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Antibacterial Activity of Biherbal Root Extracts on Bacterial Isolates

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ABSTRACT: This research determines the antibacterial activity of the aqueous and methanol root extracts of male and female *Carica papaya* and *Garcinia kola* individually and in biherbal formulations using the agar diffusion method. The dried roots of the two plants were powdered separately. *Garcinia kola* (100 g) and *Carica papaya* (100 g) were macerated separately in boiled water and absolute methanol; *Garcinia kola* (50 g) and *Carica papaya* (50 g) were combined (100 g) and macerated in boiled water and absolute methanol at room temperature (30°C ± 2°C) for 72 hours. They were filtered and concentrated at 60 °C. The extract's antimicrobial susceptibility and minimum inhibitory concentration against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella* species and *Pseudomonas aeruginosa* were performed using the agar diffusion methods. The aqueous root extract of *Garcinia kola*, methanol extracts of *Garcinia kola*, and its combined formulations of *Carica papaya* and *Garcinia kola*, had zones of inhibition against *Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa.* However, *Escherichia coli* and *Klebsiella species* showed no zone of inhibition in aqueous and methanol root extracts. *Staphylococcus aureus* recorded the lowest minimum inhibitory concentration followed by *Bacillus subtilis* and *Pseudomonas aeruginosa*. The root extracts of *Carica papaya* and the aqueous biherbal formulation showed no antibacterial properties, however, its methanol extract recorded significant antibacterial activity $p < 0.05$ and could be used to treat bacterial infections.

Keywords: Root, *Carica papaya, Garcinia kola,* Minimum inhibitory concentration.

Introduction

The use of medicinal plants to prevent and treat diseases is gaining more popularity due to more knowledge of plants' antibacterial potency, antibiotic resistance, and high drug costs. Plants and plant derivatives have successfully treated abnormal uterine bleeding and regulated the female reproductive cycle (Westfall, 2001; Born *et al.*, 2005). Bacterial infection is one of the causes of abnormal uterine bleeding (AUB) with a resultant effect on female health, fertility and a huge financial burden (Claessen and Cowell, 1981; William, 1994; Prentice, 1999). Cervicitis, endometritis, salpingitis and peritonitis infections are causes of AUB. An intrauterine device (IUD) increases the risk of microbial infections by facilitating the spread of bacteria along the device into the uterine cavity and tubes. The incriminated bacteria in AUB are *Escherichia coli*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae.* Antibiotic drugs are administered to patients to treat bacterial infections however, the complications associated with antibiotic use have necessitated using medicinal plants to treat bacterial infections (McEwen and Collignon, 2018). Some medicinal plants with antibacterial potency in Nigeria and other African countries with reported antibacterial activities for bacterial infection treatment are *Bidens pilosa* (Lawal *et al*., 2015; Owoyemi and Oladunmoye, 2017); *Morinda lucida* (Adomi, 2008); *Allium sativum* (Yahaya *et al*., 2017; Airaodion *et al*., 2020); *Citrus sinensis* (Abalaka *et al*., 2016; Baba *et al*., 2018; Nata'ala *et al*., 2018); *Carica papaya* (Anibijuwon and Udeze, 2009; Wemambu *et al*., 2018); *Tapinanthus* *dodoneifolius* (Aina *et al*., 2010; Ndamitso *et al*., 2017); *Zanthoxylum zanthoxyloides* (Adebiyi, *et al*., 2009); *Zingiber officinale* (Akintobi, *et al*., 2013; Yassen and Ibraheem, 2016) and *Garcinia kola* (Akin-Osanaiye and Chukwu, 2018; Djague *et al.*, 2020).

Garcinia kola Heckel (Bitter kola) taxonomy; - Family: Guttiferae; Genus: Garcinia; Species: *Garcinia kola* Heckel. The Yorubas call it orogbo; Hausa cida goro; Igbos Aku ilu or Ugugolu (Dalziel, 1937), and in Edo, edun. It is widely distributed in the South East, West, and Edo State of Nigeria (Otor *et al*., 2001). *Garcinia kola* seed *in vitro* antibacterial, antimicrobial, antiviral and trichomonacidal activities were reported (Adegboye *et al*., 2008; Ofokansi *et al*., 2008; Gabriel and Emmanuel, 2011). The chloroform and ethanol root extracts of *Garcinia kola* had antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* (Idu *et. al*., 2014). The hydro-ethanol (1:4 v/v) and absolute methanol of *Garcinia kola* leaves, roots and stem bark extracts exhibited varying antibacterial activity against *Salmonella typhimurium*, *Salmonella enteritidis and Salmonella typhi* (Djague *et al.*, 2020). Extraction solvents affected the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the seed and leaf extracts of *Garcinia kola* against gram-negative and gram-positive bacteria (Ezeanya and Daniel, 2013; Akin-Osanaiye and Chukwu, 2018).

