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Original research

Antibacterial and antifungal activity of Eurasian water-milfoil collected from lentic and lotic water body in Central Black Sea Region, Turkey

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Abstract: The natural compounds obtained from plants are very important as antioxidant and antimicrobial agents. However, researchers were mostly focused on terrestrial plants. In this study, the antimicrobial effects of the submerged macrophyte *Myriophyllum spicatum* L. was investigated, an invasive species in freshwater and having a strong allelopathic potential. In the direction of research, the samples were collected from Lake Ulugöl (Ordu) and the Terme Creek (Samsun). Ethanolic extracts from dried and milled plants were prepared. The antimicrobial activities of the extracts were tested against test microorganisms by means of disk diffusion method. Four Gram-positive bacteria, four Gram-negative bacteria, one mold and one *Candida* were used tested microorganism. Our data showed that Gram-positive bacteria were more sensitive than Gram-negative. The most sensitive microorganisms were *Candida albicans, Aspergillus brasiliensis, Bacillus subtilis,* and the lowest efficacy on *Escherichia coli. Myriophyllum spicatum* samples taken from the Terme Creek showed higher antimicrobial activity than Lake Ulugöl. *Myriophyllum spicatum* is a macrophyte which is widespread and which grows naturally in all kinds of freshwater. Thus, this macrophyte and similar macrophytes with high therapeutic potential can play a significant role in pharmaceutical process and drug discovery.

Keywords: *Myriophyllum spicatum*, microbial pathogen, antimicrobial activity, aquatic plants, spiked water-milfoil

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Introduction

Plants, herbs, and ethnobotanicals have been used since the early days of humankind and are still used throughout the world for health promotion and treatment of disease (Ertürk, 2017). Also, medicinal and aromatic plants have been used for many years and they are still being used in many areas such as spices, drugs and cosmetics. Active ingredients of many drugs produced synthetically are similar to chemicals isolated from plants. At least one fourth of the active ingredients of drugs produced pharmacologically are obtained from plants (WHO, 2013). Recently, there has been an increase in medicinal plant consumption demands. According to World Health Organization (WHO), 80% of the population living in developing countries of the world generally depends on plant-based traditional medicine for their basic health needs. Drug companies which start off from the traditional use of plants define and synthesize the active ingredients of some of these and make safer dose standardization. However, synthetic and chemical containing drugs still have side effects. The reasons for increased demand for plants from which drugs can be obtained in both developed and developing countries are the facts that they are cost efficient, they do not have side effects or they have few side effects and they are produced naturally (Sekar and Kandavel, 2010). Medicinal characteristics of plants result from the presence of chemical substances which are present in their structure and which have physiological effects of living beings. Natural compounds which are obtained from plants draw a lot of attention as antimicrobial, anti-inflammatory, antitumor and anticancer agents (Ya, 2015). Secondary metabolites in plants are mostly responsible for antimicrobial activities (Savoia, 2012). The primary groups of phytochemicals with antimicrobial characteristics are phenolics and polyphenols (flavonoids, quinones, tannins, coumarins), terpenoids, alkaloids, lectins and polypeptides (Cowan, 1999; Savoia, 2012; Upadhyay, 2014). Antibiotic resistance has become one of the major problems of human beings since the late 20th century (Givonyan et al., 2017). This situation makes treatment difficult. Plant materials are shown to be one of the most promising resources for the exploration of antimicrobial compounds (Cowan, 1999; Parekh et al., 2005; Abreu et al., 2012; Savoia, 2012). Plant based antimicrobials are thought to be safer when compared with synthetic compounds since they are natural (Rajeh et al., 2010; Upadhyay, 2014). Studies conducted show that for a more effective treatment, natural antimicrobial agents obtained from plant extracts includes compounds with inhibitor effect and that these can be used for therapeutic purposes (Ya, 2015; Givonyan et al., 2017; Elyashevich and Buzuk, 2010).

