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Research Article

Characterization of the Microbes Isolated from *Salacia reticulata* Raw Material and its Extract: A Simple Method to Reduce the Microbial Load in Herbal Products

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Abstract

The study investigated and characterized the microbes that contaminate *Salacia reticulata* herbal extracts used as an antidiabetic herbal formula. Also investigated was the microbial load of these herbal powders by pour plate technique and HiChrome agar spread plate methods. The characterization of the isolated microbes confirmed the dominance of members of the genus *Bacillus* by 16 sRNA sequencing. The fungal sps was found to be *Aspergillus* that was present in the raw material and also in the dry powder extract of *Salacia*. While the bacterial count was in the range of 10^4 to 10^5 cfu/g, the fungal count of *Salacia* extract ranged from 2.0 x10^1cfu/g to 1.9 x10^2 cfu/g. The isolated bacteria showed sensitivity towards Tetracycline, Gentamycin, Tobramycin, Kanamycin, Streptomycin, Ampicillin, Doripenum, Tripethoprim, Rifampicin and Fusidic acid and resistance towards the action of Azithomycin, Penicillin G, Carbenecillin, Moxifloxacin, Vancomycin and Nalidixic acid. Since tetracycline is a heat-labile antibiotic, we carried out a downstream process of extraction of one of the herbal raw material of *Salacia reticulata* with water with and without tetracycline. The obtained *Salacia* extract powders were then subjected to microbial counts and the results indicate >99.99% reduction in the bacterial and yeast and molds load by the proposed manufacturing process. Similar rate of microbial load reduction was seen when the *Salacia* extract with the microbial load of >10^4 cfu/g was treated with tetracycline. The residual tetracycline in the final extract preparations was lesser than 5% to the original concentration used. This study emphasizes the need for constant monitoring and control of the standards of herbal medicines and discloses a simple method to reduce the microbial load present in the herbal samples using antibiotics so that after treatment, the microbial load are well within WHO standard.

Keywords: *Aspergillus;* WHO; Microbes; *Salacia reticulata*

Introduction

Medicinal plants harbor potential endophytic microbes such as bacteria, yeast and fungi and benefit from their bioactive compounds. The microbiological background of such microbes, present in the roots, shoots and endosphere [1,2], depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations.

According to a World Health Organization (WHO) survey, about 70 to 80% of the world's populations, particularly in the develop-

ing countries, rely on non-conventional medicines, mainly from herbal sources, for their primary healthcare including elderly population [3,4]. With the advent of global expansion of the herbal medicines market, there is increase demand in herbal products and hence concerns around their composition and safety are being recognized. In traditional medicines, medicinal plants have been in use since years since they represent a very important source of bioactive molecules that display antibacterial, antiviral, and antifungal properties [5].

Like conventional medicines, there is a great deal of effort put by many manufacturers to ensure safety, quality, and efficacy of herbal medicines, while some lack standard practice to manufacture quality products due to lack of stringent regulatory requirements resulting in free selling of such products in the market with little or no restraint. The safety of traditional and herbal medicines has therefore become a major concern to both national health authorities and the general public [6,7].

Most of the herbal raw materials support some form of microbial growth, depending on its nutritive properties and moisture contents. Since there are no obvious signs of spoilage of such raw materials, it is advisable to have knowledge of the microbial contents of all the herbal drugs and medicines so that appropriate caution is practiced during manufacturing and packaging and there is constant quality assessment of herbal materials in the market in order to ensure that medicinal plant materials and products are suitable for human consumption [8]. Many of the medicinal plant materials carry a large number of microbes originating from the soil that may be introduced during harvesting, handling, and production of various herbal remedies [9-11]. *Escherichia coli* and *Bacillus cereus* are some of the bacteria identified in herbal medicinal powders and molds such as *Penicillium* species, *Fusarium* species, and *Aspergillus* species are reported [12].

In developing countries, herbal extracts are recognized as pharmaceutical medicine and are used as complementary medicine and since consumptions of such extracts are in high volumes, adequate supervision is required before these are released directly into the market [13,14].

Salacia reticulata is known for the treatment of diabetes in ayurvedic medicine. The roots and stem of this plant is prone to microbial contamination and insect infestation during storage and transportation causing concerns in maintenance of quality and prevent economic loss [15,16]. This study evaluates the microbial load in *Salacia* root extract and characterizes pathogens by sequencing and examined its antibiotic susceptibility. The microbial diversity in the *Salacia reticulata* extract was examined by aerobic plate count method and microbial isolates were identified based on their morphological, cultural, biochemical characteristics and 16 sRNA sequencing. This is the first report on identification of microbes in the raw material and extract of *Salacia reticulata* roots and opens up a simple and cost-effective method to reduce the microbial load to acceptable limits for human consumption.

Materials and Methods

Chemicals and reagents

Biolog GEN III micro plates for aerobes (Cat. No. 1030, Biolog), and Inoculating Fluid B (Cat. No. 72402, Biolog) were purchased from Biolog, Hayward, CA 94545, USA.

