

Isolation and Characterization of Contaminating Bacteria from *Garcinia cambogia* Extract: Methods to Reduce Microbial Load and Its Anti-Obesity Effect in Wistar Rats

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Abstract

Objectives: This study aimed to identify the contaminating bacteria in the extract of *Garcinia cambogia*, which is regularly used as a dietary supplement for addressing obesity in humans. **Methods:** The *Garcinia cambogia* extract was used and experiments were conducted to isolate the contaminating bacteria and antibiotic susceptibility was tested. The organism was identified using BIOLOG system. Such an extract was used in a placebo-controlled animal study when 6 eight adult male rats weighing between 200 and 220 g were randomly distributed into three groups (n = 3) and in test group 1, a single dose of 100 mg/kg bw of *Garcinia cambogia* extract was given while in the test group 2, 100 mg *Garcinia cambogia* extract + 116 mg *Picrorhiza kurroa* extract were administered through oral gavage. The normal control rats were given distilled water, and the treatment lasted for 30 days. Blood plasma and liver tissues were prepared for biochemical analysis and histology studies. **Results:** Nearly $\sim 10^3$ cfu/g of *Bacillus atrophaeus* was present in the *Garcinia cambogia* extract and we demonstrate >99% reduction in the microbial load with tetracycline. Such an extract at a dose of 100 mg/kg, showed weight loss in Wistar rats when administered orally for 1 month with no significant changes in liver histopathology. *Picrorhiza kurroa*, also known for its hepatoprotective properties, has been administered at a dose of 116 mg/kg along with *Garcinia* extract at 100 mg/kg orally and found to improve levels of hepatic enzymes as similar to control animals, although not statistically signifi-

cant. **Conclusions:** The study revealed that *Garcinia cambogia* could prevent weight gain in Wistar rats when given orally and the weight gain in *Garcinia*-treated animals was almost 4 times less (7.31%), as against weight gain of 25.36% seen in vehicle control animals. The antibiotic susceptibility data indicated that the isolated bacterium is resistant to many antibiotics with a strong susceptibility to tetracycline.

Keywords

Garcinia cambogia, Hydroxy Citric Acid, *Picrorhiza kurroa*, Liver Health, ATP Citrate Lyase

1. Introduction

Garcinia cambogia is an evergreen tropical shrub of the family Clusiaceae commonly known as *Garcinia gummi-gutta* or *Malabar tamarind* [1]. It is a tree that is native to southeastern Asia and also distributed in tropical Asia, Polynesia and, Africa [2]. *Garcinia cambogia* fruits are used to treat rheumatism, edema, irregular menstruation, constipation, piles, and intestinal parasites in many south Asian countries [3]. The *Garcinia* extract exhibits several biological properties viz. appetite-suppressant, anti-obesity, hypolipidaemic, antidiabetic, anti-inflammatory, antinociceptive, antioxidant, anticancer, antihistaminic, anti-ulcerogenic, vasodilator, hepatoprotective, nephroprotective, and cardioprotective, hepatoprotective, anthelmintic and diuretic activity [4].

Garcinia cambogia, *Garcinia indica*, and *Garcinia atroviridis* are rich in hydroxycitric acid (HCA) and predominately used as a dietary supplement for weight loss and anti-obesity agent [5] [6] [7]. The weight loss due to intake of *Garcinia* extract is attributed to its property to inhibit ATP-citrate-lyase involved in fatty acid biosynthesis, inhibition of pancreatic alpha-amylase, intestinal alpha-glucosidase, thereby leading to a reduction in carbohydrate metabolism and also reducing the availability of serotonin in the brain, causing to appetite suppression [8] [9]. Probiotics and *Garcinia cambogia* extract alleviate weight gain and adiposity, in part via differentially modulating the composition of gut microbiota in high-fat diet (HFD) fed mice, and obesogenic bacteria such as *Clostridium aminophilum* are reported to be suppressed by the combination of probiotics and *Garcinia cambogia* extract [6].

The increasing use of herbal medicines has created a new public health issue due to ineffective surveillance on the quality of these products, their efficacy, and toxic effects due to fungal and bacterial contaminants [10]. As quality control of herbal drugs has been at the discretion of health policies of each country, the average microbial load of herbal drugs varies drastically from country to country [11] [12]. Microbial contamination has become a hurdle in developing herbal products; therefore certain quality control and assurance measures have been sought out to avoid the health risk due to the use of contaminated herbal medicines. Also, the microbial contaminants adversely affect the quality of the extraction process and

decrease the yield and potency of active constituents in herbal extracts [13].

The microbiological quality of medicinal plants is dependent upon intrinsic factors such as presence of microbial endophytes, nature of the plant and natural barriers and structure of the plant and plant composition [14]. The external factors such as climatic conditions, packaging, storage conditions, humidity, harvesting methods, and environmental microbial contaminants also affect the quality of herbal drugs [15]. It was reported that 51.5% and 35.6% of the herbal medicines used in Macapa, Brazil are contaminated with bacterial species and fungal strains respectively. The prevalent species of bacteria found in herbal products were *S. aureus* (49.2%), *Salmonella* Spp. (34.8%), *E. coli* (25.8%) and *P. aeruginosa* (14.4%). The consumption of such contaminated herbal products will adversely affect the health of people due to lack of microbial quality standards [16]. Agarwal *et al.* [17] reported that the high moisture content of herbal raw materials favors growth of microbial contaminants which in turn decreases the effective concentrations of active constituents present. Different fungal and bacterial sps identified in the extracts of *Glycyrrhiza glabra* strains have been tested and high moisture content was the reason attributed for the presence of these bacterial species (10^3 to 10^7 cfu/g).

There are several hepato-protective medicinal plants reported of which *Picrorhiza kurroa* has been commonly used and well investigated for the treatment of jaundice [18]. *Picrorhiza kurroa* belonging to the family Scrophulariaceae, a small perennial herb, growing at an elevation of 3000 - 5000 meters, is used to treat fever, dyspepsia, and liver and respiratory disorders [19]. The active component Picroliv has been shown to have hepato-protective effects in mice [20] [21] [22]. Hence, it was considered desirable to study if *P. kurroa* along with *Garcinia* would provide additional advantages in an animal model.

2. Material and Methods

2.1. Materials

BIOLOG GEN III microplates for aerobes (Cat. No. 1030, BIOLOG), and Inoculating Fluid B (Cat. No. 72402, BIOLOG) were purchased from Biolog, Hayward, CA 94545, USA. The soybean casein digest agar (SCDA) and nutrient agar were purchased from Hi-media, Mumbai, India. SGPT (ALT), SGOT (AST), alkaline phosphate (ALP) and Bilirubin kits. Picroside I and II were procured from Natural Remedies, Bangalore, India and other chemicals and reagents used for the study were of analytical grade.

2.2. Plant Material

The fruit rinds of the *Garcinia cambogia* and *Picrorhiza kurroa* were collected from different regions of India and identity was confirmed at Durva Herbal Centre, Chennai, Tamilnadu, India. The collected samples were dried and stored at room temperature (RT) protected from direct sunlight. Commercial samples of *Garcinia* extract were collected from the production unit, Sava Healthcare, Malur, Karnataka.

