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Antibacterial, antifungal and enzyme inhibitory effects of selected plants from Turkey

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Abstract: In this study, antibacterial, antifungal, antihyaluronidase, anticollagenase and antielastase activity of *Hypericum bithynicum, Malva neglecta*, *Morus alba*, *Rubus discolor*, *Sambucus ebulus* and *Smilax excelsa* were investigated. Methanol extracts of *M. neglecta* and *R. discolor* and all extracts of *H. bithynicum* were more active against *Staphylococcus epidermidis.* Similarly, water extracts of *M. alba* and *S. ebulus* were more active against *Streptococcus pneumonia.* Additionally, *S. ebulus* and *S. excelsa* had prominent antifungal activity on *Candida albicans*. Besides, methanol extract of *M. neglecta* and *n*-hexane extract of *H. bithynicum* were determined to have significant antihyaluronidase activity. Only *R. discolor* showed significant antielastase effect.

Keywords: Antibacterial activity, antifungal activity, antihyaluronidase activity, antielastase activity, anticollagenase activity.

INTRODUCTION

The acquisition of resistance of microorganism against antibiotics has become a growing threat for last few decades in modern medicine. Unfortunately, the number of new antibiotics is not increasing at same rate. The development of new antibiotics has become even more important at the present time. Therefore, the plants that used with ethnobotanical purposes are very important sources for new antibiotic researches in Turkey as well as all over the world.

Ethnopharmacological studies in Turkey have been increasing in recent years. The folk medicines of Düzce province (Turkey) was studied by our research group and over a hundred medicinal plants have been ascertained as folk medicine in the treatment of various diseases (Gürbüz *et al.*, 2019). In the present study, six of these medicinal plants which have utilizations associated with dermal infections were selected. In addition, some of these plants have been well known as foodstuff in Turkey. For example, aerial parts of *Malva neglecta* Wallr. is consumed after cooking with bulgur, rice, egg, yoghurt and onion (Gümüş, 1994; Baytop, 1999; Ertuğ, 2000; Şimşek *et al.*, 2004; Elçi and Erik, 2006), while the fruits of *Morus alba* L. and *Rubus discolour* Weihe & Nees are consumed as fruit (Ertuğ, 2000; Duran *et al.*, 2001; Doğan *et al.*, 2004).

To research antibacterial and antifungal effect of these plant extracts, bacteria and fungi that could cause skin infections were selected. Mentioned antibacterial activity study was carried out against standard strains of 8 bacteria (*Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus*, *S. epidermidis, Streptococcus pneumoniae* and *S. pyogenes*), besides 3 fungus species (*Candida albicans*, *C. parapsilosis* and *C. krusei*) by microdilution method. In compliance with Clinical and Laboratory Standards, susceptibility testing was done (CLSI, 2006; CLSI, 2008). Their in vitro hyaluronidase, elastase and collagenase inhibitory activities were determined as well.

MATERIALS AND METHODS

Plant materials

The plants were collected from various villages of Düzce Province (Turkey) during the folk medicine research (Gürbüz *et al.*, 2019) in 2008-2009. Collected plant species were identified by Prof. Dr. Galip Akaydın and herbarium materials are preserved in Gazi University Faculty of Pharmacy Herbarium (GUEF). Collection sites and folk medicinal usages in Düzce could be seen in table 1.

Extraction procedure

Plants were dried in the shade at room temperature, then crushed coarsely. Considering the used parts and preparation as folk medicine (table 1), plants were extracted with distilled water and methanol. Additionally, *n*-hexane extract was prepared with *H. bithynicum.* Weights of the all extracts and % yields are given in the table 2.

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Table 1: Selected plant species, their collection sites and ethnobotanical usages.

Scientific name (Family)	Used part	Preparation and administration	Collection sites (date)	Herbarium No.
Hypericum bithynicum Boiss. (Hypericaceae)	Aerial parts	For wounds: macerated in olive oil and applied on wound.	Esmahanım, Akçakoca, Düzce (July, 2009)	09 DZ 124
Malva neglecta Wallr. (Malvaceae)	Leaf	For wounds; cooked with milk to prepare a poultice and applied on wound.	Muhapdede, Gölyaka, Düzce (July, 2009)	09 DZ 177
Morus alba L. (Moraceae)	Leaf	For wounds on animals; wounds are washed with decoction.	Bekiroğlu, Gölyaka, Düzce (July, 2009)	09 DZ 176
Rubus discolor Weihe & Nees (Rosaceae)	Leaf	For incisions and wounds; applied on affected area. For wounds located inside of the mouth; gargle with decoction.	Dutlar, Yığılca, Düzce, (July, 2009)	09 DZ 154
Sambucus ebulus L. (Caprifoliaceae)	Leaf	For wounds; boiled with pine resin to prepare an ointment, than applied on affected area.	Çayağzı, Akçakoca, Düzce (May, 2009)	09 DZ 142
Smilax excelsa L. (Liliaceae)	Fresh shoot	For wounds; applied on affected area.	Akbıyıklar, Düzce center (July, 2009)	09 DZ 150