Plant materials

Stem and roots of *Salacia reticulata* were collected from different regions of India. The identity of *Salacia* was confirmed at Durva Herbal Centre, Chennai, Tamilnadu, India. The freshly collected samples of stem and roots were stored at room temperature, protected from light and humidity before processing. Commercial samples of *Salacia* extract was collected from the production unit, Sava Healthcare, Malur, Karnataka.

Preparation of *Salacia* root extracts

100 g of *Salacia* roots was extracted with 4 volume of DM water w.r.t batch size for three times (each 3h) at temperature 80°C. After extraction, all three extraction fluids were pooled and concentrated on rotary evaporator at 55-60°C to obtain a dry powder of *Salacia* extract.

Isolation of the endophytic bacteria and fungus from *Salacia reticulata* roots

10 g of *Salacia reticulata* extract was mixed with 100 mL sterile distilled water and dissolved completely. The solution was brought to boiling for 5 min and 1 mL of sample was taken in a sterile petri dish. 5-20 mL of dextrose peptone agar was poured into petri dish and incubated at 55°C for 36-48 h. The isolated colony from DPA plate was selected and streaked on Soybean casein digest agar/Nutrient agar incubated at 55°C for additional 36-48 h.

For isolation of the fungus, 10 g of the *Salacia* extract was taken and added to 90 mL of Sabouraud's Dextrose Broth and homogenized for 2 min. 1 mL of such a homogenized sample was taken into sterile petri dish and 15-20mL of Sabourad's dextrose agar was poured and plates incubated at 20-25°C for 3-5 days. Selected isolated colony from SDA plate was streaked on Sabourad's dextrose agar at 25°C for 3-5 days and stored appropriately for further analysis.

For isolation of bacteria at room temperature, one gram of *Salacia reticulata* root extract or *Salacia reticulata* raw material (powdered) was suspended in 9 mL of sterile saline solution and mixed by vortexing for 5-10 min. 50 µL of powder suspension was transferred in sterile petriplate and 20-25 mL of sterile molten Hi-chrome universal differential agar medium was poured. Agar plates were allowed to solidify and the plates incubated at 37°C for 24h. After incubation, plates were observed for different colored colonies. Different types of colonies were selected and purified by sub culturing on Hi-chrome universal differential agar. The isolated cultures SBC1, SBC2, SBC3 and SBC4 were subjected to PCR sequencing as described before.

Identification of the bacterial strain by 16sRNA sequencing

Bacterial culture

The culture plates were sub-cultured on nutrient agar at 55 ºC for 24 hours. Upon sub-culturing based on morphology two different bacterial colonies were observed and each colony was subjected to 16S rDNA sequencing individually.

DNA extraction

DNA was extracted from bacterial culture using the QIAmp DNA Minikit (Qiagen) as per manufacturer's instructions, with a final elution volume of 50 µL. Extracted DNA was stored at 4 ºC until required for PCR.

PCR amplification

The 1 µL of bacterial DNA extract and controls were amplified with 1µL of 10µM primers (16s FP: 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer (TCTACGCATTTCACCGCTAC), 3.5 µL of GoTaq Green Mastermix (Promega), 3.5 µL of nuclease free water. Amplification conditions for the PCR assay was as follows: 5 min at 94°C to denature the DNA, followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 10 sec and strand extension at 72°C for 30 sec on a Eppendorf thermalcycler. PCR products were separated on a 2% agarose gel and DNA bands were visualized with ethidium bromide.

Primers and excess nucleotides were removed from the amplified DNA using a PCR clean-up kit (Qiagen). The amount of DNA in the cleaned-up product was quantified by comparing the intensity of the band to bands of known intensity in a HyperLadder marker (Bioline). Spiked samples were included with each PCR to determine whether or not the sample was inhibited. Spiked samples contained 1 cfu/ml of *E. coli* culture.

PCR sequencing and sequence analysis

Purified PCR products were sequenced in the forward and reverse direction in separate reactions. Each reaction contained 1 µL of template DNA, 1 µL of the appropriate PCR primer (0.5 µM), 1 µL of nuclease water and 2 µL of Big Dye Terminator v3.1 Ready Reaction Mix (Applied Biosystems). Each reaction was heated to 96°C for 1 min, followed by 35 cycles at 96°C for 10 sec, 55°C for5 sec and 60°C for 1 min. The sequencing products were purified using an ethanol precipitation method to remove unincorporated reagents and ensure a neutral charge. To the sequencing reaction 0.5µL of 0.5M EDTA was added and vortexed briefly for homogenization of the reaction mixture. The homogenized reaction mixture, was further washed by adding 21µL of ethanol precipitation mix (1µL of 3M sodium acetate and 20µL of absolute ethanol) and centrifuging at 4000 rpm for 15 min. The washing step was repeated twice. The pelleted DNA was dried by incubating in concentrator for 5 min at room temperature under vacuum condition. The dried DNA pellet was reconstituted in 10µL of formamide and denatured by heating the samples at 95°C and then snap cooling over ice. The denatured samples were loaded onto a 3730xl Genetic Analyzer Capillary Array for detection (Applied Biosystems). One forward and one reverse sequences for each sample were aligned using BioEdit to obtain a composite sequence. The quality of each sequence trace was visually assessed and poor quality sequence was edited and removed. Organisms were identified for each assay by comparing consensus sequences to a database library of known 16S rRNA gene sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) by multiple sequence alignment. The bacterial source of the sequence was identified by matching it with a sequence with the highest maximum identity score from the GenBank database. Where more than one bacterial species had the same highest score,

the species identification of the test organism was deduced based upon the taxonomy hits.