In most of the studies conducted in Turkey, which are tested against microorganisms, terrestrial plants have been used, while aquatic plants have been ignored. Our country is very rich in terms of different types of aquatic ecosystems. There are various hydrophytes in the form of emerged, submerged and floating-leaved. Some types (for example, *Myriophyllum spicatum* L., Eurasian water-milfoil) can be over dispersed as in Ordu Ulugöl Lake (Taş et al., 2018a). There are antimicrobial studies conducted on marine macroalgae rather than aquatic plants (Demirel et al., 2009; Ertürk and Taş, 2011; Gümüş and Ünlüsayın, 2016; Ertürk et al., 2017; Gümüş et al., 2018) and studies conducted with aquatic plants commonly found in fresh waters are even less (Özbay and Alim, 2009; Gülçin et al., 2010; Savaroğlu et al., 2011; Taş et al., 2018b, c). Aquatic plants are more commonly used in the medicine of European and Asia peoples (Ya, 2015). The investigations carried out with aquatic plants are mostly reported from Asia countries (Swapna et al., 2011; Biswas et al., 2012; Shirshova et al., 2012; Vladimirova and Georgiyants, 2013; Amin et al., 2016).

The purpose of this study is to examine the antibacterial and antifungal effects of *M. spicatum*, which is an invasive species in freshwater and which is known to have strong allelopathic potential. The study also aims to compare the antimicrobial effects of the same plant which lives in different localities with different ecological structures.

Materials and Methods

Plant materials: The material in our study is *Myriophyllum spicatum* L. (Plantae, Tracheophyta, Magnoliopsida, Haloragales, Haloragaceae) species. *M. spicatum* was identified according to the Altınayar (1988) and Seçmen and Leblebici (1996). *M. spicatum* is a submerged type, living completely under water, the roots of which are attached to the ground (Figure 1). It can grow as tall as 2-3 m. Its green leaves are lined in circles on the stem. *M. spicatum* is a perennial, herbaceous non-endemic type. It can be found in lakes, irrigation canals and slow moving rivers from 0 to 2000 m (Altınayar, 1988; Seçmen and Leblebici, 1996; TÜBİVES, 2018). *M. spicatum* is found in freshwater lakes, rivers, streams, canals and ditches. This species prefers wetlands which include high resolution inorganic carbon, nitrate, nitrite and alkali water systems and hydroxyl groups containing high phosphate and organic matter. *M. spicatum* is used in some areas of Asia to help cleaning dirty water. In addition, it can be used in pools and aquariums for landscape purposes. The greatest threat on *M. spicatum,* which is a prevalent invasive species, is the disappearance of wetlands by drying up. IUCN category of the species is LC (IUCN, 2018).

Sampling localities: *Myriophyllum spicatum* samples, which are widespread in lentic and lotic systems in Central Black Sea Region of Turkey, were collected from two different localities. Samples were collected from both localities in September 2017.

Ulugöl Lake: Ordu province, Gölköy town, Ulugöl Nature Park (40°37'44.53" N, 37°32'44.69" E, altitude: 1203 m), lentic system (Fig. 1). *M. spicatum* species is among submerged plants which are common in this lake (Taş et al., 2018a).

Terme Creek: Samsun province, Terme town (41°12'31.40"N, 36°58'25.77"E, altitude 4 m), lotic system. It has been determined 160 species and infraspecific taxa belonging 61 families and 141 genera which can be used for medicinal purposes in the Yeşilırmak Delta (Mumcu and Korkmaz, 2018).

