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جداسازی و شناسایی مولکولی دو قارچ اندوفیت تولیدکنندهٔ روتین از گیاه کبر (*Capparis spinosa* L.)

محسن رجبی: دانشجوی دکتری گروه مهندسی تولید و ژنتیک گیاهی، دانشکدهٔ کشاورزی، دانشگاه زنجان، زنجان، ایران، m.rajabi@znu.ac.ir
محمدرضا عظیمی مقدم*: دانشیار گروه مهندسی تولید و ژنتیک گیاهی، دانشکدهٔ کشاورزی، دانشگاه زنجان، زنجان، ایران، azimi@znu.ac.ir
علی عزیزی: دانشیار گروه باغبانی، دانشکدهٔ کشاورزی، دانشگاه بوعلی‌سینا، همدان، ایران، azizi@basu.ac.ir
جلال سلطانی دانشیار گروه گیاه پزشکی، دانشکدهٔ کشاورزی، دانشگاه بوعلی‌سینا، همدان، ایران، soltani@basu.ac.ir

چکیده

مقدمه: فلاونوئیدها گروهی از ترکیبات پلی‌فنولیک‌اند که در اندام‌های مختلف گیاهان عالی به‌طور طبیعی یافت شده‌اند. بسیاری از فلاونوئیدها پایه ساخت داروهای گیاهی هستند و اثرات فارماکولوژیکی مؤثری دارند. روتین نیز یکی از فلاونوئیدهایی است که به‌عنوان معرف در مطالعات فلاونوئیدها کاربرد داشته است و نیز یک آنتی‌اکسیدان محسوب می‌شود. این مطالعه به‌منظور جداسازی و شناسایی مولکولی قارچ‌های اندوفیت تولیدکنندهٔ روتین از گیاه کَبَر انجام گرفت.

مواد و روش‌ها: نمونه‌برداری از ۱۰ منطقه ایران و از بافت‌های مختلف (شامل برگ، ساقه، ریشه و میوه) انجام شد. نمونه‌ها پس از ضدعفونی سطحی روی محیط کشت PDA قرار گرفتند و جدایه‌های قارچی به‌دست‌آمده خالص‌سازی شدند. شناسایی مولکولی جدایه‌ها با توالی‌یابی ناحیه ITS1-5.8S-ITS2 انجام شد. شناسایی مورفولوژیکی قارچ‌ها نیز با روش تهیه اسلاید میکروسکوپی و کلیدهای شناسایی قارچ‌ها انجام شد. عصاره متانلی قارچ‌ها به روش خیساندن، تهیه و وجود روتین با استفاده از تکنیک HPLC بررسی شد.

* نویسندهٔ مسئول مکاتبات

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نتایج: در مجموع ۲۴ قارچ شناسایی شدند. تجزیه و تحلیل HPLC نشان داد پیک فلاونوئیدهای استاندارد شامل روتین، کوئرستین و آپی‌جنین به‌ترتیب در ۳/۸۲، ۷/۶۰ و ۱۲/۴۰ دقیقه ظاهر شد. نتایج نشان دادند تنها دو جدایه قارچی قادر به تولید روتین بودند. مقایسه میانگین تولید روتین تفاوت معنی‌داری را نشان داد. قارچ *Alternaria alternata* M28 بیشترین میزان تولید روتین را داشت (۲۲/۹۴ پی‌پی‌ام). همچنین نتایج نشان دادند تولید روتین در بافت میسلیوم بیشتر از محیط خارج سلولی است؛ به‌طوری‌که در *A. alternata* M28 و *Paecilomyces maximus* M7 به‌ترتیب حدود ۶۷/۵ و ۵/۵۶ برابر روتین در میسلیوم تولید می‌شود.

بحث و نتیجه‌گیری: روتین قارچی موجود در عصاره‌های متانولی با HPLC مشخص شد. این نخستین گزارشی است که نشان می‌دهد قارچ‌های اندوفیت پتانسیل پذیرفتنی برای تشکیل روتین از گیاه *C. spinosa* دارند و تحقیقات بیشتر روی این جدایه‌ها می‌تواند میزان تولید روتین را در آنها افزایش دهد و احتمالاً به تجاری‌سازی منجر شود.

واژه‌های کلیدی: فلاونوئیدها، HPLC، روتین، کَبَر

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Isolation and Molecular Identification of Two Rutin-producing Endophytic Fungi from Caper (*Capparis spinosa* L.)

Mohsen Rajabi
Department of Plant Production and Genetic, Faculty of Agriculture, University of Zanjan, Zanjan, Iran, m.rajabi@znu.ac.ir
Mohammad Reza Azimi Moghadam*
Department of Plant Production and Genetic, Faculty of Agriculture, University of Zanjan, Zanjan, Iran, azimi@znu.ac.ir
Ali Azizi
Department of Horticulture, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran, azizi@basu.ac.ir
Jalal Soltani
Plant Pathology Section, Plant Protection Department, Bu-Ali Sina University, Hamedan, Iran, soltani.soltani@basu.ac.ir

Abstract

Introduction: Flavonoids are a group of polyphenolic compounds naturally found in plants. Many flavonoids are the basis of herbal medicines and have effective pharmacological effects. Rutin is one of the flavonoids used as a reagent in the study of flavonoids and is also considered an antioxidant. This study was conducted to isolate and identify rutin-producing endophytic fungi from *C. spinosa*.

