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Evaluation of *Ageratum houstonianum* Mill leaves extracts against phytopathogenic fungi

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Ageratum houstonianum Mill. (Asteraceae) leaves extract (in distilled water and methanol) was evaluated against five phytopathogenic fungi: *Alternaria brassicae, Botrytis cinerea, Fusarium oxysporum, Phytophthora capsici* and *Sclerotium rolfsii* at different concentrations (50, 100, 150, 200, and 250 mg/mL). The phytochemical screening depicted the presence of terpenoids, saponins, flavonoids, tannins and alkaloids. The activity test of extracts against fungi was determined by poisoned food technique and linear mycelium growth reduction (LMGR) percentage was calculated. Methanol crude leaf extract had higher antifungal potential than the distilled water extract. Aqueous and methanolic extracts of leaves of *A. houstonianum* greatly reduced the mycelium growth of tested fungi, which can be used for the disease management.

Keywords: *Ageratum houstonianum* Mill., Antifungal activity, Linear mycelium growth, Phytopathogens.

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Introduction

Fungal pathogens are the second most important organisms which cause severe crop losses all over the world. One-third of global agriculture production is destroyed each year by different pest and diseases[1]. Mostly, different chemicals are used to control diseases. The use of synthetic chemicals has been found very effective in controlling fungal diseases but some major problems threatened to limit the continued use of fungicides. The synthetic fungicides usually take long periods of time to be degraded completely causing heavy toxicity to human beings and domestic animals[2]. Some fungi have developed resistance to chemicals which become difficult to control. In nature, many secondary metabolites play an important role in the protection of the plants as antibacterial, antiviral, antifungal and insecticidal agents[3]. Extracts of many allelopathic plants are known to exhibit antifungal properties. The active ingredients found in allelopathic plants can be synthesized, or used in the form of extracts. The plant extracts are rapidly degraded in soil by reducing the impact on the environment, and they can have an effective role in sustainable agriculture[4].

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A. houstonianum is an annual ornamental shrub of 30–70 cm height. It is commonly known as floss flower and native to southeastern Mexico, Central America. The whole plant of *A. houstonianum* is used medicinally in traditional Chinese medicine to clear away heat and toxic materials. People in Central America (Ecuador) use this plant as an antiphlogistic to relieve swelling and pain in the throat[5]. In the previous reports, various flavonoids, triterpenoids, steroids, pyrrolizidine alkaloids, and benzofuran derivatives (chromenes) have been isolated and identified in the plant[6-9]. It is reported that extracts derived from the aerial parts (leaves) of *A. houstonianum* exhibit antimicrobial, acaricidal, and mosquitocidal activity as well as repellency against mosquitoes[10-12]. However, a survey of literature has revealed that there is no report on the antifungal activity of *A. houstonianum* against phytopathogenic fungi. The present research was therefore undertaken for phytochemical screening and to investigate the activity of the aqueous and methanolic extracts against five phytopathogenic fungi.

Material and Methods

Collection of plant material

Leaves of *A. houstonianum* were collected from different areas of Sauraha, Chitwan, Province 3, (27°34′29″ N, 84°29′37″ E, altitude: 150 m above sea

level**)** in the month of July-August 2012. Plant samples were identified by the expert of the Central Department of Botany, Tribhuvan University. Herbaria of the samples were prepared and deposited in the herbarium of Central Department of Botany, Tribhuvan University (AH No. 13).

Drying and preservation of plant samples

Fresh and healthy leaves were collected and washed properly with tap water. The leaves were cut into small pieces and were shade dried. The dried leaves were ground into a fine powder with the help of an electric grinder. The ground plant samples were preserved into a zipper bag for further analysis.

Preparation of extract

The ground plant leaf sample of 25 g was soaked in 250 mL of distilled water and methanol (99%) separately in a conical flask for 72 h. Each mixture was stirred at 24 h interval using a sterilized glass rod. The samples were filtered using three layers of muslin cloth. Distilled water extract was evaporated on a heating mantle using water bath till the thick residue was formed[13] and methanol was evaporated using a rotary evaporator at 60 ^{o}C. It was made into semisolid form by evaporation using a water bath. After the solvent evaporation, each of the solvent extracts was weighted and preserved in airtight bottles until further use in the refrigerator at temperature 4-10 ^{o}C.

Phytochemical screening

The phytochemical screening of crude extracts from the leaves of *A. houstonianum* was carried out to determine the presence of active secondary plant metabolites. The plant extracts were screened for the presence of tannins, saponin, cardio-glycosides, terpenoids, steroids, flavonoids and alkaloids according to the established procedures. Preliminary qualitative phytochemical screening was carried out on the powdered samples applying the following standard procedures described by several researchers[14-16] and for the result, a sharp change in colour was noted.

