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*ANTIMICROBIAL ACTIVITY AND THE PHENOLIC PROFILE OF FIVE *SCROPHULARIA* L. SPECIES

BEŞ *SCROPHULARIA* L. TÜRÜNÜN ANTIMIKROBIYAL AKTIVITESI VE FENOLIK PROFILI

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ABSTRACT

Scrophularia L. genus (Scrophulariaceae) has 310 taxa worldwide. Some *Scrophularia* species are used in folk medicine for the treatment of different skin inflammatory diseases and as antibacterial. The aim of the study was to investigate the antimicrobial activity and determine the phenolic profile of *S. kotscyhana* Bentham, *S. cinarescens* Boiss., *S. catariifolia* Boiss. & Heldr., *S. chrysantha* Jaub&Spach and *S. scopolii* var. *adenocalyx* Hoppe. ex Pers. Phenolic components were analyzed by an HPLC-DAD method. Disk diffusion method and microtiter-plate assay were used to determine the antimicrobial activities of the aerial parts methanolic extracts of the species. Our results revealed that all of the tested species contain caffeic acid and ferulic acid in varied concentrations and none of them have chlorogenic acid. All of the plant species exhibited moderate activity against *Candida albicans* (MIC values; 0.3125 mg/mL). Besides, *S. cinarescens* exhibited moderate activity against *Enterococcus faecalis* (MIC value; 0.3125 mg/mL) and *S. chrysantha* exhibited moderate activity against *Pseudomonas aeruginosa* (MIC value; 0.3125 mg/mL). The results of this study showed that further studies were needed to identify the secondary metabolites of the species and evaluate their antimicrobial activities.

Keywords: Antimicrobial activity, HPLC-DAD, microtiter-plate assay, medicinal plants, Scrophularia species.

ÖZ

Scrophularia L. cinsi (Scrophulariaceae) dünya üzerinde 310 taksona sahiptir. Bazı *Scrophularia* türleri halk arasında çeşitli iltihaplı cilt hastalıklarının tedavisinde ve antibakteriyel olarak kullanılır. Çalışmanın amacı, *S. kotscyhana* Bentham, *S. cinarescens* Boiss., *S. catariifolia* Boiss. & Heldr., *S. chrysantha* Jaub&Spach ve *S. scopolii* var. *adenocalyx* Hoppe. ex Pers. türlerinin antimikrobiyal aktivitesini araştırmak ve fenolik profilini belirlemektir. Fenolik bileşenler bir HPLC-DAD yöntemi ile analiz edildi. Türlerin toprak üstü kısımlarının metanol ekstrelerinin antimikrobiyal aktivitelerini belirlemek için disk difüzyon yöntemi ve mikrodilüsyon yöntemi kullanıldı. Bulgularımız, test edilen tüm türlerin, çeşitli konsantrasyonlarda kafeik asit ve ferulik asit içerdiğini ve hiçbirinin klorojenik asit içermediğini ortaya koymuştur. Çalışılan tüm türler, *Candida albicans*'a (MIC değerleri; 0.3125 mg / mL) karşı orta derecede aktivite göstermiştir. Ayrıca, *S. cinarescens*, *Enterococcus faecalis*'e karşı (MIC değeri; 0.3125 mg/mL) ve *S. chrysantha Pseudomonas aeruginosa*'ya karşı (MIC değeri; 0.3125 mg/mL) orta derecede aktivite gösterdi. Bu çalışmanın sonuçları, türlerin sekonder metabolitlerinin aydınlatılması ve antimikrobiyal etkinliklerinin değerlendirilmesi için daha ileri çalışmalara ihtiyaç duyulduğunu gösterdi.

Anahtar kelimeler: Antimikrobiyal aktivite, HPLC-DAD, mikrodilüsyon yöntemi, tıbbi bitkiler, *Scrophularia* türleri

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1. INTROTUCTION

Medicinal plants have been a popular choice for treating various diseases for years. It is important to evaluate the traditional uses of medicinal plants and to provide resources for pharmaceutical raw materials in the direction of proven efficacy. Additionally, the use of medicinal plants or finding new natural sources for the treatment of microbial infections as an alternative to synthetic medicines where many infectious microorganisms are resistant, is very promising.

Scrophularia L. genus that belongs to Scrophulariaceae family has 310 taxa worldwide and represented by 78 species and subspecies in Turkey (1-3). *Scrophularia* species which are primarily located in the Irano-Turanian and Mediterranean regions, has been used as folk medicine since ancient times (4).

