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Abstract

Background: *Sclerotium rolfsii* Sacc. is a destructive soil-borne plant pathogen that infects over 500 plant species and causes significant yield losses in many economically important plant species. Synthetic fungicides used to combat the menace also pollute the environment and cause health hazards. In order to search environmental friendly alternatives from natural resources, methanolic extracts of three leguminous tree species namely *Acacia nilotica* (L.) Willd. ex Delile subsp. *indica* (Benth.) Brenan, *Prosopis juliflora* (Sw.) DC. and *Albizia lebbek* (L.) Benth. were evaluated for their antifungal activity against *S. rolfsii* and *A. nilotica* subsp. *indica* exhibited the maximum fungicidal potential.

Materials and Methods: Two hundred grams dried leaf material of each of the three test plant species were extracted with methanol for two weeks. After filtration, methanol was evaporated on a rotary evaporator. Malt extract broth was used to make various concentrations of the crude methanolic extracts and their antifungal potential was determined by comparing the fungal biomass in various treatments with control. Chemical composition of methanolic leaf extract of *A. nilotica* subsp. *indica* was determined through GC-MS analysis.

Results: Methanolic leaf extract of *A. nilotica* subsp. *indica* showed the highest fungicidal activity. Fungal biomass was decreased by 17-55% due to various concentrations of this extract over control. Different concentrations of *P. juliflora* reduced fungal biomass by 3-52%. Fourteen compounds were identified in methanolic extract of *A. nilotica* subsp. *indica*. 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- (16.59%) was the most abundant compound followed by 1-pentanol, 2 methyl-, acetate (14.80%); hexanedioic acid, dimethyl ester (13.10%) and cyclotriaconta- 1, 7, 16, 22-tetraone (10.28%).

Conclusion: This study concludes that methanolic leaf extract of *A. nilotica* subsp. *indica* can be used for management of *S. rolfsii*.

Key word: Antifungal activity, GC-MS analysis, leaf extracts, leguminous trees, *Sclerotium rolfsii*.

Introduction

Sclerotium rolfsii is a versatile soil-borne pathogen of the tropics, subtropics and other warm regions of the world that may cause damping off of seedlings, collar or stem rot, foot rot, crown rot, wilt and blight (Punja, 1985). Over 500 plants species are affected by *S. rolfsii* (Agrios, 2004), due to its capacity to produce phytotoxin (oxalic acid), cell wall degrading enzymes and polysaccharides degrading multiplex enzymes system in host (Gubitz et al., 1996; Tang et al., 2015). Penetration of *S. rolfsii* in host plant results in production of abundant white mycelia and formation of appressoria with tissue necrosis and later on advancement in hyphal growth causes tissue death (Tang et al., 2015). The melanized outer layer of sclerotia enables the fungus to survive in harsh condition for more than 3 years (Punja, 1985). Chemicals recommended to manage *S. rolfsii* include copper oxychloride, flutolanil, Pentachloronitrobenzene, tebuconazole, carbendazim, mancozeb and difenconazole etc. (Casinos, 1989; Hagan et al., 2004; Madhavi and Bhattiprolu, 2011; Khan and Javaid, 2015). However, these chemical fungicides found to be inappropriate due to poor adaptation of cultivars, high cost (Punja, 1985; Yaqub and Shahzad, 2009; Amin et al., 2014) and persistent outcome on all form of life (Dias, 2012).

