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Research Article

Microbiological Contamination and Mutagenicity of Herbal Products Used in Ayurvedic Medicine

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Abstract

Ayurveda is a traditional medicine diffused worldwide, consisting principally of herbs and regulated under various legislation in each country. The Ayurvedic products are perceived as safe by consumer because of natural ingredients, but their use is not free from risks. This study aimed to assess the microbiological contamination and mutagenicity of some herbal products commonly used in Ayurvedic medicine, produced in India, according to place of purchase, namely shops or drugstores in India or Italy. Thirteen Ayurvedic drugs bought in different Indian shops and three products bought in Italy, all containing *Withania somnifera*, *Gymnema sylvestre* or *Phyllantus niruri*, were tested for microbiological contamination (total viable aerobic count and presence of *Escherichia coli*) and mutagenicity (Ames test on *Salmonella typhimurium*). Microbiological contamination results suggested that the products of industrial origin, bought in Italian herbalist's shops and Indian drugstores, are less contaminated than those from local production, bought in Indian herbalist's shops and street markets, which showed great bacterial and fungal contamination and sometimes the presence of *Escherichia coli*, too. The Ames test underlined differences between Italian and Indian products. Almost all the Indian samples showed high genotoxicity, with values of net revertants from 20.55 to 25,764, whereas the Italian samples showed only modest or null mutagenicity. The Ayurvedic products tested are of some concern for consumer, especially those sold in Indian herbalist's shops and street markets. The risk seem to be not related to the original plant, but probably to contamination of the products by bacteria and fungi and possibly by chemical substances derived from the production process. Products of industrial origin seem to be substantially safe, suggesting that complying with standard controls procedures during production and distribution process leads to better product quality and safety.

1. INTRODUCTION

Traditional, complementary, alternative, or non-conventional medicines are used by 70-95% of the global population, in both developing countries, especially in Asia, Africa, Latin America and the Middle East, and developed nations, such as Canada, France, Germany and Italy[1].

Ayurveda is one of the traditional medicines. It originated in India more than 2000 years ago, is recognized officially by the Indian Government and the WHO, and is taught at Medical Schools in India and other countries. Ayurvedic remedies, most of which consist of herbal products, are used by approximately 80% of the Indian population and are also widespread in developed countries, where they are commonly sold as self-medication, home remedies, dietary supplements, health foods, functional foods or phytoprotectants[1]. According to each country's laws, these drugs are subject to various types of certification, which often do not require proof of their efficacy or safety[2,3].There is a general lack of information on the safety of herbal medicines[4].

Despite the lack of information and regulation, Ayurvedic products, like other traditional remedies, are often perceived as "safe" because they contain plant or other "natural" ingredients and have been used by millions of people for millennia. Their use, however, is not free from risks or adverse effects, due to their actual health effects[5] or their interaction with conventional Western medicine drugs[4,5,6]. Product contamination is another well-documented concern. Metals or metalloids, as well as other chemicals, including persistent organic pollutants, radionuclides and solvents have been found in Ayurvedic products[3,7-11]. Even bacteria, fungi and parasites have been found in herbal medicines, mainly because the raw material is exposed to microbial contaminants before harvesting and during handling and storage[12]. Various medicinal plant samples imported from India have been found to be contaminated by fungi or contain mycotoxins beyond the tolerance level[11,13,14,15]. However, limited research has been carried out on bacterial contamination of Ayurveda herbal medicines[4,14,16], although it may represent a serious risk for human health.

Although various plant products have shown anti-mutagenic and anti-oxidant properties in *in-vitro* experiments[17,18,19,20], the possible genotoxic effects of Ayurvedic remedies have been poorly investigated so far. This is in contrast with worldwide government regulatory policies, which now recommend that all newly produced natural or synthetic substances undergo genotoxic/mutagenic screening[21].

This study aimed to assess the microbiological contamination and mutagenicity of some herbal products commonly used in Ayurvedic medicine, produced in India, according to place of purchase, namely shops or drugstores in India or Italy.