Fungal genomic DNA isolation

The DNA from the isolated fungus was isolated using Macherey Nagel Nucleospin kit, as per manufacturer's instructions. Fungal ITS region gene was amplified using standard PCR reaction using forward (ITS1: 5' TCCGTAGGTGAACCTGCGG 3') and reverse primers (ITS4: 5' TCCTCCGCTTATTGATATGC 3'). The primer pair ITS1 and ITS4 was used in a PCR reaction with an annealing temperature of 45°C. The PCR conditions included an initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation, annealing and extension at 95, 45 and 72°C respectively for 30 sec each. After a finale extension of 7 min at 72°C, the PCR amplicon (600 bp) products was purified by using exosap it kit (Invitrogen) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at Blast N site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequencing was performed using one of the PCR primers.

Phenotypic characterization of *Salacia* bacterial isolate (SBC 0)

The *Salacia* bacterial culture was streaked on the soybean casein digest agar (SCDA) and incubated at 35 °C for 24 h. The protocol B was used for the phenotypic characterization of SBC 0. Isolated colonies of SBC 0 were suspended in inoculating fluid (IF B) to get inoculum of recommended cell density. 100 µL of 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 SBC 0 was compared with Biolog database.

Antibiotic sensitivity of the isolated bacteria by agar diffusion assay

Pre-grown culture of bacterial isolate was used as inoculum for antibiotic susceptibility assay. The colonies of bacterial strain were suspended in sterile saline solution (5 mL) to get inoculum for agar diffusion assay. The turbidity of the inoculum was adjusted to 0.5 McFarland standard. The suspension spread on agar surface of SCDA plates. Antibiotic sensitivity discs were placed on the agar surface using sterile forceps. Agar plates were incubated at 35 ˚C for 18 h. After incubation of 18 h, plates were observed for zone of inhibition against bacterial strain and diameters of zone of inhibition were recorded.

Salacia trials

The powdered raw material of *Salacia reticulate* roots was processed with water extraction and the extraction fluids was treated with and without tetracycline at 50 and 100 µg/mL final concentration. After 16 h, the treated and the untreated materials were treated with activated charcoal at 0.5% final concentration and the liquids were evaporated in a rotary evaporator. The extracts, thus prepared were tested for microbial load. Similar process was followed for *Salacia* extract treated with and without tetracycline.

Chromatographic conditions for HPLC analysis of tetracycline

Buffer solution Preparation

Dissolved 0.14 gm of potassium dihydrogen phosphate (KH_2PO_4) in 900mL of water, adjusted pH 2.8 with 0.02 % ortho-phosphoric acid, made the final volume up to 1000 mL with water, mixed, degased and filtered through 0.45µ filter before use.

Chromatographic conditions

The chromatographic separation, using HPLC, was performed with gradient elution of mobile phase A (KH_2PO_4 buffer) and mobile phase B (pure acetonitrile). The gradient program was set as (time/% B) 0/10, 10/20, 20/50, 25/90, 30/90, 33/10, 35/10 which pumped the phases at a rate of 1 mL/min. The column used was Inertsil ODS, 3V, 250 x 4.6 mm and 5 µm, with the column temperature of 30°C. The injection volume of the samples was kept at 20 µL and the tetracycline peak was observed at 277 nm. The run time was 35 min and the tetracycline peak was seen at 10 min.

Standard and sample preparation

10 mg of Tetracycline hydrochloride (Hi-media) was weighed and dissolved in 10 mL of water. For sample preparation, 10 mg of the herbal extract was taken in a 10 mL volumetric flask, re-suspended in 5 mL of water and sonicated to dissolve the antibiotic completely. After making up the volume to 10 mL with water, the solution was filtered through 0.45 µ sterile filter and used for HPLC injections.

Results

Genomic identification of the isolated organisms

Based on sequence alignment analysis over NCBI BLAST server, the test isolate SBC0 was identified as *Bacillus licheniformis* with a sequence similarity of >98.65% while SBC1, SBC2, SBC3 and SBC4 are *Bacillus subtilis*, *Bacillus zanthoxyli*, *Bacillus subtilis* and *Bacillus aryabhattai*, respectively (Supplementary Figures S1, S2, S3 and S4). The fungal colony isolated from *Salacia* extract was identified as *Aspergillus* sps. (Supplementary Figure S5).

