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Antimicrobial activities of five endemic *Hypericum* species from Anatolia compared with *Hypericum perforatum*

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ABSTRACT: Different crude extracts of some endemic *Hypericum species* (*H. thymbrifolium, H. spectabile*, *H. pseudolaeve, H. neurocalycinum, H. malatyanum*) and *H. perforatum* were analyzed using a microdilution assay for antimicrobial activity against several microorganisms. It is observed that all extracts showed activity against tested Gram positive bacteria (*Staphylococcus aureus*, Meticillin resistant *S. aureus* and *Streptococcus epidermidis*). The most active extracts were from *H. neurocalycinum* and *H. malatyanum* which showed potent activity with lowest MIC (4.8 µg/mL) value against more tested Gram positive bacteria. Additionally, it was also found that some extracts of *H. spectabile* and *H. pseudolaeve* had antifungal activities against *C. albicans*. These endemic species were evaluated for antimicrobial activity for the first time in the literature.

KEYWORDS: Endemic; *Hypericum*; antimicrobial activity; microdilution.

1. INTRODUCTION

Hypericum has nearly 100 taxa grouped under 19 sections in Turkey, among them, 45 taxa are endemic [1]. The genus *Hypericum* L. (Hypericaceae) has been used for the treatment of burns, wounds, bacterial and viral infections and ulcers in Turkish traditional medicine [2-4]. The antimicrobial effect of *H. perforatum* has been known for many years, and oleate was extracted from the upper parts of the flowering plants in the 17th century by physicians to clean surgical and inflammatory wounds [5]. In a study conducted in India in 1999, the antibacterial effect of the extracts obtained from the different parts of the *H. perforatum* were examined, giving a map for the isolation of the compounds which were responsible for the antibacterial effect. The isolation and structure determination studies revealed that this compound was hyperforin. This lipophilic material was found in the petroleum ether extract; however, antibacterial activity studies were interrupted for a period of time, due to the low stability of purely isolated hyperforin. Using the agar dilution method, hyperforin at a concentration of 0.1 to 100 µg/mL exerted no activity against the Gram-negative bacteria. The activity of hyperforin against the Gram-positive bacteria including methicillin- and penicillin-resistant *S. aureus* strains were examined, and the proliferation of all strains were found to be inhibited by hyperforin within a concentration range of 1 to 100 µg/mL. Hyperforin has also antibacterial activities against *Corynebacterium diphtheriae* (E 6040) at a concentration of at least 0.1 µg/mL, *Streptococcus agalactiae* B (D 595), and *S. pyogenes* A (E 12449) at a concentration of 1 µg/mL. In addition, several studies have reported that hyperforin has an antibacterial effect at higher concentrations [6-8]. In another study, methanol, petroleum ether, chloroform, and ethyl acetate extracts obtained from the upper parts of the flowering plant of *H. perforatum* spp. were examined for antibacterial activity. The authors reported that the ethyl acetate extract had the highest activity and it contained flavonoid compounds, hypericin, and hyperforin, as assessed by high-performance liquid chromatography (HPLC) method [9]. Antibacterial activities of phloroglucinol-derived compounds which are isolated from *H. perforatum* spp. such as deoxyhyperforin, furohyperforin,

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furoadhyperforin, furohyperforin A, pyranohyperforin against *S. aureus, Bacillus subtilis* spore form of Gram-positive strains and *Escherichia coli* strains from the Gram-negative strains were examined through the agar diffusion method. It showed no effect against the Gram-negative strains and *B. subtilis* spore form of the Gram-positive strains, while it exerted furohyperforin A effect on *S. aureus* strain [10]. According to literature survey, the antimicrobial activities of the many kind of extracts of *Hypericum* species which are growing in Turkey have been determined previously. These species are namely *H. perforatum*, *H. pamphylicum*, *H. scabrum* , *H. lysimachioides* var. *lysimachioides*, *H. retusum, H. capitatum, H. heterophyllum*, *H. hyssopifolium* subsp. *elongatum* var. *elongatum*, *H. linarioides, H. heterophyllum*, *H. hyssopifolium* subsp. *elongatum* var. *elongatum, H. kazdaghensis, H. avicularifolium subsp. depilatum var. depilatum* and *H. salsugineum* [1, 11-16].

