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Article

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Antifungal activities and phytochemical screening of two invasive alien species of Nepal

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Abstract

The antifungal activities and phytochemical screening of two invasive alien species: *Ageratina adenophora* and *Ipomoea carnea* ssp. *fistulosa* of Nepal were studied. Distilled water and methanolic extract of the leaves of plants were tested against five phytopathogenic fungi viz. *Alternaria brassicae*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phytophthora capsici* and *Sclerotium rolfsii* at five different concentrations (50 mg/ml, 100 mg/ml, 150 mg/ml, 200 mg/ml, 250 mg/ml). The antifungal activity was performed by poisoned food technique; and linear mycelium growth reduction (LMGR) percentage was calculated. A qualitative phytochemical screening was performed for constituents such as tannins, saponins, terpenoids, alkaloids and flavonoids. The methanolic extract was more efficient as compared to the distilled water extract. *A. brassicae, B. cinerea* and *S. rolfsii* were found the most resistant fungi and *F. oxysporum* was found the most susceptible fungus. Out of two plants, *A. adenophora* was found more active against selected pathogens than *Ipomoea carnea* ssp. *fistulosa.* It can be concluded that the tested plants possess antifungal properties which may be used as alternative fungicides after further investigation.

Key words – *Ageratina adenophora* – *Ipomoea carnea* ssp. *Fistulosa* – LMGR – Plant extracts

Introduction

An alien species is a species that is non-native, non-indigenous, exotic, foreign and/or introduced to an ecosystem other than its natural home. Those alien species that colonize unmanageably out-compete the native species are known as invasive alien species. Invasive alien species reduce biodiversity, replace economically important native plant species and decrease the investment in agriculture and silviculture, disrupt prevailing vegetation dynamics, alter nutrient cycling and cause changes in the pattern of plant succession (Tiwari et al. 2005). Several exotic plants have invaded the high-value biodiversity areas and have adversely affected the natural and semi-natural vegetation/ecosystems (Tripathi 2009). Once established some alien species have the ability to displace or replace native plant species the problem will likely worsen with time because of climatic changes that promote species migration worldwide. The diverse bioclimatic zones of Nepal range from tropical to alpine favor the introduction of several alien species. These species have been spreading aggressively by colonizing several landscapes and ecosystems displacing the native ones.

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From the earlier reports (Dabur et al. 2007, Mdee et al. 2009) it is evident that some of the invasive plants have antifungal compounds which do have the capacity to inhibit the fungal pathogens. Plant fungal pathogens in particular, pose a major threat to economically valuable crops. Plant pathogenic fungi attack most crops in the field and also post-harvest thereby decreasing production and shelf life of many agricultural crops (Agrios 1997) .The most important method of protecting plants against fungal attack is the use of fungicides. The development of resistance of pathogenic fungi towards synthetic fungicides is of great concern. There is, therefore, a need to find safe, efficacious and environmentally friendly fungicides. Many plants produce antifungal agents by secondary metabolism to protect themselves from fungal attack, and therefore many plant species possess substantial antifungal activity (Rashmi & Rajkumar 2011). Thus, the use of plant extracts with inhibitory activity against fungal plant pathogens could lead to the development of environmentally acceptable fungicides based on the availability of natural products. If fungal pathogen plays an important role in the growth or establishment of plant species, invasive species may have better resistance against plant pathogens (Mdee et al. 2009).

Many invasive plant species release chemical compounds into the environment, which are not generally harmful to them, but those chemicals suppress the growth of other plant species growing in close proximity of such invasive species. Prime importance can be given for the bioprospecting of novel active compound which can be utilized for the management of several plant diseases. Many reports are available on inhibiting fungal pathogen from plant extracts (Dabur et al. 2007, Bindu & Kumar 2009, Jack & Orubike 2008, Satish et al. 2007, Vital & Rivera 2009, Rashmi & Rajkumar 2011, Devkota & Das 2016, Devkota & Sahu 2017). The invasive plants are easily available throughout the year and are found growing around the crop lands. This will enable the farmers to use these plants in crop protection against various phytopathogenic diseases. Utilization of invasive species against phytopathogenic disease, not only control the disease but also for proper management of invasive plants as well. Therefore, the present work is aimed to investigate two invasive plant species for antifungal activity against plant pathogens in order to develop a useful plant product.

