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Antifungal Potential of *Achyranthes Aspera* Tannins and Ginger Extracts: A Sustainable Approach to Combat Seed-Borne Fungal Contamination

MADIEHA AMBREEN[1], SAFDAR ALI MIRZA[2], ZAHIDA BANO[3] and MUHAMMAD TALHA HASSAN[4]

[1] School of Biology, Botany section, Minhaj University, Lahore, Pakistan, [2] Department of Botany, GCU, Lahore, Pakistan,[3] University of Doha for Science and Technology,[4]Frontier Medical College Abbottabad, Pakistan,

**To whom correspondence should be addressed, Madieha Ambreen*

*madiha ambreen(drmadieha.bot@mul.edu.pk) *, Safdar Ali Mirza (safdaralimirza@gcu.edu.pk) *, Zahida Bano (zahida.qau@gmail.com) *, Muhammad Talha Hassan (drtalhahassan92@gmail.com) **

Abstract

Seed-borne fungi can be detected through various methods, including in vitro cultures. Synthetic fungicides are commonly used for contamination control, but they pose environmental risks. This study explored the antifungal potential of *Achyranthes aspera* callus cultures, along with Ginger and Neem extracts, to mitigate seed-borne fungal contamination in a sterile environment. Fungal-induced seed discoloration was assessed using the Blotter and Towel methods. *Aspergillus niger* caused the most severe black spot discoloration, followed by dark brown discoloration from *Aspergillus alternata*, dark black from *Mucor hiemalis f. corticola*, and creamy discoloration from *Pseudomonas aphidis. A. niger* dominated the seed coat, while *M. hiemalis f. corticola* was prevalent in the embryo. To control fungal contamination, fresh Ginger and Neem extracts were tested alongside a synthetic fungicide. Additionally, the antifungal activity of *A. aspera* extracts from leaves, stems, root seedlings, and callus cultures grown on different media and extracted using various solvents was evaluated against *A. niger* and *F. solani*. Traditionally valued for its medicinal properties, *A. aspera* is a promising candidate for bioactive antifungal compound development. Callus cultures, known for their controlled production of secondary metabolites, exhibited potent antifungal activity, particularly due to tannins. Methanol and petroleum ether extracts from leaf and root callus cultures grown in MSDN and MSDI media showed varying inhibition levels. The petroleum ether extract of MSDN leaf callus culture exhibited the highest inhibition against *A. niger*, while the chloroform extract of MSDI root callus culture had the lowest inhibition against *F. solani*. Overall, *A. aspera* callus cultures and fresh plant extracts demonstrated strong antifungal potential, offering eco-friendly alternatives to synthetic fungicides and supporting their further investigation for sustainable agriculture.

Keywords: Fresh plant extracts, seed-born fungi, tannins, minimum inhibitory concentration

Introduction

Seed-borne fungal contamination poses a significant threat to agricultural productivity and food safety. The presence of phytopathogenic fungi in crops not only leads to reduced yields and decreased nutritional value but also increases the risk of mycotoxin contamination in grains (Peng et al., 2021; Satish et al., 2007). Chemical pesticides have been traditionally employed to combat these fungal infections; however, the emergence of pesticide-resistant pathogens and the adverse effects associated with pesticide use have necessitated the exploration of alternative strategies. In recent years, there has been a growing interest in the use of natural plant extracts as potential alternatives to chemical treatments (Shahbaz et al., 2022). Various plant species, including Ginger, Garlic, Neem, Mint, Yarrow, and Turmeric, have

demonstrated promising antimicrobial properties (Parham et al., 2020), making them valuable resources for managing pathogen infections in crops. Among these plants, Neem and Ginger extracts have been commonly utilized to control seed-borne fungal diseases (Ojaghian et al., 2012; Wylie et al., 2022), offering a safer and environmentally friendly approach. Another avenue for combating fungal infections and developing new antimicrobial agents is the exploration of phytochemicals derived from medicinal plants. Tannins, a group of naturally occurring plant polyphenols, have attracted attention due to their ability to form stable compounds with proteins and other polymers. With their larger molecular structures and distinct surface characteristics, tannins exhibit diverse biological activities and have been studied for their potential therapeutic applications (Smeriglio et al., 2017; Rajasekar et al., 2021). These polyphenolic compounds interfere with fungal growth by disrupting cell membranes, inhibiting spore germination, and interfering with key metabolic pathways. Their antifungal effects are primarily attributed to their ability to form complexes with fungal proteins, enzymes, and cell wall components, thereby impairing cell integrity and inhibiting pathogenic activity (Zhu et al., 2019). Tannins extracted form various plants have been analysed for antimicrobial activity (Mailoa et al., 2014; Vu et al., 2017), however, the antimicrobial or antifungal properties of tannins from *A. aspera* callus culture have not been extensively investigated yet.

