

## MICROBIAL EVALUATION OF A MARKETING HERBO-MINERAL FORMULATION *TRIBHUVAN KIRTI RAS*

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### ABSTRACT

Ayurvedic herbal medicines and their finished herbal products have been used since ancient times to treat a wide range of diseases. WHO survey shows 70-80% of world population rely on non-conventional medicines mainly herbal products for their primary health care. The microbial contaminations of these medicines may reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patients. *Tribhuvan Kirti Ras* is one of the commonly used herbo-mineral formulation for acute or chronic fever related with respiratory tract infections, Present study was performed to assess the pathogenic proliferation in the locally available commercial *Tribhuvan Kirti Ras*. The pathogenic load was compared

with the microbiological standard given by the WHO. The TVAC was found to be  $9.3 \times 10^4$ ,  $4.5 \times 10^4$  and  $5.8 \times 10^4$  CFU/g in three samples respectively, All three samples were found to be contaminated with the fungi species of *Glodidium*, *Papulospora*, *Geomyces* and *Rhizopus* as well as Gram positive and Gram negative bacteria. The presence of bacteria and fungi in these samples suggested that there is need to improve the quality of manufacturing and labelled the condition of product.

**KEYWORDS:** Microbial condition, *Tribhuvan kirti ras*, Herbomineral formulation.

### INTRODUCTION

Ayurvedic herbal and herbomineral products have been used since ancient times to treat a wide range of diseases.<sup>[1-3]</sup> The World Health Organization (WHO) survey indicated that about 70-80% of the world population particularly in the developing countries rely on non-conventional medicines mainly of herbal origins for their primary health care. This is because

herbal medicines are relatively accessible and cheaper than the synthetic drugs and free from resistance and hazardous side effects.<sup>[4]</sup> However, microbial contamination of these medicines may affect human health. Microbial contamination of medicinal herbal can 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. In order to improve the purity and safety of the products, observation of basic hygiene during preparation, standardization of some physical characteristic such as moisture content, pH and microbiological contamination levels are desirable.<sup>[5,6,7]</sup>

Biological contamination may involve living microbes such as bacteria and their spores, yeasts and moulds, viruses, protozoa, insects (their eggs and larvae), and other organisms. However, products of microbial metabolism such as toxic, low-molecular-weight metabolites from moulds are important chemical which originate contaminants.<sup>[8]</sup> The main microbial contamination of plant materials, in general, are attributed to total aerobic mesophilic, enterobacterial, yeast and mould.<sup>[9]</sup> With the ever increasing use of herbal medicines and the global expansion of the herbal medicines market, safety has become a concern for both health authorities and the public. This is because many contaminants and residues that may cause harm to the consumers have been reported.<sup>[10]</sup> Although bacterial endospores and fungal spores can be regarded as the two dominating groups of contaminants associated with medicinal plants, a broad diversity of bacterial, fungal cells and viruses can be found either in or on the plant material.<sup>[11]</sup> Although enterobacteria can be found in nature, this family possesses some indicative value towards faecal contamination. The presence of enterobacteria and *E. coli* reflect the situation regarding faecal contamination.<sup>[12]</sup> Together with the group of coliforms, it can be taken as an indicator for undesirable hygiene conditions, although this conclusion has to be related to the magnitude of viable count measured.<sup>[13]</sup> *Staphylococcus aureus* is not common contaminant of this type of plant material and relatively rarely found. The risk of the presence of microorganisms in a herbomineral product depends on this finality of the use, its nature and its potential damage that may be caused to the consumers. Although high fungal load may be accepted due to the natural origin of those products, they indicate the potential for spoilage and mycotoxigenesis.

There are different preparation techniques which may influence on the microbial quality. The production of an herbal medicine generally involves the steps in which a herb was subjected

to unfavourable conditions to survival of microorganisms like **Drying process** is the process of decreasing of plant moisture content, aimed at preventing enzymatic and microbial activity, and consequently preserving the product for extend shelf life.<sup>[14]</sup> The optimization of the drying process contributes to physical, chemical and microbiological stability of the medicinal herbs. **Extraction methods** Water is almost universally the solvent used to extract activity. The microbiological contamination and controlling microbial contamination can be difficult in aqueous extracts.<sup>[15]</sup> **pH influence** The pH value is one of the main factors influencing the quality of medicine. It always controls many chemical and microbiological reactions.<sup>[16]</sup> When the pH value is low (presence of acidic substances), the bacterial count could be low, but at neutral or higher pH the level of contamination of the herbal preparations could observed to be higher. This suggests that a neutral or alkaline pH favoured high contamination levels of the herbal preparations. This agrees with the observation that bacterial growth is optimal at more or less neutral pH, around pH 5-8.5.<sup>[17]</sup> **Storage** Most pre-storage processing of plant material, such as that involving drying, heat, cooling and packaging, can prevent the degradation of plant material during storage.<sup>[18]</sup>