Materials & Methods

Collection of plant materials and preparation of extracts

Fresh and healthy leaves of two invasive alien species (IAS) *Ageratina adenophora* and *Ipomoea carnea* ssp. *fistulosa* were collected in their natural habitat from different location of Nepal (Table 1). Leaves were washed thoroughly under running tap water and then dried under shade. The dried plant materials were grind in electric mixed grinder and stored in air tight zipper bag.

Table 1 List of plant species

S.N.	Plants	Common name	Family	Part used	Place of collection
I	*Ageratina adenophora*	Crofton weed	Asteraceae	Leaves	Kirtipur, Kathmandu
Ii	*Ipomoea carnea* ssp. *fistulosa*	Shrubby morning glory	Convolvulaceae	Leaves	Jhojhi Kataiya, Dhanusha

Twenty five gram of each powder samples were added in 250 ml of distilled water and methanol (95%) solvents respectively then kept them for 72 h at room temperature. The mixture were stirred at 24 h (Alagesaboopathi 2011). The extracts were filtered through a triple layered cotton cloth. Water content of distilled water was evaporated by water bath process till solution reduced to semisolid form (Bhattarai & Shrestha 2009). The methanolic extract was evaporated

under reduced pressure using rotary evaporator and then transferred into sterile labeled bottles and they were made into semisolid form by evaporation to water bath at 50º C. The final crude extracts were weighted and made the bottles air tight and kept in the refrigerator at 4º C for experimental use (Mahida & Mohan 2007).

Antifungal screening by poisoned food technique

Alternaria brassicae, Botrytis cinerea, Fusarium oxysporum, Phytophthora capsici and *Sclerotium rolfsii* were used for the antifungal test. Poisoned food technique used to assess the antifungal activity of plant extracts by applying the method of Nene & Thapliyal (1979). For fungal culture potato dextrose agar (PDA) media was applied. 1 ml. of each concentration was aseptically poured into the well labelled and sterile petriplates and then 9 ml of melted PDA (at 50º C) was added and was swirled gently to achieve through mixing of the contents (Singh & Singh 2013a). The plates with distilled water or methanol was served as negative control while fungicide Bavistin (Systematic fungicide) and Mancozeb (contact fungicide) were used as 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 center of the PDA. Seven replicates of each extract was incubated for seven days at temperature 26±1º C for fungi. The fungal growth was measured on the 7th day of incubation (Singh & Singh 2013b). The percentage of linear growth reduction of pathogenic fungi compared with control was calculated using the formula as given by Khalil & Dababneh (2007).

$$\textbf{Linear growth reduction}(\%) = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Phytochemical Screening

Preliminary qualitative phytochemical screening of the distilled water and methanol extracts was done by using standard phytochemical screening methods described by Harborne (1973), Sofowara (1993), Trease & Evans (1989) with slight change for saponins, terpenoids, tannins, alkaloids, and flavonoids. Change in color was noted for the result.

Tannins – About 0.5 g of the dried powdered sample was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

Saponin – 1 gram of the powdered samples were boiled in 10 ml of water and methanol separately in a water bath and filtered. 5 ml of the filtrate was mixed with 2.5 ml of distilled water and shaken vigorously for a stable persistent froth.

