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Full Length Research Paper

Antimicrobial Activity of *Garcinia kola* (Heckel) Seed Extracts and Isolated Constituents Against Caries-causing Microorganisms

Ajayi T. O[a], Moody J.O[a], Fukushi Y[b], Adeyemi T.A[c], Fakeye T.O[d]**

[a]*Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan. Nigeria.*
[b]*Research Faculty of Agriculture, Hokkaido University. Sapporo, Japan.*
[c]*Department of Preventive Dentistry, College of Medicine, University of Ibadan, Nigeria.*
[d]*Department of Clinical Pharmacy and Pharmacy Administration, Faculty of Pharmacy, University of Ibadan. Nigeria.*

ABSTRACT

Garcinia kola Heckel (Guttiferae) seed, has found use in folkloric medicine of Southern Nigeria for the treatment of toothache and prevention of dental caries. The crude ethanolic extract, chromatographic fractions and isolated constituents of *Garcinia kola* seed against clinical strains of dental-caries-causing and related microorganisms is being evaluated. Antimicrobial evaluations were done by testing different concentrations of the crude extract, vacuum liquid chromatographic (VLC) fractions and pure isolates against *Streptococcus mutans*, *Streptococcus viridans* and *Staphylococcus aureus* in already set blood agar with gentamicin as the reference standard. The zones of inhibition and minimum inhibitory concentrations (MIC) were determined as appropriate. Fraction N, eluted with (hexane: ethyl acetate 70: 30), exhibited the highest activity with MIC's of 1.50 $mgml^{-1}$ and 0.33 $mgml^{-1}$ while the pure isolates **1** (cycloartenol) and **2** (24-methylenecycloartanol) gave MIC's of 0.17 $mgml^{-1}$ and 0.38 $mgml^{-1}$ against *Streptococcus mutans* and *Streptococcus viridans* respectively. Isolate **3** (garcinianin) gave MIC of 1.0 $mgml^{-1}$ against *Streptococcus mutans* but there was no significant activity against *Streptococcus viridans* and *Staphylococcus aureus.* The results provide justifications for the folkloric use of *Garcinia kola* Heckel (Guttiferae) for dental caries-related health problems while the isolated compounds may also serve as templates for future antimicrobial drug development.

Key words: *Garcinia kola*, 24-methylenecycloartanol, cycloartenol, *Streptococcus*, garcinianin.

INTRODUCTION

Dental caries, also known as tooth decay is an infectious disease that damages the structures of teeth (Suddick and Norman, 1990). The disease can lead to infection, pain, tooth loss, and in severe cases, death. Tooth decay becomes fatal when the micro-organism (*Streptococci*) enters the blood stream via small wounds and causes sepsis or leads to abscesses forming in the throat, lungs or liver. It may even cause life threatening cardiovalvulitis and heart disease

*Corresponding author:
E-mail: tayomiajayi@yahoo.com
Tel: +234 7030308600

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(Suddick and Norman, 1990). Following *Streptococci*, a high incidence of infective endocarditis caused by *Staphylococcus* species, especially *Staphylococcus aureus* has been reported (Hoen *et al.,* 2002).

Garcinia kola Heckel (Guttiferae) seed has been used extensively in traditional medicine for the treatment of various diseases (Taiwo *et al*., 1999). The seeds are chewed in Southern Nigeria and parts of West Africa as a masticatory and are also used to prevent or relieve colic, headaches, chest colds and cough. The seeds are also chewed as an aphrodisiac and find use in the treatment of dysentery, bronchitis and throat infections (Braide, 1990). The stem bark is used as a purgative and for the treatment of malignant tumors. The sap is used for parasitic skin disease while the latex (gum) is used internally for gonorrhea treatment. It is applied externally to treat fresh wounds (Tona *et al*., 1999). The plant has also been employed in the treatment of liver disorders while the fruit yield favorite bitter chewing sticks sold in small bundles in local markets across West Africa (Iwu, 1993).