Prolonged storage in poorly ventilated storehouse usually increases sample moisture content in the bulk due to heat exchange capacity, rendering herbs more susceptible to molds growth and toxin production. Fungi are the predominant contaminants of herbs, but most such microbial populations are probably regarded as commensal residents on the plant that survived drying and storage. Most fungi are present on plants, which develop after harvest if relative humidity is not controlled during storage.<sup>[19,20]</sup> Moulds are responsible for biodeterioration of a number of substrates including raw materials of some medicinal plants. These moulds reduce raw herbal drugs shelf life and market value. The fungal deterioration adversely affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs.<sup>[21]</sup>

The microbiological quality of medicinal products became noteworthy in 1966 when over 200 cases of salmonellosis were reported from consumption of contaminated thyroid tablets, demonstrating that microbial contamination of medications can result in clinical infection. Fungal contamination has been reported to affect the chemical composition of the raw materials and thereby decrease the medicinal potency of herbal drugs. The most prominent fungal toxins reported are aflatoxins, zearalenone, ochratoxin and patulin, which are collectively known to cause hazards to the liver, nervous system, muscular system,

respiratory organs as well as digestive and genital systems. Based on this fact, we demonstrated the bacterial and fungal population in the locally available herbal medicines. Contamination in finished product may lead to deterioration of product, loss of potency, pyrogen reactions, infection of patient with secondary spread, loss of patient compliance, inadequate drug delivery and transformation of therapeutic agents leading to alteration of pharmacological activities.

Microorganisms by virtue of their adaptive nature can degrade any kind of substrate, which may lead to product spoilage and a loss of drug efficacy. In recent years, there has been increase in the usage of herbal medicinal products and Ayurvedic herbomineral preparations for the treatment and prevention of diseases. The public awareness about the purity, microbial quality and side effects of herbal drugs is also increasing day by day. Taking in to account these factors safety, efficacy and quality of such products has acquired importance for health professionals.

According to the WHO technical guidelines for the assessment of microbial quality of herbal preparations, determination of microbiological contaminants and limit tests for total viable aerobic bacteria and fungi indicate the quality of herbal preparations.

The specification of WHO for total aerobic microorganisms are not more than  $10^7$  CFU/g for the plant material for use as teas and infusions and at most  $10^5$  CFU/g for internal use. The specification of WHO for yeasts and molds are at most  $10^4$  CFU/g for the plant material for use as teas and infusions and at most  $10^3$  CFU/g for internal use. Present study medicine *Tribhuvan Kirti Ras* is a herbomineral contemporary medicine use in large scale mainly for the respiratory ailments and available at all retail Ayurvedic shops. Maximum companies are preparing *Tribhuvan Kirti Ras* for marketing.

*Tribhuvan Kirti Ras* is one of the commonly used herbo-mineral formulation for acute or chronic fever related with respiratory tract infections, it is available in the form of tablets in the market. It contains *Shuddha Hingula* (Purified Cinnabar – Ore of Mercury), *Shuddha Vatsanabha* (Purified Aconitum ferox), *Shunti* (Ginger rhizome –Zingiber officinalis), *Maricha* (Black pepper – Piper nigrum), *Pippali* (Long pepper – Piper longum), *Tankana Bhasma* (Borax), *Pippalimoola* (Long pepper root – Piper longum) all above ingredients are finely powdered and mixed in equal quantity. Further it is processed in Juice extract of *Tulasi* (Holy Basil – Ocimum sanctum), *Dhattura* (Datura metel), *Adrak* (Wet Ginger rhizome –

*Zingiber officinalis*) in sufficient quantity three times each one after another. The dough of above mixture than parted into 125mg of tablet and dried well and packed for dispensing. The dose of *Tribhuvan Kirti Ras* is 60-125 mg with the *anupan* of *Adrak* juice+ honey; *Tulsi Patra* juice, *Bilva phant* or with any *jwaraghna kwath*, in *pittaj* condition it would be used with *praval pisthi*, *abhrak bhasma* and *makshik bhasma*. In *kaphaj* condition it can be used with *godanti bhasma*, *shrunga bhasma*, *abhrak bhasma* and *chandramruta ras*. In *dwidoshaj* or *sannipataj* condition it also used with *tankan bhasma*, *apamarg kshar* and *dashmool kwath*.<sup>[22]</sup> The pharmacological action of the *Tribhuvan Kirti Ras* is act as *Jwaraghna*(antipyretic), *Swedajanana* (induces sweating),*Vedanahara* (analgesics). It is commonly indicated in Influenza, tonsilitis, pharyngitis, laryngitis, bronchitis, pneumonia and measles like condition.

