



Microbial Quality Assurance of Dried Roots of *Chlorophytum borivillianum* Sant F (Safed Musli)

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Authors' contributions

This work was carried out in collaboration among all authors. Author MS performed the study, wrote the protocol, analysed the results and wrote the first draft of the manuscript. Authors RS and PS designed the study, supervised the experiment and wrote the final version of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2021/v32i930413

Editor(s):

(1) Dr. Patrizia Diana, University of Palermo, Italy.

(2) Prof. Marcello Iriti, Milan State University, Italy.

Reviewers:

(1) Satyajit Panda, India.

(2) Goli Penchala Pratap, India.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/73687>

Original Research Article

Received 01 July 2021
Accepted 11 September 2021
Published 18 September 2021

ABSTRACT

Aim: To assess total bacterial load and detect *E. coli* and *Salmonella* in *Chlorophytum borivillianum* (Safed Musli), cultivated and processed in the Bundelkhand region.

Study Design: Quality assurance of medicinal plant raw material is essential for the preparation of good quality herbal medicines. The dried roots of Safed Musli were collected from different regions of the Bundelkhand and total aerobic microbial count was measured.

Methodology: We collected 10 samples of Safed Musli dried roots from different regions of Bundelkhand. A stock suspension was prepared using sterile peptone water and plated on a nutrient agar medium which was used to calculate total aerobic bacterial load as colony-forming units per gram (CFU/g). Further, biochemical tests were performed to confirm the identification of *E. coli* and *Salmonella*.

Results: The total aerobic bacteria count in dried roots of Safed Musli was within the permissible limit (10^5 - 10^7 CFU/g of dried raw material) as per globally recognized pharmacopoeia and other

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regulatory agencies such as the Central Council of Research in Ayurvedic Sciences (CCRAS), Ministry of AYUSH, Govt. of India, United States Pharmacopoeia (USP), Brazilian Pharmacopoeia (BP), European Pharmacopoeia (EP), World Health Organization (WHO), American Herbal Products Association (AHPA) and National Science Foundation/American National Standards Institute (NSF/ANSI). Although 2 out of 10 samples were found to have contamination of *E. coli* and *Salmonella* within the permissible limit of WHO, EP and NSF/ANSI.

Conclusion: The results indicated that the Safed Musli cultivated and processed in the Bundelkhand region is suitable for the preparation of herbal medicines and food supplements.

Keywords: *Chlorophytum borivilianum*; roots; microbial quality assurance; *E. coli*; salmonella.

1. INTRODUCTION

The use of herbal medicines (HM) is gaining widespread popularity all over the world by being a pivot of complementary and alternative medicine. It has been a major component of primary healthcare in many rural communities and also contributes a vital part of the culture of many civilizations of the world [1]. It is estimated that approximately 80% of the population in developing countries uses traditional herbal medicines as part of their prime wellbeing [2]. HM largely contains crude plant drugs with a complex mixture of chemical compounds that are responsible for pharmacological activities and correspond to health benefits [3]. It is widely accepted due to its low costs, low toxicity, and efficacy in multifactorial diseases. With the continually escalating global use of HM for chronic or serious ailments, relevant quality assurance methods are crucial for the affirmation of the therapeutic value and avoiding any toxic manifestation. Quality assurance of herbal products might be assured by the proper control of the herbal ingredients by means of good agricultural practices and manufacturing processes. These are the essential steps for quality assessment of herbal products and play a fundamental role in assuring the stability of herbal products.

The presence of microbial contamination is a major issue which not only reduces the therapeutic activity of medicinal products but also adversely affects the health of consumers. Therefore, microbial load assessment of herbal preparations is of paramount importance from a regulatory perspective. The main safety risks associated with herbal products/medicines are contamination by various microorganisms from soil, air and water during cultivation and also during post-harvest processing. The microbial load may also be influenced by environmental factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods, handling practices and the

storage conditions of crude and processed medicinal plant materials. The microbial contamination involves bacteria and their spores, fungus, yeasts and moulds, viruses, protozoa, insects (their eggs and larvae), and other organisms [4]. The presence of pathogenic bacteria (*Staphylococcus aureus*, *Shigella* spp., *Salmonella*, *Escherichia coli*, *Clostridium perfringens*) and fungi (*Aspergillus*, *Penicillium*, *Mucor*, *Candida* and *Trichosporium*) have been reported in the medicinal plant products [5–7].