Biochemical characterization of *Salacia* bacteria SBC 0

Only the *Salacia* bacteria isolated at 55 °C was processed for generating a metabolic/phenotypic fingerprint with Biolog's database. The biochemical characterization of SBC 0 revealed that it was able to utilize substrates such as dextrin, D-maltose, D-trehalose, D-cellulobiose, gentibiose, sucrose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, D-glucose, D-mannose, Fructose, D-mannitol, myo-insitol, glycerol, pectin, D-galactouronic acid, gluconic acid, D-glucornic acid, L-lactic acid, citric acid and D-malic acid.

Antibiotic sensitivity of the isolated organisms

Table 1 gives the antibiotic sensitivity of the isolated bacteria from *Salacia* extract. It is evident from this table that the isolated organisms are sensitive to tetracycline, gentamycin, tobramycin, kanamycin, streptomycin, ampicillin, doripenum, tripethoprim, rifampicin and fusidic acid while it was resistant to the action of Azithomycin, Penicillin G, Carbenecillin, Moxifloxacin, Vancomycin, Nalidixic acid. The antibiotic sensitivity pattern of all the organisms was found to be different supporting the differences seen from genomic characterization studies. Figure 1 (panel a) shows the growth of SBC0, SBC1, SBC2, SBC3 and SBC4 bacteria isolated from *Salacia* raw material which on Hichrome agar plates which are identical to the bacteria isolated from the *Salacia* extract (Figure 1, panel b).

SR No.	Antibiotic	SBC 0		SBC 1		SBC 2		SBC 3		SBC 4	
		DZI (mm)	Antibiotic Susceptibility	DZI (mm)	Antibiotic Susceptibility	DZI (mm)	Antibiotic Susceptibility	DZI (mm)	Antibiotic Susceptibility	DZI (mm)	Antibiotic Susceptibility
1	Tetracycline	31	Sensitive	16	Sensitive	23	Sensitive	18	Sensitive	21	Sensitive
2	Azithromycin	-	Resistant	15	Intermediate	19	Sensitive	17	Sensitive	19	Sensitive
3	Tobramycin	23	Sensitive	15	Intermediate	17	Sensitive	16	Sensitive	18	Sensitive
4	Gentamycin	18	Sensitive	23	Sensitive	17	Sensitive	-	Resistant	16	Sensitive
5	Streptomycin	20	Sensitive	-	Resistant	18	Sensitive	07	Resistant	17	Sensitive
6	Kanamycin	22	Sensitive	21	Sensitive	18	Sensitive	-	Resistant	17	Sensitive
7	Ampicillin	21	Sensitive	17	Sensitive	11	Resistant	11	Resistant	-	Resistant
8	Doripenam	47	Sensitive	35	Sensitive	29	Sensitive	31	Sensitive	29	Sensitive
9	Penicillin -G	-	Resistant	-	Resistant	-	Resistant	05	Resistant	-	Resistant
10	Carbenicillin	-	Resistant	16	Intermediate	11	Resistant	20	Sensitive	14	Resistant
11	Moxifloxacin	-	Resistant	21	Sensitive	18	Sensitive	15	Intermediate	26	Sensitive
12	Nalidxic acid	-	Resistant	10	Resistant	14	Resistant	11	Resistant	14	Resistant
13	Vancomycin	-	Resistant	14	Resistant	16	Sensitive	10	Resistant	16	Sensitive
14	Trimethoprim	49	Sensitive	21	Sensitive	21	Sensitive	22	Sensitive	23	Sensitive
15	Rifampicin	23	Sensitive	23	Sensitive	13	Resistant	18	Sensitive	11	Resistant
16	Fusidic acid	19	Sensitive	18	Sensitive	16	Sensitive	12	Resistant	11	Resistant

Table 1: Comparative study of antibiotic susceptibility of *Salacia* bacterial isolates.

DZI: Diameter of Zone of Inhibition.

Salacia trials

Figure 2A and 2B shows the flow chart of processing of *Salacia* raw material and the extract respectively. It is clear from Table 2A that reduction of the microbial load of *Salacia* is dependent on the concentration of the tetracycline used for inactivating the microbes while Table 2B shows the residual yeast and mold levels after these

Figure 1: Growth of all bacterial isolates from *Salacia reticulata* raw material (panel A) and *Salacia* extract (panel B) on Hichrome Universal differential agar plates.

treatments. For achieving maximum killing of the inherent bacteria in *Salacia*, one needs tetracycline in the concentration of 100 µg/mL. Hence, for further experiments, tetracycline was used at 100 µg/mL final concentration, unless mentioned otherwise. Table 3 shows the residual microbial load with and without tetracycline when *Salacia* raw material and *Salacia* extract as used as starting materials respectively.

