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# Antibiofilm Potential of Aqueous Extracts of *Garcinia kola* against *Salmonella* species in Greater Cane Rat (*Thryonomys swinderianus*, Temmink 1827) from Badagry, Lagos State, Nigeria

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**ABSTRACT**

Biofilms encompass a cluster of microorganisms encased in a slimy matrix of extracellular polymeric substances, which imbibe antibiotic resistance. *Salmonella* specie is a major bacterial cause of food-borne diseases in both humans and animals. The prevalence of *Salmonella* species is increasing which may form biofilm, and bacteria that form biofilm may be more resistant to antimicrobial agents. This study is intended to determine the presence and antimicrobial susceptibility patterns of *Salmonella* species among greater cane rats and carry out the biofilm formation of isolated *Salmonella* species and dispersion using *Garcinia kola*. A total of 20 cane rats were obtained from a farm in Lagos state and roadside hunters. Samples of rectal swabs were collected aseptically for bacterial culture. Bacteria isolation was done with selective media using standard bacteriological techniques following pre-enrichment of the samples with buffered peptone water. Gram staining and biochemical tests were used for confirmation of the organisms. An antimicrobial susceptibility test was performed following standard protocol. Zones of inhibition were measured and categorized as totally sensitive and resistant. Nine (9) isolates of *Salmonella* species were obtained from rectal swabs of greater cane rats. A modified crystal violet assay was employed to develop biofilm and test the effect of a crude extract of *Garcinia kola* on biofilm dispersal. Data obtained from this study was subjected to statistical analysis using descriptive statistics and paired t-test at a level of significance of $P \leq 0.05$. The overall prevalence of *Salmonella* species obtained in this study was 45%. Antimicrobial study showed susceptibility of 77.8% for Chloramphenicol and 66.7% for Cefuroxime while all isolates were 100% susceptible to Amikacin and Gentamicin. The effect of *Garcinia kola* extract on different isolates at 430nm showed a significant difference ($p= 0.0348$) in biofilm formation in isolates with *Garcinia kola* extract when compared with isolates without extract. The effect of *Garcinia kola* extract on different isolates at 650nm elicited significant ($p = 0.0216$) biofilm inhibitions across the isolates. The antibiofilm activity displayed by *Garcinia kola* suggests its potential to serve as an alternative antimicrobial agent to combat drug resistant bacteria such as *Salmonella* species. However, further investigations should be carried out on the individual bioactive compounds to know the exact metabolites responsible for the antibiofilm activity of this bacteria species.

**Keywords:** Antimicrobial susceptibility; Biofilm; *Garcinia kola;* Greater Cane Rats, *Salmonella* species,

## INTRODUCTION

Greater cane rats (GCRs), commonly called grasscutters are perceived as pests of economic importance in agriculture. Their meats are consumed widely and often regarded as one of the best bush meats sold and used for ceremonial purposes, especially in West Africa (Opara, 2010). Based on the current trend of domestication of GCRs, there is a need to study the normal fecal flora and pathogenic organisms that could present danger for the farmers and final consumers. GCRs are sources of food for humans and therefore, possible harbor grounds for certain pathogens present in food which are often implicated in human gastrointestinal infections; such as *Salmonella* species and *Campylobacter species* (Abebe *et al.,* 2020).

*Salmonella* species are zoonotic bacteria that stand as a leading cause of human gastroenteritis worldwide, resulting in severe enteric fever which requires the use of

antimicrobial therapy, especially among infants, and the elderly population who are immunocompromised (Jajere, 2019; Abebe *et al.,* 2020; Orum *et al.,* 2022). Some of these bacterial species cause infections where they reside in the digestive tracts of many farm animals. A proper understanding of their life cycles and occurrence would aid an in-depth grasp of their role in human transmission. Also, the zoonotic potency of these pathogens in food, and their tendency to produce toxins that can lead to death is a pointer for public health (Keerthirathne *et al.*, 2022). This is particularly in the aspect of occupational health distinct to farmers and hunters whose occupation it is, and eventually to the larger population.