World Health Organization (WHO) has recommended technical guidelines to indicate the microbial quality of herbal preparations, determine microbiological contaminants and test for total viable aerobic bacteria and fungi. The total aerobic bacterial count is an important determinant of the microbial load. It is generally influenced by different factors, such as the distance of the economically important plant part from the soil and the plant surface area to plant weight ratio [8]. Regarding the permissible microbial limits, regulatory guidelines have been formulated by official compendia such as World Health Organization (WHO), United States Pharmacopoeia (USP), European Pharmacopoeia (EP) (<https://www.intechopen.com/books/latest-research-into-quality-control/microbial-quality-of-medicinal-plant-materials>), Brazilian Pharmacopoeia (BP) (<https://www.gov.br/anvisa/pt-br/assuntos/farmacopeia/farmacopeia-brasileira/arquivos/8025json-file-1>), Tea and Herbal Infusions Europe (THIE) (<https://thie-online.eu/activities/food-safety.html>), American Herbal Products Association (https://www.ahpa.org/Portals/0/PDFs/Policies/14_0206_AHPA_micro_limits_comparisons.pdf), and Central Council for Research in Ayurvedic Sciences (CCRAS), Ministry of AYUSH, India (<https://www.ayush.gov.in/docs/guideline-drug-development.pdf>) for permissible limits of bacterial load in crude plant drugs and processed herbal formulations (Table 1).

Chlorophytum borivillianum, commonly known as 'Safed Musli'[9] is an important medicinal plant of high commercial value. The plant possess hepatoprotective [10] anti-inflammatory, antioxidant [11], analgesic, immunomodulatory [12], anti-diabetic [13], anti-ulcer [14], antimicrobial [15], anti-stress [16], anti-cancer [17], and sexual function enhancement activities [18]. The roots (tubers) of Safed Musli are found to be rich in alkaloids, vitamins, minerals, saponins and steroids. The present work is aimed at the microbial quality assurance of roots of Safed Musli cultivated and processed by farmers in the Bundelkhand region.

2. MATERIALS AND METHODS

2.1 Collection of the Sample

The peeled and dried roots of Safed Musli (10 samples) of the same season were collected from the farmers in Baruasagar (District, Jhansi, Uttar Pradesh) and Prathvipur (District Niwari, Madhya Pradesh) of Bundelkhand region, during October 2019 (Fig. 1). The roots were collected in a sterile screw-capped, pre weighted falcon tube and labelled. The samples were transferred to the laboratory, refrigerated at 4 °C and processed within 24 hours of receiving.

2.2 Total Aerobic Microbial Counts

The weight of the falcon tube was recorded to measure the amount of sample available in each tube. Sterile peptone water was added to the tubes to prepare a stock suspension (1g/10ml).

Seven serial dilutions were prepared from each tube for estimation of bacterial load in from of colony-forming units (CFUs). Nutrient Agar (NA) plates were prepared as per suppliers instructions. By using a calibrated micropipette, 100 µl volumes of stock solution and serial dilutions (up to 10⁻⁶) of the bacterial suspension were poured on the NA plate. With the help of a glass rod spreader, the sample was spread evenly on the NA plates. The plates were incubated at 35-37 °C for 18 to 24 hours and the bacterial colonies were counted using a colony counter. By multiplying the average number of colonies with dilution factor, total aerobic bacterial load was calculated and reported as colony-forming units per gram (CFU/g) of a sample [19,20]. The obtained CFU/g from samples was compared with the standards provided in Table 1.