Preparation of different concentration

Distilled water and methanol semisolid leaf extract were used for the preparation of concentrations viz. 50, 100, 150, 200, and 250 mg/mL. These concentrations were diluted in distilled water and methanol separately hence, distilled water and methanol were used as the negative control.

Antifungal activity

The pure fungal strains were collected from Nepal Agriculture and Research Council (NARC), Khumaltar, Kathmandu. The five strains used for the test were *Sclerotium rolfsii, Phytopthora capsici*, *Alternaria brassicae, Fusarium oxysporum* and *Botrytis cinerea*. Poisoned food technique used to assess the antifungal activity of plant extracts by applying the method of Nene & Thapliyal[17]. For fungal culture, potato dextrose agar (PDA) media was applied. Exactly 1 mL of each concentration was aseptically poured into the well labelled and sterile Petri plates and then 9 mL of melted PDA (at 50 ºC) was added and was swirled gently to achieve thorough mixing of the contents[18]. The plates with distilled water or methanol served as negative control while fungicide Bavistin (Systematic fungicide) and Mancozeb (Contact fungicide) were used as a positive control. After solidification, seven-day-old fungal culture was cut aseptically with a sterile needle of generally 5 mm diameter and inoculated upside down on the centre of the PDA. Seven replicates of each extract were incubated for seven days at temperature 26±1ºC for fungi. The fungal growth was measured on the 7th day of incubation[19]. The percentage of linear growth reduction of pathogenic fungi compared with control was calculated using the formula as given by Khalil & Dababneh[20].

Results

Phytochemical screening

Plant species showed the highest reaction for cardiac glycosides, terpenoids, steroids, alkaloids in methanol extract. Moderate and weak reactions were shown in distilled water extract (Table 1).

Mean linear mycelium growth (LMG)

In distilled water crude leaf extract of *A. houstonianum*, *S. rolfsii* had the largest (100 mm) mean LMG at concentrations 50, 100, 150, and

Table 1 — Preliminary phytochemical screening of tested samples

Plants	Phytochemical Constituents							
	Solvent	Tannin	Saponin	C. glycoside	Terpenoides	Steroids	Flavonoid	Alkaloid
Ageratum houstonianum	Distilled water	+	++	+	-	-	++	+
	Methanol	-	-	+++	+++	+++	++	+++

200 mg/mL and lowest (57 mm) at 250 mg/mL respectively (Table 2). *P. capsici* had 100 mm LMG at all the concentrations, 100 mm growth was also observed in the negative control. *A. brassiceae* had the largest (65 mm) LMG in 50 mg/mL and lowest (36 mm) in 250 mg/mL concentration, while growth in negative control was 72 mm. The concentrations 200 mg/mL and 250 mg/mL had lower LMG than the fungicide bivastin (45 mm). *F. oxysporum* had highest (66 mm) LMG at 50 mg/mL concentration and lowest (17 mm) at 250 mg/mL concentrations, having 77 mm growth in the negative control. These values are higher than the fungicides bivastin and mancozeb having LMG 13 mm. *B. cinerea* showed LMG of 23 mm at 50 mg/mL, 13 at 250 mg/mL concentrations and 27 mm growth in the negative control. LMG at 250 mg/mL was found equal to that in mancozeb (13 mm). This value was also lower than bivastin having 16 mm LMG (Table 2). There was significant (P <0.01) difference between the LMG of the tested fungi and the different concentration used. There was a significant difference among the different concentrations also which are indicated by different letters (Table 2). In methanol crude leaf extract, *S. rolfsii* had the highest (64 mm) LMG at 50 mg/mL and the growth was inhibited at the concentrations 150, 200, and 250 mg/mL while the fungus had LMG of 47 mm in bivastin, 21 mm in mancozeb and 100 mm in negative control (Table 3). *P. capsici* had highest (74 mm) LMG in 50 mg/mL whereas at 250 mg/mL concentration the mycelium growth was inhibited. The LMG was also found to be lower than bivastin (100 mm) and negative control (100 mm). Concentrations 200 mg/mL and 250 mg/mL had lower (26 and 0 mm) LMG than mancozeb having LMG 33 mm. *A. brassiceae* at all the concentrations had lower (36 mm, 25 mm, 23 mm, 21mm and 16 mm) LMG than bivastin with LMG 45 mm while negative control had 51 mm growth. *F. oxysporum* had highest (19 mm) LMG at 50 mg/mL and the growth was inhibited at 200 and 250 mg/mL concentrations while the fungus had 13 mm LMG in fungicides bivastin and mancozeb. Similarly, in negative control, 31 mm growth was observed (Table 3). *B. cinerea* had the