*Garcinia kola*, commonly known as bitter cola, has been dubbed a "wonder plant," as findings have revealed that every part of it is of great medicinal value (Ekene and Earnest, 2014). The use of antimicrobial drugs is instrumental in the management of infectious diseases which aids the protection of animal and human health (FAO, 2021). However, the indiscriminate use of antimicrobial drugs in agricultural, animal medicine and human health has been associated with the sprouting and persistence of bacterial resistance which leads to ineffective management of infectious diseases and raising serious concerns in public health (FAO, 2021).This has led to increasing attention being paid to the adoption of medicinal plants used in folkloric treatments as potential substitutes for antibiotics resistant to multiple bacterial organisms (Adegboye *et al*., 2008).

Some noticeable resistance to antibacterial agents is developments that arise from biofilms which are microorganism clusters that affix to cells in an autogenic pattern of Extracellular Polymeric Substances (EPS) (Maier, 2021; Hemmati *et al.,* 2021; Wong *et al.*, 2021). Under certain environmental conditions, *Salmonella species* can develop biofilms on contact surfaces and on food, thereby raising a cause for public health attention(Seo *et al.,* 2020). This study was conducted to investigate the occurrence, antimicrobial susceptibility profiles and explore the antibiofilm activity of *Garcinia kola* against *Salmonella* species in GCRs.

## MATERIALS AND METHODS

### Design and Sampling Method

An observational cross study was conducted to analyze the occurrence of *Salmonella* species, its multidrug-resistance, biofilm formation of the *Salmonella* isolates and possible dispersion by *Garcinia kola* in GCRs. The GCRs were bought from Panvemgo Grasscutter Farm and hunters in Badagry, Lagos State, Southwest Nigeria with a coordinate of 6°25′N 2°53′E.

### Sample Collection

We procured 20 GCRs from both an animal farm and roadside hunters in Lagos. Upon restraining the animals, we collected their rectal swab samples which were safely placed in polythene plastic bags to minimize spilling and avoid cross-contamination. Samples were properly labeled and then placed in cold icebox containing ice packs. They were promptly transported to the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, University of Ibadan for processing.

### Isolation of *Salmonella* species

The isolation of S*almonella* species was conducted according to the International Organization for Standardization standard 6579 guidelines specifically designed for characterizing *Salmonella* species (ISO, 2017). The process began with pre-enrichment, where each fecal swab was immersed in 10 ml tubes containing buffered peptone water. By utilizing a vortex machine, the samples were homogenized and then incubated at 37°C for approximately 20 hours. After the pre-enrichment step, 0.1mL of the broth culture was subculture into 10mL of Rappaport Vassiliadis broth to ensure selective enrichment of the bacteria. This selective enrichment was maintained at a temperature of 37°C for 18 to 24 hours. Following the overnight enrichment, a loopful size of the culture was inoculated into xylose lysine deoxycholate agar, this isolation process was carried out for 24 to 26 hours at a temperature of 37°C. To obtain pure Salmonella cultures, a subculture procedure was performed at 37°C for 24 hours. The focus was on colonies that appeared reddish with dark centers when inoculated in xylose lysine deoxycholate agar. The pure colonies were subsequently inoculated onto nutrient agar slants and incubated at 37°C for 18 to 24 hours. Finally, to maintain the purity of the isolates in nutrient agar slants, they were preserved in a refrigerator at 4°C.

### Morphological Identification and Biochemical Tests

We identified *Salmonella* species based on morphology of colonies, which was further conceptualized and characterized with Gram stain technique and other biochemical test like indole, citrate, methyl red, urease, catalase, Voges Proskeur and triple sugar iron. In addition, sugar fermentation tests were conducted: glucose, sucrose, mannitol, lactose, and xylose.

