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BIOPESTICIDAL ACTIVITY OF *Calotropis procera* L. AGAINST *Macrophomina phaseolina*

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Abstract

Background: Mungbean [*Vigna radiata* (L.) Wilczek] is an important pulse crop globally. This imperative crop is severely affected by charcoal rot disease caused by *Macrophomina phaseolina* (Tassi) Goid. In the present study, the leaves of *Calotropis procera* L. were tested for their antifungal potential against *M. phaseolina*.
Materials and Methods: Various concentrations i.e. 1%, 2.5%, 4%, 5.5% and 7% of methanolic extract of *C. procera* leaves were prepared and their *in vitro* bioactivity was examined against the test fungus. Methnolic leaf extract was partitioned using n-hexane, chloroform, ethyl acetate and n-butanol and antifungal activity of each fraction was evaluated. n-Hexane fraction was subjected to GC-MS analysis.
Results: The higher concentration of methanolic leaf extract (7%) caused maximum inhibition in the diameter of *M. phaseolina* i.e. 38%. The *n*-hexane fraction of methanolic leaf extract was found to be the most effective against *M. phaseolina*. Seven compounds belonging to classes of chlorocarbon, aromatic hydrocarbon, azocompounds, aromatic carboxylic acids and fatty acids were identified in GC-MS analysis of n-hexane fraction.
Conclusion: Antifungal activity of the methanolic leaf extract of *C. procera* might be due to the presence of the identified compounds in n-hexane fraction of methanolic leaf extract.

Keywords: Antifungal activity; bioassay; *Calotropis procera;* GC-MS; *Macrophomina phaseolina;* methanolic extract; phytochemicals.

Introduction

Mungbean is a widely grown pulse crop in Pakistan. Several factors such as low seed quality, inadequate plant protection, improper patterns of planting and excessive fertilizer use are major growth limiting factors (Khattak et al., 1999; Khattak et al., 2000). Amongst biotic elements, fungal diseases are the most problematic. *Macrophomina phaseolina* the causative agent of charcoal rot is the pest that reduces yields up to 60% (Gaige et al., 2010). It is a highly virulent pathogen and causes diseases in many hosts including mungbean, okra, cucurbits, rice, maize and cotton (Shehzad et al., 1988). It is a soil-borne pathogen and infects root by producing tuber or cushion shaped black sclerotia which are crucial for survival (Kaisar, et al., 1988). Farmers commonly prefer chemical pesticides for the control of *M. phaseolina*. However, excessive usage of pesticides damages the environment and human health (Pal and Gardener, 2006). Plant derived pesticides and plant metabolites have been shown as the best substitutes of synthetic pesticides (Javaid and Akhtar, 2015; Khaliq et al., 2016).

They have less harmful impact on environment and less hazardous to users in contrast to chemical fungicides (Hanekamp and Kwakman, 2004; Mishra et al., 2012). Constituents of several plants have potential to control plant diseases, proved safe and non-cytotoxic contrasting to chemical fungicides (Al-Samarrai et al., 2012; Verma et al., 2012). There are reports that crude extracts of many plant species namely *Datura metel, Imperata cylindrica* and *Eclipta alba* can effectively control growth of *M. phaseolina* (Javaid and Saddique, 2012; Banaras et al., 2015; Javaid et al., 2015).

Common milk weed (*Calotropis procera* L.), family Asclepiadaceae, is known to possess antisyphilitic, antimicrobial, antirheumatic, diaphoretic and antidysentric properties (Moustafa et al., 2010). It contains triterpenes, triterpenoids, alkaloids, cardinolides, procerursenyl acetate, proceranol and phytosterol (Gupta et al., 2000; Verma et al., 2012). Keeping in view the bioactive potential of *C. procera*, this study was designed to explore its potential for the control of *M. phaseolina*, the cause of charcoal rot of mung bean.

Materials and Methods

Collection of plant material

Leaves of test plant *C. procera* were collected from Wapda Town Housing Society, Lahore, Pakistan. These leaves were first washed with tap water then surface sterilized with 1% sodium hypochlorite and lastly with distilled water. After washing, leaves were dried up in an electrical oven at 40 ^{o}C and powdered. Culture of *M. phaseolina* was isolated from stem of a diseased mungbean plant using potato dextrose agar (PDA) medium.

