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Juniperus macrocarpa endophytes isolated on standard- and plant extract supplemented-culture media - and evaluation of their antimicrobial activity

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ABSTRACT:
This study aimed to investigate the endophytes of *Juniperus macrocarpa* collected from Çesme in İzmir, Turkey, using a culture-dependent approach and to evaluate their antimicrobial activity for the first time. Since endophytes interact with phytochemicals of the host plant, in addition to the standard culture media, a *J. macrocarpa* extract supplemented culture media was also used for isolation to enhance the cultivability of the endophytes. Six bacteria out of twelve and three fungi out of seven were isolated from the plant extract supplemented culture media. The genotypic identification of the bacterial and fungal isolates was determined based on 16S rDNA and Internal Transcribed Spacer (ITS) sequence analysis, respectively. The genus *Juniperus*, which has ethnopharmacological uses, is rich in phytochemicals with multiple bioactivities. Since *Juniperus* spp. is listed as a priority natural habitat, it is necessary to find alternative resources that could replace the bioactive compounds of these plants. Endophytes of *Juniperus* spp. might be good candidates as antimicrobial producers. From this point of view, the antimicrobial activity of the crude fermentation liquid of the *J. macrocarpa* endophytes, and also aqueous and methanolic extracts of *J. macrocarpa,* were evaluated using a disc diffusion assay against a panel of test microorganisms, including antibiotic resistant ones. One fungus and seven bacteria showed remarkable antimicrobial activity against at least one test microorganism. These results indicated that some endophytes of *J. macrocarpa* had antimicrobial properties like their host plant and could substitute these plants as a source of antimicrobials.

Keywords:
antimicrobial activity, endophytes, *Juniperus macrocarpa*, plant extract supplemented culture media

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INTRODUCTION

Endophytes are microorganisms which spend the whole, or at least part of their life cycle, living within the cells or the intercellular spaces of the healthy tissues of host plants without causing any immediate adverse effects (Stone *et al*. 2000; Tan & Zou 2001). Culture-dependent analysis of endophytes is a common approach to investigating the culturable part of microbial communities. This method has some advantages such as obtaining pure cultures of culturable microorganisms and evaluating their potential bioactivities and possible applications (Khunnamwong *et al*. 2018). The choice of isolation media in culture-dependent studies is important because it determines the cultivability of some endophytes, and consequently affects not only the number, but also the diversity of the isolated endophytic microorganisms (Eevers *et al*. 2015). Using standard culture media such

as Tryptic soy agar (TSA) for the isolation of endophytic bacteria or Malt extract agar (MEA) for the isolation of endophytic fungi is a common approach (Verma *et al*. 2019). However, these media contain nutrients of animal origin and thus may not be sufficient for the cultivation of endophytes which interact with host plants and their phytochemicals throughout their life cycle. For that reason, nutrients of plant origin may be needed to isolate some endophytes (Sarhan *et al*. 2019). Therefore, to mimic the plant environments chemically, plant extract supplemented culture media containing plant extract, or plant-based culture media composed of solely plant extract, have been developed to improve the cultivability of endophytes in recent years (Eevers *et al*. 2015; Sarhan *et al*. 2016; Mourad *et al*. 2018).

Medicinal plants are an important source of phytochemicals with therapeutic potential. However, increasing needs for these plants and their metabolites may cause overutilisation and this could be considered as a threat to medicinal plants (Sandhu & Gupta 2015). On the other hand, due to the long-term symbiotic relationship between endophytes and the host plant, they possess the ability to synthesize some of the plant-associated metabolites and are capable of adapting to the host's microenvironment. Moreover, endophytes protect the host plant from herbivores and pathogens by synthesizing various secondary metabolites (Zhang *et al*. 2006). For these reasons, endophytes are evaluated as sustainable resources which could serve as a substitute for some medicinal plants (Sandhu & Gupta 2015).

The genus *Juniperus*, belonging to the Cupressaceae family, has gained attention in both traditional and modern medicine. Ethnopharmacological uses of *Juniperus* species are very common in Indian, Turkish, Nepalese, Tunisian and other remedies to treat various diseases such as eczema, wounds, pharyngitis, diabetes and others. *Juniperus* spp. are rich in essential oils, terpenoids, flavonoids, lignans, and phenolic acids which have antimicrobial, anti-inflammatory, antioxidant, cytotoxic, and antidiabetic activities. Due to their traditional and pharmacological applications, *Juniperus* species seem to be a good candidate for the development of new drugs (Seca *et al*. 2015). However, the genus *Juniperus* may not be a sustainable resource for these bioactive metabolites because the natural habitats of *Juniperus* spp. have conservation status, as stated in the Habitats Directive 92/43 EEC (Council of the European Commission 1992). The genus *Juniperus* is represented by eight species in Turkey and one of them is *J. macrocarpa* (Kandemir 2018).

Juniperus macrocarpa, which is also named *J. oxycedrus* subsp. *macrocarpa* (Sibth. & Sm.) Ball., grows on coastal dunes, paleodunes and cliffs in the Mediterranean basin, from southwestern Spain to western Turkey (Diez-Garretas & Asensi 2014). It is a threatened species due to the local distribution and sensitive habitats. *J. macrocarpa* is naturally distributed in the west coastal Aegean region (in the Çanakkale, İzmir and Muğla provinces) in Turkey (Kandemir 2018). The main population of *J. macrocarpa* is distributed in the Çeşme district in the İzmir province. Due to the habitat preference of dunes at the seaside, *J. macrocarpa* populations are mostly degraded by tourism activities. As a result, the anthropogenic effects are gradually leading *J. macrocarpa* populations towards extinction in Turkey.

