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Original Article

The Antimicrobial Activity of Endophytic Fungi Isolated from *Thymus* spp.

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ABSTRACT

Medicinal plants have been known to act as a harbor for endophytic fungi, owing to their being able to produce bioactive compounds similar to those of their host. *Thymus* is a member of the Lamiaceae family. It enjoys a long history of traditional and modern medicine as a disinfectant that possesses antimicrobial properties. The pharmaceutical properties of this plant can be attributed to its endophytes. In this research, 89 endophytic fungi of *Thymus* spp. were tested and examined to investigate their biocontrol effects against the plant pathogenic fungus *Botrytis cinerea*, the plant pathogenic bacteria *Xanthomonas arboricola* pv. *juglandis* and *Streptomyces scabies* and human pathogens *Escherichia coli* ATTCC 25922 and *Staphylococcus aureus* ATCC 33591. Thereafter, to control fungal and bacterial pathogens, the extracellular metabolites of the endophytic fungi were extracted and used in seven different concentrations. The effect of endophytic fungi on the growth of *B. cinerea* suggested that the M24 isolate (*Fusarium subglutinans*) was the one with the greatest percentage of inhibition. Out of the 89 isolates tested against bacteria, only one isolate affected *X. arboricola*, three of them affected *E. coli*, and eight isolates showed biocontrol effect on bacterium *S. aureus.* In the case of *S. scabies*, all *Fusarium* isolates prevented its growth. Among other isolates, only M32 and M33, which belong to mycelia sterilia, affected the growth of this bacterium. Endophytic extracellular metabolites had great potential to control plant pathogens as well.

INTRODUCTION

Endophytes are microorganisms that colonize the internal, healthy tissues of plants without causing any symptoms [1]. These microorganisms are rich sources of bioactive compounds [2-4] that are produced as secondary metabolites, which, in turn, are of great value in the pharmaceutical industry. In some cases, endophytes are known to produce compounds similar to those of their host [5-8]. Besides, these microorganisms can produce unique compounds, such as secondary metabolites, which protect their host from fungal and pest attacks [9]. The ability of endophytic fungi to protect the host against pathogens and, in specific cases, to increase systemic resistance of the host plant is regarded as a basis to control plant diseases [10]. In this regard, several research groups have used endophytic fungi and their natural products to control plant and human pathogens [10-15]. Nowadays, it seems necessary for researchers to make an endeavor to discover beneficial compounds that reduce various types of diseases, and this is because newer types of antibiotic-resistant bacteria are emerging everyday [16]. To achieve this goal, scientists have resorted to natural products, which have crucially helped scientists in their efforts to discover and develop drugs. Therefore, endophytic fungi have been regarded as alternative sources for the extraction of antimicrobial compounds due to their specific ability to produce bioactive compounds [12,17].

Thymus, an increasingly important plant, belongs to the Lamiaceae family [18]. Essence is the effective material of this plant, the most important parts of which contain phenols, monoterpene hydrocarbons

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and, alcohols [18,19]. Thymol is the main part of phenolic compounds in *Thymus* and carvacrol is an ancillary part [20]. Scientists have known the essence of *Thymus* to be a microbicide since the 16th century, and they have attributed its antimicrobial effect to the presence of thymol and carvacrol [21–23].

Researchers have previously proved the level of influence that the essence and extract of *Thymus* have on controlling plant pathogens of *Xanthomonas arboricola* [24] and *Botrytis cinerea* Pers.: Fr. [25,26] and human pathogenic bacteria of *Escherichia coli* (Migula) Castellani and Chalmers and *Staphylococcus aureus* Rosenbach [22,27,28].

Due to the importance of secondary metabolites produced by endophytic fungi as well as the antimicrobial properties of *Thymus*, in this study, the antimicrobial effect of *Thymus* endophytic fungi on plant pathogens was investigated. *X. arboricola* pv. *juglandis* (Xaj) and *E. coli* ATTCC 25922 as the representative of Gram-negative, and *Streptomyces scabies*, Lambert and Loria, and *S. aureus* ATTCC 33591 as the Gram-positive bacteria, and fungus *B. cinerea*, were used to assay the bioactivity of endophytic fungi isolated from *Thymus*.