A. aspera, a medicinal plant belonging to the Amaranthaceae family, is well-documented for its diverse therapeutic properties, including antibacterial, antifungal, and anthelmintic effects (Ndhlala et al., 2015: Singh et al., 2019). Traditionally, it has been used in the treatment of headaches, skin diseases, asthma, pneumonia, and leukoderma, among other ailments (He et al., 2017: Singh et al., 2019). As part of the on-going search for novel antifungal agents, the potential of tannins derived from *A. aspera* callus cultures presents a promising avenue for exploration. Callus cultures, which are undifferentiated cell masses generated through plant tissue culture techniques, provide a controlled and sustainable platform for the production of bioactive secondary metabolites, including tannins. The use of callus cultures for tannin production offers several advantages in antifungal applications. Unlike field-grown plants, callus cultures provide a controlled and optimized environment for producing bioactive secondary metabolites, ensuring a higher yield of potent tannins (Ambreen et al., 2024). Additionally, plant tissue culture techniques allow for the manipulation of growth conditions, such as the use of elicitors and plant growth regulators, to enhance tannin biosynthesis and maximize their antifungal efficacy.

This study aims to assess the antifungal properties of Ginger, Neem and tannins extracted from callus cultures of *A. aspera* and evaluate their effectiveness in inhibiting fungal contamination in seeds. The antifungal potential of these tannins was determined through bioassays, including fungal growth inhibition studies, and zone of inhibition tests against common seed-borne fungal pathogens. By investigating the therapeutic effects of callus-derived tannins, this study seeks to establish their role as a natural and eco-friendly alternative for controlling fungal infections in crops. The findings could contribute to the development of sustainable antifungal treatments, with potential applications in agriculture and food security.

Materials and methods

Procurement of plant materials and tannin extraction

Healthy seeds of *A. aspera* L. (Voucher No. GC.

Herb. Bot. 3492) were procured from the market and identified by taxonomist of GC University Lahore and compared with herbarium specimen, Voucher specimens were submitted for further research. The whole research was performed at Plant Biotechnology Laboratory GC University Lahore. Tannins were extracted from leaf, stem and roots of the plants using different solvents including chloroform, P. ether, methanol and distilled water as explained in (Ambreen et al., 2024) and quantified using Folin-Ciocalteau Colorimetric Method (Adegbusi et al., 2022).

Seed discoloration

Blotter Method

In the Blotter Method, a total of eighty to ninety seeds were used. To initiate the process, filter paper pieces were immersed in sterile water and placed in Petri dishes. Each Petri dish contained twenty seeds. To ensure surface sterilization of the seeds, a five percent Sodium hypochlorite solution was used. The seeds were immersed in this solution and then placed on the Petri plates.

The Petri plates were incubated for seven days at a temperature of 25 ± 2°C. During incubation, a 12-hour alternating cycle of UV light and darkness was maintained. Seeds were observed under microscope after incubation to evaluate the proportion of seed-borne fungi. The observation aimed to determine the presence and distribution of these fungi in different parts of the seeds, including the seed husk, seed coat, and embryo.

Towel method

Two square sheets of blotter paper were moistened with distilled water, ensuring sufficient margins. In three separate replicates, a total of fifty discoloured seeds were subjected to surface sterilization for a duration of 2 min using a 5 percent sodium hypochlorite solution. These sterilized seeds were then evenly distributed evenly between blotters.

The blotter sheets were folded along one edge and rolled up after placing the seeds. These rolls were stored in a plastic bag during the incubation period to maintain the desired conditions. Incubation was carried out at a temperature of 28°C, utilizing a fluorescent tube light source. The incubation setup followed an alternating pattern of 12 hours of darkness and 12 hours of light. After a 14-day incubation period, the resulting seedlings were carefully examined. The investigation focused on determining the number of normal seedlings, identifying seed rot, and categorizing any atypical seedlings as either blighted or stunted.