Preliminary antifungal bioassay

Powdered leaves of *C. procera* (500 g) were soaked in methanol (1 L) for seven days at room temperature and thereafter filtered with autoclaved muslin cloth. The obtained filtrate was evaporated on rotary shaker and 6 g methanolic leaf extract of *C. procera* was obtained as gummy mass. Stock solution (20%) was prepared by the resuspension of 6 g methanolic gummy mass of *C. procera* leaves in 30 mL distilled water. *In vitro* bioassay was performed using 20% stock solution of *C. procera* leaves. PDA (2%) was prepared in 250 mL conical flasks by dissolving 1.2 g of PDA in 39, 43.5, 48, 52.5, 57 and 60 mL of distilled water. These medium containing flasks were autoclaved at 121 °C for 30 min and five concentrations viz. 1.0, 2.5, 4.0, 5.5, and 7.0% were prepared by adding 3.0, 7.5, 12, 16.5 and 21 mL stock solution, respectively, in flasks containing 57, 52.5, 48, 43.5 and 39 mL PDA. Final volume (60 mL) for each concentration was prepared in this way and sixty milliliter medium was used as the control treatment and it was without any plant extract (Javaid and Samad, 2012). To avoid bacterial contamination fifty milligrams of chloromycetein

capsule was added in each flask. Experiment was conducted in Petri plates with three replicates for each concentration. Five millimeter disks were cut from seven days old culture of *M. phaseolina* with the help of surface sterilized cork borer and one disk was placed in the center of each Petri plate. These plates were incubated at ± 25 °C for seven days in an incubator. Thereafter, fungal colony diameter in each plate was measured and percentage decrease in colony diameter of *M. phaseolina* due to various extract concentrations over control was calculated using the following formula:

$$\text{Growth inhibition } (\%) = \frac{\text{Growth in control - Grwoth in treatment}}{\text{Growth in Control}} \times 100$$

Bioassays with organic solvent fractions of methanolic leaf extract

In preliminary antifungal bioassay, *C. procera* leaves methanolic extract was proved effectual in inhibiting the fungal growth. Hence this was subjected to further fractionation and bioassay. For this 400 g of *C. procera* dry leaf powder was extracted with 1 L of methanol at room temperature as before. The methanolic extract was evaporated on a rotary evaporator at 40 °C resulted in 5.41 g extract. The partitioning of this extract was done successively with 50 mL n-hexane, 50 mL chloroform, 50 mL ethyl acetate and 50 mL n-butanol in separating funnel (Jabeen et al., 2011). These fractions were separated in separate beakers and evaporated under vacuum at 40 °C on rotary evaporator. After evaporation of organic solvents the gummy masses of isolated organic fraction were obtained. This evaporation gave n-hexane (1.53 g), chloroform (1.53 g), ethyl acetate (0.38 g) and n-butanol (0.07 g) fractions. Bioactivities of these fractions were checked against the test fungus by using protocol of Shadomy and Coworkers (1991).

GC-MS analysis

To identify the antifungal constituents, the most effective n-hexane fraction was selected for GC-MS analysis. For this, to 1.4 g of n-hexane fraction, 100 mL n-hexane (A. R. grade) was added and shaken for three days on rotary shaker for complete mixing. This solution was filtered by using nylon filter of 0.22 µm pore size and 47 mm diameter. The filtrate was used for GC-MS analysis using a GC-MS-QP 2010 chromatograph of 70eV ionization voltage and 55-950 Da m/z scan range fitted out with a capillary column DB-5 of 30 m, 0.25 mm and 0.25 µm. The temperature of the oven was maintained at 45 °C for 1 min using helium gas as carrier and then automated from 45-100 °C @ 5 °C min^{-1}, held for 1 min and increased up to 200 °C @10 °C min^{-1} retained for 5 min as final temperature. The detector and injector temperatures were 250 °C and 200 °C, respectively (Kumar et al., 2012). The data was qualitatively analyzed by using NIST Library 2010 word software.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used to analyze the data statistically at 5% significance level (Steel et al., 1997).

Results and Discussions

In the current study, the antifungal potential of *C. procera* leaves was examined against *M. phaseolina*. The methanolic extract of leaves of the test plant was applied *in vitro* against causative agent of charcoal rot disease of mungbean. All the applied concentrations viz. 1%, 2.5%, 4%, 5.5% and 7% of this extract of *C. procera* significantly suppressed the radial diameter of the test fungus. However, 7% concentration was found the most effective as it inhibited the *M. phaseolina* growth up to 38% as compared to control. The 5.5% concentration was also significantly retarded the growth of the target fungus up to 31% followed by 4, 2.5 and 1% concentrations (Fig. 1)**.** The results of the present study were supported by previous studies. Agoramoorthy et al. (2007) examined the antimicrobial activity of *C. procera* against some bacteria and fungi (*Aspergillus niger* and *A. flavus*) and suggested that *C. procera* has significant antifungal potential but less antibacterial activity. Yesmin et al. (2008) also reported that *C. procera* leaves have good antimicrobial potential.