Materials and Methods: Sampling was performed in 10 regions of Iran and was taken from different tissues (including leaves, stems, roots, and fruits). After surface disinfection, explants were placed on a PDA medium and the fungal isolates were purified. Molecular identification of isolates was performed by sequencing ITS1-5.8S-ITS2. Morphological identification of fungi was performed by microscopic slide preparation and fungus identification keys. Methanolic extract of fungi was prepared by the maceration method and the presence of rutin was investigated using the HPLC technique.

Results: A total of 24 fungi were identified. HPLC analysis showed that the peak of standard flavonoids including rutin, quercetin, and apigenin appeared at 3.82, 7.60, and 12.40 min, respectively. Results showed that only two isolates were able to produce rutin. A comparison of the mean production of rutin showed a significant difference between the two fungi. *Alternaria alternata* M28 (22.94 ppm) had the highest amount of rutin production. Results also showed that rutin production in the mycelial tissue was more than

*Corresponding Author

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in the extracellular medium, so *A. alternata* M28 and *Paecilomyces maximus* M7 had rutin content of about 67.5 and 5.56 times, respectively.

Discussion and Conclusion: Fungal rutin in methanolic extracts was characterized by HPLC. This is the first report showing that endophytic fungi have a potential for rutin production from *C. spinosa* and more research on these isolates can increase the amount of rutin production in them and lead to the possible commercialization.

Key words: Flavonoids, HPLC, Rutin, Caper

Introduction

Endophytes are microorganisms such as bacteria and fungi that live at least one stage of their lifecycle in plants without causing obvious damage, symptoms, or negative effects on the host. These microorganisms were known in the 19th century but were not studied extensively (1). They have been found in many plant species and have been identified as a potential source of effective natural compounds for use in medicine, agriculture, and industry (2). Some endophytes synthesize chemical compounds in plants. Many of them are able to synthesize effective compounds that can be used by plants to defend against pathogens, and some of them can be useful in discovering new drugs (2, 3). So far, effective compounds such as antibiotics, anticancer agents, biological control agents, and other functional compounds have been obtained from endophytes (4).

In general, the biological aspects and the interaction between endophytes and their hosts are exciting topics that have been extensively studied (5, 6). In fact, more studies at the molecular level should be done on the interaction and ecology of endophytes (7). The genetic relation between endophyte and host for creating an interaction is a complex and unknown process that involves the selective expression of fungal genes responsible for the production of enzymes and secondary metabolites which helps in cloning (8, 9).

Many of these secondary metabolites are antibacterial, antifungal, antiviral, anti-inflammatory, anti-tumor, and anti-malarial which belong to different classes such as alkaloids, terpenoids, flavonoids, phenolic compounds, and steroids (5, 10). Some endophytic fungi can produce secondary metabolites similar to their hosts, which are essential medicinal drugs, such as taxol and increase the likelihood of using these endophytes as an alternative and stable source for faster production of these compounds than plants (11). It has also been found that these organisms make plants more resistant to some stresses such as water shortage (3). Endophytes, on the other hand, secrete plant hormones and other substances such as enzymes and, in some cases, effective drug compounds (for human health) that are important in biotechnology and medicine (12) .Qiu et al. (13) reported two flavonoid-producing endophytic fungi such as *Aspergillus nidulans* (ST22) and *Aspergillus oryzae* (SX10) from *Ginkgo biloba*. Also, Zafari et al. (14) introduced *Chaetomium globosum Cr95* as vinblastine producers from *Catharanthus roseus.*

The Caper (*Capparis spinosa* L.) grows as a wild plant in crevices of rocks, on top of mud walls, in ruined areas, and generally in any soil with minimal nutrients. Areas with a rainfall of 350 mm per year are ideal for this plant and produce a good yield (15). Resistance to drought, salinity, heat, and the intense sun has caused this invasive plant to be distributed in different parts of the provinces of Iran (16) .This plant has rutin (17) and results of Kianersi et al. (18) suggest that rutin content and the

expression patterns of rutin biosynthesis genes in Caper can be significantly enhanced by the salicylic acid and methyl jasmonate treatments in a growth stage-dependent manner.

Therefore, the present study aimed to identify flavonoid-producing endophytes that may be isolated from *C. spinosa*.

Materials and Methods

Collecting Plant Samples: In the present study, due to the widespread distribution of *C. spinosa* in Iran, in July 2017 (summer), 10 areas were selected for study and four mature and healthy plants were selected from each study area. Sampling was carried out based on a randomized systematic sampling design (19). We collected and placed explants (including roots, leaves, stems, and fruits) in paper bags and kept in cool conditions (4-6°C), and transferred them immediately to the Plant Pathology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Bu-Ali Sina University.

Isolation of Endophytic Fungi: Explants were washed under running tap water to remove dust. Then, four pieces (about 10 mm) were cut from each organ according to the protocol of Suryanarayanan et al. (20). The samples were surface-sterilized successively with 70% ethanol for 2 min. They were then exposed to 2% sodium hypochlorite for 5 min. Then, we washed them three times with sterile distilled water for 5 min and finally, the samples were dried on sterile filtering papers. After drying, the samples were placed on plates containing a PDA medium. The plates were incubated for 2 weeks at 27±2°C and 8/16 h light/dark cycle. After growing the endophytic fungi and re-culturing, purification was performed using the hyphal tip method on a WA (Water Agar) medium. To ensure the purity of the colonies, the fungus isolation procedure was repeated three times.