MATERIAL AND METHODS

Endophytic Fungal Isolates

Eighty-nine endophytic fungi which were isolated in our previous study [29] from six different species of *Thymus*, *T. eriocalyx* (Ronniger) Jalas, *T. lancifolius* Celak., *T. fallax* Fisch. & C.A. Mey., *T. kotschyanus* Boiss. & Hohen, *T. vulgaris* L. and *T. daenensis* Celak., in the Hamedan province (Located in the west of Iran), were tested to investigate their biocontrol activities.

Test Microorganisms

Pathogenic microorganisms used in this research were Gram-positive (*S. scabies*, *S. aureus*) and Gram-negative (*X. arboricola* pv. *Juglandis*, *E. coli*) bacteria and fungus *B. cinerea.* Human pathogenic bacteria were received from the Persian Type Culture Collection (PTCC) and plant pathogenic bacteria and *B. cinerea* from Bu-Ali Sina University. Cultural media of potato dextrose agar (PDA), containing yeast extract and peptone, was used to investigate the antibacterial activity, and PDA media to investigate the antifungal activity.

Preliminary Antimicrobial Assay

Antifungal Properties of Endophytes

The effect of the endophytic isolates on *B. cinerea* was investigated using the method of Dennis and Webster [30] through dual culture. A mycelial plug of 5 mm diameter from the margin of the 10-day colony of each endophytic fungus was cultured along the margins of Petri dishes containing PDA and kept for two days in 25 °C ± 2 °C. Then, a five-millimeter plug of *B. cinerea* was placed on the other side, 5 cm away from the endophytic fungus. Moreover, a 5 mm plug of the fungus-free medium was used in the control treatment. The experiment was conducted in a completely randomized design with three replications. After four days, the radial growth of the pathogenic fungus was measured against endophyte, and the inhibition percentage was obtained by the formula of Taechowisan *et al*. [31]:

Inhibition%= [(growth radius in untreated control – growth radius in treatment) / growth radius in untreated control] × 100.

In the cases of inhibition halo formation, the radius of the halo was measured too.

Antibacterial Properties of Endophytes

Based on the modified method of Pereira *et al*. [32], first, endophytic fungi were cultured on PDA containing yeast extract and peptone. After 48 hours, a suspension of pathogenic bacteria with a concentration of 10^7 CFU/ml was spread on the plates containing endophytes. The experiment was done in a completely randomized design with three replications. The plates containing plant pathogenic bacteria and human pathogenic bacteria were incubated at 27 ºC and 35 ºC, respectively, for 24-48 hours. After incubation, the radius of inhibition halo was measured, and the potential of antimicrobial activity of endophytic fungi was calculated using the following formula:

Fungal antibacterial potential = inhibition halo radius / fungal colony radius.

Secondary Metabolites Extraction

The isolates which had antimicrobial effects in the preliminary tests were chosen to extract extracellular metabolites. These isolates were transferred to Erlenmeyer flasks of 250 ml, containing 200 ml of potato dextrose broth. Erlenmeyer flasks were then incubated for 14 days, at 27 °C, in a rotary shaker at 130 rpm in the dark. Extracellular metabolites were

extracted using the method described by Haque *et al*. [33] and Chakravarthi *et al*. [34] with a slight modification. To do so, first, the fermented broth and fungal biomass were separated using Whatman paper No. 1. The extraction of extracellular metabolites from the liquid phase was done by adding an equal volume of organic solvent (chloroform: methanol, 4:1 v/v). The resultant organic phase was evaporated at 40 ºC in an oven.

Bioeffects of Secondary Metabolites

Antifungal Properties of Endophytic Fungal Extracts

The antifungal activity of methanol extracts was tested using the well diffusion method. There were seven concentrations of 0, 6, 12, 24, 48, 96 and 192 mg/ml obtained for each extract through dimethyl-sulfoxide (DMSO). This study was done using the method of Pavithra *et al*. [13], with a slight modification. First, several wells were punched into PDA medium using a sterile cork borer of size 5 mm in diameter. Each well was then filled with 20 µl of each extract concentration. The plates were kept in a refrigerator for one hour so that the material inside the wells diffused into the agar. Afterward, a suspension of 10^5 spores/ml was provided from *B. cinerea* and spread all over the surface of the plates and were incubated at 22 ºC for 24 hours. After the growth of mycelia around negative control wells containing 20 µl of DMSO solvent and the creation of halo around positive control wells containing 20 µl of fungicide of fluconazole at a concentration of 5 mg/ml, the inhibition halo diameter was measured. Each concentration consisted of three replicates and evaluation was done in a completely randomized design.

