Biospectra : ISSN: 0973-7057, Vol. 16(2), September, 2021, pp. 129-134 **25(PS).**
An International Biannual Refereed Journal of Life Sciences **Plant Science**

ISSN : 0973-7057

Int. Database Index: 616 www.mjl.clarivate.com

Assessment of antifungal efficacy of selected botanicals by Thin Layer Chromatography bioassay

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Received : 11th June, 2021 ; Revised : 12th July, 2021

Abstract- Knowing the antifungal efficacies of large numbers of botanical extracts in short span of time were carried out by Thin Layer Chromatography bioassay against selected fungi. Methanol, Ethanol and Ethyl acetate extracts of botanical spices namely, Garlic (*Allium sativum*), Nutmeg (*Myristica fragrans*), Black pepper (*Piper nigrum*), Ajwain (*Trachyspermum ammi*), Chebulic myrobalan (*Terminalia chebula*), Fenugreek (*Trigonella foenumgraecum*) and Ginger (*Zingiber officinale*) were isolated. The obtained botanical extracts were subjected through TLC bioassay and tested against selected phytopathogens namely *Alternaria capsici* (*Capsicum*) and *Curvularia lunata* (*Oryzae sativa*). The separated compound were subjected to bioassay by spraying spore suspension (10^4cfu/ml) of selected fungi and incubated for 24-36 hrs at 25±2°C. After incubation; zone of inhibition were recorded and on that basis antifungal potential of the extracts were determined. All the organic fractions of Garlic showed antifungal properties while methanol extract of nutmeg, ethanol extracts of Ajwain and Chebulic myrobalan showed highest zone of inhibition against both the test fungi. The presence of these active principles with definite R_f value in spices extracts when prepared as bioformulations or established in crop rotation could be exploited as an organic inputs towards eco-friendly, alternatives of chemical fungicides in disease management strategies.

Key words: Thin layer chromatography, Botanical, *A. capsici, C. lunata*, Inhibition.

INTRODUCTION

Plants and plant produces are facing several stresses like pest and disease; causing annual yield loss nearly 28-30%. Among the plant diseases; seedborne, soilborne, and airborne fungal, bacterial and viral phytopathogens played important role in yield losses[1-3]. To minimize the yield loss, synthetic pesticides had become an instant & very common solution for agriculturists but its continuous use and prolonged exposure had severe after effects *viz.* health impairments, disturbances in soil & aquatic ecosystem, residual toxicity and non-biodegradability[4,5]. So, researchers are continuously engaged in searching viable, cheap, eco-friendly & very next options to chemical fungicides, in the form of botanicals, biopesticides and plant metabolites[6]. Reports suggest that the negative effects of chemical fungicides & pathogens had not been limited to only plants but also humans fell prey to their toxicity every now & then[7,8]. *A. capsici* and *C. lunata* are the common fungal pathogen causing severe yield losses, decreasing the nutritive value & storability of crop produce[9-11].

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Spices and herbs are an important group of agricultural commodities which are being used all over the world to add aroma, taste and nutritional values in the food. India is known as the 'homeland of spices'. Today, Indian spices are the most sought-after globally; because of their exquisite aroma, texture, taste and medicinal values. Spices and herbs were used for healing of various physical, mental and emotional problems mentioned in Ayurveda 'Indian traditional medical systems' and modern literatures[12]. In India, Garlic (*Liliaceae*), Ginger (Zingiberaceae), Ajwain (Apiaceae), Fenugreek (Fabaceae), Nutmeg (Myristicaceae), Black pepper (Piperaceae), are spices widely used for culinary purposes while Myrobalan (Combretaceae), Ajwain and other selected spices are known for magic medicine related to stomach ailments. Moreover, these spices contain a variety of active principals which also possess the ability to inhibit wide range of plant disease causing pathogens[12]. Maurya *et al*. (2004)[13] reported that the plant extracts of the bark of *Azadirachta indica* and *Cyperus rotundus* showed strong antifungal efficacy at very low concentration (500 ppm) while Ginger, Tulsi, Mahua, Cashew nut shell extract were also effective at 2000 ppm against powdery mildew disease of pea caused by phytopathogen *Erysiphe pisi*.

In the present study, seven botanical plants or spices namely *A. sativum*, *M. fragrans*, *P. nigrum, T. ammi*, *T. chebula*, *T. foenumgraecum*, *Z. officinale* were selected for extraction of the active components using three organic solvents based on decreasing polarity namely Methanol, Ethanol and Ethyl Acetate and their antifungal properties were screened against *C. lunata* by thin layer chromatography bioassay technique.

