Antifungal Potential of *Calotropis procera* against *Macrophomina phaseolina.*

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Abstract: The antifungal activity of *Calotropis procera* (aak) was investigated against the phytopathogenic fungus *Macrophamina phaseolina* causes charcoal rot in various economically important crops. Different concentrations of leaf and stem methanol extracts viz. 1% 2% 3% 4% 5% was applied against *M. phaseolina in vitro*. Leaf extract was found more effective & showed significant antifungal activity as its 3% concentration 16.5% reduces the fungal growth. Methanolic extracts of *C. procera* stem was promoting the growth of test fungus except 5% concentration. *C. procera* leaf extract was effectively suppressing the growth of *M. phaseolina* in screening bioassays, so this was subjected for fractional guided bioassays. Methanolic extract of *C. procera* leaves was partitioned with n-hexane, chloroform, ethyl acetate and n-butanol. Minimum inhibitory concentration (MIC) of these fractions and a commercial reference fungicide (72 %WP, Puslan) was evaluated against *M. phaseolina.* Different concentrations from (700 mg-1.36 mg mL^{-1}) were used and data was recorded after 24 and 48 hrs. n-hexane and synthetic fungicide were most effectively retard conidial germination with (1.36 mg mL^{-1}) MIC. The other fractions were comparatively less antifungal.

[Jabeen K, Waheed N, Iqbal S. **Antifungal Potential of *Calotropis procera* against *Macrophomina phaseolina.***
Life Sci J 2013;10(12s):572-576] (ISSN:1097-8135). http://www.lifesciencesite.com. 93

Keywords: Antifungal activity, Calotropis, Organic fractions, Methanolic extract, MIC

1. Introduction

Macrophomina phaseolina (Tassi) Goid. Is a soil-borne necrotrophic fungal pathogen, infecting more than 400 plant species (Dhingra and Sinclair, 1977). Under favorable conditions the fungus causes many diseases like damping off, seedling blight, collar rot, stem rot, charcoal rot and root rot in various economically important crop (Mihail and Taylor, 1995).The infectious hyphae enter into the plant through root epidermal cells or wounds and mycelium penetrates into the root epidermis and limited the intercellular spaces of the cortex of primary roots, as a result, adjacent cells collapse and heavily infected plantlets may die due to the production of fungal toxins e.g. phaseolinone (Mayek-Pérez et al., 2002). Charcoal rot reduced the crop yield upto 100% and 23-64% grain yield under favorable conditions especially in arid regions of the world (Mughogho and Pande, 1984; Hoes, 1985).

Various disease management methods have been implemented to eradicate pathogenic fungus; these methods include cultural, regulatory, physical, chemical and biological methods. All these methods are effective only when employed well in advance as precautionary measure. Once a disease has appeared, these methods become ineffective. In that situation, chemical control offers a good choice to grower to control the growth of disease. However, it has been realized now that use of chemical in agriculture is not as beneficial as chemical pose serious health hazards to an applicator as well as to a consumer of the treated material. Increasing awareness of humankind toward the ecosystem and environment has made a marked shift from synthetic material to plant bio-products. Botanical derivatives are more environmentally safe than synthetic chemicals. Several antifungal compounds present in certain plant species have been used for treating fungal infections. Plant extracts have played significant role in the inhibition of seed-borne pathogens (Mansilla and Palenzuela, 1999; Neerman, 2003).

Calotropis procera R.Br. is a large shrubby weed belongs to family Asclepiadaceae, commonly known as milkweed or swallow-wort (Singh et al., 1996). It is used as a traditional medicinal plant with unique properties (Oudhia and Tripathi, 1998a, b). *C. procera* is popular due to its large quantity of latex also contains potential secondary metabolites such as glycosides, alkaloids and calotropis. This plant has potential antimicrobial properties against microbial infections (Mosses et al., 2006; Kareem et al., 2008; El-Khawaga et al., 2013).

The present study is, therefore, designed to evaluate the antifungal potential of *Calatropis procera* against the destructive plant pathogenic fungi *Macrophomina phaseolina*, the causal agent of charcoal root rot.

2. Material and Methods

Collection of plant material

Calotropis procera leaves and stem were collected from Nespak housing society Lahore, Pakistan. These plant materials were thoroughly washed with tap water and then surface sterilized by

1% sodium hypochlorite solution followed by distilled water. After washing plant materials were dried at 40°C in an electric oven and carefully grinded to form powder.

Isolation and culturing of *pathogen*

Test fungus was isolated from diseased stem of soyabean. Diseased plant part was surface sterilized with 1% sodium hypochlorite solution followed by thorough washing with distilled water and was placed on 2% PDA (Potato dextrose agar) medium. Fungal culture was identified as *Macrophomina phaseolina* from previous literature. The isolated culture was subculture and maintained on PDA medium and was kept at 4°C in refrigerator.

