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Evaluation of Microbial Load of Herbal Raw Materials: a Necessary Quality Control Measure to Ensure Safety of Finished Herbal Preparations

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Abstract

Background: Selection of herbal raw material plays a vital role in developing herbal medicines. Various factors that affect the quality of herbal raw material include storage conditions, humidity, microflora and hygiene status of the people handling them. Various microorganisms that contaminate the herbal raw material be it roots, leaves, stems, rhizomes include pathogenic fungi, Gram positive and gram negative bacteria.

Method: We describe a simple and an effective method that involves extraction of microbes from the herbal raw material pulverized powder within 10 minutes and estimation of microbial counts by conventional plating method and using Soleris®.

Findings: Various types of organisms identified in different plant materials using Hi-Chrome Agar include *Klebsiella*, *Salmonella and Pseudomonas Spp* and some plant materials showed the presence of Proteus and *Enterococcus Spp.* The microbial load represented as cfu/g of various plants tested in this paper supports the reported values estimated by other methods.

Interpretation: A simple, rapid and a cost-effective method for estimation of microbial load in herbal raw materials is described for the first time. The method is proven to be robust and correlated well by conventional plating method as well as through the use of Soleris® instrument for the first time.

Keywords: Raw material; Liquorice; Phytomedicines; Hi-Chrome Agar; Soleris®

Introduction

Herbal medicines also called as botanical medicines or phytomedicines refers to herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or whole plant as active ingredients for the betterment of health in humans [1]. Although the use of herbal drugs dates back to nearly 60,000 years, in the last decade, the interest and emphasis on the use of herbal medicines has increased due to observed drug resistance of infectious pathogens against used antimicrobial agents and also due to considerable side effects of the used synthetic drugs and their cost of treatment [2]. Nearly 80% of people worldwide rely on herbal medicines for some part of primary healthcare and such Complementary and Alternative Medicines (CAMs) are becoming popular in countries such as UK, rest of Europe, as well as in North America and Australia [3]. Although, the use of herbal extracts as drugs and health supplements have shown promising potential to treat human diseases with the efficacy of a good number of herbal products clearly established, many of them remain untested and not monitored adequately due to lack of suitable quality controls etc. [4].

The microbial contamination in herbal products could be due to microbes coming from leaves, stems, flowers, seeds, roots, rhizhomes from which herbal extracts are prepared. Since the plant materials are procured in tonnes, sometimes merely doing microbial estimation in end products cost heavily for the manufacturing plants, hence estimation of microbial load in raw materials is a critical parameter to define the quality of raw material to be used for manufacturing of extracts. This will minimize wastage of time and cost and make the COGS attractive for all markets. Also, during handling and processing the raw

material can get contaminated with airborne bacterial and fungal pathogens; hence there is a need to verify the presence of bacterial and fungal pathogens to avoid the health risk during drug development.

The presence of endophytic microorganisms particularly bacteria and fungi in medicinal plants are reported to enhance the production of pharmacologically active secondary metabolites [5]. However, some of the microbes also contaminate the raw material and create problem in developing herbal drugs. Some of the contaminating pathogens include fungi such as *Alternaria alternata*, *Fusarium solani*, *Colletotrichum gloeosporioides*, *Zythiostroma pinastri*, *Phomopsis elaeagni*, *Verticillium dahlia*, *Cercospora traversiana* and *Puccinia coronate* [6] while the most commonly isolated bacteria from the herbal medicines include *S. aureus* (49.2%), *Salmonella spp*. (34.8%), *E. coli* (25.8%), and *P. aeruginosa* (14.4%) making them unfit for consumption [7]. Fifteen bacterial contaminants have been identified in a selected medicinal plant in South Africa, the most recurrent being *Pantoea sp*. and five strains of *Bacillus spp*. (non-pathogenic) [8]. Nearly 80% of the population in developing countries use traditional herbal medicines as part of their primary health care [9-11]. The health status of elderly consumers are more affected by the presence of microbial contaminants in herbal products due to their immunocompromised conditions, hence steps to minimize such adverse events is critical. Most of the global consumers consider herbal products to be safe, although toxic heavy metals and microbial contamination in finished herbal products have been a concern [12]. The general guidelines of ayurvedic formulations as prescribed in ASU Pharmacopoeias have specified microbial limits with the total absence of *Staphylococcus aureus*, *Salmonella Spp, Pseudomonas aeruginosa* and *Escherichia coli* and the total microbial plate count (TPC) to be in the range of 10^5/g, 10^7/g (for topical use) and the total yeast & mold the count to be ~ 10^3/g. In the light of the above facts, we have attempted to evaluate microbial load in raw material stage by a simple method of extraction and report that the estimation of microbial load in herbal raw materials described is simple, sensitive, reproducible and applicable to all forms of the plant material and is robust. We also identified the types of bacteria that contaminate such raw materials so that removing them from the raw material can be thought of and also one could ascertain if the nature of such bacteria are pathogenic or non-pathogenic.

