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Short Communication

Antimicrobial studies on three *Hypericum* species from Turkey

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The antimicrobial activity of several extracts and fractions of some *Hypericum* species (*H. rupestre* Jaub. & Spach, *H. vacciniifolium* Hayek & Siehe and *H. imbricatum* Poulter) was investigated using the disc diffusion method against *Escherichia coli* ATCC 11230, *Staphylococcus aureus* ATCC 6538P, *Klebsiella pneumoniae* UC57, *Micrococcus luteus* La 2971, *Bordetella bronchiseptica* ATCC 19395, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Corynebacterium xerosis* CCM 7064, *Mycobacterium smegmatis* CCM 2067, *Bacillus subtilis* ATCC 6633, *Aeromonas hydrophila* ATCC 49803, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9730, *Kluyveromyces fragilis* NRRL 2415 and *Rhodotorula rubra* CCY. The methanol extract and chloroform fraction of *H. vacciniifolium*, as well as the methanol extracts, butanol and chloroform fractions of both *H. rupestre* and *H. imbricatum*, showed good antimicrobial activity against especially Gram-positive bacteria and the Gram-negative bacterium *Bordetella bronchiseptica.* The methanol extracts and fractions did not have antifungal activity. The results of the study support the use of these specimens in Turkish traditional medicine to treat skin and eye infections.

Medicinal plants have for centuries constituted an important source of active bio-molecules. Although medicinal plants' potential in Turkey is quite large, ethnobotanical and pharmaceutical studies on these plants are inadequate.

Hypericum has been used for centuries in the treatment of burns, bruises, swelling, inflammation and anxiety, as well as bacterial and viral infections (Baytop 1999, Ozturk *et al.* 2002). One of the most important species of this genus is *Hypericum perforatum* L. which has been used in herbal medicine externally for the treatment of skin wounds, eczema and burns, and internally for diseases of the central nervous system, the alimentary tract and others (Rabanal *et al.* 2002).

The *Hypericum* species (*H. rupestre* Jaub. & Spach, *H. vacciniifolium* Hayek & Siehe and *H. imbricatum* Poulter) used in this study are endemic to Turkey and the Euro-Siberian area. Although other species of *Hypericum* have been found to exert antidepressant, antimicrobial and wound-healing effects (Sakar and Tamer 1990, Sokmen *et al.* 1999, Pistelli *et al*. 2000, Schwob *et al.* 2002, Couladis *et al.* 2003, Mukherjee *et al.* 2003), there is no ethnobotanical information on these species. However, it was determined that the *Hypericum* species used in this study have been used for opthalmia, eczema, swelling, bruises and wounds during our field studies. In this study, our aim was to determine the antimicrobial effects of plant extracts obtained from three endemic *Hypericum* L. species against micro-organisms. Further investigations on *Hypericum* L. species are necessary to provide additional knowledge about these plants.

Three species of *Hypericum* (*H. rupestre* Jaub. & Spach, *H. vacciniifolium* Hayek & Siehe and *H. imbricatum* Poulter) were collected from Icel, Turkey, during the months of September–October of 2001. Voucher specimens of the plants (AG 346, AG 474 and AG 302, respectively) were deposited in the Biology Department at Canakkale Onsekiz Mart University, Canakkale, Turkey.

Escherichia coli ATCC 11230, *Staphylococcus aureus* ATCC 6538P, *Klebsiella pneumoniae* UC57, *Micrococcus luteus* La 2971, 7, *Bordetella bronchiseptica* ATCC 19395, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Corynebacterium xerosis* CCM 7064, *Mycobacterium smegmatis* CCM 2067, *Bacillus subtilis* ATCC 6633, *Aeromonas hydrophila* ATCC 49803, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9730, *Kluyveromyces fragilis* NRRL 2415 and *Rhodotorula rubra* CCY were maintained in the Laboratory of Basic and Industrial Microbiology, Aegean University.

The aerial parts of the plants were dried in an oven at 40°C (12h) and powdered. The methanol extracts were obtained by maceration of the plant material with methanol for 3 days at room temperature, and this procedure was

repeated three times. The respective extracts were filtered and dried under reduced pressure at a temperature below 45°C. Then, with the methanol extract, a distribution between chloroform/water ($CHCl_3/H_2O$) was made and the chloroform and aqueous fractions obtained. Finally, the aqueous fraction was again subjected to a partitioning between n-butanol/water (n-BuOH/H_2O). The yield obtained for each extract and fraction with respect to the initial dry material were *H. rupestre*: 18.20% for MeOH ($CHCl_3$ 7.79%, BuOH 24.42%, aqueous 34.52%); *H. vacciniifolium*: 24.30% for MeOH ($CHCl_3$ 15.29%, BuOH 18.75%, aqueous 42.80%); *H. imbricatum*: 21.95% for MeOH ($CHCl_3$ 28.24%, BuOH 32.14%, aqueous 22.82%).

Infusion was prepared with 100g of crude powder and 1 000ml of water, and the volume was adjusted to a concentration of 1g ml^{-1} under reduced pressure and at 40°C.