Morphological Identification of Endophytic Fungi: Morphological traits such as colony characteristics, shape, and color of the observe and reveres view of Plate, colony diameter and margin shape after 7 days, conidia and conidiophores characteristics (body and beak length, shape and size, arrangement of conidia on conidiophores, number of conidia on conidiophores and branching pattern including, number of longitudinal and transverse septa of twenty conidia per isolate), Phialides shape, and size were studied and measured using mycological keys based on Simmons and Samson (21, 22). Preliminary identification of fungi was performed in a specific culture medium for seven days (27±2°C, 75.5±3% RH). In order to obtain a microscopic image of endophytic fungi, the method of staining with blue lacto-phenol solution (23) was used. Then, the image was observed under an optical microscope (Nikon 80i model) with 40X and 100X lenses. In this staining method, lactic acid penetrates into the mycelium of the fungus and preserves the structure of the fungus. Phenol inactivates viable cells and stains the fungal composition of the fungus, and glycerol causes the dye to become semi-permanent and possibly reduces dye deposition.

Molecular identification of endophytic fungi

DNA extraction of endophytic fungi: A liquid culture medium (Potato Dextrose Broth; QUELAB Canada) was used to extract DNA from endophytic fungi. For DNA extraction, five discs (0.5 cm diameter) were added to the Erlenmeyer containing the PDB. Then, they were placed on a shaker at 120 rpm for three weeks. After fungi growth, DNA extraction was performed based on the method of Moller et al. (24) using SDS-CTAB with some modifications. The quality of the

extracted DNA was evaluated using on agarose gel electrophoresis technique. The spectrophotometer was used for quantitative DNA analysis and the absorption rates were measured and recorded at 260 and 280 nm, respectively.

Polymerase Chain Reaction (PCR): The polymerase chain reaction (PCR) mixture was performed according to the company protocol (Sina Clone Co.) in a volume of 25 µl (25). Universal primers (ITS1 and ITS4) were used to identify endophytic fungi (Table 1). To perform the polymerase chain reaction, a thermocycler (Biorad) was used according to the following program (26): 5 min for primary DNA denaturation at 95°C, 60 seconds for secondary DNA denaturation at 95°C, 45 seconds for annealing temperature at 55°C, 60 seconds DNA extension at 72°C, and 6 min for the final extension at 72°C.

Table 1- The Sequence of Primers Used to Determine the Species of Studied Fungi

Gene name	Primer name	Nucleotide sequence	Length (base pair)
ITS1	Universal-1	5' TCC GTA GGT GAA CCT GCG G 3'	500-600
ITS4	Universal-2	5' TCC TCC GCT TAT TGA TAT GC 3'	

Phylogenetic Analysis: PCR products were sent to Microsynth Company (Switzerland) for sequencing. The quality of sequencing was checked with Chromas 2.6.6 software. The NCBI site and the BLASTN command were then used to identify the isolates. In NCBI, the successes (cases where the match occurred) are displayed graphically. In addition, a table that shows the sequence of matches with the points given to each. Using the heuristic method, the blast algorithm finds matched sequences. According to the performed blast and their comparison with the ITS region sequence of the fungi registered in the NCBI, the most similarities were examined and the desired strains were identified as genus or species (27). To classify the obtained sequences, the phylogenetic tree of the sequences was drawn using Mega X software and the neighbor-joining method (Kimura two-parameter distance calculation) (28). For each search, 1,000 replicates of random stepwise sequence addition were performed. Statistical support for the internal branches was estimated by bootstrap analysis with 1,000 replications.

Preparing the Crude Extract from Endophytic Fungi: The Maceration method was used to obtain an extract from endophytic fungi. Purified fungi were first cultured in an Erlenmeyer flask containing PDB (200 ml). The Erlenmeyer was continuously placed on a rotary shaker at a speed of 120 rpm for three weeks. The solid part containing the mycelium of the fungi was then isolated in an Erlenmeyer flask containing 150 ml of methanol and placed on a rotary shaker for 48 hours at 120 rpm. Also, 150 ml of methanol was added to the PDB. The extracts were then filtered through the Whatman filter paper. The filtered solution was poured into sterile plates and placed in an oven at 40°C for about four days. Then, extracts were dried, weighed, and kept at -20°C for later use (29).

Flavonoids Detection using HPLC-UV Analysis: In order to detect the presence of rutin, quercetin, and apigenin (as flavonoids) in the endophytic fungi isolated from *C. spinosa,* we used the HPLC analysis device (KNAUER, Germany) in the National Forest and Rangeland Research Institute of Iran. First, the standard components were prepared at a concentration of 1000 ppm. Then,

methanolic (HPLC grade) extracts of fungi were prepared at a concentration of 500 ppm and filtered with 0.45 µm syringe filters. The column in this device was C18 type (Eurosphere 5-100) based on non-polar silica gel and had a length of 250 mm (with pre-column) with a thickness of 4.6 mm. The wavelength was used to detect the compounds in such a way that at a time of 0-6 min a wavelength of 257 nm and at a time of 6-16 min a wavelength of 340 nm with a UV detector were obtained. About 80 µl of the extract solution was injected into the HPLC device according to the following program: The mobile phase consisted of methanol: aceto-nitrile: distilled water (40:15:45 v/v/v) containing 1% acetic acid and a flow rate of 1 ml min^{-1} at normal laboratory temperature.

Results

Molecular and Morphological Identification of Endophytes: In this study, the identification of endophytic fungi isolated from *C. spinosa* (including leaves, stems, roots, and fruits) was examined. For their fungal endophytic content, a total of 640 pieces were tested. Then, 72 fungal isolates were recovered from the samples after 7 days of incubation.

