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Antithrombotic activities of methanolic extract of *Garcinia kola* in rats

Sylvester C. Ohadoma[1*], Isaac Nnatuanya[2], Paul J. C. Nwosu[3], Joseph C. Enye[1] and Oluchi Nnaeto[1]

[1]Department of Pharmacology; [2]Department of chemical pathology; [3]Department of surgery
Madonna University, Elele Campus, Nigeria.

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Abstract
The antithrombotic activities of methanolic extract of *Garcinia kola* was investigated in rats. Haematological parameters including bleeding time, whole blood clotting time, and platelets count were employed in this analysis. The rats were divided into four (4) groups (A-D). 0.5ml/day of normal saline was administered orally to group A rats serving as negative control group, 250Mg/kg/day of aspirin was administered orally to group B rats. Group C and D rats received 250mg/kg/day and 500mg/kg/day of the extract respectively, which lasted for two weeks before the haematological analysis. The results indicated that group A rats had a mean value of 4.20, 2.06, and 349 for bleeding time, whole blood clotting time, and platelets count respectively. Rats in group B were taken as positive control and showed mean value of 7.05, 2.04, and 64 for bleeding time, whole blood clotting and platelets count respectively. Group C and D indicated mean values of 6.12, 2.24, 88 and 7.67, 2.29, 80 respectively for bleeding time, whole blood clotting, and platelets count. This study indicates that the methanolic extract of *Garcinia kola* definitely exhibits an antithrombotic activity that is dose-dependent.

Keywords: *Garcinia kola*, Antithrombotic activity; Rats.

Introduction

Thrombosis is simply the formation of blood clot inside the blood vessel, thereby obstructing the flow of blood through the circulatory system (Ohadoma, 2008). Thromboembolic disorders are some of the groups of medical conditions arising from disorders in blood function. They are generally implicated as some of the factors that predispose an individual to cardiovascular complications such as ischaemic heart disease, acute myocardial infarction, acute ischaemic stroke, angina pectoris, cerebral ischaemia etc, and these disorders have over the ages been effectively managed by the use of tradomedicinal practices specifically via the use of medicinal plants. These medicinal plants such as *Azadirachta indica, Garcinia kola, and Curcuma longa* had been claimed to have antithrombotic effect. (Olatunde, *et al*., 2002). Traditional medicine popularly known as tradomedicine is defined by the World Health Organization (WHO) as "health practices, approaches, knowledge, spiritual therapies, manual techniques, exercise, applied

* Corresponding author. *E-mail address*: chodraf@yahoo.com, Tel: +234 (0)8035081946
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singularly or in combination to treat, diagnose and prevent illness or maintain wellbeing" (WHO,2003). Statistical evidences have shown that traditional medicine serve as the main means of health care remedy for about 80% of the populace especially those of the third world countries, largely due to their poor economic states as well as inadequate provision of orthodox medicinal facilities (Baurmman, 1992).

Garcinia kola also known as bitter kola (English) Namiji goro (Hausa), Orogbo (Yoruba) and Akilu (Igbo) is a species of flowering plant in the Guttiferae family. According to Ebana (2000) the chemical constituent of the seeds, fruits and bark of *Garcinia kola* include Biflavonoids (Kola viron), Flavonoids, Saponin, Tannins, Phlobatannins, Polyphenols, Glucides, Alkaloids, Cardiac glycosides, Reducing compounds, Hydroxymethyl anthraquinones, Dimethylamines, methlylamine, ethylamines and isopentylamine.

The chemical composition of *Garcinia kola* seed and hull was determined using standard method (Atawodi *et al.*, 1995). Result shows that crude protein, lipid extract, ash and crude fibers ratios are: 39.52g/kg and 99.92g/kg, 43.25g/kg and 92.91g/kg, 11.92g/kg and 18.62g/kg, 114.62g/kg and 153.44g/kg respectively. The dormant saturation of fatty acid was palmitic acid (31.55g/kg and 276.01mg/kg). It also contains neutral lipid (17.3g/kg/58.0g/kg), triacylglycerol of neutral lipids (15.0g/kg/49.6g/kg), glycolipids (3.0g/kg-7.2g/kg) and phospholipids (3.7g/kg–11.2g/kg). It contains unsaturated fatty acids; Oleic acid (38.36g/kg and 52.77mg/kg) and Linoleic acid (36.16, mg/kg and 235.83mg/kg).

