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MANAGEMENT OF *Ascochyta rabiei* BY *Chenopodium album* EXTRACTS[1]

*Manejo de **Ascochyta rabiei** por Meio de Extratos de Folhas de **Chenopodium album***

SHERAZI, A.Z.[2], JABEEN, K.[2], IQBAL, S.[2], and YOUSAF, Z.[2]

ABSTRACT - *Chenopodium album*, leaves were selected to evaluate their antifungal potential against *Ascochyta rabiei* causative agent for chickpea blight. Different concentrations of methanolic extract of *C. album* leaves i.e. 1%, 2.5%, 4%, 5.5% and 7% were tested against the target fungus *A. rabiei.* Maximum reduction in the test fungal biomass (68%) was observed in 7% concentration. This methanolic leaf extract was partitioned and *n*-butanol, chloroform, *n*-hexane, ethyl acetate fractions were isolated according to their polarity. In vitro antifungal activity of these fractions was studied by serial dilution method. *n*-hexane fraction exhibited the highest antifungal potential with 55% inhibition in test fungal biomass, so this fraction was selected for Gas chromatography mass spectrometry (GC-MS) analysis. Total thirteen compounds identified in this analysis belonged to class aromatic hydrocarbons, hydrocarbons, saturated fatty acids, aromatic carboxylic acid, siloxanes, phosphonates and cardiac glycosides. These compounds might be responsible for antifungal activity of *C. album*.

Keywords: antifungal potential, bathu, GC-MS, methanolic extract.

*RESUMO - Foram selecionadas folhas de **Chenopodium album**, para avaliação do potencial antifúngico contra o **Ascochyta rabiei**, agente causal da praga do grão de bico. Diferentes concentrações do extrato metanólico das folhas de **C. album** (1%, 2.5%, 4%, 5.5% e 7%) foram testadas contra o fungo-alvo **A. rabiei**. Foi observada máxima redução (68%) na biomassa do fungo testado na concentração de 7%. O extrato metanólico das folhas foi particionado, e as frações n-butanol, clorofórmio, n-hexano, acetato de etila foram isoladas de acordo com a polaridade. A atividade antifúngica in vitro dessas frações foi analisada por meio do método de diluições em série. A fração n-hexano apresentou o maior potencial antifúngico, com 55% de inibição na biomassa do fungo testado; por isso, essa fração foi selecionada para análise de Cromatografia Gasosa acoplada à Espectrometria de Massas (GC-MS). Um total de treze compostos identificados nesta análise pertenciam às classes hidrocarbonetos aromáticos, hidrocarbonetos, ácidos graxos saturados, ácido carboxílico aromático, siloxanos, fosfonatos e glicosídeos cardíacos. Estes compostos são, possivelmente, responsáveis pela atividade antifúngica de **C. album**.*

Palavras-chave: potencial antifúngico, bathu, GC-MS, extrato metanólico.

INTRODUCTION

Chickpea (*Cicer arietinum*) is the third most important crop cultivated worldwide. Pakistan stands second in the Indian subcontinent and third in the world for cultivation and production of chickpea crops (FAO, 2014). However, per annum production of this crop is affected by fungal diseases like chickpea or Ascochyta blight. The pathogenic fungus *Ascochyta rabiei* is responsible for chickpea blight. This fungus attacks the crop in areas where humid and cool weather persists, and causes great yield loss (Pande et al., 2005). The most common and effective method for the control of chickpea blight is the application of fungicides, such as mancozeb, chlorothalonil, and triphenyltin hydroxid. However, fungicidal

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[2] Department of Botany, Lahore College for Women University, Lahore. <khajista_1@hotmail.com>.

control is uneconomical, unsustainable and hazardous to the environment (Pande et al., 2007; Christoffoleti et al., 2008; Silva et al., 2012). The best alternative to chemical pesticides is the use of plant based natural compounds (Hernandez-Terrones et al., 2007). A series of molecules with antifungal activity against different fungal strains has been found in plants (Medeiros et al., 2011). These molecules may be used directly or considered as a precursor for developing better molecules (Arif et al., 2009). *Chenopodium album*, commonly known as bathu, is biologically active against plant fungal pathogens. Chemical composition of Chenopodium showed the presence of saponins, flavonoids (e.g. phenolic amide), cinnamic acid amide, apocortinoid, alkaloids (e.g. chinoalbicin), phenols, lignans and xyloside (Agrawal et al., 2014). Therefore, the present study was planned to find out the effect of the organic extracts of *C. album* against *A. rabiei*.

