A_020_PF: ANTIFUNGAL ACTIVITY OF CRUDE EXTRACTS FROM LICHEN *Parmotrema tinctorum* (Despr. ex Nyl.) Hale AGAINST *Pythium* spp. CAUSAL AGENTS OF DAMPING-OFF DISEASE OF MARIGOLD (*Tagetes erecta* L.)

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Abstract: Damping-off in marigolds caused by *Pythium* spp. can damage young marigolds leading to collapse of seeding, and cause severe economic losses. The objective of this study was to investigate the antifungal activity of lichen crude extracts, *Parmotrema tinctorum* (Despr. ex Nyl.) Hale, against *Pythium* spp. causal agents of damping-off disease of marigold (*Tagetes erecta* L.). Two hundred grams of the investigated lichen thalli were cut and macerated in 500 mL of acetone for 48 hrs. The extracts were concentrated with rotary evaporator at 40 °C. The lichen crude extract was dissolved in dimethyl sulphoxide (DMSO) and tested against two isolated strains of *Pythium* spp. (Py-R7, Py-RM4) by poisoned food method with five different concentrations; 3,000, 1,000, 500, 100 and 50 µg/mL. Fungicide metalaxyl and DMSO were used as positive and negative controls. .EC_{50} was calculated using Probit analysis. The crude extract at 3,000 µg/mL inhibited 87% and 78% mycelial growth of Py-R7 and Py-RM4, respectively. In contrast, the crude extracts at concentrations of 500, 100 and 50 µg/mL were less active to reduce the growth of both fungal strains (less than 50% mycelial growth inhibition). The EC_{50} values of crude extracts on Py-R7 and Py-RM4 were 436.5 and 630.9 µg/mL, respectively.

Introduction: Lichens are symbiotic organisms of fungi (mycobiont) and algae or cyanobacteria (photobiont or phycobiont). Lichen-forming fungi produce a great number of various secondary metabolites, and most of them are unique. The lichen substances are produced by the mycobiont[1,2], and accumulate in cortex or medullary layers of lichen thalli. Approximately 1,050 secondary compounds have been reported[3] which many of them have various biological activities such as antifungal, antiviral and antiprotizoal[4].

Parmotrema tinctorum (Despr. ex Nyl.) Hale is a cosmopolitan lichen species belonging to the family Parmeliaceae. This lichen is characterized by large foliose thalli with broad lobes and the present of two lichen substances viz. atranorin and lecanoric acid. The crude extracts and lichen acids from this lichen have been reported to have antifungal activities. Kekuda and Vinayaka (2016)[5] studied methanol crude extracts of *P. tinctorum* against fungi isolated from seeds of maizes and groundnuts. Lecanoric acid was tested against the fungus *Cladosporium sphaerospermum*, a dematiaceous saprophytic fungus. The results revealed that the compound had potent fungitoxic activity[6]. Shivanna et al. (2014)[7] evaluated the antifungal activity of the acetone, ethyl acetate and methanol extracts of *P. tinctorum* against *Fusarium oxysporum* f. sp. *capsici*, causing Fusarium wilt of chili pepper. The results showed that both ethyl acetate and methanol extracts had the efficiency to reduce mycelial growth of the plant pathogen, whereas the acetone extract had no activity to control the growth of mycelial.

Marigold (*Tagetes erecta* L.) is one of the important economic flowers and normally it can resist to insect issues however, fungal diseases in marigold plants are the problems. Many diseases causing by fungi were reported such as leaf spots (*Alternaria tenuissima*) and flower blight (*Alternaria zinniae*)[8]. Damping-off is another serious problem, caused by *Pythium* spp. that damage or weaken young marigolds leading to collapse of seeding. The aim of this study was to evaluate antifungal activity of crude extracts of *Parmotrema tinctorum* against *Pythium* spp. isolated from disease marigolds.

