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Optimization of the Antifungal Activity of Essential Oil Isolated from Aerial Parts of *Thymus kotschyanus* Boiss & Hohen (Lamiaceae)

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ABSTRACT: Although utilization of synthetic chemicals is inevitable for management of economically detrimental agents, numerous side-effects such as environmental contaminations and effects of non-target organisms associated with them. Plant essential oils with low/without toxicity on mammals and as bio-degradable natural materials have been considered for different pests and fungi management in the recent years. In the present study, the essential oil of *Thymus kotschyanus* isolated by a Clevenger apparatus and its mycelial growth inhibition was measured against two phytopathogenic fungi *Botrytis cinerea* and *Fusarium graminearum*. The best models for predicting of antifungal effects were quadratic models. The essential oil showed a prospective mycelial growth inhibition against both phytopathogenic fungi. Optimization of the antifungal effects indicated that 206.207 ppm of the essential oil caused 50% mycelial growth inhibition of *B. cinerea* after 89.651 h. This value was 85.600 ppm for *F. graminearum* within 117.194 h. Results of the present study designated a great potential of *T. kotschyanus* essential oil for management of pathogenic fungi *B. cinerea* and *F. graminearum*.

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Fusarium graminearum is a cause of *Fusarium* head blight (FHB) of wheat and other cereals throughout the world (Suga *et al*., 2008). Due to this fungus activity, a poor quality product with wrinkled grains is produced and the weight of the seeds is reduced. Along with the reduction of product quality, it also creates some mycotoxins including Nivalenol, Deoxynivalenol, and Zearalenone which pose serious health risks for humans and animals (Windels, 2000). *Botrytis cinerea* with a very wide range of hosts is one of the other dangerous fungi that infect many plants before and after harvest. In terms of scientific and economic importance, this fungus has the second place among the 10 important fungi (Dean *et al*., 2012). *B. cinerea* causes gray mold disease in many fruits, vegetables and ornamental plants (Williamson *et al*., 2007; Romanazzi, 2013).

The use of synthetic chemicals is a typical method for plant protection against fungal infections. However, the overuse of such compounds has led to several negative effects, such as adverse effects on the environment, human health threats, and the emergence of resistant populations (Pimentel *et al*., 2008; Damalas and Eleftherohorinos, 2011; Cavalcanti *et al*., 2010). Therefore, the search and introduction of natural and bio-degradable agents are critical.

Plant essential oils in the form of secondary metabolites play an important role in plant protection against fungi and phytophagous insects. These bio-agents act as signaling molecules and show an evolutionary relationship with their functional role in the plants (Tholl, 2006). In recent years, several investigations have been accomplished on the use of essential oils in managing pathogenic fungi (Bakkali *et al*., 2008; Pinto *et al*., 2013). *Thymus kotschyanus* Boiss and Hohen is one of the popular medicinal plants, which is used in traditional medicine and herbal tea. Further, some biological effects including antibacterial, antifungal, and antioxidant activities of *T. kotschyanus* essential oil were recognized recently (Afshari *et al*., 2016; Sevindik *et al*., 2016).

The main goals of the present study were the evaluation of the antifungal effect of *T. kotschyanus* essential oil against two phytopathogenic fungi *Botrytis cinerea* and *Fusarium graminearum*. Further, development of mathematical models to find the optimized conditions for these bio-effects was the other objective.

MATERIALS AND METHODS

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Extraction of essential oil: Aerial parts of and *Thymus kotschyanus* were collected, respectfully, from Hassan Baroog and Sardabeh regions (Ardabil province, Iran). After drying at room temperature, the specimens were chopped with an electric grinder. Extraction of essential oil was done using a Clevenger apparatus with 100 g of each plant sample and 1500 ml distilled water within 3 hours. Obtained essential oil was dewatered using anhydrous sulfate sodium and kept in a refrigerator at 4 ° C until use.

Antifungal activity of the essential oil: Phytopathogenic fungi *Fusarium graminearum* and *Botrytis cinerea*, which were isolated respectfully from infected wheat and strawberry fruits, were considered for mycelial growth inhibition activity of *T. kotschyanus* essential oil. The fungal isolates were identified and stored in Mycology Laboratory of the Department of Plant Protection, University of Mohaghegh Ardabili, Iran. Both isolated were stored on PDA medium (Potato Dextrose Agar) at 25 °C.