Table 2 — Mean linear mycelium growth in distilled water crude leaf extract of *Ageratum houstonianum* in different test fungus

Fungal strains	Linear mycelium growth (mm)									
	Concentrations (mg/mL)					Control			*P*	F
	50	100	150	200	250	Negative	Positive			
						Dist.water	Bivastin	Mancozeb		
Sr.	100±0d	100±0d	100±0d	100±0d	57±2c	100±0d	47±1b	21±1a	.000	5.571E3
Pc.	100±0b	100±0	100±0b	100±0b	100±0b	100±0b	100±0b	33±1a	.000	1.048E4
Ab.	65±3f	60±1e	46±2d	42±5c	36±2b	72±6g	45±1cd	21±1a	.000	190.258
Fo.	66±10e	54±2d	22±1c	19±1bc	17±3ab	77±3f	13±1a	13±1a	.000	312.172
Bc.	23±2e	21±4de	20±2cd	18±3bc	13±1a	27±1f	16±1b	13±1a	.000	39.497

For each fungal strain, significance difference between mean among different concentration are indicated by different letters (Duncan multiple comparison test, P <0.01). F and p values were obtained by one way analysis of variance (ANOVA).

Sr= Sclerotum rolfsii, Pc= Phytopthora capsici, Ab= Alternaria brassiceae, Fo= Fusarium oxysporum, Bs= Botrytis cinerea, values are mean±SD of seven replicates, Dist.water= distilled water

Table 3 — Mean linear mycelium growth in methanol crude leaf extract of *Ageratum houstonianum* in different test fungus

Fungal strains	Linear mycelium growth (mm)									
	Concentrations (mg/mL)					Control			*P*	F
	50	100	150	200	250	Negative	Positive			
						Methanol	Bivastin	Mancozeb		
Sr.	64±5e	56±3d	0±0a	0±0a	0±0a	100±0f	47±1c	21±1b	.000	2.08
Pc.	74±7e	44±7d	34±3c	26±2b	0±0a	100±0f	100±0f	33±2c	.000	575.39
Alt.	36±3d	25±2c	23±1bc	21±2b	16±1a	51±4f	45±1e	21±1b	.000	204.23
Fo.	19±1c	18±1c	17±1c	0±0a	0±0a	31±5d	13±1b	13±1b	.000	206.79
Bc.	19±2e	17±1d	14±1c	12±1b	10±1a	22±1f	16±1d	13±1bc	.000	62.72

For each fungal strain, significance difference between mean among different concentration are indicated by different letters (Duncan multiple comparison test, P <0.01). F and p values were obtained by one way analysis of variance (ANOVA).

Sr= Sclerotum rolfsii, Pc= Phytopthora capsici, Ab= Alternaria brassiceae, Fo= Fusarium oxysporum, Bs= Botrytis cinerea, values are mean±SD of seven replicates.

highest (19 mm) and lowest (10 mm) LMG at concentrations 50 and 250 mg/mL. The LMG at 150, 200, and 250 mg/mL was found lower than the growth in bivastin and negative control (22 mm). The LMG, 12 mm and 10 mm at concentrations 200 and 250 mg/mL was also found to be lower than mancozeb having LMG of 13 mm (Table 3). There was significance (p <0.01) difference between mycelium growth of tested fungi and the different concentrations used. Concentrations among themselves were also found to be significantly different which is given by different letters (Table 3).

Mean linear mycelium growth reduction (LMGR) percentage

Mean Linear Mycelium Growth Reduction (LMGR) percentage in crude leaf extract of *A. houstonianum* was found higher in methanol leaf extract than in distilled water leaf extract for all the tested fungi at all the concentrations (Fig. 1). In distilled water crude leaf extract no LMGR was found for *S. rolfsii* at concentrations 50, 100, 150 and 200 mg/mL while at 250 mg/mL 43% reduction was observed. In methanol extract, 36-100% reduction was observed at 50, 100, 150, 200, and 250 mg/mL concentrations (Fig. 1) No LMGR percentage was found in *P. capsici* in distilled water while in methanol extract 26-100% linear growth reductions were observed in different concentrations (Fig.1b). In *A. brassiceae* 10-50% LMGR percentage was found in distilled water and 29-68% LMGR percentage was seen in methanol extract at 50, 100, 150, 200, and 250 mg/mL concentrations (Fig. 1c). LMGR percentage for *F. oxysporum* in distilled water extract was found to be 14-78% while in methanol it ranged from 39-100% (Fig. 1d). Similarly, in *B. cinerea* LMGR percentage in distilled water was found 13-51% while in methanol extract it was 15-54% (Fig. 1e). LMGR percentage was found increased on increasing

