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Antimicrobial Screening of *Calliergonella cuspidata*, *Dicranum polysetum* and *Hypnum cupressiforme*

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The anti-infective activities of plant-derived secondary metabolites are under investigation in recent years due to accelerating antibacterial and antifungal resistance rates of microorganisms. In this study, antimicrobial activity of *Calliergonella cuspidata*, *Dicranum polysetum* and *Hypnum cupressiforme* were screened against *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784 by using the disk diffusion method. It is observed that ethanolic extract of the moss samples have antimicrobial activity against several gram positive and gram negative microorganisms tested. But antimicrobial activity of *D. polysetum* is notable especially against *S. carnosus*. These results are the very first data about the antimicrobial activity of *C. cuspidata* and *D. polysetum*.

Key words: *Calliergonella cuspidata*, *Dicranum polysetum*, *Hypnum cupressiforme*, Bryophyte, antimicrobial activity, disk diffusion test.

Secondary metabolites synthesized by plants are not necessarily produced under all conditions and in majority of the cases the function of these compounds and their benefit to the organism are not totally known[1]. But it is determined that these compounds are synthesized especially as a defence mechanism against microorganisms, insects and herbivores[2,44-45].

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The anti-infective activities of plant-derived secondary metabolites are under investigation in recent years due to accelerating antibacterial and antifungal resistance rates of microorganisms. Since antibacterial and antifungal resistance have been developing in human pathogens against commonly used antibiotics, there is a need for a search about new antimicrobial substances[3]. In addition, increasing morbidity and mortality rates of bacterial and fungal infections also lead to a research on new antimicrobial agents[4].

Generally microorganisms, insects, snails, slugs and small mammals cannot damage bryophytes[5]. It has been known for years that Bryophytes have anti-infective activity on some microorganisms[5-16,46]. Bryophytes were also used to treat cardiovascular diseases, tonsillitis,

bronchitis, cystitis and skin infections especially in traditional Chinese medicine. Native Americans used some bryophytes, such as *Bryum*, *Mnium* and *Philonotis*, to heal burns, bruises and wounds[17].

Bryophytes are plants which define about 18,500 species[18]. In contrast to the extensive utilisation of higher plants as a source of antimicrobial substances, only minute amount of the Bryophytes are screened in terms of their anti-infective properties until now[7].

The aim of this study is to test the antimicrobial activity of some moss samples, namely *Calliergonella cuspidata*, *Dicranum polysetum* and *Hypnum cupressiforme*.

Dicranum polysetum Sw. ex anon. (Family = Dicranaceae Schimp.) is a Dicranum species with porose cells throughout the leaf and recurved leaf margin of leaf base. Its leaf is strongly undulate and coarsely spinosely toothed above.

Hypnum cuppressiforme Hedw. (Family = Hypnaceae Schimp.) is a common pleurocarpus moss. It's slender to medium sized and usually forming smooth mats. It has strongly curved leaves, which has very short and double or no costa.

Calliergonella cuspidata (Hedw.) Loeske (Family = Hypnaceae Schimp.) one of the most common moss of moist habitats. It has an erect main stem and pinnately arranged branches. With egg shaped leaves and well developed colourless angular cells it can't be confused with another moss species.

In this study, *in vitro* antimicrobial activity of *Calliergonella cuspidata*, *Dicranum polysetum* and *Hypnum cupressiforme* was investigated against *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784 by using the disk diffusion method.

MATERIALS AND METHODS

Moss Samples

All moss samples were collected from Akda Mountain, Amasya, which is located between Central Anatolia and the Middle Black Sea region. *Calliergonella cuspidata* was collected from N 40° 48.839', E 035° 57.754', 1240 m, *Dicranum polysetum* from N 40° 51.909', E 035° 47.648', 1050 m and *Hypnum cupressiforme* from N 40° 48.249', E 036° 9.532', 1060 m.

Voucher specimens were deposited for further reference in Herbarium of Ankara University (ANK) Faculty of Science, Department of Biology, Ankara, TURKEY.

