared diluent. The machine's ability to count cells was based on the principle of electronic impedance.

RESULT

PHYTOCHEMICAL ANALYSIS

Phytochemical evaluation of the methanolic extract of *Garcinia kola* revealed the presence of tannins, phlobatannins, alkaloids, glycosides and flavonoids as shown in the table below:

Figure 1

Table 1 Phytochemical profile of *Garcinia kola* stem bark

TEST	OBSERVATION	INFERENCE
Saponins	The froth formed did not persist on warming	G1 – G2 – G3 – G4 –
Tannins	Blue black colour was observed	G1 +++ G2 +++ G3 +++ G4 +++
Anthraquinone i) Free hydroxy anthraquinone	Gold colour was not observed in the ammoniacal phase	G1 – G2 – G3 – G4 –
ii) Combined anthraquinone	No pink, red or violet colour was observed in the ammoniacal phase	G1 – G2 – G3 – G4 –
Phlobatannins	A red precipitate was deposited at the base of the beaker	G1 +++ G2 +++ G3 +++ G4 +++
Alkaloid	A red precipitate was formed	G1 ++ G2 + G3 + G4 +
Terpenes	A reddish brown ring was observed at the interphase	G1 – G2 – G3 – G4 –
Glycosides i) Lieberman's Test	No colour change from violet to blue to green was observed at the interphase for most of the samples	G1 – G2 – G3 – G4 –
ii) Salkowski's Test	A reddish brown ring was seen at the interphase	G1 +++ G2 +++ G3 ++ G4 +++
iii) Keller-Kiliani's Test	A reddish brown ring was observed at the interphase	G1 + G2 +++ G3 +++ G4 ++
Flavonoids	An reddish colour was observed	G1 ++ G2 ++ G3 ++ G4 ++

LD₅₀ Determination

Figure 2

Table 2: Arithmetic Method of Karber

GROUP	DOSE (mg/kg)	DOSE DIFFERENCE	No. DEAD	MEAN DEATH	DOSE DIFF × MEAN DEATH
1	200	50 →	0	← 0.5	25
2	250	50 →	1	← 1.5	75
3	300	50 →	2	← 2.5	125
4	350	50 →	3	← 3.5	175
5	400	50 →	4	← 4	200
6	450	50 →	4	← 5	250
7	500		6		
850					

$$LD_{50} = \text{Lowest Dose that killed 100\%} - \frac{\sum \text{Dose Diff.} \times \text{Mean Death}}{N}$$

Where N= No. of Test animals per group

$$\text{i.e. } \frac{500\text{mg/kg} - 850}{6} = 500 - 142$$

$$LD_{50} = 358\text{mg/kg}$$

Figure 3

Table 3: Arithmetic Method of Reed and Muench

GROUP	DOSE	LOG DOSE	DEATHS	SURVIVALS	CUMULATIVE			MORTALITY RATIO	% MORTALITY
					DEATHS	SURVIVAL	TOTAL		
1	200	2.301	0	6	0	22	22	0/22	0.0
2	250	2.398	1	5	1	16	17	1/17	6
3	300	2.477	2	4	3	11	14	3/14	21
4	350	2.544	3	3	6	7	13	6/13	46
5	400	2.602	4	2	10	4	14	10/14	71
6	450	2.653	4	2	14	2	16	14/16	88
7	500	2.699	6	0	20	0	20	20/20	100

From the table it is seen that 50% mortality lies between 350mg/kg and 400mg/kg

Proportionate distance of the 50% point

$$= \frac{50\% - \text{Next Lowest}\%}{\text{Next Highest}\% - \text{Next Lowest}\%}$$

$$= \frac{50\% - 46\%}{71\% - 46\%}$$

$$= \frac{4}{25}$$

$$= 0.16$$

The logarithm of the dose increment is computed as follows:

$$350\text{mg/kg} = 2.544$$

$$400\text{mg/kg} = 2.602$$

$$= 0.058$$

$$\text{Then } 0.058 \times 0.16 = 0.00928$$

$$\text{Log LD}_{50} = 2.544 + 0.00928$$

$$= 2.55328$$

$$\text{LD}_{50} = \text{Antilog } 2.55328$$

$$\text{LD}_{50} = 358\text{mg/kg}$$

ASSESSMENT OF SOME HEMATOLOGICAL PARAMETERS

Treatment, by gavages with *Garcinia kola* stem extract produced dose dependent changes in hematological parameters (Hb, PCV and RBC) in rats. Treated rats showed significant ($p < 0.001$) dose dependent decrease in haemoglobin (Hb) concentration, packed cell volume (PCV) red blood cell (RBC) count in the first week and a significant reversal (increase) in the second week.

Figure 4

Table 4: Mean values of HB-Conc, PCV and RBC-count for the first 7 days of treatment with stem extract

Parameter	Control	200mg/kg	400mg/kg	600mg/kg	800mg/kg
Hb (g/dl)	14.09±0.4	13.32±0.1***	13.28±1.1***	13.35±0.2***	13.01±0.2***
PCV (%)	44.5±0.6	40.5±0.3***	38.0±1.6***	39.0±0.12***	37.50±3.1***
RBC (10 ¹² /L)	8.21±0.20	6.88±0.16***	6.79±0.02***	6.86±0.07***	6.20±0.02***

*** = $p < 0.001$ (Dunnett's Multiple Comparison Test; comparing the doses with control)

Figure 5

Table 5: Mean values of HB-Conc, PCV and RBC-count for 14 days of treatment with stem extract

Parameter	Control	200mg/kg	400mg/kg	600mg/kg	800mg/kg
Hb (g/dl)	12.34±0.17***	12.87±0.16***	13.28±0.12***	13.48±0.11***	13.45±0.20***
PCV (%)	42.34±0.55***	44.83±0.44***	47.02±0.24***	47.88±0.25***	48.95±0.36***
RBC (10 ¹² /L)	7.09±0.12***	7.87±0.17***	8.20±0.15***	8.73±0.06***	9.23±0.18***

*** = $p < 0.001$ (Dunnett's Multiple Comparison Test; comparing the doses with control)

DISCUSSION

The phytochemistry and median lethal dose (LD₅₀) of the methanolic extract of the stem bark of *Garcinia kola* (bitter cola) was investigated in this study.

