Pure Appl. Biol., 12(2):1056-1065, June, 2023
http://dx.doi.org/10.19045/bspab.2023.120108

Research Article

Phytochemical analysis and antifungal activity of selected medicinal plant extracts against *Alternaria alternata*

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Citation

Noshaba Olikh, Brian Gagosh Nayyar, Ayesha Serwer, Maryam Ajmal, Akasha Naseer and Tehseen Kaleem Hashmi. Phytochemical analysis and antifungal activity of selected medicinal plant extracts against *Alternaria alternata.* Pure and Applied Biology. Vol. 12, Issue 2, pp1056-1065. http://dx.doi.org/10.19045/bspab.2023.120108

Received: 01/02/2023 Revised: 12/04/2023 Accepted: 19/04/2023 Online First: 03/05/2023

Abstract

Fungicides are used for plant diseases but extensive use of chemicals causes serious problems related to plant and consumer health. Therefore, this is an emerging need of time to find an alternative eco-friendly approach. Four selected weeds were investigated for antifungal potential against *Alternaria alternata*. Qualitative phytochemical screening revealed the presence of different phytochemicals viz, amino acids, proteins, tannins, flavonoids and phytosterols in these plant extracts. Mycelium inhibition of target fungus by different extracts of weeds was observed using agar disc diffusion method, agar well diffusion method and food poison method. Results obtained from food poison method were appreciably good as compared to other two methods. The order of effectiveness of weeds was: *Malva neglecta* > *Parthenium hysterophorus* > *Cannabis sativa* > *Chenopodium album*. For aqueous extracts of weeds maximum fungal mycelium inhibition was recorded by *C. sativa* (91%) followed by *M. neglecta* (75.7%), *P. hysterophorus* (73.8%) and *C. album* (51.2%). The ethanolic extracts of all weeds were also found effective against *A. alternata* and maximum antifungal activity was recorded by *M. neglecta* (86.7%) followed by *P. hysterophorus* (77.1%), *C. sativa* (62.8%) and *C. album* (61.1%). Methanolic extracts of weeds also showed significant results as maximum fungal inhibition was recorded by *M. neglecta* (86.9%) followed by *P. hysterophorus* (77.1%), *C. album* (74.6%) and *C. sativa* (69.7%). The results of this study indicates that extracts of *M. neglecta* and *P. hysterophorus* have high potential which can be used to control *A. alternata* and provides data for the researchers to make plant-based fungicides as an alternate method.

Keywords: Agar Disc Diffusion; Agar Well Diffusion; Food Poison Method; Eco- friendly; Weed extract

Introduction

Phytopathogens, such as fungi are the most significant biotic stress that causes major crop loss by producing various diseases and toxins **[1-3]**. *Alternaria alternata* is an economically significant fungus for its ability to cause considerable post-harvest losses by toxin contamination of food and feed **[4]**. *Alternaria* has about 40 species which are normal agents of decay and composition that may be found in practically every habitat and are a natural element of the fungal flora almost everywhere. Among them, some species are plant pathogens that cause a range