2.3. Preparation of *Garcinia cambogia* Extracts

Garcinia cambogia fruit rinds were purchased from Aashirwad traders, Karnataka. 750 kgs of the *Garcinia cambogia* fruit rinds raw material was processed for extraction with four volumes of demineralized water thrice for a duration of 3 h at room temperature. After all the extractions, the extraction liquids were pooled and filtered through polypropylene (PP) cloth using a sparkler filter. The calcium salt of the pooled liquid extract was prepared by using aqueous solution of calcium hydroxide powder and pH adjusted to 8.5 to 9.5. The generated salt was then filtered through a polypropylene (PP) cloth using filter press and dried in a hot air oven at 110°C. This material containing ~55% HCA and a calcium content of 20% was used for animal studies without further purification.

2.4. Estimation of Hydroxycitric Acid (HCA) by HPLC

2.4.1. Chromatographic Condition

Estimation of HCA was carried out by RP-HPLC using an isocratic mobile phase for 12 min on a Luna C18 column (4.6 × 250 mm, 5 µm). The mobile phase consisted of buffer (1 mM anhydrous potassium dihydrogen orthophosphate (KH₂PO₄) with 30% ortho-phosphoric acid to achieve a pH of 2.5 with a flow rate of 1 mL/min keeping the injection volume as 20 µL. The elution was carried out at ambient temperature (27°C ± 1°C) whereas 10°C sample temperature was maintained. A mixture of 30% ortho-phosphoric acid in water (1:9) served as a blank and diluent as well. HPLC chromatograms were recorded at 215 nm.

2.4.2. Standard Preparation

For the standard preparation, HCA (2.5 mg/mL) was weighed in a 20 mL volumetric flask containing 10 mL diluent and sonicated for 15 minutes. The volume was made up to the mark of 20 mL with diluent and mixed well. The sample was then cooled at room temperature and filtered through 0.45 µm nylon membrane filter and appropriate volume (20 µL) was injected into the HPLC system.

2.4.3. Sample Preparation

For estimation of HCA, the herbal extract sample was prepared by weighing 60 mg into a 20 mL volumetric flask containing 10 mL diluent and sonicated for 15 minutes. The volume was made up to the mark of 20 mL with diluent and mixed well. The sample was then cooled at room temperature and filtered through 0.45 µm nylon membrane filter and an appropriate volume (20 µL) was injected into the HPLC system.

2.4.4. Preparation of *Picrorhiza kurroa* Extract

Picrorhiza kurroa roots were purchased from Noor Nihal herbs trading company, Karnataka, India. 500 Kgs of *Picrorhiza kurroa* roots was processed for extraction with four volumes of demineralized water for three times (each extraction for three hours) at 75°C - 80°C. After all the extractions were completed, the liquids were pooled and filtered through a polypropylene (PP) cloth using a sparkler filter. This filtered extract was concentrated to 20% - 25% total dis-

solved solids using an evaporator and then dried using a spray dryer.

2.5. Estimation of Picroside-I and Picroside-II by HPLC

2.5.1. Chromatographic Conditions

The chromatographic separation using HPLC was performed on Hypersil BDS C18 (150 × 4.65 μ) with gradient elution of mobile phase A (0.1% ortho-phosphoric acid) & mobile phase B (acetonitrile). The gradient program was set as (time/% B) 0/20, 17/20, 20/80, 32/80, 35/20 and 40/20 which pumped out both mobile phases at the flow rate of 1mL/min. The injection volume was kept as 20 μL for the standards and samples and the eluents such as Picroside-I & Picroside-II were examined at 255 nm at a fixed wavelength. The elution was carried out at 30°C whereas 10°C sample temperature was maintained. HPLC grade methanol was used as blank as well as the diluent.

2.5.2. Standard Preparation

For the standard preparation, Picroside I and Picroside II reference standards were weighed separately each 2.5 mg into 25 ml volumetric flask containing 10 mL diluent and sonicated for 15 minutes. The volume was made up to 25 mL with the diluent and mixed well. The sample was then cooled to room temperature and filtered through a 0.45 μ nylon membrane filter and 20 μL was injected in the HPLC system.

2.5.3. Sample Preparation

Samples were prepared by weighing nearly 100 mg into 100 mL volumetric flasks containing 70 mL of the diluent. The contents were sonicated for 15 minutes and the volume was made up to the mark of 100 mL with the diluent and mixed well. After cooling at room temperature, the contents were filtered through 0.45 μ nylon membrane filter and 20 μL was injected into the HPLC system.

2.5.4. Microbial Analysis of Garcinia Extract

The microbial load of the *Garcinia* extract was estimated as total viable/aerobic count by the conventional pour plate method as per the standard method given in Bacteriological Analytical Manual, 2001 [23].

2.5.5. Bacterial Isolation from the Garcinia Extract

10 g of the *Garcinia* extract was kept in a hot air oven at 55°C for 1 h and then suspended in 90 ml of sterile saline solution. The suspension was mixed well and 1 ml was transferred to sterile petri-plate. 20 - 25 ml of sterile molten SCDA was poured, allowed to solidify at RT. After 24 h of incubation at 55°C, the well isolated colony was picked and further purified by sub-culturing on SCDA.

2.6. Identification of Bacterial Isolate Using BIOLOG System and Biochemical Characterization of Garcinia Bacterial Isolate (GBC)

BIOLOG system enables microbial identification system for both, Gram positive

and Gram negative bacteria through the use of a 96 well microplate format where all the wells are filled with necessary nutrients and biochemicals along with the redox tetrazolium dye. A suspension of the culture, to be identified, is inoculated in these wells, incubated for 16 hours at 37°C and the growth results are compared with BIOLOG database. The tetrazolium dye changes color to purple as a result of cellular respiration providing a “metabolic fingerprint” which is used to identify the bacterium [24].

Isolated colonies of GBC were suspended in inoculating fluid (IFB) to get inoculum of recommended cell density. 100 µl of the inoculum was added in each well of 96 Biolog GEN III microplates and incubated at 35°C for 24 h. After incubation, the plates were analyzed using MicroStation with Biolog’s microbial identification software and phenotypic or metabolic fingerprint of GBC was compared with Biolog database.

2.7. Antibiotic Susceptibility of GBC

The isolated colonies of GBC were suspended in sterile saline solution (5 ml) to get inoculum for agar diffusion assay. The turbidity of the bacterial inoculum was normalized to 0.5 McFarland standards. The bacterial suspension was spread on SCDA plates. Antibiotic sensitivity discs were placed on the agar surface using sterile forceps and incubated at 35°C for 18 h. After incubation, plates were observed for zone of inhibition and diameters of zone of inhibition were recorded.