### Antimicrobial Susceptibility Testing

A susceptibility test for antimicrobial content was performed using agar disk diffusion method. We reported the patterns of sensitivity recorded in the cultured isolates. Next on the process was the subculture of the isolates on nutrient agar slants onto nutrient agar plates and then left for 24 hours in an incubator set at 37°C. Following this, the isolates were standardized by comparing their turbidity in normal saline to 0.5 McFarland standard (~ 108 CFU/mL). and this was done by emulsifying a colony from each agar plate into sterile saline. Next, the standardized culture was evenly spread onto Muller Hinton agar plates. Subsequently, gram negative antibiotics discs were placed on the inoculated agar surface with the use of adequately sterilized forceps after which the plates were again incubated at a regulated incubation temperature of 37°C for 18 hours. For this procedure, the antimicrobials used in this study include ciprofloxacin (5 µg), cotrimoxazole (25 µg), tetracycline (10 µg), gentamicin (10 µg), amikacin (30

µg), chloramphenicol (10 µg), meropenem (10 µg), ceftriaxone (30 µg) and ceftazidime (30 µg). We measured the various inhibition zones around each antibiotic disc and recorded the interpretations following the guidelines mapped out by Clinical Laboratory Standard Institute (2020).

**Biofilm Quantification of *Salmonella* species**

We utilized 9 isolates of *Salmonella* for development of the biofilm, with an assessment done using the crystal violet binding method. This evaluation was done in 96-well polystyrene flat bottomed microtiter plates. A 50 µL aliquot of fresh bacterial suspension in modified tryptone soy broth (mTSB) 108 CFU/ml was transferred into respective microtitre plates and then cultured for 48 hours in an incubator having a steady temperature of 37°C. Following the incubation, the supernatant was transferred out and each of the wells sterilized to get rid of free-floating cells. The plates were dried in air for half an hour, heat-fixed for 60 seconds and the formed biofilm was stained using 0.1 % aqueous solution of crystal violet for 15 minutes at room temperature. Excess stain was washed off in distilled water three times. At the end, the dye bound to cells were solubilized by adding 200 µL of 95% ethanol to each well and left for 15 minutes incubation time while at room temperature. The absorbance level was calculated using the microplate reader at wavelengths of 432 nm and 630 nm. The overall tests were conducted in three places to ensure accuracy.

**Procedure for Preparation of *Garcinia kola* Extract**

Aqueous extracts were gotten by air-drying the powdered seeds (100 g) of the plant and adding it to 500 mL of distilled water at 70°C in a water bath for a period of two (2) hours. The filtrate was evaporated to dryness, preserved in sterile glass jars and stored in low temperature freezer for future use(Fatope *et al.,*1993*)*.

**Biofilm Dispersion of *Garcinia Kola* Evaluation**

We evaluated the effect of extracts on biofilm dispersion using a 96-well polystyrene flat microtiter plates. A measure of 50 µL of fresh suspension of the test organisms in modified tryptone soy broth (mTSB) at a dilution rate of $10^8$ was aliquoted into individual microplates and then left in an incubator for 48 hours at a temperature of $37^OC$. The plates were retrieved, infused with 50 µL of 50 mg/mL of the extracts and then incubated for 24 hours to check the dispersion level of the biofilm that had been formed. Subsequently, the supernatant was removed, and all wells properly washed in distilled water to get rid of free-floating cells, after which they were allowed to air dry for thirty minutes, heat fixed for a minute and the biofilm was stained with 0.1 % aqueous solution of crystal violet at standard room temperature for 15 minutes. After the incubation, excess stain was washed off in sterile water. A measure of 200 µL of 95 % ethanol was added to respective wells and incubated at room temperature for 15 minutes to solubilize the cells. We measured the rate of absorbance by using a microplate reader at 432 and 630 nm wavelengths. The process was carried out twice to ensure a comparison (Adedeji and Adetunji, 2020).

**Data Storage Analyses**

The data were entered into Microsoft excel 2010 and analysed using the SPSS statistical software package version 20 (IBM SPSS statistics). Descriptive statistics were used to compute proportions and frequency distributions of the rate of *Salmonella* isolation. *Salmonella* isolates were further screened for susceptibility to 9 different drugs and classified as susceptible, intermediate and resistant using frequency and proportions. Multi-drug resistance (MDR) was considered if and only if one *Salmonella* isolate was resistant in three or more antimicrobial categories.

