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Microbial safety evaluation of *Terminalia bellirica* (Gaertn.) Roxb. Fruit raw material: Bacterial diversity, Fungal incidence and Ergosterol analysis

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Abstract

The microbiological safety of crude herbal drugs significantly influences the safety, efficacy and shelf life of herbal formulations. Terminalia bellirica (Combretaceae), an important medicinal fruit and a key component of Triphala, is widely used in traditional medicine and nutraceutical preparations. The present investigation aimed to evaluate the microbial status of T. bellirica fruits collected from retail herbal markets of Mysuru, Karnataka. Microbial enumeration was carried out using the serial dilution technique, followed by isolation and characterization of bacterial and fungal contaminants. Detection of indicator pathogens such as Escherichia coli and Salmonella was performed using selective media and biochemical tests. Fungal contamination was further confirmed by estimation of ergosterol using TLC and LCMS methods. Results revealed substantial microbial contamination with average bacterial and fungal loads of 1.66×10^{7} CFU/g and 1.55×10^{5} CFU/g, respectively. A total of 27 morphologically distinct bacterial isolates were obtained, of which 70% were Gram-positive. Six out of eighteen samples were contaminated with E. coli. Nineteen fungal species belonging to ten genera were identified, with Aspergillusniger and A. flavus as predominant contaminants. Ergosterol(184.49 µg/kg) was detected in 15 samples, confirming potential fungal incidence. The study highlights the need for stringent microbial quality control measures for T. bellirica fruit raw materials used in herbal drug formulations.

Keywords: Terminalia bellirica; Microbial contamination; Ergosterol; *Aspergillus*; Bio-deterioration; Microbial quality control.

Introduction

Herbal medicines constitute an important component of traditional healthcare systems and continue to be widely used across the world due to their therapeutic efficacy, affordability and natural origin[1]. However, the quality and safety of herbal drug raw materials are often compromised by microbial contamination arising during cultivation, harvesting, processing, storage and marketing[2]. The presence of bacteria and fungi in herbal drugs can lead to bio-deterioration, reduced shelf life and potential health hazards to consumers[3].

Terminalia bellirica (Gaertn.) Roxb., belonging to the family Combretaceae, is a highly valued medicinal fruit extensively used in Ayurvedic formulations. It is one of the three ingredients of the renowned polyherbal formulation Triphala and is traditionally used for digestive, hepatoprotective and rejuvenating purposes[4,5]. Due to its extensive demand in herbal and nutraceutical industries, large quantities of *T. bellirica* fruits are collected, stored and marketed for prolonged periods, making them vulnerable to microbial infestation.

Microbial contamination of herbal raw materials is of particular concern because certain bacteria serve as indicators of fecal contamination, while storage fungi may produce harmful secondary metabolites such as mycotoxins[1,6]. Species of *Aspergillus* and *Penicillium* are frequently reported from stored medicinal plant materials and are known for their spoilage potential and toxin production, including aflatoxins[7]. Therefore, monitoring fungal contamination in herbal drugs is essential to ensure their safety and quality.

Ergosterol, a sterol component specific to fungal cell membranes, has been widely used as a reliable biochemical marker for assessing fungal biomass in plant-based materials[8]. Estimation of ergosterol complements conventional culture-based methods and provides a quantitative measure of fungal contamination[9,10]. Despite the medicinal importance of *T. bellirica*, limited studies have systematically evaluated its microbial quality and fungal contamination using ergosterol as a chemical indicator. Hence, the present study was undertaken to assess the microbial load, identify bacterial and fungal contaminants and evaluate ergosterol content in *T. bellirica* fruit raw material marketed in Mysuru, Karnataka.

Materials and Methods

Sample Collection: Eighteen dried fruit samples of *T. bellirica* were procured from different herbal retail outlets across Mysuru city. Samples were collected aseptically and transported in sterile containers for laboratory analysis.

Microbial Enumeration: One gram of powdered sample was homogenized in sterile distilled water and serially diluted. Appropriate dilutions were plated on Nutrient Agar for total bacterial count and CzapekDox Agar for fungal count following standard microbiological protocols[11]. Bacterial plates were incubated at 35°C for 48–72 hours, while fungal cultures were maintained at 27±2°C for seven days. Colony forming units were calculated and expressed as CFU/g.