Terpenoids (Salkowski test) – 5 ml of each extract was mixed in 2 ml of chloroform, and 3 ml of concentrated H_2SO_4 was added carefully to form a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Alkaloid – Plant sample of 200 mg was taken in 10 ml methanol and was filtered. Mayer's Test: In 2 ml filtrate 1% HCL was added and was steamed and 1 ml of filtrate was treated with 6 drops of Mayer's reagents. Formation of yellow colored precipitate indicated the presence of alkaloids. Wagner's Test: Filtrate was treated with Wagner reagent. Formation of brown/ reddish colored precipitate indicated the presence of alkaloids. Dragondroff's Test: Filtrate was treated with Dragondroff's reagents Formation of red colored precipitate indicated the presence of alkaloids.

Flavonoids – 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of

plant extract followed by addition of concentrated H_2SO_4. A yellow coloration observed in extract indicated the presence of flavonoids. The yellow coloration disappeared after some time.

Data analysis

The antifungal assay was done in seven replicates and standard deviation (S.D.) from the mean was calculated. The effect of extracts on fungus was evaluated by a one way analysis of variance (ANOVA) using SPSS version 16. Differences of letters at $p<0.05$ were determined by the TUKEY test.

Results

Antifungal activities

Both plant leaves extract showed positive activity against *Fusarium oxysporum* by completely inhibiting its growth at higher concentrations in methanolic extract. Among five fungi D/W extract of *A. adenophora* ranged from (90–14 mm) diameter (Table 2). In distilled water leaf extracts *Phytopthora capsici* and *Sclerotium rolfsii* showed the highest LMG (90–30) mm diameter at (50–250) mg/ml concentrations and the least LMG shown by *Botrytis cinerea* (23–14) mm diameter at (50–250) mg/ml concentrations (Table 2).

Table 2 Linear mycelium growth (mm) in distilled water and methanol crude leaf extract of *Ageratina adenophora.* The values were expressed as Mean ± S. D. and statistically analysis using One Way ANOVA for obtaining F and P value. Turkey HSD multiple comparison test was done to compare the different letters at P< 0.05

Linear mycelium growth (mm) in D/W leaf extract of *Ageratina adenophora*										
	Concentrations (mg/ml)					Control				
Fungal strain	**50**	**100**	**150**	**200**	**250**	**Negative**	**Positive**			
						DW	Bavistin	Mancozeb	P	F
A.b.	56±3 d	46±4 c	37±1 b	33±2 b	21±2 a	72±6 e	44±1 c	21±1 a	.000	200.64
B.c.	23±1 e	21±.8 d	20±1 d	15±.8 bc	14±.5 ab	27±1 f	16±.7 c	13±1 a	.000	186.78
F.o.	43±.7 c	42±1 c	41±1 c	36±1 b	15±1a	76±3 d	13±1 a	15±.9 a	.000	1.21
P.c.	90±0 c	90±0 c	90±0 c	72±4 b	30±7 a	90±0 c	90±0 c	33±2 a	.000	535.07
S.r.	90±0 e	90±0 e	90±0 e	73±4 d	30±7 b	90±0 e	46±1 c	22±2 a	.000	645.91

Linear mycelium growth (mm) in methanol leaf extract of *Ageratina adenophora*										
	Concentrations (mg/ml)					Control				
Fungal strain	**50**	**100**	**150**	**200**	**250**	**Negative**	**Positive**			
						Methanol	Bavistin	Mancozeb	P	F
A.b.	33±1 e	27±.6 d	18±.3 b	16±.3 ab	13±.7 a	50±4 g	45±1 f	21±1 c	.000	424.68
B.c.	16±.6	12±.6	12±.4	11±.6	9±.9	22±1	16±.7	13±1	.000	157.97
F.o.	24±.6 e	16±.4 d	13±.5 bc	12±.8 b	0±0 a	31±4 f	13±1 bc	15±.9 cd	.000	181.81
P.c.	71±2 d	27±2 b	0±0 a	0±0 a	0±0 a	90±0 e	90±0 e	32±1 c	.000	5.89
S.r.	90±0 e	83±2 e	64±11 d	37±9 b	24±3 a	90±0 e	46±1 c	21±1 a	.000	206.63

Abbreviations: *A.b.* =*Alternaria brassicae, B.c.*=*Botrytis cinerea, F.o.*= *Fusarium oxysporum, P.c.*=*Phytophthora capsici, S.r.*=*Sclerotium rolfsii,* values are mean ± SD of seven replicates.

