Journal of Medicinal Plants Research Vol. 5(14), pp. 3286-3292, 18 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

An evaluation of the antimicrobial activity of some Turkish mosses

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Accepted 8 April, 2011

The antibacterial and antifungal activities of seven different extract from the mosses *Funaria hygrometrica* Hedw. (Funariaceae), *Hypnum cupressiforme* Hedw. (Hypnaceae), *H. imponens* Hedw. (Hypnaceae), *Polytrichum juniperinum* Hedw. (Polytrichaceae) and *Tortella tortuosa* (Hedw.) Limpr. (Pottiaceae) were tested against six bacterial and three fungal strains by agar diffusion and microdilution methods. It was determined that some of the extracts were active on both Gram-positive and Gram-negative bacteria. In addition, extract A of *T. tortuosa* possessed the highest antibacterial activity against *Pseudomonas aeruginosa* (MIC of 5.9 µg/ml). Among the five bryophytes used, *P. juniperinum* and *T. tortuosa* showed the best inhibitory effect against the bacterial and fungal species tested. The current study indicates that extracts of *P. juniperinum* and *T. tortuosa* may be exploited for antimicrobial drugs in the future.

Key words: Bryophyte, extracts, mosses, antimicrobial activity, minimum inhibitory concentration (MIC).

INTRODUCTION

It is estimated that there are between about 15,000 and 25,000 bryophyte species known in the world. They consist of three separate divisions, the Marchantiophyta (liverworts), Anthocerotophyta (hornworts), and Bryophyta (mosses) (Goffinet and Shaw, 2009). They are to be found in all ecosystems, from desert to alpine, with the exception of marine. The ecological role of bryophytes in any ecosystem is significant (Saxena and Harinder, 2004). Although bryophytes normally grow in humid habitats, they are relatively free from microbial attack and this scarcity of disease indicates that bryophytes are able to elaborate constitutive or inducible small-molecule antimicrobials. In fact, bryophytes have been proven to be a rich source of antibiotics, and attempts to find potent, nontoxic, broad-spectrum antibiotics from these sources have widely been undertaken (Xie and Lou, 2009).

Very little is known about the chemistry of Bryophytes and information concerning research results is very scattered. The reasons for this are the difficulty that researchers have with their identification, the limited amount of the same species available for analyses due to their inconspicuous position in the ecosystem, and the difficulty with which analysis can be conducted since it relies on sophisticated methods. Bryophytes are a very interesting group in botany but few studies have successfully investigated their chemistry, especially at molecular level (Asakawa, 2001; Jockovic et al., 2008; Ucuncu et al., 2010). Generally, bryophytes are known to possess extremely high amounts of terpenoids, phenolics (flavonoids and bibenzyl derivatives), glycosides, fatty acids, as well as some rare aromatic compounds (Jockovic et al., 2008; Sabovljevic et al., 2009; Sabovljevic et al., 2010; Zinsmeister and Mues, 1987; Zinsmeister et al., 1991). Very few studies concerning the biologically active constituents of bryophytes have been published (Basile et al., 1999; Dulger et al., 2005, 2009; Ilhan et al., 2006; Sabovljevic et al., 2006; Singh et al., 2007; Veljić et al., 2008; Mewari and Kumar, 2008; Bodade et al., 2008; Sabovljevic et al., 2010; Elibol et al., 2011; Oztopcu-Vatan et al., 2011; Savaroglu et al., 2011).

This study analyzed the antimicrobial activity of seven different extracts of *Funaria hygrometrica* Hedw. (Funariaceae), *Hypnum cupressiforme* Hedw. (Hypnaceae), *Hypnum imponens* Hedw. (Hypnaceae), *Polytrichum juniperinum* Hedw. (Polytrichaceae) and

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Table 1. Percentage yield rates of obtained extracts.

Species name	Me	K	As	A	B	C	D
Funaria hygrometrica	3.44	2.88	6.51	0.65	5.81	0.77	4.72
Hypnum cupressiforme	5.45	1.25	1.76	0.41	5.18	0.76	4.18
Hypnum imponens	3.00	0.89	1.32	0.44	2.57	0.47	2.05
Polytrichum juniperinum	5.17	2.04	2.93	0.89	6.66	1.47	6.10
Tortella tortuosa	4.56	1.21	2.77	0.63	3.75	0.58	3.01

Me, methanol; K, chloroform; As, acetone; A, B, C and D represent the extracts obtained from the second extraction method.