2.8. Microbial Load Reduction Trials Using Tetracycline (TE)

100 g of *Garcinia combogia* fruit rinds were suspended in 600 ml of demineralized water and extracted at 80°C for 3 h. The suspension was filtered through PP cloth for the removal of root debris. For TE treatment, a stock solution (10 mg/ml) of tetracycline hydrochloride was prepared in water. To 500 ml of liquid extracts, 5.0 ml of stock solution of TE was added to get final concentrations of 100 µg/ml and kept overnight at RT. After TE treatment, 1.5% of activated charcoal powder was added to extracts and kept for 1 h at RT then filtered through a bed of Hyflo supercel powder. To the filtrate obtained, calcium hydroxide was added (5.0%) to adjust the pH to 9.0 for the formation of the calcium salt of HCA. The resulting suspension was then filtered through PP cloth and then the residual salt was dried at RT. The microbial load in the extracts prepared was estimated by conventional method.

2.9. Estimation of Residual Tetracycline Using HPLC

The method for estimation of tetracycline in various fractions of the *Garcinia* manufacturing process was done as described before [25]. The column used in the estimation of tetracycline in this article was Hypersil BDS 150 × 4.6 and 5 µm, in place of Inertsil ODS, 3V, 250 × 4.6 mm and 5 µm that was used earlier [25] keeping all other conditions of the HPLC same.

2.10. Animals and Housing

9 - 10 weeks old male Wistar rats were procured. The animals were housed in polypropylene cages under maintained environment with temperature $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$, relative humidity 45% - 55% and 12 hr light: 12 hr dark cycle. The animals had free access to feed pellets (VRK Nutritional Solutions, Pune) and water *ad libitum*.

2.11. Animal Study Design

Wistar rats were divided into three groups. Group I (n = 6) were administered with vehicle (distilled water) orally, while group II and III animals were treated with SAVA 8A and SAVA 8B extracts re-suspended in distilled water orally for a period of 30 days, once daily. 1.16 g of SAVA 8A was dissolved in 15 ml of distilled water and 1 ml of it was administered once daily to every 250 g rat while 2.160 g of SAVA 8B was dissolved in 15 ml of distilled water and 1 ml of it was administered once daily to every 250 g rat. The volume of 8A and 8B to be administered were determined daily based on the body weight of the animal. The parameters evaluated before the start and end of the study period were body weight (day 0 and day 30); hepatic profile on day 0 and day 30 by estimation of bilirubin and levels of enzymes such as SGPT, SGOT and ALP. The liver histopathology was examined for 3 liver samples per group as per the method described by Palipoch and Punsawad [26]. Acute toxicity studies have revealed *Garcinia gummi-gutta* at a dose of 2000 mg/kg *Garcinia cambogia* extract did not produce any lethality [27]. So, a dose of 100 mg/kg, p.o. which was $1/10^{\text{th}}$ of LD_{50} was chosen for the main study.

3. Results

3.1. Identification and Biochemical Characterization of GBC

The isolated bacterial strain GBC was identified as *Bacillus atrophaeus* by comparing the metabolic fingerprint of GBC with Biolog database with a similarity index of 0.569 (Supplementary **Figure S1**). The biochemical characterization of GBC revealed that the isolate was able to utilize most of the carbon substrates such dextrin, pectin, glycerol, maltose, sucrose, mannose, fructose, glucose, mannitol, cellobiose, gentibiose, D-Turanose, β -methyl-D-glucoside, D-Salicin, N-acetyl D-glucosamine, myo-inositol, D-galactouronic acid, D-galactouronic acid lactone, D-glucuronic acid, Glucournamide, mucic acid, L-lactic acid and citric acid. GBC was also able to assimilate amino acids L-alanine, and L-serine. The isolate was sensitive to antimicrobials such as rifamycin SV, nalidixic acid, sodium bromate, lincomycin and vancomycin (Supplementary **Table S1**).

3.2. Antibiotic Susceptibility of GBC

The antibiotic susceptibility study of GBC (**Table 1**) revealed that the bacterial isolate is susceptible to antibiotics such as tetracycline, doripenem, gentamycin tobramycin, kanamycin, streptomycin, carbenicillin, vancomycin, rifampicin, moxifloxacin and trimethoprim. Azithromycin, nalidixic acid and fusidic acid

Table 1. Antibiotic susceptibility of *Garcinia* bacterial isolate (GBC).

Sr. No.	Antibiotic Disc Name	DZI*	Antibiotic Susceptibility
1	Tetracycline	32	Sensitive
2	Azithromycin	-	Resistant
3	Gentamycin	19	Sensitive
4	Tobramycin	21	Sensitive
5	Kanamycin	20	Sensitive
6	Streptomycin	23	Sensitive
7	Ampicillin	17	Intermediate
8	Doripenem	39	Sensitive
9	Penicillin-G	18	Intermediate
10	Carbenicillin	18	Sensitive
11	Moxifloxacin	38	Sensitive
12	Nalidixic acid	17	Intermediate
13	Vancomycin	22	Sensitive
14	Trimethoprim	37	Sensitive
15	Rifampicin	21	Sensitive
16	Fusidic acid	16	Intermediate

*DZI: Diameter of zone of inhibition.

were found to be ineffective against GBC (Supplementary **Figure S2**). Among all these antibiotics, since tetracycline is freely soluble in water and is cost-effective, we decided to use this antibiotic for reducing the microbial load of the *Garcinia* extract.

3.3. Microbial Load Reduction Trials

Figure 1 shows the flow chart of the *Garcinia* trials with and without Tetracycline treatment. **Table 2** shows that the total viable count (TVC) of *Garcinia* extract was 1.83×10^4 cfu /g which upon treatment with tetracycline (100 µg/ml), reduced the microbial load to ~80 cfu/g in tetracycline which is a reduction of the microbial load by 2 logs (99%). The residual content of tetracycline in the final *Garcinia* extract did not show any peak (**Figure 2(B)**) matching the tetracycline standard (**Figure 2(A)**).

3.4. Effect of *Garcinia* Extract (SAVA 8A) and *Garcinia* + *P. kurroa* Extract (SAVA 8B) on Body Weight (g) of Animals

There was no difference in the body weight of the animals of all the groups on day 0. However, there was a significant decrease ($p < 0.01$, $p < 0.001$) in the body

weight of animals treated with SAVA 8A and SAVA 8B, respectively on day 30 (Table 3). The vehicle control animals gained an average of 32% increase in body weight. The weight gain in SAVA 8A treated groups was 7.31%, which was 25.36% or 4 times less than that of the vehicle control group. However, treatment with SAVA 8B caused a 14.70% weight gain, which was 18 % less than that of the vehicle control group.