2.2.1 Identification of *E. coli* and *Salmonella* using selective media

For the identification of specific pathogens such as *E. coli* and *Salmonella*, selective media were used. ECD MUG agar was used for the identification of coliforms and *E. coli*, and *Salmonella* differential agar was used for the identification of *Salmonella* species. The media was prepared in sterile petri plates according to the manufacturer's guidelines. The stock solutions and serial dilutions of samples were spread on these media plates and incubated at 35-37 °C for 24-48 hours for the development of colonies.



Fig. 1. Collection of dried roots of Safed Musli

Table 1. Recommended microbial limits for herbal drugs (values in CFU/g)

Compendia	Herbal products details	Total aerobic microbial count	Enterobacteria and other Gram-negative bacteria	<i>E. coli</i>	<i>Salmonella</i>
CCRAS	Dried plant material	10 ⁵	NA	Absent	Absent
	Plant materials for tropical use	10 ⁷	NA	Absent	Absent
USP	Dried or powdered botanicals	10 ⁵	10 ³	Absent	Absent
	Powdered botanical extracts	10 ⁴	NA	Absent	Absent
BP	Infusions/ decoctions	10 ²	NA	Absent	Absent
	Herbal drugs submitted to hot extracting processes	10 ⁷	10 ⁴	Absent	Absent
	Herbal drugs submitted to cold extracting processes	10 ⁵	10 ³	Absent	Absent
EP	Preparation for oral use containing raw material of natural origin	10 ⁴	10 ²	Absent	Absent
	Herbal drugs submitted to hot extracting processes	10 ⁷	NA	10 ³	NA
	Herbal drugs not submitted to hot extracting processes	10 ⁵	10 ³	Absent	Absent
AHPA	Dried, unprocessed herbs for use as ingredients in dietary supplements	10 ⁷	10 ⁴	Absent	Absent
WHO	Contamination of crude plant material intended for further processing	NA	NA	10 ⁴	NA
	Pretreated plant material for use as herbal teas and infusions	10 ⁷	10 ⁴	10 ²	Absent
	Plant materials for internal use	10 ⁵	10 ³	10	Absent
NSF/ANSI	Botanicals ingredients, non-extract	10 ⁷	10 ⁴	10 ²	Absent

* CCRAS- Central Council of Research in Ayurvedic Sciences, Ministry of AYUSH, Govt. of India; USP- United States Pharmacopoeia; BP- Brazilian Pharmacopoeia; EP- European Pharmacopoeia; AHPA- American Herbal Products Association; WHO- World Health Organization; NSF/ANSI- National Science Foundation/American National Standards Institute; NA- Not Assigned

2.2.2 Biochemical tests for *E. coli* and *Salmonella*

identification of bacterial species based on biochemical activities.

For further identification of bacteria, the biochemical tests were performed immediately after the morphology observation of the microorganisms. These tests were used for the

- i) Catalase test was performed by transferring a bacterial colony to a drop of 3% H₂O₂ on a glass slide. The catalase test facilitates the detection of the enzyme

catalase in bacteria. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase accelerates the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2 + Catalase \rightarrow 2H_2O + O_2$). This reaction is evident by the rapid formation of bubbles (effervescence) [21].

- ii) Oxidase test assesses the presence of cytochrome oxidase, an enzyme also called indophenol oxidase by reducing colourless reagent to an oxidized coloured product [22]. During bacterial respiration, the electron transport chain may involve the use of the enzyme cytochrome oxidase, which catalyses the oxidation of cytochrome c while reducing oxygen to form water. The oxidase test often uses a reagent tetramethyl-p-phenylenediaminedihydrochloride (TMPD) as an artificial electron donor for cytochrome-c [23] which is oxidized by cytochrome-c, and it changes from colourless to a dark blue or purple compound indophenol blue [24]. Microorganisms are oxidase-positive when the colour changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase-positive when the colour changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the colour does not change or it takes longer than 2 minutes.
- iii) Indole test was performed to determine the ability of the microorganism to convert tryptophan into indole. The indole test displays the ability of an organism to degrade the amino acid tryptophan and produce indole, a by-product of tryptophan metabolism. When the bacterial colony is incubated in a tryptophan medium, the tryptophanase enzyme of bacteria deaminates tryptophan and releases indole. The presence of indole demonstrates that an organism can degrade tryptophan. Detection of indole relies upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) under acidic conditions and produces the red dye rosindole. A small amount of bacterial colony was inoculated in tryptophan broth and incubated at 37 °C for 24-28 hours. After incubation, Kovac's reagent was added and a colour change to pink was observed.
- iv) Methyl red test was performed to determine fermentation of glucose by