MATERIALS AND METHODS

Collection of experimental material

Leaves of *C. album* were collected from Shahpur, District Sargodha, Pakistan. Leaves were surface sterilized with 1% sodium hypochlorite solution followed by distilled water to avoid contamination and then dried at 40 °C in an electric oven. The test culture with the fungus *A. rabiei* was prepared by inoculation of the infected part (stem) of the chickpea plant on 2% MEA (Malt Extract Agar) medium (Ilyas and Khan, 1985).

In vitro bioassay

In vitro evaluation of test plant *C. album* against *A. rabiei* was carried out by using the protocol of Karim et al., (2015). Dried powder of the plant material (500 g) was soaked in 1 liter of methanol for 7 days at room temperature. After seven days, this methanol extract was filtered though muslin cloth followed by filter paper and was kept in an electric oven at 40 °C for two days. This evaporation provides 15 g methanolic gummy mass of *C. album* leaves and 20% stock solution was prepared by adding 70 mL of distilled water in this 15 g extract.

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 in flasks containing 57, 52.5, 48, 43.5 and 39 mL (2% ME) broth, respectively, to make the final volume up to 60 mL. The control treatment did not have any plant extract. Chloromycetein capsule at 50 mg 100 mL^{-1} of the medium was added to avoid bacterial contamination. Three replicates of each concentration were prepared and 5 mm mycelial discs from seven day's old actively grown culture of *A. rabiei* were placed in each flask. These flasks were placed in an incubator for 7 days at 25 °C. Total fungal biomass was observed by filtering the fungal mat through pre-weighed Whatman No. 1 filter papers. Filter papers containing fungal biomass were dried in an electric oven. Percentage growth inhibition in test fungal biomass was measured by using the formula:

$$Growth\ inibition\ (\%) = \frac{Growth\ in\ control - Growth\ in\ treatment\ x\ 100}{Grouth\ in\ control}$$

Bioassay guided fractionation

Leaves of *C. album* were subjected to bioassay guided fractionation and five hundred grams leaves were thoroughly extracted with methanol (Me OH, 1 Litre) at room temperature. This organic extract was evaporated on a rotary evaporator at 40 °C. Methanolic extract was partitioned between n-hexane and water and resultant aqueous fraction was successively partitioned with chloroform, ethyl acetate and n-butanol (Jabeen et al., 2011) by using a separating funnel at room temperature. This partitioning gave gummy mass of n-hexane (1.78 g), chloroform (0.3 g), ethyl acetate (0.3 g) and n-butanol (0.15 g).

In vitro antifungal activity of organic fractions

In vitro antifungal activity of four isolated fractions was tested against *A. rabiei t*hrough the serial dilution method given by Shadomy et al. (1991). The stock solution (20%) was prepared by adding 0.15 g of each isolated fraction into 0.75 mL distilled water. Two concentrations i.e. 0.15% and 0.25% were made from the 20% stock solution in 2% MEA

autoclaved medium. Chloromycetein capsule was added to avoid bacterial contamination. All the concentrations were replicated thrice; 5 mm mycelial discs were obtained from an actively grown culture of *A. rabiei* with the help of a sterilized cork borer, and they were placed in each Petri plate.

Isolation of bioactive compounds through Gas Chromatography Mass Spectrometry (GC-MS)

The *n*-hexane fraction which was found most effective in the bioassay guided fraction was selected for GC-MS analysis. Twenty grams of powdered leaves of *C. album* were soaked in 600 mL of *n*-hexane in a 1,000 mL conical flask and placed in an incubator shaker for 3 days for the complete extraction of secondary metabolites. Finally, the sample of solvent was filtered via membrane filter (Pore size: 0.22 µm, Diameter: 47 mm, Material: Nylon) with the help of filtration assembly and used for chemical analysis with GC-MS. The sample was analyzed using a GC-MS-QP 2010 chromatograph following the procedure given by Kumar et al. (2012) with slight amendments. Ionization voltage was 70 eV, m/z scan range 55-950 Da and equipped with a DB-5 capillary column (30 m, 0.25 mm, 0.25 mm). The oven temperature was held at 45 °C for 1 min, then programmed from 45-100 °C at a rate of 5 °C min^{-1}, held for 1 min, increased up to 200 °C at the rate 10 °C min^{-1} and was kept at the final temperature for 5 min, using He as a carrier gas. The injector and detector temperatures were 200 °C and 250 °C, respectively. The percentage composition of volatile compounds was computed from GC peak areas. Qualitative analysis was based on a comparison of retention times, indices and mass spectra with the corresponding data in the literature (NIST Library 2010 word software).