Tortella tortuosa (Hedw.) Limpr. (Pottiaceae) against some bacterial and fungal species. In addition, the presence of flavonoids, terpenoids, anthraquinone, and alkaloids in the extracts were investigated, followed by rapid screening methods.

MATERIALS AND METHODS

Plant materials

Five moss species belonging to 4 different families were collected from the Sundiken Mountains (Eskisehir, Turkey), in 2007, and identified by Dr. F. Savaroglu, Department of Botany, Eskisehir Osmangazi University. Voucher specimens have been deposited in the Herbarium of our department.

Extraction procedure

Only green or green brown shoots were used for experimental work. The plant material was carefully cleaned of attached litter and dead material under running tap water. Fresh gametophytic samples of five moss species were treated with 0.8% Tween 80 aqueous solution to remove any epiphytic hosts normally found on the surface, extensively washed in tap and distilled water, and dried on filter paper at room temperature. Dried materials were then ground with a hammer mill.

Extraction was carried out through two different processes. In the first process, 10 g of the sample in powder form was extracted with 250 ml of 80% methanol, chloroform, and acetone for 8 h using soxhlet equipment. After filtering with Whatman filter paper, all extracts were concentrated by rotary evaporation to dryness in vacuum and stored at +4°C for future use (Jones and Kinghorn, 2005).

The second extraction process was completed in four steps. First, 30 g of gametophytic plant sample in powder form was extracted with 250 ml of petroleum ether for 8 h using soxhlet equipment, and the solvent was removed under reduced pressure on a rotary evaporator. (Extract A). At the second stage, fat-free air-dried material (15 g) was extracted four times with methanol: water (70:30, v/v) at 40°C, 30 min. The extract was then concentrated to dryness in vacuum (Extract B). The third and fourth extracts were prepared as follows: fat-free air-dried material (15 g) was extracted four times with methanol: water (70:30 v/v) at 40°C, 30 min, and this was concentrated in vacuum for evaporation with methanol, and the residual aqueous phase was extracted with ethyl acetate at room temperature. This was then concentrated to dryness in vacuum (Extract C). The aqueous solution was concentrated separately by rotary evaporation to dryness in vacuum (Extract D) (Jones and Kinghorn, 2005; Ozturk et al., 2009; Tsao and Deng, 2004). The percent yields of the extracts obtained using the two extraction methods are given in Table 1. All yields were stored at 4°C for further use. Before being used, they were first weighed and then dissolved in dimethyl sulphoxide (DMSO) to a final concentration of 200 mg/ml.

Phytochemical screening

An attempt was also made to observe the presence and absence of different phytochemical constituents, alkaloids (Dragendorff's test), anthraquinones, flavonoids (using dilute ammonia and concentrated sulphuric acid), and terpenoids (Salkowski test) according to standard methods (Ayoola et al., 2008).

Microorganisms

Bacterial strains were recovered from long-term storage at –80°C in the cryobank. The bacteria were refreshed in Nutrient Broth (Merck, Germany) at 35 to 37°C, and then inoculated on Nutrient Agar (Merck) plates to check the microbial purity. The moulds were refreshed in Malt Extract Agar (Merck) at 27°C. The strain numbers and sources of the acquired microorganisms are listed in Table 2.

Antimicrobial activity

This experiment was performed according to the method described by the National Committee for Clinical Laboratory Standards (NCCLS, 2008), with some modification. The bacterial test cultures were incubated in Mueller-Hinton broth (MHB) at 35 to 37°C until they were visibly turbid. The density of these cultures was adjusted to a turbidity equivalent to that of the 0.5 McFarland standard (at 625 nm, 0.08 to 0.1 absorbance) with sterile saline. Alternatively, to induce spore formation, the molds were grown on potato dextrose agar slants at 27°C for 5 to 7 days. After being counted with the Thoma slide, the spore concentration was adjusted to 10^6 CFU/ml with sterile 0.1% Tween 80 for each mold. Mueller-Hinton agar (MHA) for bacteria and Sabouraud dextrose agar (SDA) for fungi, sterilized in a flask and cooled to 45 to 50°C, were distributed to the sterilized Petri dishes (9 cm). The entire surface of the MHA plates and Sabouraud 4% glucose medium (SGM) plates was inoculated with the bacteria and fungi by spreading them with a sterile swab dipped into the adjusted suspensions. Six wells, each 6 mm in diameter, were cut out of the agar and 20 µL of the extract solutions were placed into each well. The plates were incubated with bacteria at 37°C for 24 h or with fungal strains at 30°C for 48 h. Following these incubations, the dishes were kept at 4°C for 2 h. The diameters of the inhibition zones were measured in millimeters. Penicillin and tetracycline (Bioanalyse) were used as a positive control for bacteria, amphotericin B (Sigma) as a positive control for

Table 2. Bacterial and fungal strains used for antimicrobial activity test.