Carica papaya Linn. Taxonomy Family: Caricaceae; Genus*:* Carica*;* Species: *Carica papaya* Linn. grow in semitropical regions of the world. It is called pawpaw in Nigeria (Sentilkumaran and Shalini, 2014). The root of *Carica papaya* is used for fungal infection and pile treatment (Sentilkumaran and Shalini, 2014), to expel roundworms (Lohiya *et al.,* 2001). Doughari *et al.* (2007) reported no significant antibacterial activity in the aqueous root extract of *Carica papaya* against gram-positive and gram-negative bacteria. However, methanol extract showed the highest activity against all tested bacterial isolates. *Carica papaya* ethanol and cold-water root extracts exhibited antibacterial activity against *Klebsiella* species, *Escherichia coli*, *Salmonella* species, *Pseudomonas* species and *Staphylococcus aureus*, with ethanol extract recording higher antibacterial activity (Tiwari *et al*., 2011; Wemambu *et. al.,* 2018).

Herbal formulations can either be mono, bi or poly combinations. Bi-herbal and poly-herbal formulations have more therapeutic actions at lower concentrations over single herbs, reducing toxicity. Plants and their derivatives are prepared into formulations with positive potential activity; mutual enhancement, restraint and antagonism (Ramaiah *et al.,* 2013). Presently, drug combinations for infectious disease treatment have renewed patients' hope of living (Kajaria *et al*., 2011; Risberg *et al.*, 2011).

In Nigeria, the roots of *Carica papaya* and *Garcinia kola* are combined into a formulation used to treat protracted, heavy and intermittent bleeding between menstrual periods in females. This research aims to determine the antibacterial potential of the aqueous and methanol root extracts of male and female *Carica papaya* and *Garcinia kola* individually and in bi-herbal formulations against some gram-positive and gram-negative bacterial isolates.

Materials and methods

Plant material and authentication: The roots of *Garcinia kola* and *Carica papaya* were harvested from Ovia North East Local Government Area of Edo State in November 2020. The plants were officially identified at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State and, assigned Voucher numbers: *Garcinia kola* UBH-365 and *Carica papaya* UBH-C505.

Plant extraction: The roots of the two plants were washed, cut into small pieces, and dried in the shade for two weeks. The dried roots were further dried in a hot air oven at 60 °C for 6 h before being separately ground into powder. *Garcinia kola* (100 g) and *Carica papaya* (100 g) roots were macerated separately in boiled water and absolute methanol. For the biherbal formulation, 50 g of *Garcinia kola* and 50 g of *Carica papaya* roots were combined (100 g) and macerated in boiled water and absolute methanol. They were left at room temperature (30±2 °C) with frequent shaking for 72 h. They were filtered using a glass funnel tightly plugged with cotton wool, and the filtrates were concentrated in a hot air oven at 60 °C.

Bacterial isolates: The bacterial typed cultures, *Staphylococcus aureus* (Sa_{ATCC} 25922) and *Bacillus subtilis* (Bs $_{NCTC}$ 8236) were obtained from the Department of Pharmaceutical Microbiology at the University of Benin, while clinical isolates, *Staphylococcus aureus*, *Escherichia cola*, *Klebsiella species* and *Pseudomonas aeruginosa* were collected from the Department of Medical Microbiology Laboratory at the University of Benin Teaching Hospital in Benin City. The bacterial isolates were confirmed using the procedures outlined in Cruickshank (1970) and Cheesbrough (2000).

Antibacterial activity: A preliminary antimicrobial susceptibility test was performed using the agar ditch method. Thirty millilitres of molten nutrient agar was poured into standard-size Petri dishes and allowed to solidify. A 10 mm wide and 60 mm long ditch was made in the centre of the solidified nutrient agar plate. The base of the ditch was sealed with molten agar to prevent the extract from seeping under the agar. Diluted overnight nutrient broth cultures of bacteria isolates to match the 0.5 McFarland standard were streaked across the ditch, and each ditch was filled with 1.0 ml of 300 mg/ml BH extract. They were incubated uninverted at 37 °C for 24 h and observed for the zone of inhibition from the ditch, indicating the organism was sensitive (Itemire and Idu, 2014).

