

Efficacy of Triphala Churn ingredients against *A. niger* and potential of clove extract as herbal fungitoxicant

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Abstract

The present study explores the association of *Aspergillus niger* with stored raw and powdered ingredients of Triphala Churn, which is a 1:1:1 proportional combination of *Embllica officinalis* Gaertn. (Amla), *Terminalia bellerica* (Gaertn.) Roxb. (Baheda), and *Terminalia chebula* Retz. (Harada) respectively. Total 106 stored fruits and 68 powdered samples of *E. officinalis*, *T. bellerica* and *T. chebula*, have been analysed for their fungal association, if any. Results revealed that among all the fungal isolates, *A. niger* was found to be a frequently occurring species as well as a major contaminant. Therefore, present investigation was carried out to study the efficacy of aqueous extracts of fruits (fresh & dry) and a powdered ingredient of Triphala churn against the growth of *A. niger*. In addition, an effort has been made to evaluate the antifungal potential of aqueous extracts of *Syzygium aromaticum* (L.) Merril & Perry (Clove), *Cinnamomum zeylanicum* Blume (Cinnamon), and *Zingiber officinale* Roscoe (Ginger) as herbal fungitoxicants, to control the growth of *A. niger*. During the investigation of samples for fungal contamination, highest percent frequency (93.10%) of *A. niger* was recorded. It was observed that none of the aqueous extracts of fruits and powdered ingredient of Triphala churn was found effective against the growth of *A. niger*. However, the aqueous extract of Clove was found strongly effective to inhibit the growth of *A. niger* completely at 20% concentration (v/v). Among the other extracts Cinnamon showed 24.73% inhibition, whereas Ginger extracts was observed to be ineffective against *A. niger*. Hence, the aqueous extract of Clove can be utilized as herbal fungitoxicant to control the growth of *A. niger*.

Keywords: Raw material; Triphala churn; *A. niger*; herbal fungitoxicant.

Introduction

Aspergillus niger is a cosmopolitan fungus, commonly known as "black mold". This fungus is among the most common fungi causing food spoilage and bio-deterioration of other materials (Samson 2004). It is a filamentous ascomycete that is ubiquitous in the environment (Perfect 2001). This organism is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocelluloses and also plays a significant role in the global carbon cycle (Scott 2006). Because of their ability to produce extracellular organic acids, some of them are commonly used in food industry. Moreover, *A. niger* is one of the fungi that have been labelled with the GRAS (generally recognized as safe) status according to the US Food and Drug Administration. However, *A. niger* has been found to be an opportunistic reason for various infections of human beings. It can be dangerous to humans in sufficient quantities if intense spore inhaled, causing severe lung problems i.e. Aspergillosis. This fungus can also cause ear infections, which ultimately damage ear canal and tympanic membrane (Schuster et al. 2002). Despite of harmful effects of *A. niger* to living beings, it is also reported to contaminate herbal drugs

during harvesting, pre and post processing practices. This fungus is one of the major fungal contaminants with high relative density in raw materials of some herbal drugs (Sareen 2010), herbal teas and coffee substitutes (Tournas and Katsoudas 2008). It is also recovered as dominating fungal contaminant in Triphala churn samples analysed previously (Gautam and Bhadauria 2008) and its ingredients (Bugno et al. 2006; Gautam and Bhadauria 2009). Due to saprophytic nature, it is not only contaminating the herbal drugs during storage but can also secrete certain secondary metabolites called mycotoxins. Several studies have shown that occasional isolates of *A. niger* can produce ochratoxin A, Fumonisin B2 and aflatoxins (Schuster et al. 2002; Noonimabc et al. 2009; Al-Abdalall 2009) and the occurrence of mycotoxin contamination in herbal drugs by *A. niger* seems to be very inevitable. Contamination of stored herbal drugs with fungi not only result in discoloration, quality deterioration, reduction in commercial values but also in their declined therapeutic potential, while mycotoxins are leading cause of several ailments of liver, kidney, nervous system, muscles, skin, respiratory organs, digestive tract, genital

organs, etc (Rai and Mehrotra 2005; Trucksess and Scott 2008).