Research indicates that *J. macrocarpa* extracts have antimicrobial activity (Taviano *et al*. 2011; Lesjak *et al*. 2014). However, endophytes of *J. macrocarpa* have neither been identified nor evaluated for their antimicrobial activity to date. In the light of these facts, this study aimed to isolate *J. macrocarpa* endophytes using both supplemented plant extract and standard culture media, to identify them, and also to evaluate the antimicrobial activity and compare it with the same obtained for the extracts of the host plant.

MATERIAL AND METHODS

Sampling of the plant materials. The specimens of *J. macrocarpa* were collected and identified by the fourth author. The voucher specimens of *J. macrocarpa* are kept in the Botanical Garden and Herbarium of the Research and Aplication Centre of Ege University. After the identification of *J. macrocarpa* based on the botanical characteristics in the area of Çesme in İzmir, Turkey, the branches, stems, and roots of a healthy plant were cut aseptically to about 10 cm in length, placed in sterile plastic bags, transferred in a cold box to the laboratory at Ege University, Izmir, and processed on the same day.

Preparation of the *J. macrocarpa* extracts. The leaves of *J. macrocarpa* were detached from the branches. The stem and root samples of *J. macrocarpa* were cut into small pieces with a scalpel. The plant samples (leaves, both of the stem and root, separately) were oven-dried at 37°C for 24 h. The dehydrated plant materials were ground with a mechanical grinder. The aqueous and methanolic extracts of *J. macrocarpa* were prepared as described below:

- Aqueous extracts: The powdered leaves and stem & root samples (5 g each) were extracted twice separately by maceration with distilled water (25 ml) at 27°C, under continuous shaking (120 rpm), for 24 h. The filtrates of the leaves and stem & root extracts were combined separately and evaporated to dryness at room temperature. The crude residues were dissolved in 2 ml of methanol to obtain aqueous leaf and stem & root extracts for antimicrobial activity analysis.
- Methanolic extracts: The powdered leaves and stem & root samples (5 g each) were extracted twice separately by maceration with methanol (25 ml) at 27°C, under continuous shaking (120 rpm), for 24 h. The filtrates

of the leaves and the stem & root extracts were combined separately and evaporated to dryness at room temperature. The crude residues were dissolved in 2 ml of methanol to obtain methanolic leaf and stem & root extracts for antimicrobial activity analysis.

Isolation media. Five media were prepared and used for isolation to enhance the cultivability and diversity of the isolated endophytes. The isolation media were grouped into two categories: standard culture media and plant extract supplemented culture media containing *J. macrocarpa* extracts.

- Standard culture media *

1. Tryptic soy agar (TSA, Merck) supplemented with 100 IU/ml nystatin as an antifungal agent: TSA was prepared according to the manufacturer's instructions.

2. Actinomycete isolation agar (AIA, BD Difco™) supplemented with 100 IU/ml nystatin as an antifungal agent and 20 µg/ml nalidixic acid as an antibacterial agent (Kaewkla & Franco 2013): AIA was prepared according to the manufacturer's instructions.

3. Malt extract agar (MEA) supplemented with 25 µg/ml tetracycline as an antibacterial agent (Stone *et al.* 2004): malt extract 20 g/l; peptone 1 g/l; glucose 20 g/l; agar 20 g/l (Blakeslee 1915)

* The media were prepared and autoclaved at 121°C for 15 min. After sterilization, the media were cooled down to 50–55°C and the filter-sterilized stock solution of the antimicrobial agents was added to each media as stated above.

- Plant extract supplemented culture media

4. MEA-1 supplemented with 25% *J. macrocarpa* leaf extract: The leaves of *J. macrocarpa* were oven-dried at 37°C for 24 h and then ground with a mechanical grinder. The powder (10%, w/v) was macerated with distilled water at room temperature for one day. The extract was filtered through filter paper to separate the plant residue. The media was prepared with the same quantity of constituents as in MEA, but with 750 ml distilled water, and after autoclaving, a 250 ml filter-sterilized leaf aqueous extract (25%, v/v) was added to the media.

5. MEA-2 supplemented with 25% *J. macrocarpa* stem & root extract: The stem and root samples of *J. macrocarpa* were cut into small pieces with a scalpel and oven-dried at 37°C for 24 h. Then the dehydrated plant materials were ground with a mechanical grinder. The mixed powder (10%, w/v) was macerated with distilled water at room temperature for one day. The extract was filtered through filter paper to separate the plant residue. The MEA-2 medium was prepared exactly as MEA-1, but with *J. macrocarpa* stem and root extract.

Isolation of the endophytic microorganisms. The branches, stems, and roots of *J. macrocarpa* were washed with tap water to remove dust and dirt and cut into pieces of about 4 cm in length with disinfected secateurs. To remove the epiphytic microorganisms and sterilize the tissue surfaces, the branch and stem samples were immersed in 70% ethanol (v/v) for 3 min, 3% NaOCl (v/v) solution for 5 min, 70% ethanol (v/v) for 30 s, sequentially (Sun *et al.* 2008). A different surface sterilization protocol was applied for the root samples since the roots of the plants interact with the rhizosphere where an abundance of microorganisms live. In the first step the samples were immersed in 35% H_2O_2 (hydrogen peroxide) (v/v) for 1 min instead of ethanol, because ethanol is reported to cause the contraction of the plant tissues which may result in the entrapment of epiphytic conidia, spores, or mycelia (Sieber 2002). Afterwards they were treated with 3% NaOCl (v/v) solution for 5 min and 70% ethanol (v/v) for 1 min, sequentially. Finally, each sample was rinsed three times in sterile distilled water. To confirm the success of the surface sterilization process, 1 ml aliquot was taken from the final washing solution, inoculated in 4 ml tryptic soy broth (TSB) and malt extract broth (MEB), and examined for microbial growth at 25°C for two weeks.