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2. MATERIALS AND METHODS

2.1 Study design

Sixteen products were bought in Indian drugstores, herbalist's shops, or street markets and from local producers, and in Italian herbalist's shops. All the products contained one of three plants widely used in Ayurvedic tradition, *Withania somnifera*, *Gymnema sylvestre* and *Phyllantus niruri*, in various forms, as shown in Table 1. Microbiological contamination and genotoxicity were evaluated for each product.

Table 1: Brief description of the products investigated in the study.

Sample	Plant	Origin	Form
1	*Withania somnifera*	India, drugstore	capsules
2	*Withania somnifera*	India, herbalist's shop	powder
3	*Withania somnifera*	India, street market	powder
4	*Withania somnifera*	India, street market	roots
5	*Withania somnifera*	India, local production	powder
6	*Gymnema sylvestre*	India, drugstore	capsules
7	*Gymnema sylvestre*	India, herbalist's shop	powder
8	*Gymnema sylvestre*	India, street market	powder
9	*Gymnema sylvestre*	India, street market	leaves
10	*Gymnema sylvestre*	India, local production	powder
11	*Phyllantus niruri*	India, drugstore	capsules
12	*Phyllantus niruri*	India, herbalist's shop	powder
13	*Phyllantus niruri*	India, street market	branches
14	*Withania somnifera*	Italy, herbalist's shop	tablets
15	*Gymnema sylvestre*	Italy, herbalist's shop	capsules
16	*Phyllantus niruri*	Italy, herbalist's shop	tablets

2.2 Microbiological contamination

Since an ad hoc regulation is not available for herbal medicines, the microbiological quality of the products was evaluated by following the indications of the European Pharmacopoeia for microbiological examination of oral non-sterile products. These criteria establish a limit of 10^3 bacteria and 10^2 fungi per gram or millilitre of product and the absence of *Escherichia coli*[22]. The total viable aerobic count (bacteria and fungi) was performed as an index of overall microbial contamination, and the presence of *E. coli* was evaluated as an indicator of possible faecal contamination.

10 g of material was dissolved in 100 ml of Buffered Peptone Water 0.1% Tween and the solutions were maintained at 4°C. Afterwards, the presence of the mentioned microorganisms was searched for using different procedures.

2.2.1 Total viable aerobic count

1 ml of each sample solution was transferred to Casoy TSA Agar plates for bacteria and to Sabouraud Chloramphenicol Agar plates for fungi, two plates for each level of dilution. The plates were incubated for 5 days at 35°C for bacterial and at 25°C for fungal grown. After the incubation, colonies grown on the medium were counted, selecting the plates corresponding to one dilution and showing the highest number of colonies less than 300 for bacteria and 100 for fungi. The arithmetic average of the count was calculated and the number of colony-forming units (CFUs) per gram of sample was calculated, taking the dilution factor into account.

2.2.2 Presence of Escherichia coli

10 ml of each sample solution (corresponding to 1 g of original product) was used to inoculate 100 ml of Triptone Soya Broth, homogenized and incubated at 35°C for 48 hours. 1 ml was then transferred to 100 ml of MacConkey Broth and incubated at 45°C for 24 hours. Any changes in colour or gas production in the medium were recorded: change in colour from purple to yellowish and gas production suggest growth of *E. coli* or coliforms. One ml of this last solution was then transferred to MacConkey Agar n. 3 plates and incubated at 35°C for 72 hours. Red, non-mucoid colonies of gram-negative rods, indicating the possible presence of *E. coli*, were transferred to tubes containing SIM medium and incubated at 35°C and 44°C for 24 hours. At the end of the incubation period, 2 drops of Kovacs reagent were added to each tube. These last passages in SIM medium, detecting indole and H_2S production and motility of the organisms, can confirm the presence of *E. coli*, which typically produces indole and not H_2S.