The dormant non essential amino acid is glutamic acid (6.80g/kg and 8.10g/kg) while the dormant essential amino acid are lysine and valine (2.40g/kg and 7.10g/kg). Therefore the proportion of essential amino acids in the total amino acid is 44.52% in the hull and 35.81% in the seed. Therefore *Garcinia kola* hull has significantly higher ($p<0.05$) proteins and fibres, but has comparable values for lipid and ash. The most abundant mineral element in *Garcinia kola* seed are potassium and phosphorus (334.82mg/kg and 242.61mg/kg), while phosphorus predominates in the hull (288.61mg/kg). Carbon to Nitrogen ratio for seed and hull are 57.88 and 29.01 respectively. *Garcinia kola* seed has significant higher values of sodium, potassium, copper and cobalt while chromium, molybdenum, manganese, nickel, selenium, lead, mercury were not detected.

Garcinia kola has been demonstrated to be a versatile compound employed as a medicinal plant used to treat and manage a lot of disorders, ailments and disease. *Garcinia kola* seed is used in traditional medicine for various therapeutic purpose based on pharmacological effects of the active components (flavonoid) in the seed and other parts of the plants. It is widely consumed as a stimulant (Atawodi *et al*., 1995). Its traditional African Medicinal uses include; treatment of cough (Okunji and Iwu 1991), diarrhoea (Braide, 1999), bronchitis (Adesina *et al*., 1995), throat infection (Orie and Ekon 1998), and Liver disorders (Iwu and Igboko 1999).

The sap from *Garcinia kola* is used for parasitic skin disease while the latex is used internally for gonorrhea (Odeigah *et al*., 1999). The seeds are rich in flavonoids, which have been shown to have antimicrobial activity (Madubunyi, 1995). *Garcinia kola* has been demonstrated to be effectively against a lot of micro-organism via its inhibitory actions against, *Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Eschericha coli and Nesisseria gonorrhea* (Ebana, 2002). The *Garcinia kola* seed enjoys a folk reputation in Africa as a poison antidote (Kabangu *et al.,* 1998). In

addition the plant possess anti hepatotoxic (Braide 1999), antioxidant (Olatunde *et al*, 2004), hypoglycemic (Iwu *et al*., 2001) and aphrodisiac properties (Ajibola and Satake 1992). *Garcinia kola* seed contains biflavonoid (Kolaviron) capable of exerting anti-inflammatory properties (Braide, 2002).
In this work, we investigated the antithromobotic effect of methanolic extract of *Garcinia kola* to ascertain its acclaimed use in traditional medicine.

Experimental

Collection and preparation of plant material. Fresh *Garcinia kola* fruit was bought from Owerri main market in Imo State. The Epicarp of the *Garcinia kola* was neatly peeled with a blade, exposing the inner milkfish-white fleshy part. The seeds were sliced into very small pieces and dried under ambient temperature until their weight is about the same. The dried *Garcinia kola* seeds were further reduced in size into fairly fine, but coarse sized particles by grinding using a miller.

Preparation of methanolic extract. 50g of grinded sample was macerated in 250ml of methanol for 48hrs. The mixture was separated by sieving with a Whatman filter paper into a clean glass bottle. The extract was concentrated using a rotary evaporator, and was further dried in a hot air oven at a temperature of 50^{o}C. The dried sample was stored in a clean glass bottle.

Animal Used. Wistar Albino rats weighing 90g-138g and Wistar albino mice weighing 25g-32g were employed in this test. Both animals were mixed sexes. They were housed in clean gauzed cages and had free access to food and water.