of diseases in many economically important plants including vegetables, fruits, ornamentals and many other crops. *Alternaria* spp. are also well known as postharvest pathogens and has a very wide host of range including tomato, apple, citrus, litchi, ficus and many others [5]. The sesame crop is under the severe attack of fungal diseases specifically leaf blight disease, caused by *Alternaria alternata* around the world [6]. *A. alternata* is a destructive plant pathogenic fungus which attacks the aerial parts of the plant and may lead to complete defoliation during severe attack [7] Sesame yield loss about 30–40% reported in India due leaf blight caused by *Alternaria* spp. [8]. There are reported attacks of *Alternaria* leaf blight disease of chickpea around the world and it has caused an average loss of 5–47% in Pakistan as well [9]. Many synthetic fungicides have been recommended for the management of the fungal disease [10, 11]. However, the extensive and indiscriminate use of fungicides has resulted in a variety of issues, including environmental degradation, disruption of natural processes and ecosystem functioning, contamination of food and feed, and eventually detrimental effects on human and animal health [12]. The usage of synthetic fungicides for disease management considered to an exclusive mean since long time due to inaccessible alternative sources [13]. However, they are not regarded long-term solutions because of the concerns about environmental pollution, fungicide residues, cost, source of carcinogenicity and other human health issues [14]. Therefore, it is emerging need of time to develop alternative solutions for plant diseases and infections in minimizing reliance on synthetic fungicides [15]. From past few years, the interest in the use of medicinal plant extracts has been increased and for this purpose scientists have identified several medicinal plants as substitute which are non-toxic, eco-friendly, biodegradable, inexpensive and fit very well in the integrated disease management. The higher plants are the source of natural medicines, chemicals, metabolites, and oils with antibacterial characteristics [16, 17]. Many plants have been employed for their antimicrobial properties, due to the presence of secondary metabolites such as phenols, flavones, quinones, flavonoids, tannins, and coumarins, which act as plant defense boosters against pathogenic microbes [18-21]. The main purpose of these phytochemicals is to provide durable immunity in the form of resistance against many disease - causing pathogens [22]. Botanical extracts may be a preferable alternative to synthetic pesticides owing to their environmental benefits as compared to synthetic chemicals [23, 24]. The naturally occurring secondary metabolites present in plant extracts are of great interest for the researchers against various human and plant diseases. Throughout the world, 444,000 flowering plant species are found, in which about 4000 plants are used for their phytochemical importance. In Pakistan, 6000 plant species are present and among them about 180 plants are used for medicinal purpose [22]. Four weeds *Cannabis sativa*, *Chenopodium album*, *Malva neglecta* and *Parthenium hysterophorus* were tested against the target pathogen *A. alternata* via different *in vitro* methods using different extracts. Several workers studied antifungal potential of some plant extracts including *C. sativa*, *C. album*, *M. neglecta* and *P. hysterophorus* respectively [25, 26]. Different concentration of methanolic leaf extracts of *C. sativa* showed antifungal potential against *Aspergillus flavipes*, whereas *C. album* demonstrated antifungal activity against the fungus *Fusarium oxysporum*, which causes basal rot disease in onions. Antifungal properties of *M. neglecta* have been reported against *Fusarium solani*, *Aspergillus niger*, *Aspergillus flavus* and

Aspergillus fumigatus. *P. hysterophorus* methanolic extract shown excellent antifungal activity against *Candida kefyr*, *Candida albicans*, and *Aspergillus niger* [27, 28]. The current study is an attempt to evaluate the antifungal potential of four local weeds extracts for the bio control of leaf blight diseases caused by *A. alternata*.

Materials and Methods

Collection and preparation of plant material

Fresh leaves of *Cannabis sativa*, *Chenopodium album*, *Malva neglecta* and *Parthenium hysterophorus* was collected from different localities of district Sialkot, Pakistan. Leaves of these plants were thoroughly washed under running tap water to remove dust particles and shade-dried for 15–20 days. The dried leaves were crushed into a fine powder using a kitchen grinder and stored in zipper bag at 4°C until used [29]. Following the maceration technique, 10g of each plant powder was used for extraction in 100ml of water, ethanol, and methanol in 250ml Erlenmeyer flasks. These flasks were covered with cotton plug and sealed properly with parafilm tape and wrapped in aluminum foil to withstand at room temperature for three days until the soluble matter was dissolved. After three days, the mixture was filtered using Whatman filter paper No. 3 [30].

Phytochemical screening of selected weeds

For the screening of phytochemicals, various standard laboratory techniques were used indicating the presence or absence of secondary metabolites such as phenols, saponins, tannins, alkaloids, flavonoids, and glycosides in each plant extracts [31].

Detection of amino acids

Ninhydrin test was used for the determination of amino acid by adding ten drops of Ninhydrin solution (10mg of Ninhydrin in 200ml of acetone) into the 2ml of each plant extract. Appearance of purple color indicated the presence of amino acids.