Use of natural compounds from plants could serve as alternative strategy to manage *S. rolfsii* and other plant pathogens (Javaid and Shoaib, 2013). Natural compounds from plants such as phenolics and alkaloids possess ability to trigger plant defense mechanism by activating function of antioxidant enzymes and inactivating enzymes of pathogen (Gurjar et al., 2012). Previously, extracts of various plants have been reported to possess antifungal potential against diseases caused by *Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *cepa* and *S. rolfsii* (Javaid and Iqbal, 2014; Javaid and Bashir, 2015; Javaid and Rauf, 2015). Plants belong to family leguminoasae e.g. *Acacia nilotica*, *Prosopis juliflora* and *Albizia lebbek* are well-known for their medicinal and antimicrobial activity due to presence of number of secondary metabolites like amines, alkaloids, tannin, glycosides, flavonoid, fatty acids etc. (Malviya et al., 2011; Ikram and Dawar, 2013; Kokila et al., 2013; Amjad ur Rahman et al., 2014). Ikram and Dawar (2013) showed soil mixing with *P. juliflora* parts considerably declined root rot diseases caused by devastating soil-borne pathogens *Fusarium* spp., *Rhizoctonia solani* and *Macrophomina phaseolina* in cowpea and mungbean due to presence of active compound juliflorine. Seed, pod, root and flower extract of *A. lebbek* showed considerable antibacterial and antifungal activity (Shahid and Firdous, 2012), and many active phytoconstituents (budmunchiamines, quercitrin and isoquercitrin) have been isolated from its different parts (Kokila et al., 2013). Likewise, Rafi et al. (2015) showed that seed priming with seed, stem and leaves extract of *A. nilotica* significantly increased growth in okra, sunflower chickpea and peanut, while suppressed various root rot pathogens (*Fusarium* spp., *R. solani* and *M. phaseolina*) owing to richest sources of bioactive compounds in the extracts. Therefore, the present study was conducted to check the fungicidal potential of methanolic leaf

extracts of *A. nilotica* subsp. *indica*, *P. juliflora* and *A. lebbeck* against *S. rolfisii*. Besides, the most effective extract was analyzed through GC-MS for identification of active phytochemicals profiling.

Materials and Methods

Preparation of Methanolic Leaf Extracts

Leaves of three allelopathic leguminous trees viz. *A. nilotica* subsp. *indica*, *A. lebbeck* and *P. juliflora* were collected from University of the Punjab, Lahore, Pakistan. Plants were identified by Dr. Arshad Javaid (Assistant Professor, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan). The specimens were deposited in the herbarium of the University, with voucher no. SJ 191 (*A. nilotica* subsp. *indica*), SJ 192 (*A. lebbeck*) and SJ 193 (*P. juliflora*). After thorough washing under tap water, leaves were dried in an electric oven at 45 °C. Two hundred grams of leaves powder of each plant species were soaked in 1000 mL methanol for 2 weeks. All the soaked materials were filtered first through cheese cloth and then through Whatman filter papers followed by evaporation of solvent on a rotary evaporator at 45 °C. After evaporation, 19 g, 15.03 g and 25 g crude extract of *A. nilotica* subsp. *indica*, *A. lebbeck* and *P. juliflora* were obtained (Javaid et al., 2015).

Table 1: Compounds identified from methanolic leaf extract of *Acacia nilotica* subsp. *indica* through GC-MS analysis.

Com p. No.	Names of compounds	Group	Molecular Formula	Molecular weight	Retention time (min)	Peak area (%)
1	1-Pentanol, 2 methyl-, acetate		C ₈ H ₁₆ O ₂	144	8.844	14.80
2	Hexanedioic acid, dimethyl ester		C ₈ H ₁₄ O ₄	174	9.643	13.10
3	2, 3-Dihydro, 4-oxo- beta ionol		C ₁₃ H ₁₈ O ₂	206	13.194	4.22
4	9-Octadecynoic acid		C ₁₈ H ₃₂ O ₂	280	16.668	3.89
5	8-Octadecynoic acid, methyl ester		C ₁₉ H ₃₄ O ₂	294	17.110	1.38
6	Hexadecanoic acid, methyl ester		C ₁₇ H ₃₄ O ₂	270	17.569	8.04
7	9,12-Octadecadienoic acid, methyl ester		C ₁₉ H ₃₄ O ₂	294	19.192	3.66
8	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z,-)		C ₁₉ H ₃₂ O ₂	292	19.268	16.59
9	9,12,15-Octadecatrienoic acid, 2, 3bis (acetyloxy) propyl ester, (Z,Z,Z,-)		C ₂₅ H ₄₀ O ₆	436	19.378	2.98
10	Heptadecanoic acid, 10-methyl-, methyl ester		C ₁₉ H ₃₈ O ₂	298	19.455	2.24
11	Spirost-8-en- 11-one, 3-hydroxy-, (3-beta, 5-alpha, 14 beta, 22-beta, 25R)-		C ₂₇ H ₄₀ O ₄	428	25.002	1.82
12	Cyclotriaconta- 1, 7, 16, 22-tetraone		C ₃₀ H ₅₂ O ₄	476	25.419	10.28
13	Vitamin E		C ₂₉ H ₅₀ O ₂	430	27.177	3.79
14	Propanoic acid, 2-(3-acetoxy-4, 4, 14-trimethylandrost-8-en-17-yl)		C ₂₇ H ₄₂ O ₄	430	28.621	5.80