Decoction and infusion of *Garcinia kola* (Heckel) stem bark is used by traditional medicine practitioners in Ogoni, Nigeria, to treat dysmenorrhoea.

The result of the phytochemical screening indicated that the stem bark of *G. kola* collected from the various locations, is very rich in tannins and phlobatannins, has a good flavonoid, cardiac glycoside and alkaloid content and is devoid of saponins and anthraquinones (Table 1), this finding contrast with the work of Monago & Akhidue (2002) who reported about the phytochemistry of the seed of *G. kola*. In their report, the seed of *G. kola* has a low tannin content and high saponin content. This difference may be explained by the fact that there could be variation in the occurrence of bioactive compounds in different part of the same plant or even in the same plant parts in plants found in different environments (Elujoba, 1989)

The stem bark of *G. kola* is used by traditional healers in the Ogoni area of Southern Nigeria to treat dysmenorrhoea and burns, among other traditional uses. The phytochemical compounds, flavonoids and tannins, has been severally fingered as anti-inflammatory and analgesic active principles in medicinal plants (Liang et al., 1999; Terashima et al., 2002; Polya, 2003; Musa et al, 2008), the presence of these compounds in the extract may therefore suggest them to be the therapeutic principle behind the traditional antinoceptive use of the stem bark. Furthermore, the work of Bope et al, 1948 described the safety and effectiveness of phlobatannins and tannins in the local treatment of burns.

The high phlobatannin & tannin content of the extract therefore scientifically justify the use of pulverized *G. kola* stem bark to treat burns by the Ogoni people.

G. kola is a potential source of useful drugs. The seed of the plant has been reported to have antihepatotoxic (Braide, 1991; Akintowa and Essien, 1990), hypoglycemic (Iwu et al., 1990; Odeigah et al., 1999), aphrodisiac (Ajibola and Satake, 1992) and antioxidant (Olatunde et al., 2004) properties, among others. The stem bark has not been well researched, but from this phytochemical analysis, there is so much promising value that this part of the plant has.

The haematological studies showed that the indices examined (Hb, PCV, RBC and Platelet counts) showed a dose dependent significant reduction for the animal groups tested in the first week, and a dramatic dose-dependent rise in those tested for two weeks. This could be due to complexes which flavonoid forms with reactive metals such as iron, zinc and copper thereby reducing their nutrient absorption in the first week (Siegenberg, 1991). The formation of these complexes might have affected haemoglobin synthesis and erythropoiesis (Fairbanks, et al 1971, Esomonu et al, 2005). The physiological compensation for the reduced haemoglobin synthesis and erythropoiesis is by iron salvage as ageing erythrocytes are destroyed and the iron transported to the erythroid cells of the marrow and reutilized for new haemoglobin synthesis (Conley, 1974). This was seen in the second week as the values increased significantly.

Furthermore, the antioxidant activity of flavonoids (contained in *G.kola* stem bark powder) which ultimately maintains the haeme iron in its ferrous state (*vis-à-vis* the ferric state that is associated with production of defective methaemoglobin) seem to enhance erythropoiesis. The link between antioxidant activity and haemoglobin quality and quantity was shown by the observation that ascorbic acid (a standard antioxidant) via its action as a free radical scavenger, increased significantly the haemoglobin levels in children suffering from sickle cell anaemia (Jaja et al., 2002; Ahumibe and Braide, 2009).

In conclusion, the constituent of the methanol extract of *G. kola* stem bark extract do not impart any significant toxicological implication to erythrocytes, rather it showed the tendency of increasing the erythrocyte number over time, this property has been confirmed by Esomonu et al, 2005, in the seed of *G.kola*.

It has been argued that even if LD₅₀ values could be measured exactly and reproducibly, the knowledge of its precise numerical value would barely be of practical

importance, because an extrapolation from the experimental animals to man is hardly possible (Lorke, 1983), however, it still serve a great purpose as a first pointer to the safety or toxic potential of a substance whose toxicity profile is not yet known.

The part of the plant under investigation (stem bark of *G. kola*) has an LD₅₀ value of 348mg/kg from the result above (Table 2 and 3). This value rates it as a very toxic substance (Gosselin et al, 1984). Though the effects on the red blood cells may be beneficial over a longer time, there should still be caution in the amount of the decoction of this stem bark which traditional healers need to expose their patients to.

CONCLUSION

The result in this study validate the traditional medicinal use of the evaluated part of *G. kola* in treating gynaecological pains (especially dysmenorrhoea) and wounds from burns; but the acute toxicity result also indicate that the plant part (stem bark) has a “very toxic” profile. This calls for caution in the “dose” of the decoction given to patients in traditional medical practice. Furthermore, the results of histopathological studies, that are ongoing, will throw further light on the organs of the body that are likely to be compromised in case of toxicity.

ACKNOWLEDGEMENTS

The authors really appreciate the assistance of Mr. Danladi Bala and Nsikan Malachy Udo of Departments of Pharmacognosy and Pharmacology & Toxicology respectively, University of Uyo

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Author Information

H.D. Kagbo

Department of Pharmacology, University of Port Harcourt

D.E. Ejebe

Department of Pharmacology & Therapeutics, University of Port Harcourt