All the ingredients of *Tribhuvan Kirti Ras* are plants (herbal drugs) except *Hingula* (Purified Cinnabar) all these are collected from the market and processed to form the consumable form of medicine during this process there may be the chances of microbial contamination. Present study was designed to assess the microbiological contamination in commercial *Tribhuvan Kirti Ras* and to compare the pathogenic load with the microbiological standards for herbal preparations.

## OBJECTIVES

- 1) To determine the nature of microbial contamination of the *Tribhuvan Kirti Ras*,
- 2) To determine the extent of such contamination,
- 3) To compare the results of this study with the standards set by WHO.

## METHOD AND MATERIALS

All materials and chemical reagents were of analytical grade. Experiments were done carefully with appropriate controls for three times.

### Settings

This controlled experimental study was carried out on locally available herbomineral combination of *Tribhuvan Kirti Ras* in the department of pharmacy, of RTM Nagpur University, Nagpur, state of Maharashtra in India.

**Sampling**

Total three samples of *Tribhuvan Kirti Ras* were collected from retailer at Nagpur, state of Maharashtra in India for assessment of their microbial contamination. Sample of *Tribhuvan Kirti Ras* were collected in sterile glass beaker and special care was taken to prevent accidental contamination of the sample during its collection, transportation and processing.

**The culture media**

All the culture media were procured from Hi-Media Laboratory Pvt. Ltd., Mumbai and were proven to have no antimicrobial activity and used without any further processing or purification. Sterilization was carried out at 121°C for 15 minutes unless and otherwise specified.

**Ayurvedic herbal formulations**

The market survey was done and *Tribhuvan kirti ras* company manufactured, sold and used in India was considered for microbiological study and procured from the retail pharmacy. The different brand was randomly coded and used for microbial evaluation.

**Microbiological testing****Pre treatment of samples**

*Tribhuvan kirti ras* was subjected to grinding using a grinder, suspended and diluted in a suitable media. The antimicrobial property was eliminated by dilution and neutralization.

**a) For water soluble materials**

A 10g of grind sample was dissolved in lactose broth or buffered sodium chloride-peptone solution (in case of *P. aeruginosa* species) and the volume was made up to 100 ml with the same medium. The pH of the suspension was adjusted to 7.

**b) For non fatty materials insoluble in water**

A 10 gm of grind sample was suspended in lactose broth or buffered sodium chloride-peptone solution (in case of *P. aeruginosa* and *S. aureus*) and diluted to 100 ml with the same medium. When necessary the suspension of material was examined and homogenized mechanically. A surfactant, such as a solution of polysorbate 80 (1 mg mL<sup>-1</sup>) was added for uniform dispersion of material and the pH of the suspension was adjusted to 7.0.

**c) For fatty materials**

A 10 gm of grind sample was homogenized with 5 gm of surfactant solution of polysorbate 80 R. The suspension was heated up to 40°C with careful stirring and maintaining the temperature in a water bath, until it dissolves. To this homogenized mixture 85 ml of lactose broth or buffered sodium chloride-peptone solution was added. The solution was heated up to 40°C for 30 minutes or until an emulsion was formed. The pH of the emulsion was adjusted to 7.0.

**Dilution of pre-treated material**

A 10gm of pre treated material was diluted to 100 ml using the same medium and shaken thoroughly. 10 ml of this solution was further diluted to 100 ml using the same medium so that an expected bacterial count of solution up to 300 or fungal count up to 100 was obtained.

**Total viable aerobic count (TVAC)**

To study the TVAC, pour plate method was used.

For **bacteria**, petri dishes of 10 cm diameter were used. To each sterile dish, a mixture of 1gm of the diluted pre-treated material and 15 ml of liquefied soya bean casein agar was added when the temperature was 45°C. The petri dishes were incubated at 30-35°C for 5 days. The number of colonies obtained were counted using colony counter.

For **fungi**, petri dishes of 10 cm in diameter were used. To each dish, a mixture of 1 gm of the diluted pre-treated material and 15 ml of liquified Sabouraud dextrose agar with 0.5 ml of Gentamycin (concentration 3.2µg/ml) was added at temperature of 45°C. The petri dishes were incubated at 20-25°C for 5 days. The number of colonies was counted using colony counter. To identify various fungi, Czapek's Dox agar was also used.

**Enterobacteriaceae and certain other gram-negative bacteria  
detection of bacteria**

The pre-treated material was appropriately homogenized and incubated at 30-37°C for a length of time (2-5 hours) sufficient for revivification of the bacteria but not sufficient for multiplication of the organisms. The container was shaken and 1gm equivalent of the homogenized material was transferred to 100 ml of enterobacteriaceae enrichment Mossel (broth) and incubated at 35-37 °C for 18-48 hour. A subculture was prepared on a plate with violet-red bile agar with glucose and lactose and incubated at 35-37 °C for 18-48 hours. The



material passes the test when there is no growth of colonies of Gram negative bacteria in the plate. Gram staining was performed by routine staining method for identification of bacteria.