Material and Methods

Reagents and Chemicals

NF-TVC vials were procured from NEOGEN, Lansing, USA and Soyabean Casein Digest Agar from HiMedia Laboratories, Mumbai, India. All other reagents were of analytical grade, unless mentioned otherwise.

Raw material collection

Commercially available raw materials of plant origin were procured from Production Unit, SAVA Healthcare Ltd., Malur, Karnataka, India. Different plant parts of medicinal plants were selected viz leaves, roots, rhizome, fruit rinds, fruits and whole plant based on the reported therapeutic value from different plants for the enumeration of microbial load.

Sample preparation for microbial analysis

The method followed is given in Figure 1. In brief, one gram of the finely ground raw material was suspended in 19 ml of sterile saline solution and mixed thoroughly by vortexing for 5-10 minutes. The resulting suspension was centrifuged at 2000 rpm at RT to remove debris of raw material and supernatant was used for microbial analysis.

Microbial analysis by conventional plating method

The Total Viable Count (TVC) in the supernatant was determined by conventional method using pour plate technique as per standard US FDA BAM method [13]. Briefly, one ml of supernatant was transferred to 9 ml of sterile saline solution in screw capped tubes and mixed well by vortexing to get 10^1 dilutions. One ml of the previous dilution was transferred to fresh 9 ml sterile saline solution to obtain 10^2 dilutions. Likewise, supernatant was diluted up to 10^6 dilutions. One ml of each dilution was transferred aseptically to separate sterile petri plates and 20-25 ml of molten sterile soybean casein digest agar was poured. Agar plates were allowed to solidify and then incubated at 35 °C for 48 h. After incubation, plates were observed for colonies and the numbers of colonies were counted to enumerate microbial load (cfu/gm) of raw material.

Microbial analysis by Soleris®

One gram finely ground raw material was suspended in 9 ml of sterile saline solution and mixed thoroughly by vortexing for 5 -10 minutes and then centrifuged at 2000 rpm to remove debris of raw material and supernatant was used for microbial analysis using Soleris® Instrument, Lansing, USA. The dilutions were prepared by adding 1 ml of supernatant to 9 ml of sterile saline solution and similarly further dilutions were prepared up to 10^7. NF-TVC vials were used for enumeration of microbial load. 1 ml of each dilution was inoculated separately in TVC vials and incubated at 35 °C in Soleris® for 24 h as per protocol given by manufacturer.

Bacterial identification using Hi-Chrome Agar and method followed

Hi-chrome universal differential medium is used for presumptive identification of various groups of bacteria on the basis of color developed by their colonies.

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One gram finely ground raw material suspended in 9 ml of saline solution and mixed by vortexing for 5-10 minutes. 50 µl of the suspension transferred in sterile petri plate and 20-25 ml of molten sterilized Hi-chrome universal differential agar medium poured in petri plates. The agar medium was allowed to solidify and then incubated at 35 °C for 18-24 h. After incubation, agar plates were observed for different colored colonies.