Preparation of organic solvent extracts

Twenty gram dried, powdered leaves and stem of *C. procera* were soaked in 100 mL organic solvent (methanol) and left for three days at room temperature. Materials were filtered through an autoclaved muslin cloth followed by filter papers. Filtrate was evaporated at 35°C in an electric oven and then diluted by adding 100 mL distilled water. These stock extract was covered and stored at 4°C.

Antifungal bioassay with extracts

PDA medium was made in 250 mL flask by adding 2 g of potato dextrose agar in 100 mL of distilled water and was autoclaved at 121°C for 30 minutes. To 80 mL of PDA and adequate amount of distilled water was added in each flask of all applied concentrations of leaf and stem of *C. procera* 5% v/v concentrations of organic solvent extract was made by adding 25 mL of stock solution in 80 mL the medium. The lower concentrations of 4% , 3% , 2% and 1% were prepared by adding up 20,15 ,10 and 5 mL of the stock solutions to 80mL of PDA . The plant extracts were gently mixed with the extracts. Control treatment was without any plant extracts. Each concentrations was endowed with Chloromycetin capsule @ 50mg mL^{-1} of the medium to avoid bacterial contamination, 20 mL of each medium was poured in 9 cm sterilized Petri plate. *in vitro* antifungal bioassay was conducted with organic solvent extract. 5 mm mycelial discs were prepared using sterilized cork borer from the tip of 7 days old culture of *Macrophomina phaseolina* and were placed in the centre of each Petri plate after solidification of PDA media. Three replicates were made for each treatment. All these plates were incubated at 25 °C for one week .After 7 days fungal growth diameter was measured by taking average of three diameters taken at right angles for each colony. Percentage growth inhibition of the fungal colonies was measured by using the formula:

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

Fractionation guided bioassays

Leaf extracts of *C. procera* were found very effectual in suppressing the *in vitro* growth of *M. phaseolina.* This extract was selected for fractionation.

Partitioning of plant material

Leaves of *C. procera were* partitioned with various organic solvents in increasing order of polarity n-hexane; chloroform, ethyl acetate (EtOAc) and n-butanol at room temperature by using separating funnel. Two fifty grams of Calotropis leaves were thoroughly extracted with methanol (MeOH x 700 mL) at room temperature. The extract was evaporated under vacuum on rotary evaporator at 40°C, yield 13 g gummy mass. This (13 g) methanolic extract was partitioned between n-hexane and water. The aqueous fraction was successively partitioned with chloroform, ethyl acetate and n-butanol (Jabeen et al., 2013). This partitioning was resulted as gummy mass of n-hexane (1.7g), chloroform (0.7 g), ethyl acetate (1 g) and n-butanol (1 g) and remaining water fraction.

Minimum inhibitory concentration (MIC) evaluation of the isolated fractions

The MIC (minimum inhibitory concentration) of the four isolated fraction were tested along with a commercial synthetic fungicide (72% WP, Puslan) in test tubes by serial dilution assay (Jabeen et al., 2011) with small amendments. For MIC assay, maximum 1g of each fraction was dissolved in 1 mL of DMSO (dimethyl sulphoxide) & 1 mL of distilled water and this concentration was further serially diluted and the minimum tested concentration was 1.36 mg mL^{-1}. Seven days old culture of *M. phaseolina* was taken and freshly prepared 2% PDA medium was added in it to reach a final conidial concentration of 1×10^5, 100 µL of this was added to test tubes of 1.6 cm diameter and 15 cm length. Test tubes containing DMSO and distilled water was served as control. These test tubes were incubated at 25-30°C.after 24 and 48 hours. MIC of the fractions and fungicide was observed visually by using inverted microscope to study the fungal mycelial growth.

Statistical analysis

Data were analyzed statistically by using ANOVA followed by Duncan's multiple range tests (Steel et al., 1997).

3. Results

All the data was analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range test (DMR) computer software. The analysis of variance show significant effect of different plant parts of *Calotropis procera* solvent extracts and their different concentration on growth of fungus at ($P \leq 0.05$).

Antifungal activity of methanolic extract of *Calotropis procera* leaves

Methanolic extract of leaf exhibited strong antifungal activity against the test fungus *M. phaseolina*. Data regarding methanolic extract of *C. procera* leaves against *M. phaseolina* was presented in Fig. 1 & 2. Maximum reduction in fungal growth diameter was observed in 3% concentration i.e, 16.5 %. Other applied concentrations of *C. procera* leaves viz. 2% and 4%, showed significant effect resulting suppressing fungal growth upto 9.58 % as compared to control treatment (5.6%). Higher concentrations (5%) also effectually retard the *M. phaeolina* diameter upto 6.56%.