### Quantitative evaluation

The quantities of homogenized material prepared as described above, was diluted appropriately to form solutions containing 1.0 g, 0.1g and 0.01 g of the material being examined. The solutions were inoculated in a suitable amount of enterobacteriaceae enrichment Mossel (broth) and incubated at 35-37 °C for 24-48 hours. A subculture of each of the colony was prepared on a plate with violet-red bile agar with glucose and lactose, in order to obtain selective isolation and incubated at 35-37 °C for 18-24 hours. The growth of well developed colonies, red or reddish in colour indicated the presence of Gram-negative bacteria. The number of bacteria was determined by using following Table.

**Table- 01: Determination of enterobacteriaceae and certain other gram-negative bacteria**

Result for each quantity or volume			Probable no. of bacteria per g of material
1.0 g or 1.0ml	0.1g or 0.1ml	0.01g or 0.01ml	
+	+	+	More than 10 <sup>2</sup>
+	+	—	Less than 10 <sup>2</sup> but more than 10
+	—	—	Less than 10 but more than 1
—	—	—	Less than 1

### Detection of specific organisms

#### i. *E. coli*

A quantity of pre-treated material was homogenized in lactose broth and incubated at 35-37°C for 24-48 hours. This culture was diluted to 100 ml of Mac Conkey broth, to form solution containing 1 g of the material being examined and incubated at 43-45°C for 18-48 hours. A subculture from this broth was made on a plate with Mac Conkey agar and incubated at 43-45°C for 18-24 hours. The growth of red, non mucoid colonies (sometimes surrounded by a reddish zone of precipitation) indicated the possible presence of *E. coli*. This was confirmed by performing Gram staining and indole test.

**a) Indole test:** This test was performed in peptone water culture after 48-96 hours of incubation at 37°C. To the culture 0.5 ml of Kovac's reagent was added and shaken gently. The development of red colour indicated the presence of *E. coli*.

#### ii. *Salmonella* species

The pre-treated material was prepared and incubated at 35-37°C for 5-24 hours.



a) **Primary test:** 10 ml of the enrichment culture was transferred to 100 ml of tetrathionate bile brilliant green broth and incubated at 42-43°C for 18-24 hours. The subcultures of the sample were made separately on deoxycholate citrate agar and xylose-lysine-deoxycholate agar and incubated at 35-37°C for 24-48 hours. The secondary test was performed when any colonies produced were in confirmation to the description given in following Table.

**Table- 02: Description of *salmonella* colonies appearing on different culture media**

Medium	Description of colony
Deoxycholate citrate agar	Well developed, colourless
Xylose-Lysine-Deoxycholate agar	Well developed, red, with or without black centres

b) **Secondary test:** A subculture of any colonies, showing the characteristics described in above Table was made on the surface of triple sugar iron agar using the deep inoculation technique (first inoculating the inclined surface of the culture medium followed by a stab culture with the same inoculating needle) and incubated at 35-37°C for 18-24 hours. When a colour change from red to yellow was observed in deep culture (but not in the surface culture), it indicated the presence of *Salmonella* species.

**iii) *P. aeruginosa***

The pre-treated material was inoculated in 100 ml of soybean-casein digest broth to form a suspension containing 1 g of the material being examined and incubated at 35-37°C for 24-48 hours. A subculture from above culture was prepared on a plate of cetrimide agar and incubated at 35-37°C for 24-48 hrs. When no growth of microorganism was detected, the material passes the test. When growth of colonies with a greenish fluorescence occurs, the Gram staining was performed and oxidase test was applied. When the organism is Gram negative, oxidase test was positive, the growth in soybean casein digest medium at 42°C was tested. The material passes the test when colonies of the type described does not appear or when the confirmatory biochemical test was negative.

**iv) *S. aureus***

The pre-treated material was inoculated in 100 ml of soybean-casein digest medium to form a solution containing 1gm of the material being examined and incubated at 35-37°C for 24-48 hours. A subculture from this culture was made on Baird Parker agar and incubated at 35-37°C for 24-48 hours. The material passes the test when no growth of microorganism was detected. Black colonies of Gram-positive cocci often surrounded by clear zones indicated

the presence of *S. aureus*. For catalase positive cocci confirmation was obtained by coagulase and deoxyribonuclease tests.

**a) Coagulase test (slide coagulation test):** The black colonies of *S. aureus* from Baird Parker agar were taken on a glass slide and few drops of human plasma were added to it. The slide was observed for the coagulation.

**b) Deoxyribonuclease test:** The black colonies from the above were taken on a glass slide and few drops of para-aminodiethylaniline were added to it. The formation of deep blue colour indicated the presence of *S. aureus*.

The material passes the test when cultures of the type described do not appear or when the confirmatory biochemical tests were negative.