Results and Discussion

Figure 1 depicts the flow chart for processing of the herbal raw materials for enumeration of microbial load. Table 1 shows the herbal plants used in the study along with the plant parts that contain the active constituents for the therapeutic or nutritional value demonstrated by earlier researchers. To ensure that our described processing method does not underestimate the microbial load, we took two herbal samples namely *Salacia reticulata* roots and whole plant of *Tribulus terristris* and tested the microbial load by conventional plating method. The results indicated that irrespective of the time of extraction, the microbial values (cfu/g) remain the same. Hence for all further experiments we adhered to using 10 minutes extraction time (Table 2). For the evaluation of microbial contamination in randomly selected 12 raw materials, the total number of viable bacterial colony-forming units per gram (cfu/g) were determined (Table 3) by conventional plating method and also by using the Soleris® system from the material processed as shown in Figure 1. As evident from Table 3, the cfu values/g of the pulverized raw material is similar when tested by either of the methods.

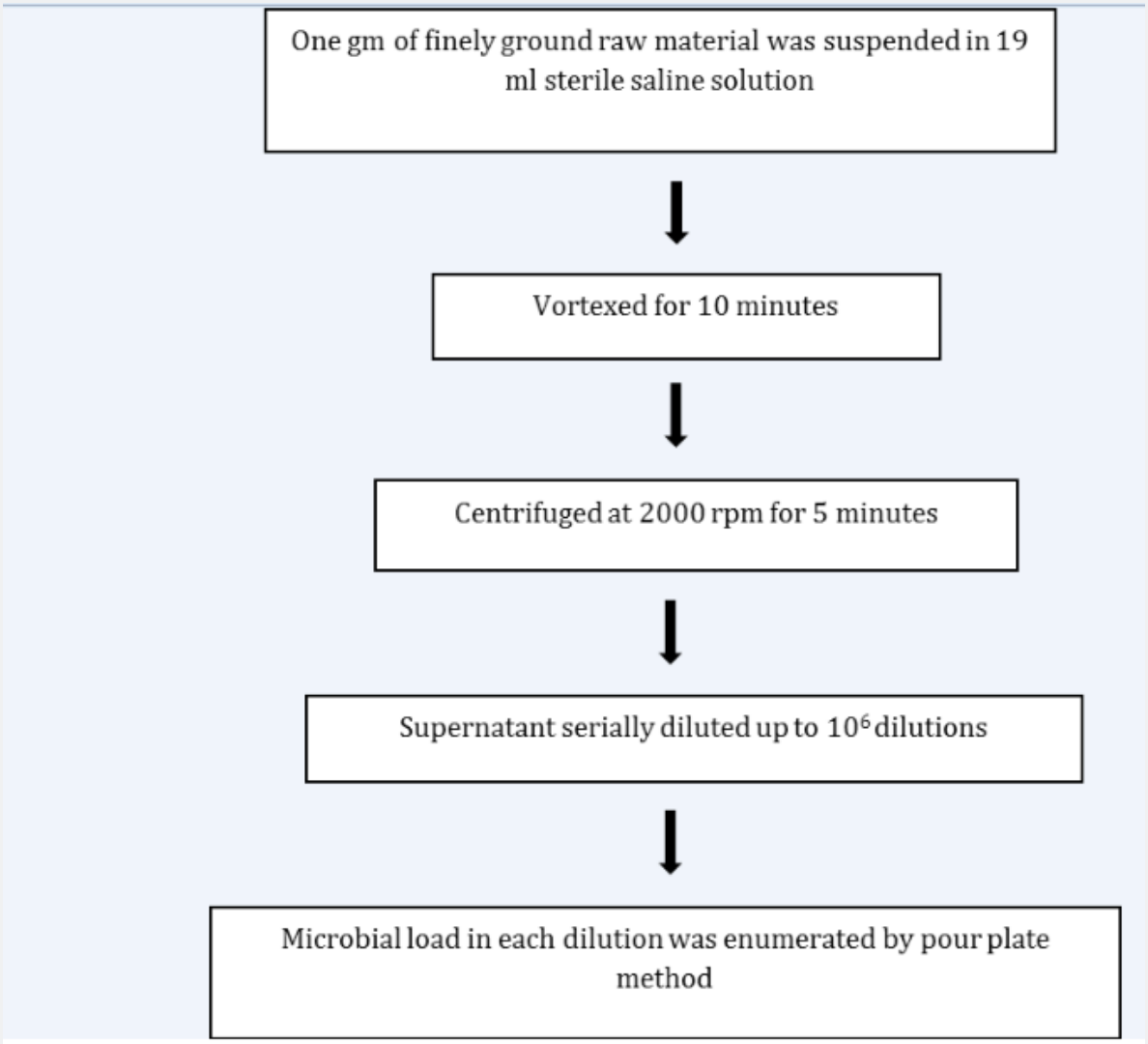

Figure 1: Flow chart for processing of herbal raw material for enumeration of microbial load.

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Table 1: Selected plants used for enumeration of microbial load.

