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Microbial and Heavy Metal Analysis in Herbal Formulation for the treatment of Fungal Infection

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Abstract
The use of traditional herbal medicinal products has been increasing worldwide due to the readily available raw materials and low cost compared to the synthetic industrial preparations. Medicinal herbs have been reported to be contaminated with agents and microorganisms indigenous to the soil and plants where they are grown. Confounded with poor unhygienic and handling pactices, the microbial safety and quality of these products is a public health concern. The present investigation was study aimed at evaluating microbial and heavy metals analysis in herbal formulation [dusting powder] containing selected herbs viz., Neem, Bakuchi, Tulsi, Bhunimba, Bhasma, Ajwoin, Nimbu and Starch. Results of the study indicates that the total aerobic viable count, total yeast and mould, *E. coli* and *Salmonella* Species in herbal formulation were lower than the limit prescribed in The Ayurvedic Pharmacopoeia of India. Results showed that concentration of lead and cadmium in herbal formulation was found within the limit. The presence of heavy metal in herbal formulation was less than the limit prescribed in The Ayurvedic Pharmacopoeia of India.

Key-words: Herbal Formulation, Microbial Analysis, Heavy Metals, Fungal infection, Powder

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Introduction

Fungal infection, also known as mycosis, is a disease caused by fungi. Fungal infection occurs after spores are either breathed in, come into contact with skin or enter the body through the skin such as via cut, wound, or injection. Fungi that cause infection in humans include yeasts, molds, and fungi that are able to exist as both mold and yeast. Some fungal infections are contagious and hence it is important to wash hands after touching other people or animals. Sports clothing also be washed after use. Since fungi thrive in warm, moist environments, fungal skin infections can often develop in sweaty or damp areas that don't get much airflow. Some examples include the feet, groin, and folds of skin. Treatment depends on the type of fungal infection and usually requires topical or systemic antifungal medicines. [1] Anti-fungal herbal formulation (dusting powder) is an herbal remedy made up of herbs like Neem, Bakuchi, Tulsi, Bhunimba, Bhasma, Ajwoin, Nimbu and Starch and was crafted to alleviate itching, irritation, and fungal infections. Infused with potent antifungal and antibacterial properties, this powder is adept at both preventing and treating conditions like ringworm infections. Offering a refreshing and cooling sensation, it may also soothe discomfort caused by sweat irritation and prickly heat. [2] According to research, approximately 80% of people in developing countries use traditional herbal remedies as their primary form of healthcare. Contamination by microorganisms of various types that may be adherent to the leaves, stems, blossoms, seeds, and roots from which herbal medicines are manufactured. Microorganisms can also be added throughout the processes

of harvesting, handling, open-air drying, preserving, and manufacturing. Due to consumers uncompromising conditions and microbial infections, the presence of microbial contaminants in herbal products might negatively impact their health status, posing a global health issue. [3] Therefore, it is essential to ensure that users of herbal products are safe. Thus, the present study was undertaken which deals with the analysis of microbes and heavy metal in herbal formulations.

Material and Methods

Material: Anti-fungal herbal formulation (dusting powder) made by fine powder of herbs like Neem, Bakuchi, Tulsi, Bhunimba, Bhasma, Ajwoin, Nimbu and Starch

Determination of Microbial Content [4]

Total viable aerobic count

Preparation of sample: Dissolved 10g herbal formulation being examined in buffered sodium chloride peptone solution pH 7.0 and adjust volume 100ml with same medium.

Examination of sample: Total viable aerobic count in the sample was examined by using the plate count method by Digital colony counter by Chemiline.

C. For bacteria: Petri dishes of 10cm diameter were used; 1ml pretreated mixture was added to each dish and 15ml liquefied casein soyabean digest agar at a temperature not more than 45˚C. Two Petri dishes for each sample were prepared using the same dilution and incubated at 30- 40˚C for 3days. The number of colonies form was calculated using the digital colony counter. Results were calculated using plate with the greatest number of colonies but taking 300 colonies per plate as the maximum consistent with good evaluation.

