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Antimicrobial Activity of *Thuidium delicatulum* (Bryopsida) Extracts

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Abstract: This study aims to observe the antimicrobial activity of *Thuidium delicatulum* extracts. Moss samples were collected from Kastamonu - TURKEY and eight different extraction solvents were used in the study. All the extracts were investigated for *in vitro* antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Yersinia enterocolitica* O3, *Salmonella enteretidis* ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11230, *Candida albicans* ATCC 95071, *Escherichia coli* O157:H7 and *Listeria monocytogenes* ATCC 7644 by using the disc diffusion method. The results were supported with the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) tests. Six reference antibiotics were used as positive controls to compare the results. As a result, the extracts present activity against *Yersinia enterocolitica* O3, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 95071.

Keywords: *Thuidium delicatulum*, antimicrobial activity, disc diffusion method, MIC, MBC, MFC

Thuidium delicatulum (Bryopsida) Özütlerinin Antimikrobiyal Aktivitesi

Özet: Bu çalışmada, *Thuidium delicatulum* özütlerinin antimikrobiyal etkisinin gözlenmesi amaçlanmaktadır. Çalışmada kullanılan karayosunu türleri Kastamonu civarından toplanmış ve bu türlerden özütlerin elde edilmesi amacıyla sekiz farklı çözücü kullanılmıştır. Bütün özütlerin *in vitro* antimikrobiyal etkileri *Bacillus subtilis* ATCC 6633, *Yersinia enterocolitica* O3, *Salmonella enteretidis* ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11230, *Candida albicans* ATCC 95071, *Escherichia coli* O157:H7 ve *Listeria monocytogenes* ATCC 7644 suşlarına karşı disk difüzyon yöntemi kullanılarak denenmiştir. Disk difüzyon metodu ile elde edilen sonuçlar, minimum inhibisyon konsantrasyonu (MIC), minimum bakterisit konsantrasyonu (MBC) ve minimum fungisit konsantrasyonu (MFC) testleri ile desteklenmiştir. Çalışmada ayrıca sekiz adet referans antibiyotik pozitif kontrol olarak kullanılmıştır. Sonuç olarak, elde edilen özütlerin *Yersinia enterocolitica* O3, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 95071 suşlarına karşı etkili olduğu gözlenmiştir.

Anahtar kelimeler: *Thuidium delicatulum*, antimikrobiyal aktivite, disk difüzyon yöntemi, MIC, MBC, MFC

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Introduction

The healing powers of plants have been used for hundreds of years (Jones 1996). In many developing countries about 80% of the available therapeutic substances are originated from medicinal plants (Baytop 1999, Keleş et al. 2001). Scientists examine plants having medicinal properties for their biological activities ranging from antimicrobial to antitumor (Rajakaruna et al. 2002).

The antimicrobial activity of plants has many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans 1997, Reynolds 1996).

The Bryopsida is a large group, consisting of about 14,500 species (Veljic et al. 2008). It is interesting that only some birds and insects feed on mosses but the other organisms avoid them (Saxena and Harinder 2004). In contrast to the extensive utilisation of higher plants as a source of antimicrobial substances, Bryophytes have rarely been considered for this (Basile et al. 1998).

Although there are several evidences about using Bryophytes as medicinal plants more than 400 years in China (Asakawa 1990); it has been started to be documented in the last decades (Ando and Matsuo 1984, Garnier 1961). Since drug resistance develops in human pathogens against commonly used antibiotics, there is a need for a search about new antimicrobial substances from other sources including plants (Erdogrul 2002).

The aim of this study is to observe the antimicrobial activity of *Thuidium delicatulum* extracts.

Materials and Methods

1. Plant Material

Thuidium delicatulum (Schimp.), 1852 samples used in this study were collected from Kastamonu-TURKEY and identified by Professor Dr. B. Çetin and Associated Professor G. Uyar. Voucher specimens were deposited for further reference.

