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MICROBIAL LOAD IN THE RAW MATERIALS OF NIŚĀKATAKĀDI KAṢĀYAM

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Abstract: The raw drugs used in āyurvedic medicines have naturally a high load of microorganisms in them. A study was done to analyze the microbial load in the raw materials of the popular āyurvedic formulation Niśākatakādi kaṣāyam. All the ingredient herbs found carrying high level of microbes. Various bacterial species including *Escherichia coli, Salmonella, Pseudomonas, Staphyloçoccus* and fungal species like *Aspergillus, Mucor,* etc. were detected. These contaminants can be removed by proper cleaning, treatment with ethylene oxide and irradiation that effectively kill the microbes.

Introduction

Āyurveda employs many dosage forms for delivering medicinal principles. Kaṣāyam or decoction is one among them. This is a liquid obtained by boiling herbal material, which may include stems, roots, bark, seeds and rhizomes. Niśākatakādi kaṣāyam is usually prescribed for diabetes mellitus (Vaidyan and Pillai, 2011). This kaṣāyam is made up of eight dried raw materials. As these raw materials are of natural origin, they can be easily colonized by fungi and bacteria (Stevic *et al*, 2012). Some groups of bacteria are able to thrive under adverse conditions and are therefore particularly dangerous (Sousa *et al*, 2011). Aerobic sporulating bacteria frequently predominate in this to which additional contamination and microbial growth occur during harvesting, handling and production. (Kulkarni, 2010).

The significance of contamination in a processing industry is determined by a number of factors as well as properties of the microorganism(s) concerned, the product and environmental factors. Environmental factors such as pH and water activity of the product or raw material, available nutrients and the ambient temperature, determine what microorganism would be the dominant contaminant and the level of spoilage of the product or raw material. Microorganisms of concern are often present in small numbers as part of the natural microflora of raw materials and could not be totally eliminated. Factors contributing to multiplication of microorganisms to unacceptable levels may include improper storage conditions and improper handling by the workers. Organisms originating from raw materials can contaminate hands of workers and then be transferred to the

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product and equipment. (Bandaranayake, 2006).

There are reports to show that 10% of spices are contaminated with mesophilic aerobic microorganisms and 20% with entero-bacteriaceae. The contamination level in aromatic herbs was 26% for both these kinds of bacteria. The study detected the presence of bacteria from the genuses *Acinetobacter (A. calcoaceticus), Enterobacter* and *Shigella.* Species of microorganisms such as *Yersinia intermedia, Staphylococcus aureus* and *Hafnialvei* were also detected (Isabel *et al*, 2010).

The microbial limit of raw materials has to conform to WHO standards (Anonymous, 2007). According to WHO standards, values of the microbial limits should not exceed 10^5/g for total aerobic bacteria, 10^3/g for yeast and moulds, 10/g for *E. coli* whereas *Salmonella, Staphylococci* and *Pseudomonas* should be totally absent (Table 1). But the standards are not normally maintained by manufacturers. This fact cannot be ignored, and efforts should be made to enforce microbiological quality of raw materials.

The ingredient drugs of Niśākatakādi kaṣāyam are common in many āyurvedic preparations. Considering the importance of maintaining microbiological quality of the ingredients, a study of the microbial flora in the eight raw materials of Niśākatakādi kaṣāyam [niśā (*Curcuma longa*), kataka (*Strychnos potatorum*), nellikka (*Emblica officinalis*), tecci (*Ixora coccinia*), pāccoṭṭi (*Symplocos racemosa*), bhadrika (*Aerva lanata*), ekanāyakam (*Salacia oblonga*) and rāmaccam (*Vetiveria zizanioides*)] was carried out.

TABLE 1
Microbial limits for raw materials set by W.H.O. and U.S.F.D.A.

