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BIOCHEMICAL CONTROL OF CHICKPEA BLIGHT PATHOGEN BY METHANOLIC FRUIT EXTRACT OF SYZYGIUM CUMINI

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A B S T R A C T

In vitro bioassays were carried out to investigate the antifungal activity of methanolic fruit extract of *Sizygium cumini* (L.) Skeels and its organic solvent fractions against *Ascochyta rabiei* (Pass.) Lab., the cause of chickpea (*Cicer arietinum* L.) blight. Different concentrations of methanolic extract (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg mL^{-1}) reduced the fungal biomass by 44-66%. Methanolic extract was portioned using four organic solvents viz. *n*-hexane, chloroform, ethyl acetate and *n*-butanol. Different concentrations viz. 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.562 mg mL^{-1} of these fractions were tested against the fungal pathogen. All the organic solvent fractions reduced fungal biomass to variable extents. There was 31-58%, 22-66%, 49-66% and 50-67% suppression in biomass of the fungus due to different concentrations of *n*-hexane, chloroform, ethyl acetate and *n*-butanol fraction, respectively. The present study concludes that methanolic fruit extract has substantial antifungal potential against *A. rabiei*.

Keywords: *Ascochyta rabiei,* chickpea blight, natural fungicides, *Sizygium cumini*.

INTRODUCTION

Chickpea is an important leguminous crop that ranks third after *Phaseolus vulgaris* L. and *Pisum sativum* L. (Kanouni *et al.,* 2011), and is a major source of protein in developing countries (Pande *et al.,* 2010). In Pakistan, it is cultivated on an area of 1053.8 thousand ha with total production of 496 thousand tonnes and an average yield of 471 kg ha^{-1} (Anonymous, 2011). This valuable crop is vulnerable to blight disease caused by *Ascochyta rabiei* (Kimurto *et al.,* 2013; Sarwar *et al.,* 2013). It is a highly destructive disease in most chickpea growing areas of the world (Saxena and Singh, 1987; Taran *et al.,* 2013). On the host plant, the pathogen grows asexually while its perfect stage *Didymella rabiei* (Kovachevski) Arx. has been reported on over wintering chickpea debris (Kaiser, 1995). Mostly, blight disease affects all parts of shoot of chickpea plants, producing lesions and breaking the shoots (Bayraktar *et al.,* 2007), causing heavy yield losses. In severely affected fields, yield losses may be up to 100% (Singh and Reddy, 1990; Kimurto *et al.,* 2013).

Several foliar fungicides namely captafol, dithianon, captan, mancozeb, ferbam, maneb, chlorothalonil, penconazole, propiconazole, Bordeaux mixture, thiabendazole and propineb have been used to reduce attack of Ascochyta blight (Nene and Reddy 1987; Pande *et al.,* 2005; Shtienberg *et al.,* 2006; Ahmed *et al.,* 2008). Likewise some systemic fungicides namely azoxystrobin, difenoconazole and tebuconazole have been found effective against this disease (Shtienberg *et al.,* 2000, 2005). However, the use of synthetic fungicides pollute the environment and cause health hazards (Chiejina and Ukeh, 2012). Due to the ill effects of these chemicals, scientists are in search of natural alternatives from plants for disease management (Iqbal and Javaid, 2012; Javaid and Samad, 2012; Rauf and Javaid, 2013). The present study is a continuation of these efforts. This study was designed to evaluate the potential of methanolic fruit extracts of *S. cumini* and its various organic fractions to control *A. rabiei.*

MATERIALS AND METHODS

Ripened fruits of *S. cumini* were collected from Quaid-e-Azam Campus, University of the Punjab, Lahore, Pakistan during July 2011. After washing thoroughly under tap water, fruits were dried in sunlight. Five kilograms of dried fruits were crushed thoroughly and soaked in 7.0 L

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of methanol at room temperature for two weeks. After that, materials were filtered through a muslin cloth. Residues were again extracted with methanol for one week and filtered. Methanol extract of the two batches was combined and evaporated under vacuum in a rotary evaporator to obtain crude methanolic extract of *S. cumini* fruits.