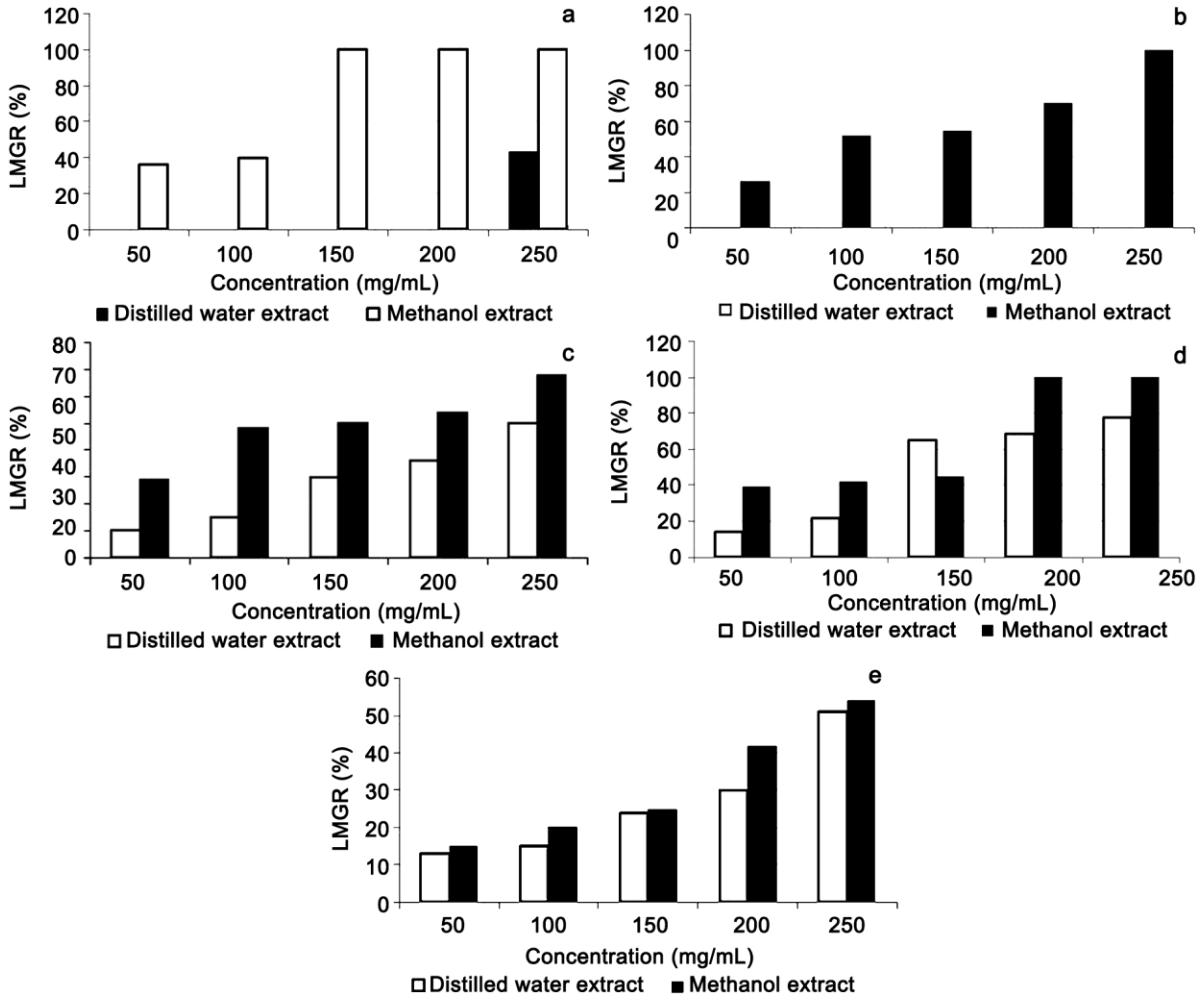

Fig. 1 — Mean Linear Mycelium Growth Reduction (LMGR) percentage in distilled water and methanol crude leaf extract of *Ageratum houstonianum*. a) in *S. rolfsii*, n=7 b) *P. capsicii*, n=7 c) *A. brasiceae*, n=7 d) *F. oxysporum*, n=7 e) *B. cinerea*, n=7

the concentration in both distilled water and methanol extract (Fig. 1).