Sr. No.	Name of Plant	Botanical Name	Plant Part used
1	Gurmar	*Gymnema sylvestre*	Leaves
2	Shevaga	*Moringa oleifera*	Leaves/stem
3	Licorice	*Glycyrrhiza glabra*	Roots
4	Kutki	*Picrorhiza kurroa*	Roots
5	Salacia	*Salacia reticulata*	Roots
6	Ashwagandha	*Withania somnifera*	Roots
7	Jatamansi	*Nardostachys jatamansi*	Roots
8	Gokhru	*Tribulus terrestris*	Whole plant
9	Brahmi	*Bacopa monnieri*	Whole plant
10	Green Chiretta	*Andrographis paniculata*	Whole plant
11	Amla	*Phyllanthus emblica*	Fruits rinds
12	Fenu-greek	*Trigonella foenum-graecum*	Fruits

Table 2: Effect of extraction time on microbial load of raw material.

Sr. No.	Raw material	Time used for extraction (min)	Microbial load (cfu/g)
1	*Salacia reticulata roots*	10	3.6×10^5
		30	1.90×10^6
		60	1.78×10^6
2	*Tribulus terrestris* whole plant	10	7.6×10^6
		30	8.2×10^6
		60	7.6×10^6

Table 3: Enumeration of microbial load of raw materials by conventional method and Soleris.

Sr. No.	Botanical Name	TVC by conventional Method			TVC by Soleris®		
		Dilution Factor	No. of colonies	cfu/gm	Dilution Factor	Detection Time (hr)	cfu/gm
1	*Salacia reticulata*	10^1	TNTC	3.6×10^5	10^5	9.2	$<10^6$
		10^2	133				
		10^3	18		10^6	ND	
		10^4	3				
2	*Picrorhiza Kurroa*	10^1	15	3.0×10^3	10^3	14.9	$<10^4$
		10^2	1				
		10^3	Nil		10^4	ND	
3	*Glycyrrhiza glabra*	10^1	TNTC	1.3×10^5	10^5	13.2	$\geq 10^6$
		10^2	65				
		10^3	5		10^6	13	
		10^4	Nil				
4	*Moringa oleifera*	10^1	TNTC	1.62×10^5	10^5	ND	$<10^5$
		10^2	81				
		10^3	5		10^6	ND	
		10^4	Nil				