Antibacterial Properties of Endophytic Fungal Extracts

To investigate the antibacterial activity of endophytic fungal extracts, the diffusion method of Pavithra *et al*. [13] was used. Wells of 5 mm diameter on PDA containing yeast extract and peptone, were filled with 20 µl of each extract concentration. Then, a suspension of 10^7 CFU/ml was provided from each type of bacteria, and 500 µl of the suspension was spread on the media. Three replications were considered for each concentration. Plates were incubated at 27 °C for plant pathogens and 35 °C for human pathogenic bacteria. After 24-48 hours, the inhibition halo diameter was measured, and the data were analyzed using a completely randomized design.

RESULTS

Bioeffects of Endophytes

Antifungal Properties of Endophytes

The effect of endophytic fungi on the growth of *B. cinerea* was investigated in a completely randomized design with three replications. There was a significant difference between treatments based on the Duncan test ($P\leq 0.01$). The highest amount of inhibition was attributed to the M24 isolate (*Fusarium subglutinans* (Wollenw & Reinking) P.E. Nelson, Tousson & Marasus) (61.33%). However, there was no statistically significant difference between M24 isolate and M22, M15, M12, and M73 (*Fusarium)* as well as M75 (*Phoma* Sacc.) (Fig. 1).

In the cases of inhibition halo formation, the radius was measured, and there was a significant difference among isolates for this trait ($P\leq 0.01$). The mean comparison of data showed that the greatest halo belonged to M35 and M33, with a significant difference compared to the other isolates (Fig. 2).

Antibacterial Properties of Endophytes

Out of the 89 isolates tested against *X. arboricola*, only one isolate, M35 (*Chaetosphaeronema* sp. Moesz), showed high activity (antibacterial potential 1.1) compared to the control ($P\leq 0.01$) (Fig. 3). The remaining isolates did not have any effect on this bacterium. The investigation of the antibacterial property of endophytic isolates on *S. scabies* illustrated that all *Fusarium* isolates prevented its growth. From amongst the remaining isolates, only M32 and M33 could influence the growth of this bacterium with a significant difference ($P\leq 0.01$) (Fig. 3). Three isolates, i.e., M32, M35, and M33, affected *E. coli*, and there was a significant difference among them ($P\leq 0.01$) (Fig. 3). Out of 89 fungal endophytes, eight isolates showed biocontrol effect on the human pathogenic bacterium *S. aureus* with a significant difference at 99% probability level. The greatest antibacterial activity was for M33 without any significant difference between it and M32 (Fig. 3).

Endophytic Fungal Metabolites

Antifungal Properties of Endophytic Fungal Extracts

The extracts of 14 endophytes, M24, M22, M15, M12, M75, M73, M87, M50, M35, M33, M32, M29,

M89, and M67 isolates, which showed biocontrol potential against *B. cinerea* in the dual culture test and inhibition halo formation, were used in seven concentrations of 0, 6, 12, 24, 48, 96, and 192 mg/ml. Neither the growth of fungus nor the production of inhibition halo was affected in the concentrations of 6, 12, 24, and 48 mg/ml. In the concentration of 192 mg/ml, nine out of 14 isolates formed inhibition halo, and data analysis revealed that there was a significant difference between them at 99% probability level. Following the positive control, fluconazole fungicide, M87 (*Alternaria alternate* (Fr.) Keissl.) with a halo diameter of 16 mm had the most effect on *B. cinerea.* Isolates M89 (*F. lateritium* Nees), M29 (*Fusarium* sp.), and M32 (sterile mycelium) were placed in the next group (Fig. 4). The least significant effect was related to M22 (*F. oxysporum* Schlecht.) with a halo diameter of 8 mm (Fig. 4). In a dual-culture experiment, M22 affected *B. cinerea* growth with no inhibition halo, while M89, M29, and M32 displayed remarkable inhibition halo.

Among the tested extracts in the concentration of 96 mg/ml, the extracts of M89 (*F. lateritium*), M32 (sterile mycelium), M29 (*Fusarium* sp.), and M24 (*F. subglutinans*) resulted in the reduction of growth of the fungal colony. They displayed inhibition halo, with a significant difference ($P \leq 0.01$). Following the positive control, M89 (*F. lateritium*) showed the greatest effect and located in a higher statistical group (Fig. 4). In this concentration, the extracts of fungal isolates which produced inhibition halo in a dual-culture test outperformed.