2. Extraction Procedure

Moss samples were extensively washed with sterile distilled water (sdH_2O). In order to reduce the particle size to ease the extraction procedure, moss samples were grounded with liquid nitrogen and a powder was obtained by crushing the dry, frozen material. 50 mg of grounded moss samples were extracted subsequently with 2 millilitres of chloroform, benzene, diethyl ether, ethyl alcohol, methyl alcohol, ethyl acetate, sdH_2O and 0.5M Tris-HCl buffer (pH:8.0). After 1 hour, all extracts were centrifuged at 4000 rpm and the supernatants were transferred into empty test tubes which were used for further analysis (Basile et al. 1998).

3. Microorganisms

Bacillus subtilis ATCC 6633, *Yersinia enterocolitica* O3, *Salmonella enteretidis* ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11230, *Candida albicans* ATCC 95071, *Escherichia coli* O157:H7 and *Listeria monocytogenes* ATCC 7644 were used in the study (Ankara University, Department of Biology, Bacteriology Laboratory Culture Collection).

4. Prepartion of Inocula

All bacterial strains were incubated at 37 ˚C for 24 hours and *Candida albicans* ATCC 95071 at 27 ˚C for 48 hours. Inocula were prepared by transferring morphologically similar colonies of each organism into 0.9% sterile saline solution until the visible turbidity was equal to 0.5 McFarland standard having approximately 10^8 cfu/ml for bacteria and 10^7cfu/ml for *Candida albicans* ATCC 95071 (Hammer et al. 1999).

5. Disc Diffusion Method

Disc diffusion test was performed as described previously in BSAC (BSAC

2003). The culture medium was poured into 120 mm sterile Petri dish to give a mean depth of 4.0 mm ± 0.5 mm. 20 µl aliquots of each extract was applied on sterile paper discs (HiMedia SD 067) of 6 mm diameter end up with 1 $mg.\mu l^{-1}$ sample on each disc (Mahasneh and El-Oqlah 1999). To get rid of any residual solvent which might interfere with the results, discs were left to dry overnight (Silici and Koc 2006). The surface of the plates was inoculated using previously prepared inocula containing saline suspension of microorganisms. Inoculated plates were then left to dry for 5 minutes at room temperature before applying the discs. Discs were firmly applied to the surface of the plate which had an even contact with the agar. Plates were incubated and inhibition zone diameters were expressed in millimetres.

6. Determination of MIC

Broth dilution method for Minimum Inhibitory Concentration (MIC) determination as described in Basile et al. (1998) was performed. Serial 2-fold dilutions were made to obtain a concentration range of 0,0039 - 2 $mg.ml^{-1}$. The MIC was defined as the lowest concentration of extract inhibiting any visible bacterial growth.

7. Determination of MBC and MFC

The Minimum Bactericidal Concentration (MBC) and the Minimum Fungicidal Concentration (MFC) determination were performed by sub-culturing suspensions from non-turbid MIC test tubes to agar medium. The MBC and MFC were defined as the lowest concentration of extract inhibiting bacterial and fungal growth.

8. Controls

All extraction solvents and empty sterile discs were used as negative controls. In addition to this, six standard antibiotic discs Cephalothin (30µg), Gentamicin (10µg), Cefuroxime (30µg), Ampicilline (10µg), Sulfamethoxazole - Trimethoprim (23,75 - 1,25 µg) and Vancomycin (30µg) were used as positive control.

9. Statistics

All extracts were tested in triplicate and MACANOVA (version 5.05) was used for statistical analysis of the data. *P* values of <0.05 were considered statistically significant.

Results

The main aim of this study was to identify the antimicrobial activity of *Thuidium delicatulum* (Hedw.) Schimp. extracts. To do this, the first test performed was "disc diffusion test". In this test, extracts were loaded on empty sterile discs and these discs were then applied on a culture medium inoculated with microorganisms. If the extracts were active against microorganisms, they have caused an inhibition zone. The diameter of the inhibition zones recorded as the diameter of the zones in millimetres for *Thuidium delicatulum* samples are given in Table 1.