Detection of flavonoid

Ferric chloride test was used for flavonoids detection. Each weed extract (1ml) was dissolved in 1ml of 10% $FeCl_3$ solution. The Mixture was shaken vigorously for 3 minutes by hand. Flavonoids give a dull green/reddish brown color which confirmed the presence of flavonoids.

Detection of phytosterols

Phytosterols were detected by Salkowski test. Each plant extract (1ml) was added into 1ml of conc. H_2SO_4. Solution was shaken vigorously by hand and cooled at room temperature which slightly changed the solution color. Red color in test sample indicates the presence of phytosterols.

Detection of protein

Xanthoproteic test was used for protein detection. Each weed extract (1ml) was added into 1ml of conc. HNO_3. Mixture was allowed to cool down at room temperature; afterwards 1ml of 40% NaOH was added into test tube which changed the color of tested sample. Dark yellow / orange – red color was observed for the presence of protein.

Detection of tannins

For tannins, NaoH (10%) test was performed in which 0.5ml of each plant extract was added into 4ml of NaoH solution. The mixture was shaken vigorously by hand for 5 minutes and left at room temperature for the emulsion formation which indicates the presence of tannins in sample solution.

Collection of fungus

Alternaria alternata (accession no. FCBP-PTF-1176) was collected from the First Fungal Culture bank of Pakistan (FCBP), Institute of Agricultural Science (IAGS), University of the Punjab, Lahore, Pakistan. This fungus was refreshed and preserved at 4 °C until used.

Effect of different weed extracts on mycelium growth of *A. alternata*

Plant extracts were tested for their efficacy

against the *A. alternata* using three methods viz., Food Poison Method, Agar Well Diffusion Method, and Agar Disc Diffusion Method. For *Food poison method*, 20ml Potato dextrose Agar (PDA) containing antibiotic (streptomycin / 100mg/1000ml) was poured into sterilized petri dishes, PDA was amended with 2ml of weed extract and the content was agitated into circular motion to mix the extracts in PDA homogenously. After the solidification of PDA, these petri dishes were inoculated with 5 mm diameter agar plugs containing active mycelium (6–7 days old culture) of *A. alternata* in the center of petri dishes and incubated at 28±1 ºC for 7 days. The colony diameter of *A. alternata* was measured and recoded after incubation period (3, 5 and 7days) [32]. *Agar disc diffusion method* was performed by soaking sterilized 6mm disc of filter paper Whatman No. 3 in each weed extract and carefully placed on the sterilized agar surface. After solidification of PDA, these petri dishes were inoculated with 5 mm diameter agar plugs containing active mycelium (6–7 days old culture) of the test fungus in the center of plates and incubated at 28±1 ºC for 7 days. The colony diameter of *A. alternata* was measured and recoded after incubation period (3, 5 and 7days) [33]. For *Agar well diffusion method*, 20ml of PDA was added in petri dishes, after solidification PDA was inoculated with 5mm fungus culture. Four wells (5mm) in these petri dishes weremade using cork borer No. 4 at a certain distance and the agar discs were removed. Sterilized micropipettes were used to add 20µl extract of each plant into the wells and then these petri dishes were incubated at 28±1 ºC for 3 days [34]. Three replicates were used within each treatment and PDA with *A. alternata* was considered as positive control and Topsin M (fungicide) served as negative control. The efficacy of extracts of selected four weeds and percentage of mycelium growth of test pathogen over the control was measured by using following formula:

%inhibition= [(C-T)/C] × 100

Here "C" represents the diameter of control while "T" represents the diameter of treatment.

Statistical analysis

All the data obtained from the experiments were analyzed using analysis of variance (ANOVA) for comparison of the means of treatment. Fisher's least test (LSD) was used for comparison and separation of means at 0.05% level of significance.