bacteria that produces several organic acids. The bacterial colonies were inoculated in MR-VP (Methyl Red/Voges-Proskauer) broth and incubated at 37°C for 24 hours. After incubation, a pH indicator (methyl red) was added and a colour change to red was observed. This test is based on the detection of acidic metabolic by-products of carbohydrate metabolism. All members of *Enterobacteriaceae* are glucose fermenters.

- v) Citrate utilization test uses Simmons citrate agar which is a defined medium to determine an organism that can use citrate as its carbon source. A slant was prepared with inoculation of a small amount of bacterial colony and then incubated at 37 °C for 48 hours. An appearance of Prussian blue colour was observed. This test is used for the differentiation of members of *Enterobacteriaceae* based on citrate utilization as the sole carbon source. Citrate is the sole source of carbon in the Simmons citrate medium which consists of magnesium sulphate, ammonium dihydrogen phosphate, dipotassium phosphate, sodium citrate, sodium chloride, agar and bromothymol blue (pH indicator). Ammonium dihydrogen phosphate and sodium citrate serve as the sole nitrogen and carbon source respectively. When these salts are metabolized by the bacteria, the medium becomes alkaline which was indicated by a change in colour of the pH indicator from green to Prussian blue.

3. RESULTS AND DISCUSSION

3.1 Total Bacterial Load

The results of the bacterial load of the different samples of Safed Musli are presented in Table 2. The samples showed a total aerobic bacterial count in the range from 4.6×10^3 to 2.6×10^5 CFU/g (Fig. 2). The minimum value of total aerobic bacterial count was shown by sample-6 and the maximum count was indicated by sample-1. The total aerobic count was under prescribed limits according to the various standards set by CCRAS, USP, BP, EP, AHPA, WHO and NSF/ANSI (Table 1).

Since Safed Musli is one of the most widely used plants in various herbal formulations and food supplements, we assessed the total bacterial load and presence of the two most common food

pathogens, *E. coli* and *Salmonella* in the roots of this plant. The acceptable total aerobic microbial load in raw unprocessed herb for preparation of HM ranges from 10^5 - 10^7 as per different pharmacopoeia and regulatory agencies (Table 1). The total aerobic bacterial load in 10 samples in Safed Musli in this study ranged from 4.6×10^3 - 2.6×10^5 CFU/g. The findings indicated that the bacterial load is within the permissible limits. Safed Musli is harvested in September- October in the Bundelkhand area. After the monsoon season, there is a brief span of dry and hot weather, before the onset of winter. This is the best weather for peeling and drying Safed Musli. The climate provides an excellent opportunity for sun drying of Safed Musli, a natural and cost-effective method. In a previous study from India, total aerobic microbial counts of 2.5×10^5 CFU/g has been reported in the roots of Safed Musli from Haridwar, Uttarakhand [25].

3.2 Detection of Coliforms in Safed Musli

ECD MUG Agar was used for the quantification of total coliforms in the samples. This is a rapid assay to detect *E. coli* by incorporating 4-methylumbelliferyl- β -glucuronide (MUG) into

lauryl tryptose broth. When grown in this medium, *E. coli* produced the enzyme glucuronidase that hydrolysed MUG to yield a fluorogenic product that was detectable under a long wave (366 nm) UV light. Sample no. 3 and 9 have shown the presence of fluorogenic product yielded by *E. coli* under UV light (Fig. 3) and their CFU was observed in the range from 6.5×10^3 to 8×10^4 .