In methanol crude leaf extract of *A. houstonianum*, the highest LMGR percentage was found in *S. rolfsii*, *P. capsici* and *F. oxysporum* i.e.100% (Figs.1a,b,d) and least LMGR percentage was found in *B. cinerea* (15-54)% (Fig. 1e) and in distilled water extract *F. oxysporum* had the highest (Fig. 1d) while *S. rolfsii* and *P. capsici* had no LMGR percentage at 250 mg/mL concentration (Fig. 1a,b).

Discussion

The methanol extract of *A. houstonianum* showed the highest antifungal activity by completely inhibiting the growth of *P. capsici, S. rolfsii,* and *F. oxysporum* at different concentrations (Table 3). A similar result was given by Javed and Bashir[21] where crude *n*-hexane extract of *Ageratum conyzoides* completely inhibited the growth of *S. rolfsii* and significantly inhibited the growth of *F. solani. A. houstonianum* showed highest antifungal activity against *F. oxysporum* and *B. cinerea* (Tables 2 and 3). All the tested fungi were found more resistant to methanol extract than distilled water extract (Tables 2 and 3) which can be attributed to antimicrobial properties of the plant parts or the whole plant vary with the type of solvents used to prepare the extracts from respective plant parts[22]. There was a significant difference (P <0.01) between the linear mycelium growth and the different concentrations for leaf extract and the tested fungus. The plant crude extracts at concentrations 150-250 mg/mL were found more effective in reducing the growth of the fungus than the synthetic fungicides used in the study showing the plant extracts at higher concentrations might have fungicidal properties.

All tested concentration of leaf extract showed a varying degree of antifungal activity which might be correlated to the various phytochemicals present in their respective extract[23] and also this may be due to the reason that the agrochemicals present in the plants are the supply of natural fungicides, insecticides and pesticides[24-25]. Similarly, Bajpai *et al*.[26] also reported antifungal activity of Invasive Alien plants species against *Magnaporthe oryzae, Rhizoctonia solani, B. cinerea, Phytophthora infestans, Puccinia recondita, Blumeria graminis* f. sp. *hordei, Colletotrichum coccodes.*

Linear mycelium growth reduction

The result showed that the LMGR percentage was found higher in methanol leaf extract of plant than in distilled water leaf extract for all the test fungus at all the concentrations which might be due to the reason that methanol solvent is known with its ability to isolate more antimicrobials compounds from plants than water solvent extracts[27]. Plant extract of *A. houstonianum* was found most effective against *A. brassiceae* by limiting its growth to 50 and 68% in distilled water and methanol extract respectively at 250 mg/mL concentrations among the three plants used (Fig 1).

A. houstonianum showed the highest antifungal activity. The several important chemical constituents present in the leaves of *A. houstonianum* have antifungal activity[28-29] *P. capsici, S. rolfsii* and *F. oxysporum* were found the most susceptible fungus while *A. brassiceae* and *B. cinerea* were the most resistant fungus to the extract at higher concentrations. A similar result for *A. brassiceae* was observed in the methanol leaf of the extract of *Terminalia catappa*[30]. The resistance of fungi to the tested extracts might be due to the presence of more complex cell wall with rigidity and also might be due to the reason that the fungi differ in optimum growth conditions such as pH, production rate of manganese and lignin peroxidases and their resistance to toxic chemicals[31]. Also, this may be due to their ability to produce extracellular enzymes that helps them to degrade and metabolize substrate such that the extract becomes a source of food to the fungi instead of inhibiting their growth after they have been rendered nontoxic due to degradation[32] and also fungi are able to degrade chemicals extracellularly using ligase and manganese- dependant enzymes[33,34]. Increasing the concentration of the plant extract had increased the LMGR percentage of the test fungus under the study as evident from shorter mycelium length at higher concentration. Similarly, the present result was found similar to the results of Suleiman[35]; who found that the inhibitory action of the extracts of neem and tobacco on mycelial growth of three fungal pathogens of tomato increased with increase in concentrations. Bajpai *et al*.[26] also reported that disease severity increased as the concentrations of the plant extract increased in all tested pathosystem.

Conclusion

This study suggests that *A. houstanianum* has great antifungal potential. Leaves of *A. houstanianum* have fungitoxic chemicals against phytopathogenic fungi- *Sclerotum rolfsii, Phytopthora capsici, Alternaria brassiceae, Fusarium oxysporum,* and *Botrytis*

cinerea. Aqueous and methanolic extracts of leaves of *A. houstonianum* greatly reduced the mycelial growth of tested fungi, which can be used for the disease management. Further investigation of the isolation of active antifungal compound should be done from different parts of the *A. houstonianum*, and the isolated antifungal compounds should be checked against other pathogenic fungi to control the different diseases.

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