Parameter	WHO	USFDA
Total aerobic plate count	10^5/g	3000
Total yeast and mould count	10^3/g	100
E. coli	10/g	0*
Salmonella	0	0
Staphylococcus	0	0
Pseudomonas	0	0

*0 - Should be absent

Materials and methods

Raw materials and culture media

Dried samples of *Curcuma longa* (rhizome), *Stryçhnos potatorum* (seed), *Emblica officinalis* (pericarp), *Ixora coccinia* (root), *Symplocos racemosa* (bark), *Aerva lanata* (root), *Salacia oblonga* (root) and *Vetiveria zizanioides* (root) obtained from the market were tested for microbial load. The procedures prescribed as per AYUSH/WHO guidelines (Lavekar, 2010) and Bacteriological Analytical Manual (Maturin and Peeler, 2001; Tournas *et al*, 2001) were followed. Nutritional media used for evaluation of microbial limits were procured from Hi-Media Laboratories Ltd. and were ready-to-use dehydrated media.

Aerobic plate count (Bacteria, yeast & mould)

All the raw materials were powdered finely. 10 g of each powder was suspended in 90 ml of buffered peptone water and mixed homogeneously. Using separate sterile pipettes, decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, of the samples were prepared by transferring 10 ml of previous dilution to 90 ml of diluents. 1 ml of each dilution was pipetted into separate, duplicate, appropriately marked petri dishes. 12-15 ml of plate count agar (cooled to 45 ± 1°C) was added to each plate within 15 min of original dilution. Immediately sample dilutions and agar medium were mixed thoroughly and uniformly by

alternate rotation and back-and-forth motion of plates on flat level surface. Solidified petri dishes were inverted, and incubated promptly for 48 ± 2 h at 35°C for bacteria and 25°C for yeast and moulds for 5-6 days.

Colonies were counted and expressed in Colony Forming Units (CFU). Plates with 25-250 CFU were counted and CFU/g was calculated using the formula: $N = \frac{\Sigma c}{(1 \times n_1)+(0.1 \times n_2) \times (d)}$ where,

N=Number of colonies per ml or g of product; Σc=Sum of all colonies on all plates counted; n_1= Number of plates in first dilution counted; n_2 = Number of plates in second dilution counted; d = Dilution from which the first counts were obtained

Detection of specific organisms and fungi

Methods prescribed in AYUSH/WHO guidelines were used to test microbial quality. Four specific pathogens viz. *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* sp. were also checked for their presence. Isolated organisms were identified using morphological, cultural characteristics and biochemical tests. Fungi were stained using Lacto phenol cotton blue stain (Forbes *et al*, 2007).

Identification of microbes

The specific organisms obtained after testing were further confirmed by biochemical testing and other selective media. The fungi were stained to study the morphology. Most of the bacteria answered the biochemical tests including IMViC tests leading to their confirmation (Danladi, 2009).

Results and discussion

The study showed that all the eight raw materials carried a high load of bacteria and fungi (Table 2). Out of the eight, only *Ixora coccinia*

TABLE 1

Microbiology of the raw materials of Niśākatakādi kaṣāyam

Raw drugs	Total aerobic count (values in cfu/g)		Total yeast/mold count (in cfu/g)		*E. coli* (value in cfu/g)		*Salmonella* sp.		*P. aeruginosa*		*S. aureus*	
	Observed	Limit	Observed	Limit	Observed	Limit	Observed	Limit	Observed	Limit	Observed	Limit
Aerva lanata	98180	-	106360	10^5	$<10^4$	10^4	Present	-	Present	Absent	Present	Absent
Ixora coccinia	22818	-	50900	10^5	$<10^4$	10^4	Present	-	Present	Absent	Present	Absent
Salacia oblonga	327200	-	9360	10^5	$<10^4$	10^4		-		Absent		Absent
Strychnos potatorum	1845000	-	1318000	10^5	$<10^4$	10^4		-		Absent		Absent
Curcuma longa	2400000	-	TFTC*	10^5	$<10^4$	10^4		-		Absent		Absent
Symplocos racemosa	2627200	-	23272	10^5	$<10^4$	10^4		-		Absent		Absent
Vetiveria zizanioides	2227200	-	44181	10^5	$<10^4$	10^4		-		Absent		Absent
Emblica officinalis	200000	-	TFTC*	10^5	Absent	10^4	Absent	-	Absent	Absent	Present	Absent

*Within limit (TFTC - too few to count)

contained lesser bacterial count. The fungal and yeast counts were within limit, except for *Strychnos potatorum*.

