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FOOD SAFETY AND QUALITY - RESEARCH PAPER

Detection and characterization of pathogenic *Bacillus haynesii* from *Tribulus terrestris* extract: ways to reduce its levels

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Abstract
Plant parts such as roots, bark, leaves, flowers, and fruits that hold ethnopharmacological significance are naturally prone to microbial contamination, influenced by environmental factors like moisture and humidity. This study focuses on assessing the microbial load in the raw material of *Tribulus terrestris* (TT). The primary bacterium isolated from the pulverized raw material was identified as *Bacillus haynesii* through 16S rRNA sequencing. Biochemical assays revealed the organism's ability to utilize lysine and ornithine, produce urease, and generate hydrogen sulfide. The bacterium exhibited resistance to multiple antibiotics and caused 21.5% hemolysis in RBC lysis assays. To reduce microbial contamination, Glutaraldehyde (GA) and polyhexamethylene biguanide (PHMB) were tested, with GA at 1% reducing the microbial load by 99% without affecting the yield (0.5%) or bioactive saponin content. High-Performance Liquid Chromatography (HPLC) confirmed the absence of residual GA, ensuring an eco-friendly and safe process. This highlights the importance of quality control measures, including Hazard Analysis and Critical Control Points (HACCP) regulations, in maintaining the integrity of herbal extracts.

Keywords Hemolysis · Glutaraldehyde · HPLC · Polyhexamethylene biguanide · Anti-bacterial susceptibility · RBC lysis

Introduction

In recent times, there has been a significant surge in the utilization of herbal extracts and remedies for human health, largely due to their perceived minimal to nonexistent side effects. As per the World Health Organization (WHO), approximately 60% of the global population utilizes herbal medicine, with nearly 80% of individuals in developing nations relying primarily on it for their essential healthcare [1]. Among many such herbal remedies, one such ethno-pharmacologically important plant viz., *Tribulus terrestris* (TT) is widely used as therapy for several disease indications.

This plant belongs to the Zygophyllaceae family and is commonly referred to as puncture vine or Gokharu. It has a rich history of use in both Chinese and Indian medicinal traditions for treating diverse conditions, including diabetes, diuretic effects, hypolipidemia, hepatoprotection, cardiotonic properties, analgesic effects, anti-inflammatory actions, antibacterial activity, immunomodulation, antispasmodic effects, anti-cancer properties, larvicidal activity, anthelmintic effects, and prevention of dental caries [2]. The plant, encompassing its various parts like fruits, is a source of numerous bioactive components such as saponins, chlorogenin, diosgenin, ruscogenin, 2–5 D-spirosta-3-5-diene, gitogenin, kaempferol, and tribuloside. Recent years have witnessed extensive research aiming to substantiate its therapeutic efficacy, particularly in addressing skin-related and central nervous system (CNS) disorders, as documented by Chattre et al. [3].

However, a key safety issue linked with herbal medicines involves the risk of being contaminated by diverse microorganisms. These microorganisms might inhabit the

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leaves, stems, flowers, seeds, and roots utilized in crafting herbal remedies. Alternatively, contamination can transpire throughout the stages of harvesting, handling, open-air drying, preservation, and manufacturing [4]. The occurrence of microbial contaminants in herbal products can have detrimental effects on the health of consumers, particularly individuals with weakened immune systems who are susceptible to microbial infections, thus contributing to a global health issue [5, 6].

Glutaraldehyde (GA) is a highly effective disinfectant that is characterized by low toxicity, high stability and efficiency. This water-soluble di-aldehyde is known for its broad-spectrum disinfectant action and is used in various industries such as food, pharmaceuticals, leather, poultry, cosmetics, and sterilization processes [7]. When GA is alkalinized and applied at a concentration of 2.4% for sufficient time, disinfection takes place. Once activated, the 2.4% GA solution retains its activity for 14 days, provided no further dilution is carried out [8]. Its capability to inhibit pathogenic bacteria, including *Staphylococcus aureus* and *Escherichia coli*, as well as spores from *Bacillus* and *Clostridium* species, is well-documented [9]. Recently, use of GA in reducing bacterial load in *Salacia chinensis* extract has been demonstrated [10].

Similarly, polyhexamethylene biguanide (PHMB) is another disinfectant that exhibits broad-spectrum antimicrobial properties. It effectively targets bacteria, fungi, parasites, and specific viruses, with a favorable therapeutic index. PHMB finds extensive application in clinical, domestic, and industrial settings [11]. Nonetheless, information is scarce regarding the efficacy of GA and PHMB in specifically eradicating or inhibiting pathogens and disease-causing bacteria found in herbal extracts or raw materials. The objective of this study is to develop a cost-effective strategy for mitigating microbial contamination in the pulverized raw material (PRM) of *Tribulus terrestris* (TT) using glutaraldehyde (GA) and polyhexamethylene biguanide (PHMB). Specifically, we aim to isolate and characterize the contaminating microorganisms, evaluate their pathogenicity through hemolysis assays and antibiotic susceptibility tests, and analyze the saponin content following treatment with GA and/or PHMB.