Bioassays were carried out with a fraction of the crude methanolic extract. For this purpose, 14.4 g methanolic extract was dissolved in 6 mL dimethyl sulphoxide (DMSO) and volume was raised to 18 mL by adding sterilized distilled water to prepare a stock solution. Likewise, a control solution was prepared by dissolving 6 mL DMSO in 12 mL distilled water to get the same concentration of DMSO as in the stock solution. Seventy six milliliters of malt extract broth was autoclaved and different quantities of stock solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mL) and control solution (3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5, 0 mL), respectively, were added to malt extract broth to raise the volume to 80 mL. The control treatment received 4 mL of control solution only. The medium was divided into four equal parts so that each 100-mL flask contained 20 mL medium to be used as replicates. One actively growing fungal plug of 5 mm diameter was added to each flask. Flasks were arranged in a completely randomized design in an incubator maintained at 26 °C. After 10 days incubation, fungal biomass from each flask was filtered, dried and weighed.

The remaining fraction of the crude methanolic extract was mixed with 450 mL distilled water and the mixture was extracted with 450 mL of *n*-hexane. There after, the remaining mixture was successively extracted with chloroform, ethyle acetate and *n*-butanol in order of increasing polarity. The extracted fractions were evaporated on a rotary evaporator to get 15 g *n*-hexane, 19 g chloroform, 10 g ethyl acetate, 8 g *n*-butanol and 350 g of aqueous fraction of methanolic fruit extract of *S. cumini* fruit (Javaid and Saddique, 2011).

For *in vitro* antifungal evaluation of different fractions of methanolic extract, 1.6 g of the each fraction was dissolved in 1 mL DMSO and volume was raised to 8 mL by adding growth medium. Half of this stock solution of 200 mg mL^{-1} concentration was used for bioassays and rest of the solution was double diluted serially by adding growth medium to prepare 100, 50, 25, 12.5, 6.25, 3.125 and 1.562 mg mL^{-1} concentrations. For the control, a mixture of DMSO and malt extract broth (1:7) was successively double diluted so that different control treatments had the same concentrations of DMSO that was present in different extract treatments. Bioassays were carried out by taking 1 mL of the growth medium in 10-mL test tubes. Measured volume (10 µL) of *A. rabiei* conidia was added to each test tube. Each treatment was replicated four times. Test tubes were incubated at room temperature. Biomass of the fungus in each test tube was filtered on pre-weighed filter papers after seven days and fungal dry biomass was recorded (Javaid and Samad, 2012).

All the data were analyzed by analysis of variance followed by Duncan's Multiple Range Test to compare the treatment means at 5% level of significance (Steel *et al.,* 1997).

RESULTS AND DISCUSSION

The effect of different concentrations of methanolic fruit extract on growth of *A. rabiei* is presented in Fig. 1. The effect of DMSO on fungal growth was not very pronounced as indicated by difference in negative and positive control treatments. All the extract concentrations significantly reduced fungal biomass over negative as well as positive control treatments. Fungal biomass was gradually decreased with the increase in extract concentration. There was 46-67% and 44-67% reduction in fungal biomass due to different concentrations of methanolic fruit extract as compared to negative and positive control treatments. *S. cumini* fruit concentrate has a very long history of use for various medicinal and antimicrobial purposes (Jagetia and Baliga, 2002; Migliato, 2005). Various chemicals such as phenolics and flavonoids isolated from *S. cumini* (Lima *et al.,* 2007) may be responsible for its antifungal activities (Ahn *et al.,* 2005; Meragelman *et al.,* 2005).

All the concentrations of *n*-hexane fraction of methanolic extract of *S. cumini* fruit significantly reduced the biomass of *A. rabiei.* There was 31-58% reduction in fungal biomass due to different concentrations of *n*-hexane fraction of methanolic extract (Fig. 2 & 7). Similarly, chloroform fraction of methanolic extract exhibited pronounced antifungal activity against the target fungal pathogen. The adverse effect of all the extract concentrations was significant. The effect of 12.5 to 200 mg mL^{-1} concentrations was more pronounced than the effect of lower concentrations of 1.562 to 6.25 mg mL^{-1} (Fig. 3). There was 61-66% and 22-25% reduction in fungal biomass over corresponding control treatments due to 12.5 to 200 mg mL^{-1} and 1.562 to 6.25 mg mL^{-1} extract concentrations, respectively (Fig. 7).