Results

The leaf powder extracts of four weeds revealed the presence and absence of various phytochemicals viz., amino acids, flavonoids, proteins, phytosterols and tannins in different extracts of weeds (Table 1). The results of qualitative phytochemical analysis revealed that, amino acids detected in *C. album* and *P. hysterophorus* while they were not detected in aqueous and ethanol extract of *C. sativa* and *M. neglecta* respectively. Proteins were detected in all the extracts of four weeds. Tannins were not detected in the ethanol extract of *C. sativa* while detected in rest of the weed extracts. *P. hysterophorus* are rich in flavonoids but flavonoids were not detected in the ethanol extracts of *C. sativa*, *C. album* & *M. neglecta* and detected in other extracts weeds. Phytosterols detected in all extracts of *C. sativa* and *M. neglecta* and not detected in ethanol extract of *P. hysterophorus* while in *C. album* phytosterols were not detected in ethanol and methanol extract but detected in aqueous extract of *C. album*. Phytochemical screening results dependent on the type of solvent, process of extraction and chemicals used for it.

For the sustainable and eco-friendly management of *A. alternata*, the present study tested the antifungal activity of

aqueous, ethanol and methanol extracts of four weeds and their traditional use against other fungal pathogen of plants. It was found that almost all weed extracts were effective in inhibiting the fungal mycelium growth of *A. alternata*. Food Poison Method was found most effective as compared to other two methods because the results were visible and consistence. Maximum percent inhibition was observed for aqueous, methanolic and ethanolic extract of *C. sativa* (91%), *P. hysterophorus* (86.7%) and *M. neglecta* (86.7%) respectively. Aqueous extracts of *C. album* and *P. hysterophorus* did not show significant results (Table 2). Results of Agar Disc Diffusion Method indicated that the maximum percent inhibition was observed for aqueous, methanolic and ethanolic extracts of *M. neglecta* (75.7%), *P. hysterophorus* (77.1%) and *M. neglecta* (77.7%) respectively. Aqueous extracts of *C.album* did not show significant results (Table 2). Results of Agar Well Diffusion Method revealed that maximum percent inhibition was observed for aqueous, methanolic and ethanolic extracts of *P. hysterophorus* (72.4%), *C. sativa* (69.7%) and *M. neglecta* (61.4%) respectively. Plant extracts of *C. album*, and *P. hysterophorus* did not show any significant results (Table 2).

Table 1. Qualitative phytochemical screening of *C. sativa, C. album, M. neglecta* and *P. hysterophorus*

Weeds	SolventType	Aminoacid	Protein	Flavonoids	Phytosterols	Tannins
Cannabis sativa	Ethanol	+	+	–	+	–
	Methanol	+	+	+	+	+
	Aqueous	–	+	+	+	+
Chenopodium album	Ethanol	+	+	–	–	+
	Methanol	+	+	+	–	+
	Aqueous	+	+	+	+	+
Malva neglecta	Ethanol	–	+	–	+	+
	Methanol	+	+	+	+	+
	Aqueous	+	+	+	+	+
Parthenium hysterophorus	Ethanol	+	+	+	–	+
	Methanol	+	+	+	+	+
	Aqueous	+	+	+	+	+

***Key**: (+) = Present, (–) = Absent*

Table 2. Percentage growth inhibition of *A. alternata* in the presence of different weed extracts

Weeds	Food Poison Method			Agar disc diffusion method			Agar well diffusion method		
	Aqueous	Ethanol	Methanol	Aqueous	Ethanol	Methanol	Aqueous	Ethanol	Methanol
C. sativa	91%	54.6%	73.5%	48.1%	50.8%	44.3%	58%	62.8%	69.7%
C. album	34.25%	73.1%	38.3%	51.2%	50.1%	74.6%	46.1%	61.1%	55.1%
M. neglecta	61.75%	86.7%	86.9%	75.7%	77.7%	70.5%	59.1%	55.4%	61.4%
P.hysterophorus	25.5%	86.7%	86.7%	73.8%	77.1%	77.1%	72.4%	41.1%	50.5%