Characterization of Bacterial Isolates: Distinct colonies were purified and characterized based on morphological attributes including colony form, margin, elevation, pigmentation, and surface texture. Gram staining and biochemical assays were performed for differentiation[12].

Detection of Indicator Pathogens: Selective and differential media were used to screen for *Escherichia coli* and *Salmonella* spp. MacConkey agar and EMB agar were used for *E. coli* and Selenite F broth, brilliant green agar and bismuth sulphite agar were used for *Salmonella.* Biochemical confirmation was conducted as recommended in pharmacopeial guidelines[12].

Fungal Identification: Fungal isolates were identified by examining macroscopic colony features and microscopic structures such as conidial heads, spore morphology, and hyphal organization using standard taxonomic manuals[13].

Ergosterol Extraction and Analysis: Ergosterol was extracted using methanolic solvent treatment. Preliminary detection was achieved through Thin Layer Chromatography, followed by confirmation using LC–MS analysis. Compound identification was based on retention time comparison and molecular ion peak detection, consistent with established fungal sterol characterization methods[14].

Results and Discussion

Microbial Analysis: Microbial analysis of test samples revealed the presence of both bacterial and fungal contaminants in all eighteen samples of Terminalia bellirica fruits. The average bacterial load was 1.66×10^7 CFU/g, whereas the average fungal load was 1.55×10^5 CFU/g (Table-1). Such elevated microbial loads indicate significant exposure to environmental contaminants during post-harvest handling and storage. Similar levels of microbial contamination in marketed herbal raw materials have been reported from tropical countries where open drying, prolonged storage, and poor hygienic handling are common practices[15,16]. According to the World Health Organization guidelines, crude herbal materials exceeding permissible microbial limits may pose risks to consumer safety1. A wide diversity of bacterial flora was recorded. Isolation of twenty-seven distinguishable bacterial colonies was done, and their morphological characteristics are presented in Table 2. Among these, Gram-positive bacteria predominated (19 isolates), while 8 isolates were Gram-negative. The predominance of Gram-positive organisms is consistent with previous findings in dried plant materials, as their thick peptidoglycan layer enhances resistance to desiccation and environmental stress[17]. The occurrence of Gram-negative bacteria further suggests contamination from water, soil, or human handling during processing[13].

Table-1: Total bacterial and fungal counts in *Terminalia bellirica* (CFU/g).

Microbe	Colony-forming units (per gram)
Bacteria	$1.66\text{x}10^7\pm4.70$
Fungi	$1.55\text{x}10^5\pm0.41$

Colony morphology showed marked diversity in form, margin, elevation, pigmentation, and surface texture. Circular and irregular colonies were equally predominant, followed by punctiform and rhizoidal forms. Entire margins were common, with raised and pulvinate elevations frequently observed. Most colonies were whitish, with fewer yellow and rare orange pigmented forms. Broth growth patterns included sediment, pellicle formation, and uniform turbidity. Such heterogeneity indicates a complex microbial community capable of contributing to biodeterioration and reduction in shelf life[13]. Mixed microbial populations in stored herbal materials are known to accelerate degradation of phytoconstituents through synergistic enzymatic activity[18].

Detection of *Escherichia coli* and *Salmonella:* All samples were screened for fecal indicator pathogens. Among the 18 samples analyzed, 6 (33%) were contaminated with *Escherichia coli*, while none tested positive for *Salmonella*. *E. coli* formed pink to reddish colonies accompanied by bile salt precipitation on MacConkey agar, and displayed the typical metallic green sheen when cultured on EMB agar. Confirmation was achieved through biochemical tests including indole production, motility, nitrate reduction, β-galactosidase activity, and carbohydrate utilization patterns. The occurrence of *E. coli* is of particular concern as it serves as a recognized indicator of fecal contamination and reflects inadequate sanitary practices during harvesting and storage. Although *Salmonella* was not detected, the detection of *E. coli* alone renders the raw material microbiologically unacceptable under international herbal drug quality standards[1]. These findings highlight the necessity for strict implementation of Good Agricultural and Collection Practices (GACP) and Good Manufacturing Practices (GMP).

