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PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL EVALUATION OF N-HEXANE EXTRACT OF *GARCINIA KOLA* HECKEL [*BITTER KOLA*] SEED

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ABSTRACT

The patented flavonoid extract of *Garcinia kola* seeds for the treatment of liver disorders has been shown to have anti-oxidant and scavenging properties. This study, therefore, evaluated the phytochemical composition of *Garcinia kola* seed and antimicrobial activity of the N-hexane extract of *Garcinia kola* seed against *Pseudomonas aeruginosa; Staphylococcus aureus; Bacillus subtilis; Escherichia coli; Klebsiella pneumoniae* and *Candida albicans.* The zones of inhibition of the N-hexane extract concentrations (100mg, 50mg and 25mg) against the growth of the organisms were determined by the cup-plate agar method. A Marcfarland Standard of each of the bacteria was used to seed sterile molten nutrient agar medium maintained at 45^0 C and Sabourand dextrose agar plate was seeded with *Candida albicans*. Results of phytochemical screening tests showed that *Garcinia kola* seed powder contained starch, protein, glycosides, flavonoids, tannin, saponins, sterols and triterpenoids. The anti-bacterial activity of 100mg extract was highest and was in the order: action on *Bacillus subtilis* > *Pseudomonas aeruginosae* >*Staphylococcus aureus* and *Klebsiella pneumoniae* with zones of inhibition of 10.66mm, 9.66mm, 9mm, 9mm, respectively while that of the 50mg concentration was of the order: *Bacillus* subtilis> *Pseudomonas aeruginosa* and *Staphylococcus aureus* >> *Krebsiella pneumoniae* with zones of inhibition of 8.33mm, 8. 33mm, 8.33mm and 4.66mm, respectively. The 100mg, 50mg and 25mg concentrations of the extract were all poorly inhibitory to *Candida albicans* and were resisted by *Eshcerichia coli.* The triterpenoids found in the extract (possibly in conjunction with the glycosides) are suggested to be responsible for the anti-bacterial and anti-fungal activity of the N-hexane extract of *Garcina kola* seed obtained in this study.

KEY WORDS: Phytochemical, antimicrobial, N-Hexane Extract, Garcinia kola, Seeds.

INTRODUCTION

Maceration methods are based on the immersion of the drug in a bulk of solvent with modification to multiple stage [double or triple] extraction to increase the yield if necessary. In this study, milled *Garcinia kola* seeds were extracted through multi-stage extraction of the macerated powder.

Garcinia kola is chewed in traditional usage to relieve cough, catarrh or for general health promotion. It is also compounded in traditional drug mixtures as a body tonic. *Garcinia kola* seed extract is known to change the taste of any substance eaten after it has been eaten.

Aqueous extract of *Garcinia kola* seed demonstrated inhibitiory effects on gastric acid secretion. [1 ,2] *Garcinia kola* seed extract similarly exhibited antispasmodic [3] and anti-diabetic effects.[4] Its flavonoid constituents have been shown to have liver-protective and antioxidant properties.[4,,5, 6, 7, 8]

This study carried out the phytochemical screening of *Garcinia kola* seed powder to determine its

phytochemical constituents and evaluated the minimal inhibitory concentration of the extract (made from macerated *Garcinia kola* seed powder) to *Pseudomonas aeruginosae; Staphylococcus aureus; Bacillus subtilis; Escherichia coli; Klebsiella pneumoniae* and *Candida albicans.*

MATERIALS AND METHODS

Materials: Materials included *Garcinia kola* seeds (obtained locally) which were powdered after removing the testae, N-hexane extract of the powdered seeds, N-hexane (BDH, Poole, England), all reagents were of Analar grade, Saborandi Dextrose agar (BDH, Poole, England) and bacteria (all of Marcfarland Standard).

Methods: The methods established by [9] were used in the undermentioned tests.

Preparation of *Garcinia kola* seed powder: Peeled *Garcinia kola* seeds were dried under the sun to constant weight, in a clean tray. The dry seeds were broken to pieces and pulverized into a fine coarse powder. The *Garcinia kola* seed powder was subjected to phytochemical screening tests, macerated and extracted to produce the extract whose minimum inhibitory concentration against selected laboratory bacteria and *Candida albicans* was investigated.