Bacterial strains	Fungal strains
[a]*Bacillus subtilis* NRRL B-209	[a]*Aspergillus fumigatus* NRRL 163
[b]*Enterococcus faecalis* ATCC 29212	[d]*Fusarium solani* (wild type)
[b]*Escherichia coli* ATCC 25922	[d] *Geotrichum candidum* (wild type)
[b]*Pseudomonas aeruginosa* ATCC 27853	
[c]*Salmonella typhimurium* ATCC 14028	
[b]*Staphylococcus aureus* ATCC 25923	

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fungi, and DMSO was used as the negative control. All assays were done in duplicate.

Minimum inhibitory concentration (MIC)

MIC was determined by the micro dilution method using a 96 well plate according to NCCLS (NCCLS, 2008). Primarily, 100 µL of MHB or Sabouraud dextrose broth (SDB) was placed in each well. The stock solutions of the extracts were diluted and transferred into the first well, and serial dilutions were performed so that concentrations in the range of 1.5 to 1500 µg/ml were obtained. The inoculums were adjusted to contain approximately 10^5 CFU/ml bacteria and 10^4 CFU/ml fungi, as described previously. One hundred µL of the inoculums was added to all the wells and the plates were incubated at 37°C for 24 h for bacteria or at 30°C for 48 h for fungi. MIC values were detected by adding 20 µL of 0.5% trifenil tetrazolium chloride (TTC) aqueous solution. The MIC value was taken as the lowest concentration of the extract that inhibited any visible bacterial or fungal growth, as indicated by TTC staining after incubation (NCCLS, 2008). Penicillin and tetracycline were used again as the reference antibiotic control.

RESULTS AND DISCUSSION

Thirty-five crude extracts from five species of bryophytes were screened for antimicrobial activity against six bacterial and three fungal strains (Table 2). Agar diffusion method was used for screening process. The crude extracts obtained with two extraction methods from five moss species were applied by the agar diffusion method (Table 3). With the exception of extract D, all the crude extracts assayed demonstrated visible antibacterial activity against the Gram-positive bacterium *B. subtilis*. Similarly, 77% of extracts showed activity against the Gram-negative bacterium *P. aeruginosa*. However, while the rate of extract activity against the Gram-negative bacterium *E. coli* and two other Gram-positive bacteria - *E. faecalis* and *S. aureus* - were lower (6, 17 and 34%, respectively), none of the extracts showed any activity against the Gram-negative bacterium *S. typhimurium*. It is significant that all extracts of *P. juniperum* and *T. tortuosa* showed an inhibition effect against the Gram-negative bacterium *P. aeruginosa*. Typically, antibiotics are generally more active against gram-positive than gram-negative bacteria. However, the antibacterial activity of mosses was found to be active against gram-negative bacteria. This clearly makes the selection of bryophytes as antimicrobial agents advantageous.

The preliminary results of screening showed that according to the extraction method used, the mosses species exhibited a distinct difference in antibacterial activities. In this study, two extraction methods were used. In the first method, three different solvent extracts (with methanol, chloroform, and acetone) were obtained for each moss species, and four sequential extracts (A, B, C and D) were obtained for each moss species in the second method. According to the size of the inhibition zones of different crude extracts, the second method gave results that were more appreciable. In other words, while the crude extracts considerably inhibited growth when the second extraction method was used, the crude extracts from the first extraction method inhibited none or less of the same test organisms. It can therefore be concluded that the second method was a functional method to detect small amounts of active ingredients recovered with some solvents of different polarity. A similar method has been employed in many studies (Ozturk et al., 2009; Tsao and Deng, 2004). In this study, the methanolic extracts from mosses demonstrated a poor effect against the selected bacteria species in general while chloroform, acetone, and ethyl acetate (C) extracts had a higher potency. Russell reported that none of the mosses investigated in a study showed any antibiotic activity when methanolic extracts were used (Russell, 2010). In addition, Bodade et al. (2008) stated that ethanolic, acetone, and chloroform extracts of Bryophytes were found to be more effective than methanolic extract.