Evaluation of Antifungal Activity of Methanolic Leaf Extracts

Nine grams methanolic leaf extract of each the three leguminous plant species were dissolved in 5 mL dimethyl sulphoxide (DMSO). Appropriate quantity of autoclaved distilled water was added to make final volume of each stock solution to 15 mL. DMSO (5 mL) was added in 10 mL of distilled water to make control solution. Autoclaved malt extract broth (55 mL) was used to make five concentrations viz. 1, 2, 3, 4, and 5% of each of the three extracts with mixing of 4, 3, 2, 1 and 0 mL of control solution and rising up to 60 mL. Four replicates were finally prepared by equally dividing 60 mL of each concentration in each 100-mL volume flask. Control treatment contained 5 mL control solution in 55 mL autoclaved malt extract broth to keep same amount of DMSO in control and experimental treatments. Actively growing *S. rolfisii* culture was used to inoculate the flasks with 5 mm diameter discs. Inoculated flasks were kept for incubation (27 °C) for 10 days. Afterwards, the filtered biomass of the fungus in each replicate flask was dried in an electric oven (60 °C) and weighed (Javaid et al., 2015). Data regarding fungal biomass were subjected to ANOVA followed by mean separation by LSD test at 5% level of significance using computer software Statistics 8.1. Relationship between different concentrations of methanolic extracts and fungal biomass was found out by drawing trend lines using MS Excel 2007.

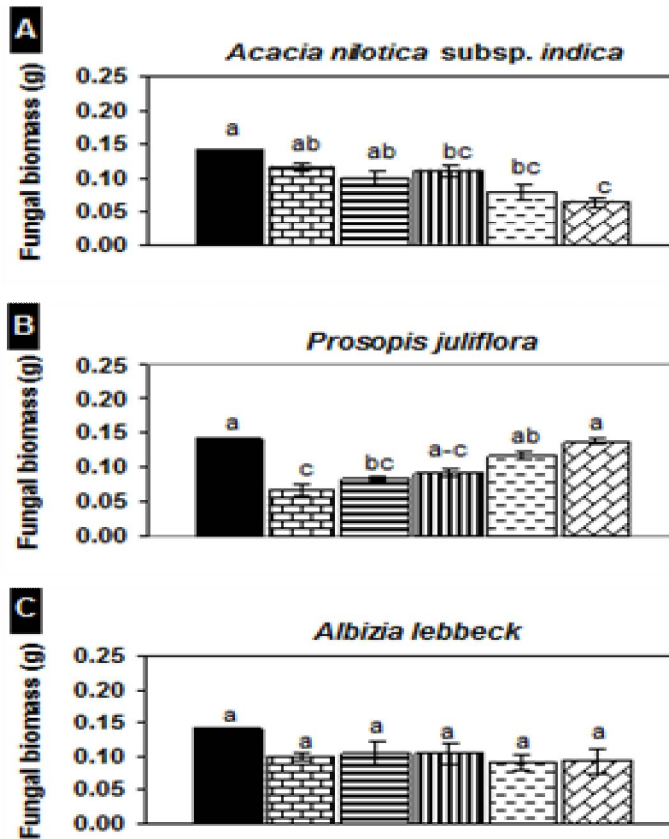
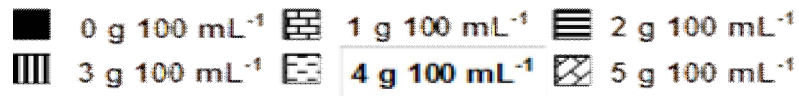


Figure 1: Effect of different concentrations of methanolic leaf extract of leguminous trees on growth of *Sclerotium rolfsii*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P < 0.05$) as determined by Tukey's HSD Test.