Table 2: Weight and % yield of the prepared extracts from selected plants.

Plant material	Water extract		Methanol extract		*n*-Hexane extract	
	Weight[a]	Yield %[b]	Weight[a]	Yield %[b]	Weight[a]	Yield %[b]
H. bithynicum	0.79	15.80	0.87	17.40	0.21	4.20
M. neglecta	0.92	18.40	0.06	1.20	-	-
M. alba	1.34	26.80	0.52	10.40	-	-
R. discolor	0.69	13.80	0.38	7.60	-	-
S. ebulus	1.22	24.40	0.96	19.20	-	-
S. excelsa	0.85	17.00	0.48	9.60	-	-

[a] gram; [b] g extract/g dried plant material

Table 3: Antibacterial activities of the extracts and the reference drugs against studied bacteria as minimum inhibitory concentration (MICs; in µg/mL).

Test material	Type of extract	Gram-positive bacteria				Gram-negative bacteria			
		S. aureus	*S. epidermidis*	*S. pneumoniae*	*S. pyogenes*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*A. baumannii*
H. bithynicum	MeOH	32	8	32	64	32	32	32	32
	H_2O	32	8	32	32	32	32	32	32
	n-Hexane	32	8	32	32	32	32	32	32
M. alba	MeOH	32	16	32	32	32	32	32	32
	H_2O	32	16	8	16	32	32	32	32
M. neglecta	MeOH	32	8	32	32	32	32	32	32
	H_2O	32	16	32	32	32	32	32	32
R. discolor	MeOH	32	8	32	32	32	16	32	32
	H_2O	32	16	32	32	32	32	32	32
S. ebulus	MeOH	32	16	32	32	32	16	32	32
	H_2O	32	16	4	32	32	32	32	32
S. excelsa	MeOH	32	16	32	32	16	16	32	32
	H_2O	32	16	32	32	32	16	32	32
AMP[a]		<0.12	<0.12	<0.12	<0.12	-[e]	-	-	-
LFX[b]		0.25	0.25	0.25	0.25	<0.12	1	<0.12	0.12
GM[c]		-	-	-	-	NA[d]	0.5	NA	NA

[a]AMP: Ampicillin®; [b]LFX: Levofloxacin®; [c]GM: Gentamicin®; [d]NA: No activity observed on studied concentrations; [e]-: Not studied.

Table 4: Antifungal activities of the extracts and the reference drugs as minimum inhibitory concentration (MICs; in µg/mL).

Test material	Type of extract	Fungi		
		C. albicans	*C. parapsilosis*	*C. krusei*
H. bithynicum	MeOH	32	32	64
	H_2O	32	32	64
	n-Hexane	32	32	64
M. alba	MeOH	32	8	64
	H_2O	32	8	64
M. neglecta	MeOH	32	8	64
	H_2O	32	32	64
R. discolor	MeOH	32	8	64
	H_2O	32	8	64
S. ebulus	MeOH	8	32	64
	H_2O	8	32	64
S. excelsa	MeOH	8	32	64
	H_2O	32	32	64
KET[a]		1	1	4
FLU[b]		2	4	64

[a]KET: Ketoconazole®; [b]FLU: Fluconazole®

Table 5: Hyaluronidase inhibitory activities of the extracts

Material	Concentration (µg/mL)	Inhibition (%) ± S.E.M.[a]
H. bithynicum (*n*-hexane)	100	38.70 ± 2.28**
H. bithynicum (MeOH)	100	14.25 ± 1.34
H. bithynicum (H_2O)	100	6.09 ±0.92
M. alba (MeOH)	100	18.31±1.75
M. alba (H_2O)	100	8.07±1.13
M. neglecta (MeOH)	100	25.43 ± 2.17*
M. neglecta (H_2O)	100	7.12 ± 1.53
R. discolor (MeOH)	100	4.12 ± 2.68
R. discolor (H_2O)	100	5.96 ± 3.03
S. ebulus (MeOH)	100	8.35 ± 1.72
S. ebulus (H_2O)	100	3.17 ± 0.98
S. excelsa (MeOH)	100	10.24 ± 1.93
S. excelsa (H_2O)	100	2.08 ± 0.76
Tannic acid	100	79.13 ± 0.71***