Figure 1. General appearance of invasive aquatic plant *Myriophyllum spicatum* species in Lake Ulugöl

Preparing the plant extract: The samples brought to laboratory were washed well first with tap water and then distilled water and they were cleared from the shells and epiphytes on them. The plant which was broken into small pieces was dried for about a week in the dark and under room temperature. Later, it was ground into powder with mechanical grinder (Waring® 8011 EB Blender, Waring Commercial, USA). The samples were weighed as 2 g each with precise scale (Radwag AS220.R2), they were transferred to 50 mL falcon tubes and 10 mL 99.8% ethanol was added on them. The samples were kept waiting in the refrigerator at +4 °C for extraction. Later, extraction was speeded up by keeping them for 30 minutes at ultrasonic cleaner (Daihan WiseClean® WUC-A02H, Daihan Scientific, S. Korea). The samples were vortexed for 1-3 minutes and distilled with vacuum filtration from Whatman® No:1 filter paper. After the alcohol in the extract was removed completely in room temperature, about 3-5 mL plant extract was obtained. The samples were kept in the refrigerator at +4 °C until antimicrobial tests were conducted.

Determination of antimicrobial activity: Strains of bacteria and fungi were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Four Gram positive bacteria (*Bacillus cereus* ATCC® 11778™, *Bacillus subtilis* ATCC® 6051™, *Clostridium perfringens*, ATCC® 313124™ and *Enterococcus faecalis* ATCC® 29121™), four Gram negative bacteria (*Citrobacter freundii* ATCC® 43864™, *Escherichia coli* ATCC® 25922™, *Klebsiella pneumoniae* ATCC® 13883™ and *Pseudomonas aeruginosa* ATCC® 27853™), one mold (*Aspergillus brasiliensis* ATCC® 16404™) and one *Candida* (*Candida albicans* ATCC® 10231™) were used as test microorganism.

Antimicrobial activity was determined using ten different pathogens according to disk diffusion method. Mueller Hinton Agar (Merck) or Mueller Hinton Broth (Merck) and Sabouraud Dextrose Broth (Difco) or Sabouraud Dextrose Agar (Oxoid) were used for growing bacterial and yeast or fungal cells, respectivelly. Prepared agar was 1.5 atm pressure and 121 °C, 15 min it was sterile. For each petri dish, as much as 20 mL agar was poured into aseptic conditions (Esco Airstream® Class 2 Biological Safety Cabinet) and frozen. Bacteria, yeast and fungi samples used in the study were set to 0.5 McFarland turbidity density using the McFarland instrument. The test microorganisms were planted in at least three different ways with the spread plate method on the plate. Six empty discs (Oxoid, USA) were equally spaced for each petri dish. From plant ethanol extracts 25 µL with taking (active substance concentration of 0.144 mg/mL at 25 µL in the Ulugöl Lake sample and 0.152 mg/mL at the Terme Creek sample) were dripped onto empty antibiogram discs. After these sterilized procedures, the bacteria are incubated for 24 hours at 37 °C and cork cultivated petri dishes were incubated in two different ovens (BINDER GmbH, Germany) for 48 hours at 30 °C taken (Ertürk, 2006). The inhibition zones formed on the plate at the end time were measured in millimeter (mm) using a caliper. The experiments were repeated three times in parallel and the arithmetic mean of the three obtained data was taken. For the bacteria ampicillin and cephazolin, for the fungi and yeast nystatin were used as a positive control. Ethanol was used as negative control.

Results and Discussion

The ethanol extracts of *M. spicatum* species collected from Ulugöl Lake and Terme Creek were tested on Gram positive and Gram negative bacteria, fungi and yeast through disc diffusion method. The results of antibacterial and antifungal effects are summarized in Table 1. Active matter concentration at 25 μL in Ulugöl Lake sample of *M. spicatum* species is 0.144 mg/mL, while it is 0.152 mg/mL in Terme Creek sample. According to antibiogram analysis results conducted, it was found that ethanol extracts of *M. spicatum* species had the highest effect on fungus among test microorganisms (19.9-22.0 mm; 20.9 mm/25 μL, on average). The highest fungal effect was shown on Terme Creek sample *Candida albicans* (22.5 mm). *M. spicatum* ethanol extract showed the larger inhibitory zone on Gram positive bacteria among tested bacteria (min. 18.1 mm – max. 21.2 mm; 19.6 mm/25 μL, on average). The highest inhibition zone was measured on *Bacillus subtilis* (24.5 mm). On Gram positive bacteria, Terme Creek sample was found to be more effective than Lake Ulugöl sample. On Gram negative bacteria, Ulugöl Lake sample (19.0 mm, average) was recorded to be more effective than Terme Creek sample (17.9 mm, average). Ethanol extract of *M. spicatum* from Ulugöl Lake showed the highest antibacterial effect on *Citrobacter freundii* (21.5 mm).