Antimicrobial substances may have cidal or static type of activity. Cidal agents have a capability of killing microorganisms, where static agents have a capability of inhibiting the growth or reproduction of microorganisms. The disc diffusion test alone is not enough to decide whether the activity type is cidal or static. In order to identify the type of activity the disc diffusion test should be followed by MIC and MBC/MFC tests. Cidal agents have MFC values that are close to the MIC values. For static agents, the MIC values are much lower than the MFC values.

The MIC values, which were defined as the lowest concentration of extract inhibiting any visible microorganism growth stated as $mg.ml^{-1}$ are given in Table 2. The MBC and MFC values which were defined as the lowest concentration of extract inhibiting bacterial and fungal growth after sub-culturing suspensions from

non-turbid MIC test tubes to agar medium stated as $mg.ml^{-1}$ are given in Table 3.

All the results are compared with standard antibiotic discs. The disc diffusion test results for the standard antibiotic discs are given in Table 4. There is no activity observed for the negative controls; solvents and empty sterile discs.

Discussion

In the study eight different solvents were used. Among these eight different solvents; chloroform is the most effective solvent in terms of extracting antimicrobial substances against tested organisms. An activity was also observed against *Candida albicans* ATCC 95071 in ethyl alcohol, methyl alcohol, ethyl acetate and 0.5M Tris-HCl buffer (pH:8.0) extracts. But this activity is less than the activity of chloroform extracts.

According to Cowan (1999) chloroform can extract flavonoids and terpenoids from plant samples. But the intersection of the active substances those can be extracted by ethyl alcohol, methyl alcohol and chloroform are only terpenoids (Cowan 1999).

In the light of this information, if the extracts presenting antimicrobial activity against *Yersinia enterocolitica* O3 and *Staphylococcus aureus* ATCC 25923 are analysed, it can be concluded that this active substance can only be a flavonoid, because no activity was observed in ethyl alcohol and methyl alcohol extracts too. It can also be concluded that the active substance showing antimicrobial activity against *Candida albicans* ATCC 95071 can be a terpenoid, because an activity was observed not only for the chloroform extract, but also for ethyl alcohol and methyl alcohol extracts as well.

When the results of MIC and MBC/MFC tests are compared, the MIC values and MBC values for the chloroform extract of *Thuidium delicatulum* used against *Yersinia enterocolitica* O3 and *Staphylococcus aureus* ATCC 25923 are observed that they are the same. It can be concluded that these extracts have cidal effect against these microorganisms. Also the chloroform, ethyl alcohol and buffer extracts of *Thuidium delicatulum* used against *Candida albicans* ATCC 95071 have a cidal effect, but since the MFC values are higher than MIC values for the methyl alcohol and ethyl acetate extracts have, these extracts a static effect against the same microorganism.

Fig. 1 shows the comparison of the disc diffusion test results of the standard antibiotic discs with the *Thuidium delicatulum* extracts. According to this figure it can be concluded that the chloroform extract has higher antimicrobial activity than Cephalothin and Ampicillin against *Yersinia enterocolitica* O3.

The chloroform extract has also lower antimicrobial activity against *Staphylococcus aureus* ATCC 25923 when compared to all standard antibiotic discs, but it has an effect close to Vancomycin. Chloroform, ethyl alcohol, methyl alcohol, ethyl acetate and 0.5M Tris-HCl buffer (pH: 8.0) extracts have lower antimicrobial activity against *Candida albicans* ATCC 95071 when compared to all standard antibiotic discs.

The amount of the extract loaded on the empty sterile antibiotic discs was 1 $mg.\mu l^{-1}$. As a result of this study it is obvious that with an increase in the amount of extract loaded on discs, *Thuidium delicatulum* extracts, especially chloroform extracts, can be used as an antimicrobial agent against *Yersinia enterocolitica* O3, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 95071.