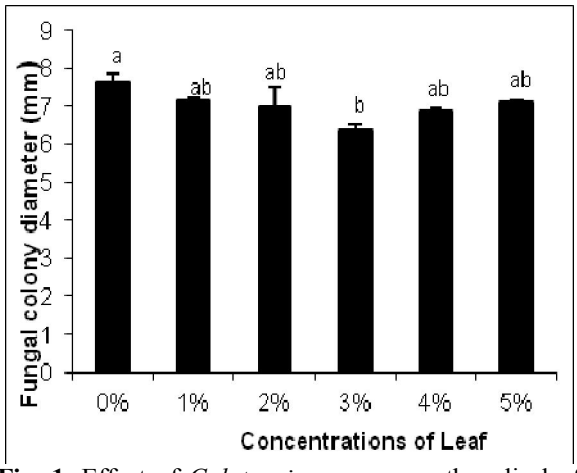

Fig. 1: Effect of *Calatropis procera,* methanolic leaf extracts on *in vitro* growth of *Macrophomina phaseolina*. Vertical bars show standard error of means of three replicates. Values with different letters show significant difference as determined by DMR Test.

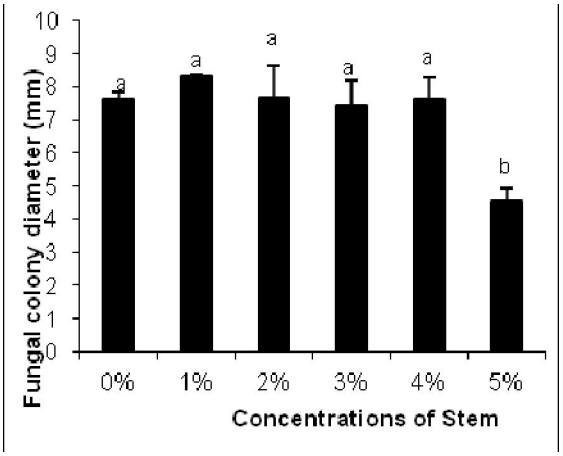

Fig. 3 Effect of *Calatropis procera,* methanolic stem extracts on *in vitro* growth of *Macrophomina phaseolina* Vertical bars show standard error of means of three replicates. Values with

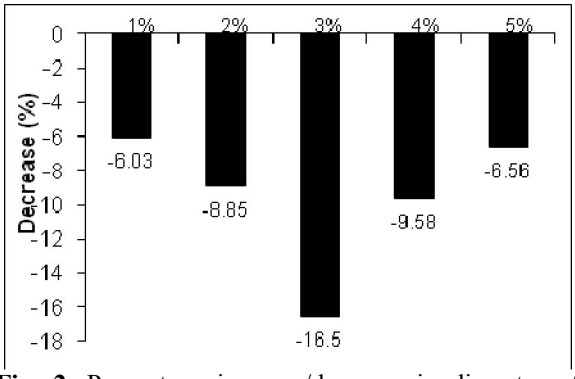

Fig. 2. Percentage increase/decrease in diameter of *Macrophomina phaseolina* dueto different concentrations of methanolic leaf extract of *Calatropis procera.*

Antifungal activity of methanolic extract of *Calotropis procera* stem

Methanolic stem extract of *C. procera* stem showed significant antifungal potential against *M. phaseolina* after 7 days incubation period. The highest concentration (5 %) was highly effective against the target fungus as it retarded fungal growth 40.2% as compared with control followed by the 1% concentration with a reduction of 9.31 %. other applied concentrations of *C. procera* stem extract also significantly suppressed the *in vitro* growth of *M. phaseolina* (Fig. 3 & 4).

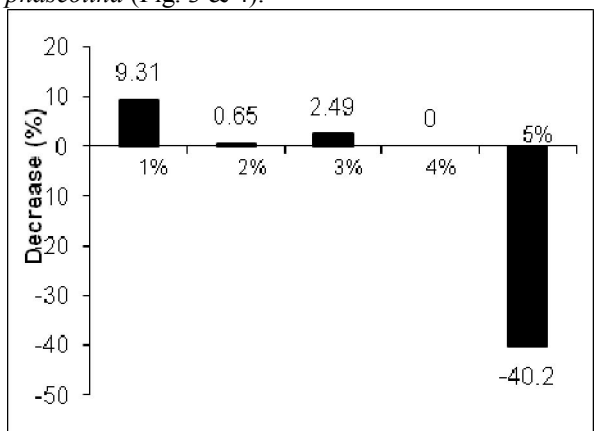

Fig. 4. Percentage increase/decrease in diameter of *M.phaseolina* due to different concentrations of methanolic stem extract of *C. procera*. different letters show significant difference as determined by DMR Test.