**Ethical Statement**

Ethical clearance was obtained from the Animal Welfare and Research Ethical Review Committee of the Ebonyi State Ministry of Agriculture and Natural Resources.

**RESULTS**

**Isolation Rate and Morphological Identification of *Salmonella* species**

Presumptive Salmonella colonies were observed to be red with a black center on xylose lysine deoxycholate agar. Prior to biochemical testing and gram staining, 12 (55%) out of the 20 samples yielded presumptive isolates during the culture phase, of which 9 (45%) were subsequently confirmed positive following biochemical analysis and gram staining. Gram staining revealed the isolated *Salmonella* species to exhibit small, Gram-negative rod-shaped morphology, appearing singly. Positive sugar fermentation was evidenced by a pink coloration observed for mannitol, sucrose, glucose, lactose, xylose, maltose, and fructose. Effervescence was noted as a positive reaction in the catalase test and in the Triple Sugar Iron (TSI) test. The appearance of three distinct colors (black, yellow, and red) during the Triple Sugar Iron (TSI) test indicated a positive result for Salmonella spp. The overall isolation rate of *Salmonella* species in this study was 45% (Table 1).

**Table 1:** Isolation rate of *Salmonella* species in Greater Cane Rat in Southwestern Nigeria

| | Frequency | Percentage |
|---|---|---|
| Presumptive Isolates | 12 | 55% |
| Confirmed Positive Isolates | 9 | 45% |
| Total Sample Analyzed | 20 | 100.0% |

**Antimicrobial Resistance Tests**

Antimicrobial resistance testing of the isolates revealed diverse resistance patterns. Tetracycline resistance was observed in 66.67% of isolates, while 44.44% exhibited resistance to Cotrimoxazole, Ceftazidime, and Chloramphenicol. Resistance rates of 62.5% were recorded for ceftriaxone, 22.22% for ciprofloxacin, and 33.33% for meropenem. However, none of the isolates displayed resistance to Gentamicin or Amikacin, with all isolates exhibiting 100% susceptibility to these antimicrobials (Figure 1).

**Effect of *Garcinia kola* Extract on Different Isolates at 430nm**

Figure 2 shows various rates of inhibition across the different isolates. The results showed a statistically significant difference (p= 0.0348) in biofilm formation in isolates with *Garcinia kola* extract and compared with isolates without extract. Marked inhibitions were observed in isolates 2, 4 and 5. However, isolate 3 showed no obvious inhibition when compared to other isolates.

**Effect of *Garcinia kola* Extract on Different Isolates at 650nm**

The extract elicited significant (p = 0.0216) biofilm inhibitions across among the isolates. Isolates 5,7,8 and 9 had minimal biofilm formation while isolates 3 and 4 showed no significant inhibition when compared to other isolates (Figure 3).

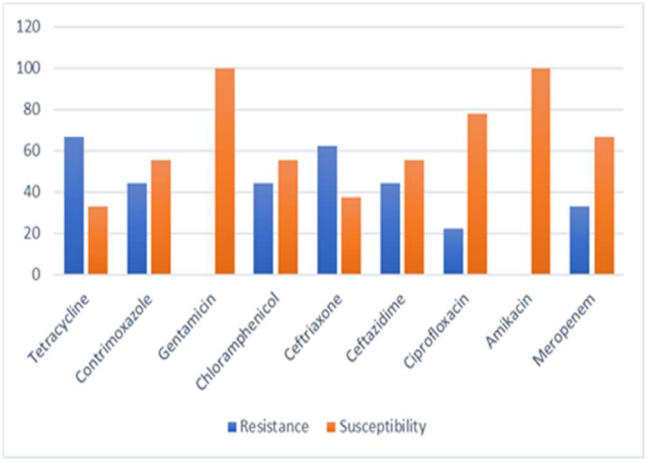


**Figure 1**: Antibiotic susceptibility pattern of *Salmonella* species isolated in Greater Cane Rat in Southwestern Nigeria.