### Validation of the tests for specific microorganisms

The test strains listed in Table below were separately grown on the nutrient agar and incubated at 30-35°C for 18-24 hours. The portions of each of the cultures were diluted using sterile buffered sodium chloride-peptone solution pH 7, so as the test suspensions contains about  $10^3$  microorganisms per ml of suspension. Approximately  $10^2$  microorganisms of each strain was used as an inoculum of *E. coli*, *P. aeruginosa*, *S. aureus* and *Salmonella* species, in the presence of sample and in the absence of the sample (Control) being examined.

## RESULT AND DISCUSSION

**Table- 03: Test strains and culture media used in validating the tests for specific microorganisms**

Microorganisms	Strain number	Medium
<i>E. coli</i>	MTCC 443	Lactose broth
<i>P. aeruginosa</i>	MTCC 424	Soybean-casein digest medium
<i>S. typhimurium</i>	Not recommended	Lactose broth
<i>S. aureus</i>	NCTC 3750	Soybean-casein digest medium

Every experiment was performed in triplicate and average is given as result.

### Microbiological evaluation of *tribhuvan kirti ras*

#### 1. Total viable aerobic count (TAVC)

All the three sample of *Tribhuvan kirti ras* showed satisfactory results with respect to total viable aerobic count, as none of the sample showed microbial load above the prescribed limit of  $10^5$  CFU/g.

**Table. 04 TVAC in *Tribhuvan kirti ras***

Sr. No.	Product Code	Number of Bacterial CFU/g	Number of Fungal CFU/g	TVAC CFU/g
1.	S <sub>1</sub>	$9.0 \times 10^4$	$3.0 \times 10^3$	$9.3 \times 10^4$
2.	S <sub>2</sub>	$4.0 \times 10^4$	$5.0 \times 10^3$	$4.5 \times 10^4$
3.	S <sub>3</sub>	$5.5 \times 10^4$	$3.0 \times 10^3$	$5.8 \times 10^4$

## 2. Tests for specific microorganisms: enterobacteriaceae and other gram negative bacteria

### a. Detection of bacteria

All three samples were separately evaluated for *E. coli*, *S. typhi*, *S. aureus*, *P. aeruginosa*. In all three samples of *Tribhuvan kirti ras* *E. coli* contamination was found.

**Table- 05 Detection of specific bacteria in *Tribhuvan kirti ras***

Sr. No.	Product Code	<i>E. coli</i>	<i>S. typhi</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
1.	S <sub>1</sub>	+	—	—	—
2.	S <sub>2</sub>	+	—	—	—
3.	S <sub>3</sub>	+	—	—	—

‘—’ absence of specific bacteria

‘+’ presence of specific bacteria

### b. Quantitative evaluation of enterobacteriaceae and other gram negative bacteria

All the three sample of *Tribhuvan kirti ras* showed growth of colonies only in 1 g quantity of each sample taken, indicated contamination with less than 10 but more than a bacteria/g of sample.

**Table- 06 Quantitative evaluation of enterobacteriaceae and other gram negative bacteria**

Sr. No.	Product Code	Quantity or volume			Probable no. Of bacteria / g of products
		1.0 g or 1.0 ml	0.1g or 0.1 ml	0.01 g or 0.01 ml	
1.	S <sub>1</sub>	+	—	—	Less than 10 but more than 1
2.	S <sub>2</sub>	+	—	—	Less than 10 but more than 1
3.	S <sub>3</sub>	+	—	—	Less than 10 but more than 1

‘—’ absence of enterobacteriaceae and other gram –ve bacteria

‘+’ presence of enterobacteriaceae and other gram –ve bacteria

**c. Evaluation of bacteria and fungi in *Tribhuvan kirti ras***

All the three sample of *Tribhuvan kirti ras* showed bacterial contamination, with two types of colonies of Gram positive bacteria. Whereas all the three sample of *Tribhuvan kirti ras* showed fungal contamination with *Gliocladium*, *Papulospora*, *Geomyces* and *Rhizopus* species.

**Table- 07 Evaluation of bacteria and fungi in *Tribhuvan kirti ras***

Sr. No.	Product Code	Gram +ve Bacteria	Gram –ve Bacteria	Species of Fungi detected on SDA and CDA
1.	S <sub>1</sub>	++	+	<i>Gliocladium</i> , <i>Papulospora</i> <i>Geomyces</i> , <i>Rhizopus</i>
2.	S <sub>2</sub>	++	+	<i>Gliocladium</i> , <i>Papulospora</i> <i>Geomyces</i> , <i>Rhizopus</i>
3.	S <sub>3</sub>	++	+	<i>Gliocladium</i> , <i>Papulospora</i> <i>Geomyces</i> , <i>Rhizopus</i>