MATERIALS & METHODS

Extraction of botanical extracts

Cold percolation technique was used for extraction of botanical extracts by using methanol; ethanol and ethyl acetate separately for each botanical seeds/plant parts[14]. Initially, each spice was weighed up (100g) and grinded by the use of pestle and mortar and then soaked in selected organic solvent separately and shaking was done thoroughly after 3-4 hrs interval a day. After 7-8 days of soaking, extraction was done by filtering with non-absorbent cotton pad while further evaporation of organic solvents was done in water bath (60°C). After evaporation semisolid oily liquid was obtained, weighed (0.89-3.15) and stored in collection bottle (extraction scheme 1).

For Thin layer chromatography

TLC plates were cut into strips of 2 cm × 7 cm of 80-120 mesh chromatographic pre-coated silica plates which acted as stationary phase[15]. With the help of pencil, a line was drawn, approx 0.5 cm from the bottom. It was 2-3 mm deep so that the solvent and the line were not submerged together. The slurry containing the extract (sample) was gently loaded at a point on the line with the help of micropipette i.e. 10 µl was loaded 2-3 times in short interval of time (>1s). The resulting spot was 1-2 mm in diameter. This ensured that the sample did not dissolve into the solvent and would travel up to TLC plate with the solvent. Based on the relative polarity, the solvent mixture i.e. Chloroform and Methanol (50:50) were selected. The solvent system selected was then poured into the glass chamber. The TLC strip was made to stand in the glass container with the solvent system as mobile phase. After few hours when the solvent front was 0.5-1 cm from the top of the plate, the plate was removed from the chamber and the solvent left on the TLC plate was allowed to evaporate in hot air oven for 5 min at 100°C. Different spots which were visible under white light and UV light (366 nm) were marked with pencil while to visualize the colourless components iodine flakes were used for photo documentation. R_f value was calculated as:

$$R_f = \frac{\text{Distance travelled by solute front}}{\text{Distance travelled by solvent front}}$$

Antifungal efficacy of selected extracts of spices

For spore germination bioassay, the selected pathogens were collected from the experimental research farm of ICAR-RCER-RC, Palandu, Ranchi district of Jharkhand (Fig a) & after washing with 2% $NaOCl_2$ solution for 1 min & subsequently by distilled water were cultured in test tube slants (Fig b). After obtaining pure cultures of the test pathogens *viz. C. lunata* and *A. capsici* were taken and a loopful of spores were mixed per ml of distilled water to get zoospore suspension. The spore suspensions were then immediately sprayed uniformly on developed chromatogram using atomizer. After spraying; the chromatograms were kept in closed glass trays fitted with moist paper for 24 hr at 24±2°C[16]. The screening was done for most potent organic plant extracts as the zone of inhibition was observed on developed chromatogram while the maximum surface on TLC plate area was covered by spore growth.

RESULT & DISCUSSION

Extraction Scheme 1.

Dried samples of botanical/plants/spices in measured amount (100g)

⇩

Course grinded with pestle and mortar

⇩

Soaked in selected organic solvent (w/v)

⇩

Gently shaking every 3-4 hrs interval a day

⇩

After 7-8 days of soaking, filtered with the help of cotton pad & separating funnel

⇩

Evaporation of the solvent water bath (60°C)

⇩

Semi solid oily liquid extract (in gm.) was collected and stored in sample collection bottle.

⇩

Tween 80 (4-8 drops) were used for ethyl acetate extracts (*viz.* garlic, ginger) as an emulsifier for slurry preparation.

Table 1. Qualitative antifungal efficacy of different organic extracts of spices against selected phytopathogenic fungi.

Plant extracts	Antifungal Efficacy of different organic extracts					
	Ethyl acetate (ETHAC)		Methanol (MEOH)		Ethanol (ETOH)	
	C. lunata	*A. capsici*	*C. lunata*	*A. capsici*	*C. lunata*	*A. capsici*
T. ammi	-ve	-ve	+ve	+ve	+ve	+ve
T. chebula	-ve	-ve	+ve	+ve	+ve	+ve
M. fragrans	-ve	-ve	+ve	+ve	-ve	+ve
T. foenumgraecum	+ve	+ve	-ve	-ve	-ve	-ve
P. nigrum	-ve	-ve	-ve	+ve	-ve	+ve
A. sativum	+ve	+ve	+ve	+ve	+ve	+ve
Z. officinale	+ve	+ve	+ve	-ve	+ve	-ve

+ve = Positive inhibition against test fungal growth, -ve = Non-effective against test fungi.