[a]S.E.M.: Standard error of the mean. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

Methanol and n-hexane extracts

5 g dried and crushed materials were kept in 100 mL methanol or *n*-hexane at room temperature for 24 hours. After this process, materials were filtrated and residues were subjected to same procedure. Obtained extracts were combined and dried under reduced pressure in the temperatures not exceeding at 45°C.

Water extract

5 g dried and crushed materials were kept in 100 mL distilled water in room temperature for 24 hours. After this process, materials were filtrated and residues were subjected to the same procedure. Obtained extracts were combined and lyophilized.

Anti-microbial activity studies

Test materials

The extracts were dissolved in dimethylsulphoxide: ethanol (20:80) and sterilizing by filtration. Antibacterial agents that used as positive control were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in phosphate buffer solution or water according their solubility (Karakaya *et al*., 2013).

Microorganisms

Standard strains of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145, *Klebsiella pneumoniae* RSKK 574, *Acinetobacter baumannii* RSKK 02026, *Staphylococcus aureus* ATCC 25923,

Table 6: Collagenase and elastase inhibitory activities of the extracts

Material	Concentration (µg/mL)	Collagenase inhibition (%) ± S.E.M.[a]	Elastase inhibition (%) ± S.E.M.
H. bithynicum (*n*-hexane)	100	16.27 ± 0.93	20.04 ± 1.07
H. bithynicum (MeOH)	100	9.24 ± 1.62	17.35 ± 1.13
H. bithynicum (H_2O)	100	8.46 ± 0.71	9.13 ± 2.42
M. alba (MeOH)	100	9.12 ± 1.73	12.33 ± 1.81
M. alba (H_2O)	100	11.38 ± 2.03	19.41 ± 1.54
M. neglecta (MeOH)	100	7.84 ± 0.95	11.72 ± 1.46
M. neglecta (H_2O)	100	12.18 ± 1.07	9.26 ± 1.28
R. discolor (MeOH)	100	19.43 ± 0.56	54.07 ± 0.85**
R. discolor (H_2O)	100	13.30 ± 1.12	23.15 ± 1.57
S. ebulus (MeOH)	100	9.18 ± 1.75	10.52 ± 1.20
S. ebulus (H_2O)	100	6.22 ± 1.93	7.28 ± 0.98
S. excelsa (MeOH)	100	5.15 ± 0.34	12.25 ± 1.96
S. excelsa (H_2O)	100	7.08 ± 0.92	6.71 ± 1.38
Epigallocatechin gallate	100	31.13 ± 0.61**	70.92 ± 0.92***

[a]S.E.M.: Standard error of the mean. * : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$

Staphylococcus epidermidis ATCC 12228, *Streptococcus pneumoniae* ATCC 19615, *Streptococcus pyogenes* ATCC 13615, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 90028 and *Candida krusei* ATCC 6258 were used for the determination of antimicrobial activity (ATCC; American type culture collection, RSKK; Culture collection of Refik Saydam Central Hygiene Institute). *Antibacterial and Antifungal Tests:* To determine the anti-microbial activities, broth microdilution method was used. Details of used method were given in previous studies (Özçelik *et al*., 2009; Orhan *et al*., 2012).

In vitro wound healing activity

Determination of hyaluronidase inhibitory activity: Bovine hyaluronidase solution was prepared by dissolving 50 µL bovine hyaluronidase (7900 units/mL) in 0.1 M acetate buffer (pH 3.6) and mixed with 50 µL extract solutions (in 5% dimethylsulphoxide) in different concentrations. As control, 5% dimethylsulphoxide was used instead of the extract solutions. After incubating for 20 minutes at 37°C, 50 µL 12.5 mM calcium chloride solution was added and incubated for 20 minutes at 37°C. Incubating for 40 minutes at 37°C was performed after 250 µL sodium hyaluronate solution (1.2 mg/mL) addition. Subsequently, 50 µL 0.4 M sodium hydroxide and 100 µL 0.2 M sodium borate put in to the mixture. Afterwards this mixture incubated throughout 3 minutes in boiling water bath. After cooling to room temperature, reaction mixture was treated with 1.5 mL *p*-dimethylaminobenzaldehyde solution and incubated at 37°C for 20 minutes. The absorbance of developed colour was measured at 585 nm (Lee and Choi, 1999; Sahasrabudhe and Deodhar, 2010; Süntar *et al*., 2012).