The main objectives of this study were to evaluate the stored fruit and powdered ingredients of Triphala churn for *A. niger* contamination and their antifungal potential against this fungus. In addition, efforts have been made to find out the effects of aqueous extracts of *Syzygium aromaticum* (L.) Merrill & Perry (Clove), *Cinnamomum zeylanicum* Blume (Cinnamon), and *Zingiber officinale* Roscoe (Ginger) to control the growth of *A. niger*.

Materials and Methods

Sample collection

A total of 106 fruit samples and 68 powdered samples of *E. officinalis*, *T. bellerica* and *T. chebula* stored commercially for 6 to 8 months by local suppliers after harvesting, were randomly collected from different locations of Gwalior (India) market during the year 2007-2009. These samples were collected in sterilized polythene bags to avoid further contamination, transported immediately to Mycology laboratory at School of Studies in Botany, Jiwaji University Gwalior, and stored in airtight containers at room temperature until further analysis.

Measurement of moisture content

For moisture content, weighed amount of individual samples were dried at 80°C for 24 h and the difference in weight was calculated (Essono et al. 2007).

$$MC = [(W_i - W_f)/W_i] \times 100$$

Where MC = Moisture content; W_i = Initial weight and W_f = Final weight

Mycological analysis

For isolation of fungi, a 10% (w/v) herbal powder suspension was prepared in sterilized distilled water and small pieces of fruit samples were used for the isolation of mycoflora. The suspension was diluted to 10^6 times. Enumeration of fungi was performed by pour plating method for powdered sample. Moreover, in case of fruit samples, small pieces were inoculated using Potato-Dextrose Agar (PDA) and Czapek Dox Agar (CDA) media (Mandeel 2005; Gautam and Bhadauria 2008). Triplicate of each sample (both, powder and fruit) were incubated at $25 \pm 2^\circ\text{C}$ for 7 days and examined daily. The mean number of fungal colony-forming units (cfu) was recorded. After incubation, the plates were

examined visually as well as under a compound light microscope. Identification of fungal species was done on the basis of cultural and morphological characteristics (Gilman 2001). Most frequently occurring fungal species were further validated by culture at Indian Agricultural Research Institute (IARI), New Delhi, India. The percent relative density, occurrence frequency and percent incidence of *A. niger* in each samples was calculated (Giridher and Ready 1997; Verma and Dubey 2001; Mandeel 2005).

Preparation of aqueous extracts

100 gm dried fruit and powdered samples of *E. officinalis*, *T. bellerica* and *T. chebula* were macerated with 100 ml sterile distilled water in a blender for 10 min. The macerate was first filtered through double-layered muslin cloth followed by centrifugation at 4000 rpm for 30 min at room temperature. The supernatant was filtered through Whatmann No. 1 filter paper and sterilized, which served as the mother extract (Satish et al. 2007). For evaluation of antifungal activity of the extracts, percentage dilutions i.e. 5%, 10% and 20% of extract were obtained by adding appropriate of standard basic stock solution to stock media.

Antifungal activity assay

For screening of antifungal activity of fruit and powdered ingredients of *E. officinalis*, *T. bellerica*, and *T. chebula* Poisoned food technique was followed (Sinha et al. 1993). Czapek Dox Agar (CDA) medium was prepared and sterilized. The medium was supplemented with different serial dilutions of aqueous fruit extracts i.e. 5, 10, & 20% (stock solution). About 15 ml of this medium was poured into each petriplate and allowed to solidify. Five mm disc of 7-day-old culture of the *A. niger* were placed at the centre of the each petriplate and incubated at $25 \pm 2^\circ\text{C}$ for seven days. After incubation, the colony diameter was measured in millimeter (mm). For each treatment group (for a given percentage of extract) three replicates were maintained. CDA medium without the aqueous extract was taken as control. The fungitoxicity of the extracts in terms of percentage inhibition of mycelial growth was calculated by using the following formula

$$\% \text{ inhibition} = dc - dt/dc \times 100$$

Where dc = Average increase in mycelial growth in control, dt = Average increase in mycelial growth in treatment (Singh and Tripathi 1999).

Evaluation of antifungal activities of Clove, Cinnamon, and Ginger against *A. niger*

The aqueous extracts of Clove, Cinnamon, and Ginger were prepared (Satish et al. 2007). The antifungal activities of the aqueous extracts were recorded at 5%, 10%, and 20% concentrations obtained by adding appropriate amount of standard basic stock solution to the stock CDA media as per the poisoned food technique (Sinha et al. 1993).

