Wageningen Academic Publishers

Quality evaluation of commercially available Triphala powder: a renown dietary supplement of Indian system of medicines

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Received: 6 January 2014 / Accepted: 18 August 2014

RESEARCH ARTICLE

Abstract

Use of herbal formulations is increasing day by day. Triphala also witnessed the increase in demand due to its various therapeutic uses. This led to the availability of a number of brands in the market. Being a plant based formulation it is highly vulnerable to adulteration and contamination that can finally alter the efficacy of the product and may pose serious health risks for consumers. In this study various physicochemical and phytochemical parameters were studied to assess the quality of the marketed product. Morphological and microscopic characteristics of the Triphala powder samples showed the adulteration of powdered endocarp of the ingredients. A variation was also observed in pH and moisture values. Excluding one or two samples, ash values were found within permissible limits. Samples of all categories of manufacturers were found contaminated with various fungal species and the majority of them exceeding the permissible limit of 10^3 spores/g for the medicinal formulation of internal uses as set by the World Health Organization. A remarkable variation in therapeutically important phytoconstituents was also observed among the samples of popular brands. Findings of this study suggest for formulation of stringent quality control guidelines for herbal formulations so that maximum benefits can be obtained from these traditional formulations.

Keywords: Triphala, adulteration, contamination, phytochemical variation

1. Introduction

Triphala powder is a widely used poly-herbal formulation of Ayurvedic system of medicine. It is a powdered mixture of dried fruit pulp of three important myrobalans, i.e. *Emblica officinalis* Gaertn., *Terminalia belerica* Roxb. and *Terminalia chebula* Retz. in equal proportion. It is easily available in the global market as a dietary supplement. This formulation is considered as an important *rasayana* (herb that confers youthfulness and cure diseases) in Ayurvedic system of medicine. The recipe for this Ayurvedic formulation is described in the ancient books on Ayurveda, the *Charak Samhita* and *Susruta Samhita* which date back to 1500 B.C. (Gupta, 2010). Triphala is an important formula that have balancing and rejuvenating effects on the three constitutional elements of the body that Ayurveda believes govern human life (Gupta, 2010). Triphala is a mild, non-habit forming, safest and most strengthening laxative and purgative formulation hence recommended for all age groups. Ayurvedic doctors and practitioners recommend this formulation for the treatment of the constipation, weight loss (Hashimoto and Nakajima, 1997), enhances intelligence, strength, youth, lustre, sweetness of voice and vigour (Gupta, 2010). In addition, daily use of Triphala creates a favourable chemical environment for the proliferation of beneficial intestinal bacteria and an unfavourable environment for non-beneficial intestinal bacteria (Gupta, 2010). It is also used as rejuvenating agent and widely recommended by herbal practitioners (HP) for various ailments of the human body and often called elixir of life.

In the present context use of herbal medicines is increasing day by day, probably due to the belief of people that consumption of natural products is healthier than conventional medicines. Among various herbal formulations; Triphala powder also witnessed the increased demand. This increased demand may lead to

indiscriminate and unscientific collection, misidentification of ingredients and adulteration, making the quality of product below standard. The raw material used by the drug industry and communities in large cities, towns and regions is generally procured through market channels and is sometimes found adulterated. Being a plant based formulation; Triphala powder is also very sensitive to fungal invasion and contamination. Storage conditions, moisture content, pH of the samples also plays significant role in the determination of the quality of the products. The adulteration and microbial contamination may result in remarkable rapid quality deterioration and can render the herbal formulations unfit for the export and consumption. Adulteration can also alter the bioefficasy of the products (Gunasekaran and Anita, 2010). Research has already shown a steady and marked presence of fungi in various herbal formulations and their raw material. A number of fungal species have been reported worldwide from various herbal products (Bugno *et al.*, 2006; Gautam and Bhadauria, 2008, 2009a,b; Halt, 1998; Singh *et al.*, 2008;). Secondly, the quantity of important phytoconstituents also determines their therapeutic potential. Depending upon the mode of processing, formulation/drugs possess theses active constituents in specific amounts, generally lower than the raw materials. But inferior raw material, adulteration and microbial contamination can generate large variations among the brands of the formulations in term of quantity of phytoconstituents. Thirdly, due to increased demand and good results, adulterations of herbal formulations with synthetic drugs has now become a very common problem (Bogusz *et al.*, 2006; Campbell *et al.*, 2013; Cianchino *et al.*, 2008; Ernst, 2002; Feng *et al.*, 2007; Kua *et al.*, 2003; Lau *et al.*, 2003; Snyman *et al.*, 2005).

Therefore in views of the wide concern over the quality and poor efficacy of the herbal formulations, it is essential to access the quality of the marketed Triphala powder. In this study an attempt was made to analyse the physicochemical and phytochemical parameters of Triphala powder that are directly associated with the quality of the product.

2. Materials and methods

Material

Sixty Triphala powder samples were randomly collected from retailers and HP of the selected sites (Maharaj Bada, Morar, Dabra, Pichhor, Bhitarwar and Gwalior trade fair) of Gwalior region (Madhya Pradesh, India) in two consecutive years, i.e. 2009 and 2010. On the bases of manufacturer, collected samples were categorised in to samples of internationally recognised manufacturers (IRM), regional manufacturers (RM), local manufacturers (LOC) and HP. Collected samples were subjected to morphological, microscopic, physicochemical and phytochemical analysis.