In the present study, different extracts obtained from flowering aerial parts of endemic species (*H. thymbrifolium, H. spectabile*, *H. pseudolaeve, H. neurocalycinum, H. malatyanum and H. perforatum)* from Turkey were tested against *Staphylococcus aureus* ATCC 6538, *Meticillin Resistant Staphylococcus aureus* (MRSA) ATCC 33591, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 1539, *Proteus mirabilis* ATCC 14153 and *Candida albicans* ATCC 10231 for antimicrobial activity. Minimum inhibitory concentration (MIC) values were determined using the standardized broth dilution method. *H. thymbrifolium, H. spectabile*, *H. pseudolaeve, H. neurocalycinum, H. malatyanum* endemic species were evaluated for antimicrobial activity for the first time.

2. RESULTS

Results of antimicrobial activity of petroleum ether (PE), diethyl ether (DE), chloroform, acetone, methanol and total methanol (TM) extracts of *H. spectabile, H. psedolaeve, H. thymbrifolum, H. neurocalycinum, H. malatyanum* and *H. perforatum* are shown in Table 1. According to our results, the most active extracts were PM, DE and TM for *H. spectabile* which showed inhibitory activity at 4.8 µg/mL against *S. aureus*; the most active extract was TM for *H. pseudolaeve* which showed inhibitory activity at 19 µg/mL against *S. aureus*; the most active extract were PE, DE, chloroform and TM for *H. thymbrifolium* which showed inhibitory activity at 4.8 µg/mL against *S. epidermidis*; the most active extract were PE, TM for *H. neurocalycinum* and *H. malatyanum* which showed inhibitory activity at 4.8 µg/mL against all studied Gram positive strains. Also, the most active extracts were acetone, methanol and TM for *H. perforatum* which showed inhibitory activity at 4.8 µg/mL against *S. aureus.* The results are compatible with previous studies [17-19]. On the other hands, we determined that PE, DE, chloroform extracts from the *H. spectabile,* and PE, methanol, TM extracts of *H. pseudolaeve* had antifungal activity against *C. albicans.*

3. DISCUSSION

Many *in vitro* studies have shown that extracts obtained from the flowering parts of *H. perforatum* have antibacterial effects. It has been also demonstrated that the decoction has activity against *Staphylococcus, Shigellae*, and *Escherichia* coli strains., while ethanol extract is more effective against the Gram-positive bacteria (*Enterococcus faecalis* and *S. aureus* ATCC 1112) than Gram-negative bacteria, and it has lower or no activity against Gram-negative bacteria (*Salmonella thypi* ATCC 1595, *Shigella dysenteriae* ATCC 1188, *Yersinia enterocolitica* ATCC 1151, *E. coli* ATCC 1330, and *Pseudomonas aeruginosa* ATCC 1074). Methanol extract has been shown the highest activity against Gram-positive bacteria, and its MIC value is 50 µg/mL [8, 9, 20]. In a study conducted with the PE extract of the flowering parts, *H. perforatum* showed a high activity against the MRSA strains, and hyperforin, a lipophilic compound, was transferred to this extract at a high concentration, explaining the underlying cause of its effect [21]. In a study conducted in Turkey, anti-*Helicobacter* activity of the extracts obtained from the flowering parts of *H. perforatum* against the clinically and normatively isolated *Helicobacter pylori* strains was examined, and the authors reported that butanol and chloroform extracts had a higher activity [4]. A another study showed that *H. perforatum* teas diluted with water had an activity against Gram-positive bacteria, particularly against the MRSA strains [21].

Table 1. Results of antimicrobial activity of petroleum ether, diethyl ether, chloroform, acetone, methanol and total methanol extracts of *Hypericum* species.