After surface sterilization, the branch, stem, and root tissues were cut into small pieces, separately, with a sterile scalpel in a laminar flow cabin under aseptic conditions. Three pieces of each tissue were placed onto five different isolation media. The plates were incubated at 25°C for 10 weeks in a sealed plastic box in a humidified atmosphere (Kaewkla & Franco 2013). The plates were observed periodically during incubation. If any colony appeared on the media, it was transferred to fresh TSA and MEA, for bacteria and fungi, respectively, and incubated at 25°C until pure cultures were obtained.

Identification of the endophytes. The polyphasic approach was employed to identify the endophytic microorganisms. The phenotypic features of the bacterial isolates were determined by Gram staining, endospore staining, and catalase and oxidase tests applying standard methods (Lanyi 1987; Tindall *et al.* 2007). The macroscopic and microscopic characteristics of the fungal isolates were observed after incubation on MEA at 25°C for 7 days (Pitt 2000; Watanable 2002). The genotypic identification of the bacterial and fungal isolates was performed by determination based on 16S rDNA and ITS sequence analysis, respectively. For this purpose, the genomic DNA of each bacterial isolate was extracted using a Geneaid Presto™ Mini gDNA Bacteria Kit according to the manufacturer's instructions. On the other hand, the genomic DNA of the fungal isolates was isolated using the manual method described by Liu *et al.* (2000) with some modifications. Genomic DNA concentration and purity were analysed using a Nanodrop 2000c UV-Vis Spectrophotometer (Thermoscientific). PCR amplifications of 16S rDNA and ITS genes were performed with a thermal cycler (Bio-Rad T100). While universal bacterial primers 27 F (5'-AGA GTT TGA TCC TGG CTC AG-

3') and 1492 R (5'-GGT TAC CTT GTT ACG ACT T-3') were used for 16S rDNA amplification (Lane 1991), the primers used for the amplification of the ITS region were ITS1 forward (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 reverse (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.* 1990). 25 µl of PCR reactions contained 12.5 µl of Taq 2x Master Mix (Ampliqon, Taq OptiMix CLEAR), 0.6 µl of 10 pmol of each primer, 6.3 µl of PCR-grade H_2O, and 5 µl of 10–50 ng/µl of template DNA. The PCR conditions for 16S rRNA amplification were: initial denaturation at 94°C for 5 min, 30 amplification cycles consisting of denaturation (94°C for 30 s), primer annealing (49°C for 30 s), primer elongation (72°C for 1 min), and final elongation at 72°C for 7 min. The PCR conditions for ITS amplification were as follows; initial denaturation at 94°C for 3 min, 35 amplification cycles consisting of denaturation (94°C for 30 s), primer annealing (58°C for 40 s), primer elongation (72°C for 40 s), and final elongation at 72°C for 5 min. The PCR products were visualised after electrophoresis on GelRed™ stained 2% agarose gel and positive PCR products were purified and sequenced commercially (MedSanTek Co. Ltd, Izmir, Turkey). The nucleotide sequence data were aligned with the sequences in the GenBank database using the nucleotide BLAST (Basic Local Alignment Search Tool) programme on the NCBI (The National Center for Biotechnology Information) website and reference species were determined. Phylogenetic analysis was conducted using MEGAv7 software.

Fermentation of the endophytes. Fermentation of the endophytic microorganisms was performed in 250 ml flasks containing 50 ml media at 25°C in an orbital shaker incubator rotating at 150 rpm. An agar plug of fungal mycelia (1 cm in diameter) activated on MEA was inoculated in MEB and incubated for 5 days. A loopfull of bacteria activated on TSB was inoculated in LB (Luria Bertani) broth and incubated for 3 days. While the fungal biomass was separated from the fermentation broth by filtering through a filter paper, the fermentation medium of bacteria was centrifuged at 5000 rpm for 20 min in order to separate the bacterial cells.

Antimicrobial activity of the *J. macrocarpa* extracts and the endophytes crude fermentation liquids. The antimicrobial activity of the aqueous and methanolic extracts of *J. macrocarpa* and also the cell-free crude fermentation extracts from the endophytes were determined by the disc diffusion assay as described by the Clinical and Laboratory Standards Institute (CLSI 2007) against *Escherichia coli* O157:H7 (RSKK 234), methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 10876 and *Candida albicans* DSMZ 5817. The bacteria and *C. albicans* DSMZ 5817 were incubated on Mueller-Hinton Agar (MHA) and Sabouraud dextrose agar (SDA), respectively, for 24 h at 37°C. The activated cultures were diluted in 0.85% saline solution and the optical densities of the bacterial suspensions were adjusted to 0.08–0.1 at 625 nm. 100 µl of each culture was inoculated in MHA and spread on the agar media with sterile swabs. Blank paper disks (6 mm in diameter) (Oxoid™), impregnated with 40 µl of the crude fermentation and *J. macrocarpa* extracts, were placed on the inoculated plates and the plates were incubated at 37°C for 24 h. A10 µg tetracycline antimicrobial susceptibility disc (6 mm in diameter) (Oxoid™) for bacteria and a blank paper disc impregnated with 100 Units nystatin for *C. albicans* were used as the positive controls. The diameters of the inhibition zones, including the diameter of the disk, were measured in millimetres (mm) with a ruler.

RESULTS

Isolation and identification of the endophytes. A total of 12 bacterial and 7 fungal endophytes were isolated from the surface-sterilized branches, stems, and roots of *J. macrocarpa* and identified. Only the bacterial and fungal colonies, which emerged from the *J. macrocarpa* tissues, were isolated and purified (Fig. 1). In addition, no microbial growth was observed in the sterility control broths after incubation, which validated the success of the surface sterilization protocols.