Previous studies have revealed that *Garcinia kola* Heckel contains a mixture of phenolic compounds including xanthones and benzophenones (Locksley *et al*., 1971; Hussain *et al*., 1982). Flavonoid derivatives including apigenin, fisetin and biflavonoids such as ametoflavone have also been isolated from *Garcinia kola* Heckel seeds (Iwu *et al.*, 1982). Kolaviron, an extractive from the seed has also been shown to possess remarkable prophylactic and therapeutic action on damaged stomach (Ibironke et al, 1997; Olaleye, 2005), liver (Farombi, 2000; Iwu, 2001; Adaramoye *et al*., 2005;), colon (Ige et al, 2010; Farombi et al, 2013) and brain (Ijomone et al, 2012)

In the present study, a bioactivity-monitored fractionation and isolation of antimicrobial constituents of *Garcinia kola* Heckel seed with activity against dental caries-causing microorganisms was carried out.

MATERIALS AND METHODS

Plant material: Samples of *Garcinia kola* Heckel seeds were obtained from Kasumu village Ibadan, Oyo State and was Identified and authenticated at the Forest Research Institute of Nigeria, Ibadan where a voucher specimen (FHI 108266) was deposited.

Extraction and thin layer chromatographic analysis: Grated and dried powdered seed samples (1.44 kg) were macerated in 96% ethanol for 72 hours. The extracts were filtered, dried and evaluated for antimicrobial activity using *Streptococcus mutans, Staphylococcus aureus* and *Streptococcus viridans* as test organisms. Thin layer chromatographic analysis of the extracts on silica gel plates (0.25 mm thickness) with appropriate solvent systems and chromogenic reagents was also carried out to detect the secondary metabolites present.

Isolation of compounds: The crude ethanolic extract was chromatographed on silica gel (Vacuum Liquid Chromatographic) (VLC) and eluted with solvents of increasing polarity (hexane- methanol gradients), followed by monitoring with thin layer chromatography. Similar fractions were pooled together and labeled K, M, N, O and P. Fraction N (11.68 g) from the VLC fractionation was chromatographed on silica gel (column chromatography) and on further purification with preparative TLC (toluene: ethyl acetate: acetone 8:2:1) yielded compounds **1, 2** and **3**. The pure isolates were subsequently characterized using spectroscopic means (Infra Red, Nuclear Magnetic Resonance, and Mass Spectroscopy).

Antimicrobial screening of crude extracts and chromatographic fractions: Different concentrations of the crude extract ranging from 400 $mgml^{-1}$ to 25 $mgml^{-1}$ was prepared and was used to fill the bored wells made in already set blood agar containing *Staphylococcus aureus*, *Streptococcus mutans* and *Streptococcus viridans* in different Petri- dishes and incubated at 37 ^{0}C for 24 hours. The zones of inhibition were measured while gentamicin (2.5 μgml^{-1}) was used as positive control as shown in Table 1.

Determination of Minimum Inhibitory Concentration (MIC) of chromatographic fractions and isolated compounds: Serial dilutions of the chromatographic fractions K, M, N, O, P and isolates were made ranging from 25 $mgml^{-1}$ to 0.04 $mgml^{-1}$, placed in wells of the already set blood agar containing test organisms and incubated at 37 ^{0}C for 24 hours so as to know the minimum concentration at which each fraction and isolated compounds will inhibit the growth of the microorganisms. The minimum inhibitory concentrations of the chromatographic fractions M, N, Q and P against *Streptococcus mutans and Streptococcus viridans* which are the causative organisms of caries were determined.

All data were expressed as mean of ±SEM (n=3). The mean of the different groups were evaluated using student's t- test significant at $p < 0.05$.

RESULTS

Three compounds were isolated by column chromatography and identified using IR, MS and NMR.

Compound **1** was obtained as an off-white powder with molecular formula $C_{30}H_{50}O$, R_f 0.81 (toluene: ethylacetate: acetic acid, 8:2:1) and was identified as cycloartenol (**1**).

IR V_{max} cm^{-1}: showed bands at 468 w, 1100 w, 1466 w, 1638 w, 2854 w, 2927 w, 3448 br. ^{1}H-NMR spectral data ($CDCl_3$, 270 MH_Z acetone-d6) showed resonances at δ_H (0.33 d, 0.55 d, 0.81 s, 0.81 s, 0.90 s, 0.98 s, 1.0 s, 1.1 s, 3.28 dd, H-3, 1.45 d, 5.08 t).

