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Antimicrobial *in Vitro* and *in Vivo* Potential of Five Lichen Species on *Fusarium Equiseti* and *Pectobacterium Carotovora* Pv. *Carotovora* Causal Agents of Potato Rots

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Abstract

Potato is one of the main crops which is suffering from the great losses in storage and the most conventional method of its tuber rot is using hazardous chemicals. Using antimicrobial potential of lichens can be one of the safe, green, environment friendly methods for plant diseases management. In this study, antimicrobial activity of acetone, methanol, chloroform extracts of *Ramalina capitaat*, *Xanthoparmelia stenophylla*, *Umbilicaria cylindrical*, *Rhizoplaca crysoleuca* and *Anamylopsora pulcherrima* were evaluated against two potato rot agents vic. *Fusarium equiseti* and *Pectobacterium carotovora* pv. *carotovora* in laboratory and storage conditions. Bioassay tests were disc diffusion and minimum inhibitory and bactericide concentration methods. Dimethyl sulfoxide solvent on paper disc was used as negative control. Positive controls were considered as %0.2 benomyl and gentamicin antibiogram discs for fungus and bacterium, respectively. There were no effect of lichens extract on fungus. *R. chrysoleuca* especially methanol and chloroform extracts of had remarkable bactericide and bacteriostatic effect on bacterium. The study with selected extracts in storage condition showed preventive effect of 80% of extracts on bacterium. The most protective effect was observed in methanol extract of *R. chrysoleuca*. Therefore, lichen extract would be promising biological product would be a potential replacement instead of chemicals.

Keywords: Antimicrobial effect, MIC, MBC, Natural product, Potato rot

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Introduction

Post-harvest diseases are destroying 10-30% of storage crops in developing contries (Agrios, 2005). Potato tuber and seed segments rot is the most important and destructive post-harvest and storage disease (Rowe, 1993). *Fusarium equiseti* is a wide-spread fungus through the world and in different climates which has quantitative losses and it is toxic due to generated mycotoxins effected on human and animals (Rowe, 1993; Marasas *et al*., 1984). *Pectobacterium carotovora* pv. *carotovora* is also causing potato tuber rot in storage ((Hooker, 1980). Environmental pollutions from chemicals threating human health, microorganisms resistant, public tendency to natural products forcing researchers to world on new green materials. Lichens are one of the promising unique sources of antimicrobial substances which possess natural products such as carbohydrates, proteins, lipids, phenolics and pigments which might have medicinal and antimicrobial potential (Ahmadjian and Hale 1973; Elix and Stocker-Wörgötter, 2008; Afzal *et al.*, 1997). These biochemical and organic substances are accumulated in fungal part of lichens, specially (Nash, 1996; Tomas, 2008). There are several organic materials in lichens which are toxic against fungi and bacteria inhibiting their growth (Romagni *et al*., 2004). Study on antibiotics derived from lichens get started by in 1994 (Burkholder *et al*., 1994) and several significant antibiotics were discovered by now which are effective on wast ranged of plant pathogens such as gr^+ and gr^- bacteria and some fungi (Ahmadijanm and Hale, 1973; Burkholder *et al*., 1994). The current research are investigating some native Iranian lichens' antifungal and antibacterial potential to prevent two fungal and bacterial potato rot agents.

Materials and methods

F. equiseti and *Pectobaterium carotovora* pv. *carotovora* fungal and bacterial potato dry and soft rot was prepared from Iranian Research Institute of Plant Protection.

Lichens are collected from Meshkinshar (Ardabil province, Iran) and Jolfa (East Azarbaijan province, Iran) from the rocks as substrate (Table 1) and transferred into paper bags.

Table 1- Collected lichens and their geographical features

lichens	collection region	longitude	latitude	Altitude (m)
Anamylopsora pulcherrima	Jolfa - Daran	45° 12' 42"	38° 25' 76"	1300
Ramalina capitaat	Meshkinshahr - valaviz	38° 19' 03"	47° 40' 26"	1800
Xanthoparmelia stenophylla	Meshkinshahr	38° 20' 28"	47° 40' 28"	1790
Umbilicaria cylindrica	Meshkinshahr - Movil	38° 15' 55"	47° 44' 21"	2805
Rhizoplaca crysoleuca	Meshkinshahr	38° 20' 28"	47° 40' 28"	1790

The lichens transferred to Islamic Azad University, Miyaneh Branch's Plant Protection Laboratory and washed, air-dried and powdered by blender. Extraction was done by soxhlet. Twenty g of powdered lichens was used with 250 ml of solvents vic. acetone, methanol and chloroform. The dry extract was isolated by rotary system after extraction. Fifty mg of dry extract was solved in 2 ml of dimethyl sulfide (DMSO) and sterilized by injection filter 0.2 µm.