2.3 Mutagenic activity

For the mutagenicity test, two types of sample solution were prepared. For the first one, 0.2 g of sample material was diluted in 4 ml of sterile distilled water to obtain a concentration of 5 mg/ml. These solutions were maintained at 4°C, stirred for the entire duration of the test, since they were very thick, and tested in a wide range of doses (0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mg/plate). Since these sample solutions showed strong microbial contamination, making the colony count very difficult, a second type of solution was prepared for each sample: 0.02 g of material was diluted in 4 ml of distilled sterile water (concentration 0.5 mg/ml), filtrated on a 0.2 µm filter to remove bacterial and fungal contamination, and tested in a limited range of doses (0.01, 0.1 and 1 mg/plate). The concentration of this second solution was lower because the density of the first solution did not allow the filtration.

Mutagenic activity of the 16 samples of Ayurvedic products was tested by means of the Ames test, which detects gene mutations in *Salmonella typhimurium*[23]. TA98 and TA100 strains were used to detect two different mutations of DNA sequence, frame-shift mutation and base-pair substitution. The test was performed with and without microsomal activation (S9 mix, 4%) to search for the presence of indirect and direct mutagenic substances. 2-nitrofluorene for TA98 without S9 (10 µg/plate), sodium azide for TA100 without S9 (10 µg/plate) and 2-aminofluorene for both strains with S9 mix (20 µg/plate) were used as positive controls. Distilled water was used as a negative control.

We computed the average of the number of colonies on triplicate plates according to guidelines for performing and interpreting bacterial mutation assays[24,25]. We considered the results positive if two consecutive dose levels or the highest non-toxic dose level produced a response at least twice that of the solvent control and when at least two of these consecutive doses showed a dose–response relationship. Only in this case it is possible to calculate the number of net revertants/mg of drug by means of a linear regression analysis of revertants/plate on 1 mg of the sample tested.

3. RESULTS

3.1 Microbiological contamination

Microbial contamination analysis was not performed on the 3 samples in the form of raw material (4, 9 and 13) because they showed strong mycotic contamination. The results of the total viable aerobic count and *E. coli* investigation of the 13 tested samples are shown in Table 2.

Four of the five products obtained from Indian herbalist's shops and local producers, showed a fungal count higher than the limit of 100 CFU/g (samples 2, 5, 7 and 10) and four had a total bacterial count higher than the limit of 1000 CFU/g (samples 2, 5, 7 and 12). Three of them (samples 2, 5 and 7) were highly contaminated by both fungi and bacteria. The fungal count was also over the limit for one of the two products obtained from Indian street markets (sample 3). All the products from Indian drugstores (samples 1, 6 and 11) and

Italian herbalist's shops (samples 14, 15 and 16) showed contamination levels under the limits for both bacteria and fungi.
As regards the faecal contamination index, *E. coli* was found in four products only: one from Indian street markets (sample 3) and three of the five products from Indian herbalist's shops and local producers (samples 2, 5 and 7). Again, none of the products from Indian drugstores and Italian herbalist's shops showed *E. coli* contamination.

Table 2. Microbiological contamination. Total viable aerobic counts (fungi and bacteria) are expressed as colony-forming units (CFUs) per gram of products. Samples showing *Escherichia coli* contamination were considered positive (+) to the test.

Source	Sample	Fungi CFU/g	Bacteria CFU/g	*E. coli*
Indian street markets	3	> 100	99.30	+
	8	4.95	29.70	-
Indian herbalist's shops and local producers	2	> 100	> 1000	+
	5	> 100	> 1000	+
	7	> 100	> 1000	+
	10	> 100	546.45	-
	12	14.72	> 1000	-
Indian drugstores	1	28.65	119.39	-
	6	0	23.43	-
	11	14.65	87.89	-
Italian herbalist's shops	14	0	14.84	-
	15	0	0	-
	16	0	0	-
	Limit[a]	100	1000	absence