Statistical analysis

All the data were statistically analyzed by using the Co-stat software for analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at a P≤0.05 significance level (Steel et al., 1997).

RESULTS AND DISCUSSION

In the present study, the methanolic leaf extract of *C. album* was checked against the target fungus *A. rabiei*. Various concentrations of leaf extract (1%, 2.5%, 4%, 5.5% and 7%) significantly decreased the test fungal growth. The maximum reduction (68%) in *A. rabiei* biomass was obsereved in 7% concentration as compared to control set. Other concentrations were also significantly retarded the fungal mycelium 28-60% (Figure 1 and 2). Many workers suggested that the Chenpodium species are biologically active and have strong antifungal and antimicrobial properties. Like essential oils of aerial parts of *C. botrys* posses significant fungicidal and bactericidal activities (Maksimoviæ, 2005). The antimicrobial activity of various organic solvents of *C. album* was tested by Pandey and Gupta (2014) and suggested that methanolic extract of *C. album* showed maximum antibacterial activity as compared to other

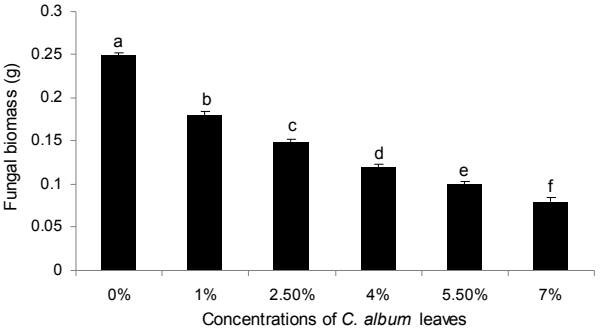

Figure 1 **-** Effect on *in vitro* growth of *A. rabiei by* methanolic leaf extracts of *C. album*. Vertical bars show standard error of means and different letters show significant differences as calculated by the DMR test.

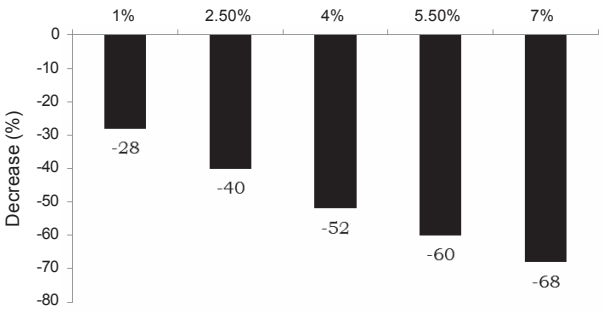

Figure 2 **-** Decrease (%) in dry biomass of *A. rabiei* as a result of various concentrations of applied extract of *C. album*.

extracts. Methanol and *n*-hexane extracts of stem, leaf, root and inflorescence of *C. album*, *C. murale* and *C. ambrosioides* were assessed against *Macrophomina phaseolina* by (Javaid and Amin, 2009). Their findings indicated that all the tested *Chenopodium* species effectively suppressed the growth of the test fungus.

The methanolic leaf extract of *C. album* was further partitioned in *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions. The antifungal activity of each fraction was observed and the best results were seen in the *n*-hexane fraction, as its two concentrations (0.15% and 0.25%) inhibited fungal growth up to 49% and 55%, respectively, as compared to the control treatment (Figures 3 and 4). Previously, Shah (2014) isolated various organic fractions from the stem of *C. ambrosioides* and found that all isolated fractions expressed antifungal and antibacterial potential against various fungal and bacterial strains. Rauf and Javaid (2013) reported that *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions isolated from methanolic extract of inflorescence of *C. album* have antifungal properties against *Fusarium oxysporum*.