Materials and methods

Chemicals and reagents

2,4-Dinitrophenylhydrazine (DNPH) from Sigma Aldrich (USA), Glutaraldehyde solution (AR, 50%) from Tokyo Chemical Industry Co. Ltd. (Japan), Soya casein digest agar (SCDA) and broth (SCDB) from TM Media (Delhi, India), Luria Bertani broth, Mueller-Hinton broth, Agar powder, Icosa G-I-Plus, Biochemical test kit (KB001 and KB002), Phosphate buffered saline (PBS) and 5% sheep blood agar plates from HiMedia (India), polyhexamethylene biguanide 20% (PHMB) from Shiv Shakti group (Gujrat, India), respectively. All other used chemicals and solvents were of analytical and HPLC grade.

Obtaining and processing plant material

The commercial raw material of *Tribulus terrestris* (TT) was sourced from the production unit of SAVA Healthcare in Malur, Karnataka, India. The collected samples (fruit) underwent drying and pulverization, resulting in a semi-powdered form. Approximately 50 g of this powder was mixed with 250 mL of the extraction solvent (water) in a ratio of 1:5. The mixture was stirred continuously for 3 h at 80˚C, and three consecutive extractions were performed. The obtained filtrates were combined, concentrated using a rotary evaporator (Buchi, USA), and yielded a dried powder (extract) following the procedure outlined in the literature [12]. In a separate process, the TT raw material underwent washing by immersing it in a 1% GA solution for 1 h at room temperature [10]. After filtration and discarding the filtrate, the extraction was carried out with three consecutive rounds, using water as the extraction solvent. The extracts, with and without GA treatment, were stored separately for subsequent analysis and assays.

Microbial analysis of the TT

About 0.5 g of extract of TT (with and without GA treatment) were weighed and dissolved in 4.5 mL saline. For the microbial load analysis of the plant extracts, serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were sequentially prepared. About 0.1 mL suspension from each dilution was spread on the SCDA medium [13]. Test samples, positive control (growth control) and negative control (media control) experiments plates kept for incubation at 37°C for 24 h.

Isolation, purification and identification of the bacterium from TT

In this study, bacterial colonies extracted from the untreated TT extract were cultured, and their genomic DNA was analyzed through PCR and DNA sequencing methods. DNA isolation was conducted using the Macherey Nagel Nucleospin kit (Germany) according to the supplier's instructions. Standard PCR was employed to amplify the bacterial 16S region gene, with the primer pair 27 F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACCTTGTTACGACT T) annealed at 54 °C. Gel images were captured using a

BIO-RAD GelDoc-XR gel documentation system, with an expected PCR product size of approximately 1450 bp. Prior to sequencing, PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher). Purified PCR products (50 ng) were sequenced using the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The resulting reaction products were then analyzed using a DNA sequencing machine (3130 Genetic Analyzer Automated; Software version- Sequencing Analysis 5.1 and ChromasPro v3.1; Applied Biosystems, USA). Additionally, BLAST analysis was performed using the BlastN site on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST).

Maintenance of culture

Bacteria present in TT isolated contaminants was selected for our study viz., *Bacillus haynesii* (identified by 16S rRNA sequencing). The culture was maintained on Soyabean casein digest agar (SCDA) at 2–8 °C.

Biochemical analysis of TT isolate

By using a commercial Biochemical test kit (KB001 and KB002, HiMedia, India) for sugar and citrate utilization, deamination, acid production, acetoin production, lysine and ornithine decarboxylation, nitrate reduction, urease activity, phenyl alanine deamination, H_2S production. The TT isolate was inoculated in Mueller-Hinton broth (MHB) and incubated overnight at 37 °C. 50 µL of this pre-inoculum was added onto the wells of the Biochemical test kit and further incubated at 37 °C for 18–24 h. Further procedures for testing were followed according to the manufacturer's protocol.

Hemolysis test

For the presence of hemolysin in the TT isolate, the organism was aseptically streaked onto a 5% sheep blood agar plate and incubated for 18–24 h at 37 °C and observation noted [14].

RBC lysis assay

The hemolytic activity of *B. haynesii* was tested using the RBC lysis assay given by Sæbø et al. [15] with slight modifications. Briefly, 3 mL of fresh human blood from a healthy volunteer was collected into centrifuge tubes containing 150 µL K_2EDTA (from 30 mg/mL stock). This was provided generously by Mprex Healthcare Pvt. Ltd, Pune. The collected blood was then aliquoted into 2 mL centrifuge tube and centrifuged at 3000 rpm for 5 min to remove the serum. The RBCs were washed with Phosphate buffered saline (PBS) 2–3 times and suspended in PBS until further use. For the assay, 50 µL of the suspended RBC's along with 100 µL (0.5 McFarland standards) of *B. haynesii* overnight culture was added and volume was made up to 1 mL and incubated at 37˚C for 18–24 h. PBS was kept as negative control while Triton X was kept as positive control. The released hemoglobin was recorded at 540 nm by UV-vis spectrophotometer. The assay was performed in duplicate and the percentage hemolysis of the mean value was calculated by:

$$Hemolysis\,\% = \frac{(OD\ of\ sample\ -\ OD\ of\ blank)}{(OD\ of\ positive\ control)} \times 100$$

Antibacterial susceptibility test

Antimicrobial susceptibility tests were performed using a commercial 20 different antibiotic disks for Gram positive organisms (ICOSA G-I-Plus, HiMedia, India). The TT isolate was suspended in sterile MHB and adjusted to a turbidity of 0.5 McFarland units. The TT Isolate was then inoculated onto a MH agar plate (200 mm diameter) with sterile cotton swab. A disk containing 20 different antibiotics discs of 6 mm diameter on its projections was placed onto the plate, and incubated overnight at 37 °C. The diameter (mm) of each inhibition zone was measured [16].