In methanolic extract of *Ageratina adenophora*, *S. rolfsii* showed the highest LMG (90 mm–24 mm) diameter at 50–250 mg/ml concentrations. The lowest mycelium growth was observed for *P. capsici* and *F.oxysporum,* 0 mm at higher concentrations (Table 2). It inhibited fungi more than synthetic fungicide Bavistin and Mancozeb on some fungi at higher concentrations of both

methanolic and DW extracts. ANOVA result showed that there was significant differences in mean value of LMG of fungi in different concentrations of plant leaf extract (Table 2).

Linear Mycelium Growth Reduction of D/W extract of *Ipomoea carnea* ssp. *fistulosa* ranged from (90–11 mm) diameter at 50 mg/ml to 250 mg/ml concentrations among selected 5 fungi (Table 3). *Phytophthora capsici* and *Sclerotium rolfsii* showed the highest LMG (90 mm) at 50 mg/ml to 250 mg/ml and *B. cinerea* showed the lowest LMG (25–17 mm) at concentrations 50 mg/ml to 250 mg/ml.

Table 3 Linear mycelium growth (mm) in distilled water and methanol crude leaf extract of *Ipomoea carnea* ssp. *Fistulosa.* The values were expressed as Mean ± S. D. and statistically analysis using One Way ANOVA for obtaining F and P value. Turkey HSD multiple comparison test was done to compare the different letters at P< 0.05

Linear mycelium growth (mm) in D/W leaf extract of *Ipomoea carnea* ssp. *fistulosa*										
	Concentrations (mg/ml)					Control				
Fungal strain	**50**	**100**	**150**	**200**	**250**	**Negative**	**Positive**			
						DW	Bavistin	Mancozeb	P	F
A.b.	47±1 e	43±4 de	38±.5 cd	34±1 c	27±.4 b	72±6 f	45±1 e	21±1 a	.000	192.26
B.c.	25±1 e	22±.7 d	21±.9d	19±.7 c	17±.8 a	27±1 f	16±.7 b	14±1 a	.000	171.69
F.o.	51±.6 f	40±1 e	29±1 d	21±1 c	11±1 a	77±3 g	13±1 ab	15±.9 b	.000	1.13
P.c.	90±0 b	90±0 b	90±0 b	90±0 b	90±0 b	90±0 b	90±0 b	32±1 a	.000	7.61
S.r.	90±0 c	90±0 c	90±0 c	90±0 c	90±0 c	90±0 c	46±1 b	21±1 a	.000	9.79
Linear mycelium growth (mm) in methanol leaf extract of *Ipomoea carnea* ssp. *fistulosa*										
	Concentrations (mg/ml)					Control				
Fungal strain	**50**	**100**	**150**	**200**	**250**	**Negative**	**Positive**			
						Meth.	Bavistin	Mancozeb	P	F
A.b.	31±.8 e	27±1 d	24±1 c	23±.6 bc	18±.6 a	50±4 g	44±1 f	21±1 b	.000	297.76
B.c.	20±.6 e	18±.9 de	17±.6 cd	15±.7 b	13±.6 a	22±1 f	16±.7 bc	13±1 a	.000	88.22
F.o.	20±1 d	15±.7 c	11±.5 b	0±0 a	0±0 a	31±4 e	13±1 bc	15±.9 c	.000	219.31
P.c.	90±0 c	90±0 c	90±0 c	90±0 c	83±7 b	90±0 c	90±0 c	32±1 a	.000	403.92
S.r.	90±0 d	90±0 d	90±0 d	85±2 d	70±6 c	90±0 d	46±1 b	21±1 a	.000	667.41

Abbreviations: *A.b.=Alternaria brassicae, B.c.=Botrytis cinerea, F.o.= Fusarium oxysporum, P.c.=Phytophthora capsici, S.r.=Sclerotium rolfsii*, values are mean ± SD of seven replicates.