A total of twenty samples of Triphala powder belonging to the top ten most popular brands (10 samples of selected types in year 2009 and 10 samples of the same types in year 2010) were also subjected to phytochemical analysis of therapeutically important phytoconstituents like total phenolic, tannin and ascorbic acid.

Physicochemical analysis

Preparation of standard Triphala powder

Fruit pulp was scraped from the surface sterilised ingredient fruits of Triphala powder and dried in oven at 60 °C (Ambassador, B.P. Industries, Delhi, India). After drying, fruit pulp was ground to fine powder and stored in airtight containers for further use.

Morphological examination of collected samples

Collected samples were examined morphologically under magnifying glass for their physical appearance like colour, powder fineness and the presence of any visual clumps.

Microscopic examination

Selected brands of Triphala powder were successively evaluated during the years 2009 and 2010 for the presence of foreign objects like: moulds, insects, animal excreta, small pebbles and stony parts of fruits (endocarp), etc. by microscopy.

Small amounts of Triphala powder (in triplicate) were placed on a glass slide containing lacto-phenol-cotton blue stain and visualised for the presence of mould hyphae. Glycerol mounted samples were observed under a Trinocular microscope (Metzer, Mumbai, India) at different magnification for the presence of dead insects/ their remains, animal excreta, small pebbles and stony parts (endocarp) of fruits, etc.

Stony parts of the fruits were detected by comparing the Triphala powder samples with the standard Triphala powder, prepared from the dried fruit pulp only.

Determination of total ash/acid insoluble ash

Triphala powder samples collected during the year 2009 were evaluated for the total ash and acid insoluble ash content by the standard method as suggested by the World Health Organization (WHO, 2005).

2 g of air dried material was taken in previously ignited, weighed silica crucible and ignited at 500 to 600 °C until material turn white, indicating absence of carbon. The crucible was cooled in a desiccator (ASGI Industries, Agra, India) and weighed (Shimadzu Corporation, Kyoto,

Japan). After weighing, the ash was further treated with concentrated HCL for the analysis of acid insoluble ash.

Total ash was calculated by the following formula:

Total ash = final weight of crucible containing ash – initial weight of ignited crucible (1)

All samples were treated in triplicate and results were expressed in g/100 g.

To the crucible containing the total ash, 25 ml of hydrochloric acid was added. Covered with a watch-glass and made to boil gently for 5 minutes. A watch-glass with vapours was rinsed with 5 ml of hot distilled water and this water was added to the crucible. Insoluble matter was collected on an ashless filter-paper and washed with hot water until the filtrate became neutral. Filter-paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in suitable desiccators for 30 minutes, and then weighed without delay. Content of acid-insoluble ash was calculated in g/100 g of air-dried material.

Measurement of total moisture content

2 g powder of Triphala was taken in a previously dried and tarred flat weighing bottle in triplicate and kept in an oven at 105 °C. Samples were dried again and again until the weight of the sample in two consecutive weighing did not differ by more than 5 mg.

Measurement of pH

5% suspension of each sample was prepared in distilled water, shaken constantly for one hour. pH of the suspension was measured using an electronic pH meter (MK-V; Systronics, Ahmedabad, India). Each sample was analysed in triplicate.

Isolation and identification of mycobiota

Various fungi were isolated from the Triphala powder samples by pour plate technique, using Czapek Dox agar media (pH 7.3; Himedia, Mumbai, India) supplemented with chloramphenicol. Under aseptic condition, 1 g sample was suspended in 9 ml of sterilised distilled water and serial dilutions were made up to 10^{-5}. One ml aliquot of each sample from appropriate dilution was poured in sterilised petri plates (in triplicate) and appropriate amount of media was added in petri plates and mixed well. After solidification, plates were incubated at 25±2 °C and growth of fungal colonies was recorded at various time intervals. After seven days of incubation period, pure culture of each fungal isolate was prepared by using potato dextrose agar media (Himedia) for identification purpose.

Colony forming units per gram (cfu/g) were calculated using the following formula:

$$cfu = N \times 10^{-n} \quad (2)$$

Where N = total number of colonies; n = dilution.

Isolated fungal species were identified on the basis of morphology (shape, size, growth rate and colour of the colonies) and microscopic characteristics (characteristics of mycelium, size, shape, colour and arrangement of conidia, spore, conidiophores, sporangiophores, vesicle, sterigmata, etc.) as described by Thom and Raper (1945), Gilman (1971), Barnett (1969), Jamaluddin *et al.* (2004) and Samson *et al.*(2007a,b).

Phytochemical analysis

Therapeutically important phytoconstituents (total phenolics, tannin and ascorbic acid) were analysed during the study. Analysis of total phenolics and tannin was carried out in accordance with the Makkar *et al.* (1993), whereas ascorbic acid was estimated by a titrimetric method as suggested by Roe (1954). All the results were expressed in percentage on dry matter basis.

Calculations

Total incidence/abundance, frequency of occurrence of fungi isolated from the Triphala powder samples and total moisture content was calculated by the formulas in Box 1.

$$\text{Frequency of occurrence} = \frac{\text{number of samples containing a genera/fungal species}}{\text{total number of sample evaluated}} \times 100 \quad (3)$$

$$\text{Total incidence} = \frac{\text{number of isolates of the species}}{\text{total number of isolates}} \times 100 \quad (4)$$

$$\text{\% moisture content} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100 \quad (5)$$

Box 1. Formulas.