Mycelial growth inhibitory of *T. kotschyanus* essential oil was investigated by mixing of the essential oil concentration with culture medium. Concentrations of 75, 150, 300, 600 and 1000 ppm were prepared in 0.05% aqueous Tween-80. Flasks (250 ml) containing a PDA medium were placed at room temperature to reduce the temperature to 45-42 °C after autoclaving and the concentrations were added to them. The flasks stirred to form a uniform emulsion and the resulting media was immediately poured into 9 cm Petri dishes and allowed to become solid. The fungal discs were then prepared from the young cultures of the pathogenic fungi and positioned in the middle of the Petri dishes. Mycelial growth of each fungus was measured daily until the surfaces of the culture media in Petri dishes were fully absorbed. Aqueous Tween-80 without any essential oil concentration was used for control groups and three replicates were considered for each treatment. Inhibition Percentage (IP) of different concentrations of essential oil was determined using the following formula:

$$\mathrm{IP} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} \times 100$$

Where C is the mean diameter of the mycelial disc in the control group and T is the mean diameter of the mycelial disc in the treatments.

Modeling of insecticidal and antifungal effects of the essential oil and statistical analysis: The data were analyzed by Design Expert versions 7.0.0 (2007, Stat-Ease company, USA). The coded independent variables are the essential oil concentrations (X_1) and time (X_2) in 5 and 3 levels, respectively, and 3 replications. Mathematical models between the independent variables [Concentration (ppm) and time (h)] and dependent variables [Inhibition Percentage (%)] evaluated by means of multiple linear regression analysis in the following form:

$$\mathrm{Y} = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} {X_i}^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n-1} \beta_{ij} X_i X_j + \mathrm{e}$$

where β0, βi, βii, βij are constant coefficients of regression, Xi and Xj are the independent variables, Y is the dependent variable, n is a number of independent variables and e is the random error term. The relationships between the responses were checked by correlation coefficients of determination (R^2), adjusted R^2, and predicted R^2. A good model will have a large predicted R^2 and a low PRESS. The significance was analyzed with a confidence level of 95% ($P < 0.05$).

RESULTS AND DISCUSSION

The results showed that the studied concentrations of *T. kotschyanus* essential oil had pleasing antifungal activity on *F. graminearum* and *B. cinerea* fungi and the mycelial growth of pathogenic fungi has decreased with increasing time (Figure 1). The results of analysis of variance for mycelial growth inhibitory of *T. kotschyanus* essential oil shows that the effect of exposure time (A) and essential oil concentration (B) along with AB, A^2 and B^2 were significant *F. graminearum* and *B. cinerea* fungi. The greatest inhibition percentages on the mycelial growth of fungi were obtained by essential oil concentration factor (59% and 85%, respectively), which indicate that this factor is very important as compared to other factors (Table 1).

According to the models presented in Table 2 and the coefficient of variations, it can be concluded that these are the best models for estimating of the antifungal effects of *T. kotschyanus* essential oil. The coefficients of the independent variables (concentration and time) are positive: an increase in each of the variables is an incremental effect on the response variable. The negative sign of the variables in each model indicates the decreasing effect of the variable on the amount of the mycelial growth inhibition percentage of fungi.

Time and concentration of *T. kotschyanus* essential oil for 88.31% of mycelium growth inhibition in *F. graminearum* fungus were 96 hours and 600 ppm, respectively. Also, 52.92% growth inhibitory in *B. cinerea* fungus requires 74.98 hours and a concentration of 7446.20 ppm of the essential oil. To inhibit 50% mycelial growth in the *F. graminearum* fungus, the concentration of 85.66 ppm and the time of 19.117 hours would be sufficient. These values for *B. cinerea* were 216.2 ppm and 89.65 hours. So, *F. graminearum* was more susceptible than *B. cinerea* to the essential oil of *T. kotschyanus* (Table 3).

Antifungal effects of the essential oils of *Thymus kotschyanus*, *Ocimum basilicum* L. and *Rosmarinus officinalis* L. were ascertained on two major phytopathogenic fungi *Penicillium expansum* and *B. cinerea* (Jalili-Marandi *et al*., 2011). In the other study, antifungal effects of the essential oils isolated from some medicinal plants were investigated against phytopathogenic fungi *Fusarium oxysporum*, *Aspergillus flavus* and *Alternaria alternate* (Mohammadi *et al*., 2014). It was found that the essential oils of *T. kotschyanus*, *Stachys pubescens* Ten. and *Bupleurum falcatum* L. exhibited an appropriate antifungal activity against these fungi. According to the susceptibility of *B. cinerea* and *Fusarium oxysporum* fungi to the essential oil of *T. kotschyanus*, results of mentioned works are in parallel with results of the present study. However, the optimization and modeling of these antifungal effects were measured in our work for the first time.