Preparation of fresh plant extracts

Five grams of fresh Neem leaves and Ginger rhizome were separately blended with 100 ml of autoclaved distilled water to prepare fresh plant extracts. The resulting mixture was filtered through a nylon cloth and centrifuged at a speed of 3000 rpm for a duration of 15 min. The supernatant obtained was filtered again using Whatman Filter Paper No.1 and autoclaved for 20 min to ensure sterility. Fifty seeds were washed and soaked in the Neem and Ginger extracts for different durations: 0.5, 1, and 2 hours for germination experiment. After the soaking period, the seeds were placed for germination in jars containing cotton pads, inside a growth room maintained at a temperature of 26±2°C and a photoperiod of 16 hours. The germination process was monitored and recorded. Meanwhile, a control group of seeds that were not treated with the extracts was also included in the experiment for comparison. Mean seedling length was measured in centimeter by

help of ruler. Seed germination percentage was calculated using the following formula.

Germination percentage (%) = Total germinated seeds/ Total no. of seeds x100 (ISTA, 1996)

The Seedling Vigor Index (SVI) was determined by using equation (Abdul-Baki and Anderson, 1970):

(SVI)= [seedling length (cm) x germination percentage]

Antifungal activity

Collection of antifungal strains

The fungal strains were collected from Department of Microbiology, GC University, Lahore.

Preparation of plant samples

Tannins (0.01g) extracted from callus and plant powder were transferred to glass vials. The volume in each vial was then adjusted to 10 ml using the suitable solvent. The glass vials containing the tannin extracts were kept at a temperature of 20°C, resulting in stock solutions with a concentration of 10 mg/ml.

Preparation of medium plates

Thirty nine grams of PDA were dissolved in 1 liter of distilled water to make potato dextrose agar medium (PDA). The mixture was heated while being continuously stirred to ensure a homogenous mixture. Afterward, the medium was allowed to cool to a temperature of 50°C, and its pH was carefully set to 5.6. Subsequently, the medium underwent autoclaving at a temperature of 121°C under a pressure of 15 psi for a duration of 15 min. Finally, the prepared medium was dispensed into Petri plates in quantities of 20 mL each.

Agar well diffusion method

The Agar Well Diffusion method (Jorgensen et al. 2015) was used to investigate antifungal potential of said plant extracts. The dilute inoculum of fungi was prepared according to 0.5 % McFarland turbidity grade and mixed in a Petri plates. Standard wells were made by using cork borer no. 4 in PDA plates. One milliliter of each extract was poured in each well of each Petri plate, reclose and sealed with cling film. The plates were kept in incubator at 37± 2^{o}C for 48 hours for antifungal activity. All the experiments were performed in triplicates to obtain precise conclusions.

Determination of Minimum Inhibitory Concentration

The least concentration was recorded by spectrophotometer as described by Andrew (Andrew 2001).

Relative percentage inhibition

Relative percentage inhibition (RPI) was calculated according to standard formula.

RPI= 100(A-B)/(C-B)

A= Total area of inhibition of plant extract

B= Total area of inhibition of solvent

C= Total area of inhibition of positive control (standard drug)

Statistical analysis

All data was expressed as mean ± SE at $p \leq 0.05$ using LSD.

Results

This research aims to identify a natural fungicide using Ginger and Neem while also assessing the antifungal potential of tannins extracted from callus cultures of *A. aspera*. Tannins were extracted from the leaves, stems, and roots tissue and callus tissue of *A. aspera* using various solvents, including petroleum ether, chloroform, methanol, and distilled water as explained in Ambreen et al., (2024) and tested against *A. niger* and *F. saloni*

Application of fresh plant extracts against seed born fungi

Tables 1, 2, and 3 illustrate that fungal infections lead to a range of discolorations, each with varying degrees

of severity. Different types of fungi were observed in different regions of the seeds. Notably, seeds exhibiting the highest percentage of black spot discoloration (16%) were found to be infected with *A. niger*, while seeds with 10% dark brown discoloration were attributed to *A. alternata* and 7% of dark black were seen in case of *Mucor r hiemalis f. corticola.* Additionally, *P. aphidis* was responsible for a creamy discoloration affecting 5% of the seed surface.