The dried roots of *S. ningpoensis* Hemsl. have been reported to be used in Vietnam for antipyretic, antibacterial and cancer treatment (5,6). It has been reported that the decoction made from the aerial parts or roots of *S. canina* L. in southern Italy was used as an antiseptic and cicatrizant against the wounds of sheep and cattle (7). Some other *Scrophularia* species are also used in traditional medicine for the treatment of skin diseases like abscess, lichen infections wounds, urticaria, and bacterial-viral infections (8-11).

S. nodosa L., *S. auriculata* L. and *S. canina* L. species of which antiseptic properties were attributed to the presence of phenolic acids, have been used for scrofula and several dermatoses (4,12,13). The phenolic fractions of the aerial parts of *S. frutescens* L. and *S. sambucifolia* L. were reported to have potent antibacterial activity especially against Gram-positive bacteria and antibacterial activity of these species were also attributed to the presence of phenolic acids (15).

The present study describes in vitro antimicrobial activity of methanol extracts obtained from the aerial parts of five *Scrophularia* species; *Scrophularia kotscyhana* Bentham, *Scrophularia cinarescens* Boiss., *Scrophularia catariifolia* Boiss. & Heldr., *Scrophularia chrysantha* Jaub & Spach, *Scrophularia scopolii* (Hoppe. ex) Pers. var. *adenocalyx* Somm. & Lev.

There are increasing number of publications on medicinal plants and their biological activies. The results of these researches showed that the phenolic compounds present in medicinal plants play important role in antibacterial activity besides the essential oils (16). The phenolic compounds which may be responsible for the antimicrobial activity was identified by high-performance liquid chromatography with diode array detection (HPLC/DAD).

2. MATERIAL AND METHODS

2.1. Plant material

The aerial parts of plant species were collected from different districts of Turkey during the flowering stages and voucher specimens were deposited in the Herbarium of Hacettepe University Faculty of Pharmacy (HUEF) and at the private collection of one of the researchers (ST). *S. kotscyhana* was collected from Trabzon Macka, Altindere village, Sumela Monastery in 11.05.2014 (HUEF 15002); *S. cinarescens* was collected from Erzurum-Pasinler road in 20.06.2015 (HUEF 15004); *S. catariifolia* was collected from Trabzon, Araklı, Arpalı village in 14.08.2009 (HUEF 13031); *S. chrysantha* was collected from Trabzon Caykara, Mogalakamboz plateau in 12.07.2015 (HUEF 15007); *S. scopolii* var. *adenocalyx* was collected from Ordu in 21.06.2013 (ST 38). Plant materials were cleaned to remove impurities and stored in air-tight containers until use.

2.2. Extraction

Each 10 grams of dried and powdered aerial parts of *Scrophularia* species were extracted with MeOH (3x200 mL) for 30 min under reflux at 40°C. After filtration and evaporation, crude methanolic extracts were obtained. All extracts were stored under refrigeration for further analysis.

2.3. Antimicrobial activity

A disc diffusion test was performed for pre-screening to measure the antimicrobial activities of the extracts, and the microplate method was used to determine the concentration of the extracts inhibiting the expression of the microorganisms (MIC). Bacterial strains used in the experiment have been determined considering morphological differences and physiological requirements. Bacterial and fungal strains (*Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC35218, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC60193) were selected from Gram positive, Gram negative, coccus, bacillus, aerob and facultative anaerobes.

Standard bacterial strains were plated out of stock and pre-cultured to a single colony in appropriate solid medium. At the end of the one-day incubation period, a single colony was removed from culture plates and seeded in 3 ml of Mueller Hinton Broth (MHB) and Sabouraud Liquid (SDB) medium. A bacterial working suspension was obtained by incubation at 37 ° C in a shaking water bath until equilibrium (0.5×10^8 bacteria / ml) of McFarland's standard 0.5. The media used in the study were prepared from the commercial products purchased in lyophilized form by applying the proportions stated above. During the test phase, the surface of the media was dried by standing for 15 minutes, and the bacterial suspensions of turbidity equivalent to McFarland's 0.5-inch tube were spread homogeneously on the media with the rub-bar. Petri dishes were allowed to stand for 5 minutes for drying of the food surface.

