Research Article

Application of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. &Thoms Leaf Essential Oil for Preservation of *Buchnania lanzan* Spreng seeds

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ABSTRACT

Mycological analysis of 20 samples of stored kernels of *Buchnanialanzan* revealed presence of sixteen fungal species viz., *Alternaria alternata, Aspergillus candidus, Aspergillus flavus, A. niger, A. ochraceous, A. phoenicis, A. tamari, A. terreus, A. sydowi, Fusarium moniliforme, F. oxysporum F. solani, P. glabrum, Rhizopus nigricans, Trichoderma viride* and *Trichothecium roseum* in both agar plate as well as blotter paper methods of study. *In vitro* activity of 20 essential oils and 2 commercial fungicides was assessed for their antifungal activity against all isolated fungi. The leaf oil of *Tinospora cordifolia*, (Willd.) Miers ex Hook. F. & Thoms exhibited absolute toxicity (100%) against dominant fungi *Aspergillus flavus, A. niger* at400ppm concentration. It was found fungicidal at 500ppm.It showed MIC against 10 fungi at 400ppm, controlled 15 fungi at 600 ppm. This could not show any ill effect on different treatments viz., temperature treatment up to 100° C, autoclaving at 15 lb/square inch pressure at 120° C and storage up to 105 days on activity of leaf oil. *In vivo* studies depict that oil of *T.cordifolia* as seed dressing agent and as a fumigant have potential to preserve seeds up to 105 days separately in polyethylene containers with minimal changes in organoleptic behaviour of fruits during storage. Thus *T.cordifolia* leaf oil can possibly be exploited in the management of seed-borne pathogenic fungi and prevention of biodeterioration of *Buchnania* seeds in an eco-friendly way.

Keywords: *Chiraunji, T.cordifolia* leaf oil, biodeterioration.

INTRODUCTION

Buchnania lanzan Spreng., popularily called Chiraunji have been originated in the Indian sub-continent found in dry deciduous forest of India as an excellent fruit tree for forestry which can withstand adverse climatic conditions. Its ripe fruits flesh is very palatable and is largely eaten raw or roasted. The oily kernels are the most important part and are used in preparation of puddings. Mesocarp of fruit is edible and cherished by children and very good juice may be prepared from the pulp of chirounji fruits. However, its kernels get bio-deteriorated by fungi and decreasing its economic value. There are synthetic fungitoxic chemicals for protection but has side effects[1-2]. The kernels are highly nutritious, have protein (25.0-30.0%) and yields sweet oil, which can be used to substitute olive and almond oil. Kernel contains 33.50 % oil, 1.90 % of which is unsaponifiable. The saponifiable part contained 20.00 % of linoleic acid. Chironji oil is non-repellant and non-toxic and is suitable for human consumption [3].

The use of essential oils as fungitoxic agent provides an alternate to avoid synthetic preservatives. Therefore, *Tinospora cordifolia*, (Willd.) Miers ex Hook. F. &Thom leaf oil was explored during storage. This is commonly known giloyguduchi or heart-leaved moonseed and is an herbaceous vine of the family Menispermaceae indigenous to the tropical areas of Indian subcontinent and highly medicinal[4-6]. The aqueous extract of *T.cordifolia* stem has shown to produce immunological activity due to the presence of arabinogalactan. Root is a powerful emetic and used for visceral obstruction; its watery extracts is used in leprosy[7]. Therefore mycofloral analysis of *B. lanzan* seeds were carried and *in vivo* preservative activity trials were conducted with most active *T.cordifolia leaf* oil.

MATERIALS AND METHODS

The grocery stores of farmers were visited in 2014-2016 of Panchgaon, Gurgaon for collection of 20 seed samples of *B. lanzan*. The mycological diversity studies were done on these seeds through agar plate[8] and standard blotter[9]techniques with minor modification following Kumar[10].In agar plate technique, 200 seeds were equidistantly spread out having czapeksdox agar medium in separate petri dishes, each containing 5 seeds. In blotter test, the seeds were similarly plated on three layered moistened blotter pads in sterilized petridishes. The assay plates were then incubated at 25 ± 2 °C and for isolation of fungal species and observed daily up to 7 days. The fungal colonies appeared on the seeds were isolated, purified and identified on the basis of by fungal literatures.