Table 1. Percentage of seed born fungi associated with different discoloration of seed born fungi (Blotter method). Letters a, b and ab indicate statistically significant differences at P<0.05

Fungi species	Discoloration	Percentage
Aspergillus niger	Black spot	16^{a}
Aspergillus alternate	Dark brown	10^{ab}
Mucor r hiemalis f. corticola	Dark black	7^{a}
Pseudozyma aphidis	Creamy	5^{a}

In Table 2, it is evident that *A. niger* accounted for the highest fungal infection percentage on the seed coat, totaling 16%, while *Mucor rhiemalis f. corticola* was responsible for only 2% infection in the embryo.

Table 2. Percentage of location of seed borne fungi associated with seeds (Blotter method).

Fungi species	Seed coat	Husk	Embryo
Mucor r hiemalis f. corticola	10^{ab}	4^{ab}	16^{a}
Aspergillus niger	17^{a}	7^{a}	14^{a}
Aspergillus alternate	12^{a}	9^{a}	11^{ab}
Pseudozyma aphidis	7^{a}	5^{a}	2^{a}

From Table 3, it becomes apparent that among the various seedling outcomes, 15% were characterized as having normal growth with black spot discoloration, while abnormal seedlings constituted 16%, and a significant 23% exhibited stunted growth with heavy black discoloration. In comparison to other types of seed discoloration and healthy seeds, creamy seeds yielded the lowest percentage of normal seedlings at 6%, followed closely by dark brown seeds at 7%. Remarkably, dark black seeds resulted in a high incidence of blight i.e., 16%.

Table 3. Effect of discoloration on seedling features percentage by Towel method

Fungi species	Discoloration	Seedling percentage		
		Normal seedling	Abnormal seedling	
			Stunt	blighted
Aspergillus alternata	Dark brown	7	21	47
Aspergillus niger	Black spot	11	33	60
Mucor r hiemalis f. corticola	Dark black	15	23	16
Pseudozyma aphidis	Creamy	6	71	34

Achyranthes aspera seeds were discovered to be contaminated with *A. niger*, a seed-borne fungus. To combat this fungal issue, various natural extracts and a fungicide called Fluconazole (100 mg) were examined. However, due to its environmental impact, the fungicide was deemed non-eco-friendly. As an eco-friendly alternative, aqueous crude extracts from Neem and Ginger were explored. Among the tested treatments, the application of Fluconazole 100 mg at a concentration of 0.1g/ml for a duration of 2 hours proved to be the most effective in controlling fungal growth on *A. aspera* seeds. In the case of fresh plant extracts, Ginger extract applied for 1 hour showed significant activity in inhibiting fungal growth. The difference between the activity of Ginger extract and fluconazole may be due to differences in their mechanisms of action, potency, or stability. Fluconazole, being a synthetic antifungal, may require a longer duration to effectively inhibit fungal growth, whereas Ginger extract may act more quickly through its bioactive compounds.

A longer exposure time for Ginger extract could potentially enhance its antifungal effectiveness by allowing more time for its active components to interact with fungal cells. However, this would depend on factors such as the stability of the extract, potential degradation over time, and the ability of the fungal cells to develop resistance or adapt. Further studies would be needed to determine the optimal exposure time for maximum efficacy.

Table 4. Effect of duration of different plant extracts on type of fungi on seeds.

Sr. No	Treatment	Duration(hours)	Germination	Fungi	Name of fungi
1	Ginger	0.5	Blighted growth	+	*A. Niger*
2	Ginger	1	Full growth	-	-
3	Ginger	2	Stunt growth	+	*Mucor r hiemalis f. corticola*
4	Neem	0.5	Blighted growth	+	*A. alternata*
5	Neem	1	Stunt growth	+	*A. niger*
6	Neem	2	Full growth	-	-
7	Fungicide	0.5	Stunt growth	+	*A. niger*
8	Fungicide	1	Full growth	-	-
9	Fungicide	2	Full growth	-	-