Antibacterial Properties of Endophytic Fungal Extracts

This study was carried out by the extracts of endophytic fungi, which outperformed the other isolates in a dual-culture test. Several isolates without antibacterial effect were also included in this experiment. The isolates tested against *X. arboricola* and *S. scabies* were M32, M33, M24, M22, M12, M15, M75, M73, M87, M50, M67, M89, M29, M56, M34, M38, M30, M35, and M77. The isolates tested against *E. coli* and *S. aureus* were M32, M35, M33, M73, M89, M67, M29, M24 and M22. The diameter of the inhibition halo was measured after 24–48 hours.

The concentrations of 6, 12, 24, 48, and 96 mg/ml did not affect bacterial growth and produced no inhibition halo.

In the concentration of 192 mg/ml, six isolates significantly controlled *S. scabies* ($P \leq 0.01$). The extract of M75 showed the best effect related to the formation of inhibition halo with a diameter of 34.3 mm (Fig. 5). Neither of the extracts tested in this study could control *X. arboricola*, while no inhibition halo was formed. At the same concentration, the isolates M67, M29, and M89 produced an inhibition halo against *S. aureus* that was significantly different from the negative control ($P \leq 0.01$). The extract obtained from M67 had more effects on *S. aureus*, which was located in a higher statistical group (Fig. 5). In this concentration, it was only the extract of M73 which formed an inhibition halo against *E. coli*, that had a significant difference with the negative control ($P \leq 0.01$) (Fig. 5).

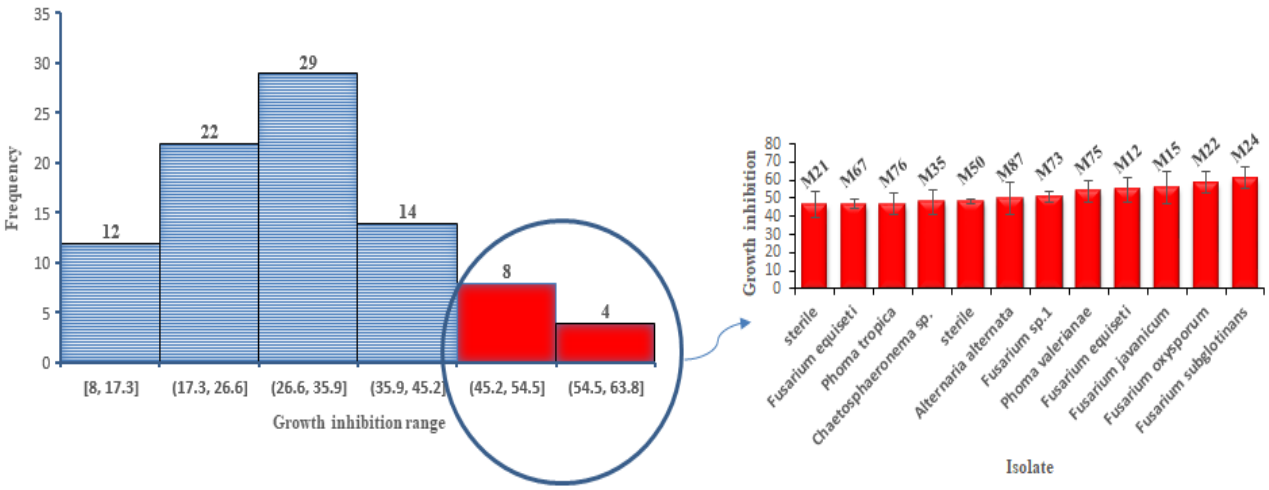

Fig. 1 Frequency of the mycelial growth inhibition of *B. cinerea* by endophytic fungi isolated from *Thymus* and showing mean comparison of the most effective isolates. Error bars represent standard error (n = 3)