LMGR of Methanol extract of *Ipomoea carnea* ssp. *fistulosa* ranged from 90 mm – 0 mm diameter at 50 mg/ml to 250 mg/ml concentrations among selected 5 fungal strain (Table 3). *Phytophthora capsici* showed the highest LMG (90 mm–83 mm) diameter at 50 mg/ml to 250 mg/ml concentrations respectively, and the lowest LMG observed in *Fusarium oxysporum* (20 mm–11mm) at 50 mg/ml–150 mg/ml and at 200–250 mg/ml concentrations the mycelium growth of fungus completely inhibited. It showed higher activity than synthetic fungicide Bavistin and Mancozeb on some fungi at higher concentrations of both methanolic and DW extract. ANOVA result showed that there was significant differences in mean value of LMG of fungi in different concentrations of plant leaf extract (Table 3).

Linear Mycelium Growth Reduction (LMGR) Percentage

Ageratina adenophora was found most effective against *Phytophtora capsici* at 150 mg/ml to 250 mg/ml and *F. oxysporum* at 250 mg/ml by inhibition the growth 100%in methanol extract. No LMGR was found in *Phytophthora capsici* and *S. rolfsii* at 50 mg/ml to 150 mg/ml in D/W leaf extract of *A. adenophora*. Distilled water extract had lower LMGR percentage than methanol extract at all concentrations for all fungi except *F. oxysporum* which was found with higher LMGR

(44%–79%) in distilled water crude extract than LMGR (22%–67%) in methanol crude extract of plant at all concentrations. *Ipomoea carnea* ssp. *fistulosa* was found the most effective against *F. oxysporum* by inhibiting 100% LMGR in methanolic extract at 200 mg/ml–250 mg/ml. *P. capsici* and *S. rolfsii* had no LMGR percentage at 50 mg/ml-250 mg/ml in distilled water leaf crude extract.With the increasing concentration of both methanol and distilled water leaf crude extract the linear mycelium growth reduction was also increased. The methanolic extract was found higher than distilled water for LMGR at all concentration for all fungi.

In this study between two plants methanol extract of *Ageratina adenophora* was observed more active against selected fungi followed by methanol extract of *Ipomoea carnea* ssp. *fistulosa*.

Fusarium oxysporum was more susceptible fungus being inhibited by methanol extract of both IAS tested at least at higher concentration (250 mg/ml). The growth of *A. brassicea,B.cinerea* and *S. rolfsii* was also decreased with increasing concentration but they were not inhibited completely by any concentration of both methanol and distilled water extract of both tested IAS. Distilled water extract was not found completely inhibiting any selected fungus but with increasing concentration of plant extract the fungal growth decreased by both methanol and distilled water crude extract of plant.

Phytochemical screening

The phytochemical screening was done to find the presence of active chemical constituents such as terpenoids, saponins, steroids, Cardiac glycosides, flavonoids, reducing sugar, tannins, phlobatannins and alkaloids.

Distilled water extract of *Ageratina adenophora* revealed the presence of high concentration of terpenoids, tannin and alkaloid while methanolic extract revealed high concentration of saponins, flavonoids, tannins and alkaloids (Table 4). Distilled water extract of *Ipomoea carnea* ssp. *fistulosa* revealed the presence of high concentrations of saponins and while had highest reaction for saponins, flavonoids and alkaloids in methanolic extract (Table 4).