Results of sequence blasting showed that the endophytic isolate identified as *Paecilomyces maximus* M7 (MW136453.1) had 100% sequence similarity with all *Paecilomyces maximus* 3 (MH345843.1), *Paecilomyces maximus* FB2 (MW940811.1) *Paecilomyces maximus* M7 (MW130831.1), and *Paecilomyces formosus* (MK179407.1) (Table 2). Also, the results of the dendrogram along with similar sequences showed that this fungus was in the close group with *Paecilomyces maximus* FB2 (MW940811.1) and caused confirmation (Figure 6).

Also isolated endophyte identified as *A. alternata* M28 (MW130846.1) had 100% sequence similarity with all *A. alternata* DT18108-A (MW009034.1), *A. alternata* UASB2 (MT742643.1), and *Alternaria tenuissima* DT1865-A (MW009017.1) (Table 2). Also, the results of the dendrogram with 11 sequences with the highest similarity showed that this fungus was placed in the same branch with *A. alternata* DT18108-A (MW009034.1) and confirmed the desired genus (Figure 7).

Morphological results also showed that *Paecilomyces maximus* M7 endophytic fungi had a yellow to brown smooth colony on PDA. The radial growth of the colony on PDA for 7 days was 2.5 cm. Conidiophores were irregularly branched with one or more branched Phialides. Phialides were flask-shaped with cylindrical bases. Conidia was one-celled, chain-like, lemon-brown, and elliptical to cylindrical with a truncated tip. Chlamydospores have thick walls, round or oval- shapes, and brown colors with sizes 3.97-6.07× 3.94-5.18 µm (Figure 4).

Also, *A. alternata* M28 had a gray and velvety colony. Conidiophores were simple, branching, and sporulation occurred directly from the surface of the mycelium. Conidiophores formed branching chains with free branches (Figure 5).

Screening the Flavonoids Producer Endophytes: By separating and purifying the obtained endophytic fungi, finally, 24 isolates were identified from the *C. spinosa*. Endophytic fungi were completely different based on morphological characteristics such as colony shape, color, colony margin, and growth rate. A comparison of dry weight in the studied fungi showed that these fungi had different growth rates so that some of them grew faster and grew rapidly in 21 days after culturing in a PDB medium and formed a dense mass. *P. maximus* M7 also had a growth rate of about twice in comparison with *A. alternata* M28 (Figure 1).

In total, 48 extracts were tested for the presence of metabolites (flavonoids). In

certain cases, the peaks observed in the extracts correspond well to the retention time of the standard compounds. The results of HPLC analysis showed that the peak of standard flavonoids including rutin, quercetin, and apgenin appeared at 3.82, 7.60, and 12.40 min after injection into the column, respectively. Examination of fungal samples indicates that some of these samples have a specific peak at the time of peak belonging to the rutin, which results show that rutin was present in these two fungi. Examination of 24 isolated endophytic fungal isolates showed that only two of them were able to produce rutin. Analysis of mean comparison (using independent t-test) showed that there was a significant difference in rutin content between the two fungi. Among the endophytic fungi producing rutin, *P. maximus* M7 produced 18.532 and 3.334 ppm of rutin in mycelium and medium, respectively (Figures 2a and 2b) (Table 3).

A. alternata M28 with the production of 22.947 ppm had the highest amount of rutin production (Figures 3a and 3b). The results also showed that the rate of rutin production in the mycelial tissue of endophytic fungi was more than in extracellular fluid culture medium. So, in *A. alternata* M28 (about 67.5 times) and *Paecilomyces maximus* M7 (about 5.56 times) respectively with production of 22.612 ppm and 18.532 in mycelial tissue had rutin production compared to 0.335 ppm and 3.334 ppm in liquid culture medium. Also, the total rutin content in both fungi was similar and not significant (Table 3). There was a significant difference between the two fungi related to fungal and liquid medium extracts ($P<0.01$).

It is important to emphasize that metabolites were observed only in mycelial extracts, suggesting that the compounds may be produced intracellularly or related to the surface of the fungal cell wall.

Table 2- Characteristics of Rutin Producer Endophytic Fungi Sequences and their Access Number in Gene Bank

Code	The closest identified phylogenetic species (GenBank species with the highest percentage of similarity*)	Organs	Similarity percentage	E-value	The length of the sequence (Base pair)	Sampling area (Province)	Gene Bank accession Number (NCBI)
M1	*A. alternata* M28	Fruit	99.22	0.00	570	Ahvaz (Khuzestan)	MW130846
M2	*P. maximus* M7	Root	100	0.00	590	Khoda Afarin (East Azarbaijan)	MW136453

*GenBank species with the highest similarity (99%) to Caper endophytes based on megablasts of ITS1 and ITS4 sequences. In all megablast searches, GeneBank changes were identified with E-values = 0.