Minimum inhibitory concentration (MIC)

Antifungal activity of *Calotropis procera* leaf extract's isolated fractions with a reference syntheticfungicide (Puslan, 72 WP) against *Macrophomina phaseolina* was determined by MIC assay after 24 and 48 hrs incubation periods. *n*-hexane fraction andPuslan were found to be extremely

antifungal as clear from the results, its highest as well as lowest (700 mg - 1.36 mg mL^{-1}) concentrations completely inhibits the conidial germination after 48 hrs incubationperiod. Other fractions were comparatively less antifungal, among these n-hexane fraction is least effective. Effectiveness order was n-hexane > n-butanol > chloroform and ethyl acetate. The results were highly pronounced when compared with controls of water & DMSO, in both treatments (water & DMSO) conidial germination starts within the 24 hrs incubation and visual mycelium was observed (Table. 1).

Table 1: MIC values of different organic fractions of methanolic leaf extract of *C. procera* and synthetic fungicide Puslan against *M. phaseolina*, after 24 and 48 hrs incubation periods. (Mycelium present (+), Mycelium Absent (-).

Fractions	mg mL^{-1}									
	700	350	175	87.5	43.7	21.8	10.9	5.46	2.73	1.36
24 hours after incubation										
Control (Water)	+	+	+	+	+	+	+	+	+	+
Control (DMSO)	+	+	+	+	+	+	+	+	+	+
n-hexane	-	-	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-	-	-
Ethyl Acetate	-	-	-	-	-	-	-	-	-	-
n-butanol	-	-	-	-	-	-	-	-	-	-
Puslan 72 WP	-	-	-	-	-	-	-	-	-	-
48 hours after incubation										
Control (Water)	+	+	+	+	+	+	+	+	+	+
Control (DMSO)	+	+	+	+	+	+	+	+	+	+
n-hexane	-	-	-	-	-	-	-	-	-	-
Chloroform	-	+	+	+	+	+	+	+	+	+
Ethyl Acetate	-	-	-	-	+	+	+	+	+	+
n-butanol	-	-	-	-	-	+	+	+	+	+
Puslan 72 WP	-	-	-	-	-	-	-	-	-	-

4. Discussion

Macrophomina phaseolina is a soil borne fungal pathogen, reducing crop yield especially in arid regions of the word (Srinivasan et al., 2009). Various disease management methods have been implemented to combat and eradicate pathogenic fungi but it remains to be a challenging task in terms of management, because these methods are useful only when they are employed well in advance as precautionary measures. Chemical pesticides have been in use since long and they provide quick effects to control. But as the use of chemicals in agriculture is not beneficial because of health hazards, pesticides also kill various beneficial organisms.

In the present study methanolic leaf & stem extracts of *Calotropis procera* were investigated against the target fungus *Macrophomina phaseolina.* Different applied concentrations of leaves (1-5%) significantly reduced the fungal growth; lower concentration of leaf was found more effective as 3% concentration 16.5% as compared to control set. Other concentrations were also significantly retarded the fungal colony diameter. Stem extracts also effectively suppresses the growth of test fungus *M. phaseolina.* These findings are supported by the literature as Mosses et al., (2006) reported that *C. procera* has potential antimicrobial properties and its different parts (root, stem, leaf) are used against fungus attack. This antifungal activity is due to different types of secondary metabolites such as glycocides, alkaloids, and calotropin.

C. procera leaves were selected for further bioassays and different organic fractions viz: n-hexane, chloroform, ethyl acetate and n-butanol were partitioned from the crude methanol leaf extract. The four organic solvent extract showed marked variation in antifungal activity, this could be due to different chemical natures of the four solvents. It is likely that different types of compounds were dissolved in different solvents that resulted in variable activity of the extracts of different solvents. Minimum Inhibitory Concentration (MIC) of the four organic solvent extracts of *C. procera leaves* was evaluated against *M. phaseolina* and compared with a known commercial fungicide Puslan. The MIC assays were conducted through broth serial dilution method; various concentrations were made ranges from 700 mg to 1.36 mg mL^{-1} (Rangasamy et al., 2007). All the four organic solvents were found variably effective against the test fungal specie. n-hexane fractions and the synthetic fungicide was much effectively inhibits the germination of *M. phaseolina* spores after 48 hrs incubation period, while chloroform, ethyl acetate & n-butanol fractions were found least effective. The

plant allelochemicals are best than the synthetic chemicals in having antifungal activity as three novel tetracyclic triterpenoids; bioactive compound were isolated from the methanolic extracts of the leaves of *Calotropis procera* that showed pronounced antifungal activity (Hua et al., 2008; Verma et al., 2012).

Conclusion

The present study concludes that different parts of *Calotropis procera* contain different antifungal substances that showed variable antifungal activity.

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11/14/2013