Statistical analysis

One-way ANOVA at $P<0.05$ followed by Tukey HSD test was used to determine the significance of the data using Minitab-16 software (Minitab Inc., Pennsylvania, USA).

3. Results

Results of various experimental parameters revealed remarkable changes in characteristics of the analysed samples of Triphala powder. Individual parameters showed the marked difference between the various brands and categories of Triphala powder samples at morphological, physicochemical and phytochemical levels. A large number of fungal species were also recorded from the samples of Triphala powder.

Morphological analysis

Morphological examination revealed notable variations in colour, powder fineness and texture of collected samples (Table 1). The powder fineness of Triphala varied from brand to brand and sample to sample. As it is evident from the Table 1, the majority of the samples (25/60) were moderately coarse. Whereas 13/60 samples were moderately fine, 10/60 were fine, 7/60 were very fine and only in 3 samples, the powder was coarse. Remaining two samples, one from each IRM and RM were in tablet forms. On the basis of category of powder, out of seven samples of the very fine powder category, six samples were of RM and one was of IRM. This was followed by fine category powder, where six samples were of IRM and four of RM; moderately fine category had four samples of IRM and nine samples of RM. In moderately coarse category, three samples of IRM, eleven samples of RM, nine samples of LOC and two samples of HP were recorded. While only one sample of regional and two samples of HP were recorded in the coarse category of powder.

Depending upon the period of storage, the colour of Triphala powder sample was also found to vary from brand to brand. Various shades of brown varying from khaki to dark brown were observed during morphological analysis. Out of sixty analysed samples, the majority of the samples (45%) were light brown in colour followed by 22.66% with khaki colour, 20% saddle brown and only 8.33% samples were dark brown in colour. It was observed that generally fresh samples were khaki in colour and with the increased storage period it changes to light brown, saddle brown and finally dark brown. On the basis of manufacturing category, 6 and 10 samples of IRM and RM, respectively, were khaki in colour. Whereas 4 samples of IRM, 13 of RM, 9 of LOC and 1 sample of HP were light brown in colour. While saddle brown colour was observed in 4 samples of IRM, 5 RM and 3 samples of HP. Similarly dark brown colour was recorded from, 1 sample of IRM and 4 of RM.

During morphological investigation, 12 Triphala powder samples (4 IRM, 6 RM, 1 LOC and 1 HP) with solid clumps were also observed. Clumps were generally recorded from the samples with prolonged storage period. Generally Triphala with coarse, saddle brown to dark brown powder with clumps is regarded as substandard in quality.

Microscopic analysis

On the basis of popularity, top ten most popular brands of Triphala powder were analysed for the presence of any foreign matter (moulds, insects, animal excreta, small pebbles, etc.) and stony parts of fruits (endocarp) by microscopic method in two consecutive years i.e. 2009 and 2010 (Table 2). The study revealed the presence of mould, insects, animal excreta, insect's remains and hairs in five samples one each from local and herbal practitioner collected during the year 2009 and 3 samples one each from regional, local and herbal manufacturer collected during the year 2010. While all the samples of (15/15) IRM were free from foreign matter.

Table 1. Morphological characterisation of Triphala powder samples.[1]

	Powder texture						Colour				Clumps
	Very fine (n=7)	Fine (n=10)	Moderately fine (n=13)	Moderately coarse (n=25)	Coarse (n=3)	Tablets (n=2)	Khaki (n=16)	Light brown (n=27)	Saddle brown (n=12)	Dark brown (n=5)	(n=12)
IRM	1/7	6/10	4/13	3/25	-	1/2	6/16	4/27	4/12	1/5	4/12
RM	6/7	4/10	9/13	11/25	1/3	1/2	10/16	13/27	5/12	4-5	6/12
LOC	–	–	–	9/25	–	–	–	9/27	–	–	1/12
HP	–	–	–	2/25	2/3	–	–	1/27	3/12	–	1/12

[1] IRM = internationally recognised manufacturers; RM = regional manufacturers; LOC = locally prepared Triphala powder; HP = Triphala powder collected from herbal practitioner; n = total number of samples.

Table 2. Microscopic analysis and ash contents of selected Triphala powder samples collected in 2009 and 2010.[1]

Sample	Foreign matter		Stony parts of fruits		Total ash (g/100 g)	Acid insoluble ash (g/100 g)
	2009	2010	2009	2010		
IRM-D	–	–	–	+	4.40±0.68	0.61±0.38
IRM-B	–	–	+++	+++	4.05±0.63	1.00±0.32
IRM-Z	–	–	+	+	4.70±0.425	0.883±0.18
IRM-DI	–	–	+	+	4.85±0.32	0.68±0.12
RM-B	–	–	+	+	3.85±0.13	0.43±0.07
RM-U	–	+	++	++	3.38±0.24	0.316±0.07
RM-S	–	–	++	+++	4.53±1.49	0.616±0.057
LOC-1	–	+++	++++	++++	3.35±0.04	0.805±0.04
LOC-2	++	–	+++	+++	4.83±0.25	0.332±0.04
HP	+	++	++	+++	8.90±0.26	5.11±0.66

[1] IRM = internationally recognised manufacturers; RM = regional manufacturers; LOC = locally prepared Triphala powder; HP = Triphala powder collected from herbal practitioner; – = absent; + = minute; ++ = moderate;+++ = high; ++++ = very high.