Checkerboard assay to assess synergy between PHMB and GA

The Checkerboard broth microdilution assay was performed in a 96-well plate using LB broth with the final volume of 200 µL modified to this specific combination against *Bacillus haynesii*. This assay applied the combination of two compounds GA diluted from 1 to 0.0156% and PHMB diluted from 10 µg/mL to 0.0097 µg/mL to provide a final classification of the combined compounds based on a Fractional Inhibitory Concentration (FIC) Index (FICI) as follows: S, synergy (FIC$\leq$0.5); A, additive (FIC$>$0.50 and 1 and $\leq$4). Appropriate controls were also incubated [17].

Estimation of total saponin content in TT

The dry fruit extract of TT (5.0 g) was treated with 90% v/v ethanol in a round bottom flask, and refluxed for 30 min using a soxhlet apparatus. By decantation, the solvent was separated and cooled. The procedure was repeated till the alcoholic extract becomes colorless. Combine the alcoholic extracts and evaporate on water bath to a soft residue. The soft extract was subjected with petroleum ether treatment and refluxed at 60–80 °C. The same soft extract was treated similarly by ethyl acetate and chloroform. After cooling,

the solvents were discarded; the soft extract was kept in the same flask. Subsequently, the same extraction procedure was followed 3 times. Then the soft extract was dissolved in 25 mL of methanol and was concentrated to 5 mL. About 25 mL of acetone was added drop by drop in above methanolic part followed by continuous stirring to get saponins in precipitated form. The precipitate was collected, filtered, and dried at 105 °C [12, 18, 19]. The following formula was used to estimate the % of total saponin content:

$$Total\ Saponins\ (\%) = Weight\ of\ residue \times\ 100/Sample\ Weight$$

Estimation of GA concentration (before and after inactivation of GA in TT extracts) by HPLC

The estimation of GA was performed by the method published by our research group earlier and one can refer it for detailed method [10]. Briefly, to determine residual glutaraldehyde, HPLC analysis was performed on TT extract. 10 mg of dried TT powder was dissolved in 10 mL acetone, vortexed, sonicated, and filtered through a 0.45-micron membrane filter. Chromatographic separation was done using a BDS Hypersil C-18 column, and data were analyzed with Chromeleon™ (version 7.2). The HPLC system (Waters 2998, e2695) was set to 2000 psi, 30 °C, with a 30-minute run time. Samples (20 µL) were injected at a flow rate of 1 mL/min, with peaks detected at 255 nm. Each sample was analyzed in triplicate and peak areas were recorded.

For inactivation of glutaraldehyde, GA-treated TT extract (10 mg) was dissolved in acetonitrile, and 2.2-fold sodium bisulfite, based on the test sample weight, was added. The solution was vortexed for 15 min and sonicated. HPLC analysis with DNPH derivatization was then performed following the method of Jordan et al. (1996) [28].

Table 1 Biochemical testing for TT isolate

Sr. No.	Biochemical parameter	Identification index
1	Indole	-
2	Methyl red	-
3	Voges Proskauer's	-
4	Citrate Utilization	-
5	Glucose	-
6	Adonitol	-
7	Arabinose	-
8	Lactose	-
9	Sorbitol	-
10	Mannitol	-
11	Rhamnose	-
12	Sucrose	-
13	Lysine	+
14	Ornithine	+
15	Urease	+
16	Phenyl alanine deaminase test by TDA	-
17	Nitrate reduction	+
18	H_2S production	-

*(+) indicated the organism's positive for the test while (-) indicated the isolate to be negative for the performed test

Results and discussions

The intention of the present study was to estimate the microbial load in the TT extract using the serial dilution and spread plate method. But, during incubation, bacterial growth was observed on the plate containing the TT water extract. The dominant bacterial colony exhibited a jelly-like texture and was subsequently isolated, purified, and cultured on SCDA. We conducted basic biochemical tests in the laboratory to determine the genus-level identification of the isolated bacteria. These tests evaluated sugar and citrate utilization, among other parameters. The isolate was found to be capable of utilizing lysine and ornithine. Additionally, it tested positive for urease activity and nitrate reduction. The tests and their results are listed in (Table 1).

The identification of the bacterium was performed using 16S rRNA analysis. The 16S rRNA gene was amplified via PCR (Fig. 1) and sequenced (Fig. 2). A neighbor-joining phylogenetic tree (Fig. 3) was constructed based on the 16S rRNA gene sequences of our TT isolate, along with closely related sequences retrieved from NCBI. The sequences were aligned using Clustal W, and bootstrap consensus reliability was evaluated through 1,000 replicates, applying the neighbor-joining distance method in MEGA 11 software. Based on the phylogenetic analysis, the predominant species in the TT extract was identified as *Bacillus haynesii*.