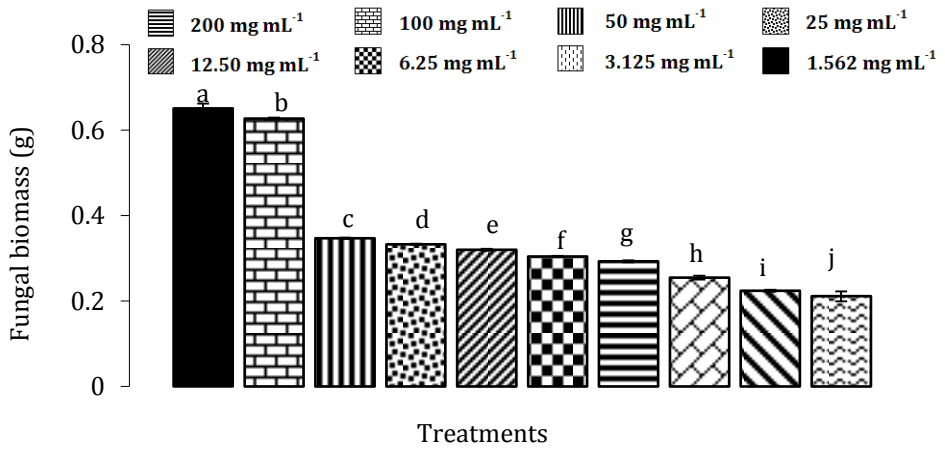

Fig. 1: Effect of different concentrations of methanolic fruit extract of *Syzygium cumini* on growth of *Ascochyta rabiei*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

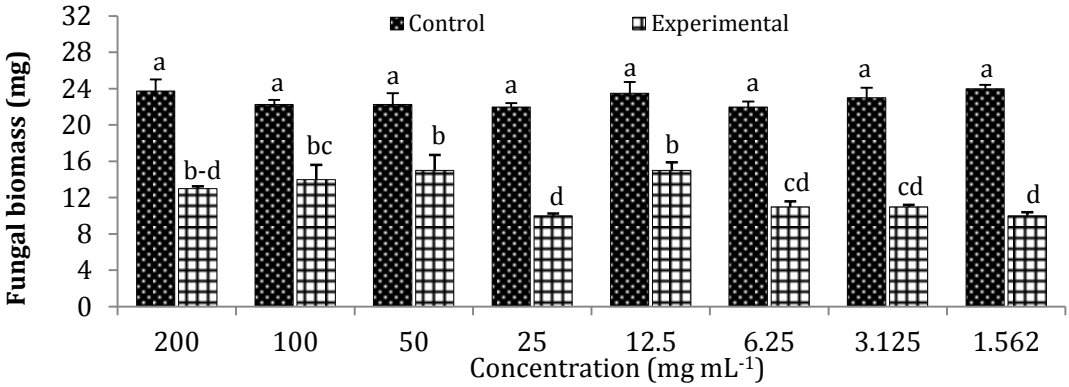

Fig. 2: Effect of different concentrations of *n*-hexane fraction of methanolic fruit extract of *Syzygium cumini* on growth of *Ascochyta rabiei*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

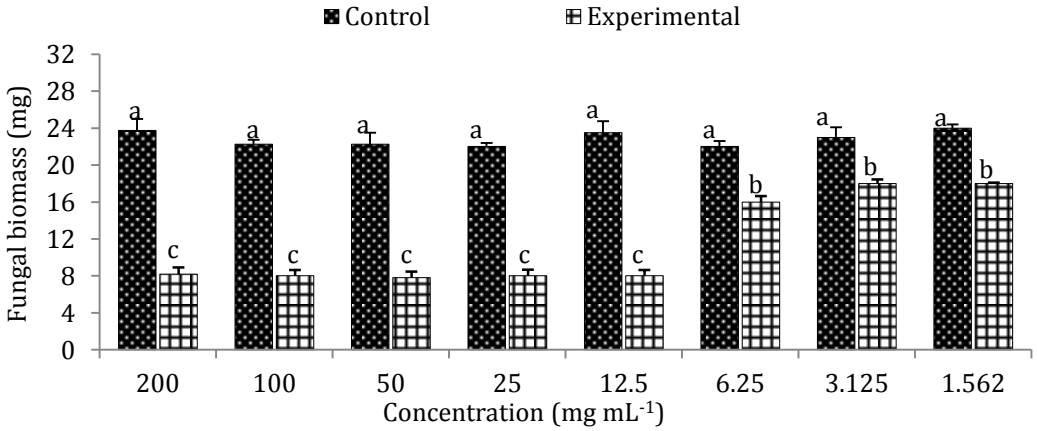

Fig. 3: Effect of different concentrations of chloroform fraction of methanolic fruit extract of *Syzygium cumini* on growth of *Ascochyta rabiei*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

