IJPSR (2017), Volume 8, Issue 10 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 14 March, 2017; received in revised form, 19 May, 2017; accepted, 27 May, 2017; published 01 October, 2017

IN VIVO PRESERVATIVE POTENTIAL OF NEEM GILOY-*TINOSPORA CORDIFOLIA* (WILLD.) MIERS EX HOOK. F. AND THOMS LEAF ESSENTIAL OIL FOR GROUNDNUT (*ARACHIS HYPOGAEA*) SEEDS

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Keywords:

Groundnut, *T. cordifolia* leaf oil, biodeterioration

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ABSTRACT: The mycological analysis of stored seed of groundnut (*Arachis hypogaea*) through agar plate as well as blotter paper methods of study revealed presence of 16 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*. Out of 20 essential oils extracted from leaf of plants and assessed for their antifungal potential only leaf oil of *Tinospora cordifolia*,(Willd.) Miers ex Hook. F. & Thoms exhibited absolute toxicity (100%) against dominant fungi *Aspergillus flavus, A. niger* at 400 ppm concentration. This showed fungicidal activity at 500ppm.It showed MIC against 10 fungi at 400ppm, controlled 15 fungi at 600 ppm. There was no ill effect of physical factors *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 was able to preserve seeds of groundnut up to 105 days separately in polyethylene containers with minimal changes in organoleptic behaviour during storage.

INTRODUCTION: The food grain production is dependent on quality seeds in a country. A quality seed is directly related with the production of healthy crop. For disease free crop Healthy and pathogen free seeds are the basic requirements. Seeds are stored for a considerable period of time and 25% of the world's crops are affected by mould or fungal growth. In India around 82% of groundnut produced is used for edible oil production, 12% as seed and 5% as feed.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(10).4291-97

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (10).4291-97

For disease transmission seed is responsible because they carry a number of pathogens in the field or in the post harvest storage condition.

Groundnut is the major oilseed of India. Its seeds are rich sources of edible oil (43-55%) and protein (25-28%).It accounts for around 25% of the total oilseed production of the country [1]. Annual production of Indian Peanuts and Indian Peanuts oil are around 5-8mln and 1.5 mln tons respectively. Regional estimates for production are *viz*., Gujarat (1-3.5 million tons), Tamil Nadu (1million tons), Andhra Pradesh (1-2 million tons), Karnataka (0.5 million tons), Maharashtra (0.5 million tons).

Synthetic chemicals are present in market to control post harvest biodeterioration but has been restricted due to their carcinogenicity,

teratogenecity, high and acute residual toxicity, hormonal imbalance, long degradation period, environmental pollution and their adverse effects on food and side effects on humans [2, 3, 4].

The studies on essential oils have shown that they are readily biodegradable and less detrimental to non-target organisms as compared to synthetic pesticides. Application of essential oils as plant products is a very attractive method for controlling post harvest pathogens. Therefore, *Tinospora cordifolia*, (Willd.) Miers ex Hook. F. & Thoms (family Menispermaceae) leaf oil was explored during storage which is commonly known as amrita, guduchi and is widely used in indigenous systems of medicine [5, 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 mycological analysis of Groundnut seeds were carried and *in vivo* preservative activity trials were conducted with most active *T.cordifolia* leaf oil.

MATERIALS AND METHODS: During 2014-2016 grocery stores of Panchgaon, Gurgaon were surveyed for seeds samples of Groundnut. The seeds were analysed for their mycoflora following Kumar [8] through agar plate and standard blotter techniques. For this 100 seeds were equidistantly kept on separate petri plates containing 10 ml czapeks dox agar medium in agar plate method. In each plates 5 seeds were kept.

In blotter method three layered moistened blotter pads in sterilized petriplates inspite of medium and the seeds were similarily plated separately. The assay plates were then kept at 25±2°C in incubator and observed daily up to 7 days for appearance of fungal colonies. They were isolated, purified and identified with the help of fungal keys.

In order to know the internal seed mycoflora, the groundnut seeds were first surface sterilized with 0.1% sodium hypochlorite for one minutes washed with double distilled water and then subjected to agar plate and standard blotter techniques for isolation of the fungi. Excess water was removed from the seed using folds of sterilized blotters.

The seeds samples of groundnut were analysed for percent occurrence of each fungal species as per formula-

$$\text{Per cent occurrence} = \frac{\text{Number of colonies of a particular fungus X 100}}{\text{Total number of colonies of all the fungi}}$$

With the help of Clevenger's apparatus essential oils were extracted from leaf of 20 plants separately up to 7 hrs at 90±2 ^{0}C through hydrodistillation. Each essential oil was dried over anhydrous sodium sulphate and was stored at 4 ^{0}C under sterilized condition for further use. The antifungal activity was evaluated separately by Inverted Petri plate technique at 500 ppm concentration for both essential oils and two commercial fungicides Copper oxychloride and Carbondazim against fungal species.