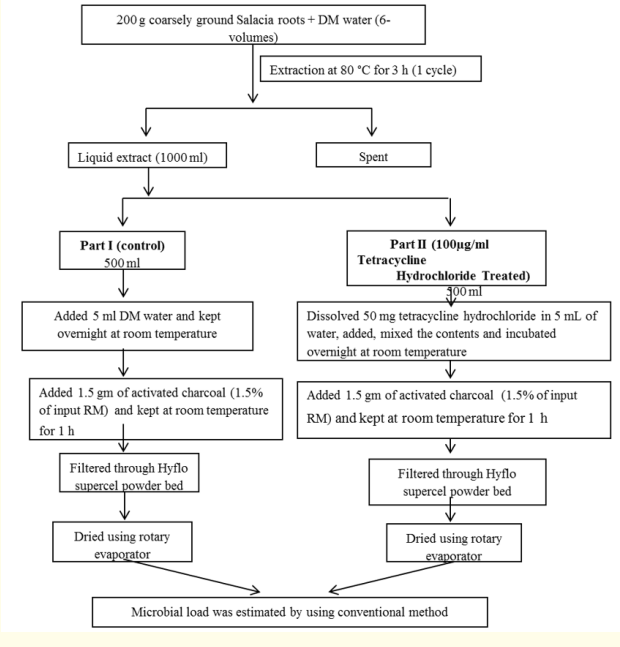


Figure 2A: Flow chart of process of *Salacia reticulate* raw material with and without tetracycline.

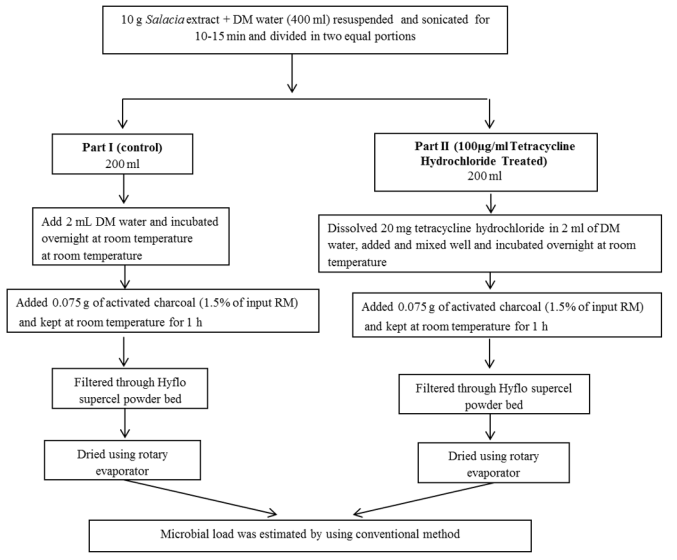


Figure 2B: Flow chart of process of *Salacia reticulata* extract with and without tetracycline.

Sr. No.	Sample Name	Dilution Factor	No. of colonies	TVC (cfu/gm)	% cfu reduction
1	*Salacia reticulata* control extract	10^2	59	1.18 x 10^4	---
		10^3	07		
		10^4	Nil		
2	Tetracycline (50 µg/ml) treated *Salacia* extract	10	35	3.5 x 10^2	97%
		10^2	05		
3	Tetracycline (100 µg/ml) treated *Salacia* extract	10	02	20	99.8%
		10^2	Nil		

Table 2A: Effect of different concentration of tetracycline on manufacturing process of *Salacia* on the residual microbial load.

Estimation of residual tetracycline

The residual tetracycline content in the *Salacia* extracts when measured by HPLC showed > 95% reduction in comparison to what was used in the starting of the process. Figures 3, 4 and 5 shows the HPLC chromatograms of standard tetracycline and residual tetracycline in *Salacia* extract made after following flow chart 2 and 3 respectively.

Sr. No.	Sample Name	Dilution Factor	No. of colonies	TVC (cfu/gm)	% cfu reduction	Fold reduction in Tet concentration %
1	*Salacia reticulata* control extract	10^2	TNTC	8.2×10^4	---	---
		10^3	41			
		10^4	05			
2	Tetracycline treated *Salacia* extract followed by charcoal treatment	10^1	Nil	<10	99.99%	95
		10^2	Nil			
		10^2	Nil			
3	*Salacia reticulata* control extract (from raw materials)	10^2	TNTC	1.76×10^6	---	---
		10^3	TNTC			
		10^4	98			
4	Tetracycline treated *Salacia* extract followed by charcoal treatment (from raw materials)	10^1	05	~100	99.99%	97
		10^2	Nil			

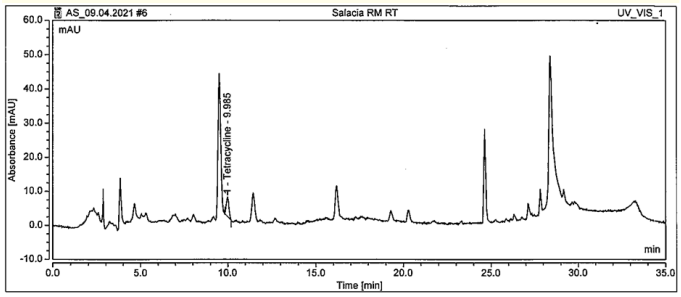
Table 3: Effect of tetracycline treatment on microbial load of *Salacia* extract.