Table 1. Inhibition zones (mm) formed by *Myriophyllum spicatum* ethanol extracts in different localities against test microorganisms. The numbers on the table are average ±standard deviation values obtained as a result of experiments repeated three times

		Samples					
		Ulugöl Lake	Terme Creek	Ampicillin	Cephazolin	Nystatin	Solvents
Gram positive bacteria	*Bc*	16.20±0.36	19.50±0.56	26.00± 9.7	28.66± 3.4	NT	-
	Bs	20.70±8.32	24.50±6.24	35.06±0.28	37.01±0.28	NT	-
	Cp	16.30±4.00	18.30±3.20	38.06±0.27	40.01±0.20	NT	-
Gram negative bacteria	*Ef*	19.50±0.28	22.50±2.34	35.26±6.46	28.00±8.28	NT	-
	Cf	21.50±2.90	18.40±0.20	16.10±2.28	15.10±0.28	NT	-
	Ec	15.30±0.42	17.60±5.24	19.00±0.0	19.00±0.0	NT	-
Fungus	*Kp*	19.00±0.564	16.50±0.653	15.20±0.10	17.20±0.10	NT	-
	Pa	20.50±2.34	19.00±0.28	35.26±6.46	28.00±8.28	NT	-
	Ab	20.50±2.45	21.50±4.32	NT	NT	17.10±4.28	-
	Ca	19.30±3.22	22.50±2.80	NT	NT	17.00±0.0	-

Note: -: no inhibition, NT: Not tested, Microorganisms: *Bc*: *Bacillus cereus* ATCC®11778™, *Bs*: *Bacillus subtilis* ATCC® 6051™, *Cp*: *Clostridium perfringens* ATCC® 313124™, *Ef*: *Enterococcus feacalis* ATCC® 29121™, *Cf*: *Citrobacter freundii* ATCC® 43864™, *Ec*: *Escherichia coli* ATCC® 25922™, *Kp*: *Klebsiella pneumoniae* ATCC® 13883™, *Pa*: *Pseudomonas aeruginosa* ATCC® 27853™, *Ab*: *Aspergillus brasiliensis* ATCC® 16404™, *Ca*: *Candida albicans* ATCC®10231™

According to the results of maximum inhibition zone measurement results conducted, the antimicrobial effects of *M. spicatum* plant extract from different localities on test microorganisms are as follows, respectively: *B. subtilis* > *E. faecalis* ≥ *C. albicans* > *A. brasiliensis* ≥ *C. freundii* > *P. aeruginosa* > *B. cereus* > *K. pneumoniae* > *C. perfringens* > *E. coli*. It was found that the highest effect obtained from the plant extracts obtained from *M. spicatum* species was on Gram positive bacterium *B. subtilis*, while the lowest effect was on Gram negative bacterium *E. coli*. Inhibition zones of *M. spicatum* ethanol extracts applied on microorganisms during the trial can be seen in Fig. 2.