Table 4 Preliminary phytochemical screening of leaf extract of *A. adenophora* and *I. carnea* ssp. *fistulosa.* Responses to various tests were denoted by +, ++ and +++ signs indicating weak, moderate and strong reactions respectively while - for no reaction

Plant spp	**Phytochemical constituents**									
	Distilled water					**Methanol**				
	Tannin	Saponins	Terpenoids	Alkaloids	Flavonoids	Tannin	Saponins	Terpenoids	Alkaloids	Flavonoids
Ageratina adenophora	+++	++	+++	+++	-	+++	+++	-	+++	+++
Ipomoea carnea ssp. *fistulosa*	+	+++	+	++	-	+	+++	-	+++	+++

Discussion

The present investigation has demonstrated the antifungal activity of both tested plants. This work proves that some invasive plants have potential and could be useful in combating plant fungal pathogens (Mdee et al. 2009). The use of plant extracts could enable the development of inexpensive and environmentally acceptable fungicides based on locally available natural products (Rashmi & Rajkumar 2011). Considering this, strict regulation should be set in place to avoid complete eradication of these plants. Isolation and characterization of active compounds is in progress.

It was revealed in this study, that the antifungal activity of the extracts was enhanced by increase in the concentration of the extracts. The similar result was found by Bobbarala et al. (2009) on *Aspergillus nigar* examined by forty nine different medicinal plants. It was found that the fungi were inhibited by the extract and the inhibition was directly proportional to the increasing concentration of the extract (Goel & Sharma 2013). In this study the plant extracts by methanol

provided more consistent antimicrobial activity compared to those extracted by distilled water. Similar result was found by Parekh & Chanda (2007) on five microorganisms by methanolic and distilled water extract of twelve species of Indian medicinal plants. Methanol has a high polarity index (Cowan 1999).The result has showed that the crude extract of selected IAS have potential antifungal activity against tested fungi. *A. brassicae* and *B. cinerea* were found most resistant fungi against plant extracts while *F. oxysporum,* was found most susceptible fungi. The fungi differ in optimum growth conditions such as pH, production rate of manganese and lignin peroxidases, their resistance to toxic chemicals and complex cell wall (Rucksdeschel & Renner 1986).

From preliminary phytochemical screening in distilled water and methanolic extract of leaves of selected IAS revealed the presence of high concentrations of terpenoids, saponins, flavonoids, tannins, and alkaloids. Distilled water extract of *A.adenophora* revealed the presence of high concentration of terpenoids, tannin and alkaloid. Methanolic extract of *A.adenophora* revealed high concentration of saponins, flavonoids, tannins and alkaloids (Table 4). Baral & Maharjan (2011) reported the presence of Glycosides but negative result was observed for saponins and alkaloids. This difference in result may be due to the presence of phytochemicals varies based on the solvents used, climatic conditions of plant grown and preliminary phytochemical screening methods (Srinivas et al. 2011). Distilled water extract of *Ipomoea carnea* ssp. *fistulosa* revealed the presence of high concentrations of saponins. Methanol extract of *I. carnea* ssp. *fistulosa* had highest reaction for saponins, flavonoids and alkaloids (Table 4). Sahayaraj & Ravi (2008) reported that the distilled water extract of *I. carnea* ssp. *fistulosa* revealed the presence of phenolic compound, saponins, tannins and flavonoids but negative result was observed for steroids and alkaloids. Chloroform extract revealed the presence of steroids, alkaloids, phenolic compounds, tannins and flavonoids but there was negative result for saponins. Benzene extract revealed the presence of phenolic compounds, saponins, tannins and negative result was observed for steroids.

Conclusion

Results of the present study show that the aqueous and methanol extracts of both plants significantly varied in their antifungal potential. Methanol extracts were more active than distilled water extracts. This research concludes that both IAS can be used against the phytopathogenic fungi and the identified phytochemical compounds may be the bioactive constituents and these plants are proving to be an increasingly valuable reservoir of bioactive compounds of substantial medicinal merit. Therefore, plant extracts or plant secondary metabolites which are not toxic and specific in their action could be considered as an alternative to synthetic fungicides based on the availability of materials.

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