Species	The extracts of *Hypericum* species	MIC values of the extracts (µg/mL)							
		S.a.	***MRSA***	***S.e.***	***E.c.***	***K.p.***	***P.a.***	***P.m.***	***C.a.***
H. spectabile	Petroleum ether (PE)	4.8	312	312	NA	NA	NA	NA	156
	Diethyl ether (DE)	4.8	39	78	NA	NA	NA	NA	156
	Chloroform	39	156	78	NA	NA	NA	NA	156
	Acetone	39	78	78	NA	NA	NA	NA	NA
	Methanol	39	312	156	NA	NA	NA	NA	NA
	Total methanol (TM)	4.8	4.8	39	NA	NA	NA	NA	NA
H. pseudolaeve	Petroleum ether	39	312	625	NA	NA	NA	NA	156
	Diethyl ether	156	78	156	NA	NA	NA	NA	NA
	Chloroform	39	156	312	NA	NA	NA	NA	NA
	Acetone	312	156	156	NA	NA	NA	NA	NA
	Methanol	39	156	312	NA	NA	NA	NA	156
	Total methanol	19	156	78	NA	NA	NA	NA	156
H. thymbrifolium	Petroleum ether	39	4.8	4.8	NA	NA	NA	NA	NA
	Diethyl ether	39	4.8	4.8	NA	NA	NA	NA	NA
	Chloroform	4.8	78	4.8	NA	NA	NA	NA	NA
	Acetone	19	312	156	NA	NA	NA	NA	NA
	Methanol	39	78	78	NA	NA	NA	NA	NA
	Total methanol	39	4.8	4.8	NA	NA	NA	NA	NA
H. neurocalycinum	Petroleum ether	4.8	4.8	4.8	NA	NA	NA	NA	NA
	Diethyl ether	4.8	39	4.8	NA	NA	NA	NA	NA
	Chloroform	4.8	78	78	NA	NA	NA	NA	NA
	Acetone	78	39	78	NA	NA	NA	NA	NA
	Methanol	4.8	78	156	NA	NA	NA	NA	NA
	Total methanol	4.8	4.8	4.8	NA	NA	NA	NA	NA
H. malatyanum	Petroleum ether	4.8	4.8	4.8	NA	NA	NA	NA	NA
	Diethyl ether	4.8	78	4.8	NA	NA	NA	NA	NA
	Chloroform	4.8	78	4.8	NA	NA	NA	NA	NA
	Acetone	78	156	4.8	NA	NA	NA	NA	NA
	Methanol	39	156	312	NA	NA	NA	NA	NA
	Total methanol	4.8	4.8	4.8	NA	NA	NA	NA	NA
H. perforatum	Petroleum ether	78	312	312	NA	NA	NA	NA	NA
	Diethyl ether	78	156	156	NA	NA	NA	NA	NA
	Chloroform	78	78	156	NA	NA	NA	NA	NA
	Acetone	4.8	78	78	NA	NA	NA	NA	NA
	Methanol	4.8	78	156	NA	NA	NA	NA	NA
	Total methanol	4.8	39	78	NA	NA	NA	NA	NA
Controls	Cefuroxime-Na	1.2			4.9	4.9		2.4	
	Vancomycin		2						
	Cefuroxime			9.8					
	Ceftadizime						2.4		
	Cloritmazole								4.9

S.a.: *Staphylococcus aureus* ATCC 6538; **MRSA**: Methicillin-resistant *Staphylococcus aureus ATCC33591;* **S.e.**: *Staphylococcus epidermidis* ATCC 12228; **E.c.**: *Escherichia coli* ATCC 8739; **K.p.**: *Klebsiella pneumoniae* ATCC 4352; **P.a.**: *Pseudomonas aeruginosa* ATCC 1539; **P.m.**: *Proteus mirabilis* ATCC 14153; **C.a.**: *Candida albicans* ATCC 10231 ; NA: Not active

In addition, *H. perforatum* prepared with the percolation had shown a higher activity rather than the ethanol extract of which activities against the MRSA and methicillin-sensitive *S. aureus* (MSSA) strains of water and ethanol extracts, and it was more active against MRSA rather than MSSA [8]. Due to the fact that *H. perforatum* extracts have antibacterial activity, some authors conducted studies using different *Hypericum* species. Previous studies investigated the antibacterial activities of the extracts and pure compounds obtained from *H. brasiliense, H. scabrum, H. hircinum, H. hookerianum, H. canariense, H. maculatum, H. mysorense* and *H. patulum*, and found an activity in many of the extracts; however, pure compounds showed either poor activity or no activity [22-27]. There are antibacterial preparations known as novoimanine and imanine which contain *H. perforatum* extract, and are widely prescribed in Russia. *In vivo* and *in vitro* studies have shown that these preparations are more effective for *S. aureus* infections than sulphanilamide, and MIC value of the preparation, namely novoimanine, which contains hyperforin, is 0.1 µg/mL [8]. In Germany, there are ointments containing extracts of *H. perforatum* flowers, and they are widely used thanks to their antiseptic properties. Several studies have shown that when these ointments are used in the treatment of second- and third-degree burns, the healing process is three-fold faster than common treatments. The effect of another ointment containing standardized extract of *H. perforatum* in the treatment of atopic dermatitis was also compared to,

and it showed a significant advantage against placebo in the treatment of mild to moderate atopic dermatitis [28]. Activities of the three lipophilic ointment formulations containing 30%, 40%, and 50% *Hyperici* oleum against the *Streptococcus pyogenes*, *Streptococcus viridans*, *Micrococcus luteus* ATCC 9341, *Moraxella catarrhalis* and *Lactobacillus acidophilus* strains in the dermal and vaginal application were investigated, and an activity against strains except of *Lactobacillus acidophilus* existing in the natural flora of the vagina was observed. In addition, as the amount of *Hyperici oleum* in the content of ointment increased, antibacterial activity increased accordingly [8].