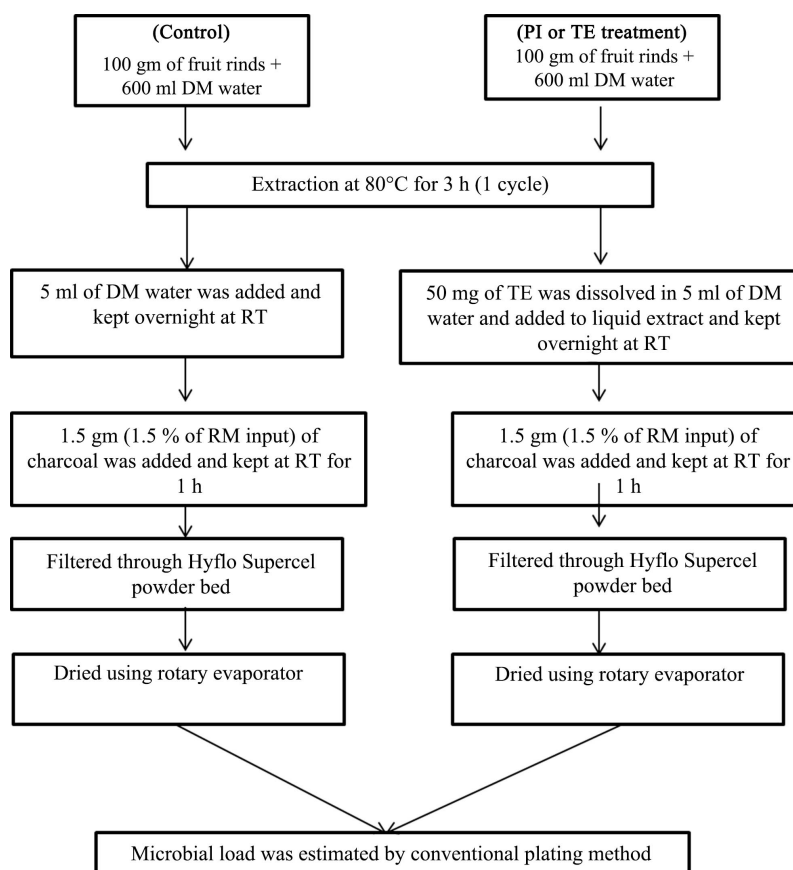
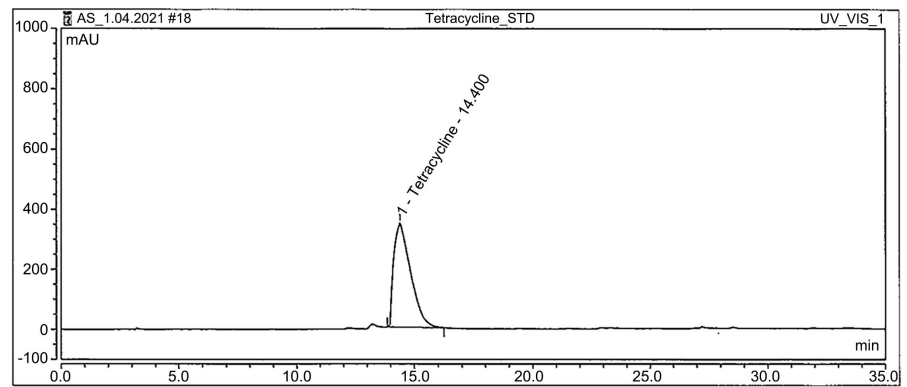


Figure 1. Flow chart for Garcinia trials with and without tetracycline. Experimental details can be found in material and methods section.

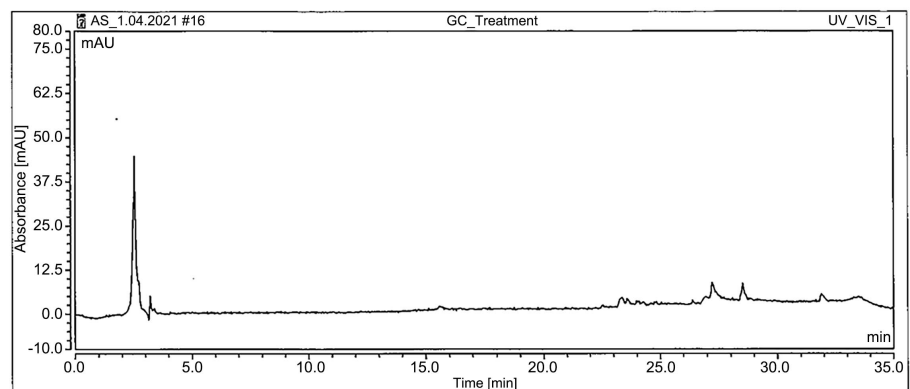
Table 2. Microbial load reduction trial using tetracycline.

Sr. No.	Sample Name	Dilution Factor	Total viable count (TVC) (cfu/g)		
			No. of colonies	TVC	% cfu reduction
1	<i>Garciniacambogia</i> control extract	10 ¹	TNTC		
		10 ²	79	7.9 × 10 ³	-
		10 ³	11		
2	<i>Garciniacambogia</i> TE treated extract (100 µg/ml)	10 ¹	08		
		10 ²	01	80	99
		10 ³	Nil		

TNTC: Too Numerous To Count.



(A)



(B)

Figure 2. (A) HPLC chromatogram of standard tetracycline (1000 ppm). Note the retention time of 14.4 min. The X axis denotes the time in (min) while the Y axis denotes the absorbance (mAu). The wavelength chosen for the HPLC runs was 277 nm; (B) HPLC chromatogram of residual tetracycline after treatment of *Garcinia* raw material as per the process depicted in Figure 1. Note the absence of tetracycline peak in the sample.

Table 3. Effect of SAVA 8A and SAVA8B on hepatic profile in rats.

Parameters	Days	Vehicle Control	SAVA 8A (116 mg/animal)	SAVA 8 B (216 mg/animal)
Body Weight (g)	Day 0	252 ± 2.53	242.20 ± 2.16	244.50 ± 3.34
	Day 30	334 ± 12.00	259.10 ± 7.41***	279.70 ± 10.78**
SGPT (U/L)	Day 0	56.17 ± 10.66	55.4 ± 3.68	49.8 ± 10.88
	Day 30	58.67 ± 10.59	59 ± 3.78	53.4 ± 1.922
SGOT (U/L)	Day 0	103.7 ± 13.56	112.7 ± 5.37	106 ± 1.64
	Day 30	106.21 ± 13.46	116.3 ± 5.51	109.6 ± 1.70
ALP (IU/L)	Day 0	149.3 ± 24.54	147.6 ± 2.33	130.3 ± 5.51
	Day 30	151.8 ± 24.52	151.2 ± 2.25	133.9 ± 5.62
Total Bilirubin (mg/dL)	Day 0	0.08 ± 0.01	0.12 ± 0.00	0.10 ± 0.00
	Day 30	0.13 ± 0.01	0.15 ± 0.00	0.12 ± 0.00
Liver weight (g)	Day 30	10.00 ± 0.58	8.80 ± 1.40	9.60 ± 1.18

Values are expressed as mean ± SEM, n = 6 for SAVA A and n = 10 for SAVA B. One way ANOVA followed by Dunnett's t test ** $p < 0.01$, *** $p < 0.001$ when compared to vehicle control.