We further performed hemolysis assay to understand the isolates pathogenicity hence, the isolate was streaked on 5% sheep blood agar plate to check for hemolysis. In (Fig. 4) β-hemolytic zone was clearly observed.

To further confirm the hemolysis result, we performed RBC lysis assay (Fig. 5). This assay confirms the haemolytic activity of test substance on human red blood cells based on release of hemoglobin as measured spectrophotometrically (Table 2). The culture was incubated with washed RBC overnight to quantify the hemolytic activity. According to the American Society for Testing and Materials (ASTM), <5% hemolysis is considered as null, 5–10% hemolysis is assumed as low and beyond 10% is apparent hemolysis [20].

The percentage hemolysis was measured for each sample, including the blank control (PBS), *Bacillus haynesii*, and the

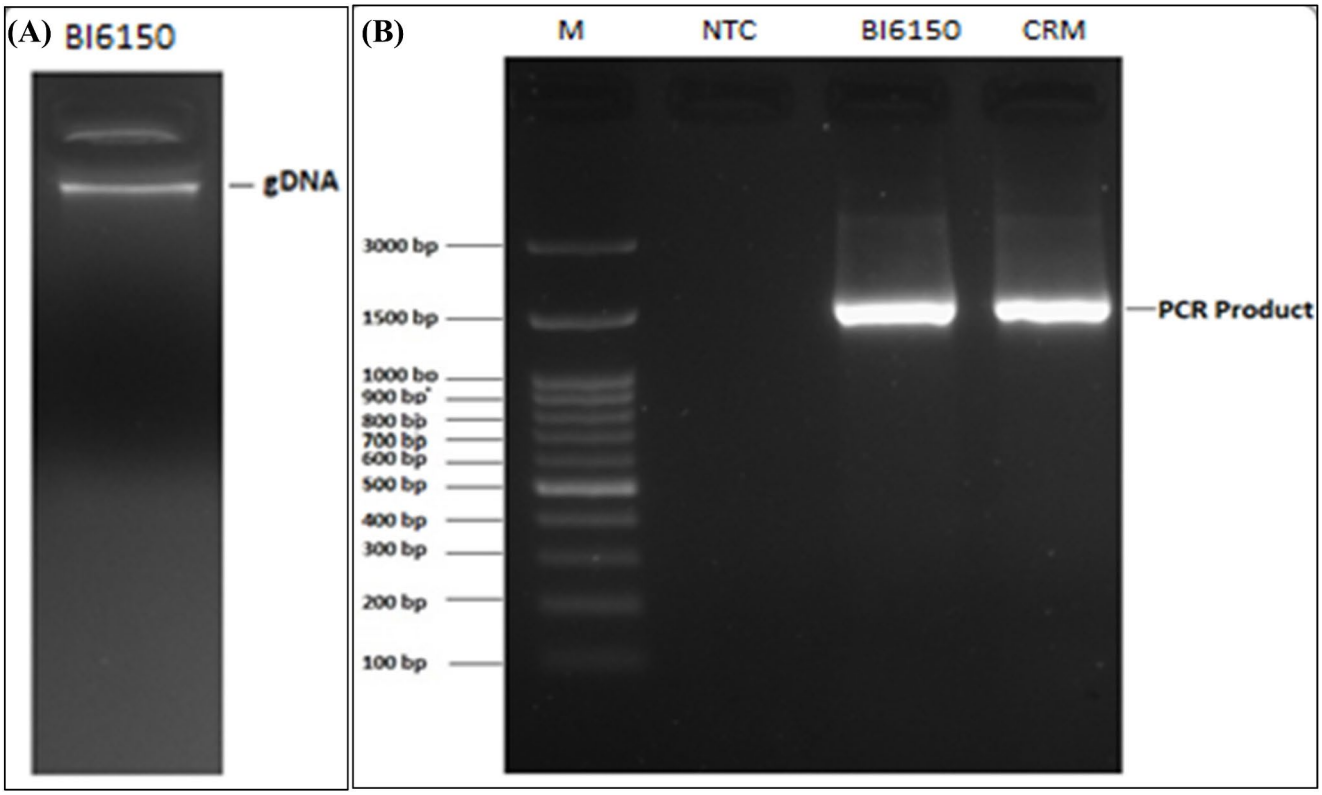

Fig. 1 Agarose gel electrophoresis of genomic DNA and PCR amplified samples of the bacterial isolate from the *Tribulus terrestris* (TT) extract. **A**. Depicts the agarose gel electrophoresis of genomic DNA performed on 1% (w/v) gel and 5 µL of sample of genomic DNA (gDNA) was loaded in the well. **B**. Depicts the agarose gel electrophoresis of PCR amplified sample. The wells M represents 100 bp to 3000 bp molecular marker; NTC represents no template control; BI6150 includes BI 6150 with primers 27-1492R PCR products; CRM denotes the positive certified reference material

>BI6150

GTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCT
AATACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACC
CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAA
AGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAG
TACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGAT
GTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGA

Fig. 2 16S rDNA FASTA sequence of the bacterial isolate from *Tribulus terrestris* (TT) extract without GA treatment. BLAST analysis identified the isolate as *Bacillus haynesii*

positive control (Triton X). The antibacterial susceptibility testing for gram positive organism was carried out for *B. haynesii* to check if the organism displays any resistance towards any antibiotics. The commercial panel of 20 antibiotics aided in the antibiotic susceptibility testing of gram-positive microorganisms. The results for antibacterial susceptibility, including the zone of inhibition in millimeter (mm), are listed in (Table 3). It was alarming to observe our TT isolate to show the resistance or non-susceptibility towards Cephalothin, Clindamycin, Erythromycin, Penicillin, Ampicillin, Oxacillin, Azithromycin, Clarithromycin, Methicillin, and Amoxyclay from the tested panel of antibiotics (Fig. 6).