In order to investigate the internal seed mycodiversity, the seeds were first surface sterilized with 0.1% sodium hypochlorite solution for two minutes and washed with double distilled water. They were then incubated to agar plate and standard blotter techniques for mycological analysis. With the use of folds of sterilized blotters excess water was removed from the seed.

The per cent occurrence of each fungal species associated with seeds samples of *B. lanzan*. was calculated as per formula-

Percent occurrence=

Number of colonies of a particular fungus X 100
Total number of colonies of all the fungi

From leaf of 20 plants the essential oils were extracted separately up to 7 hrs through hydrodistillation in Clevenger's apparatus at 90±2°C. Each isolated essential oil was dried over anhydrous sodium sulphate and was stored at 4°C under aseptic condition. The antifungal activity of essential oils and two commercial fungicides Copper oxychloride and Carbondazim against fungal species was evaluated separately by Inverted Petri plate technique at 500 ppm concentration following Kumar[10].

Fungi toxic properties of *Tinospora cordifolia* leaf oil

The minimum inhibitory concentration of most active oil *T.cordifolia* leaf oil was determined by preparing different concentration of the *T.cordifolia* leaf oil ranging from 200 to 600ppm were prepared by dissolving requisite amount of leaf oil in 0.5ml acetone and then mixing with 9.5mlczapeksdox agar medium separately following Kumar[10].In control sets the petriplates having acetone and medium without *T.cordifolia* leaf oil were used. Fungal discs (5mm diam) obtained from periphery of seven d old culture of each of test fungi were aseptically inoculated in each of the treatment and control sets. All these sets were incubated at 28±2°C for 6 days. The diameters of fungal colony of treatment/control sets were measured in mutually perpendicular directions on the 7th d and the average was used to calculate the percent inhibition of mycelial growth of test fungi separately.

For studying nature of the *T.cordifolia* leaf oil treated discs of the fungi showing complete inhibition of their mycelial growth up to 7d were washed with sterile water and placed again on fresh solidified medium to observe the revival of mycelia growth. The fungi toxic spectrum of the *T.cordifolia* leaf oil was studied against various fungi isolated from seeds samples of *B. lanzan*. In addition effect of temperature, autoclaving and storage on the fungi toxicity of *T.cordifolia* leaf oil was determined following Kumar[11]. Each experiment was repeated thrice and contained 3 replicates.

For fumigant potential efficacy determination , requisite amount of *T.cordifolia* leaf oil was soaked in cotton swab(100mg weight) and was introduced in separate polyethylene bags (17.0 cm dia. × 20.4 cm height) which had 200g of *B.lanzan* seed sample, so as to attain 1µl/ml (v/v) concentration. Similarly synthetic fumigants viz., aluminium phosphide (sulphos) and ethylene dibromide (EDBA ampule) were introduced at 500 ppm concentration onto samples of 200g seeds each.

To measure its protectant power as seed dressing the stock solution of 100µl of *T.cordifolia* leaf oil was prepared by dissolving 100µl of oil in 1 ml of acetone. 200 g of seed of *B.lanzan* were filled in plastic containers, treated with 1ml stock solution of the leaf oil. For proper coating *B.lanzan* seeds were dressed by continuous shaking for 7 min. Likewise, two contact fungicides such as copper oxychloride and carbendazim (1000 mg/ 200g seeds) were taken and also run parallely for comparison purpose.

For control set the seeds were dressed in requisite amount of acetone in place of the *T.cordifolia* leaf oil and fungicide. The polyethylene containers were sealed to make it airtight and kept at room temperature at 75±5% humidity. Observations for presence/absence of mycoflora were made after 15 days to 105 days separately.

