J. Crop Prot. 2020, 9 (3): 367-380

Research Article

Screening of plant extracts for antibacterial activity against *Pseudomonas tolaasii* and *Ewingella americana*, the bacterial pathogens of cultivated button mushroom

Seyedeh Fatemeh Nourbakhsh Shourabi, Fatemeh Yousefi Kopaei* and Roohollah Mohammadi Eshkaftaki

Plant Protection Department, Faculty of Agriculture, Shahrekord University, Shahrekord, Iran.

Abstract: *Pseudomonas tolaasii* Paine and *Ewingella americana* Grimont are considered as devastating pathogens in mushroom cultivation. Due to the short shelf life of button mushrooms, safe methods should be used to control these pathogens to avoid any toxic residues on the products. Plant secondary metabolites are assumed as important sources for biopesticides development. The aim of this study was to screen plant species for antibacterial properties against *P. tolaasii* and *E. americana*. Antibacterial activity of aqueous extract of 17 plant species on two pathogens was investigated *in vitro* using the disc diffusion method at 10 and 20 mg active ingredients per disc. Then the effect of extracts possessing antibacterial activity was tested on mycelial growth of button mushroom *Agaricus bisporus* (Lange) using the disc diffusion method. Analysis through measuring the diameter of growth inhibition zones revealed that the extract of *Hymenocrater longiflorus* Benth. and the other extracts including *H. longiflorus*, *Achillea millefolium* L., *Eucalyptus* sp. and *Teucrium polium* L. had significant antibacterial activity on *E. americana* and *P. tolaasii,* respectively. However, they had no inhibitory activity on mycelial growth of *A. bisporus.* The efficacy of four mentioned extracts was evaluated in the control of mushroom brown blotch disease caused by *P. tolaasii, in vivo.* Assessment of disease severity showed that all four extracts, at tested concentrations, had some level of preventive effect on *P. tolaasii* with no adverse effects on *A. bisporus*. It is noteworthy that the strength of the *A. millefolium* extract at 10% did not differ significantly from the 1% household bleach in reducing the disease severity. Therefore, it is possible that some plant extracts have the power to be considered as alternatives to chemical bleaches. Moreover, findings suggest that *H. longiflorus* extract is a promising candidate for control of *P. tolaasii* and *E. americana* in mushroom cultivation.

Keywords: *Agaricus bisporus*, brown blotch, internal stipe necrosis, plant extracts antibacterial effects

Introduction

Pseudomonas tolaasii Paine, the cause of mushroom brown blotch disease, is one of the most important pathogens of cultivated mushrooms such as *Agaricus* spp., *Pleurotus* spp., *Lentinula edodes* (Berk) and *Flammulina velutipes* (Curtis) (Tsuneda *et al*., 1995). The symptoms at first appear in the form of yellow to brown lesions on the cap and stipe which later they transform and become sunken and slimy. In favorable conditions, lesions expand rapidly and eventually cover the entire mushroom surface.

Handling Editor: Naser Safaie

* **Corresponding author**: yousefi@sku.ac.ir
Received: 17 April 2019, Accepted: 22 April 2020
Published online: 20 May 2020

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

Before and during the post-harvest storage, the disease leads to deterioration of mushrooms. This would result in consumer demand loss at the marketplace (Gill, 1995; Wells *et al*., 1996; Soler-Rivas *et al*., 1999). *Ewingella americana* Grimont, a species of the Enterobacteriales order, has been reported as mycopathogenic agent which causes major problems in the mushroom industry (Inglis *et al*., 1996). The symptoms of this disease emerge as internal stipe necrosis which could develop into the internal tissue of the caps (Reyes *et al*., 2004). Bacterial infections reduce quantity and quality of product (Fermor and Lynch, 1988). Considering the short shelf-life of mushrooms, application of chemical substances for control of pathogens could potentially threaten human health due to the existence of their residues on the product. In addition, pathogens may become resistant to these costly chemicals (McManus *et al*., 2002). Application of plant extracts and essential oils can be perceived as a safe and effective alternative to control important diseases (Sharma and Jandaik, 1994; Hammer *et al*., 1999). Essential oils of *Coriandrum sativum* L. and *Foeniculum vulgare* Miller var. *vulgare* (Miller) as natural bactericides have been documented as promising substances to control bacterial pathogens of mushrooms (Lo Cantore *et al*., 2004). Sokovic and van Griensven (2006) evaluated the antibacterial activity of essential oils extracted from *Thymus vulgaris* L., *Mentha spicata* L., *Salvia officinalis* L., *Matricaria chamomilla* L., *Origanum vulgare* L. and *Ocimum basilicum* L. against *P. tolaasii*. They reported the essential oils of *O. vulgare*, *T. vulgaris* and *M. spicata* as remarkably effective compounds. In another study, methanol extract and essential oil of *Eucalyptus camaldulensis* Dehnh. were introduced as effective treatments against *P. tolaasii* (Ansari Dezfoli *et al*., 2013). Nevertheless, none of the mentioned studies have been performed in the real mushroom cultivation field conditions. Therefore, the claimed results are just based on *in vitro* experiments or the experiments done on detached mushroom tissues (*in situ*). Moreover, little data has been documented in the literature about antibacterial activity of other plant species on bacterial pathogens of mushrooms. Consequently, the present study was conducted with two main aims: 1) Detection of plant extracts with efficient antibacterial activity on *P. tolaasii* and *E. americana* but without detrimental effects on *Agaricus bisporus* (Lange) by *in vitro* experiments, 2) Investigation of the plant extracts potential in prevention of mushroom brown blotch disease *in vivo*.

Materials and Methods

Bacterial isolates

Six mushroom, *A. bisporus,* cultivation centers in Chaharmahal-o-Bakhtiary province, Iran were visited and about 10 mushrooms with brown blotch or internal stipe necrosis symptoms were collected from each center. Small pieces of symptomatic mushroom caps or stipes were immersed in 0.5% sodium hypochlorite solution for 30 seconds and rinsed three times in sterile distilled water. The pieces were grinded in 3-5 ml sterile distilled water. After 20 min, the prepared suspension was streaked onto Nutrient Agar (NA) medium. The plates were incubated at 28 °C for 48 h and the obtained colonies were further purified by streaking on the same medium. The purified isolates were preserved in sterile distilled water at 4 °C.