Total yeast and mould count: Procedure was used as described in the test for bacteria using Chloramphenicol yeast glucose agar (CYGA) in place of casein soyabean digest agar and incubate the plates at 20-25˚C for 5days, unless a more reliable count was obtained in shorter time. Results were calculated using the plates with not more than 100 colonies by using colony counter

Test for *Escherichia coli*

Dissolved 10g herbal formulation in 90ml fluid casein digest-soya lecithin-Polysorbate 20 medium to get 1:10 dilution. 10ml mixture was added in 50ml nutrient broth in sterile screw-capped container, shake, allowed to stand for 1hr and shake again and incubated at 35-37˚C for 24hrs. After incubation, 1ml was added to 5ml of Mac-Conkey broth and incubated at 36-38˚C for 48hrs. If the contents of the tube show acid and gas indicated the possible presence of *E. coli.* Alternative test of an inoculating loop, Streak a portion from the enrichment culture (obtained in the previous test) on the surface of Mac-Conkey agar medium. Covered and invert the dishes and incubated at 45˚C for 24hrs. Growth of red, generally non-mucoid colonies of Gram negative rods, sometimes surrounded by a reddish zone of precipitation, indicated the presence of *E. coli.*

Test for *Salmonella* species Dissolved 10g herbal formulation in 90ml fluid casein digest-soya lecithin-Polysorbate 20 medium (1:10 Dilution). 10ml of mixture was added in 100ml nutrient broth in sterile screw-capped container, shake, allowed to stand for 4hrs and shake again and incubated at 35-37˚C for 24hrs. After incubation, 1ml enrichment culture to each of the two tubes containing (A) 10ml selenite F broth and (B) tetrathionate bile brilliant green broth and incubated at 36-38˚C for 48hrs. From each, streak looped on Bismuth sulphate agar and Xylose lysine deoxycholate agar media and incubated the plates at 36˚-38˚C for 24hrs. Well developed, black or green in Bismuth sulphate agar and red with or without black centers colonies in Xylose lysine deoxycholate agar indicated the presence of *Salmonella species.*

Determination of Heavy Metal Contamination

Heavy metal contamination in anti-fungal herbal formulation (dusting powder) was determined as per procedure mentioned below.

Preparation of Sample Solution (Lead, Cadmium and Iron)

Heavy metals analysis was done according to AOAC guidelines (2016) for non-volatile heavy metal. Take 5 g of the sample in a pre-weighed silica dish and keep it in a muffle furnace with initial temperature not higher than 100oC. Increase the temperature slowly to a maximum of 450°C. Allow the dish to stand at least 8 hrs. or overnight. Wet ash with 1-3 ml water and evaporate on hot plate or water bath. Place the crucible in muffle furnace at not more than 200°C and raise the temperature to 450°C. Proceed with ashing at 450°C for 1-2 hrs. or longer. Repeat procedure until the sample is completely ashed. Add 5 ml of 6 M Hydrochloric acid to crucible ensuring that all ash

comes into contact with acid. Evaporate acid on water bath or hot plate. Dissolve residue in 10-30 ml of 0.1 M Nitric acid. Cover with watch glass and let stand for 1-2hrs. Then stir solution in dish thoroughly with glass rod and filter the solution in a 100 ml volumetric flask and make up the solution upto the mark with deionised water. Analysis was done using atomic absorption spectrophotometer (GBC Avanta). The standard reference material of all the metals (E. Merck) was used for calibration and quality assurance for each analytical batch. Three replicates were analysed to assess precision of the analytical techniques, and results were averaged.