Fungitoxic properties of *Tinospora cordifolia* leaf oil: For determination of MIC different concentration of the *T.cordifolia* leaf oil ranging from 200 to 600ppm were prepared by dissolving requsite amount of leaf oil in 0.5ml acetone and then mixing with 9.5ml czapeks dox agar medium separately. In control sets the petriplates having acetone and medium without *T.cordifolia* leaf oil were used. The fungal discs (5mm diam) obtained from periphery of seven day old culture of each of test fungi were aseptically inoculated in each of the treatment and control sets. They were incubated at 28±2 ^{0}C for 6 days. 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 mycelia 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 upto 7d were washed with sterile water and placed again on fresh solidified medium to observe the revival of mycelial growth. The fungitoxic spectrum of the *T.cordifolia* leaf oil was studied against various fungi isolated from seeds samples of groundnut. In addition effect of temperature, autoclaving and storage on the fungitoxicity of *T.cordifolia* leaf oil was determined following Kumar [9]. Each experiment was repeated thrice and having 5 replicates.

Fumigant potential and protectant power was studied following Kumar[10] with slight modification. For fumigant potential efficacy determination, requisite amount of *T.cordifolia* leaf oil was soaked in cotton swab (100mg weight) and was introduced in polyethylene bags (17.0 cm dia. × 20.4 cm height) which had 200g of Groundnut seed sample separately, so as to attain 1µl/ml (v/v) concentration. Similarly, fumigant fungicides aluminium phosphide (sulphos) and ethylene dibromide (EDBA ampule) were introduced at 500 ppm concentration onto samples of 200g seeds each separately.

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 Groundnut were filled in polyethylene bags. They were treated with 1ml stock solution of the leaf oil. For proper coating groundnut seeds continuous shaking for 7 min were performed. Similarily, two contact fungicides such as copper oxychloride and carbendazim (1000 mg/ 200g seeds) were selected for comparison purpose and also run parallely.

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

Botany of *T.cordifolia*: *T. cordifolia* is popularily known as Guduchi and Giloy. It is an herbaceous vine of the family Menispermaceae and native to India. Its entire part is used in Ayurvedic medicine. The leaves are heart shaped (**Fig. 1**). The succulent bark is having deep clefts spotted with lenticels creamy white to grey in color. It puts out long slender aerial roots. Flowers grow in lax racemes from nodes on old wood and yellow in colour. Fruits drupes, turning red when ripe. The stem of this plant berberine, columbin, chasmanthin, palmarin, tinosporon, tinosporic acid and tinosporol. The compounds identified in essential oil include alcohols (32.1 %), phenols (16.6 %), aldehydes (16.2 %), fatty acids (15.7 %), alkanes (8.3 %), esters (3.2 %), terpenes (1.2 %), and other classes of compounds (4.8 %). Major components of the leaf essential oil were hydroquinone (16.6 %), 2-hexenal (14.2 %), palmitic acid (14.1 %), 2-hexen-1-ol (11.5 %) and phytol (11.4%) [11]

FIG. 1: *TINOSPORA CORDIFOLIA*

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 Tinospora oil were obtained by taking average of five replicates.

RESULTS AND DISCUSSION: There were 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 Groundnut. In which *Aspergillus flavus, A. niger, A. ochraceus* and *A. terreus* were dominant showing 29.0, 27.0, 23.0, 21.0 in blotter and 29.0, 22.0, 22.0, 30.6 % in agar plate method of study (**Table 1**).

TABLE 1: PERCENT OCCURRENCE OF MYCOBIOTA FROM JANUARY TO SEPT MONTHS (AVERAGE) ON STORED SEEDS OF *ARACHIS HYPOGAEA*

Fungi recorded	Blotter method		Agar plate method	
	US	SS	US	SS
Alternaria alternata (Fr.) Keissler	3.9±0.11	2.2±0.11	5.1±0.11	-
Aspergillus candidus Pers ex.	2.1±0.11	-	4.1	-
A. flavus Link	29.0±0.11	4.6	29.0	9.0
A. niger van Tieghem	27.0±0.11	3.7	22.0	7.0
A. ochraceous Wilhelm	23.0±0.11	4.6	22.0	7.5