Identification of bacterial isolates

Bacterial isolates were identified based on their biochemical characteristics and molecular methods. Briefly, colony morphological characteristics of isolates (size, shape, color and texture) were recorded after 24 h of growth on NA medium at 28 °C. Standard bacteriological phenotypic tests including gram reaction, growth aerobically or anaerobically, production of fluorescent pigment on King's B medium, LOPAT tests i.e. Levan production, oxidase reaction, potato rot, presence of Arginine dihydrolase and hypersensitive reaction (HR) were conducted (Schaad *et al*., 2001).

The isolates were also subjected to PCR using primer pairs Pt1A/1D which amplify the required gene for tolaasin production and

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

specifically detect *P. tolaasii* (Lee *et al*., 2002). Identification of the isolates suspected to be *E. americana* was confirmed by partial sequencing of 16S rDNA. The 16S rDNA was amplified by PCR using the 16S rDNA-universal primers (27F and 1492R) from a DNA sample of bacterial isolate (Islam *et al*., 2016). Purified PCR fragment was sequenced and compared with the strains obtained from NCBI database with the use of BLAST program.

Pathogenicity tests

Pathogenicity tests were performed on the excised cube of cap tissues and intact freshly picked basidiocarps of *A. bisporus* according to the methods previously described (Wong and Preece, 1982; Inglis *et al*., 1996). In brief, 50 µl from bacterial suspension of about 1×10^8 CFU/ml was loaded onto the excised cubes and intact caps for the isolates of *P. tolaasii* and was injected into stipe bases for the *E. americana* isolates. Inoculated mushrooms were incubated in a humid chamber at room temperature. Mushroom tissues inoculated with sterile distilled water were used as the negative control. Disease symptoms were examined up to 72 h. To fulfill Koch's postulates, the pathogenic strains were re-isolated from the diseased samples.

Preparation of plant extracts

The plant species used in this study were collected from Shahrekord, Isfahan and Bandar Abbas cities of Iran, during May 2018. The names of plant species and their characteristics are presented in Table 1. The collected plants were washed with running water and dried in shade at room temperature. The plant materials of each species were powdered using a household blender and were passed through a 50 mesh sieve. Ten grams of each powder was soaked in 100 ml of distilled water and was placed on a stirring machine at 100 rpm and at room temperature for three days. Then, the suspension was filtered through a piece of cloth and was centrifuged at 10000 rpm for 10 min. The supernatant was then concentrated at 45 °C and finally sterilized by passing through a Millipore filter (0.22 µm). In order to obtain the active ingredients amount of each extract, clean test tubes were weighted. One ml of each prepared extract was added to each test tube in three replications and then was evaporated and dried at 60 °C. The amount of active ingredients was calculated by subtracting empty test tube weight from the weight of test tube containing dried extract. The mean of three obtained numbers was taken as active ingredients per ml (Goudarzi *et al*., 2006).

Table 1 Plant species used for extraction in this study.

Plant species	Common name	Family	Used part	Sampling location
Mentha piperita	Peppermint	Lamiaceae	leaves and stems	Shahrekord
Mentha pulegium	Pennyroyal	Lamiaceae	leaves and stems	Shahrekord
Achillea millefolium	Yarrow	Asteraceae	flowers, leaves and stems	Shahrekord
Matricaria chamomilla	Chamomile	Asteraceae	leaves and stems	Shahrekord
Allium sativum	Garlic	Amaryllidaceae	leaves and stems	Shahrekord
Salvia officinalis	Sage	Lamiaceae	leaves and stems	Shahrekord
Thymus daenensis	Thyme	Lamiaceae	leaves and stems	Shahrekord
Teucrium polium	Ezovion	Lamiaceae	leaves and stems	Shahrekord
Kelussia odoratissima	Keloss	Apiaceae	leaves and stems	Shahrekord
Alyssum sp.	Alyssum	Brassicaceae	leaves and stems	Shahrekord
Rosmarinus officinalis	Rosemary	Lamiaceae	leaves and stems	Shahrekord
Lavandula stoechas	Lavender	Lamiaceae	leaves and stems	Shahrekord
Ocimum basilicum	Basil	Lamiaceae	leaves and stems	Shahrekord
Artemisia absinthium	Wormwood	Asteraceae	leaves and stems	Shahrekord
Hymenocrater longiflorus	Gol-e-Arvaneh	Lamiaceae	leaves and stems	Shahrekord
Eucalyptus sp.	Eucalyptus	Eucalypteae	leaves	Bandar Abbas
Olea europaea	Olive	Oleaceae	leaves	Isfahan

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

Evaluation of antibacterial activity of plant extracts *in vitro*

The antibacterial activity of aqueous plant extracts against *P. tolaasii* and *E. americana* was examined through disc diffusion method (Balouiri *et al*., 2016). Each plant extract was loaded on sterile blank paper disc (6.4 mm in diameter) to the final concentrations of 10 and 20 mg active ingredients per disc. Blank paper discs were purchased from Padtan Teb Company, Tehran, Iran. The suspension of each bacterium was prepared in sterile distilled water (OD_{600} = 0.15). Four hundreds µl of bacterial suspension was applied on the surface of six cm Petri dishes containing NA medium and were uniformly distributed using L-shaped spreader. Then, the paper discs (impregnated with plant extracts at 10 and 20 mg/disc) were placed at the center of plates (one disc in each plate). The paper discs containing Gentamycin (0.01 mg/disc) and distilled water were used as positive and negative control groups, respectively. The diameter of inhibitory zones (mm) around each disc was measured after a night of incubation. The experiments were conducted as two- factor completely randomized design (CRD) with four replications.

Evaluation of inhibitory activity of plant extracts on mycelial growth of *A. bisporus*

Five plant extracts with highest antibacterial activity on *P. tolaasii* were chosen and their inhibitory activity on mycelial growth of *A. bisporus* was examined. This investigation was conducted using the disc diffusion method at two concentrations; 10 and 20 mg active ingredients of plant extract per disc. A 5 mm mycelial block of *A. bisporus* was placed on the surface of Potato Dextrose Agar (PDA) medium, approximately two cm away from plate edge. Then one paper disc loaded with intended plant extracts was placed at a distance of two cm from the mycelial block. Paper discs containing sterile distilled water were used as the negative control. The experiments were performed in a two-factor completely randomized design (CRD) with four replications. Plates were kept in an incubator at 25 °C. The radial growth (mm) of *A. bisporus* mycelium was measured after ten days. The percentage of mycelial growth inhibition was calculated using the following formula:

$$A = \frac{(A1 - A2)}{A1} \times 100$$

Where, A = the percentage of mycelial growth inhibition; A1 = the radial growth of mycelium (mm) against distilled water disc (negative control) and A2 = the radial growth of mycelium (mm) against plant extract discs.