Two groups of isolation media, namely standard culture media and plant extract supplemented culture media, were used for the isolation of the endophytic microorganisms. When these isolation media were compared in terms of the number of endophytes isolated, the plant extract supplemented culture media yielded 6 bacterial

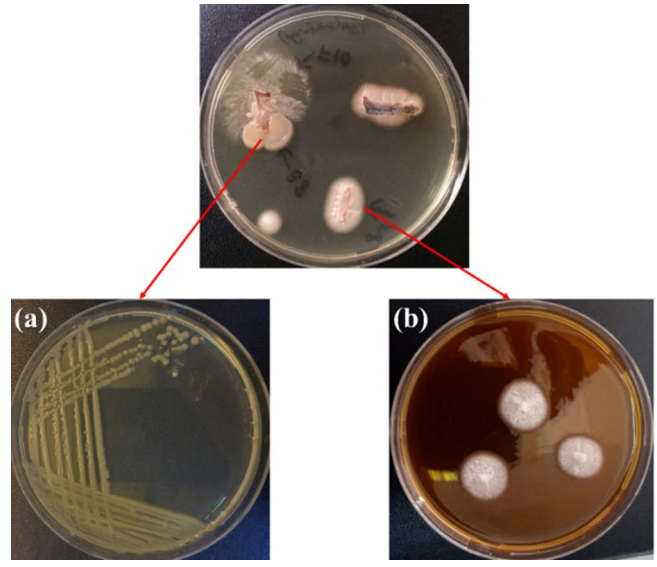

Fig. 1. The emergence of an endophytic bacterium (a) and fungus (b) on the Tryptic soy agar from the root tissues of *Juniperus macrocarpa* and the resulting pure cultures

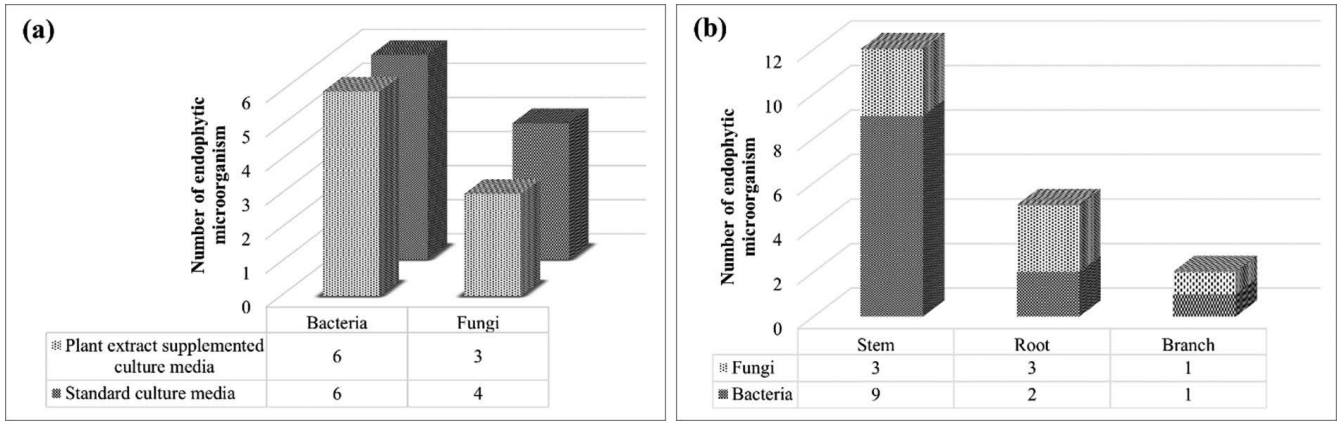

Fig. 2. The number of endophytic bacteria and fungi according to (a) isolation media and (b) tissue type

Table 1. Phenotypic characterization and 16S rDNA identification of the bacterial endophytes isolated from the branch, stem, and root tissues of *Juniperus macrocarpa*

Isolate number	Isolation media	Plant tissue	Gram Reaction	Cellular morphology	Oxidase	Catalase	16S rDNA BLASTn results	GenBank accession numbers
B1	Plant extract supplemented culture media[1]	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus subtilis*	MT706000
B2	Standard culture media	Stem	Gr (+)	Endospore-forming bacillus	(+)	(+)	*Bacillus licheniformis*	MT705990
B3	Standard culture media	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus subtilis*	MT706001
B4	Standard culture media	Root	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus* sp.	MT705993
B5	Plant extract supplemented culture media[2]	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus* sp.	MT705994
B6	Standard culture media	Root	Gr (+)	Endospore-forming bacillus	(+)	(+)	*Bacillus simplex*	MT705992
B7	Standard culture media	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus* sp.	MT705995
B8	Standard culture media	Branch	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus* sp.	MT705996
B9	Plant extract supplemented culture media[1]	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus* sp.	MT705997
B10	Plant extract supplemented culture media[2]	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus megaterium*	MT705991
B11	Plant extract supplemented culture media[1]	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus* sp.	MT705998
B12	Plant extract supplemented culture media[1]	Stem	Gr (+)	Endospore-forming bacillus	(-)	(+)	*Bacillus* sp.	MT705999

[1]MEA-1 supplemented with 25% *J. macrocarpa* leaf aqueous extract, [2]MEA-2 supplemented with 25% *J. macrocarpa* stem & root aqueous extract

and 3 fungal isolates, comparable with the standard culture media (Fig. 2a). When the occurrence of endophytes in different tissues was evaluated, it was found that 9 endophytic bacteria out of 12 were isolated from the stems, 2 were isolated from the roots, and 1 was isolated from the branches. From the total of 7 fungal isolates, 3 were isolated from the root, 3 from the stem, and 1 from the branch tissues (Fig. 2b).