Extraction Procedure

All moss samples were air-dried after collection and the samples were ground by a mortar and a pestle. Ground samples were shaken in ethanol (Merck, Germany) at 100 rpm for 3 days at room temperature to extract active substances. At the end of the extraction procedure all samples were filtered through Whatman No. 1 filter paper into evaporation flasks. The filtrate was evaporated by a rotary evaporator (Heidolph Hei-Vap Value HL/HB-G1) at 30°C [19]. After evaporation the residues were collected and used to prepare 14 mg mL^{-1} extracts for *Calliergonella cuspidata*, 21 mg mL^{-1} extracts for *Dicranum polysetum* and 10 mg mL^{-1} extracts for *Hypnum cupressiforme*.

Microorganisms

A wide range of gram positive and gram negative bacteria and yeast were selected to test the antimicrobial effect of three moss samples.

Bacillus subtilis ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC 13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784 were used in the study.

All strains used in this study were supplied by Ankara University, Faculty of Science,

Department of Biology. The strains which are not standard were all isolated from food and identified in Ankara University, Faculty of Science, Department of Biology.

Preparation of Inocula

All bacterial strains were incubated at 37 °C for 24 hours and *C. albicans* was incubated at 27 °C for 48 hours. Tryptic Soy Broth (TSB) (Merck, Germany) was selected as the culturing medium for *B. subtilis*, *E. durans*, *E. faecalis* and *E. faecium*; Luria-Bertani Broth (LB) (Merck, Germany) was selected as the culturing medium for *E. coli* ATCC 25922, *E. coli* CFAI, *E. aerogenes*, *K. pneumonia*, *L. monocytogenes*, *S. enteritidis*, *S. infantis*, *S. kentucky*, *S. typhimurium* and *S. aureus*; TSB including yeast extract (TSYB) was selected as the culturing medium for *S. carnosus*, *S. epidermidis* and *S. agalactiae* and malt extract was selected as the culturing medium for *C. albicans* to enrich strains to prepare inocula.

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^{-1} for bacteria and 10^7cfu.mL^{-1} for *C. albicans*[20-22]. All inocula were prepared freshly and used right after preparation.

Disk Diffusion Method

Disk diffusion test was performed as described previously by Andrews[23]. The culture medium, Mueller Hinton Agar (Merck, Germany) was poured into 120 mm sterile Petri dish to give a mean depth of 4.0 mm ± 0.5 mm[24,25].

40 µL, 60 µL and 100 µL aliquots of *C. cuspidata* were applied on sterile paper disks, where 50 µL, 100 µL and 150 µL aliquots of *D. polysetum* and *H. cupressiforme* was applied on sterile paper disks. Amount of samples loaded on sterile paper disks were given in Table 1.

Since any residual solvent might interfere with the results, disks were left to dry overnight at 30°C in sterile conditions[26,27]. The surface of the plates was inoculated using previously prepared inocula which contain saline suspension of microorganisms. Before applying the disks, inoculated plates were left to dry for 5 minutes at room temperature. Then disks were firmly applied to the surface of the plate[28,29]. Plates were incubated and inhibition zone diameters were expressed in millimetres.

Controls

Empty sterile disks and extraction solvent (ethanol) were used as negative controls.

Statistics

All tests are conducted in three parallels and all the results given here are mean values of these parallel studies. In order to define whether the difference between results are statistically significant or not, a statistical analysis namely Kruskal-Wallis one-way analysis of variance is chosen. This method is a non-parametric method and it was chosen since the number of data compared is not enough to apply any parametric methods. As a result, a value of $P < 0.05$ was considered statistically significant.

RESULTS

Amount of moss samples loaded on sterile paper disks are given in Table 1.

The diameter of the inhibition zones recorded in millimetres is given in Table 2. No activity was observed for the negative controls; solvents and empty sterile disks.

