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ANTIFUNGAL ACTIVITY OF *EVERNIA PRUNASTRI, PARMELIA SULCATA, PSEUDEVERNIA FURFURACEA* VAR. *FURFURACEA*

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Abstract

The aim of this study was to investigate the *In vitro* efficacy of 96% alcohol extracts of *Evernia prunastri* and *Pseudevernia furfuracea* var. *furfuracea* that were in foliose-fruticose form and *Parmelia sulcata* in foliose form against important plant pathogens. The growth of fungal colonies in Petri plates amended with lichen extracts at 25°C was measured a day before covering all surface of Petri plate in control treatment. Data were analysed according to statistic analysis test LSD at $p \leq 0.05$. The *in vitro* efficacy of extracts of *E. prunastri*, *P. sulcata* and *P. furfuracea* var. *furfuracea* showed a significant inhibition against mycelia and spor growth of *Aspergillus niger*, *Botrytis cinerea*, *Fusarium culmorum*, *F. solani*, *Macrophomina phaseolina*, *Penicillium expansum* and *Rhizoctonia solani*. The level of inhibition among extracts showed variation. It was concluded that secondary metabolites of lichens may be used as biological chemicals against some plant pathogens.

Key words: Antifungal, Lichen extracts, Plant pathogen.

Introduction

Plant pathogenic fungi cause enormous economic loss in agriculture and the food industry by destroying crops in the field and during storage (Goel *et al., 2011*). The soilborne fungal pathogens play a major role in the development of root-rot disease complexes. *Fusarium*, *Rhizoctonia*, *Macrophomina* and *Sclerotinia* are major pathogenic genra that cause rots and damping on roots and foots of plants (Arslan *et al.,* 2009). In addition, postharvest diseases are one of the major causes for the postharvest loss of horticultural fresh produce during the supply chain and marketing period. The main pathogens of harvested fruits and vegetables are *Alternaria alternata* (Fr.) Keissler, *Alternaria citri* Ell. & Pierce, *Aspergillus niger* v. Tieghem, *Botrytis cinerea* Pers. Ex Fr., *Colletotrichum* spp. *Monilinia fructicola* (Wint.) Honey, *Penicillium digitatum* Sacc., *P. expansum* (Link) Thom, *Rhizopus stolonifer* (Her. Ex Fr.) Lind, *Sclerotinia sclerotiorum* (Lib.) de Bary.(Barkai-Golan, 2001).

Incidence of postharvest diseases can affect the quality and restrict the shelf life of horticultural fresh produce (Sellamuthu *et al.*, 2013). Current control of these plant diseases is primarily dependent on continued application of synthetic fungicides (Lu *et al*., 2013). However, the development of fungicide resistance in pathogens and public concern over the potential impact of fungicides on human health and environment has created interest in alternative methods of disease control. Several promising biological approaches that include the use of either antagonistic microorganisms or compounds of natural origin have been proposed as potential alternatives to synthetic fungicides (Robiglio *et al*., 2011).

Lichens are a stable and self-supporting symbiosis between fungi (themycobionts) and photoautotrophic algal partners, namely green algae and/or cyanobacteria (the photobionts/cyanobionts) (Pavlovic *et al*., 2013). They are able to survive in some extreme environment conditions (Guvenc *et al*., 2012). The substances that were produced to survive in these conditions are also unique (Zambare & Christopher, 2012). Moreover, lichen substances exert a wide variety of biological actions including antibiotic, antimitotic, antiviral, anti-inflammatory, analgesic, antiproliferative and cytotoxic effects (Kosanić *et al*., 2013).

The present study was designed to investigate the *In vitro* efficacy of ethanol extracts of *Evernia prunastri* (L.). Ach.and *Pseudevernia furfuracea* (L.) Zopf var. *furfuracea* that were in fruticose form and *Parmelia sulcata* Taylor in foliose form against important plant pathogens.