Evaluation of antibacterial activity *in vivo*

Plant extracts preparation

The dried powder of each plant species was soaked in tap water at 5% and 10% w/v. The suspension was placed on a stirrer at 100 rpm and at room temperature for three days and then was filtered through a piece of cloth.

Selecting appropriate concentration of plant extracts for in vivo assays

In order to select appropriate concentration of plant extracts which cause no change in color, taste and shape of mushrooms, a preliminary experiment was performed. The intended extracts were applied on basidiocarps and casing soil at five and 10 percent w/v. Four concentrations (0.5, 1, 2 and 4% v/v) of household bleaching liquid containing five percent NaOCl were also applied. For the main experiment, plant extracts and household bleaching liquid were used at a concentration which caused no change in basidiocarp color, taste or shape.

Application of plant extracts to control mushroom brown blotch in vivo

For mushroom cultivation, substrate growing medium inoculated with the spawn of *A. bisporus* was purchased from a local company and was distributed in two 70 × 70 × 17 cm trays. The temperature and humidity of culturing room were properly adjusted as recommended for mushroom cultivation. Bacterial suspension of *P. tolaasii* was prepared in distilled water using an overnight culture. The suspension turbidity was adjusted to 0.5 McFarland turbidity standards. At the time of

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

emerging basidiocarps, bacterial suspension was sprayed on basidiocarps and casing soil. Plant extracts, one percent household bleaching solution (as the positive control) and sterile distilled water (as the negative control) were sprayed at two different time points: 24 h before bacterial inoculation (protective application) and 24 h after bacterial inoculation (curative application). The experiments were performed in a completely randomized split plot design with two replications (each replication contained a bulk of mushroom compost). The main plots were assigned to the time of application of plant extracts and the subplots consisted of various plant extracts. Four days after inoculation, 20 samples were randomly collected from each subplot and brown blotch disease severity of each sample was scored from one to four using the assessment scale proposed by Wong and Preece (1982). In this scale, severity value assignment is measured based on the percentage of fruiting body area which is covered by lesion (1 = no lesion; 2 = 0.1% - 5%; 3: 10 - 20% and 4 = more than 25% of fruiting body surface is affected).

Statistical analysis

The data analysis was conducted using SAS 9.1 software. In order to compare the means, the least significant difference (LSD) test was used at 5% probability level.

Results

Identification of isolates

The strains isolated from mushroom tissues with brown blotch symptoms were gram negative and aerobic. They produced catalase, cytochrome oxidase, Arginine dehydrolase, fluorescent pigment on King's B medium and HR on geranium; but did not produce Levan. These strains amplified expected 449 bps fragment in PCR by specific primers Pt1A/1D (Lee *et al*., 2002). On the basis of phenotypic characteristics and also amplification of expected fragment in PCR, they were identified as *P. tolaasii.*

The strains isolated from mushrooms with internal stipe necrosis symptoms were gram negative, catalase positive and facultative anaerobic. They did not produce fluorescent pigment on King's B medium and showed negative reactions for the tests of oxidase, Levan formation, Arginine dehydrolase and HR on geranium. Considering these characteristics and also by comparison of 16S rDNA sequence with the NCBI database, these isolates were identified as *E. americana*. The comparison of 16S rDNA sequence of representative isolate with the NCBI database indicated that it has 99.9% similarity with *E. americana* strains (accession numbers: MG334385.1, KY126990.1 and KY126989.1). The sequence of this isolate was deposited in GenBank with the accession number of MK334665.

Pathogenicity tests

In pathogenicity tests, *P. tolaasii* strains induced discoloration of excised cubes and appearance of brown spots on intact caps within 24 h after inoculation. No blotch was observed on the cubes and caps inoculated with distilled water. Strains of *E. americana* were injected into the stipes of mushrooms and the symptoms of browning disorders and necrosis along the length of the inoculation point were observed after 48 h, while the stipes injected with distilled water or *P. tolaasii* were symptomless.

***In vitro* evaluation of antibacterial activity of plant extracts**

The plant extracts exhibited different antibacterial activity against *P. tolaasii* and *E. americana*. Table 2 presents the results of *in vitro* antibacterial activity of plant extracts on both bacteria. In the case of *P. tolaasii*, considering the diameter of the growth inhibition zone, the extracts of *Hymenocrater longiflorus* Benth., *M. chamomilla, Achillea millefolium* L., *Eucalyptus* sp., *Teucrium polium* L. and *Alyssum* sp. revealed the most antibacterial activity. The diameter of inhibitory zones of these six plant extracts at 20 mg/disc were 9.6, 8.3, 8.1, 8.1, 7.8 and 7.3 mm, respectively; which were significantly higher than the diameter of inhibition zone of Gentamycin disc (6.6 mm). The diameter of inhibition zone of these extracts at 10 mg/disc was also larger than that of the other plant extracts, but smaller than

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

the Gentamycin disc inhibition zone. The extracts of *Thymus daenensis* Celak., *O. basilicum*, *Mentha piperita* L. and *Kelussia odoratissima* Mozaff. had no antibacterial activity and no inhibitory zone appeared around their discs. The extract of *Olea* sp. indicated slight antibacterial activity. The diameter of its inhibition zones were 2.4 and 4.6 mm at 10 and 20 mg/disc, respectively. These values were significantly less than the value of the inhibitory zone of Gentamycin disc (6.6 mm). Other extracts had no antibacterial activity at 10 mg/disc. Nonetheless, they presented slight antibacterial activity at 20 mg/disc (Table 1).

