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Antifungal Activities of *Ailanthus altissima* Swingle and *Juglans regia* L. Leaves Against Some Cereal Fungi

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Abstract

In livestock farming, crop growing and industry, the toxic microfungi found in cereals is a very grave problem for centuries. The fungi produce toxins that cause chronic and acute toxications when their productive value of grain is decreased. Because of their susceptible nature to microbial contamination, cereals can be contaminated easily by filamentous fungi. In literatures; some *Fusarium, Gibberella* and *Cladosporium* species were given as field fungi and; some *Aspergillus, Penicillium* and *Trichothecium* species as storage fungi. The fungus species used in this study were *Aspergillus niger*, *Aspergillus parasiticus*, *Cladosporium cladosporioides*, *Fusarium oxysporum, Fusarium sporotrichioides, Gibberella fujikuroi*, *Penicillum brevicompactum, Penicillium griseofulvum* and *Trichothecium roseum.* This study was undertaken to investigate the antifungal activity of *Juglans regia* L. (walnut) and *Ailanthus altissima* Swingle (tree of heaven) leaves against these mycotoxin and spoilage producing fungus. The powder of the leaves from *J. regia* L. and *A. altissima* Swingle (10% g/mL) showed the maximum antifungal activity against *C. cladosporioides* (71±2.0% for *J. regia* L. and 51±1.52% for *A. altissima* Swingle). Ethanol and methanol extracts of *J. regia* L. completely inhibited the spore germination at 5.0 and 10 mg/mL, and ethanol extract of *A. altissima* Swingle at 10 mg/mL. The minimum inhibitory concentration value of *J. regia* L. methanolic extracts against *C. cladosporioides* (0.625 mg/mL) was less than the other tested extracts. This study demonstrates that *J. regia* L. extracts have a potential to control the fungal contamination caused by *C. cladosporioides.*

Key words: **Antifungal activity, plant extracts, *Cladosporium cladosporioides, Ailanthus altissima* Swingle, *Junglans regia* L.**

INTRODUCTION

The contamination of toxic microfungi in cereals, animal forage and food processing industry have been serious problem since centuries. By decreasing the productive value of grain for food, feed and seed, the toxins caused by fungi in the grain result both chronic and acute toxications an animals and people, and also allergic appearances. Therefore, great attention is being paid to their study and prevention in the whole world [1]. Cereals, due to their chemical, are particularly susceptible to microbial contamination, especially by filamentous fungi. These materials can be contaminated with fungi either during vegetation in the field or during storage, as well as during the processing [2]. In literatures; *Fusarium, Gibberella* and *Cladosporium* species were classified as field fungi and; *Aspergillus, Penicillium* and *Trichothecium* species as storage fungi [1] and [2].

The following fungi species were found dominate in cereal and cereal products; *Aspergillus niger* (soybean), *Aspergillus parasiticus* (soybean, maize), *Cladosporium cladosporioides* (soybean), *Fusarium oxysporum* (soybean, rice), *Fusarium sporotrichioides* (soybean), *Penicillium griseofulvum* (soybean), *Penicillum brevicompactum* (soybean) [2] *Gibberella fujikuroi* (wheat), *Trichothecium roseum* (wheat) [1]. Several of these fungal species not only bring about deterioration in the quality and quantity of agricultural produce in storage and transit but also they create health hazards in animals and human beings by producing toxic metabolites in the form of mycotoxins in the stored commodities [3].

For many years, a variety of different chemical and synthetic compounds have been used as antimicrobial agents to inhibit the plant pathogenic fungi. Antimicrobial chemicals such as benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors are often used for control of plant disease in agriculture. However, there is a series of problems against the effective use of these chemicals in areas where the fungi have developed resistance. In order to overcome this problem, higher concentrations of these chemicals were used, but this increases the risk of high-level toxic residues in the products [4].

Plant extracts are very attractive as alternative or complementary control means because of their antifungal activity, nonphytotoxicity, systemicity and biodegradability [5]. *Ailanthus altissima* Swingle commonly known as the "tree of heaven" is used in traditional medicine in many parts of Asia, including China, Japan and Korea to treat cold and gastric diseases and as an antitumor agent. Previous phytochemical studies have demonstrated the presence of quassinoids as well as indole and β-carbolin alkaloids in this plant. Extracts of the plant leaves have demonstrated antiproliferative, central nervous depressant, antifeedant and insecticide activities [6]. The leaves of *Junglans regia* L. (walnut) are widely used and known traditional medicine in Turkey [7].

This study was undertaken to investigate the antifungal activity of *J. regia* L. and *A. altissima* Swingle leaves against spoilage and mycotoxin producing some fungi.

