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EVALUATION OF ANTIFUNGAL ACTIVITY OF *SOLANUM NIGRUM* AGAINST *ASPERGILLUS NIGER*, THE CAUSE OF BLACK ROT DISEASE OF ONION

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A B S T R A C T

Antifungal activity of methanolic leaf and root extracts of *Solanum nigrum* L. was tested against *Aspergillus niger* Teigh. that causes black rot disease of onion (*Allium cepa* L.). Different concentrations of methanol leaf and root extracts viz. 1%, 2%, 3%, 4% and 5% were applied *in vitro* against *A. niger*. Root extract was proved more antifungal than leaf extract as its 5% concentration reduced the test fungal growth by 30%. Methanol root extract of *S. nigrum* was selected for further fractionation and this was partitioned by using various organic solvents viz. *n*-hexane, chloroform, ethyl acetate and *n*-butanol. MIC (minimum inhibitory concentration) of the isolated fractions along with a commercial fungicide (metalaxyl+mancozeb) was checked by serial dilution method against *A. niger.* Various concentrations (0.20 mg mL^{-1} to 0.0007 mg mL^{-1}) were used for MIC bioassay and data was recorded after 24, 48 and 72 h incubation period. Chloroform fraction and synthetic fungicide more effectively retarded spore germination of *A. niger* with MIC 0.0007 mg mL^{-1}. The other fractions were comparatively less antifungal and their order of effectiveness was ethyl acetate > *n*-hexane>*n*-butanol.

Keywords: Antifungal activity, *Aspergillus niger,* methanolic extract, MIC, *Solanum nigrum*.

INTRODUCTION

Onion (*Allium cepa* L.) is one of the important condiments widely used in all households around the year worldwide. It is a rich source for phosphorous, calcium and carbohydrates. In Pakistan, it generally grown in all provinces with an annual production of 1,701,100 metric tons (FAO, 2012). The low productivity of the crop is attributed to various biotic and abiotic factors. One of the major diseases that cause heavy yield loses in onion is black rot disease incited by *Aspergillus niger* which causes black discoloration. It is a filamentous saprophytic fungus inhabit in soil, air, organic remains and foodstuff causing mold, stem and boll rot diseases (Bobbarala *et al*., 2009). This fungus produces mycotoxins that spoil the food, cereals and grains as post-harvest disease (Gautam and Bhadauria, 2008). Although, conventional agricultural practices like crop rotation, mulching, green manures and compost, biological pest control, and mechanical control can be used in disease control of black rot disease in onion, but these measures are not so effective in protecting the crop from the disease. The best method of controlling this disease is the use of resistant cultivars (Mabrouk and Belhadi, 2012). However, resistance methods not exhibit the desire durability in resistance because of appearance of new pathotypes of the pathogen (Singh and Reddy, 1991). Black rot disease of onion can be controlled by seed dressing and foliar spraying by fungicide. Chemical fungicides or biological organisms are usually used to kill or inhibit the fungal growth or germination (Mancini *et al.*, 2008). Increasing public concern on environmental issues requires alternative disease management systems which are less pesticide dependent or based on naturally occurring compounds (Cuthbertson and Murchie, 2005; Koul, 2008). Use of biologically active natural products (bioregulators) to control plant diseases and pathogens continue to inspire research and development in many fields (Wang *et al.,* 2004; Jamil *et al*., 2007).

Solanum nigrum L. commonly known as mako or night shade plant" belongs to family Solanaceae is traditionally used as a pharmacological agent to cure fever, allay pain, skin problems and tumors, diabetes, abdominal upsets. The phytochemical screening of the crude extract

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revealed the presence of bonding structures, responsible for presence of alkyl groups, methyl groups, alcohols, ethers, esters, carboxylic acid and anhydrides. Leaves, stem and fruit containes the highest concentration of gentisic acid, luteolin, apigenin, kaempferol, and m-coumaric acid that may be responsible for its antifungal activity (Huang *et al.,* 2010). The present study was designed to investigate the inhibitory effects of methanolic extracts of *S. nigrum* against *A. niger*.