Adulteration of Triphala powder with stony parts of fruits (endocarp) was also a very common observation. Minute to very high quantity of powdered stony parts was recorded in all 10 and 9 out of 10 samples collected during the years 2010 and 2009 respectively, indicating the substandard quality of the marketed Triphala powder samples. The presence of higher amount of powderd endocarp was detected from the regional, local and HP samples as compared to the samples of IRM. Triphala powder of local manufacturer (code 'LOC-1') was found highly adulterated with stony parts of fruits whereas a sample of an IRM manufacturer, with code IRM-D, was the least adulterated (Table 2).

Ash content

During the present investigation, the value of total ash content was between 3.35±0.04 to 8.90±0.26 g/100 g. Whereas, acid insoluble ash content was observed in the range of 0.332±0.04 to 5.11±0.66 g/100 g (Table 2). The highest amount of total ash (8.9±0.26 g/100 g) and acid insoluble ash (5.11±0.66 g/100 g) was recorded from the samples of herbal practitioner category (code HP) and locally manufactured (code LOC-1), respectively. Whereas a minimum of 3.35±0.04 g/100 g (total ash) and 0.316±0.07 g/100 g (acid insoluble ash) was recorded from the Triphala powder sample of locally manufactured (code LOC-1) and a RM sample (code RM-U), respectively.

As per the guidelines of 'Ayurvedic Pharmacopeia of India' (2005), the total ash content of the individual ingredients of Triphala powder should be <5% for *T. belerica* and *T. chebula* and <7% for *E. officinalis*.

pH of the samples

Not much variation was observed among the samples collected during the years 2009 and 2010. The pH of samples collected during the years 2009 and 2010 was in the range of 3.57 to 6.24 and 3.53 to 6.25 respectively (Table 3). Overall observations revealed a strong acidic range in the majority of the samples. After analysing pH of 60 samples the values have been grouped into various ranges (Table 4). Maximum number of samples (25) showed a pH range between 3.61-3.70, followed by 3.71-3.80 (in 16 samples), 3.50-3.60 and 4.1-5.0 (5 samples each), 3.91-4.0 (in 4 samples), 3.81-3.90 (in 3 samples), and only in two samples pH was in the range of 5.1-7.0. On the basis of manufacturing category, out of 15 samples of IRM, 8 samples were in the pH range of 3.71-3.80 followed by 3 samples in 3.61-3.70 range, one sample each in pH ranges 3.50-3.60 and 3.91-4.0. In this category high pH value 6.24 and 6.25 was observed only in two samples available in tablet and powdered form, respectively. Among 32 samples of RM, 18 samples were in the pH range of 3.61-3.70 followed by 3 samples in each pH range 3.50-3.60, 3.71-3.80, 3.81-3.90, 3.91-4. In only two samples the pH was 4.1 and 4.11. In locally manufactured Triphala powder, out of 9 samples, 4 were recorded in the pH range of 3.61-3.70, 3 in 3.71-3.80 and rest of two in 4.1-5.0. In case of the HP category two samples were recorded in the pH range of 3.71-3.80 and one each in 3.50-3.60 and 4.1-5.0, respectively (Table 4).

Table 3. Analysis of moisture content percentage and pH in various samples of Triphala powder samples collected during the years 2009 and 2010.

Serial no.	Year 2009		Year 2010	
	pH	% moisture	pH	% moisture
1	3.75	5.44	3.60	9.05
2	3.72	4.17	6.25	4.80
3	3.72	8.70	3.76	8.63
4	3.72	6.67	3.75	8.02
5	3.68	6.78	3.64	6.42
6	3.72	6.30	4.00	8.40
7	3.74	5.77	3.72	7.53
8	3.68	5.89	3.61	8.87
9	6.24	4.16	3.68	9.90
10	3.61	8.40	3.66	7.38
11	3.69	6.05	3.96	5.02
12	3.65	7.49	3.53	9.11
13	3.63	8.36	3.60	9.35
14	4.11	5.34	3.61	5.69
15	4.10	5.56	3.66	9.98
16	3.57	8.32	3.88	8.05
17	3.68	6.20	3.63	8.72
18	3.67	7.30	3.65	7.90
19	3.65	8.09	3.78	9.47
20	3.69	4.13	3.78	8.18
21	3.93	5.92	3.61	8.97
22	3.62	9.8	3.82	9.42
23	3.81	6.27	3.68	10.20
24	4.00	5.30	3.68	6.58
25	3.76	7.03	3.61	9.28
26	3.73	7.04	4.03	9.40
27	4.08	5.77	3.78	10.24
28	3.68	6.17	3.67	9.43
29	3.75	9.14		
30	3.73	4.94		
31	4.31	5.96		
32	3.54	8.75		

Moisture content of the samples

Collected samples of Triphala powder were analysed for their moisture content percentage. The moisture content of the collected samples was recorded in between 4.13% to 10.24% (Table 3).

A variation in moisture content was observed among the samples collected during the year 2009 and 2010. The percentage moisture content range was 4.13 to 9.14% and 4.8 to 10.24% in the samples collected during the year 2009 and 2010 respectively (Table 3). It was noted that samples collected during 2010 have comparatively higher moisture content (12 samples with >9% moisture content) than the samples collected during the year 2009 (only 2 samples with >9% moisture content).