The active principles of seven spices extracted in three organic solvents (Extraction Scheme 1) & their subjection to thin layer chromatography (TLC) plate provided fast and easy analysis of presence of antifungal metabolites, in short span of time. Using above R_f index calculation formula, the R_f value of separated compounds in seven spices and their methanol, ethanol and ethyl acetate fraction was noted (Table 2). In the ethanol extract of *T. ammi*; greenish brown colour compound with R_f value 0.92 and methanol extract containing green orange coloured compound with R_f value 0.84 were separated which also gave positive assessment of antifungal properties against *A. capsici* and *C. lunata* (Table1 & 2). Control showed full growth & spore germination of *A. capsici* and *C. lunata* on TLC plate (Fig1.a). On the colorless, dried & developed chromatogram of all the 3 organic extracts of *A. sativum*; strong & positive inhibition was observed against *A. capsici* & *C. lunata* (Fig1.f & g). However, compound separated in ethanol with R_f value 0.89 & methanol extract with R_f value 0.88 extracts of *T. chebula* (Fig1.c) showed positive inhibition against both the test fungi (Table 1 & 2). Although, the yellow coloured chromatogram from methanol extract of *M. fragrans* with R_f value 0.89 (Fig1.f) after screening showed positive inhibition against both test fungi but its ethyl acetate & ethanol extract was only effective against *A. capsici*. Similarly, only the *T. foenumgraecum* ethyl acetate extract when developed chromatogram at R_f value 0.79 showed positive efficacy against *C. lunata* (Table 1, Fig1.b). Against *A. capsici*; the developed chromatogram in ethyl acetate, ethanol & methanol extract of *P. nigrum* showed positive inhibition while it was –ve (sparse growth of spore) in case of *C. lunata* (Fig1.d). Against *C. lunata*

the ethyl acetate, ethanol (Fig 1. e) & methanol extract of *Z. officinale* with R_f value 0.79, 0.97 & 0.96 (Table 2) showed positive efficacy; but except ethylacetate extract, the other two organic extracts of *Z.officinale* failed to inhibit *A. capsici* spore growth (Table 1).

The present study based on isolation and confirmation of phytopathogenic constituents present in methanol, ethanol & ethyl acetate extracts of botanicals relates to the work of Rani *et al.* (2013)[17]; who also separated & tested antimicrobial compounds from methanol extract of fruit pulp of *Aegle marmelos*. The present Thin layer chromatography work was carried using solvent system methanol: chloroform (1:1) based on polarity very similar to the work performed by Yanpirat & Vajrodaya, (2015)[18] on botanicals like *Persicaria odorata* against *C. capsici* & Gupta *et al.* (2014)[19] who used botanical leaf extract & solvent system of Toluene: Ethyl acetate: Formic acid (3:1:2) to unveil the presence of phytochemicals. In earlier researches & even in the present direct bio-autographic assay, the confirmatory presence of bioactive & antifungal compound with distinct R_f value in the seed organic extracts of *T. ammi*, *M. fragrans*, *A. sativum* & *T. chebula* through developed chromatogram showed promising results against *Curvularia* & *Alternaria* species[20]. The spraying of spore suspension & separation of organic plant extracts together on stationary phase interlinks the concept of biological assay to be performed directly on TLC plate & searching the active principle which can lessen the pathogenic attack & become a better alternative to synthetic fungicides[21].