Phytochemical Screening of the pulverized *Garcinia kola* Seed Powder:

Test for Starch: 1.5g of the *Garcinia kola* seed powder was shaken in 5ml of distilled water and two drops of iodine were added to it. The appearance of blue –black colour indicated the presence of starch.

Test for Proteins: Two drops of Millions Reagent were added to a solution of 2.0g of the *Garcinia kola* seed Powder. The appearance of a white precipitate showed the presence of proteins.

Test for Alkaloids: 2.00g of the *Garcinia kola* seed powder was boiled with 2ml of dilute HCl in a test tube and filtered. The filterate was divided into 4 portions.

2 drops of Meyer's solution was added to portion '1'. No white precipitate was obtained which showed absence of alkaloids;

Two drops of Wagner's reagent were added to portion '2'. No brown precipitate was obtained which showed the absence of alkaloids;

Two drops of Dragendorff's solution were added to portion '3'. No orange precipitate was obtained which showed the absence of alkaloids.

Two drops of Picric Acid were added to portion '4'. No orange precipitate was obtained, which confirmed the absence of alkaloids in the *Garcinia kola* seed extract.

Test for Flavonoids: 2.00g of the *Garcinia kola* seed powder was dissolved in 5ml of distilled water in a test tube. Few drops of sodium hydroxide solution were added to the solution of *Garcinia kola* seed powder. A yellow colour was obtained which showed the presence of flavonoids.

10ml of ethyl-acetate was added to 0.2g of the *Garcinia kola* seed powder and heated on a water bath for 3minutes. The mixture was cooled, filtered and used for the following teats:

(a) Ammonium Test:

4ml of the filtrate was shaken with 1ml of dilute ammonium solution.

The yellow colour obtained in the ammoniacal layer indicated the presence of flavonoids.

(b) Ammonium Chloride Solution (1% Test):

Another 4ml of the filtrate was shaken with 1ml of 1% ammonium chloride solution and the layers were allowed to separate. A yellow colour in the ammonium chloride layer indicated the presence of flavonoids.

Test for Tannins: 1g of the *Garcinia kola Seed powder* was boiled with 5.0ml of water, filtered and used for the test.

Ferric Chloride test:

Few drops of ferric chloride were added to 3ml of the filtrate. A greenish black precipitate , indicated the presence of tannins (Elligitannins and gallitannins produced the blue-black colour while condensed tannins produced brownish-green-brown precipitate.

Test for Saponins: 20ml of distilled water was added to 0.25g of *Garcinia kola* powder in a test tube and bolied gently in a hot water-bath for 20minutes,. The mixture was filtered hot and allowed to cool. The filtrate was used for the following tests:

Frothing Test:

5ml of the filtrate was diluted with 20ml of distilled water and vigorously shaken and left to stand. Stable foam was observed in filtrate which indicated the presence of saponins.

Emulsion Test:

2 drops of olive oil was added to the frothing solution and the contents shaken vigorously. An emulsion was formed from the frothing solution which showed the presence of saponins.

Fehling's Test:

5ml of Fehling's solution [equal parts of Fehling's solution A & B] was added to 5ml of the filtrate and the content was heated in a water bath. A reddish

precipitate which turned brick red on further heating with added sulphuric acid indicated the presence of saponins.

Test for Glycosides:
Test for Reducing Sugars: 1g of the *Garcinia kola seed* powder, was boiled with 10mls of distilled water. 2mls of Fehling's solution [equal parts of Fehling's solution A & B] was added to the solution and the content heated in a water-bath for 15 minutes. A brick red precipitate was obtained which showed the presence of reducing sugars.

Sulphuric Acid Test for Glycosides:
The brick-red precipitate was filtered off with a filter paper and the supernatant collected. 3 ml of dilute sulphuric acid was added to 5ml of the supernatant and the content heated for 15minutes; cooled and neutralized with 3ml potassium hydroxide solution [20%]. 1ml equal parts mixture of Fehlings solution A & B was added to it and the resultant solution heated for 15 minutes in a water-bath. A brick-red colouration was obtained which showed the presence of glycosides.