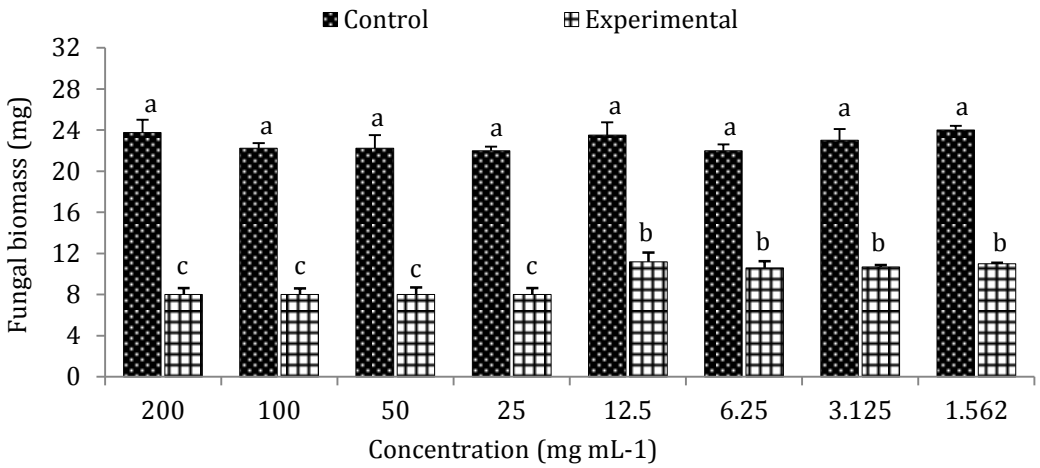

Fig. 4: Effect of different concentrations of ethyl acetate fraction of methanolic fruit extract of *Syzygium cumini* on growth of *Ascochyta rabiei*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

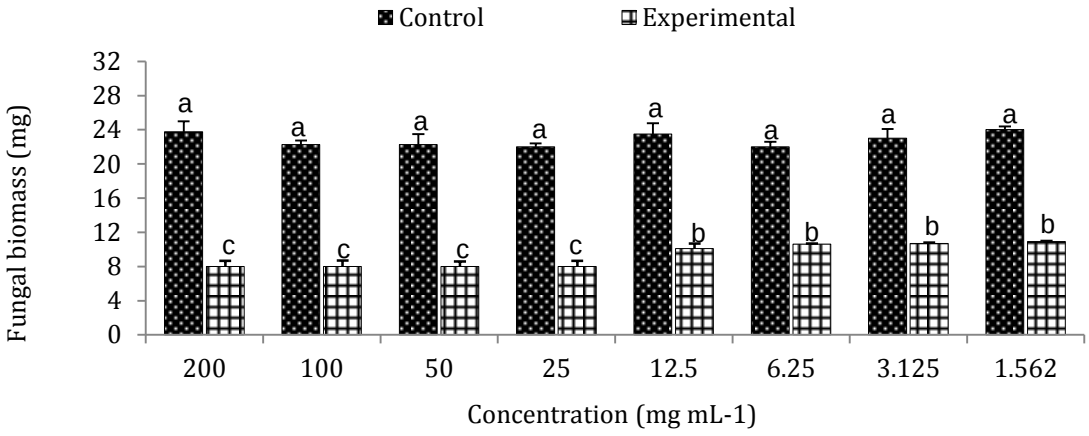

Fig. 5: Effect of different concentrations of *n*-butanol fraction of methanolic fruit extract of *Syzygium cumini* on growth of *Ascochyta rabiei*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

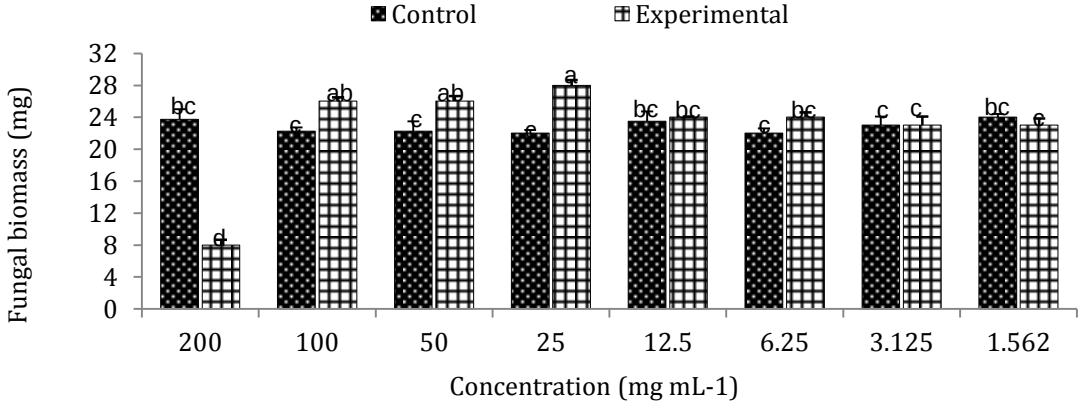

Fig. 6: Effect of different concentrations of aqueous fraction of methanolic fruit extract of *Syzygium cumini* on growth of *Ascochyta rabiei*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