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5	*Andrographis paniculata*	10^1	TNTC	6.4 x 10^5	10^5	17	<10^6
		10^2	TNTC				
		10^3	32		10^6	ND	
		10^4	3				
6	*Nardostachys jatamansi*	10^1	161	4.2 x 10^4	10^4	11.3	<10^4
		10^2	21				
		10^3	5		10^5	ND	
		10^4	Nil				
7	*Withania somnifera*	10^1	41	8.2 x 10^3	10^3	18.7	≥10^4
		10^2	9				
		10^3	1		10^4	17.2	
		10^4	Nil				
8	*Tribulus terrestris*	10^2	TNTC	7.6 x 10^6	10^6	15.4	≥10^7
		10^3	TNTC				
		10^4	38		10^7	23.6	
		10^5	4				
9	*Bacopa monnieri*	10^1	TNTC	1.96 x10^5	10^5	8.8	<10^6
		10^2	98				
		10^3	7		10^6	ND	
		10^4	1				
10	*Gymnema sylvestre*	10^1	TNTC	1.08 x10^5	10^5	3.2	<10^5
		10^2	54				
		10^3	6		10^6	ND	
		10^4	Nil				
11	*Phyllanthus emblica*	10^1	56	3.0 x 10^4	10^4	ND	<10^4
		10^2	15				
		10^3	3		10^5	ND	
		10^4	Nil				
12	*Trigonella foenum-graecum*	10^1	17	3.4 x 10^3	10^3	16.3	<10^4
		10^2	Nil				
		10^3	Nil		10^4	ND	
		10^4	Nil				

Also, we compared our microbial load values obtained for some of the raw materials with the values reported in the literature (Table 4). It is clear that for *Salacia, Moringa*, *Glycyrrhiza, Nardostachys*, *Withania*, *Andrographis*, *Trigonella* raw materials, our cfu/g values match the values reported by other workers indicating that the described method for estimation of microbial load in herbal raw materials is fast, robust and reproducible [14-17]. The organisms that have been identified from all the 12 raw materials are shown in supplementary Figure S1. At the end of the incubation period, pathogenic bacterial isolates were preliminarily characterized by colony morphology, Gram staining, and Hi Chrom agar. Hi-Chrome universal differential medium is employed for presumptive identification of various groups of bacteria on the basis of color developed by their colonies. These colors of colonies are developed due to reactions of genus or species specific enzymes with the two chromogenic substrates incorporated in the medium. While *Enterococci spp*. produces a ß-glucosidase enzyme that cleaves the chromogenic substrates resulting in the formation of bluish colonies, *Escherichia coli* produces ß-galactosidase which specifically cleaves the other chromogenic substrate resulting in the formation of purple-colored colonies. The peptone in the medium is the source of aromatic amino acids like phenylalanine and tryptophan that can be utilized by bacteria of genus *Proteus, Providencia and Morganella*, exhibits

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deaminase activity which results in the formation light pink to brown colored colonies. The *Salmonella* genus does not utilize any of these substrates, hence appear colorless while *Staphylococcus* appear golden yellow. Table 4 gives the various types of organisms identified in all the 12 extracts tested in this study.