Ferric Chloride Test for Glycosides:
3 drops of ferric chloride solution was added to another 5ml of the supernatant and boiled, cooled and filtered. The filtrate was shaken with equal volume of carbon tetrachloride (CCl_4), the lower organic layer was separated. 5ml dilute ammonia was added to filtrate/ carbon tetra chloride solution. A red coloration of the solution was obtained which showed the presence of glycosides.

Ethanol test and Lead acetate Test:
20 ml of 50% ethanol and 10ml lead acetate solution was added to 2.og of the *Garcinia kola* seed powder and heated in a water bath for 2 minute; cooled and filtered. The filtrate was extracted twice with 15ml aliquots of. The first chloroform extract was partially evaporated in a water bath and 2ml aliquots of 3,5-dinitrobenzoic acid solution and 1ml of sodium hydroxide solution were added to it. A light violet coloration was observed which showed the presence of glycosides.
The second chloroform extract was evaporated to dryness in a water bath and dissolved in 3ml glacial acetic acid in a test tube. 2drops of ferric chloride followed by carefully added (by the side of the test tube), 2ml sulfuric acid was added and the solution left to stand for 5minutes. A brownish colour which appeared at the junction of the two layers present, confirmed the presence of glycosides.

Test for Sterols and Triterpenoids: 1.0g of the *Garcinia kola* seed powder was dissolved in 5ml chloroform in a test tube. This solution was poured into a saturated solution of antimony trichloride and chloroform and heated in a water bath for 10minutes. A yellow precipitate found in the chloroform layer, showed the presence of sterols and triterpenoids.

Extraction of the *Garcinia kola* Seed Powder by Maceration:
Two-step extraction of pulverized *Garcinia kola* seeds was done.
In the first step, 800g of pulverized dry seed of *Garcinia kola* was macerated with a mixture of methanol and water in the ratio of 9:1 [1.8 liters of methanol and 200 mls of water were used for the extraction] with a maceration time of 10 hours. The marc was again macerated for 8 hours with a 1:1 mixture of methanol and water. Each extraction of the liquid extract from the plant material was done by filtering rapidly with a cotton wool plug in the neck of a filter funnel. The two extracts were then combined and concentrated to about one- third of its original volume. The resultant aqueous extract was cleared of contaminants by shaking eight times with aliquots (2.3 liters) of n-hexane in a separating funnel. The extracts were then combined and concentrated in a water-bath. The semisolid extract was then air-dried and weighed.

Evaluation of the antimicrobial Activity of the Extract of Macerated *Garcinia kola* Seed Using Measurement of Zone of Inhibition of Pathogen*:*
The antimicrobial activity of *Garcinia kola Seed* powder extract against the following standard laboratory bacteria: *Pseudomonas aeruginosae; Staphylococcus aureus; Bacillus subtilis; Escherichia coli; Klebsiella pneumoniae,* and fungi [*Candida albicans*] was evaluated using the following standard procedures.[4, 29] The zone of inhibition of 100mg, 50mg and 25mg concentrations of the extract to the growth of the organisms was determined in triplicate by making an overnight broth-culture of each bacterial organism to obtain a Marcfarland Standard of each bacterial organism which was used to seed sterile molten nutrient agar medium maintained at 45^0 C. A Sabourand dextrose agar plate was similarly seeded with the fungus used in the experiment [*Candida albicans*].
100mg/ml, 50mg/ml and 25mg/ml of the *Garcinia kola* extract were prepared in Dimethylsulfoxide [DMSO] by preparing a stock solution and carrying out double-fold dilutions on it. Three 6mm holes were bored in a 9cm diameter plate with an aseptic cork-borer when each micro-organism seeded agar plate had solidified.

With the aid of a syringe, 0.25ml (5 drops) of 100mg, 50mg and 25mg /ml dilutions of the *Garcinia kola* seed extract were filled into the three 6mm holes bored in a 9cm diameter plate with an aseptic cork-borer, when each micro-organism seeded agar plate had solidified.