Table 2 Means of growth inhibitory zone diameter obtained by disc diffusion method using two concentrations of plant extract active ingredients against *Pseudomonas tolaasii* and *Ewingella americana.*

Plant species	Plant extract concentration (mg/disc)	Growth inhibition (mm)	
		Pseudomonas tolaasii	*Ewingella americana*
Mentha piperita	10	0v	0v
	20	0v	3.8klm
Mentha pulegium	10	0v	3.1nop
	20	6.3fe	5.3g
Achillea millefolium	10	4.1jkl	1.1uv
	20	8.1b	4.3ijk
Matricaria chamomilla	10	4.3ijk	3.6lmn
	20	8.1b	4.9ghi
Allium sativum	10	0v	0v
	20	6.6fe	3.8klm
Salvia officinalis	10	0v	0v
	20	2.8opq	0v
Thymus daenensis	10	0v	0v
	20	0v	0v
Teucrium polium	10	4.6hij	3.6lmn
	20	7.8bc	5.1gh
Kelussia odoratissima	10	0v	1.1uv
	20	0v	4.1jkl
Alyssum sp.	10	3.6lmn	1.1uv
	20	7.3dc	2.3qr
Rosmarinus officinalis	10	0v	0v
	20	3.3mno	2.1rs
Lavandula stoechas	10	0v	1.8rst
	20	5.1gh	4.7ghij
Ocimum basilicum	10	0v	0v
	20	0v	0v
Artemisia absinthium	10	0v	0v
	20	3.6lmn	0v
Hymenocrater longiflorus	10	5.1gh	4.6hij
	20	9.6a	6.8de
Eucalyptus sp.	10	4.3ijk	0v
	20	8.1b	1.6stu
Olea europaea	10	2.4pqr	3.3mno
	20	4.6hij	4.6hij
Gentamycin	0.01	6.6fe	6.1f
Distilled water	-	0v	0v

The means indicated with the same letters are not significantly different at 5% probability level calculated using the least significant difference test.
Gentamycin disc and disc impregnated with distilled water were used as positive and negative control groups, respectively.

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

In the case of *E. americana,* the extract of *H. longiflorus* had the most antibacterial activity. The diameter of its inhibition zone was 6.8 mm at 20 mg/disc which was significantly higher than the inhibition zone of gentamycin disc (6.1 mm). Meanwhile, its inhibitory zone was 4.6 mm at 10 mg/disc; which was significantly higher than the inhibition zones of other extracts at the same concentration. The extracts of *Mentha pulegium* L., *T. polium*, *M. chamomilla*, *Olea europaea* L. and *Lavandula stoechas* L. were ranked in the second place. Their inhibition zones at 10 mg/disc were 3.1, 3.6, 3.6, 3.3 and 1.8 mm and at 20 mg/disc were 5.3, 5.1, 4.9, 4.6 and 4.7, respectively. *Artemisia absinthium* L.*, O. basilicum, S. officinalis* and *T. daenensis* had no antibacterial effect on *E. americana* (no inhibition zones were formed). The remaining extracts had no significant antibacterial effect at 10 mg/disc. Their antibacterial effect was increased at 20 mg/disc; however, the diameter of growth inhibition zones was much less than the inhibitory zone of gentamycin disc. Figure 1 shows the growth inhibition zones of some plant extracts against *P. tolaasii* and *E. americana*.

Inhibitory effect of plant extracts on mycelial growth of *A. bisporus*

As presented in Table 3, among five tested plant extracts (*A. millefolium, H. longiflorus, T. polium, Eucalyptus* sp. and *M. chamomilla*), the extract of *M. chamomilla* exhibited the highest inhibitory activity on mycelial growth of *A. bisporus*. The percentage of growth inhibition for this extract was 71.3 which was significantly higher than the one for the other extracts (Table 2). The radii of mycelial growth of *A. bisporus* against the paper discs impregnated with the extracts of *A. millefolium, H. longiflorus, T. polium* and *Eucalyptus* sp. at 20 mg/disc were 15, 15.5, 15.5 and 15 mm, respectively. These values did not significantly differ from each other nor from the size of 15.7 mm in negative control (distilled water disc); but they were significantly different from the size of 4.7 mm against *M. chamomilla* extract (Fig. 2). Noticeably, for all five tested plant extracts, there was no significant difference between the radius of mycelial growth at 10 and 20 mg/disc (Table 3).

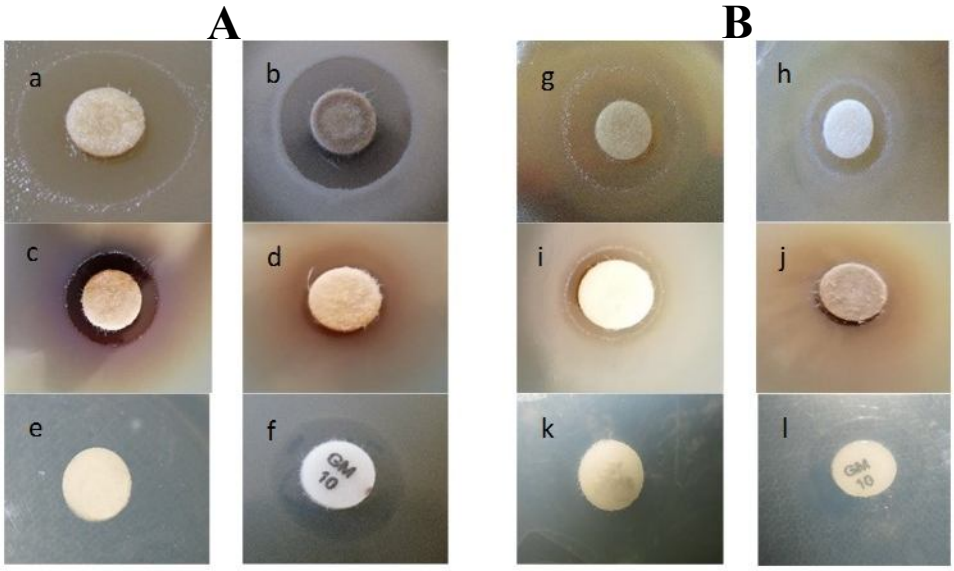

Figure 1 Growth inhibition zones of *Pseudomonas tolaasii* (panel A) and *Ewingella americana* (panel B) obtained by some plant extracts applied through disc diffusion method at 20 mg active ingredients per disc. Distilled water and gentamycin discs were used as negative and positive control groups, respectively. a: *Hymenocrater longiflorus,* b: *Teucrium polium*, c: *Olea europaea*, d: *Ocimum basilicum* (no inhibition zone), g: *Hymenocrater longiflorus*, h: *Achillea millefolium*, i: *Rosmarinus officinalis*, j: *Salvia officinalis* (no inhibition zone), e and k: Distilled water (no inhibition zone), f and l: Gentamycin (10 µg/disc).

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

Table 3 Inhibitory activity of plant extracts on mycelial growth of *Agaricus bisporus* using disc diffusion method at two concentrations; 10 and 20 mg of active ingredients per disc.