3.3 Detection of *Enterobacteriaceae* in Safed Musli

Salmonella Differential Agar was used for the identification of *Salmonella* species. This media has two parts A and B. Part A consisted of peptone, yeast extract, sodium deoxycholate, B. C. indicator, agar and Part B was propylene glycol. When samples were cultured in this medium, pink to red coloured colonies were observed that indicated the production of acid from propylene glycol which combined with the pH indicator and gave typical pink-red colour. It showed the presence of *Salmonella* species in samples no. 3 and 9 (Fig. 4) and CFU have fallen within the range of 5×10^4 to 6×10^4 .

Table 2. Total aerobic bacterial count of Safed Musli

S. No.	Samples	Total number of Aerobic Bacterial Count (CFU/g)	Fig No. 2
1	Sample-1	2.6×10^5	Plate 1
2	Sample-2	6.6×10^4	Plate 2
3	Sample-3	6.8×10^4	Plate 3
4	Sample-4	3.1×10^4	Plate 4
5	Sample-5	1.1×10^4	Plate 5
6	Sample-6	4.6×10^3	Plate 6
7	Sample-7	1.9×10^5	Plate 7
8	Sample-8	2.2×10^5	Plate 8
9	Sample-9	7.0×10^4	Plate 9
10	Sample-10	1.5×10^4	Plate 10