Statistical analysis

The results of five parallel measurements were expressed as mean ±SD. The observed data on antifungal activity, MIC, spectrum, effect of physical factors on the *T.cordifolia* leaf oil were obtained by taking average of five replicates.

RESULTS AND DISCUSSION

A total of sixteen fungal species namely *Alternaria alternata, Aspergillus candidus, Aspergillus flavus, A. niger, A. ochraceous, A. phoenicis, A. tamari, A. terreus, A. sydowi, Fusarium moniliforme, F. oxysporum F. solani, P. glabrum, Rhizopus nigricans, Trichoderma viride* and *Trichothecium roseum* were isolated by both agar plate as well as blotter paper methods on three months stored seeds of *B.lanzan*in all 20 samples. In which *Aspergillus flavus*, *A.niger*, *A.ochraceus* and *A. terreus* were dominant showing 29.1, 27.0, 23.2, 21.1 in blotter and 29.0, 22.1, 22.8, 20.6 % in agar plate method of study (Table 1).

Sharma[12] also recorded *Aspergillus flavus*, *A.niger*, *A.ochraceous* fungal species in *B. lanzan* seeds. These pathogens were disseminated predominantly by seeds and caused severe damage to seeds. These fungi produce some kind of fungal toxins like aflatoxin which have established mammalian toxic effects due to that such food is harmful for human consumption.

The review of literature reports that, plant essential oils are alternative of synthetic pesticides,(as they are known to have minimal environmental impact and danger to consumers in contrast to synthetic fungicides[13-15].

Out of 20 essential oils screened, *T.cordifolia leaf* oil displayed highest toxicity against all test fungi, showed complete inhibition (100%) of the mycelial growth at 500ppm, more effective over Copper oxychloride and Carbondazim fungicides (Table 2). It was strongly effective against *Serratia marcescens, E. coli, Streptococcus thermophilus, Fusarium oxysporium, Aspergillus niger*[16]. Narayanan *et al.*[17] found anti-bacterial activity in *Tinospora cordifolia* against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, Shigella flexneri, Salmonella paratyphi, Salmonella typhimurium, Pseudomonas aeruginosa, Enterobacter aerogene*, and *Serratia marcesenses* (Gram-positive bacteria).

Table 1: Percent occurrence of mycobiota on three months stored kernels of Buchnania *lanzan*

Fungi recorded	Blotter method		Agar plate method	
	US	SS	US	SS
Alternaria alternate (Fr.) Keissler	2.9	1.2	4.1	-
Aspergillus candidus Pers ex.	2.1	-	3.1	-
A. flavus Link	29.1	3.6	29.0	9.5
*A. niger*van Tieghem	27.0	2.7	22.1	7.3
A. ochraceous Wilhelm	23.2	3.6	22.8	6.5
A .tamarii Kita	2.1	-	2.2	-
*A. terreus*Thom	21.1	2.5	20.6	6.7
A. sydowi (Bainier and Sartory) Thom and Church	4.4	1.2	5.1	1.0
Fusarium moniliforme Sheldon	2.0	1.1	4.0	-
*F. oxy sporum*von Schlechtendal	2.2	1.3	2.1	1.3
F. solani (Mart.) Sacc.	2.3	2.0	2.1	1.1
Penicillium glabrum (Wehmer) Westling	4.0	-	1.5	-
Rhizopus nigricans Ehr.	2.2	-	0.1	-
Syncephalastrum racemosum Cohn	3.0	-	-	-
*Trichoderma viride*Pers.ex.Fr.	2.7	-	-	-
Trichothecium roseum (Persoon)	2.6	-	-	-

- Fungus not reported *Values are given as per cent of mean of 20 samples studied; US: unsterilized seeds; SS: sterilized seeds

Table 2: Activity of *T. cordifolia* leaf oil and synthetic fungicides against *Aspergillus flavus* and *A.niger*