3.5. Effect of SAVA 8A and 8B on SGPT and SGOT (U/L) Levels

On day 0, there were no significant differences in SGPT and SGOT (U/L) levels between the groups. Further, on treatment with SAVA 8A and 8B for 30 days, the treatment did not cause any significant difference in the SGPT and SGOT levels in SAVA 8A and 8B treated animals. While these enzyme levels were higher in groups treated with *Garcinia* extract, the enzyme levels were comparatively lower and closer to control group animals in groups treated with SAVA 8B, although the values were not statistically significant (Table 3).

3.6. Effect of SAVA 8A and 8B on ALP (IU/L)

On day 0, there was no significant difference in ALP (U/L) levels between the groups. Further on treatment with SAVA 8A and 8B for 30 days, did not cause any significant difference in the ALP levels in SAVA 8A and 8 B treated animals when compared with vehicle-treated rats on day 30 (Table 3).

3.7. Effect of SAVA 8A and 8B on Total Bilirubin (mg/dL)

On day 0, there were no significant differences in total bilirubin (mg/dL) levels between the groups. Further on treatment with SAVA 8A and 8B for 30 days, did not cause any significant difference in the bilirubin levels in SAVA 8A and 8 B treated animals when compared with vehicle-treated rats on day 30 (Table 3).

3.8. Effect of SAVA 8A and 8B on Liver Weight (g) of Animals

Treatment with SAVA 8A and 8B for 30 days, did not cause any significant difference in the liver weight in SAVA 8A and 8 B treated animals when compared with vehicle-treated rats on day 30 (Table 3).

3.9. Effect of SAVA 8A and 8B on Liver Histopathology

The liver histopathological changes in animals of all the groups are shown in Figures 3(A)-(C). Treatment with SAVA 8A and SAVA 8B, did not cause any change in the hepatic lobules, and the structure of hepatic lobules was intact, the hepatocyte cords were arranged radially, and there was no degeneration or necrosis of hepatocytes, no expansion or congestion in the portal area of hepatic sinuses, and no infiltration of inflammatory cells observed with SAVA 8A and 8B groups.

4. Discussion

From the present study, it was confirmed that the main contaminant in the *Garcinia cambogia* extract is *Bacillus atrophaeus*. *Bacillus* is the aerobic Gram-positive bacterium; a major genus of endospores forming bacteria [28]. The *Bacillus* Spp. is stable at a wide range of temperatures and pH enabling it to survive throughout the extraction and the herbal drug development process [29]. The *Bacillus* spores are widely distributed in the environment and resistant to killing by heat, radiation, and chemicals. Thus they can contaminate any herbal raw material or

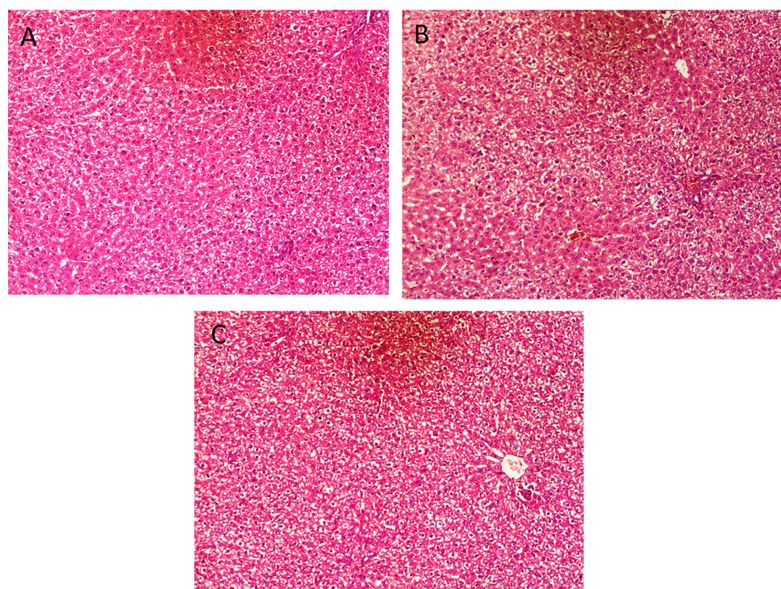


Figure 3. (A) Representative histomicrograph of transverse section of liver of a vehicle control rat showing normal architecture of liver, Hematoxylin & Eosin (H & E) stain, 100 X; (B) Representative histomicrograph of transverse section of liver of SAVA 8A extract treated rat showing normal architecture of liver, H & E stain, 100 X; (C) Representative histomicrograph of transverse section of liver of SAVA 8B extract treated rat showing normal architecture of liver, H & E stain, 100 X.

formulation [30] [31]. The microbial contaminants in herbal medicines are related to the source of raw material, contaminated environmental conditions, harvesting procedure, and improper handling, and storage [32]. Aqueous extraction is commonly used for HCA extraction from *Garcinia* fruit rinds so that there are adequate chances of survival spores in the extract prepared [33].

A similar finding of *Bacillus* contamination was reported by Fogele *et al.* [34] in the spices and herbs from local markets of Riga, Latvia. They found that *Bacillus cereus* was the major contaminant of black ground pepper and its concentration was the highest (2.49×10^{10} cfu/g) along with other contaminants including *Aspergillus* and *Penicillium* fungal species.

Vuuren *et al.* [35] reported the microbial contamination of medicinal plant species sold in Johannesburg, South Africa. The species such as *Helichrysum* sp (5.82×10^4 cfu/g) *Drimia sanguinea* (5.24×10^4), *Hydnora abyssinica* (4.22×10^5 cfu/g), *Hypoxis* sp (3.03×10^4 cfu/g) and *Acacia xanthophloea* (3.11×10^5 cfu/g) were found to be contaminated with *Pantoea* sp. and five strains of *Bacillus* spp including *B. amyloliquefaciens*, *B. lentus*, *B. megaterium*, *B. subtilis* and *B. valismortis*. The microbial load of two plant materials *Hydnora abyssinica* and *Acacia xanthophloea* exceeds the maximum microbial contamination limits set by World Health Organization (WHO) [35].

Antibiotic susceptibility study of GBC revealed that the bacterium is sensitive to several antibiotics including tetracycline. Tetracycline is a polyketide broad spectrum antibiotic, effective against both the Gram-positive and Gram-negative

bacteria and produced by *Streptomyces* genus of actinobacteria [36] [37]. It is a protein synthesis inhibitor that interferes with the translation of mRNA by reversible binding to the 30S ribosomal subunit [38]. Tetracycline hydrochloride (Hi-media) is freely water soluble up to 50 mg/ml; we have used water as a solvent for the extraction so that it can be used to reduce the microbial load of *Garcinia* extract. The used concentration of tetracycline hydrochloride (100 µg/ml) is effective to reduce the microbial load of *Garcinia* extract. The involvement of activated charcoal treatment in the extraction process ensured the adsorption of tetracycline on the bed of activated charcoal [39]. The absence of tetracycline in the TE treated extract shows the effective removal of the antibiotic by charcoal. The used activated charcoal can be heated in a high-temperature furnace, the contaminants can be vaporized to restore the carbon's original pore structure, enabling its reuse causing no environmental biohazard.