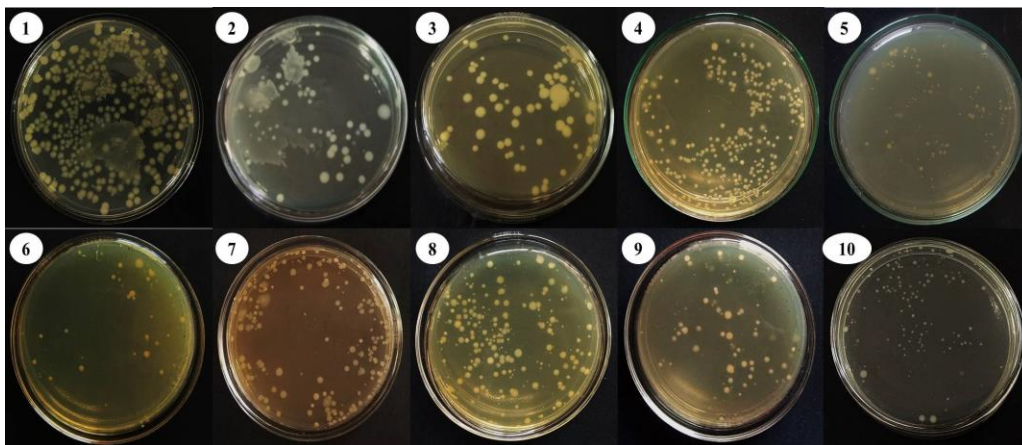


Fig. 2. Nutrient agar plates showing total aerobic bacterial growth

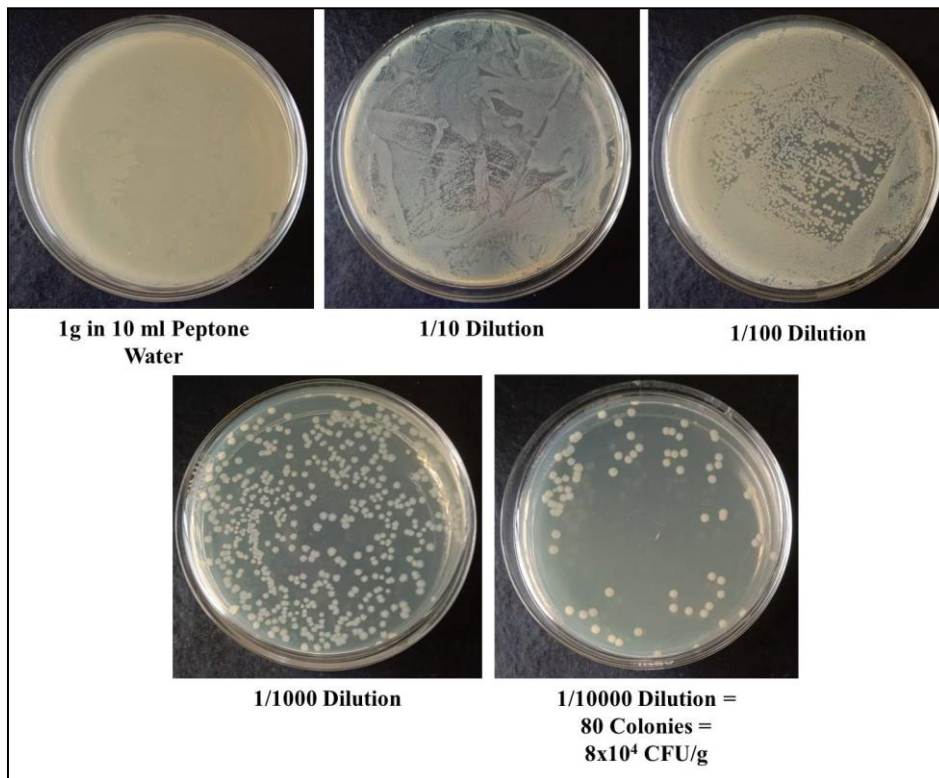


Fig. 3. Bacterial growth from Safed Musli on ECD MUG Agar

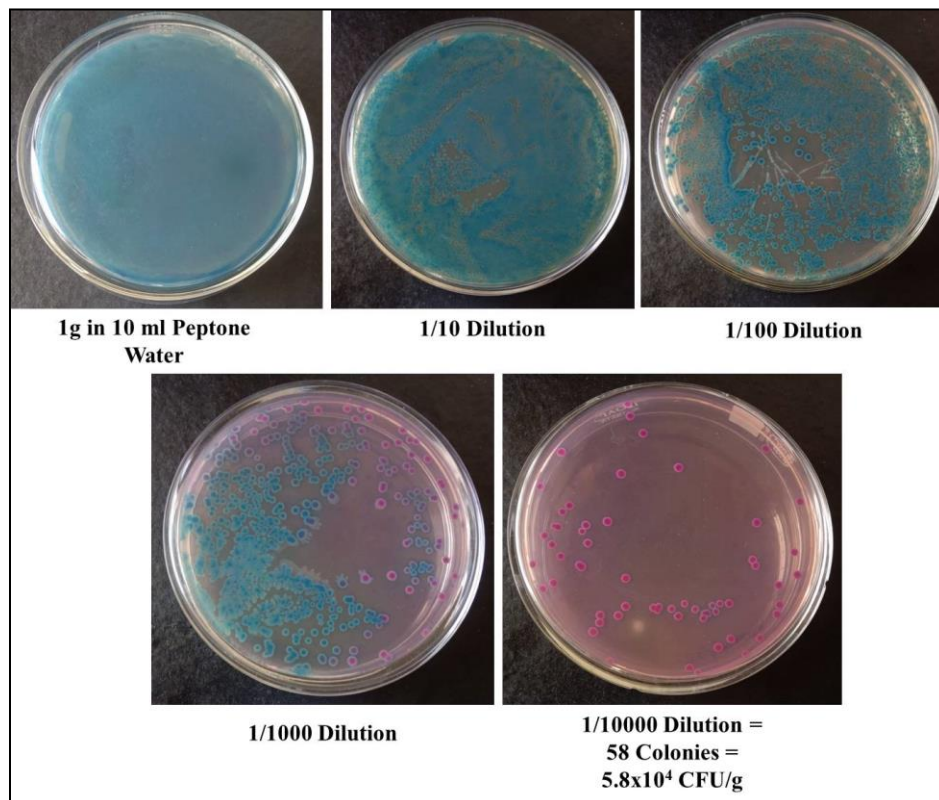


Fig. 4. Bacterial growth from Safed Musli on *Salmonella* differential agar

3.3.1 Biochemical identification of *Escherichia coli* and *Salmonella* in Safed Musli

- i) Catalase Test: Both *E. coli* and *Salmonella* formed bubbles when their colonies were picked and treated with 3% H₂O₂ (Fig. 5). Out of 10 samples of Safed Musli, two samples (Sample 3 and 9) showed effervescence, confirming the presence of *E. coli* and *Salmonella* (Table 3).
- ii) Oxidase Test: *E. coli* and *Salmonella* are oxidase negative bacteria. A smear of bacterial colonies from ECD MUG agar plates and *Salmonella* differential agar plates was prepared on filter paper and soaked in 1% TMPD. There was no colour change observed in samples 3 and 9 confirming the presence of *E. coli*. Also, samples 3 and 9 showed no colour change confirming the presence of *Salmonella* (Table 3).
- iii) Indole Test: Bacterial colonies from ECD MUG agar and *Salmonella* differential agar plates were picked, inoculated and incubated in tryptophan medium at 37°C for 24 hours. On addition of Kovac's reagent to the culture tubes after incubation, samples 3 and 9 showed a formation of a pink colour ring (Fig. 5) which detected the presence of *E. coli* and samples 3 and 9 had no appearance of the pink coloured ring, thus showed the presence of *Salmonella* in the samples (Table 3).
- iv) Methyl Red (MR) Test: When one drop of methyl red, a pH indicator was added to the test medium at the end of the period of incubation, samples 3 and 9 have shown a

colour change to distinct red which indicated the positive test for *E. coli*. Also, samples 3 and 9 gave a positive test for *Salmonella* with the appearance of red colour (Table 3). Whereas the yellowish orange colour indicated the negative test for *E. coli* and *Salmonella* (Table 3).