MATERIALS AND METHODS

Test plant parts collection: *S. nigrum* leaves and roots were collected from Lahore College for Women University Lahore, Pakistan. The plant materials were thoroughly washed with tap water and surface sterilized by 1% sodium hypochlorite solution followed by distilled water. Plant materials were dried at 40 °C in an electric oven and carefully ground to form powder.

Test fungus procurement and culturing: Pure culture of *A. niger* (accession # FCBP. 764) was procured from the First Fungal Culture Bank of Pakistan, Institute of Agricultural Sciences, University of the Punjab Lahore, Pakistan. The culture was preserved on malt extract agar (MEA) medium.

Screening bioassays: Twenty grams of dried, powdered leaves and roots of *S. nigrum* were soaked in 100 mL methanol and left for three days at room temperature. Materials were filtered through an autoclaved muslin cloth. Filtrate was evaporated at 40 °C in an electric oven for forty eight hours then diluted by adding 100 mL distilled water. The stock extract was covered and stored at 4 ºC and used within four days.

Malt extract (ME) 2% medium was prepared by adding 2 g ME in 100 mL of distilled water and autoclaving at 121 ºC and cooled to 50–55 °C. An amount of 20, 15, 10 and 5 mL of the stock solutions was added to 80 mL of ME medium to prepare the concentrations of 5%, 4%, 3%, 2% and 1% v/v of the extract, respectively. Control treatment was without any addition of the plant extracts. The plant extracts were thoroughly mixed with the medium. Chloromycin was added at the rate of @ 50 mg 100 mL^{-1} of the medium to avoid bacterial contamination. Twenty milliliters of each medium was poured into each sterilized flask.

Antifungal activity of the organic solvent extract of the plant was evaluated *in vitro*. Mycelial discs were prepared using a pre-sterilized cork borer of 5 mm diameter from the tips of 7 days old culture of *A. niger* and were placed in each experimental flask. Each treatment was replicated three times. Flasks were incubated at 25 ºC for 7 days. After 7 days the fungal biomass from each flask was filtered and dried up to stable weight in an electric oven and weighed. Percentage growth inhibition of the fungal colonies was calculated by applying the following formula:

$$\text{Growth inhibition \%} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}}$$

Partitioning of plant material: Five hundred grams of *S. nigrum* of roots were thoroughly extracted in 1000 mL methanol at room temperature under vacuum on rotary evaporator at 40 ºC. The methanolic extract (20.88 g) was partitioned between water and *n*-hexane followed by chloroform, ethyl acetate and *n*-butanol at room temperature by using separating funnel (Ahmed *et al.*, 2007). This partitioning was resulted as gummy mass of *n*-hexane (0.69 g), chloroform (0.63 g), ethyl acetate (0.2 g), *n*-butanol (0.30 g) and remaining water fraction.

Estimation of minimum inhibitory concentration (MIC) of the isolated fractions: MIC values of the isolated fractions along with a synthetic fungicide were tested in test tubes by serial dilution micro dilution examination followed by Jabeen *et al.* (2011) with small modifications. Isolated fractions were dissolved in DMSO (dimethoxy sulfoxide) and were serially diluted with water in test tubes. Maximum 0.20 mg mL^{-1} concentration was prepared by adding 2 mL of DMSO and 2 mL of distilled water, this concentration was further serially diluted and the minimum applied concentration was 0.0007 mg mL^{-1}. Test tube containing only DMSO was used as control. The culture tubes were incubated at 25-30 °C. The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth after 24, 48 and 72 hours. The mycelial growth initiation of the test fungus was visually determined.

Statistical analysis*:* All the data were analyzed by analysis of variance followed by Duncan's Multiple Range Test (DMRT) by using computer software COSTAT (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

In present study methanolic leaves and root extract of *S. nigrum* was evaluated for their antifungal activity against *A. niger*. All the applied concentrations of *S. nigrum* methanoilc leaf extract were significantly enhanced the growth of *A. niger* after 7 days of incubation period upto 85% as compared to the control treatment (Figure 1A & 1B).