Plant extract	Plant extract concentration (mg/disc)	Radius of mycelial growth (mm)	Mycelial growth inhibition (%)
Hymenocrater longiflorus	10	15.0a	4.45
	20	15.5a	1.27
Eucalyptus sp.	10	15.2a	3.18
	20	15.0a	4.45
Teucrium polium	10	15.7a	0
	20	15.5a	1.27
Matricaria chamomilla	10	4.50b	71.33
	20	4.70b	70.06
Achillea millefolium	10	15.0a	4.45
	20	15.0a	4.45
Distilled water	-	15.7a	0

The means indicated with the same letters are not significantly different at 5% probability level calculated using the least significant difference test.

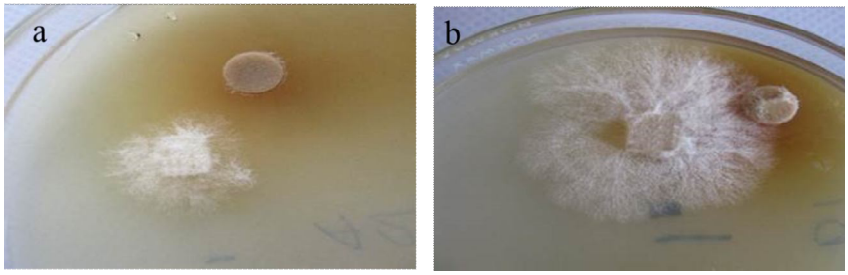

Figure 2 Mycelial growth of *Agaricus bisporus* against discs impregnated with plant extracts at 20 mg active ingredients per disc, 10 days after culturing. a: the extract of *Matricaria chamomilla* b: the extract of *Achillea millefolium*.

Selecting appropriate concentration of plant extracts for *in vivo* assays

Among all examined plant extracts, only the extract of *H. longiflorus* at 10% w/v, caused light brown discoloration of basidiocarps which reduces marketability. Moreover, household bleaching solution at 2% and 4% v/v caused some discoloration of basidiocarps. Hence, for *in vivo* assays, the *H. longiflorus* extract and household bleach were applied at 5% w/v and 1% v/v, respectively. Other plant extracts were used at 10% w/v.

***In vivo* antibacterial activity of plant extracts**

Protective application of four selected plant extracts on the basidiocarps of *A. bisporus* and covering soil, led to reduction of brown blotch disease severity (Fig. 3). Among these extracts, the maximum protective effect was obtained by *A. millefolium* extract (10% w/v). The mean score of disease severity of this treatment was 1.45 which did not significantly differ from the mean score (1.35) of positive control (1% v/v solution of household bleach). The extracts of *H. longiflorus* (5% w/v), *T. polium* (10% w/v) and *Eucalyptus* sp. (10% w/v) were ranked from the second to the fourth in terms of their potential to control brown blotch disease. Their corresponding disease severity scores were 2.25, 2.75 and 3.05, respectively which were significantly lower than the score (3.85) in negative control (Table 4). Curative application of plant extracts did not control brown blotch disease; so that, the basidiocarps treated with the four selected plant extracts were not significantly different from the basidocarps treated with sterile distilled water.

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

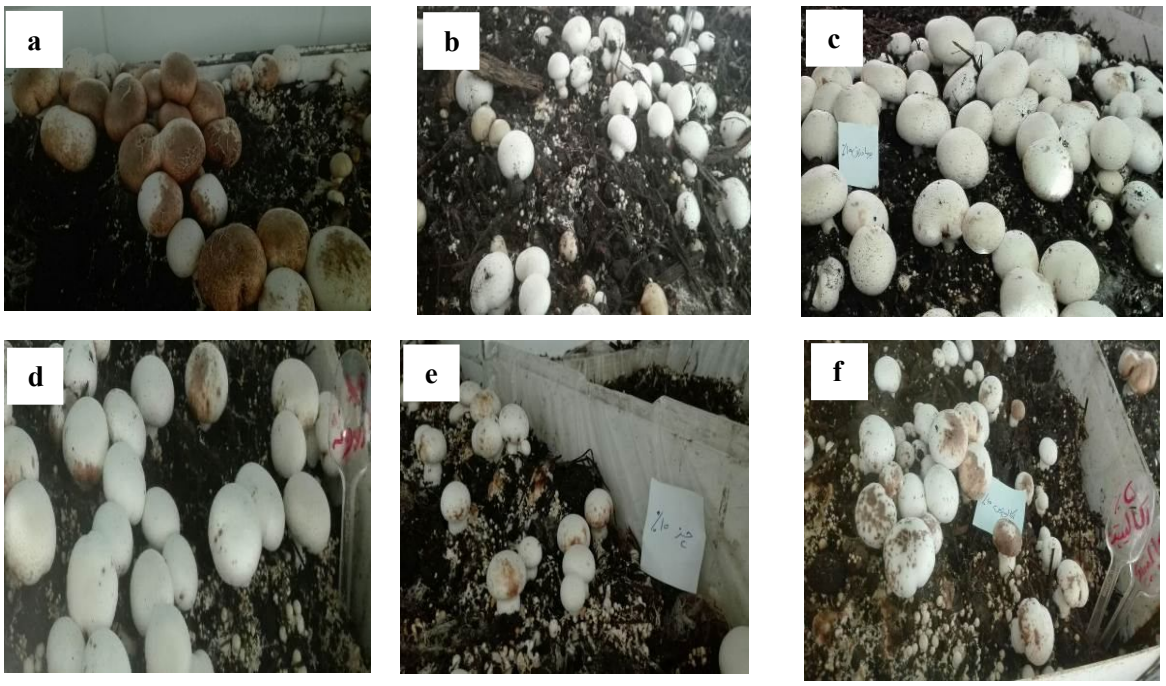

Figure 3 Protective effect of four plant extracts against *Pseudomonas tolaasii* comparing with negative (distilled water) and positive (household bleach) control groups. The treatments were applied 24 h before bacterial inoculation. a: distilled water, b: household bleach (1% v/v), c: *Achillea millefolium* (10% w/v), d: *Hymenocrater longiflorus* (5% w/v), e: *Teucrium polium* (10% w/v), f: *Eucalyptus* sp. (10% w/v).

Table 4 Brown blotch disease severity of mushrooms inoculated with *Pseudomonas tolaasii*, treated with plant extracts preventatively (24 h before inoculation) and curatively (24 h after inoculation).

Plant species	Concentration (%)	Preventive application[2]	Curative application[2]
Achillea millefolium	10 (w/v)	1.45[e]	3.85[a]
Hymenocrater longiflorus	5 (w/v)	2.25[d]	3.95[a]
Teucrium polium	10 (w/v)	2.75[c]	3.90[a]
Eucalyptus sp.	10 (w/v)	3.05[b]	3.85[a]
Household bleach[1]	1 (v/v)	1.35[e]	3.80[a]
Distilled water[1]		3.85[a]	3.95[a]

Means indicated with the same letters are not significantly different at 5% probability level calculated using the least significant difference test.