Statistical analysis

The analysis of data was performed with Microsoft Excel 2007 (Window XP) for mean and standard deviation. The statistical analysis was performed using student's t-test. The p-value <0.05 was considered significant.

Results

Incidence of *A. niger* in fruits and powdered herbal drugs

A total of 106 dried stored fruit samples and 68 powdered samples of *E. officinalis*, *T. bellerica*, and *T. chebula* were analysed for association of *A. niger*. Approximately, all the fruits and powdered samples were found to be contaminated with various fungal

contaminants. Particularly, *A. niger* (#7414.09) was recorded as the major fungal species. The mycological examination of fruit samples revealed that 93.10 % samples of *T. bellerica* are contaminated with *A. niger*, while only 63.63% of powdered samples were contaminated. The fruit samples of *T. chebula* were also found highly (75%) contaminated, whereas, in case of powdered percentage contamination was found decreased up to 38.09%. Almost all the powdered samples (92%) of *E. officinalis* were showing the association of *A. niger* while this association was observed to be 62.16% in fruit samples (Table 1).

Relative density value (%) was estimated to determine the abundance of fungal species among all samples (Table 2). The highest percentage relative density of *A. niger* was observed in *T. chebula* fruits (61.54%) while it was very low (5.2%) in its powdered samples. In case of *T. bellerica*, both fruits and powdered samples were found heavily contaminated with *A. niger* i.e. 57.06% and 48.4%, respectively. Similarly, *A. niger* were also found to be highly associated with fruits and powders of *E. officinalis*. The frequency of *A. niger* in fruits samples over powdered samples was found to be highly significant ($p = <0.05$).

Table 1. Description of the fruit and powdered samples with %age frequency of contamination with *A. niger*.

	Botanical Names	No. of contaminated samples	Frequency (%)
		No. of samples examined	
Fruits	<i>E. officinalis</i>	23/37	62.16%
	<i>T. bellerica</i>	27/29	93.10%
	<i>T. chebula</i>	30/40	75%
Powder	<i>E. officinalis</i>	23/25	92%
	<i>T. bellerica</i>	14/22	63.63%
	<i>T. chebula</i>	8/21	38.09%

Table 2. Distribution of *A. niger* (#7414.09) in fruits and powdered samples.

	Botanical Names	Density (%)	p-value
Fruits	<i>E. officinalis</i>	97 (36.88)	0.05
	<i>T. bellerica</i>	214 (57.06)	
	<i>T. chebula</i>	211 (61.54)	
	Overall mean	51.82±13.13*	
Powder	<i>E. officinalis</i>	116 (46.4)	
	<i>T. bellerica</i>	121 (48.4)	
	<i>T. chebula</i>	13 (5.2)	
	Overall mean	33.33±24.38*	

(# = confirmed at IARI, New Delhi)

Efficacy of Triphala ingredients against A. niger

Preliminary screening of antifungal potential of Triphala churn ingredients using poisoned food technique against *A. niger* are given in Table 3. The results showed that the aqueous extract of fruits and powder of *E. officinalis*, *T. bellerica*, and *T. chebula* were not found to possess any antifungal activity against *A. niger*. Even the dried fruits extracts of all the samples did not show any significant effectiveness against *A. niger*. In all cases, the treatment diameter exceeds the control. In *E. officinalis* fruits diameter of the mycelium of *A. niger* in control is 12mm, while it was 15mm, 16mm, 18mm in 10%, 15% and 20% conc. of fresh fruit aqueous extracts, respectively. Similar pattern of radial mycelial growth was observed in dry and powdered aqueous extracts of *E. officinalis*. Similarly, in case of *T. bellerica*, and *T. chebula* the diameter of the *A. niger* mycelium exceeded the control, which shows that *A. niger* may be resistant to the effect of all the aqueous extracts of fruits and their powders (Table 3).