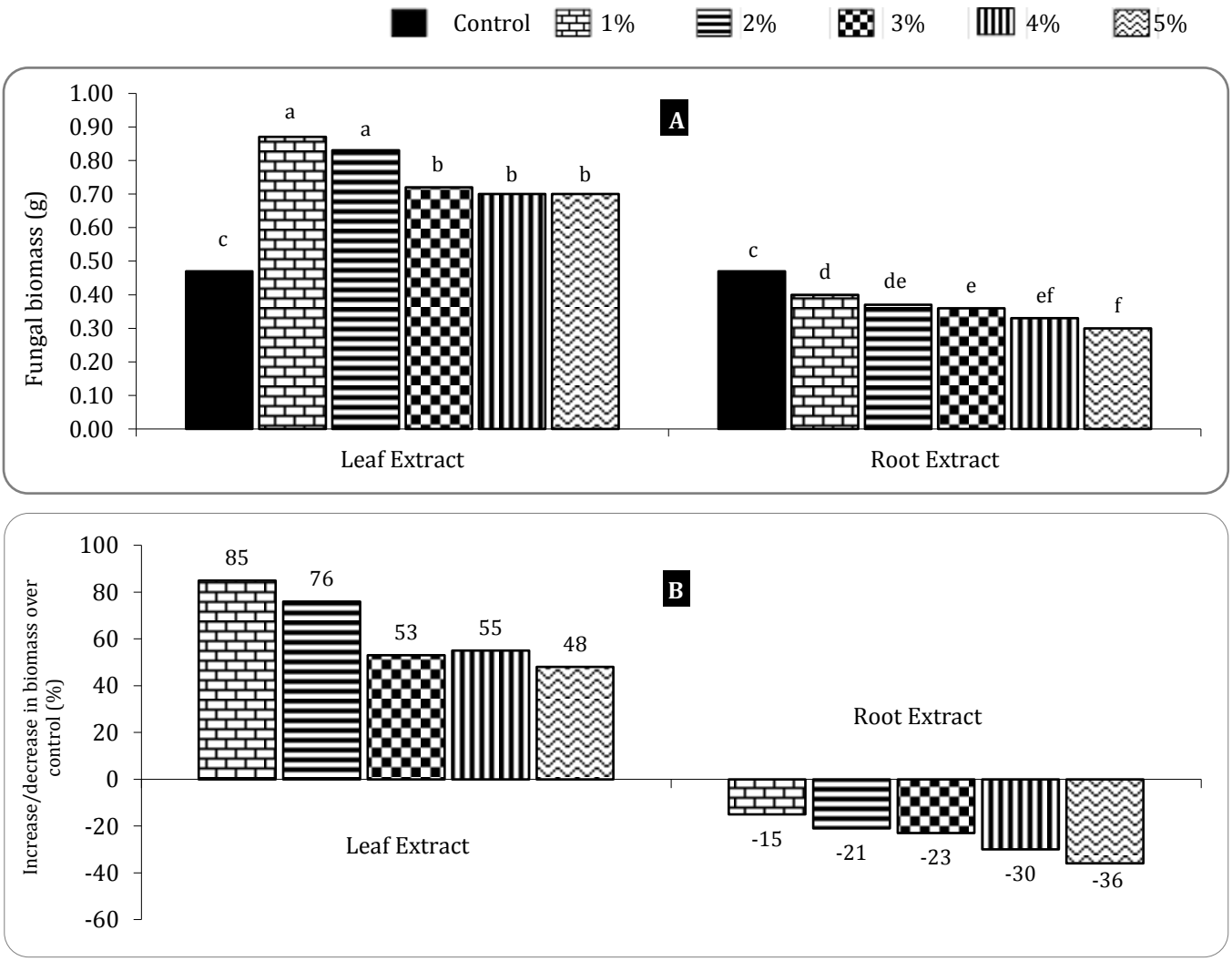

Figure 1. Effect of methanolic leaf and root extracts of *Solanum nigrum* on biomass of *A. niger*. Values with different letters at their top show significant difference (P≤0.05) as determined by DMR Test.

Methaolic extract of *S. nigrum* roots was markedly retarded the *in vitro* growth of *A. niger* (Figure 1A & 1B). Generally all the applied concentrations of root extracts showed high antifungal activity against the test fungus. The highest concentration (5%) exhibit strong fungitoxic effect and caused maximum reduction 36% in the dry biomass of *A. niger* in comparison with control treatment. Other concentrations viz. 1%, 2%, 3% and 4% suppressed the dry weight of the test fungus 15%, 21%, 23% and 30%, respectively. David *et al.* (2010) also evaluated the antifungal activity of seed extracts of *S. surattense* against *C. albicans, C. tropicalis, A. niger, A. fumigatus* and *A. flavus*, and found that methaolic extract effective inhibited the growth of *A. fumigatus, Rhizopus oryzae* and *C. albicans.* Earlier, Ahmed *et al.* (2004) also studied the anti-microbial activity of *Swert lachirata, Symplocos racemosa* and *S. nigrum* against *E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Candida albicans* and *A. niger* and found promising results.