[1] Household bleach and distilled water were applied as positive and negative control groups, respectively.

[2] Each value is the mean of diseases severity scores of 40 mushrooms. Scoring was performed four days after inoculation and based on the assessment scale proposed by Wong and Preece (1982).

Discussion

Nowadays, awareness of the threats of chemical pesticides for the environment and their potential risks for human health has generated an increasing interest in many researchers to develop safer pesticides. The growing incidence of resistance to pesticides has been also provoking the need for new pesticides. Plants and their secondary metabolites are assumed as important sources for biopesticides and development of new pesticides (Cavoski *et al*., 2011). The antibacterial activity is related to the type and concentration of components in plant extracts and essential oils (Sokovic and van Griensven, 2006; Stević *et al*., 2010).

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

Meanwhile, the concentration of antimicrobial components in each plant species can be influenced by its cultural condition environment and developmental stages (Gillitzer *et al*., 2012). In the present study, 17 plant species were screened for *in vitro* antibacterial activity against *P. tolaasii* and *E. americana*, two important bacterial pathogens of cultivated mushroom *A. bisporus*. The results confirmed that the efficacy of extracts is different based on the kind of bacterial species. Some extracts were effective on one pathogen but had no antibacterial activity against the other one. Therefore, in order to introduce a plant extract as an antimicrobial agent for a bacterial pathogen, screening tests for that pathogen is unavoidable. Considering the diameter of growth inhibition zone created by examined plant species, the extracts of *T. daenensis* and *O. basilicum* did not show any antibacterial effect against either of the two pathogens even at 20 mg/disc. In contrast, the extracts of *H. longiflorus*, *T. polium* and *O. europaea* were effective against both pathogens at 10 mg/disc and their antibacterial activity was increased at 20 mg/disc. Among mentioned three plant species, the extracts of *H. longiflorus* followed by *T. polium* had the highest level of antibacterial activities on both pathogens. The plants belonging to the genus of *Hymenocrater* spp. have been used in folk medicine all around the world (Al-Anee *et al*., 2014). Flavonoids, phenolic acids and terpenoids are important constituents of the genus *Hymenocrater* (Morteza-semnani *et al*., 2016). Antimicrobial activity of the species of this genus has been confirmed in several studies (Fazli Bazzaz and Harirzadeh, 2003; Zaidi and Crow, 2005; Ahmadi *et al*., 2010). *T. polium* is also considered as one of the most important medicinal plants used in traditional medicine in Iran. Different classes of compounds have been isolated from various parts of *T. polium*, the main groups of which are terpenoids and flavonoids. These compounds possess diverse pharmacological effects such as antioxidant, anticancer, anti-inflammatory, antibacterial and antifungal effects (Bahramikia and Yazdanparast, 2012). An increasing number of studies investigated the effects of *T. polium* against different microbes. Mosadegh *et al*., (2002) examined the effect of its aqueous extract on the growth of some bacterial and fungal pathogens. They reported that it has considerable antibacterial but no antifungal effect. This is in accordance with our findings that aqueous extracts of *T. polium* had significant antibacterial activity against both tested bacteria but did not show any antifungal activity against *A. bisporus*. The antibacterial activity of *T. polium* alcoholic extract was evaluated against some clinical bacterial pathogens such as *Bacillus anthracis* Cohn and *Salmonella typhi* and the results showed that ethanolic and methanolic extracts of this plant are effective against both gram positive and gram negative bacteria (Darabpour *et al*., 2010). *T. polium* has been also suggested as an effective medicinal plant for the treatment of infections caused by *Staphylococcus aureus* (Rosenbach) (Motamedi *et al*., 2015).

Based on the *in vitro* assays and examination on the excised tissue of mushroom cap, essential oils of *O. vulgare*, *T. vulgaris*, *M. spicata*, *Chamaemelum nobile* L. and *E. camaldulensis* were reported as effective antibacterial agents against *P. tolaasii* (Sokovic and van Griensven, 2006; Ansari Dezfooli *et al*., 2012 and 2013). The main weakness of previous research is that they have not put any attempt to evaluate the efficiency of antibacterial agents in the field (mushroom cultivation center) conditions. However, we examined the antibacterial effects of plant extracts both in the laboratory and at the field conditions. We also evaluated the effect of extracts on mycelial growth of *A. bisporus*. In order to apply any antimicrobial agent in mushroom culture centers, its inhibiting potential on mushroom growth should be also examined. In present study, despite appropriate antibacterial effect of *M. chamomilla* extract on *P. tolaasii*, it was not selected for *in vivo* experiments due to its inhibition effect on *A. bisporus* mycelial growth.

The results of *in vivo* experiments indicated that protective application (24 h prior to inoculation of *P. tolaasii*) of *A. millefolium, H.*

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

longiflorus, T. polium and *Eucalyptus* sp. extracts on the casing soil and basidiocarps has led to desirable control of the brown blotch disease. However, the most effective extract was *A. millefolium* which its efficacy was comparable with household bleach used as the positive control. The application of plant extracts 24 h after *P. tolaasii* inoculation could not control the brown blotch disease. It was because of rapid growth of bacterium such that symptoms of diseases appear within 24 h. Thus, for evaluation of the effect of essential oils and plant extracts or antagonists on brown blotch control, it is recommended that the treatment be applied before bacterial inoculation or in shorter period (6 or 8 h) after inoculation.

With regard to the ranking of antibacterial activity of plant extracts based on *in vitro* analysis, the inhibitory zone of *H. longiflorus* was larger than that of *A. millefolium* extract; while, the result of *in vivo* analysis was vice versa. This outcome was predictable because the concentration of *H. longiflorus* was half that of *A. millefolium* in the *in vivo* experiment. The 10% w/v of *H. longiflorus* extract in preliminary experiment, resulted in mushroom color change (browning), it was therefore reduced to 5% in the main experiment.

Conclusion

The present study illustrated that aqueous extracts of *A. millefolium, H. longiflorus, T. polium* and *Eucalyptus* sp. at tested concentrations are able to control mushroom brown blotch disease effectively with no adverse effects on *A. bisporus* mycelial growth or on the product quality. Consequently, they can be employed as preventive agents against *P. tolaasii*. Note that *A. millefolium* extract could be more effective than the others. Moreover, regarding our *in vitro* results, *H. longiflorus* and *T. polium* could be used in mushroom cultivation centers confronted with the mixed infection of *E. americana* and *P. tolaasii*. Obviously, in this case, further *in vivo* research is required to assess their effectiveness.