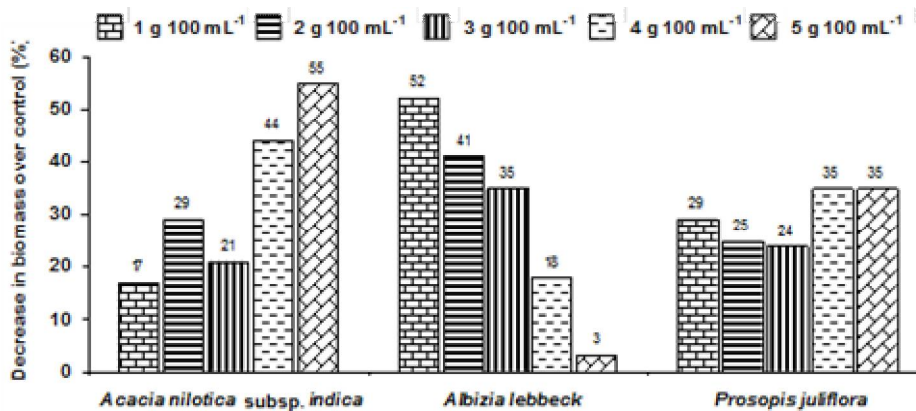


Figure 2: Percentage decrease in biomass of *Sclerotium rolfsii* due to different concentrations of methanol leaf extract of leguminous trees over control.

Methanolic leaf extract of *A. nilotica* subsp. *indica* was subjected to GC-MS analysis using Perkin Elmer Turbo Mass Spectrophotometer (Norwalk, CTO6859, and USA). About 1 μ L of the sample was injected and helium was used as a carrier gas (flow rate of 0.5 mL min⁻¹) with instrument setting at inlet at 250 °C.

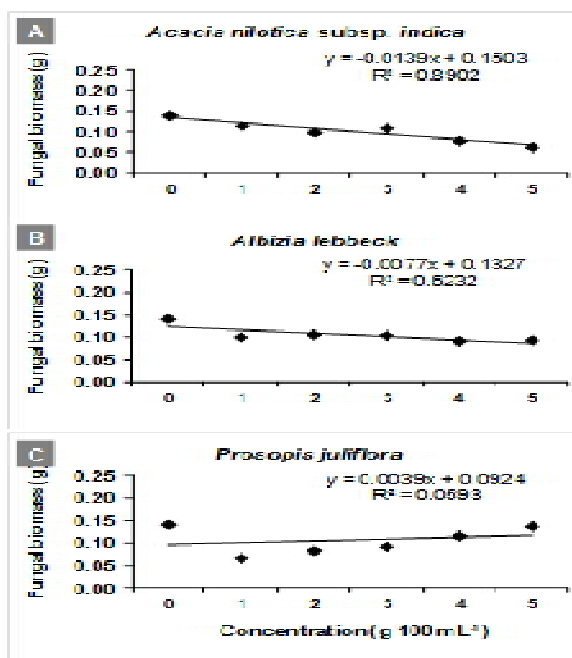


Figure 3: Relationship between different concentrations of methanolic leaf extract of leguminous trees and biomass of *Sclerotium rolfii*.