Figure 3: HPLC chromatogram of standard tetracycline (1000 ppm). Note the retention time of 9.953. 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.

Figure 4: HPLC chromatogram of residual tetracycline after treatment of *Salacia* raw material as per the process depicted in Figure 2A.

Discussion

Our present study reveals *Bacillus* spp. as the predominant bacteria in the herbal extract of *Salacia*. Our observations corroborate reports of Shah and Pokhrel [17] who assessed herbal medicines from Nepal and reported *Bacillus* sp to be a major contaminant. Similarly, Esimone., *et al.* [18] and Okunlola., *et al.* [19], reported *Bacillus subtilis* as the predominant contamination in the herbal extracts they tested and Adeleye., *et al.* [9], who detected the presence of *Bacillus* sps. in herbal raw materials of *Hippocratea indica*, *Nauclea latifolia, Enantia sp., Citrus medica var acida*, and the bark of *Mangifera indica* etc. Other species of *Bacillus*, including *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus* and *B. subtilis*, have been also isolated from the inner tissues of healthy plants, and some strains appear to have important roles in growth promotion and plant protection [20].



Peak No.	Ret.Time min	Peak Name	Area µAU*sec	RRT	Peak Type
1	9.985	Tetracycline	58360	n.a.	BMB
Total :-			58360		

Figure 5: HPLC chromatogram of residual tetracycline after treatment of *Salacia* extract as per the process depicted in Figure 2B.

Bacillus is widely distributed in the environment although the primary habitat is the soil. These organisms are usually found in decaying organic matter, dust, vegetable, water, and some species are part of the normal flora. Food-borne *B. licheniformis* outbreaks are predominantly associated with cooked meats and vegetables and toxin-producing isolates of *B. licheniformis* from industrially produced baby food have been described by Salkinoja-Salonen., *et al.* [21] and from *Vitis ninifera* plants [22].

The herbal preparations are made from plants grows on soil, which is the natural habitat of *Bacillus* sps. Improper drying, inadequate heat processing, improper handling of products and contaminated equipment are other possible sources of *Bacillus* sp in herb. The spore of *Bacillus* resists environmental stress and enables the organism to withstand adverse conditions for long term survival.

With the accumulation of 16S rRNA gene sequence data, there are now 56 genera of aerobic endospore formers of *Bacillus* and over 545 species [23]. *Bacillus licheniformis* is known to cause foodborne illness, with nausea, vomiting, diarrhoea and after consumption of foods where the microbial counts range from 10^5 to 10^8 cfu/g [21]. *Bacillus subtilis* also has been implicated in foodborne illness with vomiting as the commonest symptom with diarrhea when the bacterial loads of the organism were high (10^5–10^9 cfu/g). The resistance of endospores of *Bacillus* to heat, radiation, disinfectants and desiccation and the adhesive characters of particular spores that facilitate their attachment to processing equipment and resistance to cleaning procedures [24] result in these organisms frequently being troublesome contaminants in clinical environments, biotechnological processes and food production.

Though, our study reveals the presence of various sps. of *Bacillus* in *Salacia reticulata* roots and their extracts, we realize that two of the sps observed namely *Bacillus aryabhattai* and *Bacillus zanthoxyli* are not a major concern since they are non-pathogenic in nature [25,26]. Moreover, phylogenetic analysis based on 16S rRNA gene sequences have shown that *Bacillus zanthoxyli* is closely related to *Bacillus aryabhattai* [27]. *Bacillus zanthoxyli* has been found in Chinese red pepper so our observation of presence of such a *Bacillus* sps in *Salacia* extract also supports the observation of Li., *et al*. [27].

Aspergillus and *Penicillium* sp have been isolated from herbal extracts [28] and hence our observations of presence of *Aspergillus* in the *Salacia* raw material supports similar literature findings. Fungi causes deterioration which adversely affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs, hence having a check on their counts in herbal medicinal extracts appears to be critical.

The microbiological quality of some herbal solid dosage forms from public markets, in the city of Sari, Iran was examined by Enayatifard., *et al*. [29]. These products showed 1.1 x 10^3 cfu/g with *Salmonella* sp. predominating and no evidence for contamination of the samples by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. Our *Salacia* herbal materials also showed total absence of all the above pathogenic bacteria and hence would be safe for human consumption.

In food animals, for easier administration, the antibiotics are administered to groups simultaneously through the drinking water or feed to treat or prevent disease. Tetracycline, oxytetracycline, and chlortetracycline were approved for animals bred for fur, calves, lambs, poultry, and swine with maximum contents of active ingredient of 80 mg/ kg feed.

Tetracycline is a polyketide protein synthesis inhibitor produced by *Streptomyces rimosus* and shows broad-spectrum of activity on various infectious bacteria. Employed for their low-cost, tetracyclines have found their use in livestock feed as growth promoters for swine and poultry [30,31].