All the eight raw materials except *Emblica officinalis* carried *E. coli, Salmonella, P. aeruginosa* and *S. aureus* (Table 3). *S. aureus* seemed to be present in all the other raw materials. Identity of the organisms was confirmed by biochemical tests (Tables 4 and 5). Growth of the microbes on selected media is shown in Fig. Ia-e. Images of biochemical reactions are provided in Fig. IIa-h. Images of the various fungi isolated from the raw materials are given in Fig. IIIa-f.

Since these raw materials are being handled carelessly and not washed properly, the rate of microbial load seems to be high. There are similar situations reported from elsewhere. For example, herbal medicines sold in Kenya without control or regulations are reportedly contaminated with microbes which are potential pathogens, posing a threat to patients (Gosanjo, 2013). Human beings act as a source of contamination as in the case of handling by persons suffering from respiratory diseases. Among the fungal species detected in the samples, *Mucor, Aspergillus flavus* and *Aspergillus niger* were the predominant ones. Presence of *Aspergillus* species indicates that these organisms have colonized the samples before complete drying. Since they can grow easily in moist condition, these may result in toxic metabolite accumulation. Mycotoxins are of concern due to their potentially harmful effects on both humans and animals (Efuntoye, 2000). Therefore, it is essential to investigate the degree of contamination of the materials before accepting for production. This is all the more important with the genus *Aspergillus* (Halt, 2004).

TABLE 3
Microbial load in the raw materials

Raw material	Microorganisms			
	E.c	*S.*sp	*P.a*	*S.a*
Emblica officinalis	-	-	-	+
Ixora coccinia	+	+	+	+
Symplocos racemosa	+	+	+	+
Vetiveria zizanioides	+	+	+	+
Strychnos potatorum	+	+	+	+
Salacia oblonga	+	+	+	+
Aerva lanata	+	+	+	+
Curcuma longa	+	+	+	+

E.c - E.coli; S. sp - *Salmonella* sp.; *P.a - Pseudomonas aeruginosa; S.a Staphylococcus aureus.*
+ Present; - Absent

TABLE 5
Antibiotic sensitivity for *Pseudomonas aeruginosa*

Organism	Antibiotics		
	Genta-mycin	Cipro-floxacin	Vanco-mycin
P. aeruginosa	30mm (sensitive)	34mm (sensitive)	No zone (resistant)

Aspergillus was present in almost all the herbs. Most fungal contaminants in stored raw materials usually arise from infestations that begin in the field, although some can directly infest the harvested herbs when conditions are right. Moulds require about 12% moisture, more than 7°C, oxygen and energy for their growth. Fungal growth causes direct losses in volume and quality of raw materials and subsequently leaving behind some poisonous mycotoxin, which contaminate the raw materials and finished goods (Okoli *et al*, 2007). A recent study shows that although yeasts were the major fungi present in the cocoa bean samples, moulds were also detected. These moulds belonged mainly

TABLE 4
Biochemical confirmation

Tests	Microorganisms			
	E. coli	*Salmonella* sp.	*P. aeruginosa*	*S. aureus*
- Gram reaction	Negative rod	Negative rod	Negative rod	Positive cocci in grape like clusters
- Indole	+		-	-
- Methyl red	+		-	-
- Voges - Proskauer	-		-	-
- Citrate	-	+	+	-
- Urease	-	-	+	-
- Triple sugar	A/A (acid slant/ acid butt)	A/K(acid butt/ alkaline slant)	K/K(alkaline slant/ alkaline butt)	No change
- Catalase	-	-	+	+
- Coagulase	-	-	-	+
- Oxidase	-	-	+	-
- Motility	+	+	+	-
- H_2S	-	+	-	-
- Lactose utilization	Yellow (Acid/gas)	Orange (-)	Yellow (Acid/gas)	No change
- Growth at 42°C	-	-	+	-
- Growth in eosin methylene blue	Green metallic sheen			
- Growth in cetrimide agar	-	-	Growth with fluo-rescence at 366 nm	-
- Growth in baird Parker agar	-	-	Jet black pin point colonies with black halo	
- Growth in xylose lysine deoxycholate	Flat yellow colonies	Red colonies with black centers	-	-
- Growth in deoxycholate citrate agar	-	Colorless opaque colonies	-	-
- Growth in mac conkey agar	Pink colonies	Colourless colony	Colourless colony	Colourless colony
- Growth on mannitol salt agar	-	-	-	Yellow colony with media color changed to yelow