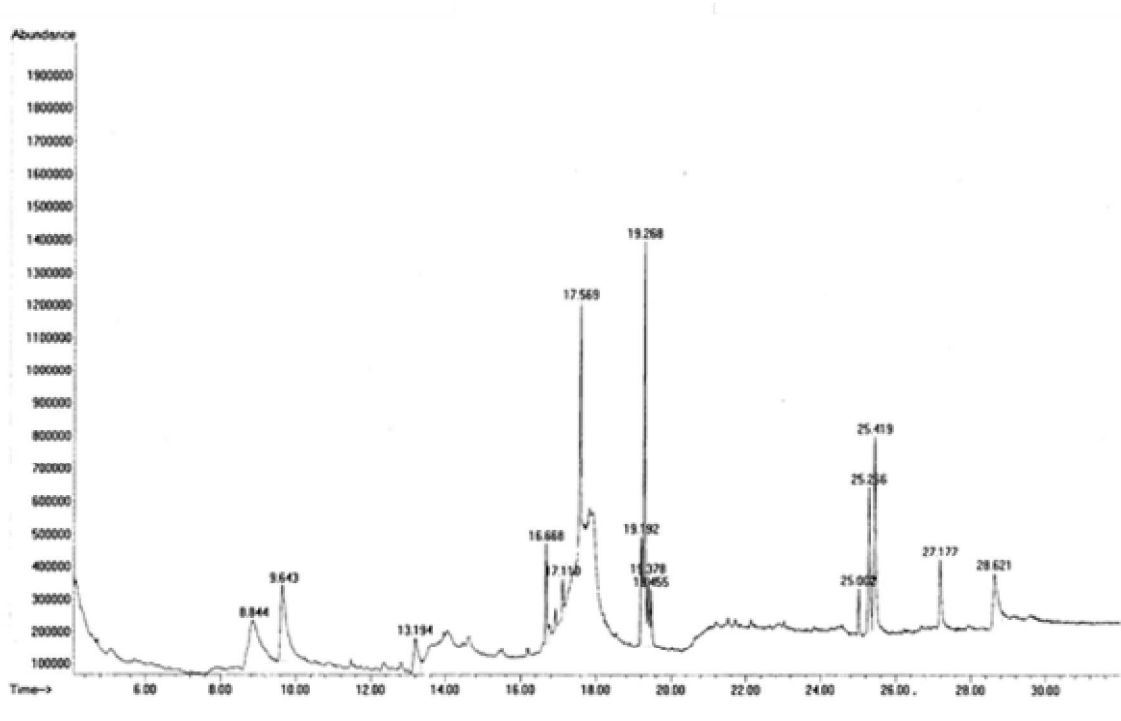


Figure 4: GC-MS analysis of methanolic leaf extract of *Acacia nilotica* subsp. *indica*.

Results and Discussion

Among the extracts of three leguminous tree species screened for their antifungal activity against the target fungal pathogen, methanolic extracts of *A. nilotica* subsp. *indica* showed pronounced antifungal activity. Different concentrations of this extract caused 17–55% reduction in fungal biomass (Fig. 1A & 2). In general, fungal biomass was gradually decreased with an increase in extract concentration. A linear relationship was recorded between fungal biomass and concentrations of methanolic extract of *A. nilotica* subsp. *indica* with $R^2 = 0.8902$ (Fig. 3A). Earlier it was shown that methanolic leaf extract of this plant is known to have significant antifungal activity against *A. flavus* (Mahesh and Satish, 2008). Likewise, leaf water extract controlled the mycelial growth of phytopathogenic fungi *Sarocladium oryzae* by 37% and *Fusarium oxysporum* by 69% while ethanolic extract showed 51% reduction in growth of *Rhizoctonia solani* (Eswaramurthy et al., 1989).

A total of 14 phytoconstituents were recorded in methanolic leaf extract of *A. nilotica* subsp. *indica* (Fig. 4, Table 1). These compounds were 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z,-) (16.59%); 1-pentanol, 2 methyl-, acetate (14.80%); hexanedioic acid, dimethyl ester (13.10%); cyclotriaconta- 1, 7, 16, 22-tetraone (10.28%); hexadecanoic acid, methyl ester (8.040%); propanoic acid, 2-(3-acetoxy-4, 4, 14-trimethylandro-8-en-17-yl) (5.80%); 2, 3-dihydro, 4-oxo- beta ionol (4.22%); 9-octadecynoic acid (3.89%); vitamin E (3.785); 9,12-octadecadienoic acid, methyl ester (3.659); 9,12,15-Octadecatrienoic acid, 2, 3bis (acetyloxy) propyl ester, (Z,Z,Z,-)(2.98%); heptadecanoic acid, 10-methyl-, methyl ester (2.24%); Spirost-8-en- 11-one, 3-hydroxy-, (3-beta, 5-alpha, 14 beta, 22-beta, 25R)- (1.82%) and 8-Octadecynoic acid, methyl ester (1.38%). Structures of these compounds are illustrated in Fig. 5. The most abundant compound 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z,-) also known as linolenic acid ester has also been found in abundance in extracts of many other plant species such as *Cucumis anguri* L. (Kumar and Kamaraj, 2010), *Wedelia chinensis* (Osbeck) Merrill (Banu and Nagarajanb, 2013) and *Saccharum spontaneum* L. (Devi and Muthu, 2014), and possibly responsible for antifungal activities of the extracts (Kumar and Kamaraj, 2010). In addition, several carboxylic acids are known to possess antifungal activity (Hwang et al., 2001).