As indicated in Table 5, more than 50% of the samples had moisture content percentage >7%. A high number of samples (in total 28, i.e. 14 in each category) were in the range of 8-9 and 9-11% moisture followed by 11 samples with 5-6%; 9 in 6-7% and 7 in 7-8% moisture range, whereas only 5 samples had <5% moisture content. It was also observed that samples of RM and LOC had a higher percentage of moisture content than Triphala powder samples of IRM. Among the samples of RM, out of 32 samples, more than 50% samples (17) were with moisture content >8%, whereas high moisture content (more than 9%) was observed only in 1 sample of IRM. In locally manufactured samples, about 44.44% of the samples (4) were with the relatively high moisture content (more than 9%). While in case of samples of HP, 2 samples showed more than 8% content (Table 5). During investigation loose clumps of powder were also present in Triphala samples with high moisture content.

Mycobiota of Triphala powder

A high level of fungal contamination was observed in the majority of the collected Triphala powder samples. As indicated in Table 6, 75% of the Triphala powder samples

Table 4. Category-wise distribution of Triphala powder samples in various pH ranges.[1]

pH range (5% suspension)	Overall samples (n=60)	IRM (n=15/60)	RM (n=32/60)	LOC (n=9/60)	HP (n=4/60)
3.50-3.60	5	1	3	–	1
3.61-3.70	25	3	18	4	–
3.71-3.80	16	8	3	3	2
3.81-3.90	3	–	3	–	–
3.91-4	4	1	3	–	–
4.1-5.0	5	–	2	2	1
5.1-7.0	2	2	–	–	–

[1] IRM = internationally recognised manufacturers; RM = regional manufacturers; LOC = locally prepared Triphala powder; HP = Triphala powder collected from herbal practitioner; n = total number of samples.

Table 5. Category wise distribution of Triphala powder samples in various moisture ranges.[1]

% moisture range	Overall samples (n=60)	IRM (n=14/60)	RM (n=32/60)	LOC (n=9/60)	HP (n=4/60)
4-5	5	2	1	–	1
5-6	11	3	6	1	1
6-7	9	4	3	2	–
7-8	7	–	5	2	–
8-9	14	4	9	-	1
9-11	14	1	8	4	1

[1] IRM = internationally recognised manufacturers; RM = regional manufacturers; LOC = locally prepared Triphala powder; HP = Triphala powder collected from herbal practitioner; n = total number of samples.

Table 6. The percentage of samples contaminated with fungi.[1]

Sample type	No. of samples	cfu range	No. (and %) of contaminated samples	No. of sample with cfu>10^3 spores/g (%)
Total collected	60	10^2-10^5	45 (75%)	68.33
IRM	15	10^2-10^5	11 (73.33%)	66.66
RM	32	10^2-10^5	25 (78.12%)	75
LOC	9	10^2-10^4	7 (77.77%)	55.55
HP	4	10^3-10^4	2 (50%)	50

[1] IRM = internationally recognised manufacturers; RM = regional manufacturers; LOC = locally prepared Triphala powder; HP = Triphala powder collected from herbal practitioner.

were contaminated with various types of fungal species with varying degree of frequency of occurrence. A total of 2,040 isolates belonging to seven different genera, i.e. *Aspergillus, Penicillium*, *Alternaria*, *Curvularia*, *Fusarium*, *Chaetomium* and *Rhizopus* were isolated during the study. In all, 16 fungal species, *Aspergillus niger* van Tieghem, *Aspergillus carbonarius* (Bain.) Thom, *Aspergillus luchuensis* Inui, *Aspergillus flavus* Link, *Aspergillus fumigatus* Fresenius., *Aspergillus nidulans* (Eid.) Winter, *Aspergillus terreus* Thom, *Aspergillus ochraceous* Wilhelm, *Aspergillus wentii* Wehmer, *Penicillium citrinum* Thom, *Penicillium chrysogenum* Thom, *Alternaria alternata* (Fr.) Keissl., *Curvularia lunata* (Wal.) Boedijn and *Fusarium* spp. Link, *Chaetomium* Kunze and Schmidt and *Rhizopus* Ehrenberg were found to be associated with various brands of samples. Among these, the *Aspergillus* group was the most dominant genera with 9 species followed by two species of *Penicillium* and species of *Alternaria*, *Curvularia*, *Fusarium*, *Chaetomium* and *Rhizopus*. *A. niger* was recorded as the most prevalent fungal species, with 63.33% frequency of occurrence followed by *A. fumigatus* (26.66%), *A. flavus* (20%), *A. carbonarius* (16.66%), *A. nidulans* (10%), *A. luchuensis* (8.33%), *A. alternata*, *C. lunata*, *Fusarium* sp. (6.66% each), *A. terreus* (5%), *A. ochraceous*, *P. citrinum*, *P. chrysogenum* (3.33% each), whereas *A. wentii* and species of *Chaetomium*. and *Rhizopus* were the least recorded fungi, with 1.66% frequency (Table 7).

While studying the overall dominance, i.e. total incidence, of isolated fungi, *Aspergillus* was recorded as the most dominant genera with a total incidence of 96.81%, i.e. out of 2,040 isolates 1,975 isolates were of aspergilli. Remaining 3.18% isolates (65/2,040) belonged to the genera *Penicillium* (20/2,040), *Alternaria* (8/2,040), *Curvularia* (9/2,040), *Fusarium* (11/2,040), *Chaetomium* (16/2,040) and *Rhizopus* (1/2,040) (Table 8).