to the species *Rhizopusstolonifer* (27%), *Aspergillus niger* aggregate (17%), *A. flavus* (31%) and *Penicillium citrinum* (13%). Other species, such as *A. carbonarius* were found to a minor extent (Amézqueta *et al*, 2008).

Several agencies provide services for microbial reduction for spice and food processing industries. Two sanitation options are of the choice - ethylene oxide (EO) fumigation and irradiation. While both effectively kill organisms, the challenges presented by the bulk packaging of spices and herbs have made ethylene oxide fumigation and gamma processing the methodologies of choice due to their efficient, high-density penetration. Often, the final destination of the spice and herb product plays a dominant role in the technology selection. Irradiation is most effective and preferred choice. Irradiation is achieved either by gamma rays, pure energy rays emitted from Cobalt-60 and similar in many ways to micro-waves, or by accelerated electrons, commonly known as electron beam (E-beam) irradiation. Approved by the FDA in 1988, gamma processing is the preferred method of food sterilization in the US and on a global basis. Less harsh and intrusive than EO, irradiation uses ionizing energy to kill bacteria, mold and insects while retaining the

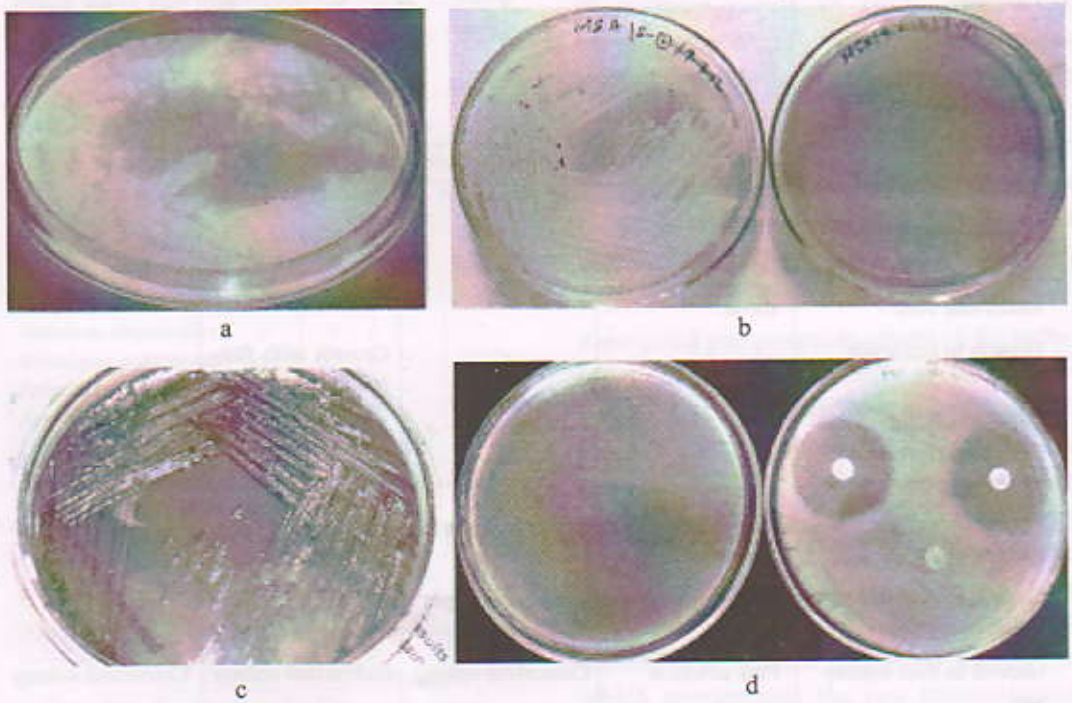

Fig. I a-e: Growth of microbes on selective media
a Fluorescence by P. aeruginosa on Cetrimide Agar; **b** *S. aureuson* Mannitol salt agar; **c** *E. coli* on Eosin methylene blue agar; **d** Sensitivity to antibiotics on Muller Hinton agar by *P. aeruginosa*

anti-oxidant properties (Rushing, 2006).

Conclusion

The study showed the occurrence of microbes in the raw materials of the Niśākatakādi kaṣāyam. Similar situations have been reported from Belgium (Devieeschouwer and Dony, 1979), France (Bernard, 1983), Germany (Frank, 1989; Kabelitz, 1996; Leimbeck 1987), Poland (Grabowska and Kedzia, 1982), U.S.A. (Lerke and Farber, 1960), and Yugoslavia (Katusin-Razem *et al*, 1988; Kolb, 1999). These reports show that herbal decoction materials have a higher level of microorganism than those found most other foodstuffs.

Reducing the level of microorganisms in herbal raw materials is difficult, as the use of EO and irradiation are controversial. EO is banned in Europe by a directive (EEC, 1998). The use of steam can loss of volatile oil (Kolb, 1999). A more reasonable suggestion is that producers of herbal materials improve their collection, cultivation and processing practices. The use of the principles of Good Manufacturing Practice, Quality Assurance and Hazard