2.3.1. Disc diffusion method

Blank antibiogram discs were sterilized in a petri dish in an autoclave and dried by standing the etch. These discs were pipetted from various concentrations of the specimens and the discs were then dried and stored at -20ºC until used. Discs impregnated with specimens prepared as described above and onto which bacteria were spread were placed in sterile conditions at 2.5 cm intervals. Ampicillin (30 µg) and fluconazole (25 µg) impregnated disc and equal volume solvent impregnated disc were used as controls. The petri dishes were incubated at the selected atmospheric environment according to the type of microorganism, temperature and time. The diameter of the inhibition zones formed at the end of

the incubation period was measured (15).

2.3.2. Microplate Method and Minimum Inhibitor Concentration (MIC)

100 µl of each sterile 96-well plate was dispensed from the prepared medium using a repetitive pipette. Samples were diluted with solvent at a concentration of 20mg/ml. From these dilutions, 100 µl was pipetted into the first wells (1A, 2A, 3A, ...) of the 96-well plates. After thoroughly mixing with the twelve-channel pipette in the first wells and pipetting the sample in the first wells, the 100 µl volumes from these are transferred to the next wells. After pipetting 5-6 times in this way, 1/2 serial dilutions of the samples (10 mg / ml - 4 µg / ml) were made by transferring 100 µl to the next wells.

McFarland 0.5 suspension of bacteria was diluted 10-2 with the medium to prepare 106 suspensions of bacteria in ml and 100 µl of each suspension was transferred from this suspension to obtain dilutions of the samples between concentrations of 5 mg / ml and 2 µg / ml. Plates were incubated at the selected atmospheric environment according to the type of microorganism, temperature and time. Following incubation, the lowest sample concentration without visual growth was recorded, as the MIC (17).

2.4. Determination of phenolic compounds by HPLC-DAD analysis

The determination of phenolic compounds in methanolic extracts of five *Scrophularia* species was based on method previously reported by Aliyazicioglu et al (18). The separation was performed using a Waters Spherisorb C_{18} analytical column (250 mm x 4.6 mm, 5 µm). The mobile phase was (A) 0.5% acetic acid in acetonitrile:water (1:1), and (B) 2% acetic acid in water. The following gradient was used; 0-2 min 95-90% B; 2-5 min 90-81% B; 5-10 min 81% B; 10-14 min 81-75% B; 14-17 min 75% B; 17-18 min 75-72% B; 18-20 min 72% B; 20-23 min 72-70% B; 23-25 min 70% B; 25-27 min 70-65% B; 27-29 min 65% B; 29-30 min 65-60% B; 30-32 min 60% B; 32-35 min 60-55% B; 35-36 min 55-50% B; 36-37 min 50-45% B; 37-38 min 45-40% B; 38-40 min 40-35% B; 40-41 min 35% B. The temperature of column oven was adjusted to 25˚C and 20 µL of sample was injected into the column. Gallic acid, caffeic acid, chlorogenic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid and *p*-coumaric acid were used as standards. The detection and quantification of phenolic compounds was performed at 232, 246, 260, 270, 280, 290, 308, and 328 nm and flow rate was 1.2 mL/min. Samples were analyzed in triplicate and mean±SD were reported in Table 2 and chromatograms were given in Figure 1.

3. RESULTS

The percentage of crude methanol extract yields of five *Scrophularia* species; *S. kotscyhana*, *S. cinarescens*, *S. catariifolia*, *S. chrysantha*, *S. scopolii* var. *scopolii* were found as 21.84, 20.92, 18.76, 17.60 and 23.60%, respectively.

The antimicrobial activity of the methanolic extracts of five *Scrophularia* species was evaluated on five microorganisms (Table 1) by disc diffusion and microplate methods. All extracts inhibited the growth of five microorganisms. MIC values for ampicillin and fluconazole which were used as a positive control in our study, ranged from 1-128 µg / ml. The antimicrobial activity of plant extracts is considered as significant when the value of MIC is smaller than 0.100 mg/mL; moderate, between the MIC values of 0.100 and 0.625 mg/ mL or weak when the MIC value is bigger than 0.625 mg/mL (19). *S. kotschyana*, *S. catariifolia* and *S. scopolii* var. *scopolii* exhibited moderate activity (MIC values; 0.3125 mg/mL) against *C. albicans*; *S. cinarescens* exhibited moderate activity (MIC values; 0.3125 mg/mL) against *C. albicans* and *E. faecalis*; *S. chrysantha* exhibited moderate activity (MIC values; 0.3125 mg/mL) against *E. coli, C. albicans* and *P. aeruginosa* (Table 1).