The results of the agar diffusion method in the present study revealed that of all the mosses species investigated, *P. juniperinum* and *T. tortuosa* had stronger potential activity against the bacterial strains tested. Moreover, it showed that extracts obtained with different

Table 3. Antibacterial and antifungal activities of five different moss extracts as inhibition zones (mm).

Mosses species	Extracts	Bacteria						Fungi		
		BS	EF	EC	PA	ST	SA	AF	FS	GC
Funaria hygrometrica	Methanol	11	-	-	-	-	9	-	-	-
	Chloroform	10	-	-	-	-	-	-	-	-
	Acetone	10	-	-	-	-	11	-	-	-
	Extract A	9	-	-	9	-	-	-	-	-
	Extract B	11	-	-	9	-	-	-	-	-
	Extract C	12	8	-	10	-	13	-	-	9
	Extract D	-	--	-	-	-	-	-	-	-
Hypnum cupressiforme	Methanol	9	-	-	-	-	-	-	-	-
	Chloroform	10	-	-	9	-	-	-	-	-
	Acetone	10	-	-	9	-	-	-	-	-
	Extract A	10	-	-	10	-	-	-	-	-
	Extract B	9	-	-	-	-	-	-	-	-
	Extract C	12	-	-	10	-	12	-	-	10
	Extract D	-	-	-	7	-	-	-	-	-
H. imponens	Methanol	8	-	-	-	-	-	-	-	-
	Chloroform	10	-	-	9	-	-	-	-	-
	Acetone	9	-	-	9	-	-	-	-	-
	Extract A	10	7	-	9	-	-	-	-	-
	Extract B	8	-	-	8	-	-	-	-	-
	Extract C	12	-	-	9	-	8	-	-	-
	Extract D	-	-	-	-	-	-	-	-	-
Polytrichum juniperinum	Methanol	13	-	-	10	-	9	-	-	-
	Chloroform	11	-	9	11	-	10	-	-	-
	Acetone	15	-	-	12	-	12	-	-	-
	Extract A	11	-	8	10	-	12	-	-	-
	Extract B	12	-	-	10	-	-	-	-	-
	Extract C	14	11	-	13	-	14	-	-	-
	Extract D	8	-	-	8	-	-	-	-	-
Tortella tortuosa	Methanol	-	-	-	11	-	-	-	-	-
	Chloroform	10	12	-	13	-	11	9	8	-
	Acetone	10	-	-	11	-	-	-	-	11
	Extract A	13	13	-	13	-	14	9	9	9
	Extract B	9	-	-	11	-	-	-	-	-
	Extract C	12	9	-	10	-	11	9	-	-
	Extract D	-	-	-	9	-	-	-	-	-
Antibiotics	Penicillin	13	27	30	30	21	35			
	Tetracycline	24	15	25	20	18	27			
	Amfoterisin B							15	13	11

BS- *B. subtilis*; EF- *E. faecalis*; EC- *E. coli*; PA- *P. aeruginosa*; ST- *S. typhimurium*; SA- *S. aureus*; AF- *Aspergillus fumigatus*; FS- *Fusarium solani*; GC- *Geotrichum candidum*, Not detected -.

polarities of solvents should be applied for screening studies. The agar diffusion assay is a qualitative non-standardized method that is useful only for the screening of large numbers of samples but not for a comparison of the antimicrobial properties of different samples. A comparison of the size of inhibition zones of different

Table 4. Minimum inhibitory concentrations (MIC, µg/mL) of selected extracts of moss species.

Bacteria	Me	K	As	A	B	C	D	Penisilin (µg/ml)	Tetrasiklin (µg/ml)
Funaria hygrometrica									
B. subtilis						750		<1.5	<1.5
S. aureus						1500			
Hypnum cupressiforme									
B. subtilis						750		<1.5	<1.5
S. aureus						1500			
Hypnum imponens									
B. subtilis						750		<1.5	<1.5
Polytrichum juniperinum									
B. subtilis	750		375		750	375		<1.5	<1.5
P. aeruginosa			750			750			
S. aureus			1500	1500		750			
Tortella tortuosa									
B. subtilis				93.8				<1.5	<1.5
S. aureus				187.5				<1.5	<1.5
P. aeruginosa		46.9		5.9				<1.5	<1.5

Me, methanol; K, chloroform; As, acetone; A, B, C and D represent the extracts obtained from the second extraction method.