The permissible limit of tetracycline in sea foods including shrimps, prawns, fish and fishery products is 0.1 ppm (0.1 mg/kg). Although, we do not see such prescribed limits for herbal medicinal products, one could target the limits set for such antibiotics as per Food Safety and Standards Regulations, 2011. Also, activated charcoal is reported to bind to tetracycline [32] and our experiments do support this literature report. Ji., *et al.* [33] report that weaker adsorption of tetracyclines on activated charcoal in comparison to carbon nanotubes and graphite. It is tempting to speculate that further reduction of tetracycline in the final herbal extracts can be achieved using such adsorption materials.

Tetracycline is bacteriostatic in nature where it prevents the growth of the bacteria by reversibly binding to the ribosomes and by inhibiting bacterial protein synthesis rather than killing the bacteria in vitro. NDM-1-producing bacterial strains have been found to be resistance to multiple antibiotics but are sensitive to combination of doripenem-tetracycline where these antibiotics display inhibition of key enzymes of NDM-1 strain [34]. It is possible that one could reduce the requirement of antibiotics to suboptimal level of MIC, when used in combination with other effective antibiotics. The microbial load in *Salacia* raw material ranges from 10^4 to 10^6 cfu/g based on time of storage and conditions of storage of the raw material. Kumar., *et al*. [15] have used gamma irradiation and achieved 4 log of reduction in microbial and yeast and mold counts. Our methodology of using a simple and cost-effective antibiotic assumes critical importance.

Extracts of *Bambusa arundinacea*, *Bidens pilosa*, *Crotton membranaceus*, *Elaeis guineensis*, *Hibiscus sabdariffa*, *Jatropha curcas*, *Mallotus oppositifolius*, *Morinda lucida* and *Spathodia campanulata* have been shown to enhance the inhibitory activity of tetracycline [35]. Since no such enhancement of tetracycline is seen in *Salacia*, it is safe to use this antibiotic to tackle the microbial load is our opinion.

Conclusion

Our present study of identification of contaminating microbes in *Salacia* will prove beneficial for researchers and manufacturers who are ensuring minimal levels of microbes in their final herbal extracts to ensure their products to be safe for human consumption. The results of a study by Agyeman-Duah., *et al*. [36] reveal significantly high amount of microbial contaminants in herbal powders sold in Ghana and Nigeria [37], constituting a health risk. Therefore, it is recommended that plants to be used in herbal preparations should be properly washed and heated as to reduce or eliminate the presence of *Bacillus* species. The study demonstrated the presence of non-pathogenic *Bacillus* sps in *Salacia* raw material and also its extract prepared using water at 80°C. Although the limits of microbial counts is acceptable as per European Pharmacopoeia, it is always safe if we have manufacturing process to reduce the presence of microbial load to bare minimum which increases the shelf-life of the product and will not affect the efficacy of the herbal medicines. Therefore, good pre-harvest and post-harvest practices together with hygienic manufacturing practices ought to be followed to minimize the level of microbial contamination in plant materials and their extracts.

In conclusion, following such processes that tackles microbes, all the samples of herbal drugs evaluated will meet the standards for microbial limits as specified in official monographs and they will also make the stability of the products better. Such products will also be safe for human consumption and the health status of consumers will be benefitted to a great extent.

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Conflicts of Interest

None declared.

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Bibliography

1. Beneduzi A., *et al*. "Plant growth promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents". *Genetics and Molecular Biology* 35 (2012): 1044-1051.
2. Berg G., *et al*. "Unraveling the plant microbiome: looking back and future perspectives". *Frontiers in Microbiology* 5 (2014): 148.
3. Akerele O. "Nature's medicinal bounty: don't throw it away". *World Health Forum* 14 (1993): 390-395.

4. Lima de Sousa CM., *et al.* "Microbial contamination in herbal medicines: a serious health hazard to elderly consumers". *BMC Complementary Medicines and Therapies,* 20 (2021): 17.

5. Castronovo LM., *et al.* "Medicinal plants and their bacterial microbiota: A review on antimicrobial compounds production for plant and human health". *Pathogens* 10 (2021): 106.

6. Kasilo OMJ and Trapsida JM. "Decade of African traditional medicine, 2001-2010". *African Health Monito* 14 (2011): 25-31.

7. Ekor, M. "The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety". *Frontiers in Pharmacol*ogy 4 (2013): 177.

8. Idu MD., *et al.* "Microbial load of some medicinal plants sold in some local markets in Abeokuta, Nigeria". *Tropical Journal of Pharmaceutical Research* 9.3 (2010): 251-256.

9. Adeleye IA., *et al.* "Microbial contamination of herbal preparations In Lagos, Nigeria". *Journal of Health, Population and Nutrition* 23.3 (2005): 296-297.

10. World Health Organization (WHO). Regulatory situation of herbal medicine: A worldwide review. World Health Organization, Geneva (1988).

11. Sofowora A. "Medicinal plants and traditional medicine in Africa". *Chichester. Wiley* (1982): 256.

12. Foote JC. "The microbiological evaluation of chamomile: A dissertation in food and nutrition". Texas Tech University, Texas (2002): 9-19.