EI-MS showed peaks at m/z 426 (59, M^+), 411 (86, M^+-15), 315 (8, M^+ -111), 286 (15, M^+-140).

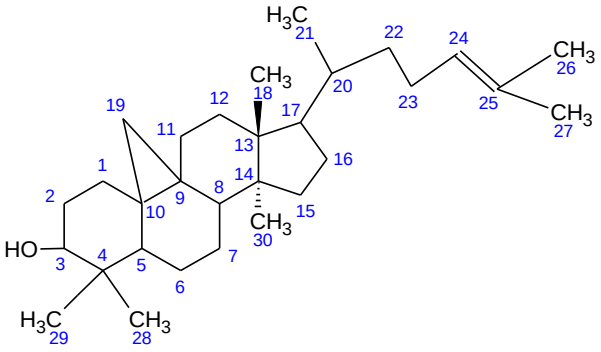

Figure 1
Cycloartenol (1)

Compound **2** was obtained as an off-white powder with molecular formula $C_{31}H_{52}O$, R_f 0.81(toluene: ethylacetate: acetic acid, 8:2:1) and was identified as 24-methylenecycloartanol (**2).**

IR V_{max} cm^{-1}: showed bands at 468 s, 1100 s, 1466 w, 1638 w, 2854 w, 2927 w, 3448 br. ^{1}H-NMR spectral data ($CDCl_3$, 270 MH_z acetone-d6) showed resonances at δ_H (0.33 d, 0.55 d, 0.81 s, 0.90 s, 0.92 s, 0.98 s, 1.0 s, 1.1 s, 4.65 s, and 4.69 s).

EI-MS showed peaks at m/z 440 (11, M^+), 425 (20, M^+ -15), 315(8, M^+ -125), 300(15, M^+ -140).

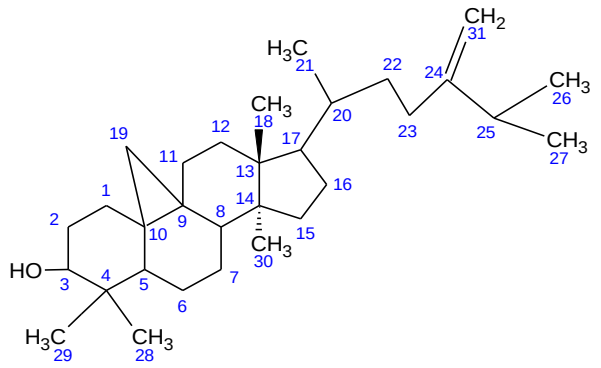

Figure 2:
24-methylenecycloartanol (2)

Compound **3** was isolated as a yellow amorphous powder with molecular formula $C_{30}H_{20}O_{11}$, R_f 0.84 (toluene: ethyl acetate: acetic acid, 5:5:0.5) and was identified as garcinianin **(3).**

IR Vmax cm^{-1} showed bands at: 472 w, 1093 s, 1638 w, 1718 w, 3448 br.

^{1}H-NMR (270 MHz, acetone- d6) showed resonances at δ_H **(**4.8 d, H-3, 5.7 d, H-2, 5.6 s, 3"-OH**,** 6.0 s, H-8, 6.4 s, H-6 or H-6", 6.5 d, 2'/3' or 2"'/3"' H-aromatic proton, 7.0 d, 2'/3' –H or 8"'/3"' H aromatic protons, 7.9 d, 3'/5' or 3'''/5'''-H aromatic protons, 8.0 s, 4'/4'''-OH, 8.2 d, H 3'/5' or 3'''/5''', 12.1-12.2 2 OH groups, 12.5 s, 5-OH or 5''-OH.

ESI-MS showed peaks at m/z 556 (M^+), 555(100, M^+-H), 541(2, M^+ -15), 429 (42, M^+ -127), 403 (2, M^+-153).