Test for antithrombotic activity. 20 Wistar rats weighing 90g-138g of both sexes were employed. The animals were grouped into 4 groups.

Group A received 0.5ml of normal saline
Group B received 250mg/kg of Aspirin (standard agent)
Group C received 250mg/kg of Extract
Group D received 500mg/kg of Extract

All administrations were a single dose of oral route. The extract, standard drugs and normal saline were administered to the rats for 14 days, and on the 15th day they were taken for hematological analysis.

Haematological analysis. Analyses carried out include: Bleeding time; Clotting time; Platelet count.

Bleeding time: Ivy's method was used. The animal was placed in a restrainer; its tail was passed through one end of the opening. The tail of the animal was massaged especially at the point where tail joins the pelvis. The tail was disinfected with spirit solution, wiped dried and pricked with a sterile lancet about 4mm deep. The stop clock was started. The oozing blood was wiped with filter paper at 15secs interval. The exercise was repeated every 15secs on fresh spots of the filter paper until the bleeding stopped. The number of blood spots on the filter paper were added and divided by 4 to get the bleeding time in minutes.

$$\text{Bleeding Time} = \frac{\text{No. of Blood Spots on Filter Paper}}{4}$$

Whole blood clotting time: The animal was placed in a restrainer and the tail was passed through one of the hole. The tail was massaged especially at the point it joins the rat pelvic. The tip of the rat tail was disinfected and wiped dry. The sterile scissors was used to clip off the tip of the rat tail. A drop of blood was gradually lifted up at 15seconds interval using a pin, this was continued until coagulation was noted.

Platelet count: Each animal in each group was anaesthetized by confirming them in an enclose container containing cotton wool clamped with chloroform. Then blood sample was collected through cardiac puncture from each animal. The blood sample

was placed in an EDTA container. In the plastic tube, 0.38ml of filtered ammonium oxalate diluting fluid was dispensed, 0.02ml of well mixed EDTA anticoagulated blood was added to the measured diluting fluid in the plastic tube and mixed properly. A cover slip was fixed on the counting chamber unit the Newton ring appeared on the edges of the chamber was filled with the sample mixture. The charged chamber and its contents was placed in a Petri dish on dampened paper and cover with a lid. The chamber was left undisturbed for 20minutes to allow the platelets to settle. The underside of the chamber was dried using the 10x objective, the ruling of the grid were focused and the central square of the chamber was brought into view. The small platelets were focused using the 40x objective and counted.

Calculation:

Counts/liter = No of cells counted x dilution factors x10^6

It should be noted that platelets are smaller than the red cells. They either appear as round, oval or elongated particles. They are refractile, stain light bluish colour and they also appear like dirt or debris. Blood is diluted 1 in 20 in a filtered solution of ammonium oxalate reagent which lyses the cells.

Dilution factor=20

Volume counted = total area of squares counted x depth.

Depth=0.1mm^3

Area of one smallest squares =1/25x1/16=1/400mm^2

Total smallest squares counted =5x16=80 smallest squares.

Let the number of cells counted=N

$$\text{Count/ liter} = \frac{Nx20x16^6}{¼ x80x0.1} = \frac{N\ x400x10^6}{80x0.1}$$

$$= \frac{Nx8000x10^6}{8} = N \times 1000x10^6/L$$

Cells counted = N x10^9/L

Results

The result of the bleeding time, clotting time and platelet count of the four groups (a-d) of the rats are as tabulated.

Discussion

Based on the result obtained as well as from further statistical evaluation, the extract of *Garcinia kola* demonstrated variability in its ability to influence the hematological indices: bleeding time, clotting time as well as platelet count when compared with values obtained from rats treated with the controls, normal saline and aspirin, ever thought in most instances the extract proves to be a good anti thrombotic agent.

On bleeding time (with a normal range of 1-6 minutes) at dose levels of 250mg/kg and 500mg/kg, the extract of *Garcinia kola* greatly and significantly increased the time it took for the rats to stop bleeding when compared with the values obtained from pretreatment with the negative control normal saline (4.200 = 0.11).