Preparation of Sample Solution (Mercury)

5-10 g of the herbal formulation was taken in a 100 ml Round bottom flask of the bethge apparatus add 3 to 4 glass beads, 10-12 ml of concentrated Nitric acid and 2-5 ml of concentrated Sulphuric acid connect the flask to the condensate receiver and reflux condenser and Put the flask in the cold condition for about 1.5 hrs. Once, remove cold condition heat the flask and collect the nitric acid in the condensate receiver continue heating till the sulphuric acid starts. Fuming and chars the sample. Remove the burner, wait for a few minute and carefully allow the nitric acid to drain into the flask. Repeat this operation to all the sample solution becomes just pale yellow colour. Cool and then remove it from the condensate receiver. Collect all the condensate in 50 ml standard flask and make the solution with deionised water. Analysis was done by using Mercury analyser MA 5840. Three replicates were analysed to assess precision of the analytical techniques, and results were averaged.

Arsenic Content

Preparation of Standard Solution (10PPM)

0.33gms of arsenic trioxide was dissolved in 5ml of 2M Sodium hydroxide solution and then diluted to 250ml with water. One volume of this was the diluted to 100 volume with water.

Preparation of Sample

Preparation of herbal formulation solution

The herbal formulation solution was prepared by means of diluting 1gm of herbal formulation to 100ml using distilled water. This is used to carryout limit test for iron and lead and also to perform qualitative test for mercury. 10ml of herbal formulation solution was pipetted out into a flask and about 10ml of concentrated nitric acid was added and evaporated to dryness on a waterbath. The residue was then dried at 130° C for 30minutes then about 10ml of hydrazine molybdate reagent was added and refluxed for 20minutes. The solution was then cooled and absorbance of both standard and test solution was measured at 800 nm usingn Perkin Elmer UV spectrophotometer.

Results and Discussion

Microbial analysis of Herbal formulation included total viable aerobic count, total yeast and mould, *E. coli, S. Typhi* count. The results are given in Table 1. Pathogenic bacteria in vegetative and non-vegetative forms are harmful to human body because they produce diseases. Hence WHO and pharmacopoeia of the advance countries have prescribed the limit for total aerobic viable count, total yeast and mould, *E. coli* and *Salmonella* species. Results indicated that total aerobic viable count was within the limit and total yeast and mould, *E. coli and Salmonella* species were absent in herbal formulation. This indicated that total aerobic viable count, total yeast and mould, *E. coli* and *Salmonella* Species in herbal formulation were lower than the limit prescribed in The Ayurvedic Pharmacopoeia of India.

Table 1: Microbial Analysis of Herbal Formulation

S/No.	Microbial Analysis	Inference
1.	Total aerobic viable count	120 CFU/gm
2.	Total yeast and mould	Absent
3.	*E.coli*	Absent
4.	*Salmonella Sp*	Absent

Analysis of heavy metal in herbal formulation like Lead (Pb), Cadmium (Cd), Mercury (Hg), Iron (Fe) and Arsenic content were carried out. Results are shown in Table 2. Heavy metals after entering in the human body can't be removed easily. On long usage they accumulated in the body ultimately produce toxicity by hindering the normal physiological activity. Hence WHO and various pharmacopoeias have prescribed the limit for heavy metals. Results showed that concentration of lead and cadmium in herbal formulation was found within the limit. The presence of heavy metal in herbal formulation was less than the limit prescribed in The Ayurvedic Pharmacopoeia of India.

Table 2: Heavy Metal Concentration in Herbal formulation

HF	**Heavy Metals (mg/kg)**				
	Lead (Pb)	**Cadmium (Cd)**	**Mercury (Hg)**	**Iron (Fe)**	**Arsenic Content (PPM)**
Herbal Formulation	3.20	0.13	0.03	1.50	2.10

Conclusion

Results of the study indicates that in herbal formulation containing herbs viz., Neem, Bakuchi, Tulsi, Bhunimba, Bhasma, Ajwoin, Nimbu and Starch the total aerobic viable count, total yeast and mould, *E. coli* and *Salmonella* Species were lower than the limit prescribed in The Ayurvedic Pharmacopoeia of India. The presence of heavy metal in herbal formulation was less than the limit prescribed in The Ayurvedic Pharmacopoeia of India.

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