Table 2 shows that ethanolic extract of *C. cuspidata* presented antimicrobial activity against *K. pneumoniae*; where *D. polysetum* presented antimicrobial activity against *B. subtilis*,

Table 1. Amount of moss samples loaded on sterile paper disks (µg.µL^{-1})

	Amount of samples loaded on disks (µg.µL^{-1})				
	40 µL	50 µL	60 µL	100 µL	150 µL
C. cuspidata	560	-	840	1400	-
D. polysetum	-	1050	-	2100	3150
H. cupressiforme	-	500	-	1000	1500

"-": Not applied.

Table 2. Disk diffusion test results (Inhibition zones in mm)

	C. cuspidata			*D. polysetum*			*H. cupressiforme*		
	40µL	60µL	100µL	50µL	100µL	150µL	50µL	100µL	150µL
B. subtilis	-	-	-	10	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-
E. aerogenes	-	-	-	-	-	-	-	-	-
E. durans	-	-	-	-	-	-	-	-	-
E. faecalis	-	-	-	-	-	-	-	-	-
E. faecium	-	-	-	-	-	-	-	-	-
E. coli ATCC	-	-	-	-	-	-	-	7	7
E. coli CFAI	-	-	-	-	-	-	-	-	-
K. pneumoniae	7	7	7	-	-	-	7	7	7
L. monocytogenes	-	-	-	-	-	-	-	-	-
S. enteritidis	-	-	-	-	-	-	-	-	-
S. infantis	-	-	-	-	7	7	-	7	7
S. kentucky	-	-	-	-	-	-	7	7	7
S. typhimurium	-	-	-	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-	-	-	-
S. carnosus	-	-	-	10	10	10	-	-	-
S. epidermidis	-	-	-	8	7	7	-	-	-
S. agalactiae	-	-	-	9	7	-	-	-	-

"-": No activity observed

S. infantis, *S. carnosus*, *S. epidermidis* and *S. agalactiae*. In addition *H. cupressiforme* presented antimicrobial activity against *E. coli* ATCC, *K. pneumoniae*, *S. infantis* and *S. kentucky*.

DISCUSSION

As far as the current literature is concerned, there have been no reports about the antimicrobial activity of *C. cuspidata* and *D. polysetum* up to now. Results given in Table 2 are the very first data about the antimicrobial activity of these moss samples. On the other hand[5,6,8,14-16] showed that *H. cupressiforme* contains several biflavonoids (hypnogenol B1 and hipnumflavonoid A), which presented antibacterial activity against several microorganisms.

Among the microorganisms used in our study *B. subtilis*, *S. carnosus*, *S. epidermidis* and *S. agalactiae* which were affected by all three extracts are gram positive; where *E. coli* ATCC, *K. pneumoniae*, *S. infantis* and *S. kentucky* are gram negative strains.

Although the antimicrobial activity against the gram negative strains tested in the study were very low, these results are very important since it is a well known fact that gram negative bacteria are in general more resistant to a large number of antibiotics and chemotherapeutic agents than gram positive bacteria[30].

Ethanolic extracts of *H. cupressiforme* showed 7 mm inhibition zone both for disks containing 100 µL (1000 µg.µL^{-1}) and 150 µL (1500 µg.µL^{-1}) extracts against *E. coli* ATCC strain. These results are matching with the previous studies conducted by *H. cupressiforme*. Inhibition zone of 8 mm was previously observed by Veljic *et al.*[5] against *E. coli* ATCC 25922 with 10 mg.mL^{-1} methanolic extracts of *H. cupressiforme*. Çolak *et al.*[14] showed that ethanolic extract of *H. cupressiforme* caused 6.9±0.7 mm inhibition zone against *E. coli* ATCC 35218, but no antimicrobial activity was observed in methanol, acetone and chloroform extracts of *H. cupressiforme* against *E. coli* ATCC 35218. Savaroglu *et al.*[16] tested methanol, chloroform and acetone extracts of *H. cupressiforme* against an *E. coli* strain and observed no antimicrobial activity against this strain. Dulger *et al.*[8] showed that the antimicrobial activity of the methanolic extract of *H. cupressiforme* (30mg.mL^{-1}) against *E. coli* ATCC 11230 was 12.2 mm. Since the strains and the

concentrations of extracts used in previous study were different, the difference in the results is very meaningful.