The HPLC-DAD analysis of methanol extracts of five *Scrophularia* species allowed the identification of eight phenolic compounds (Table 2, Figure 1). Caffeic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid and *p*-coumaric acid were previously reported in *S. frutescens*, *S. sambucifolia* and *S. buergeriana* (14, 20) while gallic acid and chlorogenic acid were determined in *S. takasimensis* Nakai (21). Regarding the HPLC profile, caffeic acid and ferulic acid were the main compounds in all of the tested species. No chlorogenic acid was determined in any of the samples. *S. chrysantha* was found to contain only three of the standards; caffeic acid, ferulic acid and *p*-coumaric acid. Among these five species, *S. cinerascens*, the richest species, was found to contain caffeic acid and ferulic acid in high amounts (Table 2).

Table 1. The MIC* values (mg/mL) of methanolic extracts of five *Scrophularia* species.

	S. kotschyana	***S. cinerascens***	***S. catariifolia***	***S. chrysantha***	***S. scopolii***
Staphylococcus aureus	0.625	0.625	1.25	0.625	0.625
Enterococcus faecalis	0.625	0.3125	0.625	1.25	1.25
Escherichia coli	0.625	0.625	0.625	0.625	0.625
Pseudomonas aeruginosa	0.625	0.625	0.625	0.3125	0.625
Candida albicans	0.3125	0.3125	0.3125	0.3125	0.3125

MIC values for ampicillin which was used as a positive control in our study, ranged from 1-128 µg / ml.

4. DISCUSSION

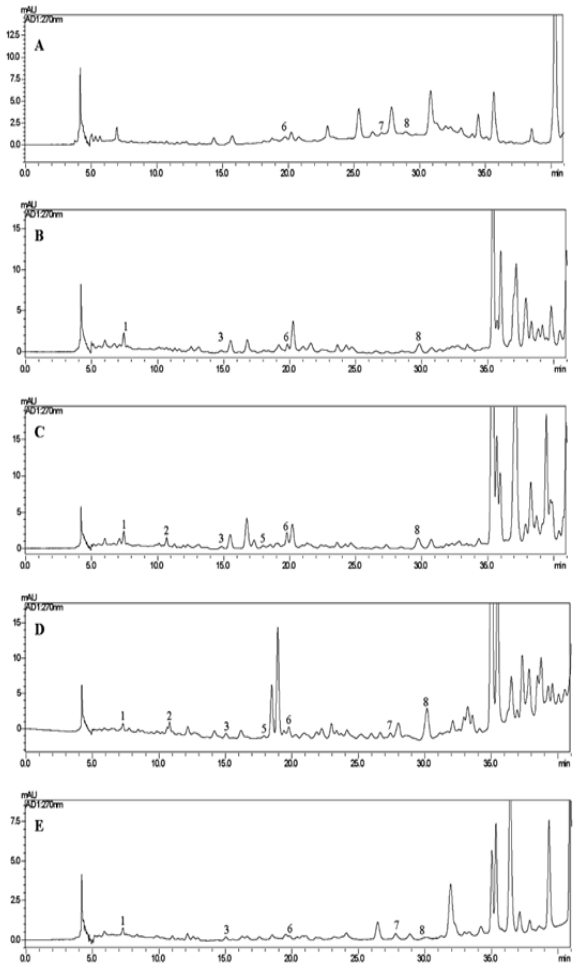
The antimicrobial activity of the plant species may be

Table 2. Phenolic compounds detected with HPLC-UV.

	Gallic acid	Caffeic acid	Chlorogenic acid	Protocatechuic acid	*p*-hydroxy benzoic acid	Vanilic acid	Ferulic acid	*p*-coumaric acid
S. kotschyana	2.18±0.10	13.10±1.28	ND	ND	93.91±1.14	ND	139.77±0.25	65.52±1.47
S. cinerascens	41.84±0.41	158.99±2.65	ND	18.82±1.78	123.42±2.84	4.18±1.68	650.61±0.91	48.11±0.52
S. catariifolia	61.90±0.58	97.55±3.45	ND	ND	90.04±0.97	ND	264.51±5.52	ND
S. chrysantha	ND	3.52±1.81	ND	ND	ND	ND	109.12±0.50	3.52±0.45
S. scopolii var. *scopolii*	75.52±0.91	99.12±0.80	ND	47.2±4.01	110.92±2.09	18.88±3.52	332.76±0.51	ND

* mean ± SD are expressed as µg phenolic compound per g dry samples (n=3). ND: non-detected.