Table 1: Result on anti-thromobotic test (Mean ± S.E) (N = 5)

Group	Dose	Bleeding Time (mins)	Clotting Time	Platelet Count
A	0.5ml N. Saline	4.20± 0.11	2.06 ± 0.01	249 ± 10.25
B	250mg/kg Aspirin	7.05± 0.14	2.04 ± 0.10	64 ± 1.44
C	250mg/kg Extract	6.12± 0.15	2.44 ±0.003	80 ± 1.55
D	500mg/kg Extract	7.67± 0.19	2.29 ± 0.08	88 ± 1.20

Table 2: Comparing negative control of Normal saline with positive control of aspirin using T-test

Parameter	Group	X	N	SS	Df	t-calc.	t-critical	p-value
Bleeding time	A	4.14	5	1.31	4	3.18	2.306	* P<0.05
	B	7.05	5	1.85	4			
Clotting Time	A	2.06	5	0.03	4	9.45	2.306	* P<0.05
	B	2.04	5	1.07	4			
Platelet Count	A	349	5	10552	4	997.2	2.306	* P<0.05
	B	64	5	210	4			

Table 3: Comparing control (Normal saline) with dose of extract (250mg/kg) using T-test.

Parameter	Group	X	n	SS	Df	t-calc.	t.critical	P-value
Bleeding time	A B	4.14 6.12	5 5	1.31 2.49	4 4	6.21	2.306	* P<0.05
	A D	4.14 7.67	5 5	1.31 3.85	4 4	11.04	2.306	* P<0.05
Clotting time	A C	2.06 2.24	5 5	0.03 0.58	4 4	6.88	2.306	* P<0.05
	A B	2.06 2.04	5 5	0.03 0.07	4 4	9.45	2.306	* P<0.05
Platelet count	A B	349 64	5 5	10552 210	4 4	997.2	2.306	* P<0.05

Table 4: Comparing control (Normal Saline) with dose of extract (250mg/kg) using T-test

Parameter	Group	X	N	SS	df	t-calc.	t-critical	P-value
Bleeding time	A B	4.14 6.12	5 5	1.31 2.49	4 4	6.21	2.306	* P<0.05
	A D	4.14 7.67	5 5	1.31 3.85	4 4	11.04	2.306	* P<0.05
Clotting Time	A C	2.06 2.24	5 5	0.03 0.11	4 4	2.00	2.306	* P<0.05
	A C	2.06 2.24	5 5	0.03 0.58	4 4	6.88	2.306	* P<0.05
Platelet Count	A D	349 80	5 5	105 52 242	4 4	1015.7	2.306	* P<0.05
	A D	349 88	5 5	105 52 162	4 4	1003.8	2.306	* P<0.05

* P<0.05 = Significant +_ P> 0.05 = there is no significant difference.

Table 5: Comparing the effect of the extract (250mg/kg) with that of the positive control aspirin, using T-test

Parameter	Group	X	N	SS	df	t-calc.	t-critical	P-value
Bleeding time	B C	7.05 6.12	5 5	1.85 2.49	4 4	0.41	2.306	* P<0.05
	B D	7.05 7.67	5 5	1.85 3.85	4 4	8.33	2.306	* P<0.05
Clotting Time	B D	2.04 2.24	5 5	0.07 0.11	4 4	9.6	2.306	* P<0.05
	B D	2.04 2.29	5 5	0.07 0.58	4 4	3.76	2.306	* P<0.05
Platelet Count	B C	64 80	5 5	210 242	4 4	1015.7	2.306	* P<0.05
	B C	64 88	5 5	105 162	4 4	25.0	2.306	* P<0.05

* _ P<0.05 = Significant +_ P> 0.05 = No significant difference,

The prolongation in the bleeding time proves to be does dependent as a dose of 250mg/kg the bleeding time for the rats is 7.05=0.19 minutes. Since bleeding time is an index used to indicate the amount of circulating platelet in blood (Ohadoma, 2008) it then implies that the extract greatly reduced the number of circulating platelets, thereby decreasing the tendency for blood to coagulate via formation of a platelet plug (one of the first processes

involved in blood coagulation that is after vasoconstriction), making it less possible to occur and consequently increasing the time it take for bleeding to stop after its initiation. Since the p>5, this proves to be an insignificant statistical result.