Determination of collagenase inhibitory activity

The extract solutions were prepared by dissolving the extracts in dimethylsulphoxide. The extract solutions and *Clostridium histolyticum* collagenase (ChC, EC 3.4.23.3) were incubated in 50 mM Tricine buffer at 25°C for 5 minutes. 2 mM N-[3-(2-furyl)acryloyl]-leu-gly-pro-ala (FALGPA) was dissolved in the Tricine buffer. 25 µL buffer, 25 µL test extract solution and 25 µL enzyme solution were mixed and incubated for 15 minutes. Decrease in the optical density (OD) at 340 nm after 50 µL substrate addition was measured immediately by using spectrometer. The ChC inhibitory activities were calculated by using following formula:

ChC inhibition activity (%)=($OD_{Control}$-OD_{Sample}) x 100/ $OD_{Control}$ (Barrantes and Guinea, 2003).

Determination of elastase inhibitory activity

The extract solutions were incubated with human neutrophil elastase enzyme (HNE, EC 3.4.21.37) in 0.1 M Tris-HCl buffer (pH 7.5) at 25°C for 5 minutes. The mixture was treated with *N*-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (MAAPVN) and incubated at 37°C for 1 hour. Subsequently, soybean trypsin inhibitor (1 mg/mL) was added to stop reaction. The optical density after formation of *p*-nitroaniline was immediately measured at 405 nm. The HNE inhibitory activities were calculated with the same formula that given for ChC inhibition activity (Melzig *et al*., 2001).

RESULTS

As seen in table 3 and 4, all extracts were showed antibacterial and antifungal activity with MICs in the range of 4 and 64 µg/mL. However, methanol extracts of *M. neglecta* and *R. discolor* as well as all three extracts of *H. bithynicum* have stronger inhibitory activity against *S. epidermidis* (MIC, 8 µg/mL). The all other extracts also demonstrated antibacterial activity at 16 µg/mL concentrations against *S. epidermidis*. Another remarkable antibacterial activity was showed in water

extracts of *S. ebulus* and *M. alba* on *S. pneumoniae* at 4 µg/mL and 8 µg/mL concentration, respectively. In addition, water extracts of *M. alba* have stronger inhibitory effect than other plant extracts for *S. pyogenes* (MIC, 16 µg/mL). All of the studied extracts were less effective against *S. aureus*.

As the MIC values against Gram-negative bacteria taken into consideration, it could be seen that any significant activity observed. However, water and methanol extracts of *S. excelsa* as well as methanol extracts of *R. discolor* and *S. ebulus* have MICs at 16 µg/mL against *P. aeruginosa.* In addition, methanol extract of *S. excelsa* demonstrated remarkable inhibition against *E. coli* at a 16 µg/mL concentration. The other extracts have lower inhibitory activities with 32 µg/mL MIC values against studied Gram-negative bacteria (table 3).

It could be seen that all of the studied extracts demonstrated stronger antifungal activity on *C. parapsilosis*, and *C. albicans* with MIC values between in the ranges of 8-32 µg/mL. But, they have less antifungal effect on *C. krusei* compared with two other tested microfungi (table 4).

Hyaluronic acid, collagen and elastin are the components which have important roles in the wound healing mechanism. Therefore, avoiding the degradation of these compounds by inhibiting hyaluronidase, collagenase and elastase could support wound healing. The *in vitro* activity results revealed that methanol extract of *M. neglecta* and *n*-hexane extract of *H. bithynicum* showed significant hyaluronidase enzyme inhibitory activity with the values of 25.43% and 38.70%, respectively. Nevertheless, only *R. discolor* methanol extract showed significant elastase inhibitory effect (54.07%) and all of the extracts did not represent any collagenase inhibition (tables 5 and 6).