Moreover, in terms of seedling length, the treatments with Fluconazole 100 mg for 2 hours and Ginger extract for 1 hour yielded favorable results (Table 4). When employing fresh plant extracts of Ginger and Neem to combat seed-borne fungal contamination, with Fluconazole 100 mg/ml serving as the control, it was observed that the 1-hour treatment with Ginger extract showed the inhibition of *A. niger*, resulting 77% of the seeds germination (Table 5). Interestingly, the fungicide treatment exhibited slightly higher growth compared to Ginger, i.e., 83%, as evidenced in Tables 5. The Neem extract shows lower antifungal efficacy compared to the Ginger. The reason for weaker antifungal activity of the Neem could be due to several factors; Neem contains various bioactive compounds such as azadirachtin and nimbin, but their antifungal effectiveness can vary based on concentration, extraction method, and fungal species targeted. Another reason could be the antifungal mechanism of Neem may be slower or less potent than the active compounds in Ginger, which have strong antimicrobial properties or certain fungal species might be more resistant to Neem's antifungal compounds while being more susceptible to the active components in Ginger.

Table 4. Effect of duration of different plant extracts on type of fungi on seeds.

Sr. No	Treatment	Duration(hours)	Germination	Fungi	Name of fungi
1	Ginger	0.5	Blighted growth	+	*A. Niger*
2	Ginger	1	Full growth	-	-
3	Ginger	2	Stunt growth	+	*Mucor r hiemalis f. corticola*
4	Neem	0.5	Blighted growth	+	*A. alternata*
5	Neem	1	Stunt growth	+	*A. niger*
6	Neem	2	Full growth	-	-
7	Fungicide	0.5	Stunt growth	+	*A. niger*
8	Fungicide	1	Full growth	-	-
9	Fungicide	2	Full growth	-	-

Identification of fungi

The seeds from infected plants were subjected to a fungal identification process based on reference (Nag, 1993) The identification of endophytic fungi and yeast was primarily conducted using morphological characteristics observed through a stereoscopic microscope. Microscopic analysis confirmed the presence of fungi by examining factors such as spore size and colony morphology. In terms of morphology, *A. niger*, a filamentous fungus, displayed smooth, colorless conidiophores and spores that were observable under a microscope. *A. alternate* featured conidiophores that were either light or dark brown in color, along with brownish conidia possessing a short or sometimes non-existent beak. These conidia had a smooth surface or were slightly warty in appearance. The hyphae of *Mucor* were typically coenocytic, but mature hyphae might contain septa. Finally, *P. aphidis* exhibited elongated, ellipsoidal blastoconidia that were found on short stalks.

Antifungal activity of tannins content

Comparative analysis of antifungal potential of extracts leaf tissue, stem, and root, in comparison to extracts from callus culture of *A. aspera*

A comparative analysis was conducted to assess the antifungal potential of extracts from leaf tissue, stem seedling root tissue and callus culture of *A. aspera* against *A. niger* and *F. solani.* Various extracts, including petroleum ether, chloroform, methanol, and aqueous extracts, were evaluated for tissue grown in various media including MSDB (T1 and T5) containing 0.5 mg/l 2,4-D and 1.5 mg/l BAP, MSDI (T2 and T6) with 2.0 mg/l 2,4-D and 4.0 mg/l BAP, and MSNB (T3 and T7) with 2.0 mg/l NAA and 2.0 mg/l BAP, MSDIB (MS media with 2,4-D and IBA) and MSDN (MS media with 2,4-D and NAA). The MS-based media were designed to influence callus induction and secondary metabolite production. The choice of these media components significantly impacted the type and yield of bioactive compounds in the callus cultures.

Heat map plot were generated using "ORIGINPRO: the Ultimate Software for Graphic and Analysis" to visualize the antifungal activity of *A. aspera* extracts using four different solvents petroleum ether, chloroform, methanol, and distilled water against two fungal species across different treatments (T0 (1) to T4 and T0 (2) to T8). Each row corresponds to a solvent, starting with distilled water (top row), followed by methanol, chloroform, and petroleum ether (P. ether) at the bottom. The color intensity in the heat map indicated antifungal activity, with blue representing lower activity, pink/red indicating moderate to high activity, and deeper red showing the highest activity levels. T0 (1) and T0 (2) served as negative controls, where no significant antifungal activity is observed. This is evident by the light blue coloration across most solvents, indicating minimal inhibition. T4 and T8 serve as positive controls, where the extracts display higher antifungal activity (Figure 1, 2 and 3). In these treatments, deeper shades of blue, pink, and red appear for certain solvents, especially petroleum ether and methanol, suggesting stronger activity.