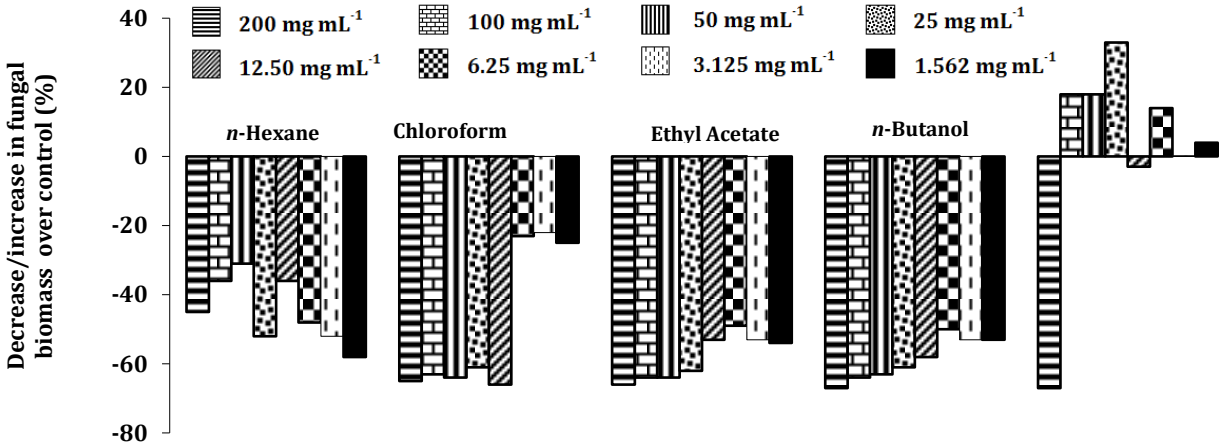

Fig. 7: Percentage decrease/increase in biomass of *Ascochyta rabiei* due to different concentrations of *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous fractions of methanolic fruit extract of *Syzygium cumini* over control.

All the concentrations of ethyl acetate fraction significantly reduced the fungal biomass over control. The effect of 25-200 mg mL^{-1} concentrations was better than the effect of lower concentrations of 1.562 to 12.5 mg mL^{-1} (Fig. 4). There was 62-66% and 49-54% reduction in fungal biomass due to 25 to 200 mg mL^{-1} and 1.562 to 12.5 mg mL^{-1} concentrations, respectively (Fig. 7). The effect of various concentrations of *n*-butanol fraction against the target fungal pathogen was similar to that of the effect of ethyl acetate fraction. In general, all the concentrations of this fraction significantly suppressed the fungal growth (Fig. 5). The 25 to 200 mg mL^{-1} concentrations exhibited similar effect resulted in 61-66% reduction in fungal biomass. Likewise, the lower concentrations of 1.562 to 12.5 mg mL^{-1} reduced the fungal biomass by 49-58% (Fig. 7). Aqueous fraction exhibited entirely different effect on fungal growth as compared to various organic solvent fractions. The highest extract concentration (200 mg mL^{-1}) significantly reduced fungal biomass by 67%. Conversely, other concentrations (25-100 mg mL^{-1}) concentrations stimulated the fungal growth resulting in significant increase of 18–33% in fungal biomass over corresponding control treatments. The effect of lower concentrations of 1.562 to 12.5 mg mL^{-1} was insignificant (Fig. 6 & 7). In the present study, a marked variation in antifungal activities among different organic fractions was noticed. Similar variable antifungal activity of different organic solvent fractions of methanolic extracts of *WIthania somnifera, Coronopus didymus, Chenopodium album* and *Datura metel* has also been reported against other fungal pathogens such as *Sclerotium rolfsii*, *Fusarium oxysporum* f. sp. *cepae* and *Macrophomina phaseolina* (Iqbal and Javaid, 2012; Javaid and Saddique, 2012; Rauf and Javaid, 2013). Variable antifungal activity in different solvents could be attributed to variation in polarity of different organic solvents. Compounds having different polarities were separated in different organic solvents and thus the isolated fractions showed different antifungal activities against *A. rabiei.* The present study concludes that methanolic fruit extracts has potential to control *A. rabiei.* Particularly, ethyl acetate and *n*-butanol fractions of methanolic extracts are highly suitable for control of *A. rabiei.*

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