Funding

This research as an MS thesis in plant pathology was financially supported by Shahrekord University.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Ahmadi, F., Sadeghi, S., Modarresi, M., Abiri, R. and Mikaeli, A. 2010. Chemical composition, *in vitro* anti-microbial, antifungal and antioxidant activities of the essential oil and methanolic extract of *Hymenocrater longiflorus* Benth., of Iran. Food and Chemical Toxicology, 48: 1137-1144.

Al-Anee, R. S. A., Sulaiman, G. M., Al-Sammarrae, K. W., Napolitano, G., Bagnati, R., Lania, L., Passoni, A. and Majello, B. 2014. *In vitro* studies of the cytotoxicity and cell cycle arrest of *Hymenocrater longiflorus* plant extract on colon cancer (RKO) cell line. Current Research in Microbiology and Biotechnology, 2: 367-372.

Ansari Dezfooli, N., Hasanzadeh, N., and Rezaee, M. B. 2013. Antibacterial activity of essential oil and extracts of *Eucalyptus camaldulensis* Dehnh. on *Pseudomonas tolaasii* under *in vitro* and *in vivo* conditions. Iranian Journal of Medicinal and Aromatic Plants, 28(4): 709-719.

Ansari Dezfooli, N., Hasanzadeh, N., Rezaee, M. B. and Ghasemi, A. 2012. Antibacterial activity and chemical compositions of *Chamaemelum nobile* essential oil/extracts against *Pseudomonas tolaasii*, the causative agent of mushroom brown blotch. Annals of Biological Research, 3: 2602-2608.

Bahramikia, S. and Yazdanparast, R. 2012. Phytochemistry and medicinal properties of *Teucrium polium* L. (Lamiaceae). Phytotrapy Research, 26(11): 1581-1593. https://doi.org/10.1002/ptr.4617.

Balouiri, M., Sadiki, M. and Ibnsouda, S. K. 2016. Methods for *in vitro* evaluating

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

antimicrobial activity: A review. Journal of Pharmaceutical Analysis, 6: 71-79.

Cavoski, I., Caboni, P. and Miano, T. 2011. Natural pesticides and future perspectives. In: Stoytcheva, M. (Ed.), Pesticides in the Modern World- Pesticides Use and Management. IntechOpen. PP. 169-190. doi: 10.5772/17550.

Darabpour, E., Motamedi, H. and Seyyed Nejad, S. M. 2010. Antimicrobial properties of *Teucrium polium* against some clinical pathogens. Asian Pacific Journal of Tropical Medicine, 3(2): 124-127.

Fazly Bazzaz, B. S. and Haririzadeh, G. 2003. Screening of Iranian plants for antimicrobial activity. Pharmaceutical Biology, 41: 573-583.

Fermor, T. and Lynch, J. 1988. Bacterial blotch disease of the cultivated mushroom *Agaricus bisporus*: screening, isolation and characterization of bacteria antagonistic to the pathogen (*Pseudomonas tolaasii*). Journal of Applied Bacteriology, 65(3): 179-187.

Gill, W. 1995. Bacterial diseases of *Agaricus* mushrooms. Japan Science and Technology Agency, 33: 34-55.

Gillitzer, P., Martin, C., Kantar, M., Kauppi, K., Dahlberg, S., Lis, D., Kurle, J., Sheaffer, C. and Wyse, D. 2012. Optimization of screening of native and naturalized plants from Minnesota for antibacterial activity. Journal of Medicinal Plants Research, 6(6): 938-949.

Goudarzi, M., Sattari, M., Najar piraieh, S., Goudarzi, G. and Bigdeli, M., 2006. Antibacterial effects of aqueous and alcoholic extracts of Thyme on enterohemorrhagic *Escherichia coli*. Yafte, 8(3): 63-69.

Hammer, K. A., Carson, C. F. and Riley, T. V. 1999. Antibacterial activity of essential oils and other plant extracts. Journal of Applied Microbiology, 86(6): 985-990.

Inglis, P. W., Burden, J. L. and Peberdy, J. F. 1996. Evidence for the association of the enteric bacterium *Ewingella americana* with internal stipe necrosis of *Agaricus bisporus*. Microbiology, 142(11): 3253-3260.

Islam, S., Akanda, A. M., Prova, A., Islam, M. T. and Hossain, M. M. 2016. Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. Frontiers in Microbiology, 6: 1-12.

Lee, H. I., Jeong, K. S. and Cha J. S. 2002. PCR assays for specific and sensitive detection of *Pseudomonas tolaasii*, the cause of brown blotch disease of mushrooms. Letters in Applied Microbiology, 35(4): 276-280.

Lo Cantore, P., Iacobellis, N. S., De Marco, A., Capasso, F. and Senatore, F. 2004. Antibacterial activity of *Coriandrum sativum* L. and *Foeniculum vulgare* Miller var. *vulgare* (Miller) essential oils. Journal of Agricultural and Food Chemistry, 52(26): 7862-7866.

McManus, P. S., Stockwell, V. O., Sundin, G. W. and Jones, A. L. 2002. Antibiotic use in plant agriculture. Annual Review of Phytopathology, 40(1): 443-465.

Morteza-Semnani, K., Ahadi, H. and Hashemi Z. 2016. The genus *Hymenocrater*: a comprehensive review. Pharmaceutical Biology, 54(12): 3156-3163. DOI: 10.1080/ 13880209.2016.1197285.

Mosadegh, M., Dehmoubed Sharifabadi, A., Nasiri, P., Esmaeili, S. and Naghibi, F. 2002. The study of phytochemical, antifungal and antibacterial effects of *Teucrium polium* and *Cichourium intybus*. Scientific Journal of Kurdistan University of Medical Sciences, 7: 1-6.

Motamedi, H., Alivand, S., Ebrahimian, M. and Moosavian, S. 2015. The antibacterial properties of methanolic extract of *Teucrium polium* against MRSA. Journal of Kermanshah University of Medical Sciences, 18: 557-562.

Reyes, J. E., Venturini, M. E., Oria, R. and Blanco, D. 2004. Prevalence of *Ewingella americana* in retail fresh cultivated mushrooms (*Agaricus bisporus*, *Lentinula edodes* and *Pleurotus ostreatus*) in Zaragoza (Spain). FEMS Microbiology Ecology, 47(3): 291-296.