Minimum inhibitory concentration (MIC): The minimum inhibitory concentration of the sensitive bacteria isolates to 300 mg/ml extracts was determined using the agar well diffusion method. Thirty millilitres of molten nutrient agar was poured aseptically into a standard Petri dish and allowed to solidify. Six wells evenly spaced were made in the nutrient agar plate with a sterile 10 mm cork borer after inoculation with the appropriate standardised bacterial isolate (0.5 McFarland standard). The base of each well was sealed with 0.025 ml of molten agar. The wells were labelled and filled with 0.2 ml of the varying concentrations of the extracts (300, 150, 75, 37.5, 18.75, 9.38 and 4.69 mg/ml). The nutrient agar plates were incubated at 37 °C for 24 h. The growth pattern was observed and the zones of inhibitions were measured using a pair of dividers and a ruler in millimetres.

Statistical analysis: A two-way analysis of variance (ANOVA) was conducted using GraphPad Prism® (version 9.5.1) to assess the effects of two factors: Extract type or bacteria species and concentration on the zone of inhibition (ZOI) across bacterial species. Tukey post hoc multiple comparison was carried out. Results were given as mean ± SEM (standard error of the mean) and presented in tables and bar graphs. Statistical significance was accepted at $p < 0.05$.

Results

Percentage yield of extracts: The aqueous extracts of male and female *Carica papaya* roots had the highest yields at 23.02 % and 33.15 %, respectively. In contrast, the aqueous extract of *Garcinia kola* root had the lowest yield of 3.38 %. The combined aqueous formulation of the root of *Garcinia kola* with male *Carica papaya* yielded 26.87 %, and the combination with female *Carica papaya* yielded 20.41 %. The methanol root extract of *Garcinia kola* yielded 9.83 %. The methanol root extract of the combined formulation of *Garcinia kola* and the male *Carica papaya* yielded 4.99%, and with female *Carica papaya,* the yield was 5.68 %.

Antibacterial activity: The aqueous root extracts of male *Carica papaya* (MCPA), female *Carica papaya* (FCPA), combined (biherbal) formulations of male *Carica papaya* and *Garcinia kola* (AA), female *Carica papaya* and *Garcinia kola* (CA), as well as the methanol extracts of male *Carica papaya* (MCPM) and female *Carica papaya* (FCPM), had no zone of inhibition against the bacterial isolates. However, the aqueous root extract of *Garcinia kola* (GKA), methanol extracts of *Garcinia kola* (GKM), the combined (biherbal) formulations of male *Carica papaya* and *Garcinia kola* (AM), and female *Carica papaya* and *Garcinia kola* (CM) showed varying sizes of zones of inhibition against *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa. Escherichia coli* and *Klebsiella species* showed no zone of inhibition in aqueous and methanol root extracts (Table 1).

Table 1: Zone of inhibition (mm) of bacterial isolates against different extracts at 300 mg/ml

Extract	***Bacillus subtilis***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella species***
GKA	20.8 ± 0.38	22.0 ± 0.93	17.5 ± 2.99	-	-
GKM	22.2 ± 0.60	21.8 ± 0.70	16.5 ± 2.22	-	-
AM	18.0 ± 0.58	20.3 ± 1.01	16.5 ± 0.50	-	-
CM	20.3 ± 0.42	23.6 ± 0.94	13.3 ± 2.33	-	-
AA	-	-	-	-	-
CA	-	-	-	-	-
FCPA	-	-	-	-	-
FCPM	-	-	-	-	-
MCPA	-	-	-	-	-
MCPM	-	-	-	-	-

Values are mean ± SEM (standard error of the mean) of 3 replicates. GKA- Aqueous extract: GK (*Garcinia kola* root) only, GKM - Methanol extract: GK (*Garcinia kola* root) only, AM - Methanol extract: GK (*Garcinia kola* root) + MCP (Male *Carica papaya* root), CM - Methanol extract: GK (*Garcinia kola* root) + FCP (Female *Carica papaya* root), AA - Aqueous extract: GK (*Garcinia kola* root) + MCP (Male *Carica papaya* root), CA - Aqueous extract: GK (*Garcinia kola* root) + FCP (Female *Carica papaya* Root), FCPA - Aqueous extract: FCP (Female *Carica papaya* root) only, FCPM - Methanol extract:

FCP (Female *Carica papaya* root) only, MCPA- Aqueous extract: MCP (Male *Carica papaya* root) only, MCPM - Methanol extract: MCP (Male *Carica papaya* root) only

Zone of inhibition of aqueous and methanol root extract of Garcinia kola: *Staphylococcus aureus* exhibited the largest zone of inhibition, followed by *Bacillus subtilis* and *Pseudomonas aeruginosa* across the different concentrations. The size of zones of inhibition reduced as the concentrations of extract decreased. Regardless of the bacteria species, higher concentrations of each extract consistently produced a larger zone of inhibition. Notably, the highest concentration of 300 mg/ml consistently yielded the largest zones of inhibition across all bacteria species. Methanol extract inhibited bacterial growth at a lower concentration (4.69 mg/ml) compared with the aqueous extract (9.38) as shown in Table 2.