During the present investigation, about 68% samples were found with a fungal load of >10^3 cfu/g. As it is evident from Table 6, out of 4 categories, samples of RM were most contaminated (78.12%), followed by samples of LOC (77.77%), IRM (73.33%) and the least contaminated were the samples collected from HP (50%). Among these categories, 75% RM, 66.66% IRM, 55.55% LOC and 50% HP samples had a fungal load of >10^3 cfu/g. As per the guidelines of the World Health Organization (WHO, 2005), total cfu of moulds should not exceed more than 10^3 fungal spores/g in medicinal plant formulations for internal use.

Table 7. Incidences of fungal contamination in Triphala powder.[1]

Sample type	Total number of samples (n=60)		IRM (n=15)		RM (n=32)		LOC (n=9)		HP (n=4)	
Isolated fungi	FO (%)	TI (%)	FO (%)	TI (%)	FO (%)	TI (%)	FO (%)	TI (%)	FO (%)	TI (%)
Aspergillus niger	63.33	74.36	66.66	84.75	59.37	63.90	77.77	78.39	50	71.66
Aspergillus carbonarius	16.66	8.82	26.66	10.96	12.5	6.95	11.11	4.93	25	12.5
Aspergillus luchuensis	8.33	2.20	6.66	0.23	12.5	4.82	ND	ND	ND	ND
Aspergillus flavus	20	4.85	6.66	0.34	25	9.30	11.11	2.46	50	7.5
Aspergillus fumigatus	26.66	2.84	20	1.03	28.12	4.03	22.22	3.08	50	6.66
Aspergillus terreus	5	1.02	ND	ND	9.37	2.35	ND	ND	ND	ND
Aspergillus nidulans	10	2.20	20	1.84	9.37	3.25	ND	ND	ND	ND
Aspergillus ochraceous	3.33	0.29	ND	ND	6.25	0.67	ND	ND	ND	ND
Aspergillus wentii	1.66	0.19	ND	ND	ND	ND	11.11	2.46	ND	ND
Penicillium citrinum	3.33	0.44	ND	ND	6.25	1.0	ND	ND	ND	ND
Penicillium chrysogenum	3.33	0.53	6.66	0.57	3.12	0.67	ND	ND	ND	ND
Alternaria alternata	6.66	0.39	ND	ND	12.5	0.89	ND	ND	ND	ND
Curvularia lunata	6.66	0.44	ND	ND	3.12	0.11	22.22	3.70	25	1.66
Fusarium sp.	6.66	0.53	6.66	0.23	3.12	0.67	22.22	4.93	ND	ND
Chaetomium sp.	1.66	0.78	ND	ND	3.12	1.25	ND	ND	ND	ND
Rhizopus sp.	1.66	0.49	ND	ND	3.12	0.11	ND	ND	ND	ND

[1] IRM = internationally recognised manufacturers; RM = regional manufacturers; LOC = locally prepared Triphala powder; HP = Triphala powder collected from herbal practitioner; FO = frequency of occurrence (%; number of samples containing a fungal species/total number of samples evaluated × 100); TI = total incidence of species isolated (%; number of isolates of the species/total number of isolates × 100); ND = not detected.

Table 8. Prevalence of fungal isolates associated with Triphala powder.

Fungal species	No. of isolates	Total incidence (%)
Aspergillus niger	1,517	74.36
Aspergillus carbonarius	180	8.82
Aspergillus luchuensis	45	2.20
Aspergillus flavus	99	4.85
Aspergillus fumigatus	58	2.84
Aspergillus terreus	21	1.02
Aspergillus nidulans	45	2.20
Aspergillus ochraceous	6	0.29
Aspergillus wentii	4	0.19
Penicillium citrinum	9	0.44
Penicillium chrysogenum	11	0.53
Alternaria alternata	8	0.39
Curvularia lunata	9	0.44
Fusarium sp.	11	0.53
Chaetomium sp.	16	0.78
Rhizopus sp.	1	0.49
Total isolates	2,040	

Phytochemical analysis

The results of the analysis of variance (P<0.05) showed significant variations among the phytoconstituents of various brands of Triphala powder analysed in two consecutive years (Table 9).

Total phenolics

During the present investigation, a significant variation was observed in the concentration of total phenolics in Triphala powder of different brands. The concentration of total phenolics (tannic acid equivalent) was found between 21.96±0.45 and 40.99±1.70%, and 21.15±1.13 amd 44.80±0.50% in Triphala powder samples collected in 2009 and 2010, respectively. A significant difference in the values of phenolics concentration was observed in the samples of the same brands collected during two successive years (Table 9). During investigation it was observed that the amount of total phenolics was much higher in samples of multinational manufacturers as compared to the sample of RM, LOC and HP, whereas samples of the same brand analysed in two consecutive years also showed large variations in the values of total phenolics.