The different extracts and fractions were diluted in dimethylsulfoxide (DMSO). The corresponding concentrations are expressed in terms of mg of extract or fraction per ml of solvent, with the exception of the infusion whose concentration is expressed in terms of mg ml^{-1} of initial dry material.

The antimicrobial studies were carried out by the disc diffusion method (Bauer *et al*. 1966). The micro-organisms to be tested were inoculated into Brain Heart Infusion Agar (Oxoid) for the bacteria, and Sabouraud Dextrose Agar (Oxoid) for the yeasts. After an incubation period of 24h at 35°C and 28°C, respectively, three or four colonies isolated from the media were inoculated into 4ml of Brain Heart Infusion Broth (Oxoid) for the bacteria and Sabouraud Dextrose Broth (Oxoid) for the yeasts and incubated for 2h at 35°C and 28°C, respectively. The cultures were adjusted with sterile saline solution to obtain a turbidity comparable to that of McFarland 0.5 standard. Petri dishes containing Mueller-Hinton Agar (Oxoid) or Bacto Yeast Morphology Agar (Difco) were impregnated with these microbial suspensions for the bacteria and the yeasts, respectively (Bauer *et al.* 1966, Jones *et al*. 1987, Rabanal *et al*. 2002).

Three concentrations were prepared for each extract and fraction: 125mg ml^{-1}, 250mg ml^{-1} and 375mg ml^{-1} and blank discs of 6mm diameter (Schleicher & Schull No 2668, Germany) were impregnated with 10µl of each (final doses obtained per disc: 1.25mg, 2.50mg and 3.75mg). The infusion were assayed at two concentrations: 1g ml^{-1} and 0.5g ml^{-1} (10mg and 5mg per disc, respectively). Blank discs impregnated with DMSO were used as negative controls. The plates were incubated overnight at 37°C (bacteria) and 28–30°C (fungi). At the end of the period, inhibition zones formed on the medium were evaluated in mm (including diameter of the disc (6.0mm)). Studies were performed in triplicate. On each plate an appropriate reference antibiotic disc was applied depending on the test micro-organisms.

Table 1 gives a summary of the antimicrobial activity by the disc diffusion method of *H. rupestre*, *H. vacciniifolium* and *H. imbricatum* methanol extracts. No activity was found against *Escherichia coli, Klebsiella pneumoniae* and *Proteus vulgaris* and the yeast cultures. In contrast, different results were obtained against *Stapylococcus aureus, Micrococcus luteus*, *Bacillus subtilis*, *Bordetella bronchiseptica, Pseudomonas aeruginosa*, *Corynebacterium xerosis, Aeromonas hydrophila* and acid-fast bacterium *Mycobacterium smegmatis*. The diameters of growth inhibition zones of extracts studied were in the range 12–22mm.

Antimicrobial activities of the aqueous, butanol and chloroform fractions obtained from the methanol extracts of the three *Hypericum* species are given in Table 2. The aqueous fraction of the three species as well as the butanol fraction of *H. vacciniifolium* showed no antimicrobial activity at all. The diameter of growth inhibition area by the extracts was in the range 10–20mm.

It could be observed in Table 1 and Table 2 that methanol extracts of the three *Hypericum* species have higher antimicrobial effect than those of the standard antibiotics P10, SAM20, CTX30 and VA30 against *Staphylococcus aureus*. When the results obtained with the methanol extracts were compared to those of some standard antibiotics, it was determined that *Bordetella bronchiseptica, Pseudomonas aeruginosa, Corynebacterium xerosis* and *Bacillus subtilis* are more susceptible compared to some of the standard references. *Micrococcus luteus* is more resistant as compared to some of the standard antibiotics. Similarly, acid-fast bacterium *Mycobacterium smegmatis* was more susceptible to the methanol extracts, as compared to P10 and CTX30 standard antibiotics.

As can be seen from Table 2, the most significant activity among *Hypericum* species was observed with the chloroform fraction from *Hypericum imbricatum*, which showed inhibition values close to those of some standard antibiotics. This fraction of *H. imbricatum* was found to show the highest activity against *Bacillus subtilis*. This situation provides a support to the traditional use of *Hypericum imbricatum* for treatment of eye infections. It is worth noting that in the case of *Hypericum vacciniifolium*, only the chloroform fraction conserved the antimicrobial effect, suggesting that the active compounds are concentrated in it.

In general, Gram-negative bacteria have been found to be more resistant to extracts (except for *Bordetella bronchiseptica*) than Gram-positive bacteria in this study, possibly because of their cell wall lipolysaccharides.

Rabanal *et al*. (2002) reported that the extracts of three species of *Hypericum* (*H. canariense, H. glondulosum* and *H. grandifolium*) showed antibacterial activity against the Gram-positive bacteria *Bacillus cereus* var. *mycoides*, *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus epidermidis* and Gram-negative bacterium *Bordetella bronchiseptica*, but no antifungal activity was seen. In addition, the chloroform fraction was found to be very effective against the tested micro-organisms. The findings obtained from this study are similar to those stated above.