Antifungal activity of four isolated organic fractions from root of *S. nigrum* along with reference synthetic fungicide (metalaxyl+mancozeb) against *A. niger* is presented in Table 1.

Synthetic fungicide and chloroform fraction explored high antifungal activity as their lowest concentration (0.0007 mg mL^{-1}) inhibits spore germination of *A. niger* even after 72 h incubation period. Higher concentrations of *n*-hexane and ethyl acetate, ranges from 0.20 mg - 0.0062 mg were effectively inhibit spore germination after 24, 48 and 72 hrs of incubation period, while the rest concentrations fails to inhibit spore germination. *n*-butanol fraction was found least effective.

Table 1. MIC of different organic fractions isolated from S. nigrum and synthetic fungicide (metalaxyl+mancozeb) against A. *niger* after 24, 48 and 72 h incubation periods. Mycelium present: +, Mycelium absent: -

Fractions	Concentration (mg mL^{-1})								
	0.20	0.10	0.05	0.025	0.0125	0.0062	0.0031	0.0015	0.0007
24 hours after incubation									
Control (H_2O)	+	+	+	+	+	+	+	+	+
Control (DMSO)	-	-	-	-	-	-	-	-	-
n-Hexane	-	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-	-
Ethyl acetate	-	-	-	-	-	-	-	-	-
n-Butanol	-	-	-	-	-	-	-	-	-
Metalayl+mancozeb	-	-	-	-	-	-	-	-	-
48 hours after incubation									
Control (H_2O)	+	+	+	+	+	+	+	+	+
Control (DMSO)	+	+	+	+	+	+	+	+	+
n-Hexane	+	+	+	+	+	+	+	+	+
Chloroform	-	-	-	-	-	-	-	-	-
Ethyl acetate	-	-	-	-	-	-	+	+	+
n-Butanol	+	+	+	+	+	+	+	+	+
Metalayl+mancozeb	-	-	-	-	-	-	-	-	-
72 hours after incubation									
Control (H_2O)	+	+	+	+	+	+	+	+	+
Control (DMSO)	+	+	+	+	+	+	+	+	+
n-Hexane	+	+	+	+	+	+	+	+	+
Chloroform	-	-	-	-	-	-	-	-	-
Ethyl acetate	+	+	+	+	+	+	+	+	+
n-Butanol	+	+	+	+	+	+	+	+	+
Metalayl+mancozeb	-	-	-	-	-	-	-	-	-

The results showed that both control and water and DMSO treatments promote spore germination of *A. niger* and visual mycelium was observed after 24 h incubation period. Earlier, Venkatesan *et al.* (2009) evaluated the phytochemical constituent and ethanolic extract antimicrobial activity of *S. nigrum.* The phytochemical analysis showed the presence of alkaloids, reducing sugars, tannins, flavonoids, phlobatannis, and steroids, which might be responsible of its antifungal activity.. Our results also supported from the previous experiments as (Versha *et al.*, 2003) studied the effect of petroleum ether, chloroform, ethyl acetate and methanol extracts of *Alstonia scholaris* leaf and found that chloroform extract exhibit strong antifungal activity against *A. niger* and *A. flavus*. Similar results were reported by another study in which Shihabudeen *et al.* (2010) studied the MIC of methanol extracts of *Eugenia jambolana* and *Cassia auriculata* against *S. aureus, S. epidermidis, Escherichia coli*, *K. pneumoniae*, *P. aeruginosa, C. albicans* and *A. niger. E. jambolana* exhibit the highest MIC value at the minimum concentration (0.75 mg mL^{-1}).

This investigation concludes that chloroform fraction of *S. nigrum* root exhibit high antifungal effect against the fungal causal agent of onion black rot disease *A. niger.*

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