**DISCUSSION**

The bacterial genus *Salmonella* is responsible for causing various gastrointestinal diseases in humans and animals. It is crucial to identify and characterize *Salmonella* species in different animal populations to comprehend their potential role as reservoirs and sources of transmission to humans. In this study, an overall isolation rate of 45% was obtained for *Salmonella* species from rectal samples of greater cane rats, indicating a relatively high isolation rate of *Salmonella* in this species. The presence of *Salmonella* in the rectal samples suggests that greater cane rats could potentially be a carrier of the bacteria, posing a transmission risk to humans and other animals. A similar study was conducted to investigate the occurrence of *Salmonella* in cane rats, which reported a lower prevalence rate of 32% for *Salmonella* isolates in comparison to our study (Oboegbulem and Okoronkwo, 1990). When compared to another study conducted on zoo animals in Nigeria, the isolation rate of *Salmonella* was significantly higher in greater cane rats. The zoo animal study reported an isolation rate of only 5% among the tested samples, which is considerably lower than the isolation rates found in our study (Oludairo *et al.*, 2013). The differences in the isolation rates can be explained by several factors, including variances in animal populations, sample sizes, geographical locations, and potential differences in animal care practices. Additionally, it is possible that the unhygienic conditions observed on farms, inadequate biosecurity measures, and the transmission of these pathogens from humans to wildlife through hunting activities have contributed to this inconsistency.

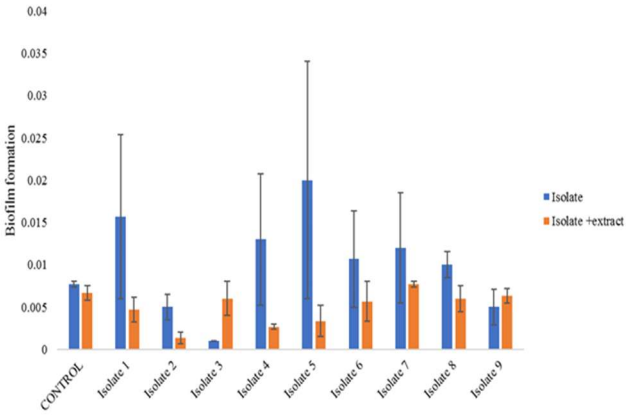


**Figure 2.** Inhibitory effects of *Garcinia kola* extract on *Salmonella* species biofilm formation at 430nm

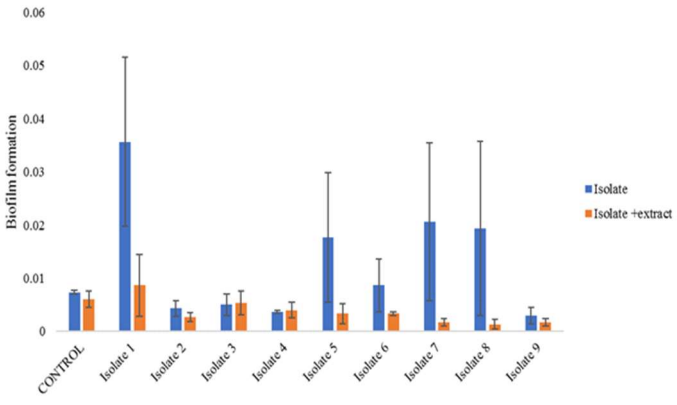


**Figure 3.** Inhibitory effects of *Garcinia kola* extract on *Salmonella* species biofilm formation at 650nm

Antimicrobial resistance of *Salmonella* isolates has been reported in living species and food samples in certain regions of Africa (Abdi *et al.*, 2017; Marami, Hailu and Tolera, 2018; Orum *et al.,* 2022). This may be because of indiscriminate antimicrobial abuses and interactions