Bioassay was done by disc diffusion method. Potato dextrose agar medium was used for fungus and inoculated by 100 µl of 10^6 of conidium suspension. In the case of bacterium, Muller Hinton agar medium was used and inoculated with 100 µl half McFarland bacteria suspension. Blank sterilized discs with 6 mm diameter purchased from Pad Tan Teb were placed on growth media in three replications 20 minutes after inoculation by fungus and bacterium suspension and 20 µl of extracts were poured on each disc. Inhibition zone diameter generated around discs were recorded after incubation of 6 days at 22ºC and 24 hours at 37ºC in the case of fungus and bacterium, respectively (Boonywanich and Panutat, 1998). Positive control was %0.02 Benomyl 50WP (Behavarshimi, Iran) for fungus and Gentamicin (Pad Tan Teb, Iran). Also, DMSO was used as negative control. The diameter of inhibition zone was measured

after mentioned periods. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) also were evaluated for bacterium. MIC was carried out by dilution tubes method. One ml of Muller Hinton broth was poured in 9 sterilized experimental tubes. One of the tubes was considered as positive control containing growth medium and bacterium. Negative control was growth medium only. Number 1 to number 7 tubes had 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 mg/ml of lichen extracts, respectively. Twenty ml of bacterium suspension (half MsFarland concentration) was added to all tubes expect negative control. After 24 hours incubation of tubes in 37 ºC, they were evaluated regarding bacterium growth. Considering disc diffusion results, five solvent-lichens treatments were selected for this experiment (Table 3). Potato tubes cv. Agria with average size of 80-98 g were obtained from Agriculture and Natural Science of Ardebil, washed with tap water and air-dried, recorded their weigh individually. Dry extracts of lichens in 10 mg/ml of DMSO were prepared. Bacterium suspension was sprayed by sterilized sprayer on potatoes injured by scalpel by length of 4 cm and depth of 5 mm. A potato tuber placed in a plastic clear dish was sonsidered as a plot and replicated in three times. Potatoes were kept in a room with 22-24 ºC temperature and 75% relative humidity for 7 days. The rot regions of potato tubers were evacuated by washing under tap water and secondary weights of all tubers were recorded. The rest percentage of tuber was calculated by following formula:

$$\text{tuber residual\%} = \frac{\text{secondary potato tuber weight}}{\text{primary potato tuber weight}} . 100$$

Experimental design was in completely randomized design with three replications. Data analysis was done by SPSS ver. 16 and means compared using Dunkan test in 5% probability level.

Results and discussion

Considering two repetitions of bioassay against fungus, there were no inhibition zone in none of the lichens extract showing there were no potential of prepared five lichens extracts on *f. equiseti*. An inhibition zone with diameter of 16 mm was observed in positive control containing Benomyl.

Table 2 – Variance analysis of inhibition zone diameter formed in *Pectobacterium carotovora* pv. *carotovora* colony effected by lichens extracts

Source of variation	Degree of freedom	Sum of Squares	Mean Square	F	Sig.
treatments	16	7849.127	490.570**	433.231	.000
Experimental error	34	38.500	1.132		
Total	50	7887.627			

There were significant variation among lichens treatments regarding their effect on *Pectobacterium carotovora* pv. *carotovora* inhibition zone diameter (Table 2). Considering compare means in disc diffusion test, no inhibition zone produced in negative control also in *A. pulcherrina* extracted by chloroform. Rest of lichens treatments significantly effected on bacterium. However, none of treatments could compete with gentamicin as positive control. The most effective effect from lichens was observed in *R. chrysoleca* in especially when extraction was done by chloroform and methanol solvents. However there was no significant difference between three solvents used for extraction. *R. capitata* had great effect also when extracted by Acetone and methanol but chloroform extract was less effective on bacterium. In the case of *X. stenophylla*, chloroform was much better than others in antibacterial materials extraction from the lichens. The weakest treatments was methanol extract of *U. cylindrical* and *X. stenophylla* (Table 3).