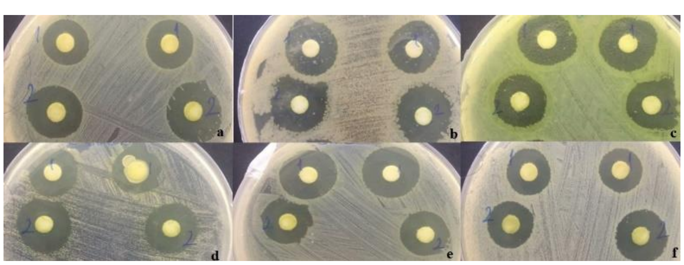

Figure 2. Zones formed by ethanol extract of *Myriophyllum spicatum* plants on test microorganisms. a: *Bacillus cereus*, b: *Enterococcus feacalis*, c: *Pseudomonas aeruginosa*, d: *Candida albicans*, e: *Citrobacter freundii*, f: *Clostridium perfiringens*; 1: Ulugöl Lake, 2: Terme Creek.

Great number of phytochemical substances within the content of extracts obtained from plants show high level of antimicrobial activity against microorganisms (Mojab et al., 2008). Plant organisms obtained by using organic solvents have higher antimicrobial activity than aquatic extracts because they include higher amounts of aromatic or saturated organic compounds (Cowan, 1999; Parekh et al., 2005). For this reason, ethanol was preferred in our study since it is not toxic. As a result of antimicrobial tests, it was found that the plant extracts obtained from *M. spicatum* species collected from different locations caused different effects on different strains. The highest antimicrobial effect against test organisms were on Fungi>Gram positive>Gram negative, respectively. According to the average values of inhibition zones, the larger inhibitory zone highest antimicrobial effect was obtained from *M. spicatum* extracts from Terme Creek (average 20.33 mm/25 μL). In traditional medicine, *M. spicatum* plant is used as an external agent for healing wounds and burns and cleaning wounds (Ya, 2015).

Myriophyllum spicatum is a widespread submerged plant specific to Europe, Asia and North Africa (Couch and Nelson, 1985). Since this species has tolerance for various aquatic conditions (oligotrophic-eutrophic, acidic-alkaline), it forms in various inland water masses (Aiken et al., 1979; Madsen, 1998; Buchan and Padilla, 2000). In studies conducted, while it was shown that low phosphor concentration did not have a limiting effect on the development of *M. spicatum* species, decrease in nitrogen was found to have a negative effect on growth (Best and Mantai, 1978). In our study, it was found the plant extracts which were obtained from Terme Creek with nutritious eutrophic conditions (Erkmen and Kolankaya, 2000) in terms of nitrogen and phosphor were more effective that those obtained from Ulugöl Lake sample (Taş et al., 2010) which had oligo-mesotrophic character. This result can show that *M. spicatum* samples which grow in Terme Creek with more nutritious water are richer than Ulugöl Lake in terms of secondary metabolites. For this, biochemical content analyses should be conducted and the plants' differences in different localities should be shown. In one study, the organic content of *Chara aspera* and *M. spicatum* plants taken from hard water and fresh water habitats were found to be different from each other. Organic content of *C. aspera* was found to be lower when compared with *M. spicatum.* Nutrient elements, chlorophyll, total phenolic compounds and anthocyanin content of plants and habitats were found to be different from each other and especially total phenolic compounds and anthocyanin content were recorded to be higher when compared with *M. spicatum* living in fresh water (Hempel et al., 2008).