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Table 1. Disc diffusion test results (Inhibition zones in mm)

	Chloroform	Benzen	Diethyl Ether	Ethyl Alcohol	Methyl Alcohol	Ethyl Acetate	sdH_2O	Buffer
Bacillus subtilis ATCC 6633	-	-	-	-	-	-	-	-
Yersinia enterocolitica O3	10.00±0.00	-	-	-	-	-	-	-
Salmonella enteretidis ATCC 13076	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC 25923	17.67±0.34	-	-	-	-	-	-	-
Escherichia coli ATCC 11230	-	-	-	-	-	-	-	-
Candida albicans ATCC 95071	8.00±0.00	-	-	8.67±0.34	8.00±0.00	7.67±0.34	-	9.00±0.00
Escherichia coli O157:H7	-	-	-	-	-	-	-	-
Listeria monocytogenes ATCC 7644	-	-	-	-	-	-	-	-

"-": no activity observed.

Table 2. MIC test results (active concentration as $mg.ml^{-1}$)

	Chloroform	Benzen	Diethyl Ether	Ethyl Alcohol	Methyl Alcohol	Ethyl Acetate	sdH_2O	Buffer
Bacillus subtilis ATCC 6633	-	-	-	-	-	-	-	-
Yersinia enterocolitica O3	1.00±0.00	-	-	-	-	-	-	-
Salmonella enteretidis ATCC 13076	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC 25923	0.50±0.00	-	-	-	-	-	-	-
Escherichia coli ATCC 11230	-	-	-	-	-	-	-	-
Candida albicans ATCC 95071	2.00±0.00	-	-	2.00±0.00	2.00±0.00	2.00±0.00	-	2.00±0.00
Escherichia coli O157:H7	-	-	-	-	-	-	-	-
Listeria monocytogenes ATCC 7644	-	-	-	-	-	-	-	-

"-": no activity observed.

Table 3. MBC and MFC test results (active concentration as $mg.ml^{-1}$)