A .tamarii Kita	3.1±0.11	-	3.2	-
A. terreus Thom	21.0±0.11	4.5	30.6	6.8
A. sydowi (Bainier and Sartory) Thom and Church	5.4±0.11	2.2	5.1	1.0
Fusarium moniliforme Sheldon	3.0±0.11	2.1	5.0	-
F. oxysporum von Schlechtendal	3.2±0.11	2.3	3.1	1.4
F. solani (Mart.) Sacc.	3.3±0.11	2.0	3.1	1.2
Penicillium glabrum (Wehmer) Westling	5.0±0.11	-	2.5	-
Rhizopus nigricans Ehr.	3.2±0.11	-	0.1	-
Syncephalastrum racemosum Cohn	4.0±0.11	-	-	-
Trichoderma viride Pers.ex.Fr.	3.7±0.11	-	-	-
Trichothecium roseum (Persoon)	3.6±0.11	-	-	-

Time to time researchers have isolated fungi *viz., Aspergillus flavus, Aspergillus niger* and *Aspergillus ruber* [12]. *Aspergillus flavus* and *Aspergillus parasiticus* [13].; *A. flavus* [14]; *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani***,** *F. oxysporum*, *Aspergillus flavus***,** *A. Niger* [15]; *Aspergillus flavus* and *Aspergillus niger* [16]; six fungal taxa, namely *Aspergillus*, *Rhizopus*, *Penicillium*, *Curvularia*, *Fusarium* and *Mucor* [17]; *Aspergillus niger*, *Aspergillus flavus*, *Alternaria dianthicola*, *Curvularia lunata*, *Curvularia pellescens*, *Fusarium oxysporum*, *Fusarium equiseti*, *Macrophomina phaseolina*, *Rhizopus stolonifer*, *Penicillium digitatum* and *Penicillium chrysogenum* [18]; *Fusarium* [19]; *Aspergillus*, *A. flavus* [20]; species of *Aspergillus*, *Penicilium*, *Fusarium*, *Rhizopus* and *Alternaria* [21].

The work recorded in 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 [22-23].

Out of 20 essential oils screened, *T. cordifolia* 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* [24]. Narayanan *et al*., [25] 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 2: EVALUATION OF *T. CORDIFOLIA* LEAF OIL AND SYNTHETIC FUNGICIDES AGAINST *ASPERGILLUS FLAVUS* AND *A. NIGER*

Plant species	Plnat part used in hydrodistillation	Per cent 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*±0.11	100*±0.11
Copper oxychloride	*Synthetic fungicide	Yogi Dye Chem Industries Satyam Complex, M. G. Road, Ghatkopar East, Mumbai - 400077, Maharashtra	91.0±0.11	92.0±0.11
Carbondazim	*Synthetic fungicide	Canary Agro Chemicals Pvt. Ltd., New Delhi	93.1	84.2

Determination of minimum inhibitory concentration (MIC) of a fungicide is necessary for prescribing its appropriate dose. High dose of fungicide increase wastage and may cause considerable loss to the quality of commodity treated. The MIC of *T. cordifolia* oil against dominant fungi *A. flavus* and *A. niger* was found to be 400 ppm (**Table 3**). It was found fungicidal at 500 ppm. It showed MIC against 10 fungi at 400ppm while at 600ppm it controlled 15 fungi (**Table 4**).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 (**Table 5**).

TABLE 3: MIC OF *T. CORDIFOLIA* LEAF OIL

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

*Fungicidal

TABLE 4.FUNGITOXIC SPECTRUM OF *T. CORDIFOLIA* LEAF OIL AT SUB LETHAL, LETHAL AND HYPERLETHAL DOSES AGAINST FUNGI ISOLATED FROM STORED SEEDS OF *ARACHIS HYPOGAEA*

Fungal species	Per cent inhibition of mycelial growth of isolated fungi			
	Sublethal 200ppm	Lethal 400ppm	Hyperlethal 600ppm	Hyperlethal 800ppm
Alternaria alternata	44.0±0.10	81.3±0.13	100.0±0.12	100.0±0.10
Aspergillus candidus	48.7±0.12	88.1±0.12	100.0±0.13	100.0±0.10
A.flavus	51.3±0.10	100.0±0.12	100.0±0.14	100.0±0.17
A.niger	34.7±0.10	100.0±0.11	100.0±0.12	100.0±0.17
A.ochraceous	41.2±0.19	100.0±0.12	100.0±0.11	100.0±0.10
A. phoenicis	47.9±0.12	100.0±0.10	100.0±0.10	100.0±0.12
A. tamari	58.3±0.12	100.0±0.10	100.0±0.10	100.0±0.12
A. terreus	55.0±0.12	100.0±0.12	100.0±0.12	100.0±0.13
A. sydowi	47.3±0.12	100.0±0.13	100.0±0.13	100.0±0.12
Fusarium moniliforme	44.1±0.12	78.4±0.13	100.0±0.13	100.0±0.12
F.oxysporum	41.3±0.12	100.0±0.13	100.0±0.13	100.0±0.12
F.solani	58.8±0.12	100.0±0.13	100.0±0.10	100.0±0.12
P.glabrum	53.2±0.12	100.0±0.13	100.0±0.10	100.0±0.12
Rhizopus nigricans	54.4±0.13	81.2±0.12	94.4±0.12	100.0±0.13
Trichoderma viride	64.0±0.13	96.1±0.13	100.0±0.13	100.0±0.13
Trichothecium roseum	64.0±0.13	96.1±0.12	100.0±0.13	100.0±0.12