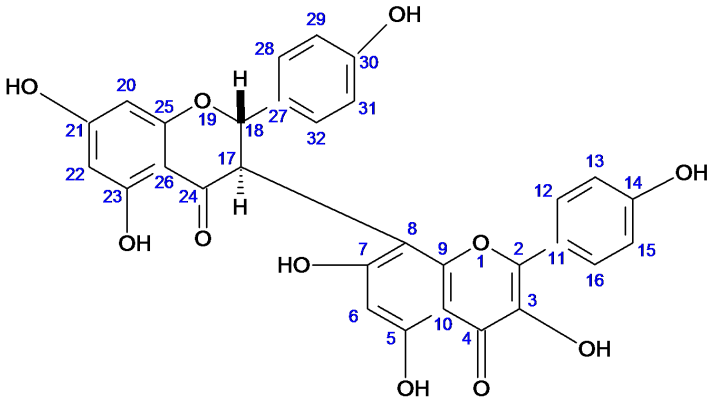

Figure 3: Garcinianin (3)

The antimicrobial screening of the crude ethanol extract of *Garcinia kola* Heckel seeds showed significant activity against *Staphylococcus aureus, Streptococcus mutans* and *Streptococcus viridans* with mean zones of inhibition in comparable range with that of Gentamicin (2.5µgml$^-$) used as a reference standard as shown in Table 1.

The MIC (Minimum inhibitory concentration) of VLC fractions of crude ethanol extract reveal that fraction N (most abundant) is the most active and on further purification with column grade silica gel 200 – 400 mesh on column chromatography yielded compounds **1**, **2** and **3**. Compounds **1** and **2** (combination) gave MIC's of 0.17 $mgml^{-1}$ and 0.38 $mgml^{-1}$ against *Streptococcus mutans* and *Streptococcus viridans* respectively. Compound **3** gave MIC of 1.0 $mgml^{-1}$ against *Streptococcus mutans* but no significant activity against *Streptococcus viridans* and *Staphylococcus aureus* as shown in Table 2. The NMR data are shown in Tables 3 and 4

Table 1:
Antimicrobial screening of crude ethanol extracts of *Garcinia kola*

Extract conc. $mgml^{-1}$	Microorganisms/Zones of inhibition ± sem (mm)			
	Staphylococcus aureus 1 PHM 001	*Staphylococcus aureus 2 PHM 002*	*Streptococcus mutans PHM 003*	*Streptococcus viridans PHM 004*
400	13.00± 1.41	14.00±1.41	14.00±1.41	16.00±1.41
200	12.00±0.07	10.50±0.07	14.00±1.41	14.50±0.71
100	10.50±0.07	9.50±0.71	13.50±1.41	12.50±0.71
50	-	-	11.50±0.71	11.50±0.71
25	-	-	11.50±0.71	12.00±1.41
Gentamicin	20.00±0.71	18.00±0.45	18.00±0.64	20.00±0.71

Key: Reference standard Gentamicin 2.5 μgml^{-1}

Table 2:
Minimum inhibitory concentration in $mgml^{-1}$ of VLC fractions of ethanol extracts of *Garcinia kola* seeds and isolated compounds **(1-3)**

Fraction/organism	K	M	N	Q	P	Reference standard*	1†and 2‡	3#
Staphylococcus aureus PHM 001	6.25	0.75	0.08	6.25	12.5	0.04	-	-
Streptococcus mutans PHM 003	-	0.75	1.50	3.07	1.50	0.04	0.17	1.0
Streptococcus viridans PHM 004	0.75	6.25	0.33	6.25	3.07	0.13	0.38	-

**Reference standard; Gentamicin 2.5 µgml ⁻¹*
†Compound 1 Cycloartenol
‡Compound 2 24- methylenecycloartanol
#Compound 3 Garcinianin

DISCUSSION

The mass spectra of compound **1** gave a molecular ion peak m/z 426 corresponding to molecular formula C_{30} H_{50} O and showed diagnostic fragmentation patterns. ^{1}HNMR spectrum of **1** showed characteristic signals assignable to a triterpenoid (Hui *et al*., 1975; Gaiko *et al*.,1976, Matsunaga *et al*., 1988; Sageer, 2003; and Basar, 2005).These fragments include m/z 315(8, M - 111) suggesting the loss of the C_8 H_{15} side chain and 286 (25, M- 140), suggesting the loss of non- aromatic ring A of the molecule. ^{1}H-NMR spectra revealed signals ranging from δ 0.30 to 5.08 ppm suggesting that compound **1** is a non- aromatic and triterpenoid in nature. The single olefinic proton appearing at δ 5.08 ppm as a triplet is attributable to proton attached to C-24. The chemical shifts at δ 0.33(d) and 0.55(d) are due to the presence of non-equivalent geminal protons of the cyclopropane ring at C- 19.