- v) Citrate Utilization Test: *E. coli* was found to be citrate negative and *Salmonella* was citrate positive by giving Prussian blue colour (Fig. 5). Samples 3 and 9 were found to be citrate negative for *E. coli* and samples 3 and 9 have given positive citrate test for *Salmonella* (Table 3).

Sample no. 3 and 9 have shown contamination of *E. coli* and *Salmonella*. Most of the pharmacopoeia and regulatory agencies have recommended that there should be no contamination with *E. coli* and *Salmonella*, however European Pharmacopoeia, WHO and American National Standards Institute have set acceptable limits of 10³, 10⁴, 10² CFU/g of *E. coli* respectively, in raw drugs. As CCRAS, Ministry of AYUSH, India standards, *E. coli* and *Salmonella* should be absent (Table 1). Cross-contamination of medicinal herbs and spices with excrements of animals or humans may be responsible for the presence of the genus *Salmonella typhimurium*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Yersinia enterocolitica*, and *E. coli* [26]. The presence of *E. coli* and *Salmonella* in 20% of the samples was probably due to the unhygienic collection, transportation or post-harvest processing of the Safed Musli roots. These findings demonstrate the need for surveillance and the establishment of proper control procedures in the production, preparation of these herbal products.

Table 3. Presence of *E. coli* and *Salmonella* observed through biochemical tests

S. No.	Sample	<i>E. coli/Salmonella</i>				<i>E. coli</i>	<i>Salmonella</i>	
		Catalase Test	Oxidase Test	Indole Test	Methyl Red Test			
1	Sample 1	-/-	+/+	-/+	-/-	+/-	Absent	Absent
2	Sample 2	-/-	+/+	-/+	-/-	+/-	Absent	Absent
3	Sample 3	+/+	-/-	+/-	+/+	-/+	Present	Present
4	Sample 4	-/-	+/+	-/+	-/-	+/-	Absent	Absent
5	Sample 5	-/-	+/+	-/+	-/-	+/-	Absent	Absent
6	Sample 6	-/-	+/+	-/+	-/-	+/-	Absent	Absent
7	Sample 7	-/-	+/+	-/+	-/-	+/-	Absent	Absent
8	Sample 8	-/-	+/+	-/+	-/-	+/-	Absent	Absent
9	Sample 9	+/+	-/-	+/-	+/+	-/+	Present	Present
10	Sample 10	-/-	+/+	-/+	-/-	+/-	Absent	Absent