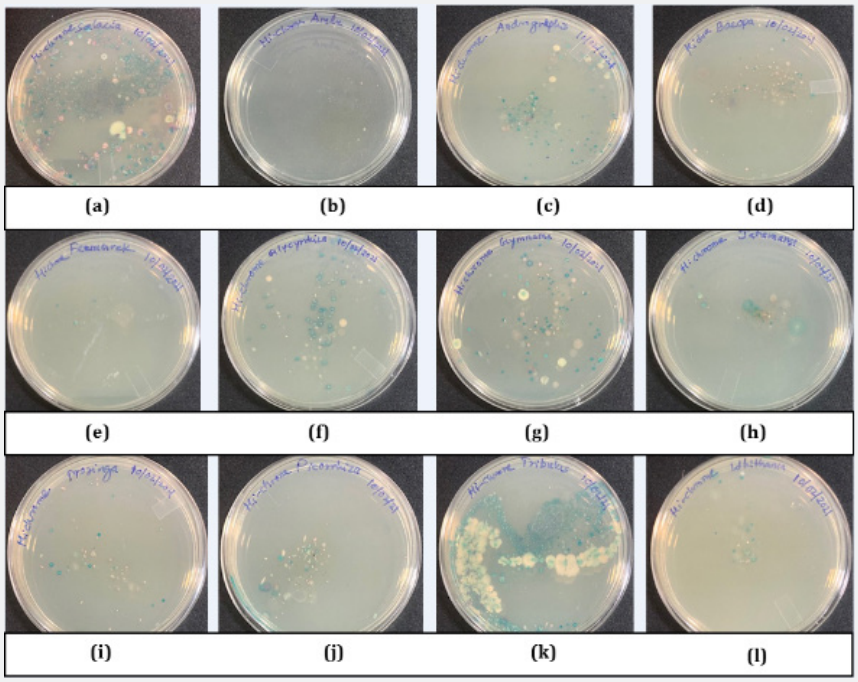

Figure S1: Differentiation of microbial load of raw materials using Hi-Chrome universal differential agar medium

Panel [a] *Salacia reticulata*; panel [b] *Phyllanthus emblic*; panel [c] *Andrographis paniculata*; panel [d] *Bacopa monnieri*; panel [e] *Trigonella foenum-graecum*; panel [f] *Glycyrrhiza glabra*; panel [g] *Gymnema sylvestre*; panel [h] *Nardostachys jatamansi*; panel [i] *Moringa oleifera*; panel [j] *Picrorhiza kurroa;* panel [k] *Tribulus terrestris*; and panel [l] *Withania somnifera*.

Table 4: Comparative data of microbial load of different herbal raw materials.

S.N.	Plant	Microbial load in raw materials (cfu/gm)		Reported in Literature	
		Conventional Method	Soleris®	Microbial load (cfu/g)	Reference
1	*Salacia reticulata*	3.6 x 10^5	<10^6	10^5	Kumar et al (2015)
2	*Picrorhiza kurroa*	3.0 x 10^3	<10^4	Not available	Not available
3	*Glycyrrhiza glabra*	1.3 x 10^5	≥10^6	10^4 to 10^7	Agarwal et al [21]
4	*Moringa oleifera*	1.62 x 10^5	<10^5	10^5 to 10^8	Adu-Gyamfi and Mahami, 2014
5	*Andrographis paniculata*	6.4 x 10^5	<10^6	4.4 x 10^5	G. Khandelwal et al. 2013
6	*Nardostachys jatamansi*	4.2 x 10^4	<10^4	6.4 x 10^5	Jha et al [15]
7	*Withania somnifera*	8.2 x 10^3	≥10^4	>10^5	Nur et al [14]
8	*Tribulus terrestris*	7.6 X 10^6	≥10^7	Not available	Not available
9	*Bacopa monnieri*	1.96 x 10^5	<10^6	Not available	Not available
10	*Gymnema sylvestre*	1.08 x 10^5	<10^5	Not available	Not available
11	*Phyllanthus emblica*	3.0 x 10^4	<10^4	6 x 10^2	Nur et al [14]
12	*Trigonella foenum-graecum*	3.4 x 10^3	<10^4	10^3-10^7	Peles et al [16]
				3.3 x 10^2	Nur et al [14]

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Microbial contamination in pharmaceuticals cause changes in their physical characteristics like turbidity or deposit, and changes in odor and color and also its quality like breaking of emulsions, thinning of creams, fermentation of syrups etc. Hence, bioburden which is the total number of microorganisms present on a product prior to sterilization and its estimation in raw materials and finished pharmaceutical products determines if the product complies with the requirements of the US Pharmacopeia. While such aspects are well recognized in allopathic medicines, there is currently no practice in estimation of bioburden in herbal raw materials and hence this article disclosing the significance of such estimation assumes critical importance. Herbal raw materials such as spices require strict control to prevent spoilage, the major causes of spoilage being improper handling, growth of spoilage microorganisms, action of naturally occurring enzymes, chemical reactions and structural changes during storage [18]. The presence of fungi in herbal preparations under certain conditions may lead to the secretion of toxic metabolites such as mycotoxins, which when ingested, inhaled, or absorbed through the skin cause illness or human and animal death [19] Table 5.