‘–’ absence of Gram positive and Gram negative bacteria

‘+’ presence of Gram positive and Gram negative bacteria

**d. Validation of tests for specific microorganisms in *Tribhuvan kirti ras***

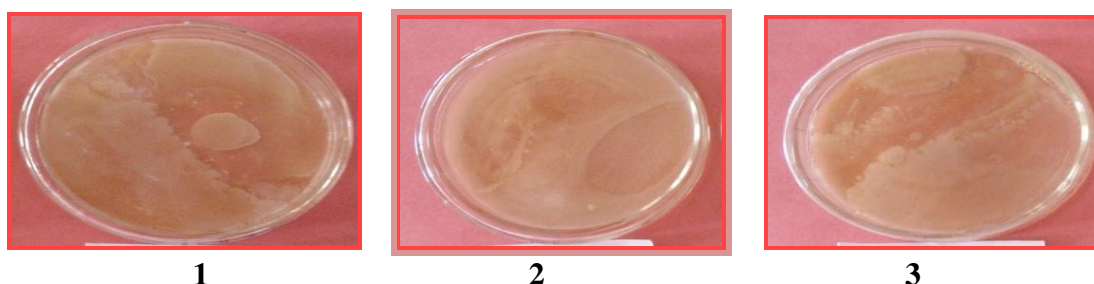
All the three sample of *Tribhuvan kirti ras* were validated for the prevention of growth of specific microorganisms compared with control, and found that all sample were positive for the growth of specific microorganism.

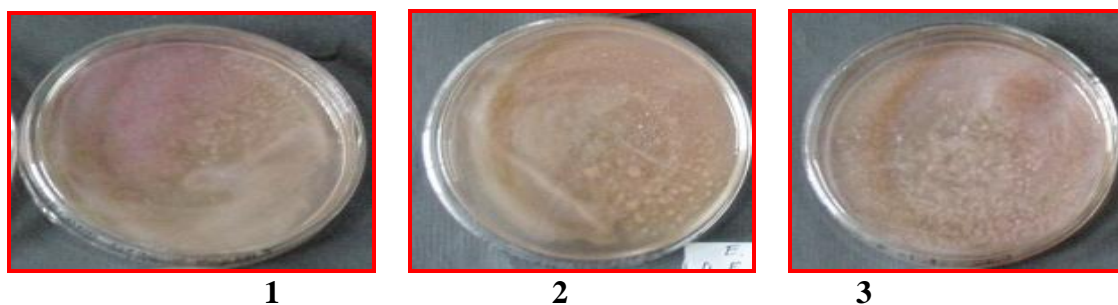
**Table- 08 Validation of tests for specific microorganisms in *Tribhuvan kirti ras***

Sr. No.	Product Code	<i>E. coli</i>	<i>S. typhi</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
1.	Control	+	+	+	+
2.	S <sub>1</sub>	+	+	+	+
3.	S <sub>2</sub>	+	+	+	+
4.	S <sub>3</sub>	+	+	+	+

‘–’ absence of specific bacteria

‘+’ presence of specific bacteria

**Plate 1 - Bacterial contamination in *Tribhuvan kirti ras***



**Plate 2 - *E. coli* contamination in *Tribhuvan kirti ras***

## DISCUSSION

**Microbial contamination:** The microbiological quality of finished product directly depends upon the microbial contamination in raw materials. Contamination occurring during manufacturing i.e. through contaminants, insects and animal vectors, products residue left in operators /or personnel, air, packaging materials and lastly during use by consumer or patients.

Every medicinal product during manufacturing has a unique set of physical and chemical conditions. Microbial contamination during manufacturing can be controlled by assessing or recognising the condition, which encourage the establishment of multiplication of microorganisms in the product which is being manufactured and to manufacturing environment.

The different source of raw material shows different type of contamination. For example, the roots and rhizomes are cultivated beneath the soil and therefore they are usually contaminated with the soil microorganisms, which may include fungal spores and bacterial spores. The surface/ grooves/ furrows on dried fruits or the presence of sugars in fruits may be the source of environmental contamination.

Microbial contamination in fresh herbal raw material can occur easily during harvest. This contamination increases from contact with workers and from physical environment like soil, water, air, hands, containers and storage conditions etc. Some herbal raw material during washing may retain water or mechanical injury occur which contribute to microbial load.

The materials get contaminated with the microorganisms, during various stages of manufacture which are carried in the final formulations. If the manufacturing processes are

harsh, the microorganisms present in the raw material get destroyed or injured. If injured, they may recover and may proliferate in the product.