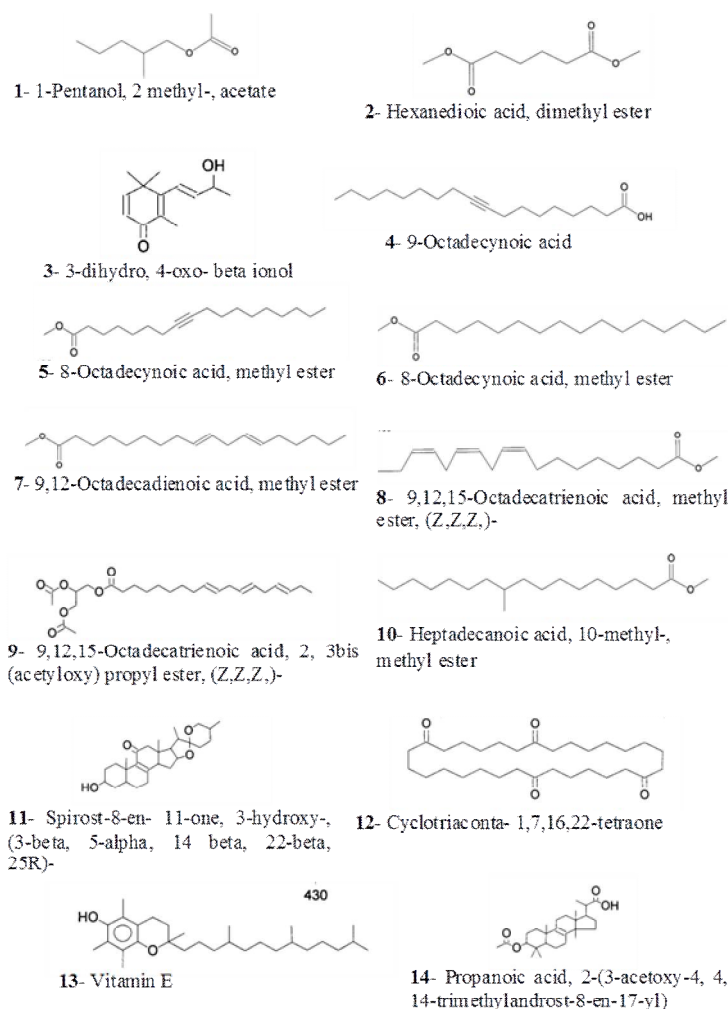


Figure 5: Structures of compounds identified in methanolic leaf extract of *Acacia nilotica* subsp. *indica* through GC-MS analysis.

Lower concentrations of methanolic leaf extract of *P. juliflora* proved inhibitory, while higher concentrations stimulated fungal growth. The inhibitory effect of 1% and 2% concentrations of the extract was significant over control however the influence of higher concentrations was insignificant. The fungal biomass was significantly declined by 3–52% due to effect of different concentrations of this extract (Fig. 1B & 2). Regression analysis indicated linear relationship ($R^2 = 0.6232$) between various concentrations of extract and fungal biomass (Fig. 3B). Previous reports indicated that lower concentrations of extracts of certain allelopathic plants such as *Dicanthium annulatum* (Forssk.) Stapf., *Toona ciliata* M. Roem and sunflower (*Helianthus annuus* L.) showed inhibition in growth of *Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *gladioli* (Riaz et al., 2008; Naqvi et al., 2012; Ashraf and Javaid, 2009). It is likely that along with antifungal compounds, growth stimulatory compounds are also present in small amount which were not effective in lower concentrations. However, as the concentration of extracts was increased, stimulatory compounds masked the inhibitory effect of antifungal compounds.

Various concentrations of *A. lebbek* reduced the fungal biomass by 24–35% over control, however, the effect was insignificant statistically (Fig. 1–3). In contrast to the present study, earlier report showed that methanolic extracts of different parts of *A. lebbek* collected from Pakistan can suppress growth of other fungal species such as *Aspergillus parasiticus*, *A. niger*, *A. effusus*, *Candida albicans*, *Fusarium solani* and *Saccharomyces cerevisiae* (Shahid and Firdous, 2012). It shows that antifungal constituents have specificity in their activity against different fungal species. The present study concludes that methanolic leaf extract of *A. nilotica* subsp. *indica* can be used for management of *S. rolfii*.

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