Plant species	Plnat part used in hydrodistillation	Percent inhibition of mycelia growth of test fungi at 500ppm		
		Family (plant part)	Aspergillus flavus	A.niger
Tinospora cordifolia (Willd.) Miers ex Hook. F. &Thoms	Leaf	Menispermaceae	100*±0.33	100*±0.27
Copper oxychloride	*Synthetic fungicide	Yogi Dye Chem Industries Satyam Complex, M. G. Road, Ghatkopar East, Mumbai - 400077, Maharashtra	90.0±0.34	94.0±0.26
Carbondazim	*Synthetic fungicide	Canary Agro Chemicals Pvt. Ltd., New Delhi	95.1±0.37	83.2±0.35

Data are mean of five replicates, ± Standard error

For appropriate dose determination, the minimum inhibitory concentration (MIC) of a fungicide is necessary to work out. Because high dose of fungicide increase wastage and may cause considerable loss to the quality of commodity treated. The MIC of *T.cordifoliam* leaf oil against dominant fungi *A. flavus* and *A. niger* was found to be 400ppm (Table 3). It was found fungicidal at 500ppm.It showed MIC against 10 fungi at 400ppm while at 600ppm it controlled 15 fungi (Table 4).

Table 3: MIC of *T. cordifolia* leaf oil

Dose of oil in ppm	*Aspergillus flavus*	*A.niger*
200	45±0.21	45±0.21
300	75±0.21	85±0.21
400	100±0.21	100±0.21
500	100*±0.01	100*±0.01
600	100±0.21	100±0.11

*Fungicidal; Data are mean of five replicates, ± Standard error

There was no ill effect of temperature treatment up to 100° C for duration 60 min, autoclaving at 15 lb/square inch pressure at 120° C and storage up to 105 days on activity of *T.cordifolia* leaf oil (Table 5).

Table 4: Fungi toxic spectrum of *T. cordifolia* leaf oil at sub lethal, lethal and hyper lethal doses against fungi isolated from *Buchnania lanzan* seeds

Fungal species	Per cent inhibition of mycelial growth of isolated fungi			
	Sublethal 200ppm	Lethal 400ppm	Hyperlethal 600ppm	Hyperlethal 800ppm
Alternaria alternata	44.0±0.11	81.3±0.21	100.0±0.21	100.0±0.21
Aspergillus candidus	48.7±0.11	88.1±0.21	100.0±0.11	100.0±0.10
A.flavus	51.3±0.11	100.0±0.11	100.0±0.10	100.0±0.10
A.niger	34.7±0.21	100.0±0.10	100.0±0.11	100.0±0.21
A.ochraceous	41.2±0.01	100.0±0.13	100.0±0.12	100.0±0.11
A.phoenicis	47.9±0.02	100.0±0.14	100.0±0.13	100.0±0.21
A. tamari	58.3±0.01	100.0±0.11	100.0±0.12	100.0±0.11
A.terreus	55.0±0.20	100.0±0.12	100.0±0.10	100.0±0.21
A. sydowi	47.3±0.11	100.0±0.13	100.0±0.11	100.0±0.11
Fusarium moniliforme	44.1±0.21	78.4±0.11	100.0±0.21	100.0±0.21
F.oxysporum	41.3±0.21	100.0±0.21	100.0±0.13	100.0±0.11
F.solani	58.8±0.31	100.0±0.11	100.0±0.31	100.0±0.11
P.glabrum	53.2±0.21	100.0±0.21	100.0±0.01	100.0±0.21
Rhizopus nigricans	54.4±0.11	81.2±0.11	94.4±0.01	100.0±0.11
Trichoderma viride	64.0±0.11	96.1±0.21	100.0±0.11	100.0±0.01
Trichothecium roseum	64.0±0.11	96.1±0.11	100.0±0.01	100.0±0.01

Data are mean of five replicates,± Standard error

Table 5: Effect of physical factors on the fungi toxicity of *T. cordifolia* leaf oil

Physical factors	Per cent inhibition of mycelial growth at its MIC
Temperature(°C) Time of treatment-60min 40°C 60°C 80°C 100°C	 100±0.31 100±0.31 100±0.31 100±0.31
Autoclaving (15lbs/sq inch pressure at 120°C) For 15 min	100±0.31
Storage in days 15 30 45 60 75 90 105	 100±0.01 100±0.11 100±0.21 100±0.14 100±0.15 100±0.01 100±0.12

Data are mean of five replicates,± Standard error

T. cordifolia oil was more effective than commercial pesticides (copper oxychloride and carbendazim) during *in vivo* experiments both as seed dressing and fumigation studies (aluminium phosphide and ethylene dibromide).