Fungal Diversity and Ergosterol Detection: Fungal investigation revealed the presence of 19 species representing 10 genera (Figure-1). Members of the genus *Aspergillus* were dominant, including *A. niger, A. flavus, A. candidus,* and *A. terricola*. Additionally, three species each of *Penicillium* and *Alternaria* were identified, along with representatives of *Cladosporium*. Among the recorded fungi, *A. niger* showed the highest relative density of 42.25%, followed by *A. flavus* (15.49%).

Table-2: Morphological features of bacterial contaminants isolates from *Terminalia bellirica.*

Colony Shape	Colony Margin	Colony Elevation	Pigmentation	Surface texture	Optical feature	Gram staining	Broth culture appearance
Circular	Entire	Raised	Yellow	Rough	Opaque	GP Bacilli	Sediment
Irregular	Entire	Flat	Whitish	Rough	Opaque	GN Bacilli	Pellicle
Irregular	Curled	Raised	White	Wrinkled	Opaque	GP Bacilli	Uniform
Rhizoidal	Rhizoidal	Flat	Whitish	Rough	Translucent	GN Bacilli	Pellicle
Punctiform	Lobate	Flat	Whitish	Rough	Opaque	GP Bacilli	Sediment
Irregular	Curled	Raised	Whitish	Wrinkled	Opaque	GP Bacilli	Pellicle
Circular	Entire	Pulvinate	Whitish	S & S	Opaque	GP Cocci	Sediment
Irregular	Curled	Raised	Yellow	Wrinkled	Translucent	GN Cocci	Sediment
Circular	Entire	Raised	Whitish	Rough	Opaque	GP Cocci	Sediment
Irregular	Entire	Raised	Orange	S & S	Opaque	GP Cocci	Sediment
Circular	Curled	Umbonate	White	S & S	Opaque	GN Bacilli	Uniform
Circular	Entire	Raised	Yellow	Rough	Opaque	GP Cocci	Sediment
Punctiform	Entire	Umbonate	Whitish	S & S	Translucent	GN Bacilli	Uniform
Irregular	Curled	Raised	Whitish	Rough	Translucent	GN Bacilli	Pellicle
Irregular	Curled	Raised	Whitish	Wrinkled	Translucent	GP Bacilli	Pellicle
Circular	Entire	Raised	White	Rough	Opaque	GN Cocci	Sediment
Punctiform	Entire	Convex	Whitish	S & S	Translucent	GP Cocci	Uniform
Punctiform	Lobate	Raised	Whitish	S & S	Opaque	GP Cocci	Sediment
Irregular	Curled	Raised	Whitish	Wrinkled	Opaque	GP Cocci	Sediment
Irregular	Curled	Raised	Whitish	Opaque	Opaque	GP Cocci	Sediment
Punctiform	Curled	Convex	Whitish	S & S	Translucent	GP Cocci	Uniform
Punctiform	Lobate	Raised	Whitish	S & S	Translucent	GP Bacilli	Flocculent
Punctiform	Entire	Pulvinate	Whitish	S & S	Transparent	GP Cocci	Uniform
Circular	Entire	Pulvinate	Yellow	Rough	Opaque	GN Cocci	Sediment
Circular	Curled	Convex	Whitish	S & S	Opaque	GP Cocci	Pellicle
Punctiform	Entire	Pulvinate	Yellow	S & S	Opaque	GP Cocci	Uniform
Circular	Entire	Convex	Yellow	S & S	Opaque	GP Cocci	Sediment

Note: S & S-Smooth and shiny; GP -Gram Positive; GN -Gram negative.