The results of the phenotypic characterization of the bacterial endophytes indicated that all of the isolates were Gram-positive and endospore-forming bacilli. In addition, while all the isolates were positive in the catalase test, 2 isolates out of 12 were positive in the oxidase test (Table 1). Molecular identification of the endophytic bacteria also revealed that all the bacterial isolates belonged to the genus *Bacillus* (Table 1). The Genbank

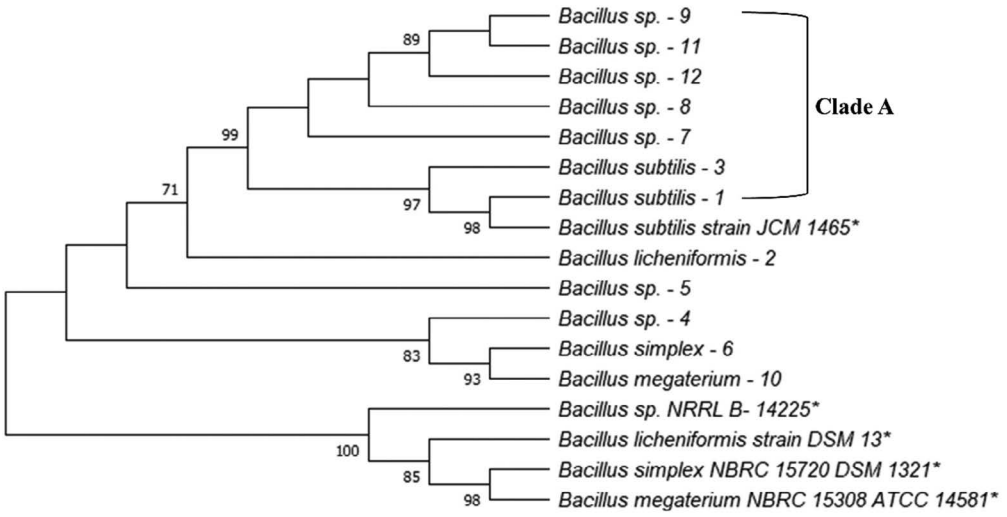

Fig. 3. The phylogenetic tree based on 16S rDNA nucleotide sequences of the endophytic bacteria isolated from *Juniperus macrocarpa* (Clade A indicated bacteria which have antimicrobial activity)

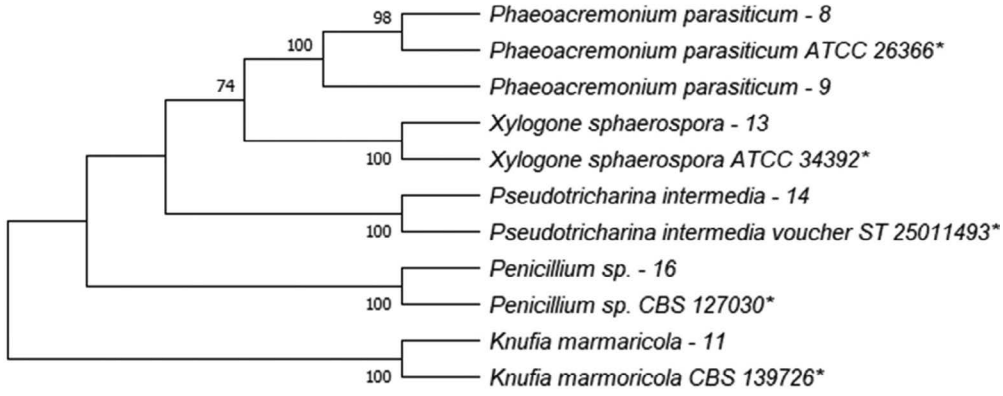

Fig. 4. The phylogenetic tree based on the ITS nucleotide sequences of the endophytic fungi isolated from *Juniperus macrocarpa*

accession numbers of the endophytic bacteria were as follows: MT706000, MT705990, MT706001, MT705993, MT705994, MT705992, MT705995, MT705996, MT705997, MT705991, MT705998, and MT705999. A phylogenetic tree was constructed to illustrate the evolutionary relationships among the bacterial endophytes of *J. macrocarpa* (Fig. 3).

Six endophytic fungal isolates out of 7 were identified based on the morphological and molecular characteristics. The pure culture of one isolate could not be identified since it could not be further cultivated and maintained under laboratory conditions. Only one fungal isolate out of 6 could be identified to the genus level (*Penicillium* sp.) using morphological techniques, while the other isolates, lacking in sporulating structures, were mycelia sterilia. According to the BLAST analysis of ITS sequences, 2 endophytic fungi isolated from the root tissues were identified as *Phaeoacremonium parasiticum,* and one as *Xylogone sphaerospora*. While two endophytic fungi isolated from the stem tissues were identified as *Knufia marmoricola* and *Pseudotricharina intermedia*, one isolated from the branches was identified as *Penicillium* sp. (Table 2). The Genbank accession numbers of the endophytic fungi were as follows: MT706008, MT706009, MT706006, MT706011, MT706010, and MT706007. The phylogenetic relationships of the identified endophytic fungi isolated from *J. macrocarpa* are provided in Fig. 4.

Antimicrobial activity of *J. macrocarpa* and the endophytes. The antimicrobial activity of the aqueous and methanolic extracts of *J. macrocarpa* is given in Table 3. All the extracts showed antimicrobial activity against *B. cereus* ATCC 10876, but the methanolic extract of the leaves was highly effective, with an inhibition zone of 14 mm. Both the aqueous and methanolic extracts of the leaves and also the stem & root methanolic extract were active against methicillin-resistant *S. aureus* ATCC 43300 and *P. aeruginosa* ATCC 27853. On the other hand, none of the extracts indicated any antimicrobial activity against *E. coli* O157:H7 (RSKK 234) and *C. albicans* DSMZ 5817. In general, the antimicrobial activity of the methanolic extracts of *J. macrocarpa* was higher than that of the aqueous extracts. Moreover, when the

Table 2. Taxonomic identification of the fungal endophytes isolated from the branch, stem and root tissues of *Juniperus macrocarpa* based on the ITS sequence analysis

Isolate number	Isolation media	Plant tissue	ITS BLASTn results	GenBank accession numbers
F8	Standard culture media	Root	*Phaeoacremonium parasiticum*	MT706008
F9	Standard culture media	Root	*Phaeoacremonium parasiticum*	MT706009
F11	Plant extract supplemented culture media[1]	Stem	*Knufia marmoricola*	MT706006
F13	Standard culture media	Root	*Xylogone sphaerospora*	MT706011
F14	Plant extract supplemented culture media[1]	Stem	*Pseudotricharina intermedia*	MT706010
F16	Standard culture media	Branch	*Penicillium* sp.	MT706007
F17	Plant extract supplemented culture media[2]	Stem	n.i.	