Table 3- Comparison of Mean (±STD) of Rutin Production and Dry Extract in Endophytic Fungi isolated from *C. spinosa*

Components	*P. maximus M7*	*A. alternata M28*	P value
Rutin content in mycelium extract (ppm)	18.532±0.87	22.612±0.98	0.008
Rutin content in liquid medium extract (ppm)	3.334±0.098	0.335±0.045	0.034
Total Rutin content (ppm)	21.8660±0.054	22.947±0.097	0.106
Fungal extract (g)	0.0203±0.0012	0.0120±0.0010	0.001
liquid medium extract (g)	0.0180±0.0085z	0.0240±0.0041	0.002
Total Extract (g)	0.0383±0.0022	0.0360±0.0014	0.057

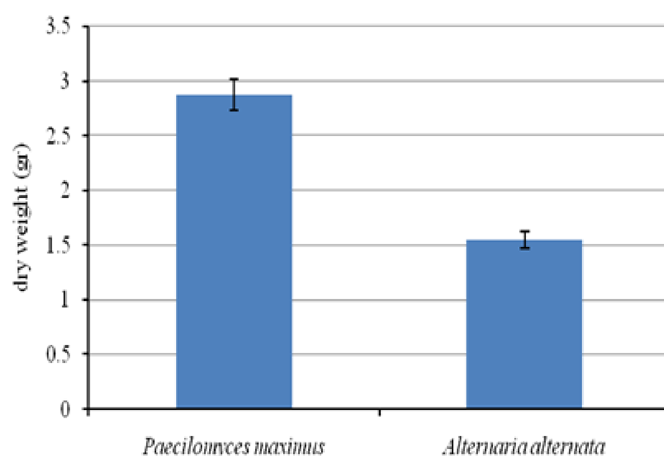

Fig. 1- Comparison of Dry Weight in Rutin-producing Fungi (Gram) on the PDB Medium

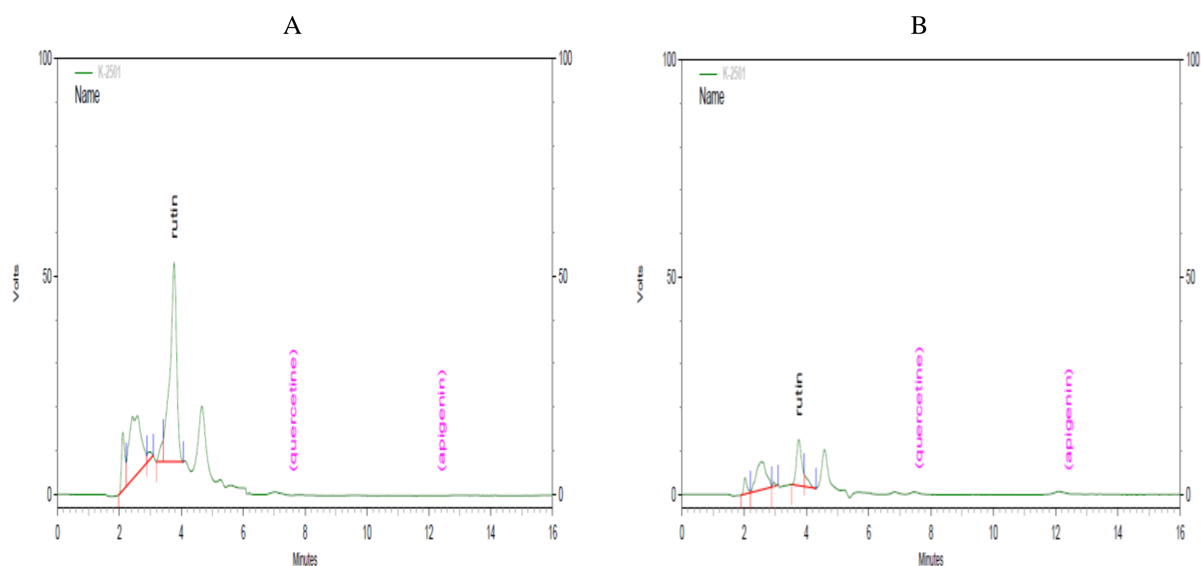

Fig. 2- HPLC Chromatogram Of Mycelium Extract (A) and Medium Extract (B) of *Paecilomyces maximus* M7

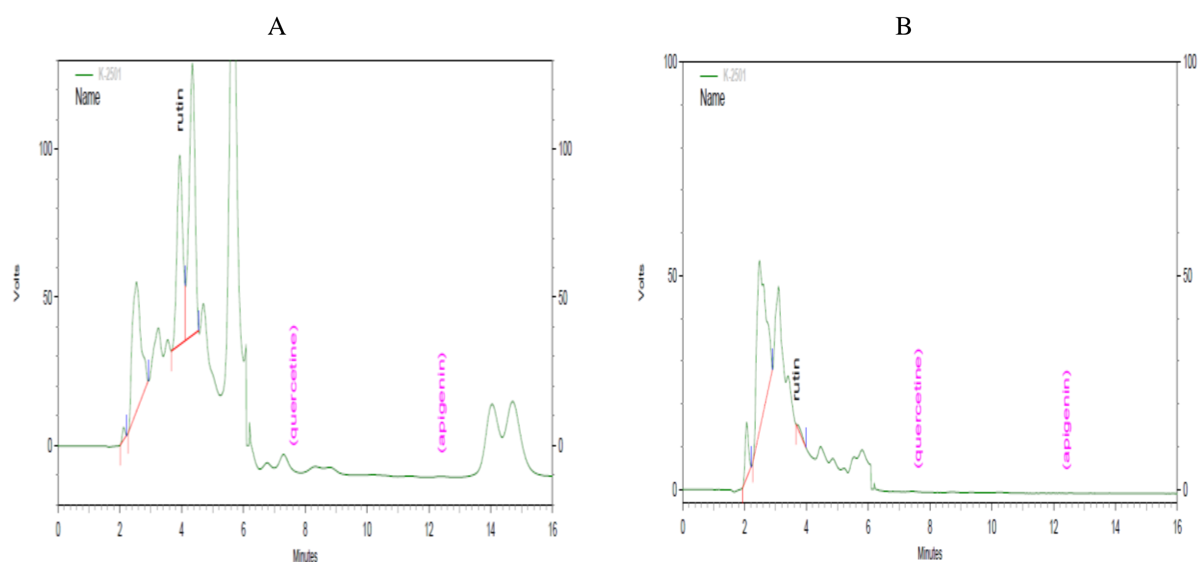

Fig. 3- HPLC Chromatogram of Mycelium Extract (A) and Medium Extract (B) of *A. alternata* M28