Materials and Methods

Lichen material: *E. prunastri*, *P. sulcata* and *P. furfuracea var. furfuracea* were collected from Bursa: Uludag, June 2009. Voucher specimens were deposited in the Herbarium of the Faculty of Biology Uludag Univercity,Turkey. Relevent literature was used for identification of samples (Brodo *et al*., 2001; Smith *et al*., 2009).

The thallus of *E. prunastri* is foliose-fruticose, lobes rather soft strap-shaped, upper surface green-grey to pale green-yellow, lower surface white, round soralia frequent or rare along the margins and on the surface of branches. It grows on trees of all kinds, more rarely on rock alls, in shade or sun. (Brodo *et al*., 2001; Smith *et al*., 2009).

P. sulcata is foliose and characterized by squarrose rhizines and a grey pseudocyphellate. Thallus often forms complete rosettes or randomly intricates; upper surface grey-white to grey-green, sometimes partly white pruinose, lower surface black. It is mostly found on bark and in both shade and sun (Brodo *et al*., 2001; Smith *et al*., 2009).

The thallus of *P. furfuracea var. furfuracea* is foliose-fruticose, composed of few to numerous hanging strap-shaped lobes, upper surface grey-white, matt, often rough with isidia lower surface usually channeled, black or pinkish with in curved margins concolurous with upper thallus. The apotecia is infrequent. It is mostly found on well lit barked wood, on conifers and mainly acid barked decidous trees. (Smith *et al*., 2009).

Preparation of the lichen ethanol extracts: The secondary metabolites of lichens are poorly soluble in water and can usually be isolated by organic dilutions such as alcohol (Cabral, 2003; Manojlović *et al*., 2012). Therefore, 96% alcohol was used in this study. Finely pulverized dried lichen thalli were extracted in 25°C for eight hours using 96% ethyl alcohol in a soxhlet extractor. Then, the extracts contracted under reduced pressure in a rotary evaporator. The dry extracts were stored at 4°C until they were used in the tests.

***In vitro* Antifungal activity**

Microorganisms and media: The following soilborne phytopathogenic and postharvest fungi were used in this study: *Fusarium culmorum* (Wm. G. Sm.) Sacc. *Fusarium solani* (Mart.) Sacc., *Rhizoctonia solani* Kühn, *Macrophomina phaseolina* (Tassi) Goid., *Aspergillus niger*, *Botrytis cinerea*, *Penicillium expansum*. The fungi were obtained from the mycological collection of the Phytopathology Lab, Department of Plant Protection Faculty of Agriculture, University of Uludag. Cultures of each of the fungi were maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA) and were stored in PDA slants at 5°C for further use and subcultured every 15 days.

Assay for the antifungal effects of the lichen extracts: Stock solutions at a rate of 10% were prepared from extracts of three different lichen species. Stock solutions were diluted to 5%, 2, 5% and 1% concentration. After centrifuging these extracts at 3000 rpm for 15 minutes, they are filtered twice in cold sterilization (Sartorius 0.2 µm). The lichen extracts at an amount of 375 µl were added to each Petri dish. Equal ratio alcohol-water mixture (10%, 5%, 2, 5%, 1%) was added to control Petri dishes.

The amended Petri dishes were left to air dry in sterile cabinets. A mycelial disc (5 mm diameter) taken from 7-day-old culture of the respective fungi was placed to the center of air dried Petri dishes including PDA and lichen extract. The plates were then sealed with parafilm and incubated at 25ºC in the dark for 2-4 days. Colony diameters were measured at two perpendicular points for 4 days incubation of *F. culmorum*, *F. solani* and 2 days incubation of *M. phaseolina*, *R. solani*. The diameters were measured in a growth stage when fungi mostly filled the Petri dish. In the evaluation of *A. niger* and *P. expansum*, a scale was used based on the number of the randomly developed colonies' formed by multiple spores in Petri dish. In the use of scale, all Petri dishes were numbered from 1 to 5 by taking into account the least and the most spore production (1: 0-20% spore production in Petri dish, 2:21-40% spore production in Petri dish 3:41-60% spore production in Petri dish 4:61-80% in Petri dish 5: 81-100% spore production in Petri dish). In the number first scale, fungal colonies cover the smallest area in Petri dish while that of fifth scale cover the largest area. The inhibition rate of mycelia growth and sporulation was calculated by comparing the growth of fungi at various lichen extracts concentrations compared with that of control.