According to Globe Newswire, the global market of herbal supplements and remedies is estimated to reach US\$208,100 million by 2027, from US\$150,270 million in 2020, at a compound annual growth rate of 4.5% during 2021-2027. Due to the COVID-19 crisis, there is an uncontrolled release of herbal formulations and supplements as immunity boosters and antiviral drugs in the market without proper assessment of the effectiveness and microbial quality of these products. The microbial contamination of several herbal raw materials and medicines was reported in the literature [40] [41]. The consumption of such contaminated drugs with pathogenic bacteria and fungi leads to other health complications. Several groups of fungi and bacteria are known to produce toxins that are potential risks for humans and animals [42] hence strategies to reduce the microbial load by alternate means are crucial for the safety of the consumption of such extracts by humans of all age groups.

Noor *et al.* [43] reported microbial contamination in herbal oral medicines in Dhaka, Bangladesh, and assessed the microbial load of 59 herbal medicines taken orally. Two samples were found to be highly contaminated with bacteria with a total aerobic count of 2.14×10^5 cfu/g. 10 samples were found contaminated with fungi, the total yeast and mold count ranging from 1.2×10^4 to 6.3×10^4 cfu/g.

The acceptable limit of herbal supplements and drugs varies from country to country and depends upon the discrimination of health policies of the government. According to the USP guidelines, the accepted microbial limits for the herbal supplement, botanical supplement, and products varies from 10^3 to 10^5 cfu/g [44]. The average microbial load of *Garcinia* extract (10^4 cfu/g) is at the higher side of acceptable limits and it is always better to reduce the microbial load to the bare minimum level in the initial steps of herbal drug development to avoid further health risks due to consumption of contaminated herbal products with pathogenic bacteria and fungi. Hence, the present article disclosing a way to reduce microbial load of *Garcinia* by 2 logs assumes critical importance.

The reports on the effect of *Garcinia* on weight loss in animals and human studies are conflicting and inconsistent. **Table 4** summarizes reports variously

Table 4. Summary of animal and human studies with *Garciniacambogia* extract.

S.N.	Details of <i>Garcinia</i> extract	Dose used	Treatment Duration	Anti-obesity effect	Reference
1	<i>Garcinia cambogia</i> extract (60% HCA) together with soy peptide and L-carnitine	3.0 g/kg/day (rat)	9 weeks	<i>G. cambogia</i> extract improved dyslipidemia in rats	Kim <i>et al.</i> (2008a)
2	<i>Garcinia cambogia</i> extract (60% HCA potassium and calcium salt)	10 g/Kg/day (mice) fed with high fat diet	12 weeks	Consumption of the <i>Garciniacambogia</i> extract effectively lowered the body weight gain	Kim <i>et al.</i> (2008b)
3	<i>Garciniacambogia</i> extract (60% HCA)	821 mg/kg/day (rat)	15 days	No different in body weight gain between control and treated animals	Ventura <i>et al.</i> (2016)
4	<i>Garciniacambogia</i> extract potassium salt (50% HCA)	150 - 310 mg HCA/kg/day (rat)	4 days	Positive effects on reduced food intake and weight loss	J. Louter-van de Haar <i>et al.</i> (2005)
6	<i>Garcinia cambogia</i> extract (50% HCA)	3 g/day (Human)	12 weeks	Failed to produce weight loss and fat mass loss	Heymssfiled <i>et al.</i> (1998)
7	<i>Garcinia cambogia</i> extract	1 g/kg/day (Rat) fat induction by dexamethasone (10 mg/kg/day)	8 days	Reduced lipid levels in fat induced rats	Mahendran and Devi (2001)
8	<i>Garcinia cambogia</i> extract (60% HCA)	33 g/kg/day (mice)	4 weeks	No significant difference in final body weight	Hayamizu <i>et al.</i> (2003)
9	<i>Garciniacambogia</i> extract containing 65% HCA	Rats fed with fat diet	after 45 day still day 75	less body weight gain in this group	Bilal <i>et al.</i> (2012)
10	<i>Garciniacambogia</i> extract with high fat diet	400 mg/kg body weight/day	10 weeks	Reduced body weight gain	Sripradha and Magadi, 2015
11	<i>Garcinia cambogia</i> extract (60% HCA)	1 g/kg/day extract and 0.5 g/kg/day probiotic mixture in HFD fed mice	9 weeks	<i>Garciniacambogia</i> extract attenuated weight gain	Heo <i>et al.</i> (2016)
12	<i>Garcinia gummi-gutta</i> extract	100 - 200 mg/kg/day (Rats- high fat diet)	4 weeks	Positive effect on weight loss	Barathane <i>et al.</i> (2020)
13	<i>Garciniacambogia</i> extract	100 mg/kg/day (rats)	4 weeks	Positive effect on weight loss. 7% weight gain with <i>Garcinia</i> treated animals as against 23% in control animals	This study
14	<i>Garciniacambogia</i> extract + <i>P. kurroa</i> extract	116 mg/kg/day (rats)	4 weeks	Positive effect on weight loss	This study

available for almost 20 years on this aspect. Supplementation of the *Garcinia cambogia* extract with a high fat diet at a dose of 400 mg/kg body weight/day for ten weeks has shown reduced body weight gain [45] (Sripradha and Magadi,

2015). Similarly, *Garcinia cambogia* extract containing 65% HCA when given to rats fed with a fat diet from 45 days till day 75, showed less body weight gain in this group [46] supporting the reports of Hayamizu *et al.* [47] who observed low body weight in the *Garcinia cambogia* group than the placebo group at both 12 and 16 weeks at a dose of 1000 mg/kg and this has been attributed to the leptin like activity of *Garcinia* [48]. This human dose of 1000 mg/kg translates to almost 1.5 g/rat, which is way above the dose that we have used (120 mg/rat) in this study. Many studies did not see any significant change in body weight upon *Garcinia* treatment [49] [50]. Our data on observations of *Garcinia* at a dose of 100 mg/kg/day to animals corroborate the recent report of similar observations by Barathane *et al.* [27] with a similar *Garcinia* dose but administered to animals fed with a high fat diet, making our study cost-effective, simple with minimizing load of a high fat on the liver of animals. Another point to emphasize here is that we have administered the *Garcinia* dose to rats only for 4 weeks and observed significant body weight reduction while the other studies report weight reduction only after a longer duration of treatment with *Garcinia cambogia* extract. These differences could be attributed to the selection of right dose, duration of treatment, or formulation of *Garcinia* extract that was used for the intended use. The reasons for better efficacy seen with our *Garcinia* extract for body weight reduction could be due to better manufacturing process and better quality of our herbal extract.

Picrorhiza kurroa is known for its efficacy as a liver tonic for centuries. A hydro-alcoholic extract of *Picrorhiza kurroa* has shown reversal of fatty changes in the liver when given in two doses viz., 200 mg/kg and 400 mg/kg twice daily [51] for 4 weeks. Hence, we included this extract at the same dose as *Garcinia* in another group of animals and found that it did improve the levels of the liver enzymes as compared to the animals fed *Garcinia* alone.