The aqueous extracts of Clove, Cinnamon, and Ginger were evaluated at 10%, 15% and 20% conc. against the growth of *A. niger*. Among all the concentrations that were tested, 20% was found to be most effective to inhibit the *A. niger* growth. The mycelial growth of *A. niger* was completely (100%) inhibited by Clove extract, followed by Cinnamon (24.73%)

and Ginger which had no antifungal effect against *A. niger*. These antifungal activities of used extracts clearly indicate that the fungicidal activity was maximum in dried flower buds of Clove. Also, aqueous extracts of cinnamon bark showed a good fungicidal activity at 20% conc. However, none of the concentrations of Ginger was found effective against *A. niger* (Table 4).

Discussion

A. niger is a filamentous fungus with the ability to grow in the wide temperature range of 6–47°C with a relatively high temperature optimum at 35–37°C and extremely wide pH range (1.4–9.8). The water activity limit for growth is 0.88, which is relatively high compared with other *Aspergillus* species. These abilities make ubiquitous occurrence of the species, with a higher frequency in warm and humid places (Palacios-Cabrera et al. 2005). Isolation of *A. niger* as a major fungal contaminant from the raw and powdered ingredients of triphala is a matter of great concern due to its allergic and mycotoxic nature. Humans are exposed to its spores every day without any clinical outcome. But, in some cases when persons are exposed to intense spore dust, hypersensitivity reactions and lung disease (primary *Aspergillus* pneumonia, aspergilloma, allergic bronchopulmonary aspergillosis, and invasive *Aspergillus*) may be observed (Schuster et al.

2002). Many researchers have reported *A. niger* as a dominant mycoflora from different substrates (Gautam and Bhadauria 2008, 2009). Reports are also available demonstrating, *A. niger* not only a causative agent of lung disease but also as a agent

secreting potential carcinogenic mycotoxins namely ochratoxin A, Fumonisin B2 and aflatoxins under optimal laboratory conditions (Battilani and Pietri 2002; Mangoli et al. 2003; Samson et al. 2004).

Table 3. Effect of different concentrations of aqueous extracts of *E. officinalis*, *T. bellerica* and *T. chebula* on mycelial growth of *Aspergillus niger*.

Fruit / powder samples		Concentration of Fruit extract / powder	Mycelial growth (mm)*	
			<i>A. niger</i>	Inhibition (%)
<i>Emblica officinalis</i> (Amla)	Fresh fruits	Control	12 mm	NE
		5%	15 mm	
		10%	16 mm	
		20%	18 mm	
	Dry Fruits	Control	6 mm	NE
		5%	10 mm	
		10%	11 mm	
		20%	12 mm	
	Powder	Control	10 mm	NE
		5%	16 mm	
		10%	20 mm	
		20%	22 mm	
<i>Terminalia bellirica</i> (Baheda)	Fresh fruits	Control	15 mm	NE
		5%	19 mm	
		10%	23 mm	
		20%	27 mm	
	Dry Fruits	Control	12 mm	NE
		5%	27 mm	
		10%	29 mm	
		20%	31 mm	
	Powder	Control	11 mm	NE
		5%	17 mm	
		10%	20 mm	
		20%	22 mm	
<i>Terminalia chebula</i> (Haritiki)	Fresh fruits	Control	9 mm	NE
		5%	17 mm	
		10%	18 mm	
		20%	19 mm	
	Dry fruits	Control	10 mm	NE
		5%	15 mm	
		10%	18 mm	
		20%	19 mm	
	Powder	Control	6 mm	NE
		5%	10 mm	
		10%	15 mm	
		20%	18 mm	

* Mean of three replicates; NE = not effective.

Table 4. Percent inhibition of mycelial growth of *Aspergillus niger*.

Fruit / powder Samples		Concentration of Fruit extract / powder	Mycelial growth (mm)*	
			<i>A. niger</i>	Inhibition (%)
<i>Syzygium aromaticum</i> (Dried flower bud)	3 rd day	Control	12mm	
		10%	10.2mm	15
		15%	5.8mm	51.66
		20%	NG	100
	4 th day	Control	16mm	
		10%	13.2mm	17.5
		15%	8.6mm	46.25
		20%	NG	100
	5 th day	Control	30mm	
		10%	20.6mm	31.33
		15%	11.2mm	62.66
		20%	NG	100
<i>Cinnamomum zeylanicum</i> (Bark)	3 rd day	Control	17mm	
		10%	14.8mm	12.94
		15%	11.6mm	31.76
		20%	8.2mm	51.76
	4 th day	Control	28mm	
		10%	25mm	10.71
		15%	21.6mm	28.85
		20%	15.6mm	44.28
	5 th day	Control	38mm	
		10%	35.8mm	5.78
		15%	32.6mm	14.21
		20%	26.8mm	24.73
<i>Zingiber officinale</i> (Dried rhizome)	3 rd day	Control	13mm	
		10%	22.2mm	NE
		15%	21mm	
		20%	20.2mm	
	4 th day	Control	24mm	
		10%	35.6mm	NE
		15%	33mm	
		20%	29.6mm	
	5 th day	Control	35mm	
		10%	45.2mm	NE
		15%	42.6mm	
		20%	41.8mm	

