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Full Length Article

Bioassay Guided Fractionation of *Withania somnifera* for the Management of *Ascochyta rabiei*

ARSHAD JAVAID[1] AND RAKHSHANDA MUNIR
Institute of Agricultural Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan
[1]Corresponding author's e-mail: arshadjpk@yahoo.com

ABSTRACT

Withania somnifera (L.) Dunal., a medicinal plant of family Solanaceae, was used to examine its antifungal activity against *Ascochyta rabiei*, the causal organism of highly damaging blight disease of chickpea (*Cicer ariatinum* L.). In laboratory screening bioassays, different concentrations (0.2, 0.4, 0.6, 0.8, 1.0 g 100 mL^{-1}) of methanolic extracts of stem, root, leaf and fruit of *W. somnifera* were evaluated for their antifungal activity against the targeted fungal pathogen. The methanolic extracts of fruit and leaf were found highly effective against *A. rabiei* causing up to 41% and 43% suppression in fungal biomass, respectively. The methanolic extract of fruit was further subjected to fractionation by using *n*-hexane, chloroform, ethyl acetate and *n*-butanol in order of increasing polarity. Different concentrations (3.125, 6.25, …, 200 mg mL^{-1}) of these fractions were used against the test pathogen. Ethyl acetate fraction was found highly effective against *A. rabiei* exhibiting complete inhibition of fungal growth. Two compounds namely A and B were isolated from this fraction through thin layer chromatography (TLC). These compounds were then appraised to find minimum inhibitory concentration (MIC) against the test fungal pathogen. Compound B was found more effective than A. The MIC values of compounds B and A were 62.5 µg mL^{-1} and 1000 µg mL^{-1}, respectively. It was concluded that antifungal compounds B could be used for the management of *A. rabiei*. © 2012 Friends Science Publishers

Key Words: *Ascochyta rabiei*; Blight; Chickpea blight; Natural fungicides; *Withania somnifera*

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important leguminous grain crop in Pakistan. The Thal desert that cannot sustain major cash crops due to low fertility and lack of artificial irrigation is well known as home to chickpea (Bibi *et al.,* 2009). In Pakistan, chickpea is cultivated over an area of 1053.8 thousand hectares giving 496.0 thousand tones of production with an average yield of 794 kg ha^{-1} (Statistics Bureau of Pakistan, 2011). It is an important source of human food and animal feed, and being a leguminous crop also helps in the increase of soil fertility due to activity of nitrogen fixing bacteria in its root nodules (Rai *et al.,* 2012). Blight is a destructive disease of chickpea plants and is caused by the fungus *Ascochyta rabiei* (Bayraktar *et al.,* 2007; Delgado *et al.,* 2012). This disease has been reported in more than 40 countries and has become one of the major limitations in chickpea cultivation (Bhardwaj *et al.,* 2010). The disease destroys chickpea crop and causes significant yield losses even up to 100% in the presence of susceptible host varieties and favorable environmental conditions (Bayraktar *et al.,* 2007).

Although some chickpea cultivars are genetically resistant against blight disease, however, the resistance is only partial (Chongo & Gossen, 2001; Iqbal *et al.,* 2005). Consequently, synthetic fungicide application approaches are required to save chickpea crops from the disease. Earlier studies showed that timely applications of effective fungicides minimized the harm caused by the disease and consequently increased the yield of chickpea (Chongo *et al.,* 2003). However, frequent applications of fungicide are not economical in many of the world's chickpea-growing regions (Tivoli & Banniza, 2007). In addition, in recent years their use is becoming more limiting due to environmental pollution, residual toxicity problems, carcinogenic effects and occurrence of microbial resistance (Rial-Otero *et al.,* 2005). Natural compounds of medicinal plants namely *Syzygium cumini* (L.) Skeels and *Melia azedarach* are very effective in the management of *A. rebiei* (Jabeen & Javaid, 2010; Jabeen *et al.,* 2011). The present study was, therefore, conducted to explore the antifungal potential of methanolic extracts of different parts of *Withania somnifera* and its fractions against chickpea blight pathogen *A. rebiei.*

MATERIALS AND METHODS

Screening bioassays: Fifty grams of dried and powdered material of each of the four different parts of *W. sominifera* viz. leaf, stem, fruit and root were soaked in 600 mL

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methanol for one week. After 7 days, materials were filtered through muslin cloth followed by filtration through filter paper. The filtrates were evaporated under vacuum in a rotary evaporator at 45°C to yield 7.55, 5.01, 2.51 and 2.53 g of crude methanolic extracts of leaf, stem, fruit and root, respectively.

Methanolic extracts (2.4 g) of *W. somnifera* were dissolved in sterilized water to prepare 3 mL of stock solutions separately. In 250 mL flasks, 79 mL malt extract broth was sterilized by autoclaving at 121ºC and cooled at room temperature. Five concentrations viz. 1.0, 0.8, 0.6, 0.4 and 0.2 g 100 mL^{-1} were prepared by adding 1, 0.8, 0.6, 0.4 and 0.2 mL stock solutions, and 0, 0.2, 0.4, 0.6 and 0.8 mL sterilized distilled water, respectively to each flask to make the total volume up to 80 mL, while control received 1 mL of distilled water. The 80 mL medium of each treatment was divided into four equal potions in 250 mL conical flasks to serve as replicates. With the help of a cork borer, 5 mm diameter mycelial discs were prepared from the edge of culture of a virulent strain of *A. rabiei* and inoculated in the flasks. There were four replicates of each treatment. Flasks were incubated at 18±2ºC for 10 days. Thereafter, fungal biomass in each flask was filtered, dried at 60°C and weighed.