Table 5: Presumptive identification of microbial load of raw materials using Hi-Chrome universal differential agar medium

S. N.	Raw material	Type of colonies observed	Identified bacterial genus/species
1	*Gymnema sylvestre*	Light blue, bluish green and white	*Kliebsella pneumoniae, Salmonella Enterococcus spp. and Pseudomonas spp.*
2	*Moringa oleifera*	Bluish green, white and light pink	*Kliebsella pneumoniae, Salmonella Enterococcus spp. and Pseudomonas spp.*
3	*Glycyrrhiza glabra*	Bluish green and white	*Kliebsella pneumoniae, Salmonella spp. and Pseudomonas spp.*
4	*Picrorhiza kurroa*	Light blue, bluish green and white and light pink	*Kliebsella pneumoniae, Salmonella Enterococcus spp. Proteus spp. and Pseudomonas spp.*
5	*Salacia reticulata*	Light blue, bluish green and white, purple and light pink	*Kliebsella pneumoniae, Salmonella, Escherichia coli Enterococcus spp. Proteus spp. and Pseudomonas spp.*
6	*Withania somnifera*	Bluish green and white	*Kliebsella pneumoniae, Salmonella spp. and Pseudomonas spp.*
7	*Nardostachys jatamansi*	Bluish green, light blue and white	*Kliebsella pneumoniae, Salmonella Enterococcus spp. and Pseudomonas spp.*
8	*Tribulus terrestris*	Light blue, bluish green and white and light pink	*Kliebsella pneumoniae, Salmonella Enterococcus spp. Proteus spp. and Pseudomonas spp.*
9	*Bacopa monnieri*	Bluish green and white	*Kliebsella pneumoniae, Salmonella spp. and Pseudomonas spp.*
10	*Andrographis paniculata*	Bluish green and white	*Kliebsella pneumoniae, Salmonella spp. and Pseudomonas spp.*
11	*Phyllanthus emblica*	White colonies	*pneumoniae, Salmonella spp. and Pseudomonas spp.*
12	*Trigonella foenum-graecum*	Bluish green and white	*Kliebsella pneumoniae, Salmonella spp. and Pseudomonas spp.*

WHO mentions that bacterial colony counts for medicinal plant materials intended for internal use, should not exceed 10^5 cfu/g while plant products intended for topical use should not exceed 10^7 cfu/g [10]. The limits of bacterial contamination given in European pharmacopoeia as reported by Okunlola et al. [20] are total aerobic bacteria (10^5 cfu/g), Enterobacteria and other Gram-negative organisms (10^3 cfu/g). Although many of the plant materials are below the prescribed limits, what appears important to understand that in many cases, these organisms will be carried forward to the extract and then the extract will be loaded with microbes which will be finally not suitable for human use.

The method followed by Vuuren et al. [8] for estimation of microbial load in herbal raw materials is lengthy. The steps include weighed plant sample in a beaker containing a measured volume of sterile water and such a samples is then agitated on a shaking incubator at 37 °C at 104 rpm for 1 h and then used for plating [8]. Our method involves merely 5 minutes vortexing and after a brief spin is ready for analysis. Hence, our method is simpler where the samples preparation does not take more than 10 minutes and the results are reproducible.

Agarwal et al. [21]. have attempted similar estimation but have carried out washing of the roots of licorice with water to remove

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dust and other contaminants. Later these washed materials were dried at 45 °C before taking for analysis. We have not done any such treatments and yet we are able to get values of TVC similar to what is reported [17]. The authors have observed variations in total microbial count from 10^4 /g to 10^7 /g based on regions from where they are procured. Our results on observations of presence of *Pseudomonas*, *E. coli*, *Salmonella* in Moringa are in conformity with the findings of Walia et al. [22] in a recent report where impact of such pathogens on infant diet for undernourished children has been debated. Also, bacteria such as *Klebsiella*, *Pseudomonas* have been seen in *Withania somnifera* tissue cultures by Kulkarni et al. [23] as seen by us in the raw material of *Withania* reported in this study.