The allowable dosage for TT fruit extract/powder is 3–6 g and for decoction it is 20–30 g as indicated in Ayurvedic Pharmacopoeia of India, Volume 1 [21]. The general guidelines for Ayurvedic formulations, as outlined

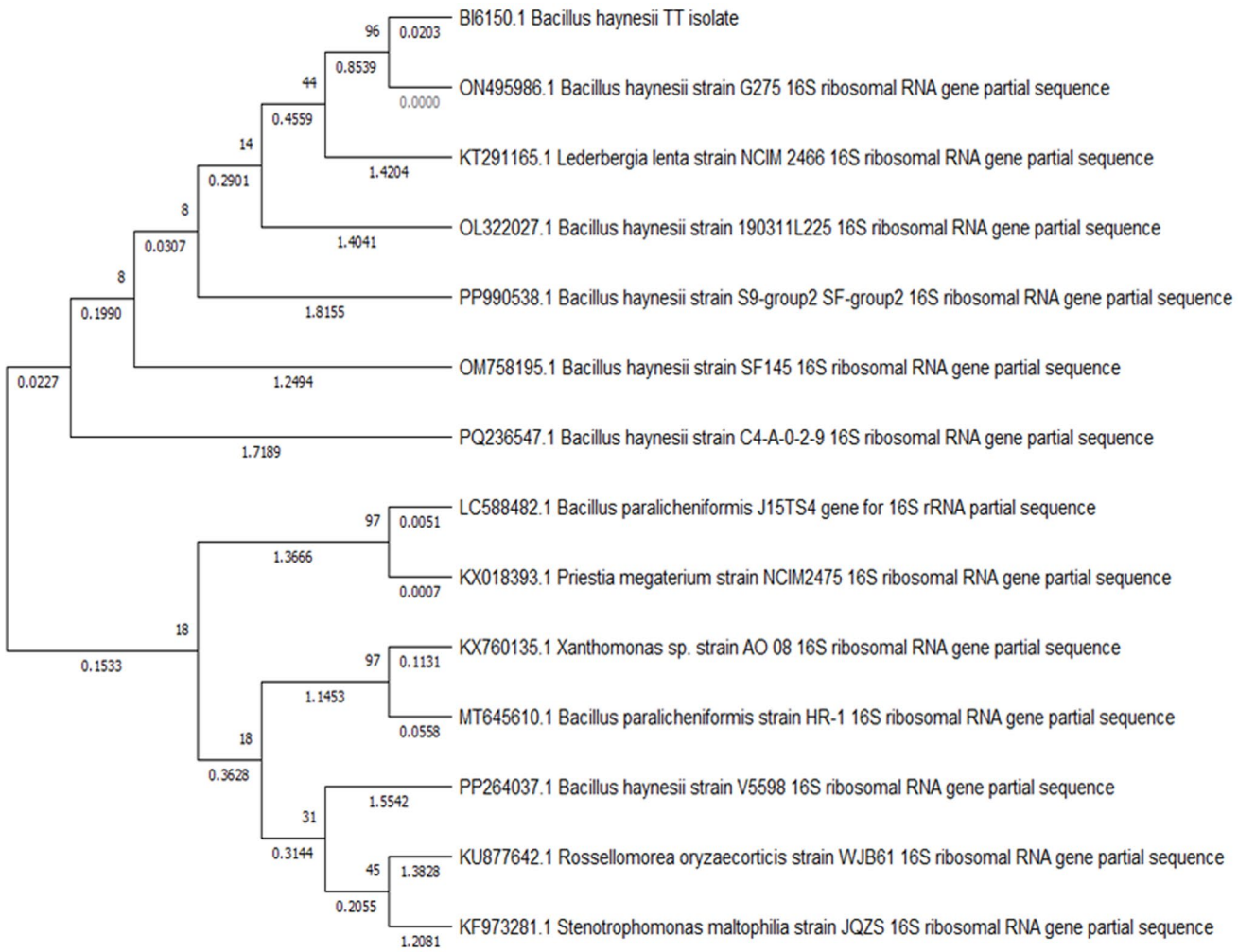

Fig. 3 Neighbor-joining phylogenetic tree constructed using 16S rRNA gene sequences of the *Tribulus terrestris* (TT) isolate and closely related bacterial species

in the Ayurveda, Siddha, and Unani Pharmacopoeias, specify microbial limits that include the complete absence of *Staphylococcus aureus*, *Salmonella species*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Additionally, the total microbial plate count (TPC) should be within the range of 10^5/g to 10^7/g for topical applications, and the total yeast and mold count should be approximately 10^3/g [22]. Having resistance to this many antibiotics, is a cause for concern as the TT extract is consumed for many different ailments.