The histopathological analysis of the liver from SAVA 8A (100 mg/animal) and SAVA 8B (216 mg/animal) treated groups was found to be devoid of any hepatotoxic property as evident by the intact architecture of the liver lobules, hepatocyte cords with no degeneration or necrosis. The study confirms SAVA 8A and SAVA 8B to be devoid of hepatotoxic potential.

Harmful effects of *Garcinia cambogia* on humans have been reported. HCA can cause a steatohepatitis by increasing hepatic collagen accumulation, lipid peroxidation, and pro-inflammatory cytokines resulting in oxidative stress. The hepatotoxicity due to *Garcinia* intake is reported in 1 of every 10,000 people in the US. The levels of liver enzymes are elevated by almost 4 - 5 times the upper normal limit. Classically AIH serology markers are negative in GC liver injury. GC liver injury can last for 2 - 3 months with normalizing of liver function tests by 5 months [52]. Reports on its angiogenic effect along with reducing brain dopamine levels in mice are recently reported [53].

Garcinia cambogia diet pills are in demand for weight loss with limited reports of side effects, but one of the toxicity symptoms due to starvation is ketoacidosis due to the effects of the hydroxycitric acid on appetite suppression [54]

[55] [56]. The effects of commercially available HCA-containing preparations such as Regulator, Citrin K, Super CitriMax HCA-600-SXS, were examined for their effect on food intake and body weight in adult male Wistar rats at a dose of 150 and 300 mg/kg, and the weight gain was found to be variable and depended on their HCA content. Since the lactone form has shown to be a very less effective inhibitor of the citrate cleavage enzyme [57], attempts to prevent HCA cyclization into lactone by using different counter-ions (such as sodium, calcium, or potassium) are reported.

Rats were supplemented orally with a single dose (1000 mg/kg) of each HCA salt. Ca-K double salt has shown better bioavailability over single salt of calcium HCA [58]. A recent work by Ghosh and Mukherjee [59] demonstrates that the high flavonoid content of HCA imparts pro-oxidant property and facilitates DNA damage at high concentrations but with no genotoxicity, hence HCA is safe consumption within the permissible dose limit. Since *P. kurroa* exhibits DNA damage protective effects [60], and to minimize the chances of such DNA damage, we included the extract of *P. kurroa* also in one group of animals and looked at the weight loss efficacy due to *Garcinia cambogia* extract. The experiments were conducted with concentrations double the maximum permissible dose (~2800 mg/day).

The size of liver weight increases does not always correlate with the amount of hepatic enzyme induction in rats [61]. Hence, the histopathological finding for associated pathology is critical to correlate with the degree of the enzymatic alterations seen. Upon damage and injury, the liver puts more AST (SGOT) and ALT (SGPT) into the blood, and hence their levels rise. As can be seen with SAVA 8A group animals, the levels of both SGOT and SGPT were more than the control animals, although the difference was not statistically significant. Interestingly, these enzyme levels were closer to levels seen in normal animals in SAVA8B group that were given *P. kurroa* extract (100 mg/kg), which is known to improve hepatic enzyme levels [51] when given at a dose of 200 mg/kg in rats.

It has been suggested that calcium reduces the solubility of HCA and hinders bio-availability, however, our studies demonstrated a quite efficacious HCA as calcium salt, and reasons for these observations is not clear [62]. Saito *et al.* [63] report that 1244 mg of HCA/kg BW/d, respectively caused potent testicular atrophy and toxicity while in our studies the HCA content is several folds less and hence appears safe but efficacious for further studies.

5. Conclusion

In conclusion, the present study demonstrates the presence of *Bacillus* sp. in *Garcinia* extract, and a simple, cost-effective method to reduce the microbial load is disclosed. One can use such methods to reduce the microbial load of other herbal extracts depending on the presence of microbial contaminants. We have used tetracycline for trials, but two or more antibiotics with synergistic combinations or other antimicrobial agents can also be used [64] [65] [66]. The

microbial quality of the herbal medicines is the basis of their efficacy, reproducibility, and safety for human consumption. The microbial contamination of pharmaceuticals and herbal supplements can create problems in the manufacturing process that can adversely affect the industry from an economic point of view [67]. The reduction in microbial load can prevent the unwanted changes produced due to microbial activities and can increase the shelf-life of herbal products to a greater extent. Finally, control majors should be employed by national authorities to release and reduce the consumption of herbal products above the minimum standards of quality. From the present study and earlier reports of *Garcinia* being safe with no side effects or adverse events in humans [68], we believe further studies with different dose ranges and with double and triple salt of *Garcinia cambogia* extracts would add value to this study.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary

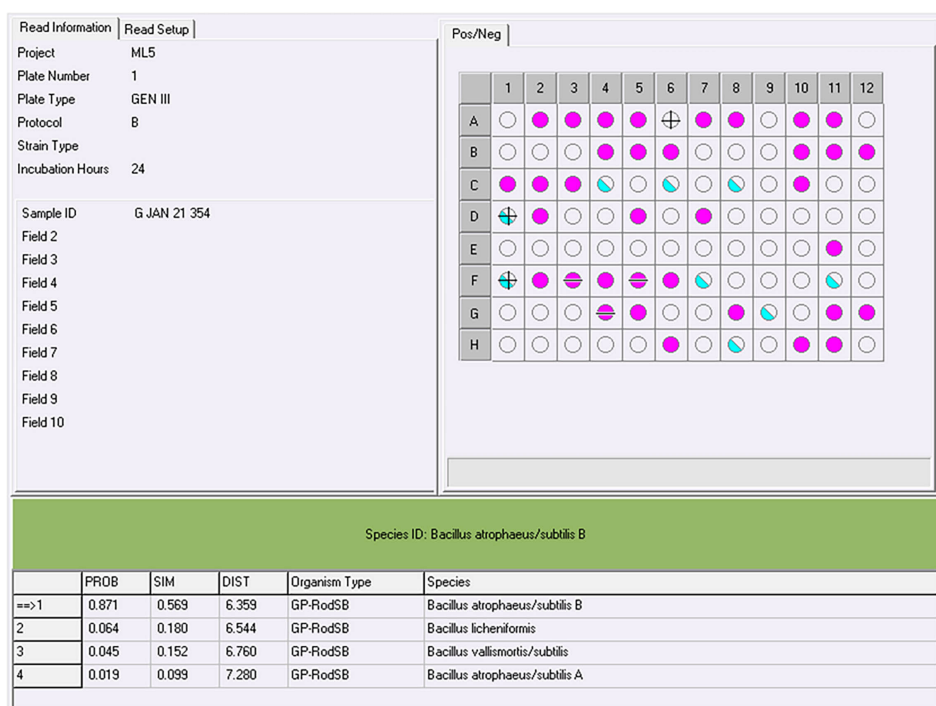


Figure S1. Phenotypic characterization and identification of *Garcinia* Bacterial culture (GBC) using BIOLOG GENIII Microplate (Violet or purple colour: Positive, half blue: Weak Positive and No colour: Negative).