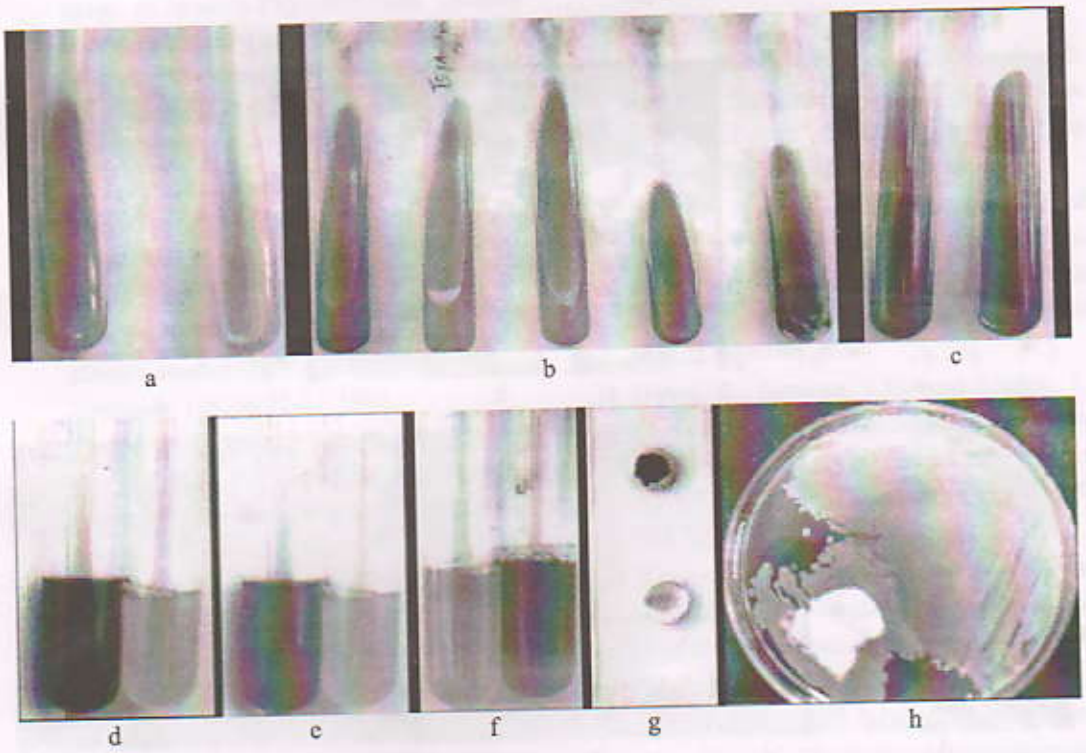

Fig. II a-h: Images of biochemical reactions
a Ureasetest; **b** Triple sugar iron agar;
c Citrate utilisation; **d** Indole test; **e** Methyl Red test; **f** Voges - Proskauer;
g Oxidase test; **h** Catalase test

Analysis of Critical Control Points can help in identifying ways to improve hygiene and reduce microbial load of the herbs. An example is the Good Agricultural Practice guideline which was agreed upon in 1999 (Kolb, 