In summary, the three *Hypericum* species used in this study showed good antimicrobial activity especially against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. This situation provides a support to the use of these species in traditional medicine for treating skin diseases such as infected wounds and abscesses and eye infections. Further phytochemical studies are required to determine the types of compounds responsible for the antimicrobial activities of these medicinal plants.

Table 1: Inhibitory activity of methanolic extracts from *Hypericum* species in the disc diffusion assay

Extract	Dose (mg/disc)	Diameter of inhibition zone (mm)														
		1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Hypericum rupestre	1.25	–	18	–	16	17	–	12	12	16	14	16	–	–	–	–
MeOH extract	2.50	–	20	–	16	18	–	13	14	18	16	18	–	–	–	–
	3.75	–	21	–	16	18	–	13	16	19	16	18	–	–	–	–
Hypericum vacciniifolium	1.25	–	17	–	13	14	–	13	12	14	14	16	–	–	–	–
MeOH extract	2.50	–	18	–	14	14	–	13	12	15	14	16	–	–	–	–
	3.75	–	20	–	14	14	–	14	12	15	14	16	–	–	–	–
Hypericum imbricatum	1.25	–	18	–	14	14	–	12	12	14	14	14	–	–	–	–
MeOH extract	2.50	–	21	–	14	15	–	12	14	14	14	16	–	–	–	–
	3.75	–	22	–	14	16	–	12	14	14	14	16	–	–	–	–
DMSO (control)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] 1: *Escherichia coli*, 2: *Staphylococcus aureus*, 3: *Klebsiella pneumoniae*, 4: *Micrococcus luteus*, 5: *Bordetella bronchiseptica*, 6: *Proteus vulgaris*, 7: *Pseudomonas aeruginosa*, 8: *Corynebacterium xerosis*, 9: *Mycobacterium smegmatis*, 10: *Aeromonas hydrophila*, 11: *Bacillus subtilis*, 12: *Candida albicans*, 13: *Saccharomyces cerevisiae*, 14: *Kluyveromyces fragilis*, 15: *Rhodotorula rubra*

(–): no inhibition zone

Table 2: Inhibitory activity of methanolic extracts from *Hypericum* species in the disc diffusion assay

Extracts/standards	Dose (mg/disc)	Diameter of inhibition zone (mm)							
		1[a]	2	3	4	5	6	7	8
H. rupestre	1.25	–	–	–	–	–	–	–	–
Aqueous fraction	2.50	–	–	–	–	–	–	–	–
	3.75	–	–	–	–	–	–	–	–
H. rupestre	1.25	12	12	13	11	12	14	13	11
BuOH fraction	2.50	10	13	12	12	10	13	15	12
	3.75	13	13	14	12	13	16	16	12
H. rupestre	1.25	14	13	13	14	13	16	14	12
$CHCl_3$ fraction	2.50	13	10	13	11	11	15	15	12
	3.75	15	14	13	11	11	20	16	12
H. vacciniifolium	1.25	–	–	–	–	–	–	–	–
Aqueous fraction	2.50	–	–	–	–	–	–	–	–
	3.75	–	–	–	–	–	–	–	–
H. vacciniifolium	1.25	–	–	–	–	–	–	–	–
BuOH fraction	2.50	–	–	–	–	–	–	–	–
	3.75	–	–	–	–	–	–	–	–
H. vacciniifolium	1.25	11	10	13	12	12	14	12	12
$CHCl_3$ fraction	2.50	13	12	13	10	14	16	13	12
	3.75	13	12	13	13	13	16	14	12
H. imbricatum	1.25	–	–	–	–	–	–	–	–
Aqueous fraction	2.50	–	–	–	–	–	–	–	–
	3.75	–	–	–	–	–	–	–	–
H. imbricatum	1.25	12	12	12	14	13	16	14	12
BuOH fraction	2.50	13	15	15	14	14	16	15	12
	3.75	14	16	15	15	14	16	16	13
H. imbricatum	1.25	16	13	13	14	14	17	14	12
$CHCl_3$ fraction	2.50	14	13	13	12	14	18	15	13
	3.75	14	13	13	13	14	20	16	13
P10	10 units	13	8	10	14	15	10	36	15
SAM20	10µg	16	10	16	12	21	12	32	16
CTX30	30µg	12	54	18	14	11	16	32	22
VA30	30µg	13	10	20	18	20	26	34	22
OFX5	5µg	24	44	28	30	32	30	28	24
TE30	30µg	26	34	26	25	24	28	22	26

[a] 1: *Staphylococcus aureus*, 2: *Micrococcus luteus*, 3: *Bordetella bronchiseptica*, 4: *Corynebacterium xerosis*, 5: *Mycobacterium smegmatis*, 6: *Bacillus subtilis*, 7: *Aeromonas hydrophila*, 8: *Pseudomonas aeruginosa*

P10: Penicillin G, SAM20: Ampicillin, CTX30: Cefotaxime, V30: Vancomycin, OFX 5: Oflaxacin, TE30: Tetracyclin, (–): no inhibition zone

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