existing in animals, environment and man (Feasey *et al.,* 2012; Usmael *et al.*, 2022). The antimicrobial profile of the 9 isolates was tested and 77.78% of the isolates were seen to resist at least one or more antimicrobial agents. 88.89% were observed to have multiple drug resistance. Amikacin and gentamicin were the most effective antimicrobials the isolates were sensitive to which was 100%. This report is similar to reports in Eastern Ethiopia (Usmael *et al.*, 2022) and Brazil (Souto *et al.*, 2017). The isolates were most resistant to tetracycline (66.67%) and ceftriaxone (62.5%) which is similar to reports in Nigeria (Orum *et al.*, 2022) and South Africa (Jaja *et al.*, 2019). This study shows the varying effects of biofilm inhibition on different isolated *Salmonella* species. This inhibitory activity against *Salmonella* biofilms formation demonstrates potential antibiotic ability of the extract. Following initial infection, *Salmonella* species has been reported to persist in the gut of the affected animals or humans due to biofilm formation (Fàbrega and Vila, 2013; Hakimi *et al.,* 2020).

Our findings in this study suggest possible antibiotic replacement therapy for resistant *Salmonella* in animals. The isolates with no significant inhibitions in this study could be because of mixed infection with organisms lacking biofilm formation ability. The increase in antimicrobial resistance has paved way for plant-based research. Several phytochemical studies on biofilm inhibition have been recorded (Sakarikou *et al.,* 2020; Hakimi *et al.,* 2020). Phytochemicals when used alone or combined, are plant metabolites that have demonstrated diverse antimicrobial activity against various infectious organisms with *Salmonella* species being inclusive (Sakarikou *et al.,* 2020). Studies on the antimicrobial action of *Garcinia kola* and other plant juices have exhibited a varying degree of susceptibility of the clinical pathogens to the juices used (Amala *et al.,* 2021). Plant essential oils have exhibited anti-biofilm properties and were suggested to be an alternative therapy for both microbial control and therapeutic treatment against pathogenic resistant bacteria (Morshdy *et al.,* 2022).

In addition to its antimicrobial activity, *Garcinia kola* have been shown to exhibit varying effects and mode of actions depending on the method of extraction, partitioning and its concentration used. Therefore, the biofilm dispersion showing varying inhibition observed at 650nm can be attributed to the biphasic concentration-dependent mechanism of action of *Garcinia kola* (Okoronkwo *et al.,* 2022).

Several phytochemical studies show that *Garcinia kola* has an antibacterial activity, which categorizes into: terpenoids and phenolics, essential oils, alkaloids and lectins, the polyacetylenes and polypeptides (Sakarikou *et al.,* 2020). The terpenoids demonstrated an antibiofilm action against both *Salmonella typhimurium* and *Salmonella enteriditis* serovars at sub-inhibitory concentrations (Amaral *et al.,* 2015).

The presence of *Salmonella* species and evidence of biofilm formation of the isolates in greater cane rats (GCRs) in this study shows that they may be sources of food contamination which is a potential threat to consumer health posing serious veterinary and public health risks. Implementation of hygienic practices, prudent use of antibiotics, and effective meat handling and preparation are crucial in preventing these zoonotic pathogens.

## Conclusions

This study reveals a high prevalence (45%) of *Salmonella* species in rectal samples of greater cane rats, indicating their potential role as reservoirs and sources of transmission to humans. The antimicrobial resistance profile of the isolates highlights the presence of multiple drug resistance, with notable susceptibility to Amikacin and Gentamicin. The inhibitory effects of *Garcinia kola* extract on biofilm formation among different isolates demonstrate its potential as an alternative antibiotic therapy. The variations in prevalence rates compared to previous studies suggest regional and environmental factors influencing *Salmonella* contamination. The findings emphasize the importance of implementing hygienic practices, responsible antibiotic use, and proper meat handling to mitigate the veterinary and public health risks associated with zoonotic pathogens like *Salmonella*. Future research can explore the phytochemical properties of *Garcinia kola* and other plant-based alternatives for combating antibiotic-resistant bacteria and improving food safety.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## Author Contributions

T.G.O. conceived and designed the study. T.T.G, J.O.O, and O.A.O. undertook the methodology of the study. T.G.O. collected, analyzed the samples and O.O.M did the statistical analysis. T.G.O. and B.M.Y, drafted the original manuscript, and E.I.G, and I.C.O. revised it. All the authors approved the final manuscript.

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