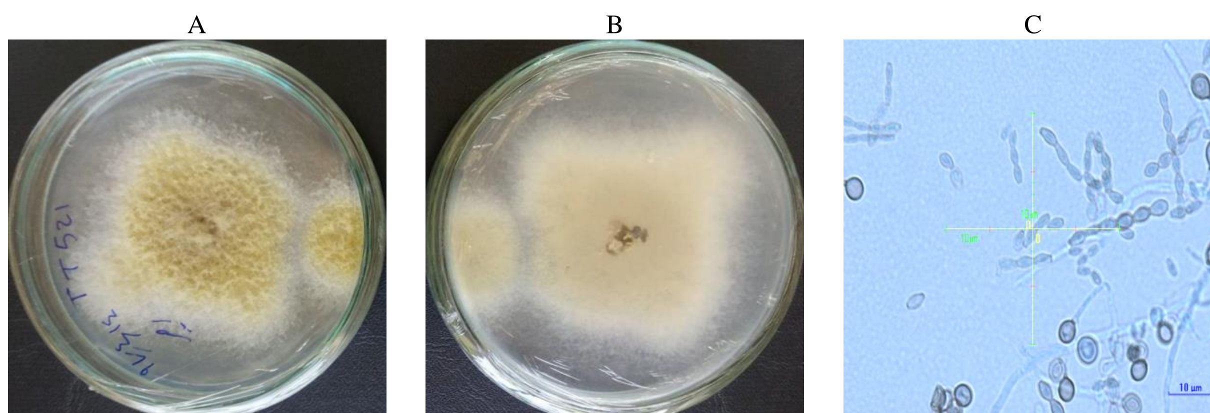

Fig. 4- The Obverse (A) and Reverse (B) of the Colony of *Paecilomyces maximus* M7 Growing on a PDA after 7 Days. C. (Conidiophores and conidia) (10 µm scale)

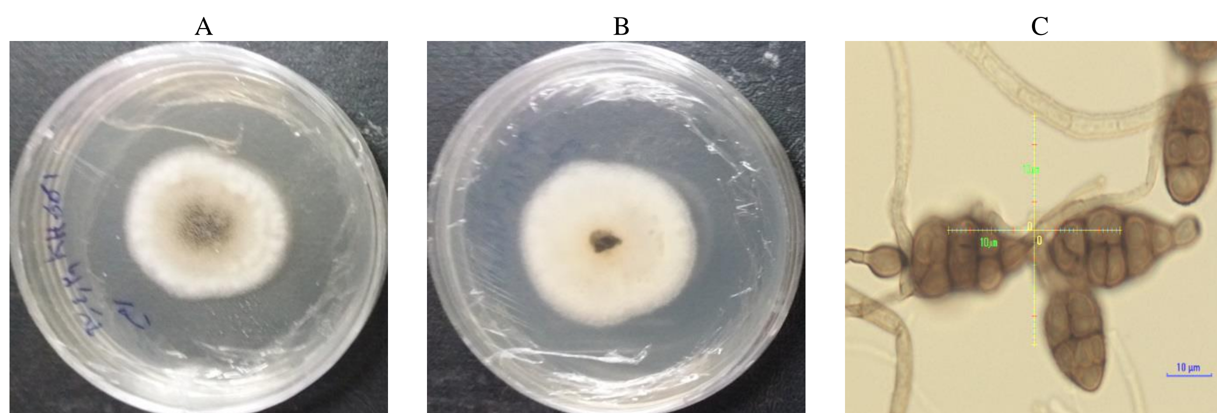

Fig. 5- The Obverse (A) and Reverse (B) of the Colony of *Alternaria alternata* M28 Growing on a PDA after 7 Days. C. (Conidiophores and conidia) (10 µm scale)