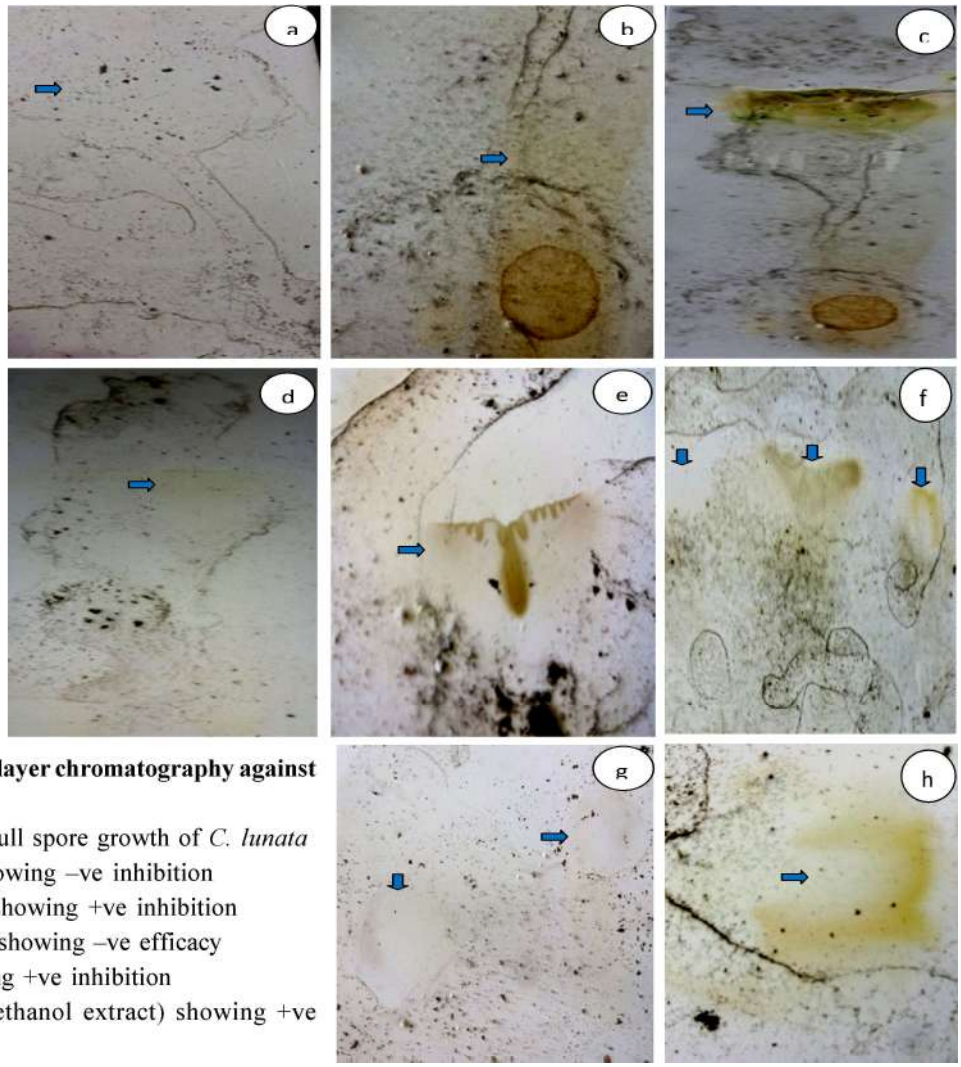

Fig 1. Antifungal bioassay by thin layer chromatography against *Curvularia lunata*.

a. TLC plate in control showing full spore growth of *C. lunata*
b. Fenugreek (ethanol extract) showing –ve inhibition
c. Myrobalan (methanol extract) showing +ve inhibition
d. Black Pepper (ethanol extract) showing –ve efficacy
e. Ginger (ethanol extract) showing +ve inhibition
f. Garlic, Ajwain & Nutmeg (methanol extract) showing +ve inhibition
g. Garlic (ethanol & ethyl acetate extract) showing +ve inhibition
h. Nutmeg (ethanol extract) +ve inhibition. (Blue arrow indicating qualitative spore germination).

Table 2. Thin Layer Chromatography: Showing R_f value, Colour and Recovery (%age) of different organic extract of spices.

Plant extracts	% recovery, retention factor and colour of the extracts								
	Ethyl acetate extract			Ethanol extract			Methanol extract		
	R_f value	Colour	Recovery (%) (per 100 gram)	R_f value	Colour	Recovery (%) (per 100 gram)	R_f value	Colour	Recovery (%) (per 100 gram)
T. ammi	0.71	Yellow orange	2.55	0.92	Greenish brown	2.26	0.84	Green orange	1.02
T. chebula	0.69	Pale green	2.04	0.89	Pale orange	2.42	0.88	Brownish green	1.71
M. fragrans	0.96	Yellow orange	1.55	0.68	Brownish yellow	2.34	0.89	Yellow	3.15
T. foenumgraecum	0.79	Pale yellow	1.38	0.81	Pale yellow	1.02	0.87	Colourless	1.78
P. nigrum	0.86	Green	1.43	0.93	Parrot green	1.98	0.91	Greenish brown	1.42
A. sativum	0.96	Colourless	1.32	0.91	Colourless	1.02	0.94	Colourless	1.62
Z. officinale	0.79	Light brown	0.89	0.97	Brown	1.44	0.96	Brown	1.96

Here, R_f = Retention factor, %age = percentage

CONCLUSION

Present study involving bioassay of selected spices confirmed the existence of antifungal compounds present within the selected spices organic extracts and which could be deployed against destructive fungi like *Alternaria* & *Curvularia*. Present work opens up the access towards extraction of the eluted phytoconstituents through column chromatography & further by HPTLC. After recognizing the secondary metabolite based on their R_f value; their formulations as contact or systemic fungicides lead to promising results even *in vivo* experiments. The screening of these botanicals against selected phytopathogens proves better alternative towards organic, natural and eco-friendly disease management than synthetic pesticides. The conversion of active ingredient or the plant extracts directly into nano particle might further enhance their efficacy by multiple times.

ACKNOWLEDGEMENT

The corresponding author is thankful to DST-INSPIRE (Govt. of India) as funding agency for Ph.D research work, and ICAR Research Complex for Eastern Region, Research centre, Ranchi, India, for providing laboratory assistance and is duly acknowledged.

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