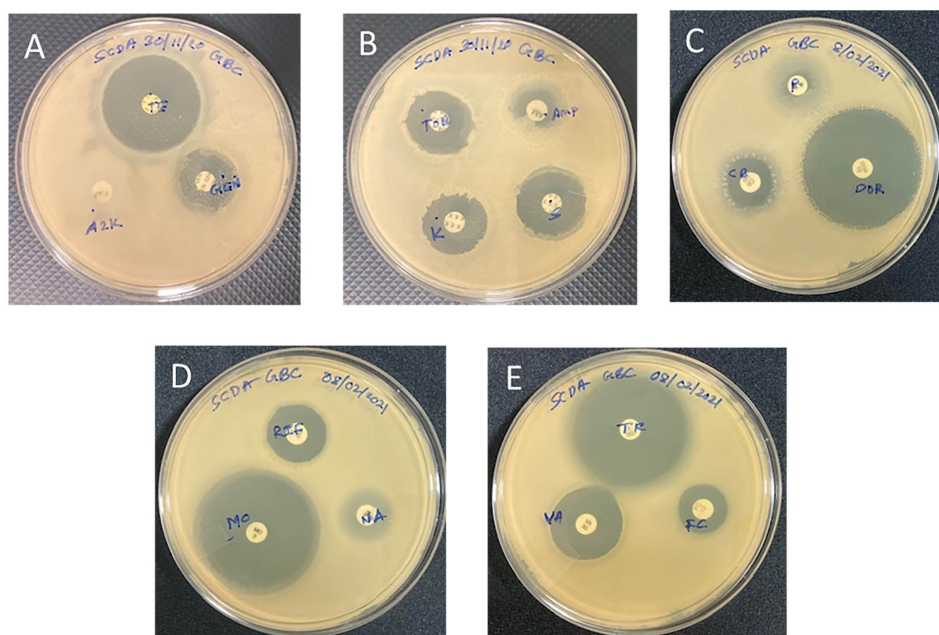


Figure S2. Antibiotic sensitivity of *Garcinia* Bacterial Culture. Panel (A) TE—Tetracycline, AZK—Azithromycin and GEN—Gentamycin; panel (B) TOB—Tobramycin, K—Kanamycin, S—Streptomycin and AMP—Ampicillin, panel (C) P—Penicillin G, CB—Carbenicillin and DOR—Doripenem, panel (D) RIF—Rifampicin, MO—Moxifloxacin and NA—Nalidixic acid and panel (E) TR—Trimethoprim, VA—Vancomycin and FC—Fusidic acid.

Table S1. Phenotypic characterization of *Garcinia* bacterial culture (Grey Color: Substrate utilization tests, Light pink color: Chemical sensitivity assay, Purple color: Positive control).

A1 Negative Control	A2 Dextrin (Positive)	A3 D-Maltose (Positive)	A4 D-Trehalose (Positive)	A5 D-Cellobiose (Positive)	A6 Gentiobiose (Negative)	A7 Sucrose (Positive)	A8 D-Turanose (Positive)	A9 Stachyose (Negative)	A10 Positive Control	A11 pH 6 (Positive)	A12 pH 5 (Negative)
B1 D-Raffinose (Negative)	B2 α -D-Lactose (Negative)	B3 D-Melibiose (Negative)	B4 β -Methyl-D- Glucoside (Positive)	B5 D-Salicin Glucose (Positive)	B6 N-Acetyl-D- Glucosamine (Positive)	B7 N-Acetyl- β -D- Mannosamine (Negative)	B8 N-Acetyl-D- Galactosamine (Negative)	B9 N-Acetyl Neuraminic Acid (Negative)	B10 1% NaCl (Positive)	B11 4% NaCl (Positive)	B12 8% NaCl (Positive)
C1 α -D-Glucose (Positive)	C2 D-Mannose (Positive)	C3 D-Fructose (Positive)	C4 D-Galactose (Partially Positive)	C5 3-Methyl Glucose (Negative)	C6 D-Fucose (Partially Positive)	C7 L-Fucose (Negative)	C8 L-Rhamnose (Partially Positive)	C9 Inosine (Negative)	C10 1% Sodium Lactate (Positive)	C11 Fusidic Acid (Negative)	C12 D-Serine (Negative)
D1 D-Sorbitol (Partially Positive)	D2 D-Mannitol (Positive)	D3 D-Arabitol (Negative)	D4 myo-Inositol (Negative)	D5 Glycerol (Positive)	D6 D-Glucose- 6-PO4 (Negative)	D7 D-Fructose- 6-PO4 (Positive)	D8 D-Aspartic Acid (Negative)	D9 D-Serine (Negative)	D10 Troleandomycin (Negative)	D11 Rifamycin SV (Negative)	D12 Minocycline (Negative)
E1 Gelatin (Negative)	E2 Glycyl-L- Proline (Negative)	E3 L-Alanine (Negative)	E4 L-Arginine (Negative)	E5 L-Aspartic Acid (Negative)	E6 L-Glutamic Acid (Negative)	E7 L-Histidine (Negative)	E8 L-Pyroglutamic Acid (Negative)	E9 L-Serine (Negative)	E10 Lincomycin (Negative)	E11 Guanidine HCl (Positive)	E12 Niaproof 4 (Negative)
F1 Pectin (Partially Positive)	F2 D-Galacturonic Acid (Positive)	F3 L-Galactonic Acid Lactone (Positive)	F4 D-Gluconic Acid (Positive)	F5 D-Glucuronic Acid (Positive)	F6 Glucuronamide (Positive)	F7 Mucic Acid (Partially Positive)	F8 Quinic Acid (Negative)	F9 D-Saccharic Acid (Negative)	F10 Vancomycin (Negative)	F11 Tetrazolium Violet (Partially Positive)	F12 Tetrazolium Blue (Negative)
G1 p-Hydroxy- Phenylacetic Acid (Negative)	G2 Methyl Pyruvate (Negative)	G3 D-Lactic Acid Methyl Ester (Negative)	G4 L-Lactic Acid (Positive)	G5 Citric Acid (Positive)	G6 α -Keto- Glutaric Acid (Negative)	G7 D-Malic Acid (Negative)	G8 L-Malic Acid (Positive)	G9 Bromo- Succinic Acid (Partially Positive)	G10 Nalidixic Acid (Negative)	G11 Lithium Chloride (Positive)	G12 Potassium Tellurite (Positive)
H1 Tween 40 (Negative)	H2 γ -Amino- Butyric Acid (Negative)	H3 α -Hydroxy- Butyric Acid (Negative)	H4 β -Hydroxy- D,L-Butyric Acid (Negative)	H5 α -Keto-Butyric Acid (Negative)	H6 Acetoacetic Acid (Positive)	H7 Propionic Acid (Negative)	H8 Acetic Acid (Partially Positive)	H9 Formic Acid (Negative)	H10 Aztreonam (Positive)	H11 Sodium Butyrate (Positive)	H12 Sodium Bromate (Positive)