Hence, to address this microbial contamination issue, many existing literatures were screened to identify an agent capable of reducing or eliminating the microbial load without compromising the bioactive contents of the extract. This search led us to investigate the antibacterial activity of PHMB and GA [10]. Through the Checkerboard experiment, the Minimum Inhibitory Concentrations (MIC) of both GA and PHMB against the isolated *Bacillus* species were determined, and found to be 0.25% and 10 µg/mL, respectively (Table 4).

In the checkerboard assay, two antimicrobiotics are tested in double serial dilutions, and the concentration of each drug is tested both alone and in combination. Thus, it is possible to determine the effect of the individual drug, but above all, the effect produced by their combination [23]. This assay utilized a blend of two substances at rising doses to determine the ultimate categorization of the combined compounds using a Fractional Inhibitory Concentration (FIC) Index (FICI), delineated as follows: synergistic (FIC $\leq$ 0.5); additive (FIC > 0.50 and < 1); neutral (FIC > 1 and $\leq$ 4) [17].

Remarkably, the concentration of PHMB required to inhibit the growth of *B. haynesii* was less than GA, indicating its efficacy as an antibacterial agent. A 2014 study by Asiedu-Gyekye et al. [24]; reported a 50% mortality rate in rats administered with 25.6 mg/kg, equivalent to 1.6 mL of a 0.4% PHMB solution (corresponding to 6.4×10^3 mg/L of a 0.1% solution). Based on this finding, the current study

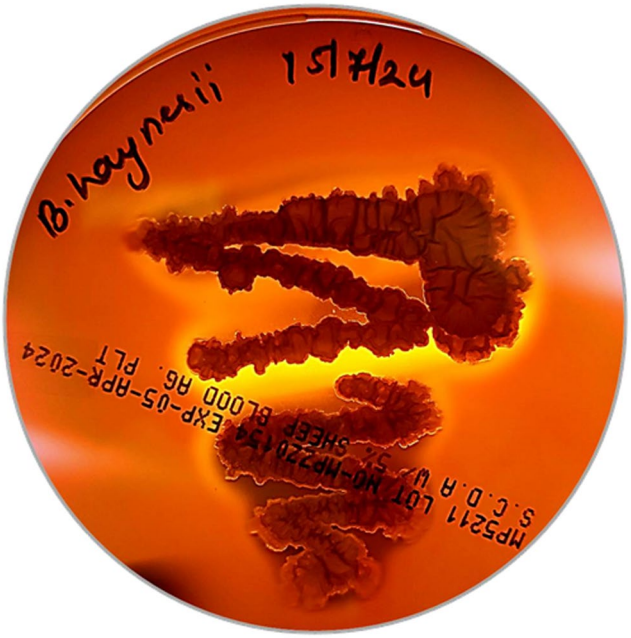

Fig. 4 β-hemolytic zone produced by *Bacillus haynesii* on a 5% sheep blood agar plate

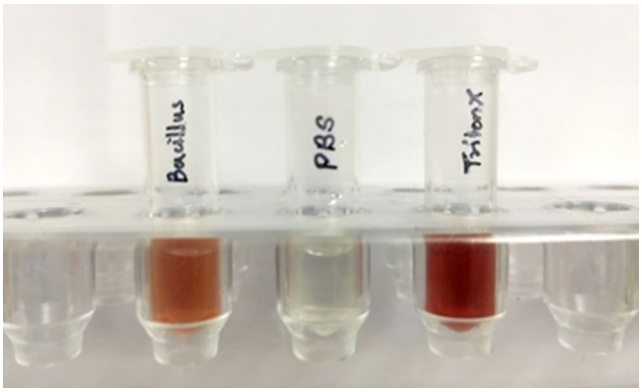

Fig. 5 RBC lysis assay of *Bacillus haynesii*, showing the lysis of red blood cells after 18–24 h of incubation

Table 2 RBC lysis assay results

Sr. No.	Sample	Percentage hemolysis
1	PBS (Blank)	0
2	*B. haynesii*	21.5
3	Triton X	100

* *B. haynesii* exhibits 21.5% RBC lysis, which exceeds the acceptable criteria established by the American Society for Testing and Materials (ASTM)

Table 3 Antibiotic susceptibility test of *B. Haynesii*

Sr. No.	Antibiotic	Concentration of disc (Lg)	Zone of inhibition (mm)	Susceptibility
1	Cephalothin (CEP)	30	< 10	-
2	Clindamycin (CD)	2	< 10	-
3	Co-Trimoxazole (COT)	25	35	+
4	Erythromycin (E)	15	< 10	-
5	Gentamicin (GEN)	10	25	+
6	Ofloxacin (OF)	5	32	+
7	Penicillin (P)	10Unit	< 10	-
8	Vancomycin (VA)	30	22	+
9	Ampicillin (AMP)	10	< 10	-
10	Chloramphenicol (C)	30	15	+
11	Oxacillin (OX)	1	< 10	-
12	Linezolid (LZ)	30	32	+
13	Azithromycin (AZM)	15	< 10	-
14	Amikacin (AK)	30	22	+
15	Clarithromycin (CLR)	15	< 10	-
16	Teicoplanin (TEI)	10	15	+
17	Methicillin (MET)	5	< 10	-
18	Amoxyclav (AMC)	30	< 10	-
19	Novobiocin (NV)	5	17	+
20	Tetracycline (TE)	30	20	+