* Mean of three replicates; NE = not effective; NG = no mycelial growth.

Because *A. niger* was recorded as dominant fungus in fruit and powdered samples, therefore attempts has been made to evaluate the efficacy of aqueous extracts of fruits and powders of *E. officinalis*, *T. bellerica* and *T. chebula* against the growth of *A. niger*. Results showed that none of the aqueous extracts were found effective in arresting the growth of *A. niger*. Although, all the aqueous extracts were found ineffective except one of the interesting observations that the fresh fruits of *E. officinalis* were found less effective than in dry. This may be attributed to the existing

water content in fresh fruits (80%) which on drying is almost eliminated. Apart from this, these variations may also be due to the differences in the presence of active principles in fresh, dry fruits and powder. The active principles present in plants are influenced by many factors including, age of plants, extracting solvents, and method of extraction and time of harvesting (Calixto et al. 2000). It has been also reported that active principles are destroyed by enzymatic processes that continue for long periods after plant material collection (Okigbo and Ogbonnaya 2006). This

might be the obvious reason for detection of *A. niger* in abundance from analysed herbal drugs in the present study.

Because all raw and powdered samples used in present study are important constituents of Triphala churn, which is used in every home regularly for curing various ailments, some antifungal agents are necessary to protect them from *A. niger* contamination. Hence, we tried to evaluate the efficacy of aqueous extracts of Clove, Cinnamon, and Ginger against *A. niger*. Modern use of synthetic chemicals as fungicide to control the fungal contamination in stored herbal drugs is a problematic issue due to their toxic effects on processors and consumers (Parekh et al. 2005). The fungicidal activity of some plant extracts in controlling different plant pathogens have been reported by several workers (Amadioha 2000; Okigbo and Nmaka 2005; Tumne et al. 2007). The results of antifungal activities of aqueous extracts in the present study clearly indicated that the fungicidal activity was maximum in dried flower buds of Clove. This may lead to the conclusion that the eugenol of this plant is responsible for this activity. There are reports in the literature on the antimicrobial activity of Clove against *A. niger* (Garg et al. 1992; Rahalison et al. 1994). Moreover, *Piper betel* extract of Clove family at the concentration of 2.25, 1.5, and 1.5 % (v/v) caused complete inhibition, 100% antifungal index, of the growth of *A. niger*. The aqueous extract of Cinnamon was also found effective against the *A. niger* growth but less than that of Clove extract. Although, chemical compounds Gingerols, shogaols and some phenolic ketone derivatives present in the Ginger have been reported to have antifungal activity (Cao et al. 1993). But, in the present study, the aqueous extract of Ginger was found ineffective against *A. niger*, which may be due to the insolubility of the active compounds in water as also mentioned by Qasem and Abu-Blan (1966) and Amadioha (2000). Because the Cloves are used in various digestive problems, we can utilize its aqueous extract to control the *A. niger* growth in Triphala and its ingredients. Therefore, the present study is an important step toward development of plant based pesticides, which are eco-friendly for the management of storage fungi, today and in near future.

Conclusion

This study showed that crude and powdered ingredients of Triphala are heavily contaminated with *A. niger*. Although, this

fungi could not be always toxic but, raw or powdered ingredients of Triphala should be carefully checked for this fungus before channelled for Triphala churn preparation. Since, none of samples showed antifungal effect against *A. niger*, hence, care should be taken during their harvesting and pre & post processing for storage. Moreover, the aqueous extracts of Clove and cinnamon could inhibit the *A. niger* growth, and therefore could be used as source of natural antifungal agents which may be added directly into raw material or incorporated in packaging materials to control the fungal contamination/s.

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