MATERIAL AND METHODS

Fungal Cultures

Fungal species used in the study [*Aspergillus niger* Tiegh., *Aspergillus parasiticus* Speare., *Cladosporium cladosporioides* Penz., *Fusarium oxysporum* Speare., *Fusarium sporotrichioides* Sherb., *Gibberella fujikuroi* (Sawada) Wollenw., *Penicillium brevicompactum* Dierckx, *Penicillium griseofulvum* Dierckx and *Trichothecium roseum* (Pers.) Link] were provided by the fungi collection of Arda Vocational School, Trakya University, Edirne, Turkey. The fungal cultures were maintained at 4°C on potato dextrose agar (PDA) with periodic sub-culturing.

The pathogen inoculums were obtained from 7-day-old cultures incubated at 25 ^{o}C. The cultures were washed with 5 mL of sterile saline solution (0.85%) and suspensions were transferred to sterile tubes, where the heavy particles were allowed to settle for 5 min. The upper homogenous suspensions were transferred with a pipette Pasteur to a new sterile tubes and vortexed for few seconds. The spore concentrations (spores/mL) were determined with aid of a heamacytometer.

Preparation of Plant Extracts

Fresh leaves of *J. regia* L. and *A. altissima* Swingle were collected from Kavaklı Campus, Kırklareli University, Kırklareli, Turkey. Leaves were cleaned, dried in the shade, then grounded to a fine powder and stored in the dark at 4 ^{o}C until use.

The powdered plant materials were separately extracted thrice at room temperature with methanol and ethanol solvents (500 mL/100 g of plant material each run). The final methanol and ethanol extracts of each plant part were filtered using filter paper (Whatman) and were evaporated under vacuum at 40 °C using rotary vacuum evaporator. All extracts were stored in sterile glass bottles at room temperature.

***In vitro* Effect of Plant Powders on Mycelial Growth**

10 g of powders of each sample were added to 100 mL of melted PDA at 40 ^{o}C. The resulting suspensions were stirred for 10 min., autoclaved for 15 min. at 121 ^{o}C and subsequently filtered through four layers of sterile cheese cloth before being dispensed into 9-cm diameter Petri plates. Pathogen grown on PDA without plant powders was used as control [8]. 5 µL of the spor suspension (1×10^{5} spore/mL) were inoculated at the centre of each PDA plates [9]. The agar plates were then incubated at 25 ^{o}C for seven days. Radial growth was determined by measuring colony size along two perpendicular axes. The antifungal activity was expressed in terms of percentage of mycelial growth inhibition (MGI) and calculated according to the following formula;

$$\% \text{ MGI} = \frac{\text{Control diameter - Plant extract diameter}}{\text{Control diameter}} \times 100$$

Effect of Plant Extracts on Spore Germination

The spore germination of *C. cladosporioides* was determined in concentrations of 0.625, 1.25, 2.5, 5 and 10 mg/mL for the extracts. Aliquots (40 mL) of sterile Sabouraud Broth with different concentrations of filter sterilized plant extracts were aseptically transferred in triplicate to sterile depression slides containing 40 mL of spore suspension adjusted to $1x10^{5}$ spores/mL [10]. Inoculated slides were placed on moist filter paper in petri plates and thereafter incubated at 25 ^{o}C for 18 h. Each slide was then fixed with lactophenol cotton blue solution to stop further germination [11]. Spore germination was determined in three microscopic fields using 10 x 40 ocular micrometers. At least 100 conidia of each replicate were observed. A spore was germinated when the length of the germ tube was at least twice the diameter of the swollen conidia [12] and [13]. The percentage of spores germination (G) was determined by using the following formula: G (%) = (Gt/Gc) x 100, where Gt and Gc represent the mean number of germinated spores in treated and control slides, respectively. Each treatment included three replicates and the test was conducted twice.

Determination of Minimum Inhibitory Concentration (MIC)

For MICs determination methanolic and ethanolic extracts were used. MIC values of *J. regia* L. and *A. altissima* Swingle leaves were determined according to Trigui et al. [14] in sterile 96-well microplates with a final volume in each microplate well of 200 µL. A stock solution of the extract (50 mg/mL) was prepared in dimethylsulfoxide (DMSO). Thereafter, a twofold serial dilution of the extract was prepared in the microplate wells over the range 0.039–10 mg/mL. To each test well 10 µL of cell suspension was added to final inoculum concentration of $1x10^{5}$ spores/mL for fungus. Positive growth control wells consisted of fungi only in their adequate medium. DMSO was used as negative control. The plates were then covered with the sterile plate covers and incubated at 72 h for fungi at 25 ^{o}C. The MIC was defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth after incubation.