Schaad, N. W., Jones, J. B. and Chun W. 2001. Laboratory Guide for Identification of Plant

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

Pathogenic Bacteria. American Phytopathological Society Press. St. Paul, Minnesota, USA.

Sharma, V. N. and Jandaik, C. L. 1994. Effect of some plant materials in controlling different moulds in *Agaricus bisporus*. Indian Journal of Mycology and Plant Pathology, 24: 183-185.

Sokovic, M. and van Griensven, L. J. 2006. Antibacterial activity of essential oils and their components against the three major pathogens of the cultivated button mushroom, *Agaricus bisporus*. European Journal of Plant Pathology, 116(3): 211-224.

Soler-Rivas, C., Jolivet, S., Arpin, N., Olivier, J. and Wichers, H. 1999. Biochemical and physiological aspects of brown blotch disease of *Agaricus bisporus*. FEMS Microbiology Reviews, 23(5): 591-614.

Stević, T., Šavikin, K., Ristić, M., Zdunić, G., Janković, T., Krivokuća-Dokić, D. and Vulić, T. 2010. Composition and antibacterial activity of the essential oil of the leaves of black currant (*Ribes nigrum* L.) cultivar Čačanska crna. Journal of the Serbian Chemical Society, 75(1): 35-43.

Tsuneda, A., Suyama, K., Murakami, S. and Ohira, I. 1995. Occurrence of *Pseudomonas tolaasii* on fruiting bodies of *Lentinula edodes* formed on Quercus logs. Mycoscience, 36(3): 283-288.

Wells, J. M., Sapers, G. M., Fett, W. F., Butterfield, J. E., Jones, J. B., Bouzar, H. and Miller, F. C. 1996. Postharvest discoloration of the cultivated mushroom *Agaricus bisporus* caused by *Pseudomonas tolaasii*, *P.'reactans'*, and *P.'gingeri'*. Phytopathology, 86(10): 1098-1104.

Wong, W. and Preece, T. 1982. *Pseudomonas tolaasii* in cultivated mushroom (*Agaricus bisporus*) crops: numbers of the bacterium and symptom development on mushrooms grown in various environments after artificial inoculation. Journal of Applied Bacteriology, 53(1): 87-96.

Zaidi, M. A. and Crow, S. A. Jr. 2005. Biologically active traditional medicinal herbs from Balochistan, Pakistan. Journal of Ethnopharmacology, 96: 331-334.

[DOR: 20.1001.1.22519041.2020.9.3.2.3]

غربال‌گری عصاره‌های گیاهی از نظر خاصیت ضدباکتریایی علیه *Pseudomonas tolaasii* و *Ewingella americana*، باکتری‌های بیمارگر قارچ دگمه‌ای

سیده فاطمه نوربخش شورابی، فاطمه یوسفی کوپائی* و روح‌الله محمدی اشکفتکی

گروه گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه شهرکرد، شهرکرد، ایران.
پست الکترونیکی نویسنده مسئول مکاتبه: yousefi@sku.ac.ir
دریافت: ۲۸ فروردین ۱۳۹۸؛ پذیرش: ۳ اردیبهشت ۱۳۹۹

چکیده: باکتری‌های *Pseudomonas tolaasii* و *Ewingella americana* از بیمارگرهای مخرب مراکز پرورش قارچ خوراکی محسوب می‌شوند. با توجه به کوتاهی زمان ماندگاری قارچ دگمه‌ای، برای کنترل این بیمارگرها باید روش‌های ایمن اتخاذ شود تا از وجود بقایای سمّی روی محصول اجتناب شود. متابولیت‌های ثانویه گیاهان به‌عنوان منابع مهمی برای توسعه آفت‌کش‌های زیستی محسوب می‌شوند. هدف از اجرای تحقیق حاضر، غربالگری گونه‌های گیاهی ازنظر خاصیت ضدباکتریایی علیه *P. tolaasii* و *E. americana* بود. اثر ضدباکتریایی عصاره آبی ۱۷ گونه گیاهی بر دو بیمارگر مذکور در شرایط آزمایشگاه (*in vitro*) به روش نشت از دیسک کاغذی در دو غلظت ۱۰ و ۲۰ میلی‌گرم ماده مؤثره در دیسک بررسی شد. سپس اثر بازدارندگی عصاره‌های دارای خاصیت ضدباکتریایی بر رشد میسلیوم *Agaricus bisporus* به‌روش نشت از دیسک ارزیابی شد. اندازه‌گیری قطر هاله بازدارنده از رشد باکتری نشان داد که عصاره *Hymenocrater longiflorus* و عصاره‌های چهار گیاه *H. longiflorus*، *Achillea millefolium*، *Eucalyptus* sp. و *Teucrium polium* به‌ترتیب روی *E. americana* و *P. tolaasii* اثر ضدباکتریایی قابل‌توجهی داشتند. با این وجود، بر رشد ریسه قارچ *A. bisporus* اثر بازدارنده نداشتند. کارایی این چهار عصاره در کنترل بیماری لکه قهوه‌ای قارچ خوراکی حاصل از *P. tolaasii* در شرایط *in vivo* مورد بررسی قرار گرفت. ارزیابی شدت بیماری نشان داد که هر چهار عصاره در غلظت مورد استفاده، بدون تأثیر سوء روی *A. bisporus* درجاتی از اثر پیش‌گیری‌کننده از *P. tolaasii* داشتند. نکته قابل توجه این‌که قدرت تأثیر عصاره *A. millefolium* (غلظت ۱۰ درصد وزن به حجم) در کاهش شدت بیماری به‌حدی بود که اختلاف معنی‌داری با محلول سفیدکننده خانگی (یک درصد) نداشت. لذا این امکان وجود دارد که برخی عصاره‌های گیاهی این قدرت را داشته باشند که بتوانند جایگزین سفیدکننده‌های شیمیایی درنظر گرفته شوند. نتایج تحقیق حاضر هم‌چنین پیشنهاد می‌کند که عصاره *H. longiflorus* گزینه‌ای امیدبخش برای کنترل *P. tolaasii* و *E. americana* در مراکز پرورش قارچ می‌باشد.

واژگان کلیدی: *Agaricus bisporus*، لکه قهوه‌ای، نکروز داخلی ساقه، اثر ضدباکتریایی عصاره‌های گیاهی

[DOR: 20.1001.1.22519041.2020.9.3.2.3]