Results of Food Poison Method reveals that all extracts of four weeds suppress and inhibits the mycelium growth *of A. alternata* at different level (Fig. 1). Some extracts inhibited more mycelium up to 91% growth of *A. alternata* as compared to the other. The order of effectiveness of different extracts of four weeds using Food Poison Method was: Aqueous extracts *M. neglecta* > *C. sativa* > *C. album* > *P. hysterophorus*, Methanol extracts *M. neglecta* > *C. sativa*> *P. hysterophorus*> *C. album*, Ethanol extracts *P. hysterophorus* > *M. neglecta* > *C. album* > *C. sativa*. The order of effectiveness of different extracts of four weeds using Agar Disc Diffusion Method was: Aqueous extracts *P. hysterophorus* >*M. neglecta* > *C. album* > *C. sativa*, Methanol extracts *M. neglecta* > *P. hysterophorus* > *C. sativa* > *C. album*, Ethanol extracts *M. neglecta* > *P. hysterophorus* > *C. album* > *C. sativa*. The order of effectiveness of different extracts of four weeds using Agar Well Diffusion Method was: Aqueous extracts *C. sativa* > *C. album* > *M. neglecta* > *P. hysterophorus*, Methanol extracts *C. album* > *C. sativa* > *M. neglecta* > *P. hysterophorus*, Ethanol extracts *M. neglecta* > *P. hysterophorus* > *C. album.*

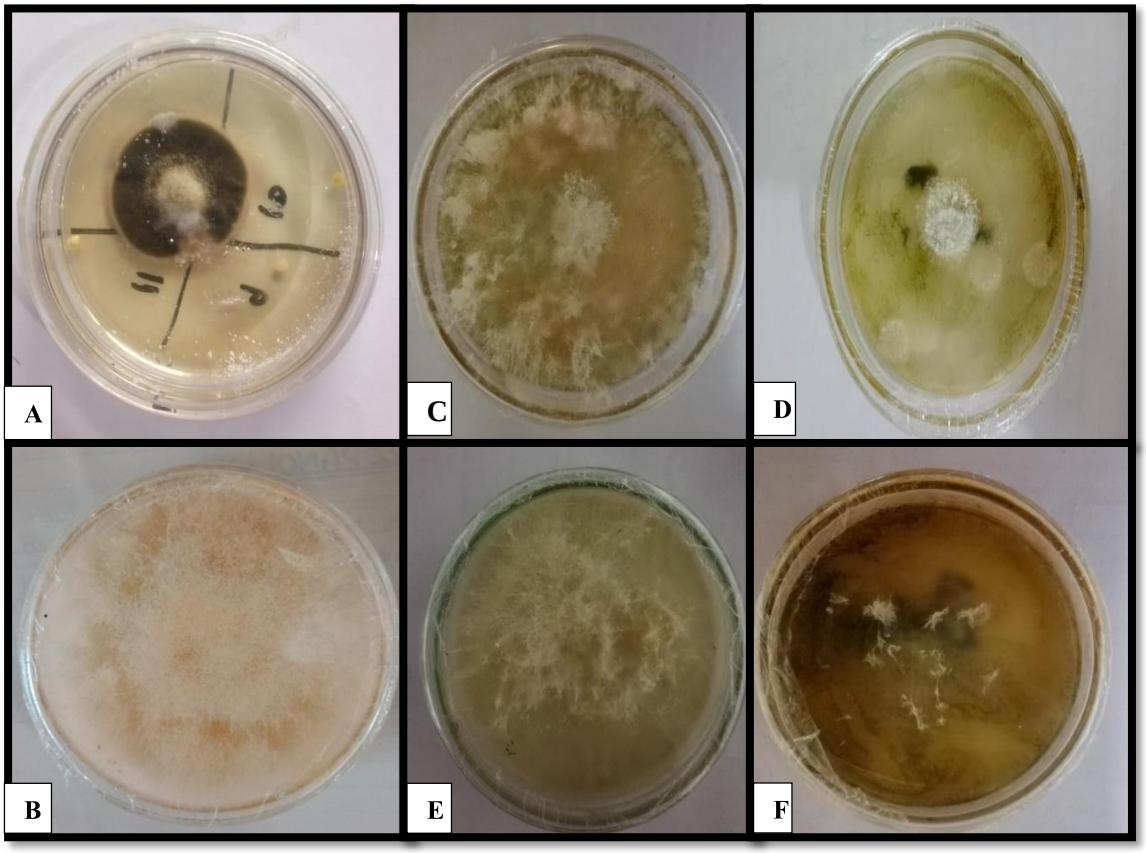

Figure 1. Bioefficacy of methanolic extracts of weeds using food poison method (A & B) Negative Control, Positive Control respectively (C–F) effectiveness of *C. sativa*, *C. album*, *M. neglecta* and *P. hysterophorus* on mycelium growth of *A. alternata* respectively