TABLE 5: EFFECT OF PHYSICAL FACTORS ON THE FUNGITOXICITY OF *T. CORDIFOLIA* LEAF OIL

Physical factors	Per cent inhibition of mycelia growth at its MIC
Temperature(°C)	100±0.17
Time of treatment-60min	100±0.14
40°C	100±0.13
60°C	100±0.12
100°C	
Autoclaving (15lbs/sq inch pressure at 120°C) For 15 min	100±0.11
Storage in days	
15	
30	
45	
60	
75	
90	
105	

T. cordifolia oil was more effective than commercial pesticides (copper oxychloride and carbendazim) during *in vivo* both during seed dressing and fumigation studies (aluminium phosphide and ethylene dibromide). The seed fumigation method was more effective than seed dressing method, protected seed of Groundnut 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 comparision 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 seeds of groundnut during storage. Since the plant *T. cordifolia* grow luxuriantly, it's essential oil is an easily available, indigenous and renewable source of fungitoxicant with no known mammalian toxicity.

TABLE 6: *IN VIVO* EFFICACY OF *T. CORDIFOLIA* LEAF OIL AND COMMERCIAL FUNGICIDES IN PRESERVATION OF SEEDS OF *ARACHIS HYPOGAEA*

Incubated period in days	Occurrence of fungal species stored seeds of *Arachis hypogaea*						
	Seed dresser efficacy			Fumigant efficacy			
	Copper oychloride	carbandazim	Leafoil	Aluminium phosphide (Sulphos)	Ethylene dibromide	leaf oil	Control set
15	-	-	-	+	-	-	+
30	-	-	-	+	+	-	+
45	-	+	-	+	+	-	+
60	+	+	-	+	+	-	+
75	+	+	-	+	+	-	+
90	+	+	-	+	+	-	+
105	+	+	-	+	+	-	+

+ Presence of fungal species, - Absence of fungal species on *Arachis hypogaea* 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 canum* Sim 500ppm [26], Jamrosa oil 0.3 ml/ ml [27]; *Cuminum cyminum* Linn. 400ppm [8]; *Adhatoda vasica* Nees 500ppm [9] and *Coriandrum sativum* L. 400 ppm [10]. The variation in the MIC of different plant oils may be due to the presence of different chemical constituents.

The fungicide must retain its fungitoxicity at the extreme of temperatures. The fungitoxicity was found to be thermostable up to 100C like *Ocimum canum* [26], *Adhatoda vasica* [9] *Coriandrum sativum* [10]. The leaf oil retained its fungitoxicity on autoclaving (15lbs/square inch pressure). So this property of oil will facilitate the isolation of their constituents in active state.

A fungicide must retain its activity during long period of its storage. The fungitoxic factor in the oil of was lost in *Ocimum canum* [26] in 90 d of storage while persisted for long period in the oil of *Adhatoda vasica* [9]; *Coriandrum sativum* [10]. The fungal toxicity was not affected by storage up to 105 days during present investigation. So this show 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 fungi toxicants than tested synthetic pesticides, thereby indicating the possibility of its exploitation as an agent for protection of seed of Groundnut during storage. This may be a fumigant as alternate of synthetic pesticides.

ACKNOWLEDGMENT: Author is thankful to the Amity University Haryana for the facilities and constant encouragement.

CONFLICT OF INTEREST STATEMENT: I declare that I have no conflict of interest.

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How to cite this article:
Kumar N: *In vivo* preservative potential of neem *Giloy-tinospora cordifolia* (willd.) Miers ex hook. F. and thoms leaf essential oil for groundnut (*Arachis hypogaea*) seeds. Int J Pharm Sci Res 2017; 8(10): 4291-97.doi: 10.13040/IJPSR.0975-8232.8(10).4291-97.