Table 2: Zone of inhibition (mm) of root extracts of *Garcinia kola*

Concentration	**Aqueous extract**			**Methanol extract**		
(mg/ml)	***B. subtilis***	***S. aureus***	***P. aeruginosa***	***B. subtilis***	***S. aureus***	***P. aeruginosa***
300	22.2± 0.80	26.5±0.87	21.0±3.51	23.0±0.89	25.8±0.84	20.0±1.15
150	20.8± 0.73	25.5±0.68	18.3±3.38	22.4±0.87	25.8±0.84	18.7±0.88
75	20.0± 0.84	24.0±0.57	16.7±3.18	21.6±0.81	24.9±0.81	16.7±0.88
37.5	19.0± 0.71	22.8±0.70	14.0±3.00	21.6±0.81	24.1±0.79	15.3±0.33
18.75	18.0± 1.05	21.5±0.65	18.5±0.50	20.4±1.03	23.3±0.86	13.7±0.88
9.38	16.0± 0.84	20.1±0.83	15.5±0.50	19.6±1.08	21.6±0.94	13.5±0.50
4.69	-	-	-	17.6±1.03	20.5±1.32	11.5±0.50

Values are mean ± SEM (standard error of the mean) of 3 replicates. There was a significant increase in the bacterial species' zone of inhibition as the extract's concentration increased $p < 0.05$.

Zone of inhibition of methanol biherbal root extract of Garcinia kola and male Carica papaya (AM) and Garcinia kola and female Carica papaya (CM): The most significant zones of inhibition were observed with *Staphylococcus aureus* compared to the other bacterial species. Higher concentrations of the extracts generally resulted in a larger zone of inhibition within each bacterial species. The highest concentration of 300 mg/ml produced the largest zones of inhibition across all bacterial species and there was a significant difference in the zones of inhibitions between AM and CM, $p < 0.05$ as shown in Figure 1.

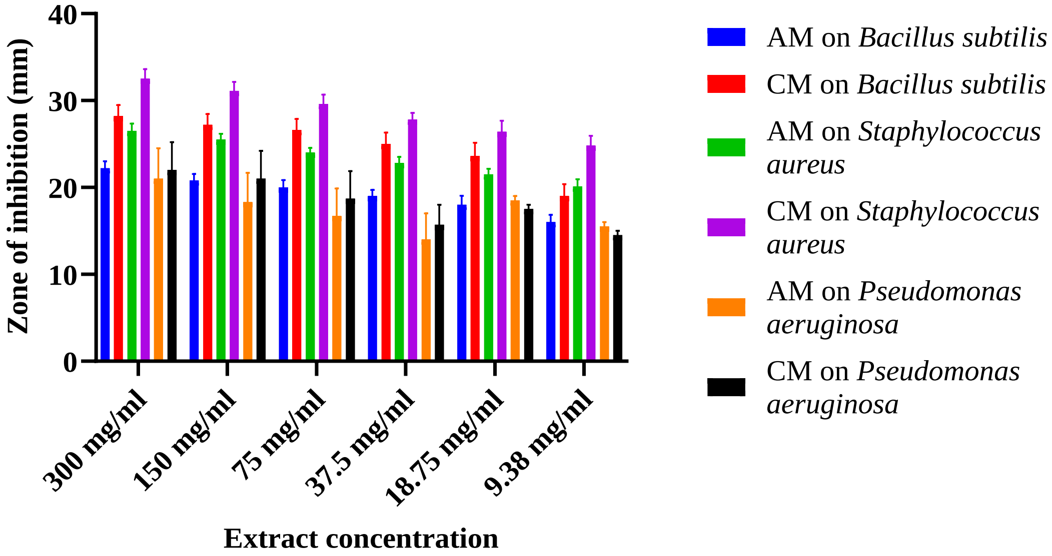

Figure 1: Zones of inhibition of methanol biherbal root extract of *Garcinia kola* and male *Carica papaya* (AM), and *Garcinia kola* and female *Carica papaya* (CM) against *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa*. There was a significant increase in the zone of inhibition of AM and CM as concentrations increased $p < 0.05$. There was a significant difference in the zones of inhibitions between AM and CM, $p < 0.05$.