Table 3 – Inhibition zone formed in colony effected by lichens methanol, acetone and chloroform extracts

lichens	solvent	Inhibition zone diameter (mm)	MIC (mg/ml)	MBC (mg/ml)	MIC/MBC
Anamylopsora pulcherrima	acetone	25^{e}	0.78	3.12	4
	methanol	14.37^{g}	1.56	6.25	4
	chloroform	6^{i}	-	-	-
Ramalina capitata	acetone	36^{c}	0.78	3.12	4
	methanol	35.5^{c}	0.78	1.56	2
	chloroform	28.33^{d}	1.56	3.12	2
Xanthoparmelia stenophylla	acetone	24^{e}	1.56	6.25	4
	methanol	12^{h}	0.78	3.12	4
	chloroform	28.67^{d}	1.56	3.12	2
Umbilicaria cylindrical	acetone	21^{f}	1.56	6.25	4
	methanol	13.67^{gh}	1.56	6.25	4
	chloroform	21.5^{f}	3.12	12.5	4
Rhizoplaca chrysoleuca	acetone	37.17^{bc}	0.78	1.56	2
	methanol	38^{b}	0.78	3.12	4
	chloroform	38^{b}	0.78	1.56	2
gentamicin	-	46.33^{a}	-	-	-
control	-	6^{i}	-	-	-

The minimum inhibitive concentration was observed in all *r. chrysoleuca* extracts, methanol extract of *X. stenophylla* and acetone and methanol extract of *R. capitata* and acetone extract of *A. pulcherrima*. The least inhibitive and bactericide effect was observed in chloroform extract of *U. cylindrical*. Also, the most bactriocide effect was in *R. chrysoleuca* acetone and chloroform extracts. Minimum 2 and maximum four times of MIC could be lethal, showing high correlation between MIC and MBC (Table 3).

The result of storage survey showed there is significant difference in 1% level among treatments (Table 4). The difference was between control and lichen treatments group. Lichen treatments could save about 60% of tubers from rotting (Table 5). There were no significant differences among lichen treatments, showing all of them could act in the same way in saving tubers from rotting.

Table 4 – Variance analysis of tuber residual inoculated by *Pectobacterium carotovora* pv. *carotovora* sprayed with lichen extracts

Source of variation	Degree of freedom	Sum of Squares	Mean Square	F	Sig.
treatments	4	9301.665	2325.416**	2262.122	.000
Experimental error	10	10.280	1.028		
Total	14	9311.944			

Table 5 - Comparison of tuber residual means affecting by different lichen treatments on potato tubers inoculated by by *Pectobacterium carotovora* pv. *carotovora* in storage condition

lichens	solvent	Primary tuber weight (gr)	secondary tuber weight (gr)	Tuber residual percentage
Rhizoplaca chrysoleuca	chloroform	94.59	87.57	91.66^{a}
Rhizoplaca chrysoleuca	methanol	83.21	79.01	91.54^{a}
Rhizoplaca chrysoleuca	acetone	92.88	86.76	92.38^{a}
Ramalina capitata	acetone	94.85	88.37	93.18^{a}

Ramalina capitata	methanol	90.65	83.61	92.23[a]	
control	-	96.55	30.20		29.45[b]

Lichens are different considering their spices, applied solvent for extraction. Also, the target microorganism in tests will effects bioassay results (Yilmaz *et al*., 2005; Rankovic *et al*., 2007). Evaluation of *Parmotrema aligherrense* on different plant and human bacterial pathogens showed no effect of aqueous extract but significantly progressive effects of other extract including ethanol and methanol and especially chloroform extracts (Sati and Joshi, 2011). Also, extracts of *Parmelina tiliacea*, *Ramalina sinensis*, *Anaptychia setifera*, *Lecanora argopholis* and *Pleopsidium gobiensis* evaluated against *P. carotovora* pv. *carotovora* and find methanol extract of *R. sicensis* to be the most inhibitive extract which act better than tetracycline and streptomycin antibiotics (Shahidi *et al.*, 2011) . In current study, chloroform extract of *R. chrysoleuca* showed the best effect against the bacterium could give a promising results in *in vitro* and *in vivo* conditions.

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