Table 9. Phytochemical profile of selected Triphala powder samples collected in 2009 and 2010.[1]

Code	Total phenolics (%)		Tannin (%)		Ascorbic acid (%)	
	2009	2010	2009	2010	2009	2010
IRM-D	36.68±0.55^{abc}	44.80±0.50^{a}	32.16±0.77^{abc}	40.37±0.57^{a}	0.084±0.006^{b}	0.164±0.002^{a}
IRM-B	40.38±1.85^{ab}	30.38±0.76^{c}	36.38±1.91^{ab}	26.74±0.83^{c}	0.042±0.004^{d}	0.049±0.001^{e}
IRM-Z	40.99±1.70^{a}	38.57±0.54^{b}	36.86±1.49^{a}	34.17±0.49^{b}	0.085±0.002^{b}	0.053±0.004^{e}
IRM-DI	39.11±0.84^{ab}	30.71±2.65^{c}	35.29±0.99^{ab}	27.83±2.36^{c}	0.053±0.003^{c}	0.106±0.005^{b}
RM-B	36.94±2.32^{abc}	43.48±0.52^{a}	33.09±2.64^{abc}	40.59±0.61^{a}	0.159±0.005^{a}	0.095±0.002^{bc}
RM-U	37.33±2.63^{abc}	24.72±0.28^{d}	33.44±2.70^{abc}	20.50±0.37^{ef}	0.076±0.005^{b}	0.087±0.002^{c}
RM-S	33.60±0.50^{c}	25.24±0.58^{d}	29.39±0.38^{c}	22.63±0.60^{de}	0.058±0.002^{c}	0.072±0.001^{d}
LOC-1	24.70±1.77^{d}	21.15±1.13^{e}	21.00±1.75^{d}	18.82±1.12^{f}	0.041±0.002^{d}	0.043±0.005^{e}
LOC-2	35.52±2.65^{bc}	25.95±0.86^{d}	31.45±1.03^{bc}	23.19±0.73^{de}	0.050±0.001^{cd}	0.101±0.008^{b}
HP	21.96±0.45^{d}	27.66±0.67^{cd}	18.83±0.61^{d}	25.36±0.75^{cd}	0.058±0.002^{c}	0.043±0^{e}

[1] IRM = internationally recognised manufacturers; RM = regional manufacturers; LOC = locally prepared Triphala powder; HP = Triphala powder collected from herbal practitioner.

Tannin

During the analysis a large variation in tannin concentration was observed in Triphala powder samples of two consecutive years ranging between 18.83±0.61 to 36.86±1.49% and 18.82±1.12 to 40.59±0.61%, respectively. A good variation in the concentration of tannin was observed among the samples of the same brands analysed in two consecutive years (Table 9).

Ascorbic acid

Ascorbic acid is a major phytochemical constituent of *E. officinalis*, one of the important ingredients of the Triphala powder. Most of the antioxidant potential of Triphala powder and its capability to fight age related problem is believed due to ascorbic acid. During phytochemical analysis of Triphala powder, concentration of ascorbic acid was found in between 0.041±0.002 to 0.159±0.005% and 0.043±0.005 to 0.164±0.002% in samples collected during the years 2009 and 2010, respectively. A significant variation in ascorbic acid concentration was observed among different brands and between the samples of the same brands analysed in two successive years (Table 9).

4. Discussion

Triphala powder is highly sensitive to various environmental factors. Most of the environmental conditions play a significant role in the determination of the quality of product at the time of processing of raw material.

During the present investigation a large variation in the morphological features (colour, and texture) of Triphala powder of different brands was observed. As compared to fresh samples, alteration in colour, texture and appearance of the powder was observed in prolonged stored samples. Instead of fine powder solid clumps were present in old stored samples indicating a change in the quality of the stored product. These findings are in accordance with the report of Gautam and Bhadauria (2009a) where authors have reported the remarkable difference in the morphological appearance in fresh and stored samples of Triphala powder and powders of its ingredients.

Microscopic analysis has also been reported as a good tool to evaluate the quality and to check the adulteration. The pharmacopeia of various countries and researchers recommends use of this technique for the assessment of the quality of the herbal medicines (Kunle *et al.*, 2012; Palanuvej *et al.*, 2007; Pandey *et al.*, 2002; Upton *et al.*, 2011; Zhao, 2010). In our study microscopic analysis revealed the presence of foreign matter in the form of hairs and insects remains in Triphala powder of RM and LOC however samples of IRM were free from any foreign matter. The samples were found adulterated with endocarp of the ingredients. Bahulikar *et al.* (2002) also noted the presence of endocarp in three samples of commercially available Triphala powder. The presence of foreign matter in samples of RM, LOC and HP may be due to non-compliance with strict quality control guidelines as their products are mainly sold in Indian market.

Quality of herbal formulation also depends on total ash and acid insoluble ash content. Total ash and acid insoluble ash was found within limits, however high value of ash is an indicator of contamination, adulteration, substitution or negligence in the preparation of herbal formulations

whereas acid insoluble ash indicates the quantity of sand and silica in the formulations as has been reported by Thitikornpong *et al.* (2011) and Ahmad *et al.* (2012) During the present investigation, except one sample, total ash and acid insoluble ash was within the limit.

The pH and moisture content of the samples are also two important factors that are reported to influence the fungal diversity in nature (Rousk *et al.*, 2010). During the present investigation, samples with a low pH range were found to harbour a good number of fungi. This is in accordance with Mateos *et al.* (2007), Rousk *et al.* (2010) and Moreno *et al.* (2011) who reported that low pH favour mycelium development, good fungal growth and recolonisation of fungi in soil. Water availability is the key factor in controlling contamination of medicinal plant material. Marin *et al.* (2009) and Copetti *et al.* (2011) suggested that diversity and overall fungal load (cfu/g) vary with the water activity value of the samples. Any availability of sufficient moisture in stored material results in microbial contamination. In present study Triphala powder samples with high moisture content were found highly contaminated and with increase in moisture content a gradual increase in fungal diversity and percentage of samples with cfu $>10^3$/g was also observed. This is in accordance with the findings of Oyebanji and Efiuvwenvwere (1999) and Quezada *et al.* (2006) who reported a gradual increase in fungal load and diversity with increase in moisture content of stored maize sample.