1999). Concerted attempt needs to be made in India to improve the quality of herbal raw materials used in ayurveda industry.

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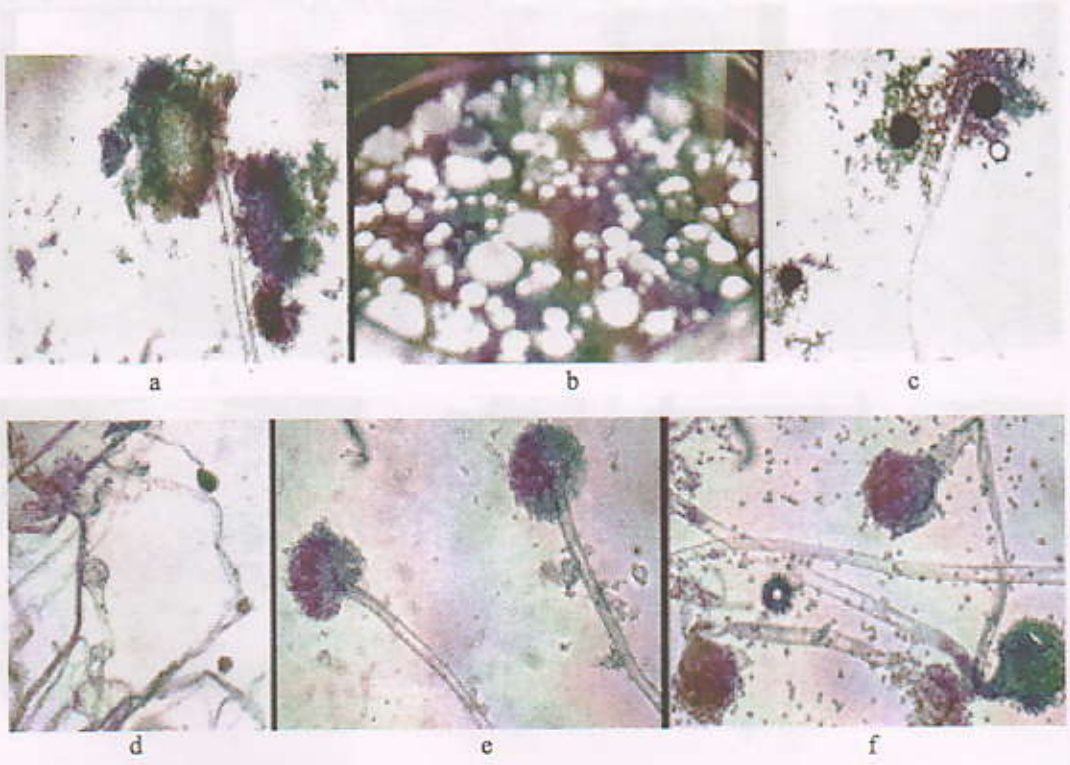

Fig. III a-f: Images of fungi isolated from raw materials
a *Aspergillus oryzae*; **b** Variety of fungi on rose bengalagar;
c *Aspergillus niger*; **d** Mucor; **e** *Aspergillus flavus*; **f** *Aspergillus* sp.

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