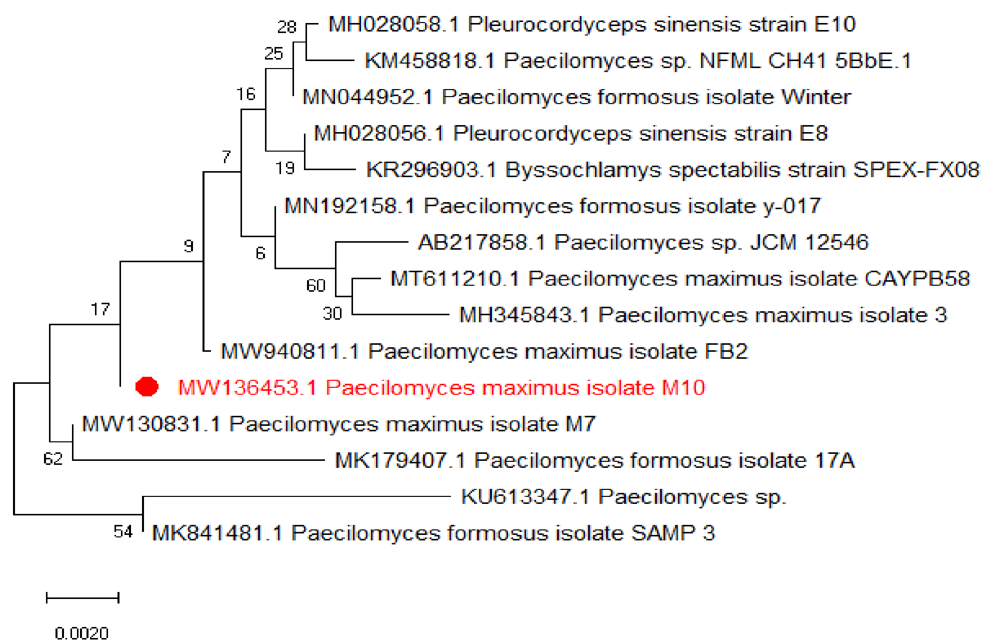

Fig. 6- Neighbor-joining tree of fungal endophyte *Paecilomyces maximus* M7 based on ITS1-5.8S-ITS2 sequences. Its phylogenetic tree was drawn with 13 sequences derived from blast results. The closest evolutionary relationship is observed with *Paecilomyces maximus* FB2.

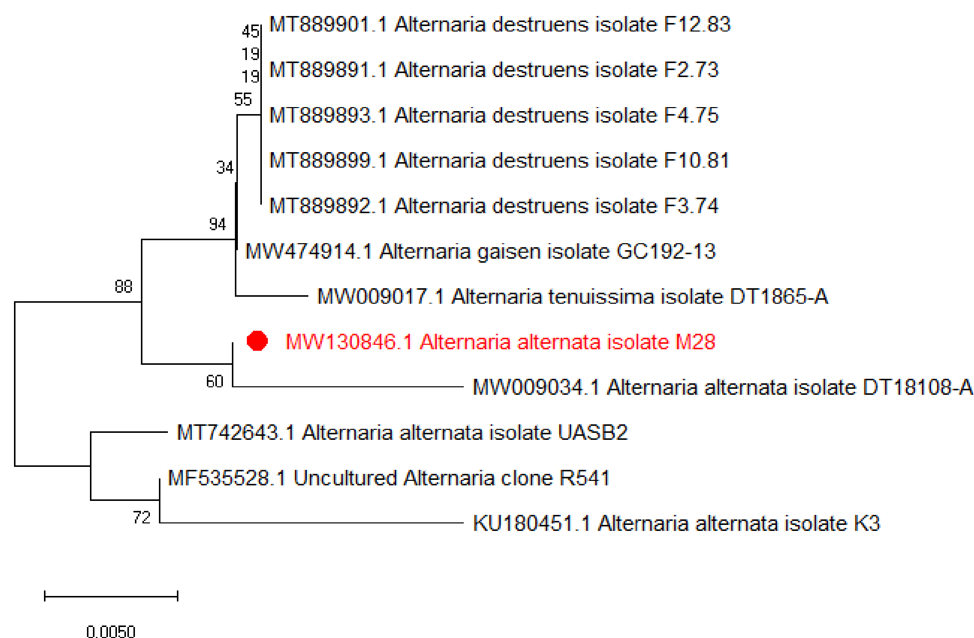

Fig. 7- Neighbor-joining tree of fungal endophyte *A. alternata* M28 based on ITS1-5.8S-ITS2 sequences. Its phylogenetic tree was drawn with 13 sequences derived from blast results. The closest evolutionary relationship is observed with *A. alternata* DT18108-A.

Discussion and Conclusion

In order to ensure the isolation of endophytic fungi and the elimination of epiphytic and pathogenic fungi, the samples were selected from completely healthy tissue and free of any symptom of the disease. Most pathogenic and non-endophytic fungi were removed by surface sterilization of explants. Also, by fixing the inner part of explants on the PDA medium, it was found that the studied fungi started their growth from plant samples and as a result, the grown fungi colonies were not caused by environmental contamination in comparison to the control factor.

Endophytes are parts of a fungal diversity that are not yet fully understood. Endophytes are an interesting field for the discovery of medicine, which is considered a hidden member of the microbial world and is a less used source for the treatment and production of new compounds (30). The study of endophytic fungi as a source of bioactive secondary metabolites first began in 1993 with the discovery of taxol. Until then, the primary sources of active natural molecules were largely isolated from plants (31). There are many possibilities for utilizing endophytic fungi to produce a wide range of important bioactive metabolites for human health. So, in the last ten years, more than 300 endophytes with the potential to synthesize metabolites with therapeutic values have been isolated and successfully cultured in laboratory conditions (32).

Endophytic fungi are capable of producing secondary metabolites similar to their host, such as Sesquiterpenoids which are a class of terpenes with significant antibacterial activity. They have been isolated from endophytic fungi capable of producing sesquiterpenes such as *Aspergillus* sp. (recovered from *Xylocarpus moluccensis*) and *Phomopsis* sp. (recovered from *Phyllanthus glaucus*) (33, 34). *A. alternata* and *Fusarium proliferatum* (Endophytic fungi) isolated from *Salvia miltiorrhiza* were able to synthesize flavonoids (35) which could confirm the results of our research. Also, they can highlight the ability of *Alternaria* to produce rutin.