Additionally, in another study investigating the antibacterial activity of the *H. kazdaghensis* acetone, chloroform, and methanol extracts against the *Bacillus subtilis*, *E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella thyphimurium*, and *S. aureus* strains compared to gentamicin demonstrated that all extracts exerted potent antibacterial effects, and were sensitive for the *Pseudomonas aeruginosa* and *Bacillus subtilis* strains [29].

Nowadays, the rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics. Unfortunately, resistance has eventually been seen to nearly all antibiotics that have been developed. The antibiotic resistance problem has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry [30]. Therefore, the investigation into herbals which have antimicrobial activity to pathogen bacteria especially to MRSA and other antibiotic resistant species are still carrying on. The present study which demonstrates the antimicrobial potential of six *Hypericum* species extract by using various solvents, reveal that the all of the extracts have antibacterial activities against Gram positive strains (*S. aureus*, MRSA and *S. epidermidis*). Additionally, it was firstly determined that *H. spectabile* and *H. pseudolaeve* have antifungal activities. The antimicrobial effects might be due to tested species contains flavonoid compounds hypericin, and hyperforin which were previously shown for *H. perforatum* [6-8]. Since due to the potential of these *Hypericum* species to have activities against Gram positive bacteria which can cause a variety of problems ranging from skin infections and sepsis to pneumonia to bloodstream infections [31] and antibiotic resistance, in both the hospital and the community has emerged as a major public health problem. It may be useful to use our *Hypericum* species to investigate on new antibacterial drugs.

4. CONCLUSION

According to our results, these tested *Hypericum* extracts possess the capabilities of being a good candidate in the future search for a natural antimicrobial agent against infections and/or diseases caused by *S. aureus*, MRSA and *S. epidermidis.*

5. MATERIALS AND METHODS

5.1. Plant materials

During the field investigations conducted in June 2010, specimens of flowering aerial parts of *H. thymbrifolium, H. spectabile*, *H. pseudolaeve, H. neurocalycinum, H. malatyanum and H. perforatum* were gathered from their natural habitats in the East Anatolia Reagion of Turkey. All plant materials were dried in the shade. The plant materials were identified by Prof. Dr. Şükran Kültür and voucher specimens were deposited in the Herbarium of the Istanbul University Faculty of Pharmacy, Istanbul, Turkey (ISTE 93194, 93192, 93193, 93195, 93196 and 93197 respectively).

5.2. Preparation of the extracts

The dried material (5 g) was extracted with methanol (100 ml) in an ultrasonic bath for 30 min. The residue was evaporated under vacuum at 40–45°C. The crude methanol extract was lyophilized and stored at -20°C. Other extracts were prepared for antimicrobial activity. The dried aerial parts (5 g) of the *Hypericum* species were extracted successively with 100 ml petroleum ether (PE), 100 ml diethyl ether (DE), 100 ml chloroform, 100 ml acetone and 100 ml ethanol (96%) in a Soxhlet apparatus. All extracts were concentrated under vacuum and stored at -20°C.

5.2. Antimicrobial activity

Antimicrobial activity against *S. aureus* ATCC 65538, MRSA ATCC 33591, *S. epidermidis* ATCC 12228, *E. coli* ATCC 8739, *K. pneumonia* ATCC 4352, *P. aeruginosa* ATCC 1539, *Proteus mirabilis* ATCC 14153 and *Candida albicans* ATCC 10231 were determined by the microbroth dilutions technique using the Clinical and Laboratory Standards Institute (CLSI) recommend Mueller-Hinton broth for bacteria, RPMI-1640 medium for yeast strain were used as the test medium [32, 33]. Serial two-fold dilutions ranging from 5000 µg/mL to 4.8 µg/mL were prepared in medium. The inoculum was prepared using a 4-6 h broth culture of each bacteria and 24h culture of yeast strains adjusted to a turbidity equivalent to a 0.5 Mc Farland standard, diluted in broth media to give a final concentration of $5x10^5$ cfu/ml for bacteria and $0.5x10^3$ to $2.5x10^3$ cfu/ml for yeast in the test tray. The trays were covered and placed into plastic bags to prevent evaporation. The trays containing Mueller-Hinton broth were incubated at 35°C for 18-20 h and the trays containing RPMI-1640 medium were incubated at 35°C for 46-50 h. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth.

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