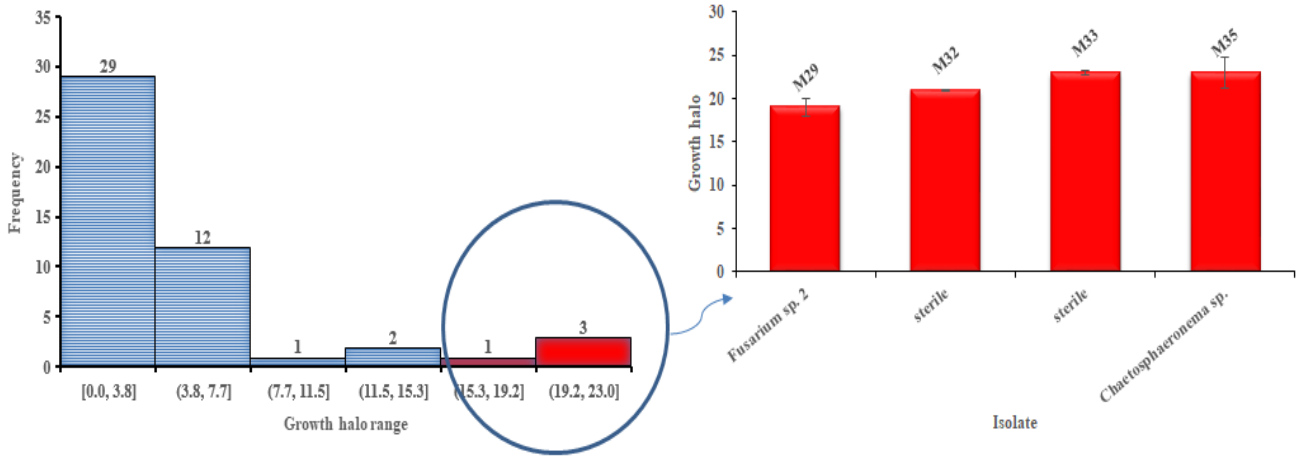

Fig. 2 Frequency of the inhibition halo in dual culture of endophytic fungi isolated from *Thymus* and *B. cinerea* and showing mean comparison of the most effective isolates. Error bars represent standard error (n = 3)

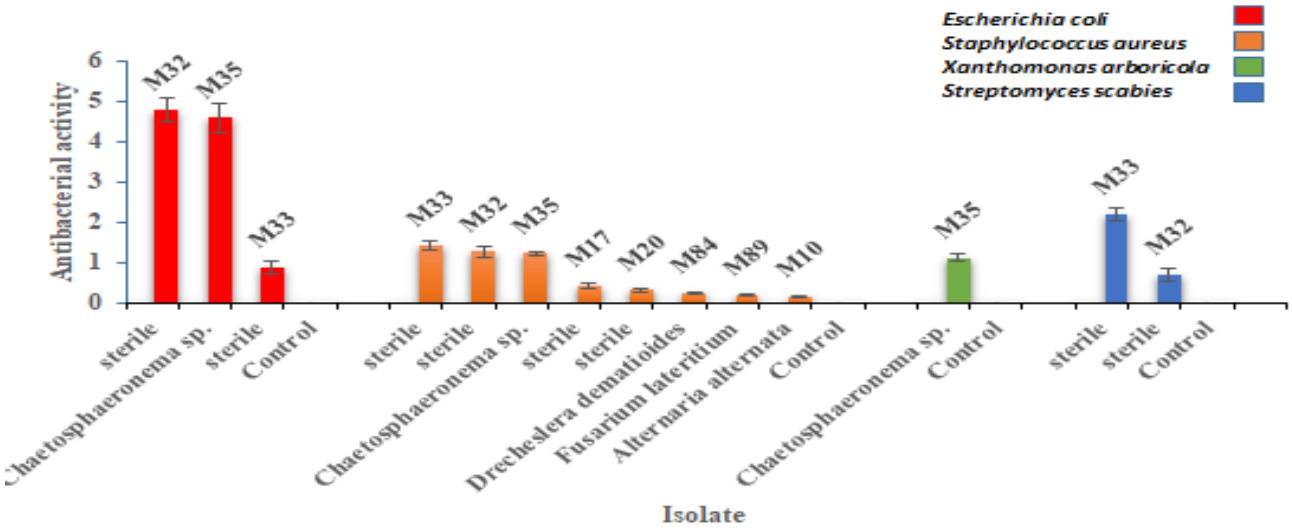

Fig. 3 Mean comparison of antibacterial activity of endophytic fungi isolated from *Thymus* sp. against *E. coli*, *S. aureus*, *X. arboricola*, and *S. scabies.* Error bars represent standard error (n = 3)