Many studies have been conducted on the production of secondary metabolites by endophytic fungi. Most of them are related to the taxol, which is due to the high importance of this compound. Strobel et al. (36) showed that among 33 endophytic fungi isolated from *T. wallachiana*, only one isolate named *Pestalotiopsis microspora* can produce 60 to 70 µg/L taxol. Also, Caruse et al. (37) isolated 150 endophytic fungi from *T. baccata* and the rate of taxol production in these fungi was very low and only in some samples, this amount reached 50-100 ng/L. Wang et al. (38) isolated 21 fungal isolates from *T. chinensis*, among which strain BT2 had the ability to produce taxol at the rate of 12-18 µg/L.

In this research, the isolation of 72 isolates of endophytic fungi from *C. spinosa* in Iran and their ability to produce rutin in 2 isolates shows that the frequency and diversity of rutin-producing endophytic fungi in the studied samples are different from other studies conducted in other parts of the world. Qiu et al. (13) reported that the concentrations of total phenolic and flavonoids in strain ST22 culture broth were 0.1413 and 0.01162 mg/ml, respectively, and the amounts of total phenolic and flavonoids in strain SX10 samples were 0.1450 and 0.01256 mg/ml, respectively (13). Meanwhile, the amount of rutin production in the isolated fungi in this research compared to the fungi isolated from other species such as *Ginkgo biloba* showed that these isolates have a potential for rutin production.

Some endophytic fungi have been determined as phenolic or flavonoid producers. Huang et al. (39) isolated 42 endophytic fungal strains from *Nerium oleander* and clarified that some phenolic compounds were found to more likely coexist with certain endophytic fungi in the same plants.

Our results indicate that the highest rate of rutin production was achieved in the mycelial tissue of fungi in 21 days, while an insignificant amount was detected in the liquid medium of the fungus, which probably led to the hypothesis that fungi do not allow the releasing of some compounds easily due to their Chitin cell wall (40). In this study, the rutin secreted in the mycelial tissue of the fungus and the medium was evaluated, because the produced rutin by the fungus is partially excreted in the medium, and rutin extraction from the mycelial tissue and medium is easier, faster, and more cost-effective. It makes the possibility of bringing its production to an economic and commercial level on a large scale using the fermenter.

However, one of the biggest problems with using fungi in rutin production is their low and unstable yield. But the cheaper medium used for the growth of fungi in comparison to the plant tissue culture and the shorter process of isolation and extraction from the fungus has drawn attention as a source of rutin production on industrial and commercial scales. By adding some precursors and elicitors, it may be possible to increase rutin production in endophytic fungi from *C. spinosa* .Therefore, it is very important to study different types and different concentrations of elicitors and precursors in order to increase the production of flavonoids, including rutin. On the other hand, benzoic acid can be mentioned, which has increased taxol production by about eight folds in very low concentrations of about 0.01 mM (41). In this study, plant samples have been selected to isolate endophytic fungi from the north, northwest, and west of Iran, so it seems necessary to study other regions of the country to isolate endophytic fungi in these areas.

The present study was based on the observations that some certain and important endophytic fungi are able to produce metabolites similar to their hosts

and therefore can be stored as new microbial sources of plant bioactive metabolites (29, 42). In our study, isolates producing rutin were identified as members of the *Alternaria* and *Paecilomyces*. *Paecilomyces* is a hyphomycete fungus with more than 100 known species. This genus is one of the string fungi that has different habitats such as soil, decaying plant materials, food, and insects and it can even cause disease in humans (43, 44). At first, this genus was described by Bainier (45) and is very similar to the *Aspergillus*. Ram (46) and Pitt (47) introduced *P. maximus* based on colony properties and conidia size. At first, *Alternaria* isolates were identified only at the genus level because members of the *A. alternata* species group including A. *tenuissima, A. arborescens,* and *A. alternata* could not be identified based on ITS sequence (48) .Finally, our results showed that based on colony properties, conidia size, and phylogenic dendrogram of this fungus is close to *A. alternata.* Sequence results of the ITS region (49), which are synonymous with *P. formosus* and actually include *P. maximus* and *P. lecythidis, P. formosus* and finally colony properties, conidia size, and phylogenic dendrogram showed that our isolated fungus is close to *P. maximus.*

Plant-related *Alternaria* species have previously been shown to be able to produce host metabolites such as methyl eugenol (50), capsaicin (51), and paclitaxel (52), but to our Knowledge, the ability to produce rutin has not been reported.

Although the number of flavonoids, especially rutin originating from the plant is relatively high, microbiological sources can offer new insights into their future industrial production. Microbiological fermentation can be stable, strong, and reproducible yields from one batch to another due to culture parameters that are well controlled .These results suggest that *A. alternata* M28 and *P. maximus* M7 can be the potential source of natural products. However, the safety of these two isolates is unknown. More research should be carried out to ensure that the isolates as the potential natural source is nontoxigenic and nonpathogenic for humans, plants, and ecosystems. Alternatively, some suitable techniques can be used to isolate the phenolic and flavonoids.

In general, it can be concluded that some endophytic fungi isolated from different plants are able to produce secondary metabolites similar to their host, which in addition to economic benefits, can prevent the destruction of rare plants. Isolation of 24 endophytic fungal isolates in this study from Caper and rutin production capability in two covered isolates showed that *A. alternata* M28 and *P. maximus* M7 have the acceptable potential for rutin production, which was obtained in their mycelial parts. More research on these two isolates can increase the amount of rutin production in them and lead to commercialization. The species identified in this study were the first introduced as endophytic fungi from *C. spinosa.* They can have high potentials for plant growth; therefore, a proper understanding of the interactions between the host and endophytic fungi and the consequences of this relationship can help increase the efficiency and growth process in certain conditions of this plant.

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