There are extrinsic and intrinsic factors that determine the microbiological quality of medicinal plants. While the intrinsic factors include the inherent antimicrobial agents present in the plant and certain beneficial microbial content for plant survival and growth, the extrinsic factors such as humidity, harvest method, post harvesting treatment, packaging and storage conditions dictate the extent and nature of microbial contaminants [24]. Since in some plants such microbial contaminations affect the concentration of active constituent available in such plants [21], it becomes imperative that accurate estimation of microbial load in raw material itself would become a crucial factor for achieving purer herbal extracts with maximum therapeutic potential.

This is the first report of a method that determines the estimation of microbial load in herbal raw materials and opens up a potential possibility of using the same as a QC releasing parameter before taking up for processing for preparation of herbal extracts. Medicinal plants, used as raw materials, for production of herbal extracts for their use as food supplements and herbal medicines, may have quality and safety issues due to deposition of fungicides, pesticides and other microorganisms in them. Since globally the herbal drugs are in high demand for primary health care purposes due to their minimal side effects, their usage is preferred over modern medicines that have adverse side effects, synthetic in nature and whose long-term side effects are unknown and in several cases are expensive [25].

The global herbal medicine market size is expected to reach US$ 550 billion by 2030 as per the recent survey by Globe Newswire. Hence, it is obvious that the demand of herbal medicines is for their therapeutic value to treat disease as well as a type of dietary supplement for enhance general health and wellbeing is increasing substantially. Several herbal extracts find their use in treatment of various diseases and disorders viz. acne vulgaris [26], hypertension [27], hyperlipidemia [28], diabetes [29], cancer [30], erectile dysfunction [31], kidney [32] to name a few. Interestingly, the synergistic effect of herbal medicines with available antimicrobial agent has been studied to overcome drug resistance in several pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Cutibacterium acnes*, *Pseudomonas aeuroginosa*, *Escherichia coli*, *Bacillus cereus* and *Klebsiella pneumoniae* [33].

Bais et al. [34] report the presence of *Escherichia coli*, *Staphylococcus aureus* and *P. aeruginosa* in *Withania* extracts available in the market and conclude that the contamination is due to poor manufacturing practices and improper storage condition of formulations. Our results presented in this article, shows the presence of *E. coli* and *Pseudomonas* in the raw material of *Withania somnifera* itself indicating that the source of such organisms is the raw material and strictly not due to the manufacturing processes adopted. The microbial load in raw materials can affect the quality and efficacy of herbal medicines. So, there is a need to assess the microbial load in early stage of development of herbal medicines. In this study, microbial load of several plant materials was analyzed by conventional method and Soleris® instrument. This comparative data would be helpful to select the raw material on the basis of microbial load so that it wouldn't create problem in latter stage of herbal drug development. This is the first report on the use of Soleris® for enumeration of microbial load of herbal raw materials directly and would be useful both for food and pharmaceutical industry.

Recent work by Silviera et al. [35] and Safa et al. [36] suggest that several herbal medicines have potential use as adjuvants in the treatment of early/mild common flu healthy adults and also in the context of COVID-19 patients. Hence, any method that would help to generate quality herbal extracts would be of immense use from clinical viewpoint and in the light of this our present article is relevant to the possible additional benefit to human subjects suffering from the current ongoing COVID pandemic. Due to the immunocompromised conditions, presence of unwanted microbes in herbal preparations creates a concern of safety of consumers of herbal products and hence this aspect needs urgent attention, especially in consumers of elderly populations. Taking these facts into consideration, we believe that regulatory agencies should come forward and take the necessary measures to ensure the safety of finished herbal preparations and encourage manufactures to adapt the analysis of microbial load in herbal raw material as a routine quality check parameter/specification.

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