Diameters of zones of inhibition of the growth of the seeded organisms [measured in millimeters] were determined after incubating the plates at 37 ^{0}C for 24 hours for the bacteria and at 25 ^{0} C for 72 hours for the fungus respectively. Three *in vitro* assays of each of the three concentrations of the extract were done.
The average of the zones of inhibition for the 100mg/ml, 50mg/ml and 25mg/ml of the *Garcinia kola* extract were found and was used to plot the graph of comparing the sensitivities of the bacterial and fungal organisms to the *Garcinia kola* extract. The Dimethylsulfoxide [DMSO] also served in the study as negative control.

RESULTS

The results of the phytochemical screening tests on the aqueous extract of pulverized *Garcinia kola* seed showed that it contained starch, protein, glycosides, flavonoids, tannin, saponins, sterols and triterpenoids.
The order of the activity of 100mg of the N-hexane extract of *Garcinia kola* seed against the tested organisms was *Bacillus subtilis* > *Pseudomonas aeruginosa* >*Staphylococcus aureus* and *Krebsiella pneumonia* with zones of inhibition of 10.66 mm, 9.66 mm, 9 mm, 9 mm, respectively . The 50mg concentration of the extract showed activity against the tested organisms in the order: *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* >> *Klebsiella pneumoniae;* with zones of inhibition of 8.33 mm, 8. 33 mm, 8.33 mm and 4.66 mm, respectively. The 100mg dose of the extract was poorly inhibitory to *Candida albicans* [inhibition zone, 3.00 mm] and not inhibitory at all to *Eschcerichia coli*. The 50mg dose was very poorly inhibitory to *Candida albicans and was also resisted by E. coli.* The 25mg dose of the aqueous *Garcinia kola* extract was fairly inhibitory to *Staphylococcus aureus* and *Bacillus subtilis* [zone of inhibition, 5.66 mm and 4.66 mm respectively] and poorly inhibitory *to Pseudomonas aeruginosae* and *Klebsiella pneumoniae* [zone of inhibition, 3.33 mm and 2.66 mm respectively]. The 25mg of the extract had insignificant action on *Candida albicans* [zone of inhibition, 0.66 mm] and no action against *E. coli*. A comparison of the sensitivities of these organisms to the *Garcinia kola* extract is shown in figure 1.

DISCUSSION

The biflavonoids and crude extracts of *Garcinia kola* seed were shown to be hepato-protective. [7, 8]
The results of the phytochemical screening tests on the aqueous extract of pulverized *Garcinia kola* seed in this study showed that the extract contained starch, protein, glycosides, flavonoids, tannin, saponins, sterols and triterpenoids.
Triterpenoids (amaraoid) found in quassia species [like *Quassia amara* (family Simaroubaceae)], a shrub growing in the Guianas, Northern Brazil and Venezuela]; are quassin, an intensely bitter lactone, neoquassin, 18-hydroxyquassin and scopoletin.[10] Quassia is used as a bitter tonic; as an insecticideand as an enema for the expulsion of worms. [10] *Quassia amara* wood is used in South American traditional medicine as an anti-amoebic, stomachic (anti-spasmodic), anti-malarial and as an anti-anaemic while other quassinoids from plants of the same family are used in South American traditional medicine for cancer, amoebic dysentery and malaria. [10] These compounds and sometimes their *associated* glycosides have been shown to have anti-leukemic, anti-viral, anti-inflammatory and anti-feedant properties. [10] Volatile oils of citrus leaves and peels have similarly demonstrated anti-feedant properties. [11]The extracts of *bitter leaf*, *Vernonia amygdalina*,a favorite soup vegetable and traditional medicinal herb in West Africa especially in Nigeria, has been shown to have antibacterial and fungitoxic; [12, 13, 14] anticancer /anti-tumor, [12, 15] anti-diabetic,[16] antihelminthic,[17], anti-leishmanic, 18] anti-oxidant,[19, 20] liver-protective [19, [17, 22] and anti-malarial properties[23].
Just like the bitter lactone quassin and the bitter sesquiterpene lactones in bitter leaf (*Vernonia amygdalina* leaf extract,[12] the bitter triterpene lactones of *bitter kola (Garcinia kola)*, are responsible for the strong antibacterial and weak antifungal effects of N-hexane crude extract of *Garcinia kola* seed obtained in this study and for the anti-spasmodic[3], anti-fungal, [24] anti-diabetic,[4] hepato-protective,[7,i8] inhibition of metabolism[3] and anti-oxidant[5] and excess gastric acid-secretion-inhibitory activities[1, 2] of bitter cola (*Garcinia kola*) seed extract found in various studies.