Also when the bleeding time values of the effect of the extract is compared to those obtained from rats pretreated with the positive control aspirin, it was seen that the rats of increment was does dependent because 250mg/kg of aspirin being an anti platelet drug produced a longer bleeding time of 7.05 0.14 minutes when compared to that extract which produced 6.12 0.15 minutes, while the extract at a dose of 500mg/kg has a longer bleeding time of 7.05= 0.19 minutes when compared with aspirin at a dose of 250mg/kg. The then implies that aspirin should reduce to a greater extent, the number of circulating platelet when compared to the extract at a dose level of both 250mg/kg and 500mg/kg. Thus, when compared statistically, there was a significant different ($p<0.05$) as there was a prolongation in the bleeding time at the dose of 500mg/kg (extract).

When comparing the result obtained from the extract, negative control (normal saline) and positive control (aspirin) on the level of platelet circulating in the blood, the results showed a highly inconsistent finding in several instances though not in all respect, as it does correlate the result obtained from the effect of these drug substances from the bleeding time of the rats used, which as an index that gives a rough estimate of the level of circulating platelets in the blood. Comparing the result as turned out by the administration of the extract to that due to the administration of normal saline, it can be observed that the extract at the different does level reduced to an appreciable extent the number of circulating platelets that is from $349+10.25x10^9k$ to $80+1.55x10^9k$ and $88+1.20\ x10^9k$ for doses of 250mg/kg and 500mg/kg respectively ($P<0.05$). this result even though consistent with the fact that the extract is capable of decreasing the plate late level in the blood, does not reflect the ability to do that a greater rate with a higher does as seen with 250mg/kg and 500mg/kg does and hence we may at a significant level of 0.05 say that the effect of the extract is does dependent as it concerns decreasing the platelet number.

Also, comparing the statistically evaluated platelet count values of the extract to those of aspirin, it can be observed that aspirin has greater capacity to decreasing the number of platelet in blood, as 250mg/kg of it has $64+1.44x10^9k$ value of platelet while extract has $88+1.20\ x10^9k$ and $80+1.55\ x10^9k$ at doses of 250mg/kg and 500mg/kg respectively. The observations on the anti-thrombotic properties of the extract with regards to the platelet number when compared with dose of aspirin and normal saline may be due to some possible experimental error or the effect of biological variables in the rats used. Some of these errors are: the ability to manipulate appropriately the experimental procedures or achieve uniformity of experimental condition related to platelet counting error arising from the counting fluid used for dilution, as well the biological variances and the rats used for each group (s).

On a different note, comparing the result obtained from measurement of clotting time (which is an index uses to evaluate a person's clotting mechanism, specially either the defectiveness of its prothrombin activator system or thromboplastin level in blood) due to the effect of the extract at the two does level used (i.e. 250 and 500mg/kg) to those of both normal saline and aspirin, it was observed that clotting time of rats. Hence the extract can be said to be its ability to prolong the respect. It then implies that the extract significantly decrease the level of circulating thromboplastin thereby decreasing the rate of formation of thrombin since prothrombin +

thromboplastin + calcium = thrombin (Ohadoma, 2008).

Conclusion

The result obtained from the pool of statistically evaluated data; shows that the extract of *Garcinia kola* possesses anti-thrombotic action, as it positively influence the hematological indices bleeding time and platelet number, while this is true, the anti-thrombotic effect of the extract however did not prove to be superior to the anti-thrombotic effect produced by the aspirin. It should be noted nevertheless that in the case of clotting time where the extract had a superior effect to aspirin, implying that the extract has a significant ability to decrease the level of circulating thromboplastin in blood. From the result obtained, *Garcinia kola* definitely has anti thrombotic property that is dose dependent.

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