The methyl signals appearing at chemical shift δ 0.81, 0.85, 0.90, 0.92, 0.98, 1.0 and 1.1 in the ^{1}H-NMR as singlets are indication of seven methyl groups in the molecule. A chemical shift at δ 3.28 (dd) assignable to H- 3 suggests the presence of OH- group at C- 3 resulting in the downfield shift of the H- 3 proton.

The molecular formula $C_{30}H_{50}O$ of compound **1**,shows only one oxygen thus, suggesting the presence of a hydroxyl group which makes the carbon to which it is attached to resonate at a shift downfield than normal because of the resultant de-shielding effect occurring at that site. The presence of a hydroxyl group is further confirmed in IR spectra with broad signal V_{max} at 3447 cm^{-1}. The ^{13}C-NMR spectra signal at δ 78.9 ppm is therefore easily assigned to C- 3 bearing the hydroxyl function. The ^{13}C-NMR of compound **1** revealed that there are seven methyl groups resonating from δ 15.4 ppm to δ 25.4 ppm. The ^{13}C-NMR spectra signals at δ 31.3 ppm and δ 30.4 ppm are assignable to C- 1 and C - 2 respectively. The chemical shift at 125.2 ppm is assignable to C-24 olefinic carbon while the quatenary olefinic carbon resonating at δ 130.8 ppm is assignable to C-25. The downfield shifts of C-24 and C-25 are due to the deshielding effect of the olefinic carbon present in compound **1** side chain.

The olefinic shift in the ^{13}C-NMR of compound **1** is confirmed by the absorption signals in the IR spectra showing at V_{max} 1637 cm^{-1}. The complete assignment of all ^{13}C-NMR signals of compound **1** compared to that of cycloartenol as reported by Consolacion *et al.*, 2004.

Compound **2** was obtained as an off-white powder which on spectral analysis gave a molecular ion peak at m/z 440 corresponding to molecular formula $C_{31}H_{52}O$.

Table 3:
^{13}C NMR data for isolated compound **1** compared with Cycloartenol. (Consolacion *et al.*, 2004)

Carbon no	Compound 1 δppm(CDCl$_3$)	Cycloartenol δppm (CDCl$_3$)
1	31.3	32.0
2	30.4	30.4
3	78.9	78.9
4	39.0	40.5
5	47.1	47.1
6	22.0	21.1
7	28.1	28.1
8	48.0	48.0
9	20.0	20.1
10	26.0	26.1
11	25.7	26.0
12	35.0	37.3
13	45.3	45.3
14	48.8	48.8
15	32.9	32.9
16	26.5	26.5
17	52.3	52.3
18	17.6	18.0
19	29.9	29.9
20	36.1	35.9
21	18.3	18.2
22	36.3	36.4
23	24.3	25.0
24	125.2	125.3
25	130.8	130.9
26	22.5	17.6
27	22.7	25.7
28	19.3	19.3
29	15.4	14.0
30	25.4	25.4

The mass spectra revealed diagnostic fragmentation patterns with ion peaks m/z 315(M-125) suggesting the loss of the side chain C_9H_{17} and m/z 300(M-140) suggesting the loss of a triterpenoid ring A. The ^{1}HNMR spectrum also showed characteristic signals assignable to a triterpenoid (Hui *et al*., 1975; Gaiko *et al*.,1976, Matsunaga *et al*., 1988; Sageer, 2003; and Basar, 2005).The molecular formula $C_{31}H_{52}O$, however shows only one oxygen and therefore suggests the presence of a hydroxyl group which makes the carbon to which it is attached to resonate at a shift more downfield (δ 78.9 ppm) than normal in the ^{13}C-NMR spectrum because of the deshielding effect occurring at that site. The presence of a hydroxyl group is further confirmed in IR spectra with a broad signal V_{max} at 3447 cm^{-1}.