[a] *European Pharmacopoeia 5.0 (2004) limit for non-sterile oral preparations.*

3.2 Mutagenic activity: Ames test

The results of the first Ames test, expressed as number of net revertants/mg of drug, are shown in Table 3. Reading of the results was difficult for all the Indian products (samples 1-13) because various types of bacterial colonies and moulds grew on the plates, hindering the count of Salmonella colonies. For one sample (9), the contamination was so high that colony count was possible only for some doses and the regression analysis could only be performed with TA100. The Italian samples (14, 15 and 16) did not display this microbial contamination, and the plates and the colonies grown on them had the typical appearance of Ames test plates.
Only two samples (2 and 15), one Indian and one Italian product, were completely free from mutagenic activity in both *S. typhimurium* strains, with and without S9 mix.
Three samples (1, 14 and 16), two of which were Italian products, showed slight genotoxicity, giving positive results with one bacterial strain only (TA98 for samples 1 and 14 and TA98+S9 for sample 16). The number of net revertants/mg of drug was low in samples 14 and 16. Regression analysis could not be performed for sample 1 with TA98, because only the highest useful dose showed genotoxicity, the number of revertants being double that of spontaneous revertants.
All the other samples (3-13) induced point mutations in both *S. typhimurium* strains, with and without S9 mix, net revertants values ranging from 20.55 rev/mg to 25,764 rev/mg. For sample 5 with TA100 and sample 11 with TA98, net revertants could not be calculated because only the highest non-toxic dose gave positive responses.
Owing to the strong contamination of the samples and the difficult reading of the first tests, a second series of tests was performed in which sample solutions were filtered before use. The solutions were diluted more to allow filtration and were tested at three doses only, corresponding to those in the first experiment. The entire second series of tests was negative, neither the Indian nor the Italian samples displaying any mutagenic activity (data not shown in tables)

Table 3: Ames test on TA98 and TA100 *Salmonella typhimurium* strains.

Source	Sample	TA98 (rev/mg)	TA98+S9 (rev/mg)	TA100 (rev/mg)	TA100+S9 (rev/mg)
Indian street markets	3	20.55	24.54	23.94	21.95
	4	77.72	90.57	83.94	10.73
	8	481.38	344.86	476.61	1,754.00
	9	ND	ND	25,764.00	ND
	13	90.42	444.76	59.39	90.84
Indian herbalist's shops and local producers	2	0	0	0	0
	5	29.45	22.09	+	33.38
	7	141.72	137.46	246.35	242.06
	10	93.91	179.49	106.28	155.54
	12	5,278.50	3,934.50	2,627.80	984.26
Indian drugstores	1	+	0	0	0
	6	103.09	160.01	245.26	108.48
	11	+	48.25	95.29	26.34
Italian herbalist's shops	14	13.81	0	0	0
	15	0	0	0	0
	16	0	29.30	0	0

The results are expressed as net revertants per mg of drug (rev/mg).
Net revertant value could not be calculated in three cases
0: no genotoxicity at all tested doses;
+: only the last non-toxic dose showed genotoxicity;
ND (not detected): no colony count because of high contamination of the plates.

4. DISCUSSION

Despite the widespread global use of herbal products, most of which are included in local traditional medical systems, particularly Indian and Chinese traditional medicine, the efficacy and safety of these products, as well as their quality, are of the highest concern for both health authorities and population[26].
This research assessed the microbiological contamination and genotoxic activity of some Ayurvedic herbal products, on sale in various places in India and Italy. Our findings rise concerns about the possible risks for human health deriving from the consumption of these products.
Microbial contamination was investigated by assessing total viable aerobic count (bacteria and fungi) and the presence of *E. coli*, as required by the European Pharmacopoeia for microbiological examination of oral non-sterile products[22]. Only seven of the sixteen samples - all the products bought in Italian herbalist's shops and in Indian drugstores and one of the two products obtained from an Indian street market - were in line with the European parameters (Table 2). One of the products from Indian street markets and all the samples from Indian herbalist's shops and local producers showed contamination by bacteria, fungi and/or coliforms. Three of these samples (2, 5 and 7) were heavily contaminated by both aerobic bacteria and fungi (over 1000 and 100 CFU/g, respectively) and also showed the presence of *E. coli*.
Biological contamination can occur at any stage of drug production: contaminants can be the result of the environment in which the medicinal plants are grown, the conditions under which they are dried and processed, the storage and transport conditions, or the manufacturing processes for ready-made medicinal products[27]. Various medicinal plant samples imported from India were reported

in recent studies to be contaminated by fungi or to contain mycotoxin levels beyond the tolerance level fixed by WHO[11-15,28,29]. Fungal contamination has been reported to affect the chemical composition of the raw materials and thereby decrease the medical potency of herbal drugs[30]. Moreover, the toxins elaborated by these fungi elicit a wide spectrum of toxic effects when the contaminated materials are ingested[12]. In contrast, limited research has been carried out on bacterial contamination of Ayurveda herbal medicines[14,16,28], but when it occurs, this contamination may represent a risk for human health, due to the type of bacterium itself, the possible toxins it produces and a decrease in the therapeutic potency of the drugs.