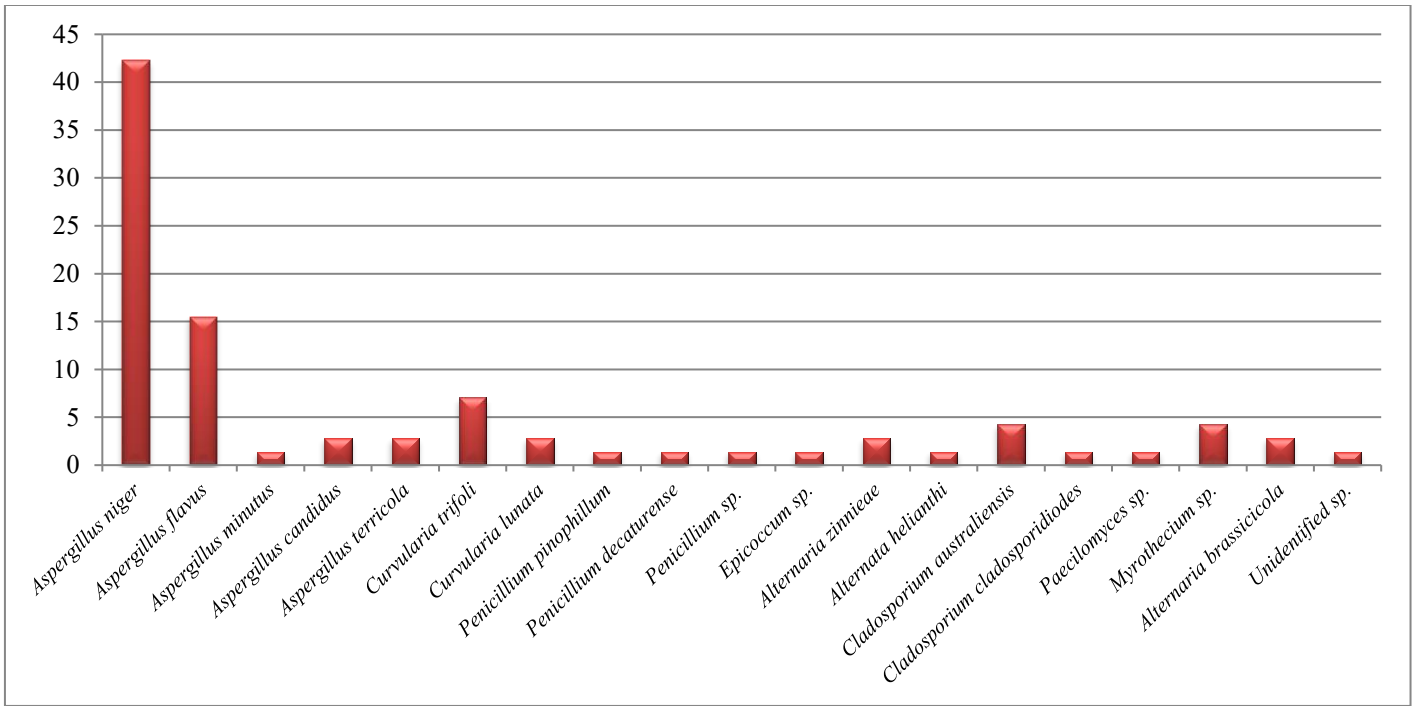

Figure-1: Percent relative density of fungi on *Terminalia bellirica* fruit.

The dominance of *Aspergillus* species in stored medicinal plants has been widely documented, particularly under warm and humid storage conditions[6,7]. Certain species such as *A. flavus* are known producers of aflatoxins, raising potential toxicological concerns. The presence of *Alternaria*, *Penicillium*, and *Cladosporium* further supports reports that storage fungi commonly colonize dried herbal materials[18].

Cultural findings were further substantiated by ergosterol estimation. Ergosterol is a principal sterol component of fungal cell membranes and serves as a reliable biomarker for fungal biomass[10]. Among 18 samples, 15 tested positive for ergosterol. TLC analysis revealed a distinct spot at an Rf value of 0.3, while LC–MS analysis showed a characteristic retention time of 3.25 minutes and a dominant ion at m/z 379.3 corresponding to the protonated molecular ion $[M+H]^+$ of ergosterol. The concurrence of cultural identification and LC–MS confirmation strengthens the reliability of fungal detection.

The average ergosterol concentration was 184.49µg/kg. Ergosterol quantification provides complementary information on fungal biomass and supports the culture-based assessment of fungal contamination[8,9]. Such integration of microbiological enumeration with chemical biomarker analysis provides a more comprehensive assessment of fungal contamination compared to culture methods alone[19].

Conclusion

The combined findings demonstrate substantial microbial contamination in marketed *Terminalia bellirica* fruits. The presence of fecal indicator bacteria, diverse spoilage microflora, storage-associated fungi, and measurable ergosterol collectively indicate compromised microbial quality. The integrative approach employing both culture-based and LC–MS–based methods strengthens the validity of contamination assessment and highlights the need for routine microbial monitoring to secure safety and therapeutic reliability of herbal raw materials.

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