Statistical analyses: Six replicate determinations were made for each concentration of lichen extracts and each replicate comprised six control Petri dishes. Experiments were conducted in duplicate. An ANOVA was applied to incidence data. Mean values were separated by using LSD test ($p \leq 0.05$).

Results and Discussion

Antimicrobial activity: Extracts of some lichens obtained by using various solvents were reported to be effective to several fungi. *Jeon et al*. (2009), indicated that lichen forming fungi might produce and excrete the enzymes responsible for cell wall degradation of the pathogenic fungi. Lichens contain a rich diversity of cell wall enzymes, including phospahates, cellulases, ureases, and also several redoz enzymes such as laccases, tryosinases, and peroxidases (Laufer *et al*., 2006; Beckett & Minibayave, 2007). The data of the present study confirmed the results of previous reports (Turk *et al*., 2006; Jeon *et al*., 2009; Kahriman *et al*., 2011; Mitrović *et al*., 2011; Guvenc *et al*., 2012; Kosanić *et al*., 2012; Zambare & Christopher, 2012). However, this is the first study reporting *in vitro* antifungal activities of ethanol extracts of the lichens *E. prunastri. P. furfuracea* var. *furfuracea* and *P. sulcata*. Antifungal efficacy of various lichen extracts and their concentrations was presented in Table 1.

The results showed that three different types of lichen extract had antifungal affect against the all tested fungal species. In contrast, the level of efficacy showed variation depending on the species of lichens.The efficacy of 1% lichen extracts was not significant compared with the control groups indicating that they were statistically in the same group (Table 2).

According to the results of this study, *E. prunasti* ethanol extracts, applied in proportionally distinct density, showed antifungal effect on mycelia inhibition and sporulation against all tested plant pathogenic fungi. In contrast to our findings Aslan *et al*. (2006), reported that methanol extracts of *Cladonia foliacea*. (Huds.) Willd., *Dermatocarpon miniatum* (L.) Mann., *Evernia divaricata*. (L.) Ach., *E. Prunastri* and *Neofuscella pulla*. (Ach.) Essl. were ineffective aganist the *F. solani*, *R. Solani* and *Penicilium* spp.

Mitrović *et al.* (2011), investigated the antioxidative, antimicrobial and antiproliferative potentials of the methanol extracts of the lichen species *P. sulcata, Flavoparmelia caperata* (L.) Hale*, E. prunastri, Hypogymnia physodes* (L.) Nyl. and *C. foliacea*. They reported that *E. prunastri* was effective against filamentous fungi, especially on *A. niger.* In our study, it was clearly observed that ethanol extracts of *E. prunastri* were the most effective treatments against all fungi.

F. solani and *M. phaseolina* were the most tolerant fungi against lichen extracts. Regarding the mycelia and spore growth inhibition rates on post-harvest and soilborne plant pathogens 10% concentration of *E. prunastri* totally inhibited the development of *B. cinerea*, *F. culmorum*, *M. phaseolina.* This was followed by *R. solani* at a rate of 98,02%, *A. niger* at a rate of 90%, *P. expansum* at a rate of 80% and *F. solani* at a rate of 73,33% (Table 2).

Table 1. Antifungal activity of *Evernia prunastri, Parmelia sulcata* and *Pseudevernia furfuracea* var. *furfuracea* extracts on soilborne and phytopathogenic postharvest fungi.