*Note <10 mm zone means not susceptible (-) towards that antibiotic

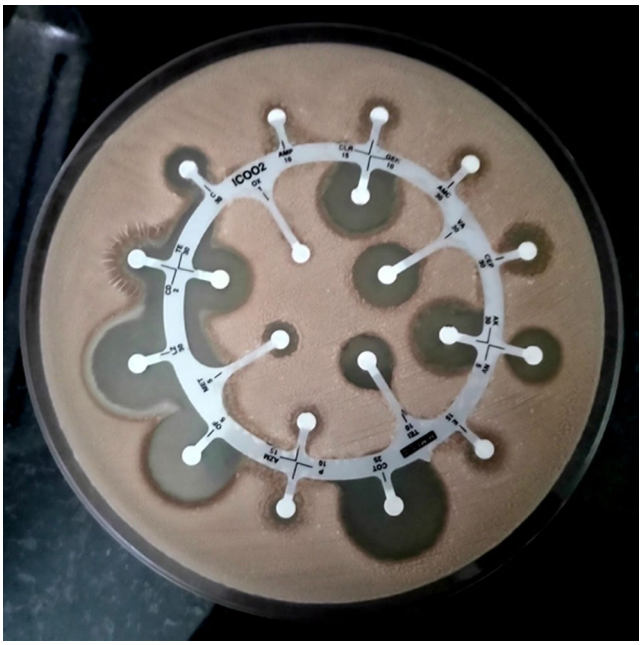

Fig. 6 *B. haynesii* antibacterial susceptibility test using Icosa-GI-Plus

Table 4 Checkerboard assay results

Name of the Sample	MIC/FIC (%/µg/mL)
Glutaraldehyde (MIC_A)	0.25%
PHMB (MIC_B)	10 µg/mL
Glutaraldehyde in combination with PHMB (A)	0.125%
PHMB in combination with Glutaraldehyde (B)	5 µg/mL
*FIC Index	1

* If FIC values are 0.5 > then synergy is observed in two compounds. If FIC values are > 4 then, antagonism is observed and for FIC value of 0.5-4, Additive or indifference is observed between two compounds

Table 5 Estimation of percent yield and total saponin (with and without GA treatment)

	Percentage of yield (%) in gram (gm).	Total saponin content in %
Without GA	7.4	31.29
With GA	6.9	30.67

Table 6 Microbial load in *Tribulus terrestris* (TT) extract, and glutaraldehyde (GA)

Reduction in the microbial load after treatment with 1% glutaraldehyde			
Extract	Initial count (cfu/g)	Final count (cfu/g)	Reduction in microbial load (%)
Tribulus terrestris (TT)	1.27×10^5	3×10^3	99.0

incorporated glutaraldehyde (GA) in the extraction process to mitigate microbial contamination of the TT extract instead of PHMB. Also, the process to neutralize GA is known.

It is reported that *Bacillus swezeyi* sp. nov. and *Bacillus haynesii* sp. nov., have been isolated from desert soil [25]. These thermophilic bacteria have the remarkable ability to withstand extreme temperatures, stress, and can form biofilms, as noted by Marín-Sanhueza et al. [26]. Our findings indicate a greater need for GA to decrease bacterial load in TT, aligning with previous literature suggesting *Bacillus* biofilms exhibit increased resistance to GA [27].

The medicinal plant extract contains saponins which exhibit strong antioxidant properties, making them promising alternatives for antioxidant use due to their ability to neutralize free radicals. In our investigation, we measured the total saponin content in the TT extracts and observed percentages of 30.67% and 31.49% for GA-treated and untreated samples, respectively (Table 5) [19]. These results suggest that GA treatment did not affect the saponin content, as indicated by the comparable percentages observed.

We also found that 1% GA effectively reduced the microbial load by > 99% and the values are represented in (Table 6). This data demonstrates that glutaraldehyde (GA) can be an effective treatment for reducing microbial load in pulverized raw materials. GA, classified as a high-level disinfectant and sterilant, is primarily used for sterilizing medical devices, equipment, and in water treatment processes. In our previous research, we reported the successful use of GA to eliminate microbial load from plant extracts, making them safe for consumption after neutralization. The current findings further validate and support our earlier work [10].

GA can cause irritation to the throat, nasal passages, and lungs if inhaled, and may result in liver damage if ingested. Therefore, it is crucial to estimate the residual GA in the TT extract before and after treatment with Sodium bisulfite (SB). This was measured using the standard HPLC method outlined earlier [10, 28]. Based on the chromatogram peak of TT extract shown in (Fig. 7), no detectable GA observed after reaction with a 2.2-fold of SB for 15 min, signifying that SB completely inactivated the GA in TT extracts via formation of a proposed GA–bisulfite complex.