Dulger *et al*[8] also studied antimicrobial activity of the methanolic extract of *H. cupressiforme* (30mg mL^{-1}) against *K. pneumoniae* and found the inhibition zone as 10.4 mm. Since the concentration of the extract used in the study was higher than the concentration we used in our study, an inhibition zone higher than 7 mm is not a surprise. In addition, the ethanolic extracts of *C. cuspidate* also showed antimicrobial activity against *K. pneumoniae*, which is equal to the activity against *E. coli* ATCC.

It was reported that antibiotics of natural origin showed >90% lacked activity against *E. coli*, although they were active against gram-positive strains[31]. It was also previously pointed out that gram negative bacteria, such as *Klebsiella* are the dominant killers among bacterial pathogens in the Intensive Care Units (*ICU*) [32]. From this point of view, having antibacterial activity against *E. coli* and *K. pneumoniae* is very important.

S. kentucky is defined as a "superbug" since it can develop resistance to some antibiotics[33]. *S. kentucky* infection was previously uncommon but an increase was observed especially after 2006 in Northeast Africa and Turkey[34]. This strain display high-level resistance to ciprofloxacin, one of the drugs used against Salmonella diseases. In addition, secondarily acquired resistances to extended-spectrum cephalosporin and trimethoprim + sulfamethoxazole were also observed[35]. An antimicrobial activity of *H. cupressiforme* against *S. kentucky* was observed in the study and these results are the very first results regarding the antimicrobial activity of *H. cupressiforme* against *S. kentucky*.

Although the results against these gram negative strains are low, probably increasing amount of extracts loaded on the empty sterile antibiotic disks may increase the activity.

Although the pathogenic potential of *B. subtilis* is generally described as low or absent[36], *B. subtilis* is known to cause disease in severely immuno compromised patients[37].

In our study, only *D. polysetum* presented antimicrobial activity against *B. subtilis* in only 50 µL sample. Interestingly 100 µL and 150 µL samples showed no activity. Since these results were true for all three parallel studies, a detailed research is needed in order to explain why only 50 µL sample presented antimicrobial activity against *B. subtilis*.

Altuner *et al.*[4] tested *Tortella tortulosa* samples against *Bacillus subtilis* ATCC 6633, and Altuner and Çetin[11] used *Thuidium delicatulum* against *Bacillus subtilis* ATCC 6633 too. But no antimicrobial activity was observed in both extracts. *D. polysetum* presented higher antimicrobial activity when compared to *Tortella tortulosa* and *Thuidium delicatulum*.

As far as the current literature is taken into account the antimicrobial activity against *S. infantis*, *S. carnosus* and *S. agalactiae* of moss samples are reported here for the very first time.

S. epidermidis is not usually pathogenic. But it often develops risk for infection for patients with a compromised immune system. These infections can be both nosocomial and community acquired, but they pose a greater threat to hospital patients. *S. epidermidis* is also a major concern for people with catheters or other surgical implants because it is known to cause biofilms that grow on these devices[38,39].

Several studies conducted on the antimicrobial activity of several higher plants against *S. epidermidis*. Mahida and Mohan[40] tested 10 mg of methanolic extracts of 23 plant extracts, but the highest zone was found to be 20 mm for *Mangifera indica*. In another study ethanolic extracts of 23 plants were tested against *S. epidermidis* and the highest inhibition zone diameter was given as 18 mm for *Stachys leptoclada*[41].

In our study we observed 8 mm inhibition zone for 50 µL where the inhibition zones are 7 mm for both 100 µL and 150 µL sample of *D. polysetum* extracts against *S. epidermidis* which is relatively low when compared to other previous studies. But it is possible to increase the activity by increasing the concentration of the extract.

As a result of the study, it is observed that ethanolic extract of three Bryophyte samples have antimicrobial activity against several gram positive and gram negative microorganims tested and antimicrobial activity of *D. polysetum* is notable especially against *S. carnosus*. But further researches are needed to be conducted in order to analyse the active substances in details.

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