[1]MEA-1 supplemented with 25% *J. macrocarpa* leaf aqueous extract, [2]MEA-2 supplemented with 25% *J. macrocarpa* stem & root aqueous extract, n.i.: not identified

Table 3. The antimicrobial activity of the aqueous and methanolic extracts of *Juniperus macrocarpa*

J. macrocarpa extracts		Inhibition zone (mm)[a]				
		BC	**EC**	**MRSA**	**PA**	**CA**
aqueous extracts	leaves	9	-[b]	8	9	-
	stem & root	8	-	-	-	-
methanolic extracts	leaves	14	-	12	13	-
	stem & root	11	-	10	11	-

BC: *Bacillus cereus* ATCC 10876; EC: *Escherichia coli* O157H7 (RSKK 234); MRSA: methicillin-resistant *Staphylococcus aureus* ATCC 43300; PA: *Pseudomonas aeruginosa* ATCC 27853; CA: *Candida albicans* DSMZ 5817
[a]Inhibition zones include the diameter of the disks (6 mm); [b]No effect

Table 4. The antimicrobial activity of the crude fermentation liquids of the bacterial and fungal endophytes isolated from *Juniperus macrocarpa*

Isolate number	Endophytic microorganisms	Positive control	Inhibition zone (mm)[a]				
			BC	**EC**	**MRSA**	**PA**	**CA**
B1	*Bacillus subtilis*		11	-[b]	-	-	-
B3	*Bacillus subtilis*		10	-	13	-	-
B7	*Bacillus* sp.		10	-	-	-	-
B8	*Bacillus* sp.		10	-	-	-	-
B9	*Bacillus* sp.		11	9	-	-	-
B11	*Bacillus* sp.		9	-	-	-	-
B12	*Bacillus* sp.		11	10	-	-	-
F11	*Knufia marmoricola*		-	-	-	-	14
		tetracycline (10 µg/disk)	15	25	27	12	
		nystatin (100 Units/disk)					25

BC: *Bacillus cereus* ATCC 10876; EC: *Escherichia coli* O157H7 (RSKK 234); MRSA: methicillin-resistant *Staphylococcus aureus* ATCC 43300; PA: *Pseudomonas aeruginosa* ATCC 27853; CA: *Candida albicans* DSMZ 5817
[a]Inhibition zones include the diameter of the disks (6 mm); [b]No effect

antimicrobial activity of the leaf extracts was compared to that of the stem & root extracts, both leaf extracts showed better antimicrobial activity, thus supporting the use of *Juniperus* spp. leaves in traditional medicine (Seca *et al.* 2015).

The antimicrobial activity of the crude fermentation liquids of the bacterial and fungal endophytes is provided in Table 4. In total, 44.4% of the endophytic microorganisms showed antimicrobial activity against at least one test microorganism. In contrast to *J. macrocarpa*, endophytic fungus *K. marmoricola* showed the highest antimicrobial activity with an inhibition zone of 14 mm against *C. albicans* DSMZ 5817. Seven endophytic bacteria out of 12 were active and all of them showed antimicrobial activity against *B. cereus* ATCC 10876, like their host plant. In addition, two isolates showed antimicrobial activity against *E. coli* O157:H7 (RSKK 234) and one against methicillin-resistant *S. aureus* ATCC 43300. As shown in Fig. 3, according to 16S rDNA sequence analysis, these isolates were clustered in Clade A in the phylogenetic tree, and thus may have similar biosynthetic gene clusters which could be linked to antimicrobial metabolite production.

DISCUSSION

Many *Juniperus* species, for instance, *J. recurva, J. virginiana, J. communis*, *J. excelsa*, and *J. procera*, have been investigated in terms of their endophytes (Kour *et al.* 2008; Zhao *et al.* 2011; Ellsworth *et al.* 2013; Hosseyni-Moghaddam & Soltani 2014; Gherbawy & Elhariry 2016). However, to the best of our knowledge, this study is the first report on the bacterial and fungal endophytes isolated from *J. macrocarpa*. Studies on the endophytes from *Juniperus* spp. have mostly focused on the endophytic fungi (Ellsworth *et al.* 2013; Hosseyni-Moghaddam & Soltani 2014; Gherbawy &

Elhariry 2016). Since the host plant species affect the diversity of endophytes, none of the fungal isolates identified in this study has been previously reported, with the exception of *Penicillium* sp. (Gherbawy & Elharir 2016). Soltani *et al.* (2016) and Zhao *et al.* (2011) investigated the endophytic bacteria of *J. communis* and *J. virginiana*, respectively. Zhao *et al.* (2011) reported a new subspecies, *Bacillus subtilis* subspecies *virginiana*, from *J. virginiana*. On the other hand, our study contributed to the understanding of the biodiversity of both endophytic bacteria and fungi associated with *J. macrocarpa* from Turkey.