CONCLUSIONS

From the findings of this study it is concluded that the *Garcinia kola* seeds used in this study contained starch, protein, glycosides, flavonoids, tannin, saponins, sterols and triterpenoids. It is also concluded that 100mg and 50mg of the N-hexane *Garcinia kola* seed extract showed strongest

antibacterial activity in the order of activity against *Bacillus subtilis* > *Pseudomonas aeruginosae* >*Staphylococcus aureus* and *Klebsiella pneumonia* [with zones of inhibition of 10.66mm, 9.66mm, 9, 9mm, respectively for 100mg and 8.33, 8. 33, 8.33 and 4.66 respectively, for 50mg of the extract]. The extract showed weak antifungal activity against *Candida albicans* and no activity against *Eschcerichia coli*. Even the 25mg dose of the *Garcinia kola* extract showed some antibacterial and antifungal activity [being fairly inhibitory to *Staphylococcus aureus* and *Bacillus subtilis* and poorly inhibitory to *Pseudomonas aeruginosae*, *Klebsiella pneumoniae* and *Candida albicans*]. None of the 3 concentrations of the *N*-hexane extract of Garcinia *kola* seed had any inhibitory action on *Escherichia coli.*

The triterpenoids found in the extract (possibly in conjunction with the glycosides) are suggested to be responsible for the anti-bacterial and anti-fungal activity of the crude N-hexane extract of *Garcina kola* seed obtained in this study.

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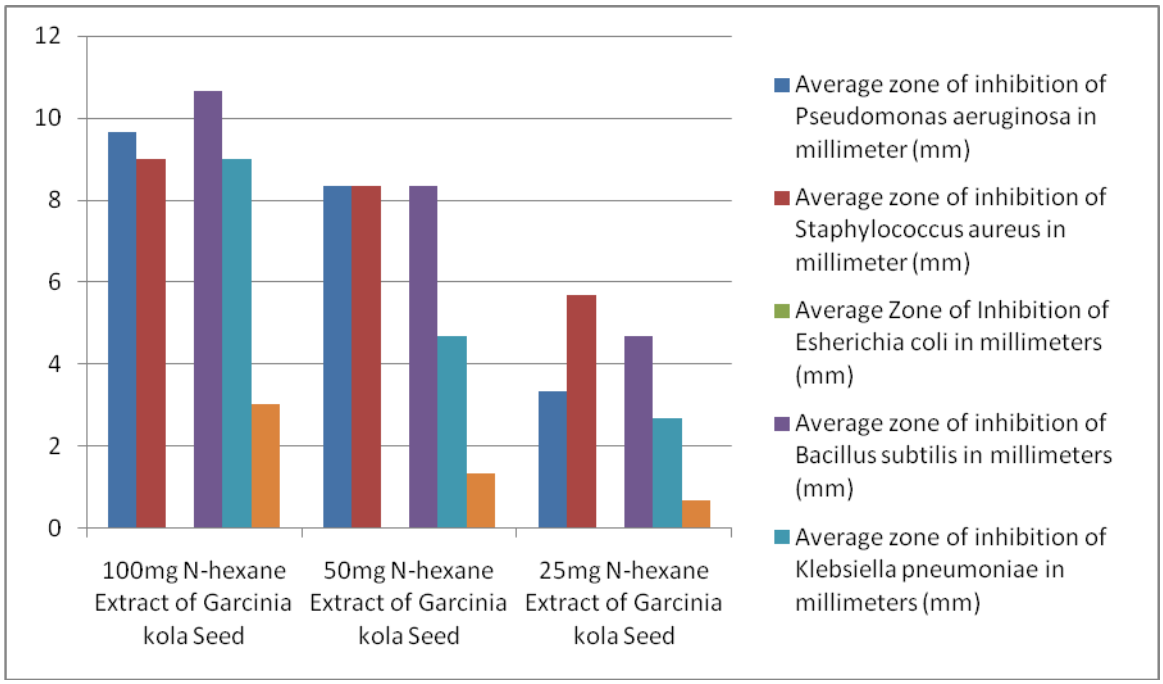