Another important factor is microbial contamination of herbal drugs, which is generally responsible for maintaining the quality and efficacy of the formulations. The reports on microbial contaminations of herbal formulations are available from all over the world (Bugno *et al.*, 2006; Efuntoye, 2004; Halt, 1998; Hitokoto *et al.*, 1978) but tropical regions are more vulnerable to microbial contamination due to favourable environmental conditions for the proliferation of microorganism especially fungi. In our study, we isolated 16 fungal species belonging to 7 genera from 60 diverse types of Triphala powder samples. Occurrence of *Aspergillus* was observed in almost all of the samples. These results were in accordance with the observations of Gautam and Bhadauria (2011), which is the only available report on the fungal contamination of Triphala powder. Several authors also reported the *Aspergillus* as most dominant genera and *A. niger* as most prevalent species in herbal drugs samples.(Choursia, 1995; Hitokoto *et al.*, 1978; Kumar *et al.*, 2009; Mandeel, 2005; Mishra *et al.*, 2012; Singh *et al.*, 2008). This is in agreement with our findings, where *A. niger* was detected as predominant fungal species of Triphala powder. This high incidence of black aspergilli can be justified on the basis of the black colour of the spores that apparently provide protection from sunlight and UV rays during sun drying (Pitt and Hocking, 1997; Romero *et al.*, 2005) Frequently occurring species of *Aspergillus* and *Penicillium*, reported as storage fungi, infest products during storage. This may be the probable reason of the high occurrence of aspergilli in stored Triphala powder samples; secondly, this high occurrence of aspergilli and low frequency or absence of other fungi may be due to high phenolic contents of Triphala powder (21 to 44% of the dry weight) as reports of various researchers suggests that phenolics are ineffective against the storage fungi especially *Aspergillus* group as compare to pathogenic and field fungi (Cowan, 1999; Miller *et al.,* 1996; Samapundo *et al.,* 2007; Sinha and Singh, 1981). These reports are in agreement with our results, where, about 96% isolates were of *Aspergillus* group.

A variation in fungal diversity and fungal load was observed in between the Triphala powder of various manufacturing categories. Although, all the categories showed almost equal percentage of fungal contamination but more than 75% samples of the RM were loaded with fungal count more than the specified limit of 10^3 cfu/g for herbal drugs of internal uses (WHO, 2005). This may be due to the violation of guidelines laid down in the Ayurvedic pharmacopeia of India/WHO by RM during manufacturing.

The study also revealed the large variation in concentration of phytochemicals among the marketed Triphala powder. Gunasekaran and Anita (2010), Patra *et al.* (2011) and Mishra *et al.* (2011) also made similar observations and reported large variations in marketed samples of Ayurvedic formulations and medicinal plants. Variation was also observed within the samples of the same brands analysed in two different years. Geographical factors may also affect the variation in the diversity of phytoconstituents. Deng *et al.* (2010) have also suggested that this variation may be due to diversity of geographical factors (soil type, sunlight, temperature and precipitation) and post growth factors (harvesting, storage, transportation and manufacturing process) associated with the ingredients of medicinal formulations. Similarly, Gao *et al.* (2011) and Kwee and Niemeyer (2011) have pointed out that the time of harvesting and type of cultivar are also capable of generating variation at the level of phytochemicals. On the other hand, Stuart and Wills (2003), Dubey *et al.* (2004), Kabelitz (2005) and Asekun *et al.* (2007) have given emphasis on storage condition, methods of drying and way of packaging of processed formulation or raw material can also generate variation at active constituent's level. It was also noted that Triphala powder samples of LOC and HP were poor in phytochemicals as compared to the Triphala powder samples of regional and multinational manufacturers. This may be due to use of prolonged stored ingredients and high incidence of adulteration of Triphala powder with endocarp of fruits. As stony part of the fruit (endocarp) is devoid of phytoconstituents, therefore the presence of stony parts/seeds results in an overall decline in concentration of phytochemicals. This is also supported by the findings of

Bahulikar *et al.* (2002) who reported the variation in tannin concentration of three marked samples of Triphala powder and predicted that this variation may be due to the presence of seeds of the ingredients that may have been powdered along with the dried flesh of myrobalans.

5. Conclusions

High adulteration, high moisture content and a wide range of pH and presence of powdered endocarp in Triphala powder shows noncompliance by manufacturers with quality control guidelines. The presence of a wide range of fungi is also of great concern as the majority of them are capable to produce mycotoxins. The study also pointed out the variation in the rate of contamination between products of different manufacturers. A large variation in concentration of therapeutically important phytochemicals may result in alteration in efficacy, thereby shatter the beliefs of common people in Ayurveda. These lacunas in quality control of commercially available herbal formulations may pose a serious risk for consumers health and can give a serious jolt to Indian herbal industry, which is already facing strong competition from the Chinese herbal industry. Therefore, it is the need of time to formulate stringent quality control guidelines for herbal formulations so that maximum benefits can be obtained from these traditional formulations.

Acknowledgements

The authors (SS and MG) are highly thankful to the School of Studies in Botany Jiwaji University Gwalior (MP) India for providing necessary facilities to carry out this research. The first author (SS) also extends thanks to retailers of herbal medicines, local manufacturers of Triphala and herbal practitioners for providing Triphala powder samples for research purpose.

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