Bioassay with different fractions of methanolic fruit extract: Two kilograms of dried and crushed fruit of *W. somnifera* were soaked in 7 L methanol for one week. After one week, the methanolic extract was filtered through a muslin cloth and residues were re-extracted with 5 L methanol. The two extracts were combined and concentrated on a rotary vacuum evaporator to obtain solvent free residue. The crude methanolic fruit extract was mixed with 300 mL distilled water and the mixture was extracted with *n*-hexane (2 × 500 mL) in a separating funnel. The *n*-hexane phase was collected and evaporated under vacuum and obtained 6.3 g *n*-hexane fraction. The remaining extract was successively fractionated with 500 mL of each of chloroform, ethyl acetate and *n*-butanol, to yield chloroform ($CHCl_3$) fraction (4.5 g), ethyl acetate (EtOAc) fraction (4.8 g), and *n*-butanol (*n*-BuOH) fraction (5.1 g). The remaining aqueous extract was evaporated and 11 g aqueous fraction was obtained.

The various fractions obtained were tested *in vitro* against the *A. rabiei.* Weighed amount of 1.2 g of each of the five fractions of methanolic extract (*n*-hexane, chloroform, ethyl acetate, *n*-butanol & aqueous) of fruit of *W. somnifera* was dissolved in 1 mL of dimethyal sulphoxide (DMSO) and added to 5 mL of malt extract broth. This stock solution (200 mg mL^{-1}) was serially double diluted by adding malt extract broth to prepare lower concentrations of 100, 50, …, 3.125 mg mL-1. In case of control, 1 mL of DMSO was mixed in 5 mL malt extract broth and serially double diluted. In 10-mL volume test tubes, 1 mL of malt extract medium was poured. Test tubes were inoculated with one drop of spores suspension of *A. rabiei* aseptically. Each treatment had three replicates. Test tubes were incubated at 18±2ºC for 10 days in an incubator. After that, fungal biomass in each test tube was filtered, dried at 60ºC and weighed.

Table I: Effect of different concentrations of *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous fraction of methanolic fruit extract of *Withania somnifera*

Methanolic fraction	DMSO (mL mL^{-1})	Extract conc. (mg mL^{-1})	Fungal biomass (mg)
Control	0.1666	0	0.1 op
	0.0833	0	0.6 kl
	0.0416	0	1.66 h
	0.0208	0	2.26 ef
	0.0104	0	3.76 c
	0.0052	0	4.26 b
	0.0025	0	5.4 a
n-hexane	0.1666	200	0.0 p
	0.0833	100	0.0 p
	0.0416	50	0.13 op
	0.0208	25	0.2 n-p
	0.0104	12.5	0.4 l-n
	0.0052	6.25	0.6 kl
	0.0025	3.125	1.1 i
Chloroform	0.1666	200	0 p
	0.0833	100	0 p
	0.0416	50	0.23 n-p
	0.0208	25	0.4 l-n
	0.0104	12.5	0.53 k-m
	0.0052	6.25	0.66 jk
	0.0025	3.125	1 i
Ethyl acetate	0.1666	200	0 p
	0.0833	100	0 p
	0.0416	50	0 p
	0.0208	25	0 p
	0.0104	12.5	0 p
	0.0052	6.25	0 p
	0.0025	3.125	0 p
n-butanol	0.1666	200	0 p
	0.0833	100	0.1 p
	0.0416	50	0.3 m-o
	0.0208	25	0.86 ij
	0.0104	12.5	1.1 i
	0.0052	6.25	1.56 h
	0.0025	3.125	2.06 fg
Aqueous	0.1666	200	0 p
	0.0833	100	0 p
	0.0416	50	0.73 jk
	0.0208	25	1.1 i
	0.0104	12.5	1.9 g
	0.0052	6.25	2.5 e
	0.0025	3.125	3.4 d

In vertical column, values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Duncan's Multiple Range Test

Thin layer chromatography: Ethyl acetate fraction was found highly effective against *A. rabiei.* This fraction was subjected to thin layer chromatography (TLC) followed by Preparative Thin Layer Chromatography (PTLC). Solvent system of chloroform and methanol in 9:1 ratio was used to separate the compounds. Two compounds namely A and B were isolated from EtOAc fraction with R_f values of 0.232 and 0.581, respectively.