Figure 1. Typical HPLC chromatograms of (A) *S. chrysanth,* (B) *S. catariifolia*, (C) *S. scopolii* var. *scopolii,*(D) *S. cinerascens*, and (E) *S. kotschyana.* Peak identification: (1) gallic acid; (2) Protocatechuic acid; (3) *p*-hydroxy benzoic acid; (4) Chlorogenic acid; (5) Vanilic acid; (6) Caffeic acid; (7) *p*-coumaric acid; (8) Ferulic acid



associated with the structure of its phytochemical components, like phenolic content which are known to be active against microorganisms. *S. cinerascens* which was the richest species in terms of phenolic content within the all five *Scrophularia* species, presented moderate activity against all of the tested microorganisms (Table 1 and 2). The other species were also presented moderate activity mainly against *E.coli*, *P. aeruginosa* and *C. albicans* (Table 1).

Results published previously about *Scrophularia* species support these findings (14). Essential oil of *S. amplexicaulis* was tested against *S. aureus* using the well diffusion method and the essential oil (100 µg/mL) was found to show comparable antibacterial activity with the positive control ampicillin (10 µg/mL) against *S. aureus* (22). The antimicrobial activity of *S. trichopoda* Boiss. & Bal., *S. candelabrum* Heywood, *S. depauperata* Boiss. and *S. mersinensis* Lall. were investigated and methanol extracts of the species showed strong antimicrobial activity against the Gram-positive bacteria and yeasts, but no activity was reported against the Gram-negative bacteria (23, 24). *S. striata* ethanolic extract exhibited higher antimicrobial activity against *S. aureus* (MIC value: 3.2 mg/mL and MLC value 6.4 mg/mL), *Staphylococcus saprophyticus* (MIC value: 1.6 mg/mL and MLC value 3.2 mg/mL), *Staphylococcus epidermidis* (MIC value: 3.2 mg/mL and MLC value 6.4 mg/mL), oral *Streptococcus* species; *S. mutans* (MIC value: 3.2 mg/mL and MLC value 12.8 mg/mL), *S. sobrinus* (MIC value: 3.2 mg/mL and MLC value 6.4 mg/mL), *S. sanguis* (MIC and MLC value 3.2 mg/mL), *Candida* species; *C. albicans* (MIC and MLC value 6.4 mg/mL), *C. glabrata* (MIC and MLC value 6.4 mg/mL) and *Aspergillus parasiticus* (MIC value: 6.4 mg/mL and MLC value 12.8 mg/mL), than methanol, aqueous and ethyl acetate extracts (25).

The antimicrobial activity of the *S. ningpoensis* leaf extracts and the isolated saponins, iridoids and flavonoids were studied against eight reference strains of bacteria by using the disc-diffusion method and micro-well dilution assay. Only Scrokoelziside A which has a saponin structure, was reported to show antibacterial activity on beta-haemolytic streptococci (MIC and MBC; 1.5 and 6.0 mg/mL respectively) (26). In another study, the effect of *Scrophularia deserti* on ethanol extract and streptomycin against *Brucella melitensis* bacteria was compared. The study concluded that the effect of ethanol extract on

B. melitensis was remarkable at increasing concentrations (27).

Previous studies have suggested that *Scrophularia* species may be considered potential antiseptic agents in bacteriological infections, particularly in the presence of Gram-positive bacteria (28). The results of this study also support these findings.

Acording to the results, caffeic acid and ferulic acid are the major compounds of *S. cinerascens* which exhibited highest activity against *E. faecalis* among the other extracts (Table 1, 2). Ferulic acid was also found to be abundant in the extract of S. chrysantha which exhibited highest activity against *P. aeruginosa* among the other extracts (Table 1, 2). The antimicrobial activity of cafeic acid and its derivatives was shown against *E. coli*, *E. faecalis*, *S. aureus*, *Listeria monocytogenes*, and *Haemophilus influenza* by previous studies in the literature and the biological activity of the molecule was attributed to its electrical charge (29, 30). The previous studies revealed that ferulic acid exerts antimicrobial activity against *P. aeruginosa* (MIC value: 500 µg/mL) (31). It was found that the phenolic acids like cafeic acid and ferulic acid affect the cell membrane structure by rigidity and alteration of the dynamics of phospholipid chains (32). These studies confirm the connection between the phenolic compounds and biological activity results of the medicinal species studied.

Conflict of interest: The authors declares that there is no conflict of interest regarding the publication of this article.

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