Methodology:
Collection and Identification of Lichen: *Parmotrema tinctorum* was collected from Khao Yai National Park -on 26 September 2017. The specimens of lichens were carefully separated from the substrate. The samples were identified based on morphological, anatomical and chemical tests (potassium hydroxide (K), calcium hypochlorite (C) and p-phenylenediamine (P)) and the secondary metabolites were characterized by thin layer chromatography[9].
Preparation of lichen crude extracts: Lichens were cleaned of substrata and shade dried at room temperature for 3 days. The dried samples were cut to small piece (ca. 2×3 mm). Two hundred grams of cut lichen were soaked with 500 ml of acetone and incubated on a shaker for 48 h at room temperature. Then the mixture was filtered through Whatman No. 1 filter paper and maceration of residue was repeated until the extractions were clear. The filtrate was concentrated in vacuo at 50 °C by using rotary evaporator. The crudes extracts were weighed and stored in a desiccator at 25 °C for further assays.
Plant pathogenic fungi isolation: The marigolds with symptoms of damping-off disease were collected from agricultural fields in Nonthaburi and Pathumthani provinces during July 2017 - October 2018. The isolation of *Pythium* spp., causal agents of damping-off disease, was made by tissue transplanting method and identified on the basis of their morphological and anatomical structures[10,11]. The pure fungal strains (Py-R7, Py-RM4) were maintained on potato dextrose agar (PDA) slants at 25 °C.
Antifungal activity assay: Antifungal activity assay was carried out by the poisoned food method. The plant pathogenic fungi were inoculated on PDA plates and incubated at 25°C for 3 days to obtain young and active colonies. The lichen crude extracts were dissolved in dimethyl sulphoxide (DMSO; the final concentration did not exceed 0.2%) and then mixed with worm PDA medium (45-50 °C) to obtain five different concentrations (3,000, 1,000, 500, 100 and 50 µg/mL). After the agar and lichen extracts were mixed together, approximately 15 ml was poured into each of 5 Petri plates and allowed to solidify at room temperature. The colony margin of fungal pathogens was cut with 5 mm diameter of sterile cork borer. Then transferred to the center of the agar plates containing different lichen extract concentrations and then the plates were incubated at 25±1 °C. Colony diameters were measured when the positive control had reached the edge of the plates. The PDA with fungicide metalaxyl and with 0.2% of DMSO were used as positive and negative controls, respectively. For each treatment was repeated five times. Percentage inhibition of mycelial growth is evaluated by comparing and measuring the colony diameter of the poisoned plates (with lichen extract) and non-poisoned plate (without lichen extract). The antifungal activity of the lichen extracts in terms of percentage inhibition of mycelial growth (PIMG) was calculated using the formula:

$$PIMG = C - T / C \times 100$$

Where, C = Average of diameter increase of mycelial growth in control plate and T = Average of diameter increase of mycelial growth in treatment plate. All observed data were analyzed by using statistical software package SPSS. The comparison differences between lichen extracts and metalaxyl against both plant pathogenic fungi were done by t-test ($P < 0.05$) and significant differences of concentrations were determined by using Duncan's multiple range analysis. The means effective inhibitory concentration (EC_{50}) against plant pathogenic fungal was calculated according to probit analysis[12].

Results and Discussion: The effects of different acetone crude extract concentrations from *Parmotrema tinctorum* (3000, 1000, 500, 100 and 50 µg/mL) on mycelial growth of *Pythium* spp., Py-R7 and Py-RM4, were shown in Table 1. The antifungal activity was found in all of the different concentrations and the activity of crude extract was significantly enhanced