The methanolic extract of *C. procera* was then subjected to sequential bioassay guided fractionation and various organic solvent fractions viz. *n*-butanol, ethyl acetate, chloroform and n-hexane were separate. *In vitro* bioactivities of these fractions were tested against *M. phaseolina*. The results showed that the n-hexane fraction was the most effectively repressed the test fungus growth (Fig. 2). Recently, Mohamed et al. (2015) studied the antimicrobial and cytotoxic effects of various fractions like n-hexane, chloroform, ethyl acetate and aqueous from latex of *C. procera* and found promising result. The methanolic, chloroform, ethyl acetate and petroleum ether fractions of *C. procera* were examined for their cytotoxic and antimicrobial potential by Ahmed et al. (2014). Results showed that all the tested fractions of *C. procera* inhibited the growth of all tested microbes viz. *Shigella*, *E. coli*, *A. niger*, *A. fumigates* and *A. flavus*. Mosses et al. (2006) reported that the presence of calotropin, glycosides and alkaloids secondary metabolites is the reason of antimicrobial activity of various parts of *C. procera*.

The n-hexane fraction of *C. procera* leaf extract being the most effective was selected for GC-MS (Gas chromatography-Mass spectrometry) analysis (Fig. 3). Seven compounds were identified in this analysis and details of the identified compounds are presented in Table. 1. Major categories of identified compounds are fatty acids, both saturated and unsaturated, hydrocarbons, aromatic hydrocarbons, aromatic carboxylic acid and catecholamine. A number of fatty acids such as pentanoic acid and octadecanoic acid were identified in our study. Antifungal activity of fatty acids is reported in previous literature as Carolina et al. (2011) reviewed that large quantities of fatty acids possess antimicrobial and antifungal potential. Agoramoorthy et al. (2007) also reported that various saturated and unsaturated fatty acid (pentadecanoic acid, hexadeanoic acid and methyl tetradecanoate) isolated from n-hexane fraction of *Excoecaria agallocha* possessed antifungal activity.

Aromatic carboxylic acid like n-benzyloxy-2-carbomthoxyaziridine also found in *C. procera* extract. Earlier (Nomiya et al., 2000) evaluated the antifungal and antibacterial activity of related carboxylic acid compounds. Butanoic acid an aromatic hydrocarbon was also detected in current study and Drobnica et al. (1967) also stated that aromatic hydrocarbons possess significant antifungal activity. Some other compounds such as amines, azocompounds and chlorocarbons (tetrachloroethylene) were also identified in the n-hexane fraction of methanolic extract of *C. procera* leaves in the present study.

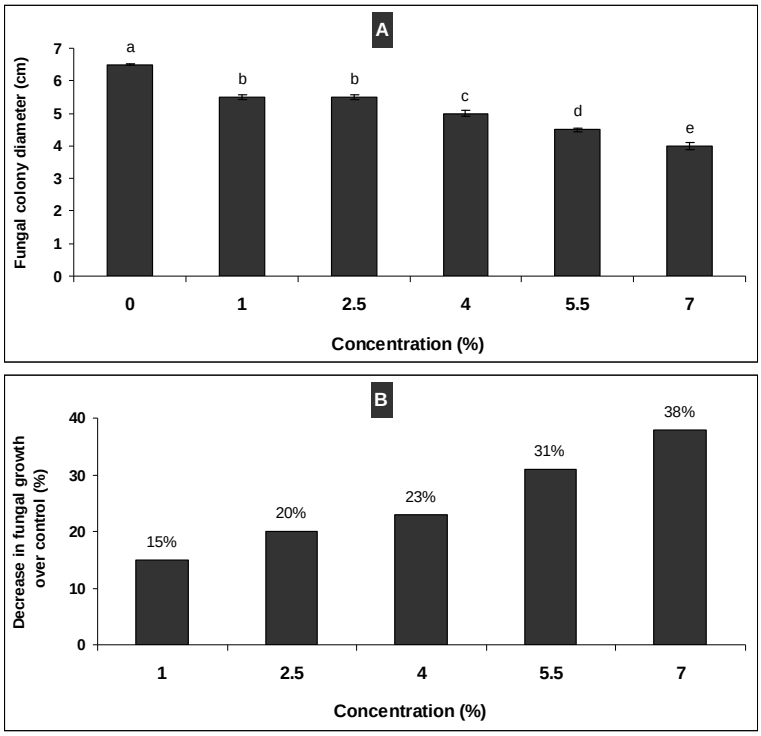

Figure 1: Effect of methanolic leaf extract of *Calotropis procera* on *in vitro* growth of *Macrophomina phaseolina*. Dissimilar letters show significant differences (P0.05) and vertical bars show standard errors of means of three replicates.