*Minimum inhibitory concentrations (MIC) of the root extracts of Garcinia kola and the biherbal extracts of Carica papaya***:** The methanol root extract of *Garcinia kola* (GKM) had the lowest MIC followed by CM and AM while GKA recorded the highest MIC against *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas* aeruginosa. The MIC for *Staphylococcus aureus* was significantly lower than *Bacillus subtilis* and *Pseudomonas* aeruginosa in all extracts as represented in Table 3.

Table 3: Minimum Inhibitory Concentrations (mg/ml) of root extracts

Extract	*Bacillus subtilis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
GKA	20.6 ± 6.89	16.4 ± 1.53	37.5 ± 0.06
GKM	3.1 ± 0.70	2.2 ± 0.56	14.1 ± 4 .69
AM	4.2 ± 0.47	2.8 ± 0.44	28.1 ± 9.38
CM	3.8 ± 0.57	2.0 ± 0.49	28.1 ± 9.38

Values are mean ± SEM (standard error of the mean) of 3 replicates. GKA--- Aqueous extract of *Garcinia kola* root, GKM- Methanol extract of *Garcinia kola* root, AM - Methanol extract of *Garcinia kola* root and male *Carica papaya* root and CM- Methanol extract of *Garcinia kola* root and female *Carica papaya* root.

Discussion

Plants are rich in therapeutic and prophylactic antibacterial compounds. *Carica papaya* root had the highest yield in the aqueous solvent while *Garcinia kola* root recorded more yield in methanol, thus suggesting bio-compounds of different polarity (Truong *et al*., 2019). Kanadi *et al*. (2019) reported *Carica papaya's* highest yield in aqueous extract followed by methanol, ethyl acetate, chloroform and n-hexane. The methanol root extracts of *Garcinia kola* and its biherbal formulation recorded a lower yield and a higher antibacterial activity. The polarity of solvents affects yield and the level of antimicrobial activities of herbal plant extracts (Padalia *et al*., 2017; Truong *et al*., 2019). Phytoconstituents, extraction solvents and methods affect the yield and bioactivity of plant extracts (Ajanal *et al*., 2012; Mahdi-Pour *et al*., 2012).

There was no antibacterial activity in the aqueous and methanol root extracts of *Carica papaya* and the aqueous bi-herbal formulation of *Garcinia kola* extract against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli***,** *Klebsiella species* and *Pseudomonas aeruginosa* which could be a result of the method of processing the root such as drying for two weeks and oven drying before grinding which could have led to the loss of antibacterial compounds. This result is comparable to the non-antibacterial activity of the aqueous root extract of *Carica papaya* reported by Doughari *et al.* (2007) however, differs from the methanol result with the highest antibacterial activity. The aqueous extract of *Garcinia kola* and methanol extracts of *Garcinia kola* and its composite (biherbal) formulation inhibited *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa. Escherichia coli* and *Klebsiella species* were not inhibited because they were resistant to *Garcinia kola* extracts at the concentrations used. At the various extract concentrations, *Staphylococcus aureus* exhibited the largest zone of inhibition and lowest minimum inhibitory concentration (MIC) in contrast to *Pseudomonas aeruginosa* with the smallest zone of inhibition and highest minimum inhibitory concentration (MIC). The minimum inhibitory concentration measures how potent a substance is in inhibiting bacterial growth, the lower the MIC the higher its antibacterial activity. The methanol extract of *Garcinia kola* was the most potent of all the extracts with the lowest MIC (*Staphylococcus aureus* 2.2 ± 0.56; *Bacillus subtilis* 3.1 ± 0.70 and *Pseudomonas aeruginosa* 14.1 ± 4 .69). The methanol biherbal extracts had higher MIC compared to the MIC of the methanol extract of *Garcinia kola* this, showed a reduction of the antibacterial activity of the biherbal extract thus, indicating the non-synergistic effect of the combination because of the non-antibacterial activity of *Carica papaya*. Antimicrobial susceptibility results of the *Garcinia kola* root were reported by Doughari *et al*. (2007) and Idu *et al.* (2014). *Staphylococcus* aureus, *Bacillus subtilis* and *Pseudomonas aeruginosa* were sensitive to the male and female *Carica papaya* biherbal extracts of *Garcinia kola,* therefore either male or female *Carica papaya* could be used in the biherbal formulation.

In conclusion, using the same extraction method, solvents affected the antibacterial activity of the plant extracts. The aqueous root extracts of the *Carica papaya* though recorded the highest yield showed no antibacterial activity. The methanol composite (biherbal) formulation root extracts recorded low yield with reduced antibacterial activity compared to the methanol root extract *Garcinia kola* with high yield and, large zones of inhibitions and low MICs. Methanol can therefore be suggested for *Garcinia kola* extraction to treat bacterial infections.

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