Figure 1 showed the antifungal potential of petroleum ether, chloroform, methanol and aqueous extracts of leaf tissue and callus culture observed against *A. niger* and *F. solani.* The petroleum ether extract, serving as the positive control and equivalent to fluconazole, exhibited the highest inhibitory activity against *A. niger*. Interestingly, the tannin content of the petroleum ether extract from the leaf callus culture containing medium MSDN (T2) displayed stronger activity against *A. niger*, indicating substantial antifungal activity. On the other hand, the aqueous extract, acting as the positive control and analogous to griseofulvin, demonstrated a minimal activity represented in light pink colour in heat map plot against *F. solani* (Figure 1).

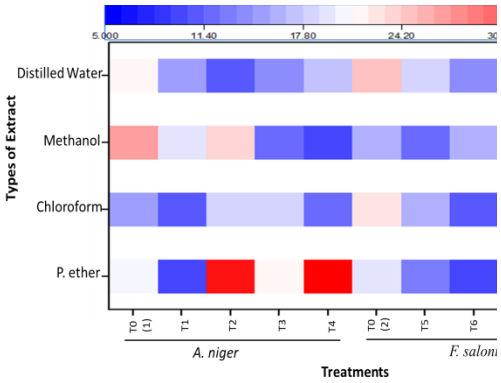

Figure 1. Comparative analysis of heat map of antifungal potential of leaf tissue and callus culture extracts of *A. aspera* against *A. niger* and *F. saloni*

Leaf explants of *A. aspera* grown on medium MSDB (T1 and T5) (0.5 mg/l 2,4-D+1.5 mg/l BAP), MSDI (T2 and T6) (2.0 mg/l +4.0 mg/l), MSNB (T3 and T7) (2.0 mg/l NAA+2.0 mg/l BAP) and leaf tissue (Controls, T0 (1), T0 (2)) and Fluconzole (T4) (positive control) and Grisofulvin (T8) (positive control). T0 (1) to T4 were treated with *A. niger* and T0 (2) to T8 were treated with *F. saloni.*

For stem seedling tissue and callus culture, the petroleum ether extract, used as the positive control analogous to fluconazole, demonstrated the highest inhibitory potential against *A. niger*. Minimal antifungal activity was observed across treatments, indicated by the light blue coloration. This suggested that water was not effective in extracting potent antifungal compounds from the stem of *A. aspera.* The aqueous extract, specifically the tannin content derived from stem seedling tissue, exhibited a minimal activity against *F. solani* as shown in Figure 2.

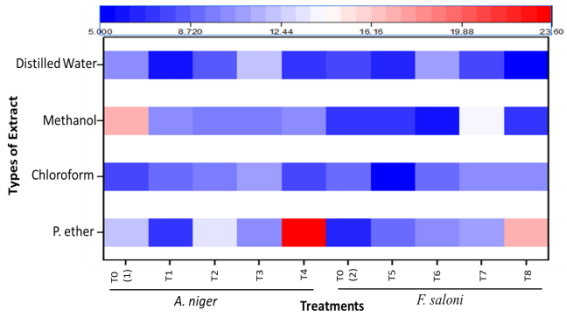

Figure 2. Comparative analysis of heat map of antifungal potential of stem tissues and callus culture extracts of *A. aspera* against *A.niger* and *F. saloni*.

Stem explants of *A. aspera* grown on medium MSDI (T1, T5) (0.5 mg/l 2,4-D+1.5 mg/l BAP) MSDN (T2 and T6) (2.0 mg/l +4.0 mg/l), MSDI (T3 and T7) (2.0 mg/l NAA+2.0 mg/l BAP) and stem tissue (Control, T0 (1), T0 (2)) and Fluconazole (T4) (positive control) and Grisofulvin (T8) (positive control). T0 (1) to T4 were treated with *A. niger* and T0 (2) to T8 were treated with *F. saloni.*

Finally, for root seedling tissue and root callus culture, the petroleum ether extract, acting as the positive control equivalent to fluconazole, displayed the highest inhibitory activity against *A. niger*, with a notable red colour in heat map plot. Among the seedling root tissue and callus culture extracts, the tannin content of the petroleum ether extract from root callus culture medium MSDI demonstrated the lowest inhibitory effect, with light blue shade in heat map plot against *F. solani*, as indicated in Figure 3.