Table 1: GC/MS profiling of n-hexane extract of *C. procera*.

Retention time (min)	Compound name	Molecular weight	Molecular formula
3.400	Tetrachloroethylene	164	C_2C_{14}
4.342	Ethylbenzene	106	C_8H_{10}
4.508	p-Xylene	106	C_8H_{10}
13.567	Diazene,bis(1,1-dimethylethyl)	142	$C_8H_{18}N_2$
19.633	Butanoic acid	102	$C_5H_{10}O_2$
23.400	Tetradecanoic acid	256	$C_{16}H_{32}O_2$
25.417	Octadecanoic acid	354	$C_{21}H_{34}O_2$

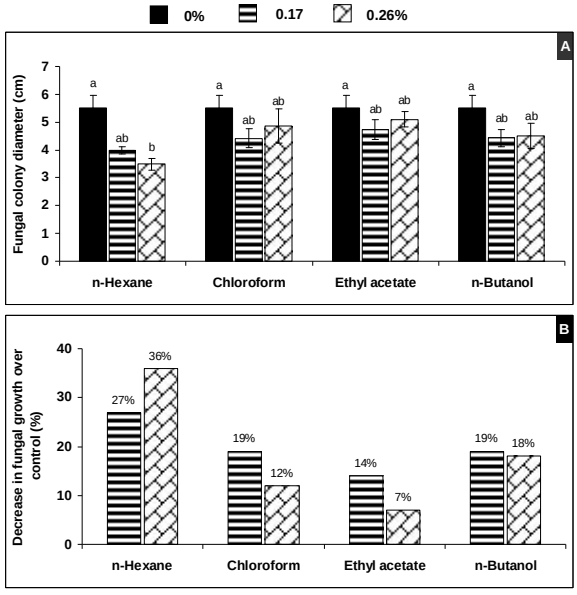

Figure 2: *In vitro* antifungal activity of various sub-fractions of methanolic leaf extract of *Calotropis procera* against *Macrophomina phaseolina*.

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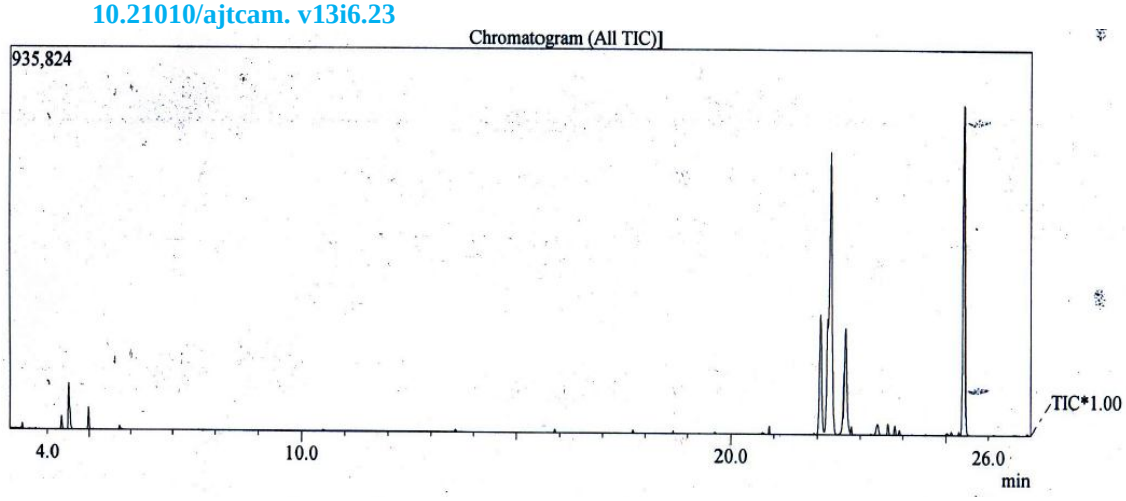

Figure 3: GC-MS chromatogram of *C. procera* leaves extract (n-hexane).

Conclusions

The present study concluded that methanolic leaf extract of *C. procera* has pronounced antifungal potential against the fungus *M. phaseolina* and the presence of large quantities of saturated and unsaturated fatty acids and aromatic carboxylic acid compounds identified by GC-MS analysis might be responsible for its strong antifungal potential against *M. phaseolina*.

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