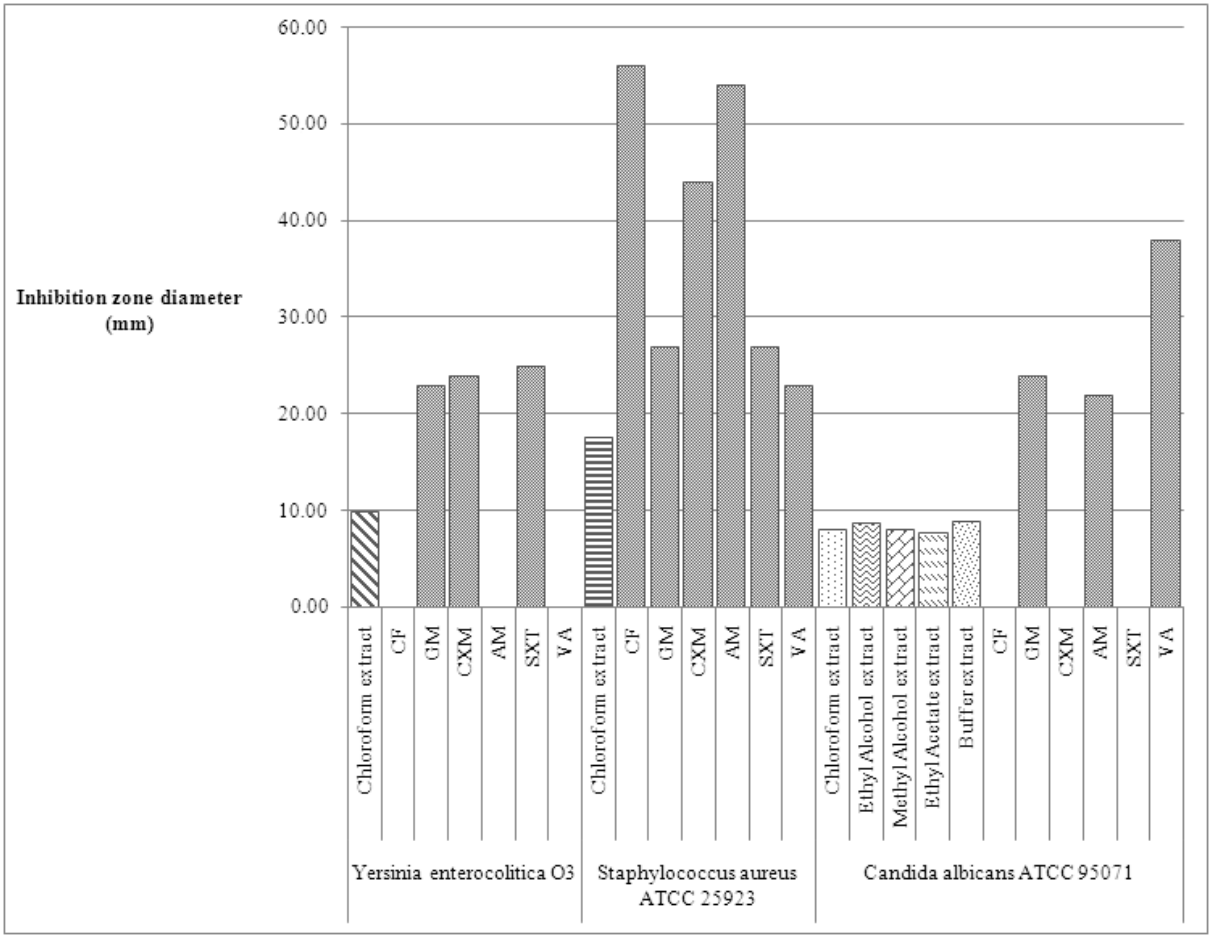
	Chloroform	Benzen	Diethyl Ether	Ethyl Alcohol	Methyl Alcohol	Ethyl Acetate	sdH_2O	Buffer
Bacillus subtilis ATCC 6633	-	-	-	-	-	-	-	-
Yersinia enterocolitica O3	1.00±0.00	-	-	-	-	-	-	-
Salmonella enteretidis ATCC 13076	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC 25923	0.50±0.00	-	-	-	-	-	-	-
Escherichia coli ATCC 11230	-	-	-	-	-	-	-	-
Candida albicans ATCC 95071	2.00±0.00	-	-	2.00±0.00	>2.00±0.00	>2.00±0.00	-	2.00±0.00
Escherichia coli O157:H7	-	-	-	-	-	-	-	-
Listeria monocytogenes ATCC 7644	-	-	-	-	-	-	-	-

"-": no activity observed.

Table 4. Disc diffusion test results for standard antibiotic discs (Inhibition zones as mm)

	CF	GM	CXM	AM	SXT	VA
Bacillus subtilis ATCC 6633	56.00	44.00	40.00	32.00	22.00	32.00
Yersinia enterocolitica O3	-	23.00	24.00	-	25.00	-
Salmonella enteretidis ATCC 13076	20.00	24.00	17.00	-	23.00	19.00
Staphylococcus aureus ATCC 25923	56.00	27.00	44.00	54.00	27.00	23.00
Escherichia coli ATCC 11230	19.00	20.00	26.00	20.00	23.00	8.00
Candida albicans ATCC 95071	-	24.00	-	22.00	-	38.00
Escherichia coli O157:H7	18.00	22.00	24.00	-	17.00	-
Listeria monocytogenes ATCC 7644	15.00	26.00	26.00	23.00	21.00	9.00

"-": no activity observed.
CF: Cephalothin, GM: Gentamicin, CXM: Cefuroxime, AM: Ampicillin, SXT: Sulfamethoxazole – Trimethoprim, A: Vancomycin



CF: Cephalothin, GM: Gentamicin, CXM: Cefuroxime, AM: Ampicillin, SXT: Sulfamethoxazole -Trimethoprim, VA: Vancomycin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