Table 4:
^{13}C NMR data for isolated compound **2** compared with 24-methylenecycloartanol. (Ufuk *et al.*, 2005).

Carbon no	Compound 2 δppm (CDCl$_3$)	24-methylenecycloartanol δppm(CDCl$_3$)
1	31.94	31.99
2	30.35	30.38
3	78.20	78.9
4	40.46	40.54
5	47.09	47.23
6	21.97	21.16
7	28.12	28.19
8	47.95	48.02
9	19.98	19.35
10	26.09	26.50
11	25.69	26.04
12	34.99	32.93
13	45.27	45.41
14	48.78	48.02
15	32.88	29.74
16	26.46	26.58
17	52.27	52.30
18	17.60	18.06
19	29.86	29.74
20	36.09	32.93
21	18.28	18.39
22	36.33	35.15
23	24.34	31.41
24	156.90	159.79
25	28.12	33.89
26	22.50	21.94
27	22.70	19.35
28	19.28	18.09
29	13.98	14.04
30	25.41	25.47
31	105.90	106.10

The ^{13}C-NMR showed that there are seven methyl groups resonating from δ 13.98 ppm to δ 25.4 ppm. The chemical shifts in the ^{1}H-NMR which appear at δ 0.33 ppm (d) and 0.55 ppm (d) due to the C- 19 protons that are non- equivalent geminal protons of cyclopropane ring while the shift at δ 4.65 ppm (s) and 4.69 ppm (s) appearing downfield are due to the presence of 24-methylene group in the molecule.

The major difference for ^{13}C-NMR shift of compound **2** as compared to compound **1** is the presence

of a olefinic carbon in compound **2** resonating at δ 105.9 ppm assignable to C- 31 and a quartenary olefinic carbon with a downfield shift of δ 156.9 ppm which is attributable to C- 24 as shown in Table 3. The complete assignment of all ^{13}C-NMR signals of compound **2** compared to that of 24- methylenecycloartanol as reported by Ufuk *et al.*, 2005.

Compound **3**, identified as garcinianin was obtained as a yellow amorphous powder with a molecular ion at m/z 556 corresponding to a molecular formula C_{30} H_{20} O_{11} as determined by electron spray mass spectroscopy (ESI-MS).The fragmentation pattern followed this order: m/z 556(M^+), 555 (100, M^+- H), 541(2, M^+ -15), suggesting the presence of methyl group in the molecule. The IR spectra of compound **3** exhibited characteristic absorptions at V_{max} 1637 cm^{-1}, 1735 cm^{-1} and 3448 cm^{-1}, suggesting the presence of olefinic, chelated carbonyl and hydroxyl functional groups respectively. The presence of chelated hydroxyl groups is confirmed by the ^{1}H-NMR spectra with downfield singlet appearing at δ 12.2(s) and 12.5(s) which are assignable to C-5 and C-5' OH groups. Other non-chelated hydroxyl groups appear between δ 5.6 ppm and δ 8.0 ppm in ^{1}H-NMR spectra.

Previous studies revealed that garcinianin (**3**) has been isolated from the stem and roots of *Garcinia kola* as well as from the leaves of *Rheedia acuminata* (Kenji *et al*., 1995; Kenji *et al*., 1999; Li *et al.*, 2002). Cycloartenol **(1)** has been isolated from a number of studies of plant sources including *Artocarpus heterophyllus* (Consolacion *et al*., 2004) and from rice bran oil (Lee *et al.*, 1991) while 24-methylenecycloartanol (**2)** has been isolated from the roots of *Salvia blepharochlaena* (Ufuk *et al.*, 2005). These three compounds are however, being reported for the first time from the seeds of *Garcinia kola* to exhibit antimicrobial activity against caries- causing organisms.

In conclusion, results obtained in this study, reveals evidence that there residues in the ethanol extract of *Garcinia kola* Heckel seeds, bioactive compounds with antibacterial activity against caries- causing microorganisms. The results provide some justifications for its use in the treatment of dental caries in Nigerian ethno-medicine (Irvine, 1961; Ajayi *et al.*, 2008).

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