by increasing of the concentrations (Table 1 and Figure 1). In addition, each concentration showed significant differences to reduce the radial growth of the tested fungi (Table 1.). At concentration 3,000 µg/mL of the crude extract showed the highest mycelial growth inhibition of both Py-R7 and Py-RM4 by 87.1% and 77.8 %, respectively. Whereas, the metalaxyl completely inhibited the mycelial growth of both fungal strains at the same concentration. Another strong mycelial growth inhibition was found at 1,000 µg/mL which reduced the growth of Py-R7 and Py-RM4 by 74.1% and 62.4%, respectively. Our results are similar to the previously reported by Kekuda et al. (2016)[13] who also found that the extracts from *P. tinctorum* had antifungal activities against several plant pathogenic fungi viz. *Helminthosporium* sp, *Curvularia* sp. *Alternia* sp., *Mucor* sp., *Penicillium* sp. and *Rhizopus* sp. The mycelial growth was suppressed lower than 50% at concentration of 500 and 100 µg/mL. In contrast, the metalaxyl reduced more than 80% of both plan pathogens at the same concentration. The two fungal strains were only slightly affected by crude extracts at 50 µg/mL (Figure 2). The comparison of the effect of different crude extract concentrations in the growth inhibition of both Py-R7 and Py-RM4 showed that Py-R7 was more significantly affected by the extract than Py-RM4 at 3000, 500 and 50 µg/mL.

The EC_{50} values of lichen crude extracts against Py-R7 and Py-RM4 were 436.5 and 630.9 µg/mL which was higher than metalaxyl with 8.32 and 7.94 µg/mL, respectively. Even though, the EC_{50} values in this study appear to be higher than the EC_{50} values of metalaxyl. However, we think the EC_{50} values obtained in this study are reasonable because lichen crude extract are generally a mixture of active and non-active compounds whereas the metalaxyl used in this investigation is one of the best fungicides to control lower fungi such as *Pythium* spp. or *Phytophthora* spp. The results of this study were concordance with Mongkolsuk et al. (2009)[14], who reported that the EC_{50} values of lichen crude extracts from *Parmotrema tinctorum* against *Pythium deliense*, causal agent of damping-off of seedling, was 548 µg/mL.

Table 1. Effect of different concentrations of acetone crude extracts from *Parmotrema tinctorum* and metalexyl on the mycelial growth of *Pythium* spp.

Concentration	Percentage of mycelial inhibition[A]				t-test[B]			
	Py-R7		Py-RM4					
	(1)	(2)	(3)	(4)	(1) vs (2)	(3) vs (4)	(1) vs (3)	(2) vs (4)
50 µg/mL	12.7±7.43[a]	86.7±1.36[a]	10.0±2.48[a]	86.7±1.36[a]	-21.886**	-60.517**	0.760	0.000
100 µg/mL	19.8±1.64[b]	88.9±0.0[a]	14.8±4.11[b]	88.0±1.22[a]	-93.770**	-38.174**	2.523*	1.633
500 µg/mL	44.2±0.92[c]	95.6±6.09[b]	45.4 ±1.13[c]	95.6±6.09[b]	-18.645**	-18.098**	-1.859	0.000
1,000 µg/mL	74.1±1.44[d]	100±0.0[c]	62.4 ±3.08[d]	100±0.0[c]	-39.959**	-27.236**	7.658**	-
3,000 µg/mL	87.1±1.68[e]	100±0.0[c]	77.8 ±0.00[e]	100±0.0[c]	-17.103**	-	12.385**	-
p-value	<0.001	<0.001	<0.001	<0.001				

[A]Values are percentage of mycerial inhibition. Means (n = 5) in each column followed by the same superscript letter(s) are not significantly different in the percentages of mycelial growth inhibition. Data reported are mean values ± standard deviation.