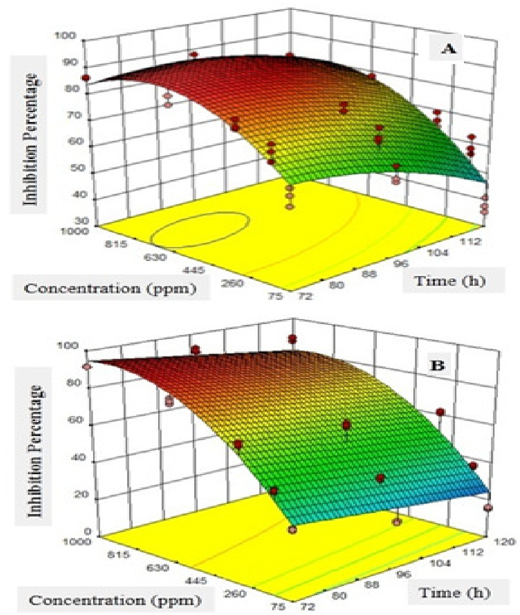

Fig 1: Three-dimensional diagrams of the growth inhibitory percentage on *Fusarium graminearum* (A) and *Botrytis cinerea* (B) by *T. kotschyanus* essential oil

Table 1: Results of the analysis of variance of data on the mycelial growth inhibitory of *T. kotschyanus* essential oil against *F. graminearum* and *B. cinerea* fungi

Pathogenic fungus	Source	Sum of Squares	df	Mean Square	F value	p-value
Fusarium graminearum	Model	6889.54	5	1377.91	61.81	< 0.0001
	A	1009.54	1	1009.54	45.29	< 0.0001
	B	3760.55	1	3760.55	168.69	< 0.0001
	AB	253.10	1	253.10	11.35	0.0017
	A^2	191.45	1	191.45	8.59	0.0056
	B^2	1196.39	1	1196.39	53.67	< 0.0001
	Residual	869.40	39	22.29		
	Pure Error	170.35	30	5.68		
	Cor Total	7758.95	44			
Botrytis cinerea	Model	22846.96	3	7615.65	159.25	< 0.0001
	A	1274.66	1	1274.66	26.65	< 0.0001
	B	19452.06	1	19452.06	406.75	< 0.0001
	B^2	2162.41	1	2162.41	45.22	< 0.0001
	Residual	1960.76	41	47.82		
	Pure Error	28.17	30	0.94		
	Cor Total	24807.73	44			

A and B, respectively, are the exposure time and essential oil concentrations.

Table 2: Estimated regression models of the mycelial growth inhibitory effect of *T. kotschyanus* essential oil on *F. graminearum* and *B. cinerea*

Pathogenic Fungus	Equation	R^2 value	Adj R^2	Pred R^2	C.V. (%)
F. graminearum	+86.860 - 6.110 A + 12.470 B + 3.960 AB - 4.380 A2 - 13.040 B2	0.8879	0.8736	0.8531	6.46
B. cinerea	+ 47.025 - 0.270 A + 0.149 B - 8.195E - 5 B^2	0.9210	0.9152	0.9057	11.48

Response variable is inhibition percentage.

Table 3: Optimization of the mycelial growth inhibitory effect of *T. kotschyanus* essential oil on *F. graminearum* and *B. cinerea*.

Pathogenic fungus	Response variable	Time (h)	Concentration (ppm)	Desirability
F. graminearum	IP = 50	117.19	85.60	100
	IP = 88.31	96.00	600.00	100
B. cinerea	IP = 50	89.65	206.21	100
	IP = 92.52	74.98	746.20	100

Conclusion: F. graminearum and *B. cinerea* are among the most damaging agents in the many countries, which have economic losses on many agricultural products. Management of such destructive agents is principally carried out by synthetic agro-chemicals but the application of these chemicals prompted several environmentally adverse effects. Therefore, the use of eco-friendly natural compounds in the management of harmful agents is required. In the present study, the mycelial growth inhibitory effect of *T. kotschyanus* essential oil was confirmed on *F. graminearum* and *B. cinerea*. Furthermore, results of the present study were announced the response surface methodology (RSM) was efficaciously applicable to the optimization of *T. kotschyanus* essential oil bio-effects.

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