The seed fumigation method was more effective than seed dressing method, protected seeds of *Buchnania* up to 105 days from fungal infestation (Table 6) thereby increasing its shelf life. The seeds of control sets showed proliferation of several fungal species after 15 days of storage. Seeds stored with leaf oil as preservative showed better smell and taste in comparison to ones stored with pesticides. This study revealed that *T. cordifolia* leaf oil was more fungitoxicants than tested fungicides, thereby indicating the possibility of its exploitation as an ideal antifungal agent for protection of kernels during storage. Since the plant, *T. cordifolia* grow luxuriantly, its essential oil is an easily available, indigenous and renewable source of fungi toxicant with no known mammalian toxicity.

Table 6: *In vivo* efficacy of *T. cordifolia* leaf oil and commercial fungicides in preservation of *B. lanzan* seeds

Incubated period in days	Occurrence of fungal species *on B.lanzan* seeds						
	Seed dresser efficacy			Fumigant efficacy			Control set
	Copper oxychloride	carbandazim	Leaf oil	Aluminium phosphide (Sulphos)	Ethylene dibromide	leaf oil	
15	-	-	-	+	-	-	+
30	-	-	-	+	+	-	+
45	-	+	-	+	+	-	+
60	+	+	-	+	+	-	+
75	+	+	-	+	+	-	+
90	+	+	-	+	+	-	+
105	+	+	-	+	+	-	+

+ Presence of fungal species, - Absence of fungal species on *B. lanzan* seeds

The leaf oil of T. *Cordifolia* showed minimum inhibitory concentration- 400ppm against both *Aspergillus niger* and *A.flavus.* The previous literature revealed that there is a marked variation in the MIC of different plant oils against *Aspergillus niger* and *A.flavus*thus-*Ocimum adscendens* Willd 200ppm[18], *Syzygium aromaticum* (L.) Merrill and Perry 200ppm[19], *Cedrus deodara (*Roxb.ex Lambert) G.Don 1000ppm and *Trachyspermum ammi* (L.) Sprague 500ppm[20], *Adhatoda vasica* 500ppm[21]. *Cuminum cyminum* 400ppm[22]. The variation in the MIC of different plant oils may be due to the presence of different chemical constituents.

Wellman[22] highlighted that a fungicide must retain its fungi toxicity at the extreme of temperatures. The fungi toxicity of seed oil of *Putrnjiva* was found to be thermostable up to 100°C like *Ageratum conyzoides*[24]; *Nardostachys jatamansi*[25] *Adhatoda vasica* oil[21] and *Cuminum cyminum*[11]. The leaf oil retained its fungi toxicity on autoclaving (15lbs/square inch pressure). This quality of oil will facilitate the isolation of their constituents in active state.

Wellman[22] reported that a fungicide should be able to retain its activity during long period of its storage. The fungi toxic factor in the oil of *Adenocalyma allicea* was lost within 21 d of storage[23] while persisted for long period in the oil of *Ageratum conyzoides*[24]; *Trachyspermum ammi*[20] and *Adhatoda vasica*[21]. The fungal toxicity was not affected by storage up to 105 days during present investigation. So this shows that the leaf oil of *T. cordifolia* can be safely stored at any ambient temperature for long periods without loss in toxicity.

CONCLUSION

The study revealed that leaf oil of T. *cordifolia* was more fungitoxicants than tested synthetic pesticides, there by indicating the possibility of its exploitation as an agent for protection of seed of *B.lanzan* during storage.

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