Table 5. Phytochemical constituents extracts of the mosses.

Test	*F. hygrometrica*	*H. cupressiforme*	*H. imponens*	*T. tortuosa*	*P. juniperinum*
Anthraquinone	-	+	+	+	+
Terpenoids	+	+	+	+	+
Flavonoids	-	+	+	+	+
Alkaloids	+	+	+	+	+

extracts cannot be used for the determination of the relative antimicrobial potency since a more diffusible but less active extract could give a bigger diameter than a non-diffusible but more active extract. The broth dilution method more accurately represents antimicrobial activity during the testing of plant extracts (Wilkinson, 2006). For this reason, we also used the microdilution method for the comparison of the antimicrobial activity of extracts, as we did in previous studies. The selection of active extracts was made with respect to the size of the inhibition zones (>12 mm) formed in the agar diffusion method.

Table 4 illustrates the MIC ranges of selected extracts against bacterial strains. The MIC values of methanol, acetone, A, B and C extracts against bacterial pathogens ranged from 5.9 to 1500 µg/ml. The results of the microdilution method showed that extract A (petroleum ether) and the chloroform extract of *T. tortuosa* possessed the highest antibacterial potency, with a MIC of 5.9 and 46.9 µg/ml against *P. aeruginosa*, respectively. Some of the extracts, those of *P. juniperinum, H. cupressiforme,* and *F. hygrometrica*, showed the lowest activity, with a MIC of 1500 µg/ml against *S. aureus*.

It was determined that the mosses species we investigated, except for *F. hygrometrica*, had alkaloids, anthraquinones, flavonoids and terpenoids (Table 5). The chloroform and petroleum ether (A) extracts of *T. tortuosa* can contain compounds such as lipids, flavonoids, and terpenoids. Such compounds have been extracted from a number of plants, including bryophytes (Cowan, 1999; Cushnie and Lamb, 2005; Iwashina, 2000). For example, the constituents that have previously been isolated from *H. cupressiforme* are hypnogenols, biflavonoids, and dihydroflavonols (Dulger et al., 2005; Sievers et al., 1992; Sievers et al., 1994). Since flavonoids are known to be synthesized by plants in response to microbial infection, it is not surprising that they have been found to be effective antimicrobial substances against microorganisms. Their

activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Basile et al., 1999; Cowan, 1999; Hahn et al., 1995; Jockovic et al., 2008).

Extract C of *F. hygrometrica* and *H. cupressiforme* showed less antifungal activity against *G. candidum*. Some extracts of *T. tortuosa* had a weak inhibition effect against *A. flavus, F. solani* and *G. candidum* (Table 3). It has been reported in previous studies that H. *cupressiforme* had antifungal activity and that it contained polycyclic aromatic hydrocarbon, hypnogenols, biflavonoids and hydroxiflavonoids (Veljic et al., 2008; Dülger et al., 2005). The data we obtained for *H. cupressiforme* seems to support the presence of these phytochemicals (Table 5).

Terpenoids represent another class of secondary metabolites which benefit the producing organisms with improved pathogen resistance (Xie and Lou, 2009). In this study, one or more of the antifungal activities observed in the extracts from *F. hygrometrica*, *H. cupressiforme*, *T. tortuosa* could be said to be active terpenoids.

Conclusion

Of the mosses investigated, the inhibition effect seen against 4 bacteria and 3 fungus by *T. tortuosa* extracts suggests that it may be used as a broad spectrum antibiotic in the future. The presence of a specific group (flavonoid and terpenoid) of compounds in extracts might be the reason for this observation. Diğer dört yosundan elde edilen the extracts demonstrated especially antibacterial activity against *B. subtilis*, *S. aureus* and *P. aeruginosa*. These results indicate that the extracts investigated should find a practical application in the prevention of and protection against both gram (+) and gram (-) bacterial infections in plants, animals and humans.

Further research is being carried out on the isolation of bioactive chemical constituents from the active fractions and their mode of action on microbial cells.

ACKNOWLEDGEMENT

This study was supported by Eskisehir Osmangazi University Scientific Research Projects Committee (Project No: 2007/19012).

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