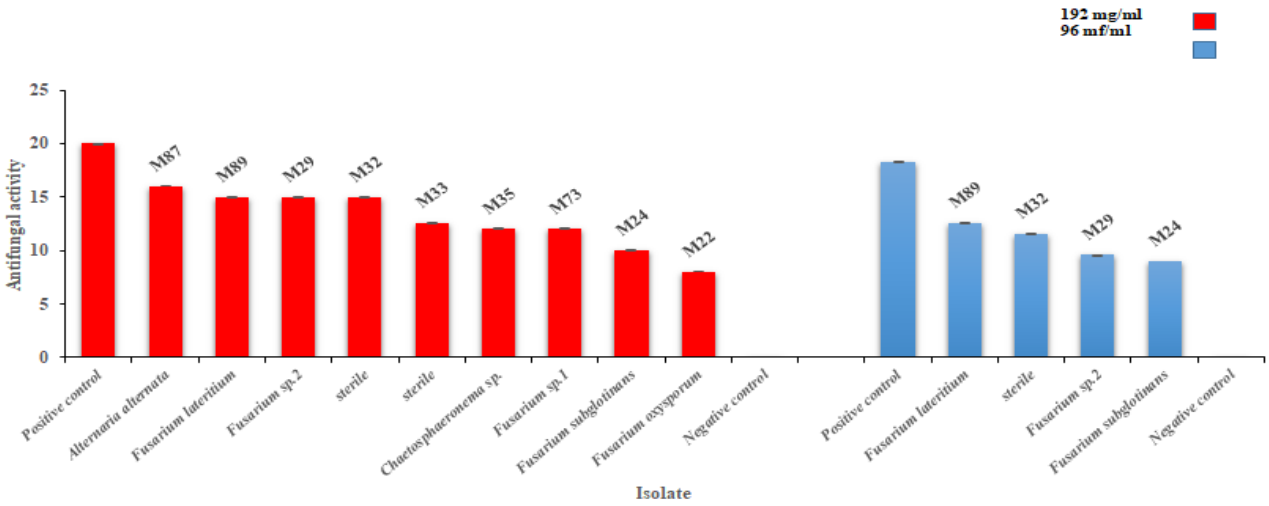

Fig. 4 Mean comparison of antifungal activity of extract of endophytic fungi isolated from *Thymus* sp. on the growth of *B. cinerea* in the concentration of 192 mg/ml and 96 mg/ml, negative control: DMSO solvent, positive control: Fluconazole fungicide. Error bars represent standard error (n = 3)

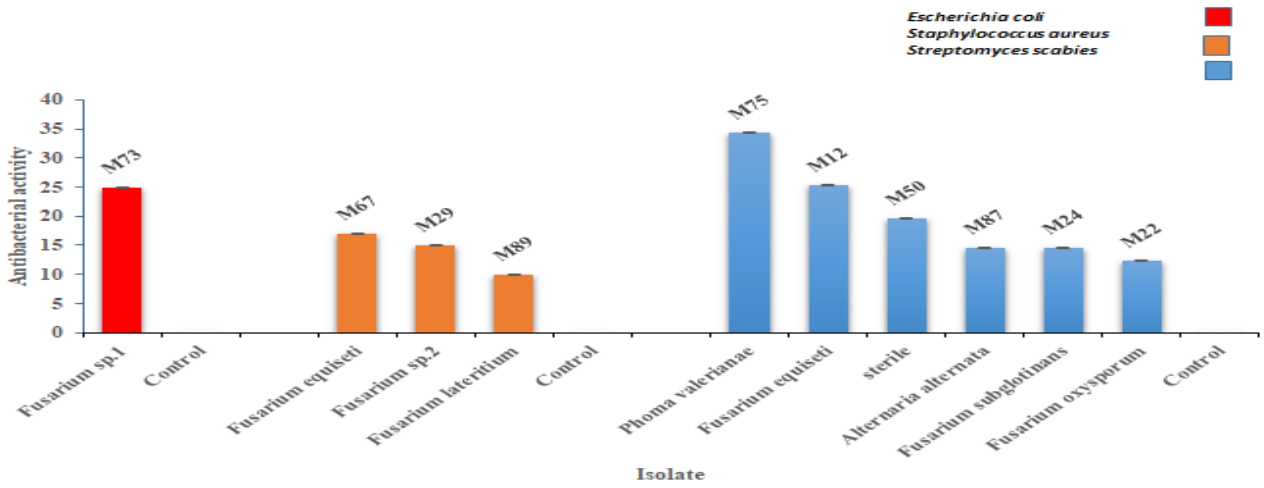

Fig. 5 Mean comparison of antibacterial activity of extract of endophytic fungi isolated from *Thymus* sp. on *E. coli*, *S. aureus* and *S. scabies.* Error bars represent standard error (n = 3)