A great number of studies conducted on freshwater macro hydrophyte *M. spicatum* species include allelopathic effects on unicellular algae. Acetone extracts of the plant have been found to show a strong inhibitor effect against various coccoid and filamentous cyanobacteria. In this study, it was reported that tellimagrandin II substance emerged as the main inhibitor substance and *M. spicatum* plant included a great amount of this compound (Gros et al., 1996). Also, in a study which examined the inhibitor effect of some allelochemical polyphenols released from *M. spicatum* species on the growth of *Microcystis aeruginosa* (toxic blue-green alga), it was reported that gallic and pyrogallic acids were more inhibitors than (+)-catechin and ellagic acid and auto-oxide products of each polyphenol were reported to show growth inhibition (Nakai et al., 2000). No research was found which examined the antimicrobial effect of *M. spicatum* plant extracts with allelochemical effect. In our study, it is a high probability that the substance inhibiting growth on microorganisms is polyphenol. In the present study, ethanol extracts obtained from *M. spicatum* plants had antimicrobial effect on all of the tested microorganism strains. The highest antimicrobial effect was shown on *Candida albicans* (22.5 mm) (Terme Creek sample, eutrophic water). The larger inhibitory zone of *M. spicatum* species was found on Gram positive bacterium *Bacillus subtilis.* This sample was collected from Terme Creek. In a study conducted by using some aquatic plants in the Northeast Anatolia Region of Turkey (*Butomus umbellatus, Polygonum amphibium, Sparganium erectum* and *S. emersum*), antimicrobial activity of methanol and acetone extracts were tested against three Gram positive, five Gram negative bacteria and one fungus. In the present study, methanol extracts of plants were not found to show inhibitor activity against any microorganism. Acetone extract was found to show significant activity only for Gram positive bacterium *B. subtilis* (Özbay and Alim, 2009). Thus, ethanol extracts of *M. spicatum* species have much higher antimicrobial effects than the aquatic plants used in the studies above. In addition, antifungal effect of *M. spicatum* ethanol extracts was found to be much higher than antibacterial effects in our study. While average inhibition zone was measured as about 21 mm, Terme Creek sample was found to show higher antifungal effect than Ulugöl Lake sample (~20 mm). In another study, it was found that *M. spicatum* ethanol extracts collected from Terme Creek showed antiparasite effect against *Acanthamoeba castellanii* trophozoite (Taş et al., 2018b). The results obtained from both trials showed that ethanolic It was also found that *Ceratophyllum demersum* plant, which is a different submerged plant, showed antiparasite effect on the same parasite (Taş et al., 2018c). The results obtained from both trials showed that ethanolic extracts of aquatic macrohydrophytes showed anti-amoebic activity against *A. castellanii* trophozoite, especially in high concentrations.

In studies conducted about the antimicrobial activity of plant compounds, it has been reported that it is more difficult for microorganisms to resist against plant drugs, that the therapeutical usage cost of plant products is lower when compared with chemical drugs and that the negative effects of synthetic substances can be decreased or eliminated with the use of plant products (Akkoç et al., 2009; Şimşek, 2013). For these reasons, it is thought that the significance of the success and/or protective role of plant compounds in antimicrobial treatments has increased gradually (Bali, 2018). At the same time, extensive studies should be conducted on a large number of aquatic plant species and research should be focus on determining biochemical structures. Aquatic plants such as terrestrial medicinal plants contain a great many active compounds. Literature information and studies conducted show that aquatic macrophytes used for medicinal, pharmacological, biological or environmental purposes have antimicrobial/antiparasitic effects. Plant extracts showed significant effect even though they were not pure antibiotic substances. We can say that plant extracts were more potent than all the standard antibiotics tested. Thus, aquatic plants can also be an important natural resource that can be assessed as medicinal plant. But, the isolation of bioactive compounds of the extracts and phytochemical characterization are necessary for future studies.

Conclusion

As a conclusion; *Myriophyllum* species produce phenolics. It is known that with its allelopathic effect, *M. spicatum* species inhibits the proliferation of cyanobacteria which cause eutrophication in freshwater with its allelopathic effect, that it has antiparasitic effect and that it is used externally for the healing of wounds. In the present study, it has been found that this aquatic plant has high antifungal effect and that it shows antibacterial effect. *M. spicatum* is a macrophyte which is widespread and which grows naturally in all kinds of freshwater. Thus, this macrophyte and similar macrophytes with high therapeutic potential can play a significant role in pharmaceutical process and drug discovery. Potential aquatic plants should be studied as new antimicrobial agents and which natural compounds they include should be found. To do this, more studies are needed on macrohydrophytes. Active ingredients of species which are found to have effect on microorganisms should be found and toxicological studies should be conducted for the potential of assessing these in pharmaceutical industry.

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