Certain plants contain natural barriers and antimicrobial substances which exert typical inhibitory effects on microbial growth and stability. It has been estimated that around 1400 herbs and spices may possess antimicrobial agents of different chemical nature as oils, peptides, liquid and organic extracts.<sup>[23]</sup> Some medicinal herbs contain essential oils which act as natural antimicrobials and may inhibit mould development and mycotoxin production.<sup>[24]</sup> Different studies have demonstrated the effectiveness of antimicrobials and their effective compounds to control or inhibit the growth of pathogenic and spoilage microorganisms.<sup>[25,26,27]</sup>

Ayurvedic formulations consist of many constituents; some of them are responsible for the inhibition of microbial growth and some for supporting microbial growth. Microorganisms are usually capable of breaking down the complex bio-molecule of raw material and utilize it either to protect themselves or as a source of nutrient. This can change the active constituents of the materials.<sup>[28]</sup> Sometimes the active ingredients present in the herbal raw material may get modified to some other form or destroyed because of microbial contamination. Therefore, it is very difficult to set the limit for microbial contamination and there are no well defined techniques of quality control or standardization.<sup>[29]</sup>

## CONCLUSION

In the present study, none of the samples were found to be contaminated with aerobic bacteria above the prescribed limit. The TVAC was found to be  $9.3 \times 10^4$ ,  $4.5 \times 10^4$  and  $5.8 \times 10^4$  CFU/g in all samples respectively. All three samples were found to be contaminated with the fungi species of *Glodidium*, *Papulospora*, *Geomyces* and *Rhizopus* as well as Gram positive and Gram negative bacteria. All the samples were contaminated with the *E.coli*, which is not in the range of acceptable pharmaceutical and microbial qualities. This contamination may be due to the use of *bhavana dravya*. The water used for making *swaras* (juice) could have been contaminated. It is also possible that the water used for cultivation of raw materials was contaminated. The process of trituration is also carried out at room temperature and if the juice is left for a longer time at room temperature, the bacteria may multiply in the juice. The personal hygiene of personnel may also contribute to these factors. If the process of drying is delayed, the organisms can proliferate. If the paper had been used during the process of manufacturing it also may be the cause of contamination.

*Gliocladium* is commonly present in soil and resembles *Penicillium* in structure. *Geomyces* frequently found in soil and decompose organic matter while *Papulospora* is found in dung, soil, rotting plant debris and on paper and textile. It is possible that the tablet formulations get contaminated when they are placed for drying on contaminated paper or cloth.

*Escherichia coli* (*E. Coli*) is a bacterium commonly found in the gut of warm-blooded organisms. Most *E. Coli* strains pose no harm to humans. However, there are six groups of *E. Coli* known for their ability to cause human illness. Several types of bacteria exist as part of the normal flora of the human gut, including *E. Coli*, this bacterium has many beneficial functions, such as production of vitamin K and B. It also prevent from harmful bacteria establishing themselves in the intestine. Although most strains of *Escherichia coli* (*E. Coli*) are harmless, some produce a toxin that makes human sick. There are several types of toxins; the group that includes 0157:H7 produces a potent toxin called *Shiga toxin* that is a harmful to the lining of the small intestine.

There is need to take some precautionary measures to prevent such microbial contaminations. The workers in the packaging should be warned to follow Good Hygiene Practices. Container and packaging material should be clean and free from dust and other contaminants. Herbalists should be trained to apply Good Manufacturing Practices(GMP), Good harvesting practice (GHP) and the safe handling and storage of herbal medicinal products. Further studies are recommended for herbal products to establish other contaminants and ways in which the contaminants can be reduced to recommended levels.

Ayurvedic formulations are available as multidose preparations and usually no preservatives are added. Frequent exposure to outer environment during use makes these products prone to microbial contamination. Thus the microbial loads should be established and the contaminants isolated and identified. In addition, alternative methods such as treatment with ethylene oxide or radiation with ionic rays lead to decontamination effects. These methods can be seen as a compromise between ensuring the microbiological safety of the product and avoiding consumer's risk and special legal permissions are required in many countries. It is evident that more detailed studies of plant species popularly used are needed in order to ensure the quality, an important concept for providing the wished security and reliability for its use.



Microbial contamination can lead to impaired performance of the product due to disruption of the stability of the formulation, modification of physical characteristics and appearance and lead to inactivation of the active ingredients and excipients in the formulation and also cause loss of confidence in the company.