RESULTS AND DISCUSSION

The systematic screening of the interactions between microorganisms and plant products is one of the widely used techniques in determining the plants' biological active component [15]. The antifungal activity of crude extracts of *J. regia* L. and *A. altissima* Swingle leaves were evaluated *in vitro* against tested fungi species. The powder of the tested leaves inhibited the growth of some fungi colonies. However, there was difference in the inhibitor effect between the two leaf samples. The powder of *J. regia* L. and *A. altissima* Swingle leaves (10% g/mL) inhibited the mycelium growth of *C. cladosporioides, G. fujikuroi* and *F. sporotrichioides in vitro* screening 71, 51; 43, 5.6; 18, 7%, respectively. The powder of *J. regia* L. leaves inhibited mycelial growth of *T. roseum* and *P. griseofulvum* 20 and 10%, respectively. The mycelial growth of *A. niger, A. parasiticus, F. oxysporum, P. brevicompactum* were not inhibited by the powder of both leaves (Table 1). The powder of *J. regia* L. and *A. altissima* Swingle leaves (10% g/mL) may have potential antifungal activity and are potentially a natural source of antifungal compounds against *C. cladosporioides* mycelial growth. It is noted that by using 10% (g/mL) of different plant powders, the mycelial growth were inhibited by 36.58-100% [16] and 21.74-100% [5]. Previous studies indicate that the effect of plant extracts on fungal pathogen may be attributed to their content on secondary metabolites, which are alkaloids, phenolics, flavonoids and terpenoids compounds, with known antifungal activity [17].

Because the powders acquired from the plants reduced the mycelial growth of *C. cladosporioides* by more than 50%, the later tests were conducted with this fungus. *J.*

regia L. ethanol and methanol extracts both at 10 and 5 mg/mL level have high fungistatic effect (100% inhibition) against *C. cladosporioides* and *A. altissima* Swingle ethanol extract had the same effect when given at 10 mg/mL concentration (Table 2). *J. regia* L. extracts were found the most effective when the spore germination inhibition of the extracts was compared to the mycelial growth inhibition. The MIC value of *J. regia* L. ethanol extracts against *C. cladosporioides* (0,625 mg/mL) was less than other extracts that were tested (Table 2). The antifungal activity of *J. regia* L. and *A. altissima* Swingle were compared to the other findings. The MIC values of *J. regia* L. extract against some *Candida sp*., Gram positive and Gram negative bacteria was found to be between 0.312-2.5 mg/mL [7] and the MIC values for *A. altissima* Swingle against food borne and spoiling bacteria was in the range between 62.5-500 µg/mL [6]. The chemical composition of plant and herbal product can be changed by origin, species, growth and the variations during harvest and processing, so it is noted that the biological activities could be different [18].

CONCLUSION

New non-synthetic preservatives are needed to destroy the problems caused by the synthetic fungicides. Research into this area has recently gained a lot of significance. Because of the effective chemical materials and the secondary metabolism products, the green plants are thought as safe non-synthetic fungicides [19]. To conclude, we propose that the extracts of *J. regia* L, are economically suitable and probably harmless to humans and the environment and probably the prime candidate in controlling the fungal contamination caused by *C. cladosporioides*.

Table 1. *In vitro* effects of plant powders (10% g/mL) on mycelial growth of some cereal fungi.

Fungi	Mycelial growth inhibition (%) of *J. regia* L. leaves	Mycelial growth inhibition (%) of *A. altissima* Swingle leaves
A.niger	---------	---------
A.parasiticus	---------	---------
C. cladosporioides	71±2.0	51±1.52
F. oxysporum	---------	---------
F.sporotrichioides	18±0.15	7±0.1
G. fujikuroi	43±5.77	5.6±0.28
P. brevicompactum	---------	---------
P. griseofulvum	10±5.77	---------
T. roseum	20±1.0	---------

Values are represented as mean ±SD of triplicate experiments.

Table 2. In vitro effect on spore germination and MICs for the plant extracts against *C. cladosporioides.*

Plant extracts	Spore germination inhibition (%)[a]					MIC[a] (mg/mL)
	Concentration of extracts (mg/mL)					
	10	5.0	2.5	1.25	0.625	
J. regia L. ethanol extract	100	100	30	20	0	0.625
J. regia L. methanol extract	100	100	36.6	0	0	1.25
A. altissima Swingle ethanol extract	100	93.3	14.29	0	0	1.25
A. altissima Swingle methanol extract	96.6	22.45	3.06	0	0	2.5

[a] Each value represents the mean of three replicated.

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