It is well known that isolation media affect the number, diversity, and cultivability of endophytes in culture-dependent analysis. For this reason, we used both plant extract supplemented and standard culture media during the isolation of the *J. macrocarpa* endophytes. In total 9 endophytic microorganisms (both bacteria and fungi) were isolated from the plant extract supplemented culture media, while 10 endophytes (both bacteria and fungi) were isolated from the standard culture media. Those isolates growing on the plant extract supplemented culture media were not observed on the standard culture media, which might be due to the fact that the plant extract promoted the growth and cultivability of some endophytes (Sarhan *et al.* 2016; Mourad *et al.* 2018). Eevers *et al.* (2015) compared the effects of 1/10 diluted 869 medium (containing glucose D+ 0.1 g/l, tryptone 1 g/l, yeast extract 0.5 g/l, NaCl 0.5 g/l, $CaCl_2.2H_2O$ 0.035 g/l, agar 15 g/l) and the plant extract supplemented medium on the isolation of bacterial endophytes from the root and shoot tissue of *Cucurbita pepo*. It was found that the medium with the plant extract yielded higher numbers of endophytes. They also demonstrated that supplementation of the medium with plant extract increased the diversity of cultivable strains from the shoot tissues, but decreased it in the case of root endophytes. In addition, Mourad *et al.* (2018) quantified bacterial endophytes in barley roots by using the cultivation-dependent method in terms of the total number of colony-forming units developed on nutrient agar (NA) and on the plant-only-based culture media prepared from plant powders of turf grass. The highest cultivability was calculated as 16.3% for the plant-only-based culture media, which was four times higher than for NA. Therefore, the use of the relevant plant extract in the isolation of endophytes in cultural studies will increase efficiency. In this study, one fungal endophyte, purified from plant extract supplemented culture media, could not be recultivated under laboratory conditions. Likewise, Eevers *et al.* (2015) mentioned that certain endophytic bacteria, which grew on the plant extract supplemented culture media during isolation, could not be recultivated under laboratory conditions after isolation, They concluded that this may have been caused by the presence of phytochemicals during isolation only, but not during further recultivation.

The antimicrobial activity of *J. macrocarpa* extracts have been investigated in previous studies (Taviano *et al.* 2011, 2013; Lesjak *et al.* 2014). Taviano *et al.* (2011) demonstrated that methanol and water extracts of *J. macrocarpa* branches showed antimicrobial activity against Gram-positive bacteria. The highest antimicrobial activity with a minimum inhibitory concentration (MIC) of 4.88 μg/ml was observed against *Staphylococcus aureus* ATCC 6538P with water extracts of *J. macrocarpa* branches. They mentioned that while the extracts were less effective against *Bacillus subtilis* P3, they were not active against Gram-negative bacteria and yeasts. The antimicrobial activity of methanol extracts of *J. macrocarpa* berries was also investigated by the same research group (Taviano *et al.* 2013). They found that the berries extract was active against Gram-positive bacteria and the highest antimicrobial activity was observed with MIC of 625 μg/ml against *S. aureus*. The extract did not show any antimicrobial activity against Gram-negative bacteria and *Candida* spp. The total phenolic, total flavonoid and condensed tannin contents of methanol and water extracts of *J. macrocarpa* branches were determined by Taviano *et al.* (2011). Taviano *et al.* (2013) also analysed the total phenolic content of the berries extract and identified the phenolic compounds by means of HPLC–MS analysis. Although Taviano *et al.* (2011) stated that the phenolic compounds of the plant extract were responsible for the antimicrobial activity by referring to other studies, Taviano *et al.* (2013) did not attribute the antimicrobial activity to the phenolic compounds in the extracts alone. They explained this by the fact that while the total phenolic content of the *J. macrocarpa* berries extract was three fold higher than that of the *J. oxycedrus* L. subsp. *oxycedrus* extract, both extracts showed the same activity against *Staphylococcus* strains (Taviano *et al.* 2013). Lesjak *et al.* (2014) investigated the antimicrobial activity of essential oils obtained from the leaves and seed cones of *J. macrocarpa*. While they both showed antimicrobial activity against *Clostridium perfringens* ATCC 13124 and *S. aureus* ATCC 11632, which are Gram-positive bacteria, the essential oils of the leaves were more effective than those of the seed cones. GC–MS analysis of the essential oils of the leaves and seed cones demonstrated that they were rich in terpenoids with high contents of monoterpenes. The results of the current study demonstrated that while all the extracts with the exception of the aqueous extract of the stems and roots showed antimicrobial activity against *S. aureus*, none of them was effective against *C. albicans*, which is in line with the literature. Additionally, all the extracts showed considerable antimicrobial activity against *B. cereus*, which is a Gram-positive bacterium. Furthermore, in contrast to the literature, all the extracts except for the aqueous extract of the stems and roots indicated antimicrobial activity against *P. aeruginosa*, which is a Gram-negative bacterium.

Research indicates that some endophytes of *Juniperus* spp. might have potential use as antimicrobial producers. Some studies have demonstrated that the crude extracts of endophytes isolated from *Juniperus* spp. showed antibacterial or antifungal activity without the identification of active metabolites (Ellsworth *et al.* 2013; Hosseyni-Moghaddam & Soltani 2014; Gherbawy & Elhariry 2016). On the other hand, some studies have shown that certain endophytes from *Juniperus* spp. produced new bioactive metabolites or the same metabolites synthesised by the host plants. Pelaez *et al.* (2000) discovered a new antifungal compound, enfumafungin, synthesised by an endophytic fungus belonging to the genus *Hormonema*, isolated from *J. communis*. Kour *et al.* (2008) isolated *Fusarium oxysporum* from *J. recurva*, while Kusari *et al.* (2009) isolated *Aspergillus fumigatus* from *J. communis,* which were producers of podophyllotoxin and deoxypodophyllotoxin, respectively, two important *Juniperus* associated metabolites with antimicrobial activity and also the precursors of three anticancer drugs.