Figure 1: Comparism of the sensitivity of *Pseudomonas aeruginosae; Staphylococcus aureus; Escherichia coli, Bacillus subtilis, Klebsiella pneumonia* to the N-hexane extract of *Garcinia kola* seed. The height of the zone of inhibition of an organism by the extract is an indication of the sensitivity of the organism to the extract. 100mg of the extract showed the highest antibacterial activity and *Bacillus subtilis* was most sensitive to it while *Staphylococcus aureus and Klebsiella pneumoniae,* the least sensitive to it. *Escherichia coli* were not susceptible to the activity of the *Garcinia kola* seed extract.

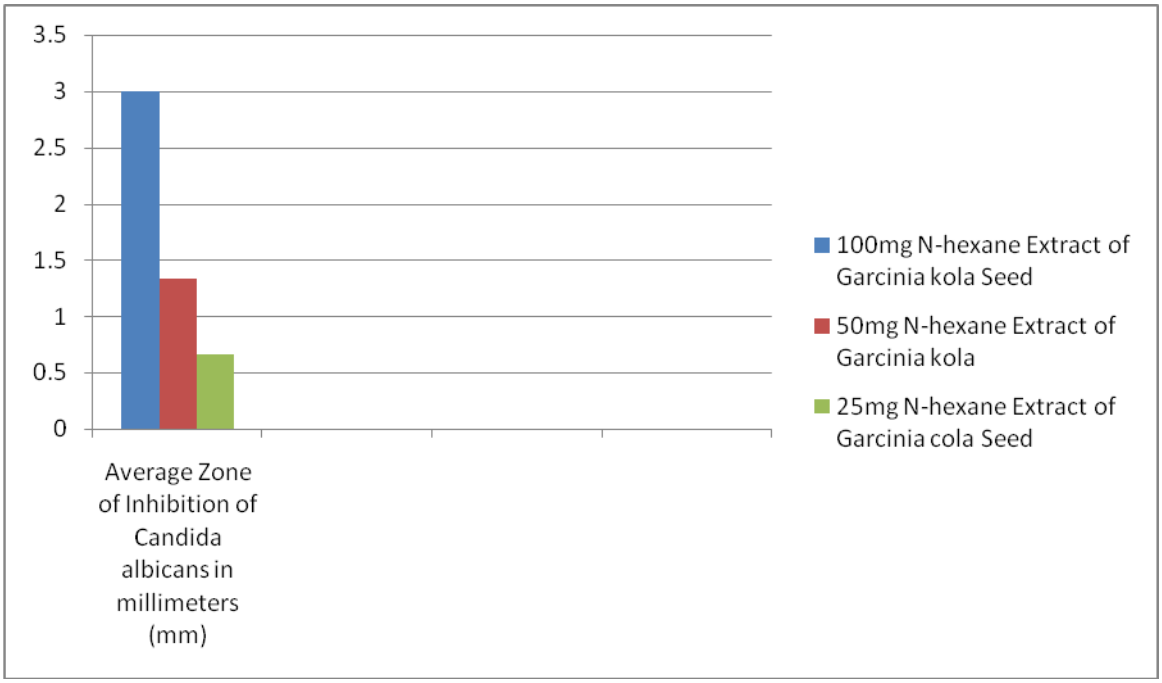

Figure 2: A measurement of the anti-fungal effects of 100mg, 50mg and 25mg concentrations of N-hexane extract of *Garcinia kola* seed. The 100mg, 50mg and 25mg concentrations of N-hexane extract of *Garcinia kola* seed all exhibited low inhibitory activity against *Candida albicans* given that 100mg of the same N-hexane extract inhibited *Bacillus subtilis* with a zone of inhibition of 10.66mm as against the zone of inhibition of 3.00mm of *Candida albicans*, in the same study.

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