Evaluation of minimum inhibitory concentration (MIC): The MIC values of the purified compounds and a reference

Table II: Antifungal activity of isolated compounds against *A. rabiei*

Treatments	Concentrations µg mL^{-1}									
	4000	2000	1000	500	250	125	62.5	31.25	15.62	7.81
Control	+	+	+	+	+	+	+	+	+	+
Mencozeb	-	-	-	-	-	-	-	-	-	-
Compound A	-	-	-	+	+	+	+	+	+	+
Compound B	-	-	-	-	-	-	-	+	+	+

+ Fungal growth appears
– No fungal growth

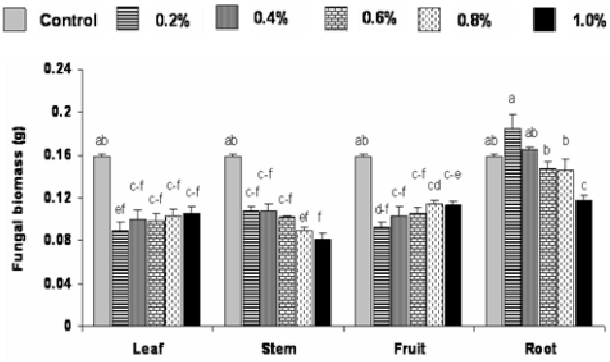
synthetic fungicide mancozeb (80% WP, KSS) were tested in culture tubes by serial dilution assay. The two isolated compounds from ethyl acetate fraction were used for MIC assays. Eight milligrams of each of the two isolated compounds and mancozeb were dissolved in 0.25 mL DMSO and 1.75 mL malt extract was added to prepare 4000 µg mL^{-1} concentration. Medium was serially double diluted with malt extract broth in culture tubes to prepare lower concentrations of 2000, 1000, …, 7.81 µg mL-1. Seven days old fungal culture was added to distilled water and filtered through muslin cloth to prepare conidial and mycelial suspension. One drop of the spore suspension was added to different concentrations of the growth medium in 5 mL volume glass tubes containing 1 mL of medium. Control treatments were prepared by adding 0.25 mL DMSO in 1.75 mL malt extract broth and serially double diluted to prepare corresponding controls for each dilution. The culture tubes were incubated at 18±2ºC. The MIC was recorded as the lowest concentration of the extract that completely inhibited fungal growth after 72 h.

Statistical analysis: All the data were analyzed statistically by applying analysis of variance. Treatment means were separated by Duncan's Multiple Range Test at $P \leq 0.05$ (Steel & Torrie, 1980).

RESULTS AND DISCUSSION

Screening bioassays with methanolic extracts: Data concerning the effect of methanolic extracts of different parts of *W. somnifera* on biomass of *A. rabiei* is presented in Fig. 1. All the concentrations of methanolic extracts significantly reduced the biomass of target pathogen. However, variability in antifungal activity among the plant parts and extracts concentrations of the test plant species was evident. There was 33-43% reduction in fungal biomass due to different concentrations of the leaf extract. Similarly, different concentrations of stem and fruit extracts significantly reduced the fungal biomass by 31-45% and 28-41% over control, respectively Fig. 1. These results supported the findings of earlier workers who reported that methanolic extracts of solonaceous medicinal plants exhibit antifungal activity (Iranbakhsh *et al.,* 2010). Root extract of *W. somnifera* exhibited the lowest antifungal activity against *A. rebiei.* Only the highest concentration of the extract significantly declined the biomass of fungal pathogen by 25%. The effect of lower concentrations of 0.2-0.8% was insignificant as compared to control (Fig. 1).

Fig. 1: Effect of different concentrations of methanol extract of leaf, stem, fruit and root on biomass of *Ascochyta rabiei*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test



Antifungal activity of different fractions of methanolic fruit extract of *W. somnifera*: Data about the effect of various fractions of methanolic fruit extract on biomass of *A. rebiei* is summarized in Table 1. Ethyl acetate fraction of methanolic fruit extract was found to be the most effective, where all the concentrations completely inhibited the growth of *A. rabiei*. Similarly, the highest concentrations of 200 and 100 mg mL^{-1} of the other four fractions of methanolic fruit extract also completely controlled the target fungal biomass. In general, *n*-hexane and chloroform fractions exhibited higher antifungal activity than the *n*-butanol and aqueous fractions. There was 79–92%, 81–86%, 62–82% and 37–56% reduction in fungal biomass due to 3.12–50 mg mL^{-1} concentrations of *n*-hexane, chloroform, *n*-butanol and aqueous fractions of methanolic fruit extract of *W. somnifera*, respectively.

Antifungal activity of isolated compounds against *A. rabiei*: All the concentrations of mancozeb completely inhibited the fungal growth. The two isolated compounds showed variable antifungal activity. Compound B showed the best antifungal activity with minimum inhibitory concentration of 62.50 µg mL^{-1}. Compound A was comparatively inhibitory against the fungal growth with MIC value of 1000 µg mL^{-1} (Table II). The isolated compounds may be with an olides as these are the constituents reported from *W. somnifera* (Jamal *et al.,* 1995). The most important with an olide is with a ferin A that is a highly oxygenated

steroidal lactone. It has cytotoxic and inhibitory action on synthesis of protein (Kalthur *et al.,* 2009).

The present study concludes that the natural compounds present in ethyl acetate fraction of methanolic fruit extract are highly potent against *A. rebiei.* The compounds A and B need to be chemically characterized.

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