The current study reported the antimicrobial activity of *J. macrocarpa* endophytes for the first time. According to our results, it was found that endophytic bacteria, which showed antimicrobial activity particularly against *B. cereus*, were clustered in Clade A in the phylogenetic tree and the closest relative of these bacteria in the tree is the strain *B. subtilis* JCM 1465. The bioactive endophytic bacteria may have similar biosynthetic gene clusters which could be linked to the synthesis of bacteriocins or bacteriocin-like substances. Members of the *B. subtilis* group have the ability to produce a wide variety of antimicrobial compounds including bacteriocins, which are ribosomally synthesized antimicrobial peptides and are mainly effective against closely related bacteria (Caulier *et al.* 2019). Additionally, it is noteworthy that Pérez-Gutiérrez *et al.* (2013) demonstrated the antagonist interactions between *Bacillus* spp. mostly caused by bacteriocin-like substances. They isolated 72 *Bacillus* spp., involved in at least seven taxonomic groups, from superficial sediment obtained from five different sites of the intermediate lagoon in the Churince water system. They found that the antagonistic effect of a certain *Bacillus* taxonomic group usually targeted *Bacillus* groups from other clades to exclude the members of different *Bacillus* spp. In parallel with our results, they indicated that the lineage, which exhibited the most antagonistic capacity, belonged to the *B. subtilis* group and the members of this group antagonized some of the isolates involved in the *B. cereus* group. Another significant finding of our results is that *K. marmoricola*, an endophytic fungus of *J. macrocarpa*, showed antimicrobial activity against *C. albicans*. There are limited studies in the literature indicating the antimicrobial activity of *Knufia* spp. Gnavi *et al.* (2016) isolated *K. petricola* from the green alga *Flabellia petiolata* and demonstrated that the intracellular fungal ethyl acetate extract was active against three bacterial strains, namely *Burkholderia metallica* LMG 24068, *P. aeruginosa* PA01, and *S. aureus* 6538P. Martínez-Arias *et al.* (2021) found that liquid filtrate of *Knufia* sp., an endophytic fungus of *Ulmus minor*, reduced the growth of *Ophiostoma novo-ulmi*, which is a fungal pathogen known to cause Dutch elm disease. On the other hand, our study is the first report on the antifungal activity of *K. marmoricola*.

To sum up, the results of the current study proved that some endophytes of *J. macrocarpa* had antimicrobial properties like their host plant and demonstrated the potential of these endophytes as sources of antimicrobial compounds.

CONCLUSIONS

This study provides insight into the diversity of endophytic bacteria and fungi associated with *J. macrocarpa* for the first time. The results contribute to understanding the importance of plant extract supplemented culture media in the isolation of endophytes, because these media promoted the emergence of certain endophytic microorganisms. A total of 12 bacterial and 7 fungal endophytes were isolated from the branch, stem and root tissues of *J. macrocarpa*. Seven endophytic bacteria and one endophytic fungi demonstrated inhibitory activity against at least one pathogen. These results showed that some endophytes of *J. macrocarpa* had antimicrobial properties like their host plant. Since the natural habitats of *Juniperus* spp. are under conservation and the over-exploitation of these plants may cause a rapid loss of these important resources, endophytes of *J. macrocarpa* could be evaluated as new resources for antimicrobial production and serve as substitutes for these plants in order to contribute to their conservation. However, further analysis is necessary to determine the active substances responsible for the antimicrobial potential of endophytes.

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REZIME

Botanica SERBICA

Endofiti *Juniperus macrocarpa* izolovani na standardnim i medijumima sa dodatkom biljnih ekstrakata, i procena njihove antimikrobne aktivnosti

Sennur Çalışkan, Pelin Gökfiliz-Yildiz, Ayşegül Özmen, Hasan Yildirim i Ataç Uzel

Cilj ove studije je da istraži endofite *Juniperus macrocarpa*, sakupljene na lokalitetu Çesme u Izmiru, Turska, koristeći pristup zavistan od kulture, kao i da se po prvi put proceni njihova antimikrobna aktivnost. S obzirom da endofiti stupaju u interakciju sa fitohemijskim jedinjenjima biljke domaćina, pored standardne podloge za kulturu, za izolaciju je korišćena podloga za kulturu dopunjena ekstraktom *J. macrocarpa*, kako bi se poboljšala kultivabilnost endofita. Šest od dvanaest bakterija i tri od sedam gljiva su izolovane iz medijuma za kulturu sa dodatkom biljnih ekstrakata. Genotipska identifikacija bakterijskih i gljivičnih izolata je određena na osnovu analize sekvence 16S rDNK i ITS analiza. Rod *Juniperus* koji ima etnofarmakološku upotrebu, bogat je fitohemijskim jedinjenjima sa mnogim bioaktivnostima. Pošto su staništa predstavnika roda *Juniperus* uvrštena u prioritetna prirodna staništa, neophodno je pronaći alternativne resurse koje bi trebalo zameniti ovim biljkama za bioaktivna jedinjenja. Endofiti *Juniperus* spp. mogu biti dobri kandidati za antimikrobne produkte. Sa ove tačke gledišta, antimikrobna aktivnost sirove fermentacione tečnosti endofita *J. macrocarpa*, kao i vodenih i metanolnih ekstrakata *J. macrocarpa*, procenjena je korišćenjem disk difuzionog eseja na panelu test mikroorganizama, uključujući i one otporne na antibiotike. Jedna gljiva i sedam bakterija pokazale su izuzetnu antimikrobnu aktivnost protiv najmanje jednog test mikroorganizma. Ovi rezultati su pokazali da neki endofiti *J. macrocarpa* imaju antimikrobna svojstva poput njihove biljke domaćina i da mogu zameniti ove biljke kao izvor antimikrobnih sredstava.

Ključne reči: antimikrobna aktivnost, endofiti, *Juniperus macrocarpa*, medijumi suplementirani ekstraktima biljaka