Lichen extracts	Extract concentration (%)	Mycelial growth (mm)					Sporulation**	
		B. cinerea	*F. culmorum*	*F. solani*	*M. phaseolina*	*R. solani*	*A. niger*	*P. expansum*
Control (Ethyl alcohol)	10	58.58 a*	58.58 a	58.58 a	58.58 a	58.58 a	4.58 ab	4.58 ab
Evernia prunastri		0 k	0 ı	15.73 j	0 ı	1.16 ı	1 ı	1 g
Parmelia sulcata		19.29 j	19.16 h	39.04 g	7.66 h	21 g	2 gh	1.16 g
Pseudevernia f. var. *furfuracea*		35.45 h	33.79 e	46.45 de	45.29 e	30.5 f	3.16 de	2.33 ef
Control (Ethyl alcohol)	5	58.5 a	58.5 a	58.5 a	58.5 a	58.5 a	4.58 ab	4.58 ab
Evernia prunastri		0 k	19.60 h	17.98 ı	14.51 g	6.39 h	1.5 h	1.36 g
Parmelia sulcata		27.08 ı	23.37 g	41.87 f	40.95 f	29.33 f	2.41 fg	2 f
Pseudevernia f. var. *furfuracea*		42.25 f	34.41 e	48.04 cd	48.5 d	46.33 c	3.41d	2.66 e
Control (Ethyl alcohol)	2.5	58.75 a	58.75 a	58.75 a	58.75 a	58.75 a	4,58 ab	4.58 ab
Evernia prunastri		36.69 gh	29.60 f	28.28 h	42 f	33.87 e	3.1 de	3.48 cd
Parmelia sulcata		38.37 g	34.83 e	44.58 e	44.95 e	36.70 d	2.83 ef	3.5 cd
Pseudevernia f. var. *furfuracea*		50.33 d	40.54 d	49.16 bc	51.25 c	49.75 b	3.58 cd	3.33 d
Control (Ethyl alcohol)	1	58.12 a	58.12 a	58.12 a	58.12 a	58.12 a	4.75 a	4.75 a
Evernia prunastri		54.58 b	54.5 b	29.79 h	54.4 b	51.51 b	4.18 b	4.08 bc
Parmelia sulcata		44.37 e	39.12 d	48.04 cd	46.04 e	38.58 d	4.08 bc	4.08 bc
Pseudevernia f. var. *furfuracea*		52.29 c	46.66 c	50.62 b	54.62 b	50.83 b	4.08 bc	4.41 ab

*Means within columns by unlike letters differ significantly according to LSD test ($p \leq 0.05$)

**Scale values (1: 0-20% spore production in Petri dish, 2: 21-40% spore production in Petri dish 3: 41-60% spore production in Petri dish 4: 61-80% in Petri dish 5: 81-100% spore productionin Petri dish)

Table 2. Inhibition of mycelial growth and sporulation of fungal species by *Evernia prunastri, Parmelia sulcata* and *Pseudevernia furfuracea* var. *furfuracea* extracts.

Lichen extracts	Extract concentration (%)	Inhibition of mycelial growth (%)					Inhibition of sporulation (%)	
		B. cinerea	*F. culmorum*	*F. solani*	*M. phaseolina*	*R. solani*	*A. niger*	*P. expansum*
Control (Ethyl alcohol)	10	0.70 k*	0.7 ı	0.70 j	0.70 ı	0.70 ı	8.33 hı	8.33 fg
Evernia prunastri		100 a	100 a	73.33 a	100 a	98.02 a	90 a	80 a
Parmelia sulcata		67.30 b	67.51 b	33.82 d	87.00 b	64.40 c	60 bc	76.66 a
Pseudevernia f. var. *furfuracea*		39.90 d	42.72 e	21.25 fg	23.23 e	48.30 d	36.66 ef	53.33 bc
Control (Ethyl alcohol)	5	0.84 k	0.84 ı	0.84 j	0.84 ı	0.84 ı	8.33 hı	8.33 fg
Evernia prunastri		100 a	66.76 b	69.51 b	75.39 c	89.16 b	70 b	72.66 a
Parmelia sulcata		54.09 c	60.38 c	29.02 e	30.57 d	50.28 d	51.66 cd	60 b
Pseudevernia f. var. *furfuracea*		28.38 f	41.66 e	18.57 gh	17.79 f	21.46 g	31.66 f	46.66 c
Control (Ethyl alcohol)	2.5	0.42 k	0.42 ı	0.42 j	0.42 ı	0.42 ı	8.33 hı	8.33 fg
Evernia prunastri		37.81 de	49.81 d	52.06 c	28.81 d	41.46 e	38 ef	30.33 de
Parmelia sulcata		34.95 e	40.96 e	24.43 f	23.79 e	37.78 f	43.33 de	30 de
Pseudevernia f. var. *furfuracea*		14.68 h	31.28 f	16.66 hı	13.13 g	15.67 h	28.33 fg	33.33 d
Control (Ethyl alcohol)	1	1.48 k	1.48 ı	1.48 j	1.48 ı	1.48 ı	5 ı	5 g
Evernia prunastri		7.48 j	7.62 h	49.50 c	7.79 h	12.68 h	16.33 h	18.33 ef
Parmelia sulcata		24.78 g	33.68 h	18.57 gh	21.96 e	34.60 f	18.33 gh	18.33 ef
Pseudevernia f. var. *furfuracea*		11.37 ı	20.9 g	14.19 ı	7.41 h	13.84 h	18.33 gh	11.66 fg