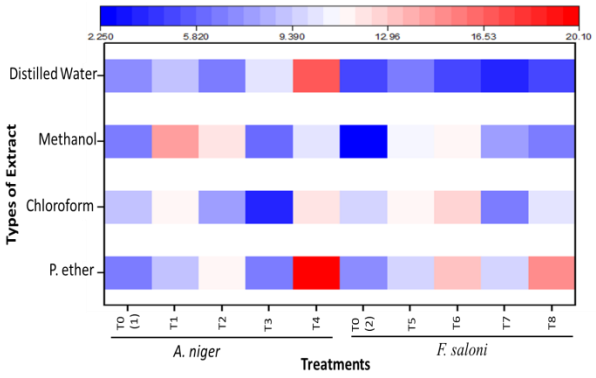

Figure 3. Comparative analysis of heat-map of antifungal potential of root tissues and callus culture extracts of *A. aspera* against *A. niger* and *F. saloni.*

Root explants of *A. aspera* grown on medium MSDI (T1 and T5) (0.5 mg/l 2,4-D+1.5 mg/l BAP), MSDN (T2 and T6) (2.0 mg/l +4.0 mg/l), MSDI (T3 and T7) (2.0 mg/l NAA+2.0 mg/l BAP) and stem tissue (Control T0 (1), T0 (2) and Fluconazole (T4) (positive control) and Grisofulvin (T8) (positive control). T0 (1) to T4 were treated with *A.niger* and T0 (2) to T8 were treated with *F. saloni.*

In terms of relative percentage inhibition, the petroleum ether extract from callus culture on medium MSDN showed the highest value, 94.87%, against *A. niger*, which was slightly lower than the positive control fluconazole at 96.07%. Conversely, the chloroform extract from callus culture containing the medium MSDIB of root exhibited the lowest relative percentage inhibition against *A. niger*, which was 13.46% (Table 7).

Table 7. Relative percentage inhibition (%) against *A. niger*

Sample types	Types of extract	Percentage inhibition
Leaf	Seedling leaf tissue P. ether extract	51.78
	MSDB (Aqueous extract)	27.90
	MSDN (P. ether extract)	94.87
	MSNB (Methanol extract)	68.08
	Fluconazol (Positive control) (P.ether extract)	96.07
Stem	Seedling stem tissue P. ether extract	83.02
	MSDB (Aqueous extract)	39.89
	MSDN (P. ether extract)	47.44
	MSNB (Methanol extract)	21.55
	Fluconazol (Positive control) (P.ether extract)	96.07
Root	Seedling root tissue P. ether extract	37.99
	MSDB (Aqueous extract)	41.23
	MSDN (P. ether extract)	23.77
	MSNB (Methanol extract)	13.46
	Fluconazol (positive control) (P.ether extract)	96.07

Table 8. Relative percentage inhibition (%) against *F. saloni*

Sample types	Types of extract	Percentage inhibition
Leaf	Seedling leaf tissue P. ether extract	32.70
	MSDB (Aqueous extract)	25.00
	MSDN (P. ether extract)	81.27
	MSNB (Methanol extract)	48.08
	Fluconazol (Positive control) (P.ether extract)	83.45
Stem	Seedling stem tissue methanol extract	61.77
	MSDIA (P. ether extract)	37.89
	MSDN (P. ether extract)	35.44
	MSDIB (Aqueous extract)	19.86
	Fluconazol (Positive control) (P. ether extract)	83.45
Root	Seedling root tissue P. ether extract	32.24
	MSDN(Chloroform extract)	30.20
	MSDB (Aqueous extract)	23.65
	MSDIB (Chloroform extract)	10.41
	Fluconazol (positive control) (P.ether extract)	83.45

Discussion

The results of this research provide valuable insights into the antifungal properties of tannin extracts from different parts of *Achyranthes aspera* plants, including leaf tissue, stem, and root, as well as from callus cultures. Additionally, the study evaluates the potential of Ginger extract in managing fungal contamination, particularly in seeds. Fungi emerged as the predominant culprits responsible for seed discoloration in this study, showcasing a range of discoloration types to varying

degrees of severity (Table 1, 2, and 3). The presence of these fungal contaminants not only resulted in lower seed germination rates but also had detrimental effects on seedling growth. Among the various discoloration types observed, the seeds with the highest incidence of black spot discoloration (16%) were attributed to infection by *A. niger* indicating its significance as a seed-borne fungus, consistent with previous studies linking it to brown discoloration in rice (Mahmud et al., 2016, Ackaah et al., 2023), and other plant species within the Amaranthaceae family.