[B]Data was analysed by *t*-test compared between (1) (2) (3) and (4)

(1) and (3) = Crude extract of *Parmotrema tinctorum*

(2) and (4) = Metalaxyl

*p<0.05, ** p<0.01

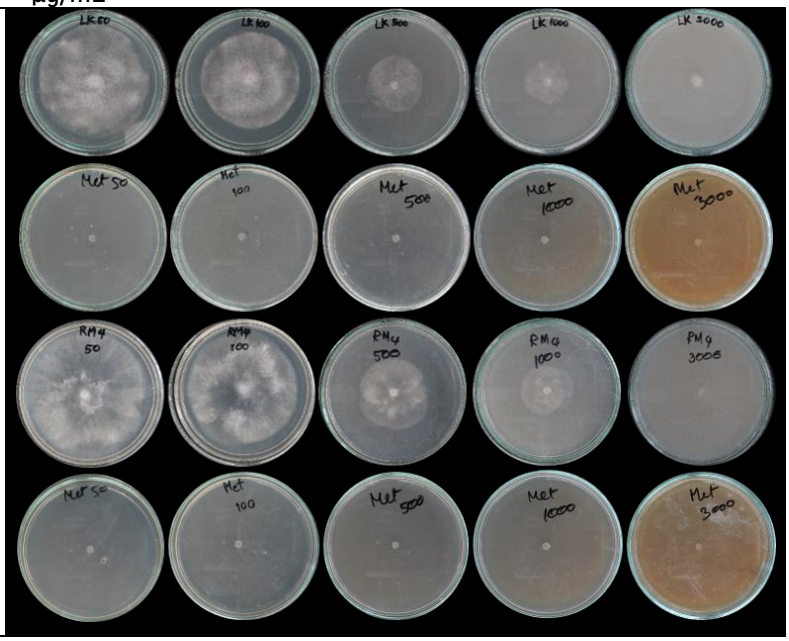
Plant pathogenic fungi	Efficacy of five concentrations of *Parmotrema tinctorum* acetone crude extracts and metalaxyls against *Pythium* spp. *in vitro*				
	50 µg/mL	100 µg/mL	500 µg/mL	1,000 µg/mL	3,000 µg/mL
Py-R7 (lichen crude extracts)					
Py-R7 (metalaxyl)					
Py-RM4 (lichen crude extracts)					
Py-RM4 (metalaxyl)					

Figure 1. Mycelial growth inhibition of *Parmotrema tinctorum* on acetone crude extract and metalaxyl PDA medium against *Pythium* spp., at 25±1 °C for 2 days.

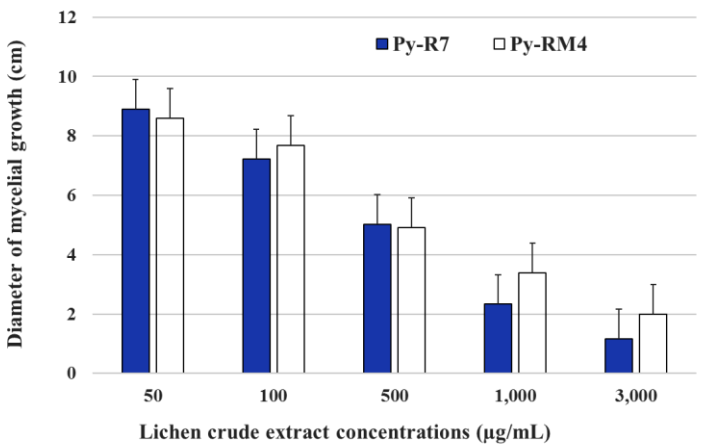


Figure 2. Growth inhibition of *Pythium* spp. (Py-R7 and Py-RM4) by acetone crude extracts of *Parmotrema tinctorum*.

Conclusion: The crude extracts of lichen *Parmotrema tinctorum* showed the antifungal activity against *Pythium* spp. causal agents of damping-off disease of marigolds. The treatments were more effective to Py-R7 than Py-RM4. The antifungal activity of lichen observed in this study could be related to the presence of bioactive secondary metabolites in the extracts. The next study should focus on the application of this inhibitory activity against *Pythium* spp. in the

condition of the potted plants and might include a larger number of fungal isolations and lichen species.

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