*Means within columns by unlike letters differ significantly according to LSD test ($p \leq 0.05$)

Compared with the previous studies, the results of this study showed similarities with the efficacy of many lichen extracts. Halama & Haluwın (2004) reported that *E. prunastri* and *H. physodes* had the highest inhibitory effects on the growth of *Pythium ultimum* Trow. and *Ustilago maydis* (DC.) Corda. The fungal growth was completely inhibited with acetone extracts of these lichens. The highest inhibitory effects were reported for ethyl alcohol extracts from obtained *E. prunastri* on *B. cinerea, F. culmorum, M. phaseolina* in our study.

Ranković *et al.* (2007) reported that the acetone, methanol and aqueous extracts of lichens *Lasallia pustulata* L. (Merat), *P. sulcata* and *Umbilicaria cylindirica* (L.) Del. Ex Duby demonstrated antibacterial and antifungal activity against the tested bacteria and fungi. In our study, the antifungal properties of lichen *P. sulcata* showed a different degree of antifungal activity depending on tested fungi species.

Turk *et al.* (2006), reported antifungal activity of ethanol, chloroform, and acetone extracts of *P. furfuracea* var. *furfuracea* against some filamentous fungi. These extracts showed antifungal activity against *A. alternata*, *Ascochyta rabiei* (Pass.) Labr., *A. niger*, *F. culmorum*, *Fusarium moniliforme* J. Sheld, *F. oxysporum* Schlect, *F. solani* and *Penicillium notatum* Westling. The results recorded for *P. furfuracea* var. *furfuracea* was similar to *A. niger, F. culmorum* and *F. solani.*

Candan *et al*. (2007), tested the antimicrobial activities of acetone, chloroform, diethyl ether, methanol, and petroleum ether extracts of the lichen *P. sulcata* and salazinic acid constituent, and all of the extracts with the exception of the petroleum ether extract showed antimicrobial activity against microorganisms. In our study, the ethanol extract of *P. sulcata* showed a moderate antifungal activity among the tested lichen species on fungi.

Variation among the antifungal activity of lichens may depend on species of lichen and extracting solvent used in the tests. Lichen species may contain different antifungal substances in different amounts Lichen secondary metabolites may be dissolved in different solvents (Gulluce *et al.*, 2006; Ranković & Kosanić, 2012; Ranković *et al*., 2012). These results mostly correspond to the literature. Previous studies conducted to test the ethanol extracts of same lichens used in this study are in agreement with our results (Halama & Haluwın, 2004; Turk *et al*., 2006; Candan *et al*., 2007; Ranković *et al*., 2007).

Our results indicated that *E. prunastri, P. furfuracea* var. *furfuracea* and *P.sulcata* extracts possessed wide antifungal spectrum against severity of major plant pathogens (Tables 1, 2). These results showed that extracts can be used as a natural biofungicides instead of synthetic fungicides. However, this was a preliminary study of the efficacy of lichen extracts on plant pathogenic fungi. The efficacy of lichen extracts should be investigated in natural conditions before use in practice.

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