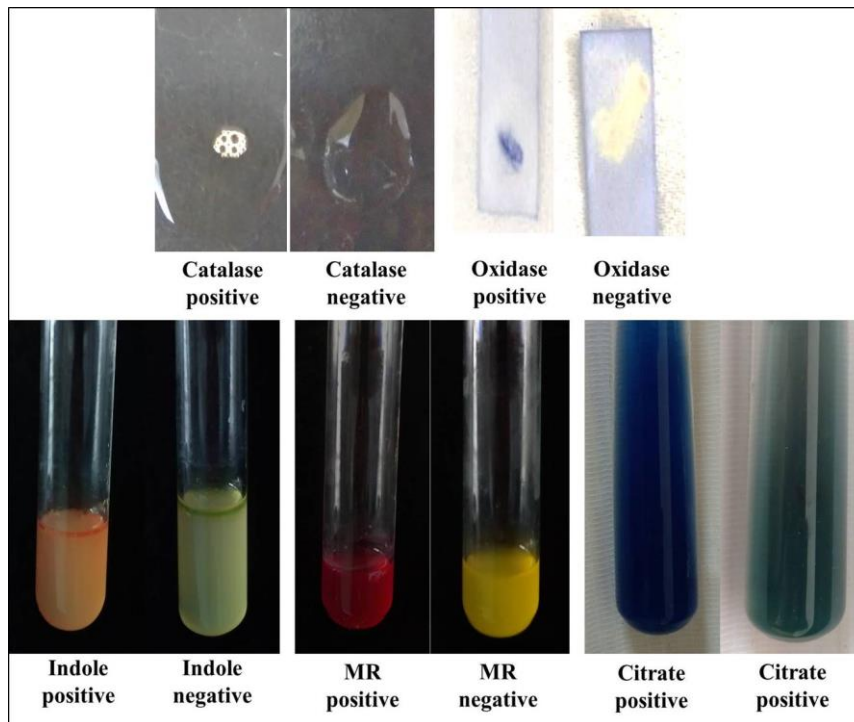


Fig. 5. Biochemical tests showing results of *E. coli* & *Enterobacteriaceae*

4. CONCLUSION

There is a need for evidence-based traditional medicine to provide suitable, safe, non-toxic and effective treatments. The quality assessment of the medicinal plant raw material is the prerequisite for HM preparations. Microbial contamination can be a prime reason for the impaired performance of the product. It can lead to the modification of physical characteristics and also, inactivation of the active ingredients in the formulation. The quality of soil and water greatly influences the bacteriological quality of raw materials which in turn affects the entire quality of herbal preparations. There may be a deficiency of basic sanitation practices associated with the management of human and animal waste assimilated into the soil or the use of contaminated water for irrigation during the cultivation of medicinal plants. Although various methods of sterilization of the herbal raw material are available, however, every method has its advantages and disadvantages. Hence prevention of microbial contamination during cultivation and post-harvest processing is the best alternative.

The aerobic bacteria load in the samples of Safed Musli was within the permissible limit set by various pharmacopoeia and other regulatory

agencies. The agricultural practises and a brief spell of dry environment between rainy season and onset of winter in the Bundelkhand is suitable for peeling and drying of Safed Musli roots. The *E. coli* and *Salmonella* in 02 samples may be due to unhygienic collection, transportation or post-harvest processing by the farmers. However, the *E. coli* and *Salmonella* bacteria load in these samples were within the acceptable limits of different regulatory agencies. The study indicated that the microbial quality assessment of Safed Musli produced in the Bundelkhand region is suitable for the preparation of HM, but it is necessary to define adequate measures of hygienic-sanitary control to guarantee the quality and safety of herbal products during collection, storage and handling.

DISCLAIMER

The products used for this research are commonly and predominantly used in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but the advancement of knowledge. Also, the research was not funded by the producing company.

ACKNOWLEDGEMENTS

The work was supported by a grant from the Department of Biotechnology, Govt. of India (No. BT/PR22867/SPD/24/784/2017) to Rambir Singh. Mansi Shrivastava was supported by a fellowship from the same grant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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