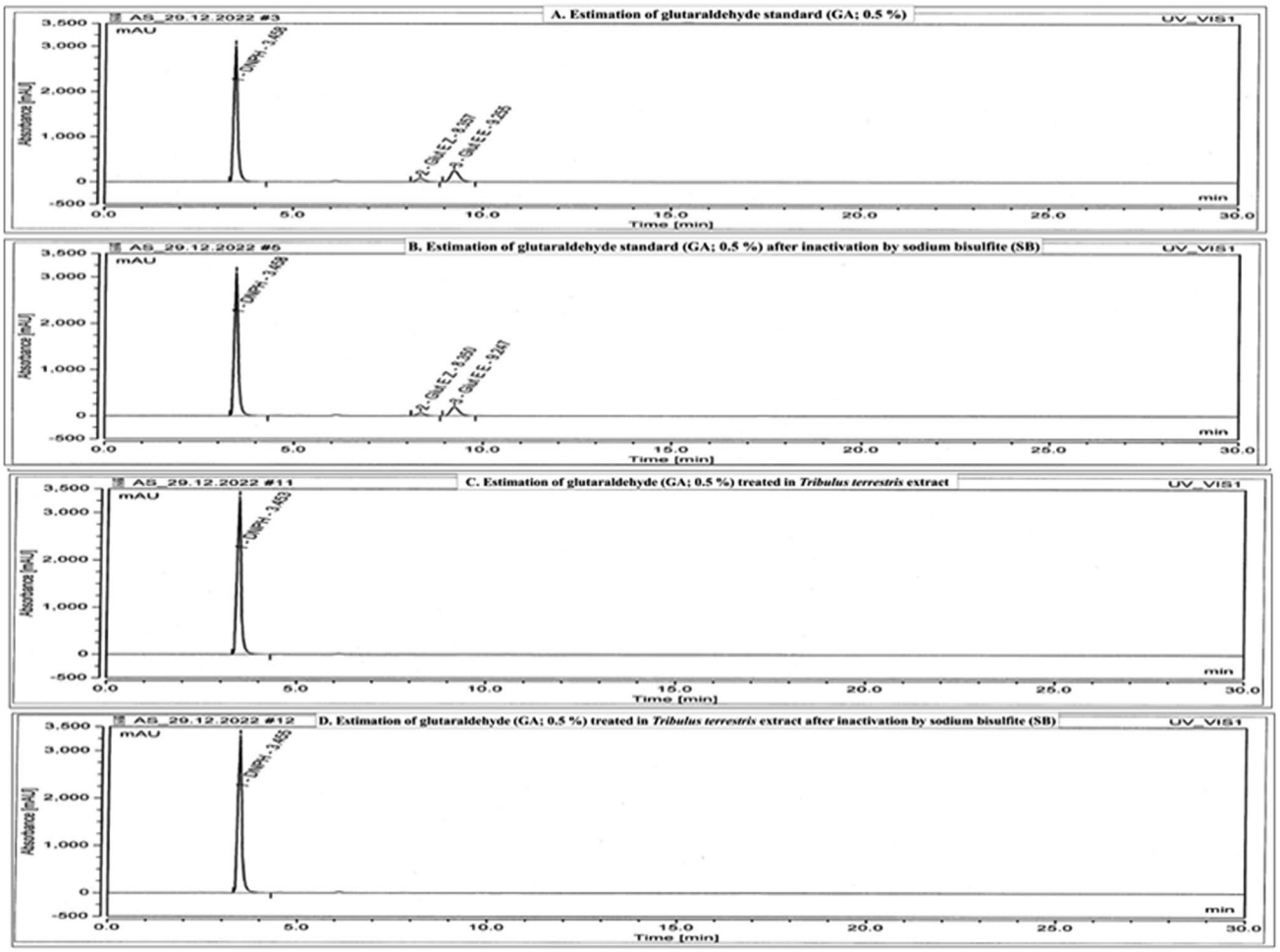

Fig. 7 HPLC Chromatogram of *Tribulus terrestris* (TT) treated with glutaraldehyde (GA; 0.5%) and inactivation of GA by sodium bisulfite (SB), as well as the reference GA (0.5%). A and B: Depicts the chromatogram of GA and inactivation of GA with sodium bisulfite (SB), respectively. While C and D: Depicts the chromatogram of TT treated with GA and inactivation of GA by SB, respectively

Conclusion

The MIC of GA and PHMB against isolated *Bacillus* sp. were determined as 0.25% and 10 µg/mL, respectively. Checkerboard assay showed no synergism between the compounds, with only a slight increase in inhibition. GA, due to its high inhibitory activity, was selected for further investigation. Analysis revealed that 1% GA-treated raw materials effectively reduced microbial load by 99% without affecting yield (0.5%). The bioactive total saponin content in TT remained unaffected post-GA treatment. Sodium bisulfite was used to neutralize residual GA, validated by HPLC analysis.

We recommend further investigation into herbal products to identify and mitigate other impurities to meet acceptable standards.

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Author contributions Dr. Priti Darne: Microbiological aspects, Data arrangement, manuscript writing and editing. Shital Palghadmal: Estimation of bioactive molecules by HPLC, and HPLC data interpretation. Nisha Mehta: Checkerboard assay and Manuscript editing, Dr. Sriram Padmanabhan: Conceptualization, Project administration, Resources, Supervision, Writing - review and editing.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest Authors declare no conflict of interest.

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