Discussion

The use of synthetic chemicals to control fungal disease creates numerous difficulties related to environmental health and pollution [35]. There is an urgent need to discover low-cost alternatives to synthetic fungicides for

the long-term treatment of plant diseases. Plant-derived antibacterial treatments might be a viable solution for this. Earlier researcher showed the efficiency of plant-based treatments in controlling plant diseases [36]. This study represents the involvement and use of extracts of four local weeds against target fungus *A. alternata* through different *in vitro* methods using different extracts prepared in water, methanol and ethanol. Before the application of the extracts their phytochemical analysis was conducted which revealed the presence of Amino acid, Protein, Flavonoids, Tannins and phytosterols. Phytochemicals are also known as secondary metabolites that are the actual source of medicinal properties of plants and which play role in defense mechanism against disease. The mechanism of disease suppression by plant products is most likely owing to secondary metabolites found in plant extracts, which may either act directly on the target pathogen or indirectly [37]. Results of this research revealed that antifungal activity of extract of weeds varied significantly, and some extracts are highly sensitive to fungus than others. Phytochemical screening results dependent on the type of solvent, process of extraction and chemicals used for it and these results are in accordance with previous researches [38, 39]. Based on the results of phytochemical analysis, selected medicinal plants extracts were subjected to the antifungal activity through different methods. *In vitro* screening of four weeds showed that they possess potent antifungal potential against the *A. alternata*. Food Poison Method exhibited satisfactory results as compared to Agar Disc Diffusion and Agar Well Diffusion Method. Maximum inhibition mycelium growth of *Alternaria alternata* was observed by food poison method while other two methods showed lesser efficacy. The research findings are in conformity with several workers [40-42]. Agar Disc Diffusion Method was found less effective as compared to Food Poison Method. Results revealed that inhibition of mycelium growth of *A. alternata* was less than the other two methods. It was noticed that the effectiveness of all the extracts depend on the concentrations of plant extracts. These results of our research are in accordance with several researchers [43, 44]. Results of Agar Well Diffusion revealed that selected weed species proved effective against leaf blight pathogen *A. alternata* but methanol extracts of *M. neglecta* and *P. hysterophorus* showed no or very slight antifungal activity. The variation in antifungal activity of different extracts of same plants could be due to differential polarity of the nature of solvents and the fact that different chemicals dissolve in different solvents used for extraction process. This is the reason behind the variation in zone of inhibition of different extracts of four selected weeds. Similar results were reported in the past by many workers [43, 45-48].

Conclusion

The findings of the current research revealed that the potent antifungal activity of selected weed extracts against leaf blight pathogen which is a serious threat to economically important plants. In order to test the antifungal potential, four local weeds extract along with commonly used fungicide Topsin-M were tested by *in vitro* methods. Among four plants, three plants *C. sativa*, *M. neglecta* and *P. hysterophorus* have significant antifungal activity and reduced the fungal mycelium up to 86.7%. Methanolic and ethanolic extracts of weeds exhibited maximum antifungal activity and significantly reduced the fungal mycelium as compared to aqueous extracts of weeds. The current investigations may be considered preliminary, but it will serve as a solid foundation in the future for the selection and identification of more promising plants and their phytochemicals with enhanced antifungal properties. Furthermore, in depth studies about the

antifungal potential of plants and extracts must be researched for the management leaf blight pathogen, which appears to have evolved various races that are resistant to fungicides.

Author's contributions

Conceived and design the experiment: N Olikh & BG Nayyar, Performed the experiment: N Olikh, Analyzed the data: N Olikh, A Serwer & M Ajmal, contributed material/ analysis/ tool: TK Hashmi, A Naseer, wrote the paper; N Olikh & BG Nayyar.

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