DISCUSSION

Microbiological researches that carried out previously on some plants that studied in this research were determined, but these investigations are differentiated from our study with some points like studied plant parts, used extracts, adopted methods or studied microorganisms. *M. alba* were the widely studied plant species for antimicrobial activity. A compound named kuwanon G was obtained from root bark of *M. alba* and inhibitory activity of this compound was searched against nine oral pathogens that two of them (*S. aureus* and *C. albicans*) same as ours. The research have demonstrated that the strong activity (8 µg/mL, MIC value) of kuwanon G on *Streptococcus mutans, S. sanguinis*, *S. sobrinus*, *Porphyromonas gingivalis* (Park *et al*., 2003). In addition MIC value against *S. aureus* and *C. albicans* are 125 and 1000 µg/mL, respectively. As seen in Table 3 and 4, MIC values of water and methanol extracts of *M. alba* on these microorganisms are 32 µg/mL and the extracts was found to have a low activity against these pathogens. So, detected activity may be caused by other compounds found in the extract.

Afterwards, antimicrobial activity of mulberrofuran G and albanol B obtained from *M. alba* root bark was studied against *C. albicans, Saccharomyces cerevisiae, E. coli, S. typhimurium*, *S. epidermis* and *S. aureus* applying the broth micro dilution method. Results of antimicrobial study were represented that mulberrofuran G and albanol B were found to be effective on *S. typhimurium*, *S. epidermis* and *S. aureus* with MIC values between 5-7.5 µg/mL (Sohn *et al*., 2004). Interestingly, the MIC values of these compounds (20 µg/mL for mulberrofuran G and 30 µg/mL for albanol B) against *E. coli* are seen to be similar with our results obtained with crude extracts of *M. alba*. As well as, Thabti *et al*. studied the antibacterial activity of water and 50% methanol extracts of *M. alba* (stem bark and leaf) against *S. aureus*, *Enterococcus faecalis, S. epidermis*, *E. coli* and *S. typhimurium* using disc diffusion method. It was stated that all hydro methanolic extracts are active at different concentration against selected bacteria except *E. coli.* But in our study, the methanol and water extracts prepared from leaf of *M. alba* were showed low activity (MIC, 32 µg/mL) against *E. coli*. Therefore, some findings of our study are not consistent with the results of Thabti *et al*. at all (Thabti *et al*., 2014). Recently, ethanol and water extracts of *M. alba* leaf were investigated against various bacteria and fungi containing *S. aureus*, *P. aeruginosa*, *E. coli* and weak activity was obtained against these tree bacteria like present study (Omidiran *et al.*, 2012).

The flowers of *M. neglecta* were investigated as antibacterial agent previously. Ethanolic extract of flowers were prepared and studied against 10 bacteria with agar disc diffusion method and found to have activity against *B. anthracis*, *S. epidermidis* and *S. aureus* at the 50 µg/mL concentration (Seyyednejad *et al*., 2010). However, we could not find any record on the antibacterial and antifungal activity on *M. neglecta* leaf in literature.

Methanolic and aqueous extracts of *S. ebulus* leaf were searched with microdilution method against six bacteria (*S. aureus*, *S. epidermidis*, *Bacillus subtilis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*) before (Mahboubi *et al*., 2012). Interestingly, five bacteria were same with present study except *B. subtilis*. It was revealed that extracts have remarkable activity against all of the tested bacteria as compatible with our results. In another study, different solvent extracts prepared from the leaf of *S. ebulus* were assessed for wound healing activity. Results revealed that methanol extract and the isolated compound, quercetin *3-O-glucoside*, displays important wound healing effect

(Peşin Süntar *et al*., 2010). In this manner, it could be said that folk medicine usages of *S. ebulus* for wound healing were supported in different mechanism of action. On the other hand, this is the first report on the antibacterial and antifungal effects of *R. discolor, S. excelsa* and *H. bithynicum* best of our knowledge.

CONCLUSIONS

In this study, antibacterial and antifungal activities of six different plants used in the treatment of wounds in Düzce were investigated. It is shown that all plants have antibacterial and antifungal activity on selected microorganism in different degrees. But the aerial parts of *H. bithynicum,* leaf of *M. neglecta*, *R. discolor* and *S. ebulus* stand out with their antibacterial activity while the shoot of *S. excelsa*, leaf of *M. alba* and *S. ebulus* were found to be considerable as antifungal agent. So, usage of these plants as folk medicine for wound healing in Düzce was thought to be at least partly related with their antimicrobial activities. Besides, *M. neglecta*, *R. discolor* and *H. bithynicum* were determined to have inhibitory effect on hyaluronidase and elastase which are the important factors on wound healing process.

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