The use of chemicals to control seed born contamination is not environmentally friendly and causes health problems to animals through environmental pollution. In response to these challenges, this study explored the use of plant extracts as eco-friendly alternatives to chemical treatments for managing seed-borne fungal contaminants. Among the two plant extracts investigated, Ginger fresh crude extract stood out as it positively influenced seedling length (Table 1). Previous research has highlighted the effectiveness of Ginger fresh aqueous extract against pathogens like *Sclerotinia* and *Sclerodia spp.* (Stangarlin et al., 2011; Nagamma et al., 2019). This effectiveness may be attributed to ginger's capacity to produce peroxidase enzymes, enhancing disease resistance, as well as its ability in inducing an accumulation of phytoalexine-3-deoxyanthocyanidin, which mitigates the effects of diseases (Nagamma et al. 2019). Additionally, caprylic acid present in Ginger may act as a bio-fungicide, further contributing to seed-borne contamination control (Anzian et al, 2020; Naz et al., 2015). In our study, the slightly higher antifungal activity observed in the standard drug compared to the Ginger extracts may be attributed to the thick cell walls of fungal strains, which contain high quantities of chitin and exhibit resistance to condensed tannins (Mathur et al. 2011). Neem extract, on the other hand, contains nimbin, azadirachtin, nimbinene, and nimbidin, which are known for their antimicrobial properties (Sidhu et al., 2004; Baby et al., 2022).

The successful control of seed-borne fungal contamination was supported by in vitro tissue culture studies of *A. aspera*, aimed at mass-producing callus for tannin extraction. The results revealed a close relationship between tannins and in vitro antifungal activity. Polyphenolic compounds like tannins have established themselves as effective antifungal agents against various fungi, including *F. solani* and *A. flavus* (Khanzada et al., 2021). The polymerization of tannin monomers is a critical factor that enhances their reactivity, and the presence of multiple OH groups in the B-ring inhibits microbial growth. Tannin content contributes to antimicrobial activity by suppressing extracellular enzymes, depriving microorganisms of essential substrates and metal ions necessary for their growth, and directly affecting microbial metabolism through the inhibition of oxidative phosphorylation (Scalbert, 1991). The heat map demonstrated that petroleum ether was the most effective solvent for extracting antifungal compounds from the stem of *A. aspera*, as evidenced by the intense red coloration in T4 and T8. Methanol also shows moderate activity, while chloroform and distilled water were less effective. The antifungal activity is most prominent in positive control treatments, confirming the potential of *A. aspera* stem extracts as a source of antifungal agents. The results highlight the importance of solvent choice in isolating and testing bioactive compounds.

The quantity of phytochemical compounds extracted in methanol could depend on several factors, including the type of bioactive compound, the polarity of the solvent used, and the fundamental bioactivity of the compounds. Leaf callus culture grown on medium

MSDN showed significant differences (Table 4). The assessment of relative percentage inhibition further emphasized the efficacy of tannin extracts in controlling fungal contamination. For instance, petroleum ether and chloroform extracts from callus cultures of leaf and root from MSDN medium exhibited the highest and lowest relative percentage inhibitions, respectively, against *A. niger* (Table 7). Similarly, the petroleum ether extract from callus culture of leaf and the chloroform extract from callus culture of root from medium MSDN showed the highest and lowest relative percentage inhibitions, respectively, against *F. solani* (Table 8).

These results align with previous studies that have demonstrated the considerable inhibitory effect of *A. aspera* tannin content against *A. niger*. The differential responses of *F. solani* and *A. niger* to tannin-rich extracts could be attributed to variations in their cell wall compositions and structures, which influence their susceptibility to antifungal agents. However, there is a lack of existing literature regarding its effectiveness against *F. solani*, highlighting the novelty of our findings.

Conclusion

In conclusion, this research sheds light on the potential of tannin extracts from *A. aspera*, Ginger and Neem for controlling fungal contamination in seeds. These natural extracts offer eco-friendly and promising alternatives to chemical treatments Moreover, the findings emphasize the importance of addressing *A. niger* as a seed-borne fungus of concern. Future studies could delve deeper into the mechanisms underlying the antifungal properties of these extracts and their applications in sustainable agriculture.

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