13. Ali N., *et al.* "Evaluation of a method to determine the natural occurrence of aflatoxins in commercial traditional herbal medicines from Malaysia and Indonesia". *Food Chemistry and Toxicology* 43.12 (2005): 1763-1772.

14. Sharafati R., *et al.* "Biological characterization of Iranian walnut (Juglans regia) leaves". *Turkish Journal of Biology* 35 (2011): 635-639.

15. Kumar V., *et al.* "Effect of gamma irradiation on the antioxidant activity of *Salacia Reticulata*". *International Journal of Traditional and Natural Medicines* 5.1 (2015): 15-22.

16. Yewale S., *et al.* "Benefits of Soleris® over the conventional method for enumeration of microbial load in *Salacia* Herbal Extract". *Polish Journal of Microbiology* 69.4 (2020): 453-462.

17. Shah B and Pokhrel N. "Microbial quality and antimicrobial activity of herbal medicine". *Nepal Journal of Science and Technology* 13.2 (2012): 191-196.

18. Esimone CO., *et al.* "Microbiological quality of Herbal preparations marketed in Southeast Nigeria". *Journal of Natural Remedies* 2.1 (2002): 42-48.

19. Okunlola A., *et al.* "Evaluation of Pharmaceutical and Microbial Qualities of some Herbal Medicinal Products in Southwestern Nigeria". *Journal of Pharmaceutical and Biological Anatomy* 2.3 (2007): 661-670.

20. Reva ON., *et al.* "*Bacillus endophyticus* sp. nov., isolated from the inner tissues of cotton plants (Gossypium sp.)". *International Journal of Systematic and Evolutionary Microbiology* 52 (2002): 101-107.

21. Salkinoja-Salonen MS., *et al.* "Toxigenic Strains of Bacillus licheniformis Related to Food Poisoning". *Applied and Environmental Microbiology* 65.10 (1999): 4637-4645.

22. Nigris GS., *et al.* "Biocontrol traits of *Bacillus licheniformis* GL174, a culturable endophyte of *Vitis vinifera* cv". *BMC Microbiology* 18 (2018): 133.

23. Logan NA and Halket G. "Developments in the taxonomy of the aerobic, endospore-forming bacteria". In: Aerobic, Endospore-forming Soil Bacteria Edition (2015): 1-29.

24. Ryu JH and Beuchat LR. "Biofilm formation and sporulation by Bacillus cereus on a stainless-steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer". *Journal of Food Protection* 68 (2005): 2614-2622.

25. Bhattacharyya C., *et al.* "Genome-guided insights into the plant growth promotion capabilities of the physiologically versatile *Bacillus aryabhattai* strain AB211". *Frontiers in Microbiology* 8 (2017): 411.

26. Li M., *et al.* "*Bacillus zanthoxyli* sp. nov., a novel nematicidal bacterium isolated from Chinese red pepper (*Zanthoxylum bungeanum* Maxim) leaves in China". *Antonie Van Leeuwenhoek* 110.9 (2017): 1179-1187.

27. Anyanwu CU. "Fungal contaminants of powdered herbal drugs sold in parts of Enugu State, Southeast, Nigeria". *Plant Product Research Journal* 14 (2010): 46 -50.

28. Enayatifard R., *et al.* "Microbial quality of some herbal solid dosage forms". *African Journal of Biotechnology* 9.11 (2010): 1701-1705.

29. Chopra I and Roberts M. "Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance". *Microbiology and Molecular Biology Reviews* 65.2 (2001) 232-260.

30. Granados-Chinchilla F and Rodríguez C. "Tetracyclines in food and feeding stuffs: From regulation to analytical methods, bacterial resistance, and environmental and health implications". *Journal of Analytical Methods in Chemistry* (2017): 1315497.

31. Choi KJ., *et al.* "Removal of tetracycline and sulfonamide classes of antibiotic compound by powdered activated carbon". *Environmental Technology* 29.3 (2018): 333-342.

32. Ji L., *et al.* "Mechanisms for strong adsorption of tetracycline to carbon nanotubes: A comparative study using activated carbon and graphite as adsorbents". *Environmental Science and Technology,* 43 (2009): 2322-2327.

33. Maryam L., *et al.* "Synergistic effect of doripenem in combination with cefoxitin and tetracycline in inhibiting NDM-1 producing bacteria". *Future Microbiology* 14 (2019): 671-689.

34. Gbedema SY., *et al.* "Modulation effect of herbal extracts on the antibacterial activity of tetracycline". *International Journal of Contemporary Research and Review* 1.4 (2010): 1-5.

35. Agyeman-Duah E., *et al.* "Microbial quality of herbal powders in Ghana". E*dorium Journal of Microbiology* 3 (2017): 10-17.

36. Christiana NC and Tope O. "Microbial quality of herbal preparations sold in some parts of Nigeria". *GSC Biological and Pharmaceutical Sciences* 6.3 (2019): 076-084.

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