Our findings with regard to microbial contamination suggest that products of industrial origin, such as those bought in Italian herbalist's shops and Indian drugstores, are less contaminated than locally produced ones, bought in Indian herbalist's shops and street markets. The better quality of the industrial products is probably related to the method of production, which adheres to higher standards regarding plant harvesting, storage, treatment and transformation and is subject to quality control. On the other hand, less attention to these aspects may be paid by small-scale local producers, who sell their products at local markets, not wholesale to the trade. The problem of the quality of herbal remedies derives mainly from the lack of regulation. For this reason, the WHO recently issued some documents for controlling the quality and safety of these products, including guidelines on research and evaluation methods, good agricultural and collection practice (GACP) and good manufacture practice (GMP)[1].

The genotoxic properties of Ayurvedic products were analysed using the Salmonella microsome/Ames test, on TA98 and TA100 strains of *Salmonella typhimurium*, with and without metabolic activation, in two different experiments. In the first experiment, almost all samples (14 out of 16) showed genotoxic activity. Substantial differences were found between the Indian and Italian samples: the former showed high genotoxic activity, inducing point mutation in both strains, with and without metabolic activation, and with very high values of net revertants, whereas the Italian samples displayed low genotoxicity. The difference between industrial and local products, was less conspicuous for mutagenic potential than that seen in microbiological tests. However, in all the Indian, but none of the Italian samples, the plates showed strong contamination caused by bacteria and moulds, which made it difficult to read the plates. We therefore decided to perform a second experiment, i.e. repeating the test after sample filtration in order to avoid this microbial contamination, with negative results for all samples.

Taken together, these findings show that these products display genotoxic activity, although this was modest or absent in the Italian drugs. The original plants do not seem to be responsible for the genotoxic effect, however, since mutagenicity disappears after sample filtration. In effect, the 0.2 µm filter used in the study allowed plant macromolecules to pass through, but not microorganisms, which contaminated most Indian samples (Table 2) and therefore are likely to have induced the genetic damage. Genotoxicity of Ayurvedic products may also be due to other substances, such as metals, persistent organic pollutants, radionuclides or solvents, deriving from the polluted environment (air, soil, water) in which the plants are grown, the use of chemical compounds in agriculture or contamination occurring at any stage of production[3,7,8,9,10,11,27]. Clearly, whatever substance induces mutations in *Salmonella typhimurium*, the effect is linked to the product itself.

Few studies have been carried out on the mutagenicity of Ayurvedic medicines. One of them investigated the mutagenic activity of a multi-herbal formulation containing *Withania somnifera* and found no mutagenicity using the Ames test[31]. Other studies investigated mutagenic potential of the raw materials (leaves, stems, etc.) and manufactured products (powder), showing no mutagenic activity when using the Ames test or other mutagenicity assays[32,33,34,35]. Only weakly positive results were observed when performing the chromosomal aberration assay on human blood cells treated with *Salaciaoblonga* extracts[34]. More research, using standardized methods of investigation, is required to ensure that these products do not constitute a threat for the consumer.

5. CONCLUSION

In conclusion, our findings suggest that some Ayurvedic products, especially those sold in Indian herbalist's shops and street markets, may be not suitable for human consumption. The health risk seems to be related not to the plant from which they derive, but rather to contamination by bacteria and fungi and possibly by chemical substances derived from the production process. In addition, products of industrial origin, particularly Italian ones, seem to be substantially safe, suggesting that complying with standard controls procedures during production and distribution process leads to better product quality and safety, and hence that an *ad hoc* regulation is urgently needed for all traditional medicine remedies.

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