DISCUSSION

Biological control, or the application of microorganisms and their secretions to prevent diseases, has increasingly become an alternative method without the harmful effects of chemical controls [35]. In recent years there has been great attention towards the endophytes as biocontrol agents. Being able to colonize an ecological niche similar to some phytopathogens, makes endophytes a good candidate for this purpose [9]. In this study, the effect of endophytic fungi isolated from *Thymus* was investigated against fungal (*B. cinerea*) and bacterial (*X. arboricola* pv. *Juglandis*, *E. coli*, *S. scabies*, *S. aureus*) pathogens. The results showed that some fungal endophytes, as well as their extracellular extract, had great potential to prevent the growth of the pathogens.

Among fungal endophytes, the bioeffects of some *Fusarium* species have been proven [36,37]. In addition, several reports are demonstrating the biocontrol potential of *Fusarium* species against *B. cinerea* [38–40]. In the present research, the results of the dual-culture test suggested that *Fusarium* isolates had remarkable effects on *B. cinerea.* Some isolates of this genus are in a higher statistical group because they have a faster growth rate and thus occupy the medium faster. This method is an existing strategy in fungi for the biocontrol of other microorganisms. *Fusarium* isolates also completely inhibited *S. scabies* growth. Metabolite production is another strategy that is used by microorganisms in biological control. Due to the slower growth of some endophytic isolates compared to pathogenic fungus, inhibition of *B. cinerea* growth may be related to the effect of extracellular metabolite leakage of these biocontrol agents. Endophytic isolates that produced a greater halo in dual-culture mostly performed better in the antifungal bioassay of extracellular extracts. Baghestan [41] believed that inhibition halo is different in bacterial and fungal biocontrol agents. In fungi this borderline is not permanent, and disappear after 3–5 days, but in bacterial biocontrol agents, the borderline is permanent. In some of the cases in this research, inhibition halo persisted in fungi even after two weeks. However, in our previous study [42], the halo was permanent at the border of endophytic bacteria and pathogen, which was in agreement with the results of Baghestan, confirming the permanency of the antagonist bacterial border.

In the study carried out by Pavithra *et al*. [13] on *Ocimum sanctum* L., 40 isolates of endophytic fungi were obtained. The antimicrobial activity of these endophytes against a spectrum of Gram-positive and Gram-negative bacteria, such as *E. coli* and *S. aureus*, was investigated, and the inhibition halo was observed at 10–22 mm. Jalgaonwala *et al*. [35] showed that endophytic fungi isolated from some medicinal plants, such as Basil, have an antibacterial effect against a spectrum of human pathogenic bacteria such as *E. coli* and *S. aureus*. Arora and Kaur [43] investigated the antimicrobial effects of *Moringa oleifera* endophytes on several microorganisms, and *S. aureus* showed sensitivity to most of them. The results obtained in this research confirm the antibacterial effect of endophytes on plant and human pathogenic bacteria. Isolates M32, M33, and M35 which produced conspicuous halo against *B. cinerea*, had significant antibacterial effects. Apparently, endophytic fungi affected the

pathogens through different mechanisms such as competition, antibiosis, and probably the production of volatile compounds.

CONCLUSION

Thymus is an antimicrobe plant that is commonly used in traditional medicine, and its antimicrobial effects can be exhibited by the endophytic fungi residing inside it. Due to the importance of secondary metabolites, which stem from endophytic fungi of *Thymus*, we decided to investigate the antimicrobial effect of *Thymus* endophytic fungi on plant and human pathogens. Our results confirmed the antimicrobial properties of thyme endophytic fungi and their metabolites. Finding the structure of these metabolites can be a remarkable first step in the discovery of new antimicrobial compounds.

Conflict of Interest

The authors declare that they have no conflict of interests.

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