## REFERENCES

1. World Health Organization (WHO). WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems, 2004. <http://www.who.int/medicinedocs/index/assoc/s7148e/s7148e.pdf>.
2. Khanyile ZC, Singh N, Smith M, Shode FO, Mngomezulz S, Dewir YH. Comparative assessment of bacterial contamination in commercial herbal products of *Lessertia futescens*. American-Eurasian J Agric. & Environ Sci, 2009; 5(4): 494- 499.
3. Kulkarni C, Deshpande A, More S. Assessment of microbial contamination in commercial herbal oral medicinal liquids. International Journal of Pharma Research and Development 1999; 2(9): 191-194.
4. Abba D, Inabo HI, Yakubu SE, Olonitola OS. Contamination of herbal medicinal products marketed in kaduna metropolis with selected pathogenic bacteria. Afr. J. Traditional Complementary and Alternative Medicines, 2009; 6(1): 70-77.
5. Abba D, Inabo HI, Yakubu SE, Olonitola OS. Contamination of herbal medicinal products marketed in Kaduna Metropolis with selected pathogenic bacteria. African Journal of Traditional Complementary and Alternative Medicines, 2009; 6: 70-77.
6. Kunle OF, Egharevba HO, Ahmadu PO. Standardization of herbal medicines- A review. International Journal of Biodiversity and Conservation, 2012; 4: 101-112.
7. Kneifel w, Czech E, Kopp B. Microbial contamination of medicinal plants- A review. Planta Medica, 2002; 5(15): 68.
8. Kosalec I, Cvek J, Tomic S. Contaminants of medicinal herbs and herbal products. Archives of Industrial Hygiene and Toxicology, 2009; 60: 485-501.
9. Kneifel W, Czech E, Kopp B. Microbial contamination of medicinal plants- A review. Planta Medica, 2002; 5(15): 68.
10. Bugno A, Almodovar AB, Pereira TC, Pinto TJA, Sabino M. Occurrence of toxigenic fungi in herbal drugs. Brazilian Journal of Microbiology, 2006; 37: 47-51.
11. Kneifel W, Czech E, Kopp B. Microbial contamination of medicinal plants- A review. Planta Medica, 2002; 5(15): 68.

12. Czech E, Kneifel W, Kopp B. Microbiological status of commercially available medicinal herbal drugs- A screening study. *Planta Medica*, 2001; 67: 263-269.
13. Kneifel W, Czech E, Kopp B. Microbial contamination of medicinal plants- A review. *Planta Medica*, 2002; 5(15): 68.
14. Rocha RP, Melo EC, Radünz, LL. Influence of drying process on the quality of medicinal plants: A review. *Journal of Medicinal Plants Research*, 2011; 5(33): 7076-7084.
15. Martins HM, Martins ML, Dias MI, Bernardo F. Evaluation of microbiological quality of medicinal plants used in natural infusions. *International Journal of Food Microbiology*, 2001; 68:149-153.
16. Liu X, Qiu Z, Wang L, Chen Y. Quality evaluation of *Panax notoginseng* extract dried by different drying methods. *Food and Bioprocess Technology*, 2011; 89: 10-14.
17. Abba D, Inabo HI, Yakubu SE, Olonitola OS. Contamination of herbal medicinal products marketed in Kaduna Metropolis with selected pathogenic bacteria. *African Journal of Traditional, Complementary and Alternative Medicines*, 2009; 6: 70-77.
18. Fennel CW, Light ME, Sparg SG, Stafford GI, Van Staden J. Assessing African medicinal plants for efficacy and safety: agricultural and storage practices. *Journal of Ethnopharmacology*, 2004; 95: 113-121.
19. Aziz NH, Youssef YA, El Fouly MZ, Moussa LA. Contamination of some common medicinal plant samples and spices by fungi and their mycotoxins. *Botanical Bulletin of Academia Sinica*, 1998; 39: 279-285.
20. Mandeel QA. Fungal contamination of some imported spices. *Mycopathologia*, 2005; 159: 291-298.
21. Kumar A, Shukla R, Singh P, Dubey NK. Biodeterioration of some herbal raw materials by storage fungi and aflatoxin and assessment of *Cymbopogon flexuosus* essential oil and its components as antifungal. *International Biodeterioration & Biodegradation*, 2009; 63: 712-716.
22. Vaidya Lakshmiapati Shastri, Bhisagratna Brahmasankar Sastri. *Yogratnakar*. 3<sup>rd</sup> ed., Varanasi; Chaukhamba Sanskrit sansthan: 1983; pp. 241.
23. Kneifel W, Czech E, Kopp B. Microbial contamination of medicinal plants- A review. *Planta Medica*, 2002; 5(15): 68.
24. Kosalec I, Cvek J, Tomic S. Contaminants of medicinal herbs and herbal products. *Archives of Industrial Hygiene and Toxicology*, 2009; 60: 485-501.

25. Tiwari BK, Valdramidi VP, O'Donnell CP, Muthukumarappan K, Bourke P, Cullen PJ. Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry*, 2009; 57: 5987-6000.
26. Wang Y, Lu Z, Wu H, Lv F. Study on the antibiotic activity of microcapsule curcumin against foodborne pathogens. *International Journal of Food Microbiology*, 2009; 30: 71-74.
27. Negi PS. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *International Journal of Food Microbiology*, 2012; 156: 7-17.
28. Blumenthal M, Brusse WR, Goldberg A, Gruenwald J, Hall T, Riggins CW, Rister RS. *The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines*, Austin TX; The American Botanical Council: 1998.
29. Ministry of Health and Family Planning. *The Ayurvedic formulary of India, Part -1*. 1<sup>st</sup> ed; Government of India: 1976: 3.