The *n*-hexane fraction which was found most effective in previous bioassay was subjected for GC-MS analysis (Table 1), and thirteen compounds were identified. Chlorothanolil a commercial fungicide which used widely to control various blight diseases and a constituent of this commercial fungicide 1,3-dichloro-2-fluorobenzene is identified in the GC-MS analysis of *n*-hexane fraction in present study. An aromatic carboxylic acid benzoic acid, 3-methyl-2-trimethylsilyloxy-trimethysilyl ester identified in present study has antifungal potential against many fungal strains (Amborabe et al., 2002). Dicyclohexyl-ethylphosphonate was also found in the GC-MS analysis of the *C. album n*-hexane fraction. Abdul Majeed et al. (2010) synthesized a series of this compound and reported that these compounds have remarkable antimicrobial properties. Guest and Grant (1991) also reported the antifungal activity of its related compounds. The derivative of the common drug benzodiazepin was detected (2H-1,4-benzodiazepin-2-one,7chloro-1,3-dihydro-5-phenyl-1-(trimethylsilyl)-3-[(trimethylsilyl)oxy]) in the present study. This drug has significant antifungal and antimicrobial potential (Kumar and Joshi, 2008). The toxic compound [1,4] dioxino [2,3-b]-1,4-dioxin,hexahydro (a cardiac glycoside) was also found in the current study. The results of the present study showed that *C. album* leaves exhibited significant antifungal potential against chickpea blight. Any of these identified compounds alone or in combination might be responsible for the fungicidal properties of *C. album* which should be explored futher for making natural fungicide against chickpea blight.

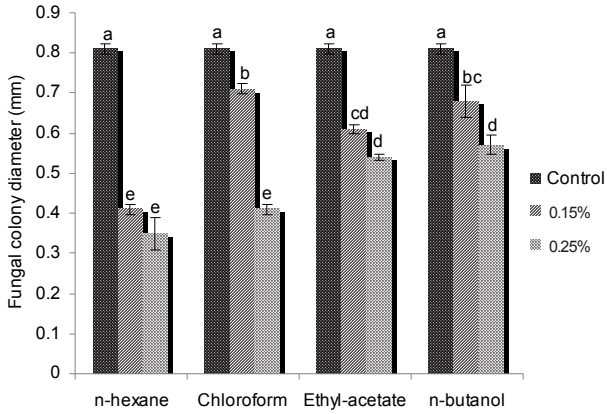

Figure 3 **-** Effect on *in vitro* growth of *A. rabiei* by different fractions of *C. album* leaf extract. Vertical bars show standard error of means and different letters show differences as calculated by the DMR Test.

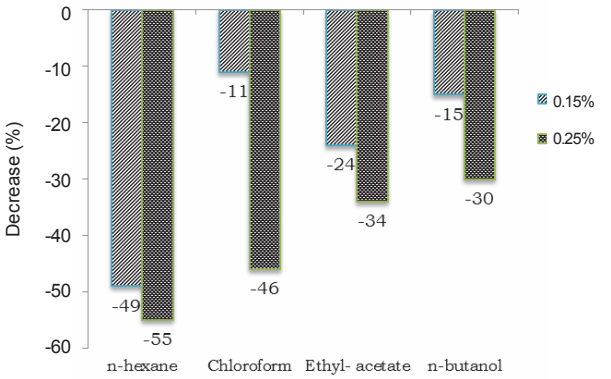

Figure 4 **-** Decrease (%) in dry biomass of *A. rabiei* as a result of different applied fractions of *C. album*.

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

R. time	Compound name	Mol. wt
	Aromatic hydrocarbon	
3.3	1,3-Dichloro-2-fluorobenzene	164
4.5	p-Xylene	106
	Hydrocarbon	
13.5	Hexane, 2,2,5-trimethyl	128
17.7	Pentane,2,2-dimethyl	100
23.8	Cyclopentane, 1,1'-(1,4-butandiyl)bis	194
	Saturated fatty acid	
22.2	Decanoic acid, 2-methyl	186
24.8	9,12,15-Octadecatrienoic acid, methyl ester	292
25.4	Hexadecenoic acid, methyl ester	270
	Aromatic carboxylic acid	
18.6	Benzoic acid, 3-methyl-2-trimethylsilyloxy-,trimethysilyl ester	296
	Phosphonates	
19.8	Dicyclohexyl-, ethylphosphonate	274
	Siloxane	
20.8	Cyclooctasiloxane, hexadecamethyl	592
	Cardiac glycoside	
25.1	[1,4]Dioxino[2,3-b]-1,4-dioxin,hexahydro	146
	Drug derivative	
22.7	2H-1,4-Benzodiazepin-2-one,7chloro-1,3-dihydro-5-phenyl-1-(trimethylsilyl)-3-[(trimethylsilyl)oxy]	430

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