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Antimicrobial Activity and Chemical Composition of *Senecio sandrasicus* on Antibiotic Resistant Staphylococci

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The antimicrobial activity of hexane, chloroform, ethyl acetate and ethanol extracts of the aerial parts of *S. sandrasicus* P.H.Davis (*Asteraceae*), endemic to Sandras mountain (Turkey), were determined. The antimicrobial activity of the extracts on microorganisms including multi-resistant staphylococci were evaluated using the disc diffusion method. The strains of multi-resistant staphylococci and the other standart bacteria were inhibited by some extracts. The volatile organic compouds of *S. sandrasicus* was determined by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). The major compounds of hexane extract were aromadendrene oxide 2 (13.3%), spathulenol (12.5%) and β-caryophylene (11.8%), respectively.

Keywords: Antimicrobial activity, *Senecio sandrasicus*, Staphylococci, GC/MS analysis.

In the last decades, the spread of antibiotic resistance in bacteria, including staphylococci, is increasing and may represent a hazard for human health [1a]. Staphylococci are responsible for a plethora of medical problems, including skin and soft-tissue infections, surgical site infections, endocarditis and hospital acquired bacteraemia [1b]. Some staphylococcal species, such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, are well known for their implications in human health diseases [1c]. Coagulase-positive *S. aureus* is a major cause of nosocomial infections, food poisoning, osteomyelitis, pyoarthritis, endocarditis, toxic shock syndrome, and a broad spectrum of other disorders [2a,2b]. Coagulase-negative staphylococci (CNS) include *S. epidermidis*, *Staphylococcus haemolyticus*, *S. saprophyticus* and a number of other species [2c]. In recent years, several species of CNS have been recognized as opportunistic pathogens and have been implicated in human infections and disease, especially in immunocompromised and seriously ill patients [2d].

Among antibiotic resistant staphylococci, multidrug-resistant *S. aureus* strains are of great public concern since resistances make more difficult the treatment of infections. Moreover, a number of CNS, such as several *S. epidermidis* strains, are important hospital-acquired infection agents and the 80–90% of these isolates are methicillin-resistant [1a].

The development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for further attempts to find new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents [3a,3b]. Various sources, including medicinal plants [3c] can yield antimicrobials.

The genus *Senecio* (*Asteraceae*) is represented by thirty-nine species in Anatolia. *S. sandrasicus* is endemic to the Sandras mountain (Mugla-Turkey) and it is a East-Mediterranean element [4a]. The aerial parts of the *Senecio* species, known as Kanarya otu, Kulluce otu in Turkey [4b]. Many species of this

genus have been used in traditional medicine and also the many members of this genus are reported to be used in Anatolian folk medicine [4c].

Several species of the *Senecio* exhibit antimicrobial [5a-5c], antibacterial [6a], antifungal [6b], antiviral [6c], antioxidant [6d], antiinflammatory [6a], antitubercular [6e] and antimalarial [6f] activities.

The information concerning the chemical composition of the *S. sandrasicus* has not been reported earlier. In the literature, there is one study on antimicrobial activity of this plant [5a], whereas the antimicrobial activity of the extracts of *S. sandrasicus*, against multi-resistant *Staphylococcus* spp., has never before been studied.

The aims of this study were to investigate the antimicrobial activity of various extracts of *S. sandrasicus* against multi-resistant *Staphylococcus* spp. Also, to investigate the chemical composition of hexane extract in order to determine of volatile organic compounds of *S. sandrasicus* by GC-MS.

In this study, 0.4 µg/disc and 3.6 µg/disc doses of ethanol, hexane, chloroform and ethyl acetate extracts of *S. sandrasicus* were investigated for their antimicrobial activities. For this purpose, 9 standard test microorganisms (*M. luteus* NRRL B-4375*, B. subtilis* ATCC 6633, *S. mutans* CNCTC 8/77, *S. aureus* ATCC 25923, *E. aerogenes* RSKK 720*, P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *C. albicans* ATCC 10239 and *C. tropicalis* RSKK 665 and 11 multi-resistant strains of various species of *Staphylococcus* were used. The antibiotic resistance patterns of the *Staphylococcus* spp. were shown in Table 1.

Table 1: Antibiotic resistance patterns of *Staphylococcus* spp.

Strains	Resistance Patterns*
S. xylosus MU 34	P, AK, DA, E, CN, OX, TEC
S. xylosus MU 35	P, DA, E, C, OX, TE
S. xylosus MU 37	P, AK, DA, E, CN, TEC, TE
S. xylosus MU 42	P, AK, DA, CN, OX, TE
S. aureus MU 38	P, AK, DA, CN, ME, TEC, TE, OX
S. aureus MU 40	P, AK, CN, C, ME, OX, TE
S. aureus MU 46	P, AK, DA, E, CN, TE, OX
Staphylococcus sp. MU 28	P, AK, DA, E, CN, TE
S. capitis MU 27	P, AK, DA, E, CN, TE
S. epidermidis MU 30	P, AK, DA, CN, OX, TEC, TE
S. lentus MU 43	P, AK, DA, CN, OX, TE

*Antibiotic resistance patterns of *Staphylococci* determined according to the recommendations NCCLS.
P: Penicillin-G (10 U); AK: Amikacin (30µg); DA: Clindamycin (2 µg); E: Erythromycin (15 µg); CN: Gentamicin (10 µg); C: Chloramphenicol (30 µg); ME: Methicillin (5 µg); OX: Oxacillin (1 µg); TEC: Teicoplanin (30 µg); TE: Tetracycline (30 µg).

Table 2: Antimicrobial activity of *S. sandrasicus* extracts (0.4 µg/disc).

Strains	Inhibition zone (mm) of the plant extracts (0.4 µg/disc)*			
	Hexane	Chloro-form	Ethanol	Ethyl acetate
E. aerogenes RSKK 720	14±0.2	15±0.2	11±0.1	10±0.2
P. aeruginosa ATCC 27853	13±0.2	11±0.2	11±0.1	9±0.2
E. coli ATCC 25922	14±0.2	9±0.1	12±0.2	11±0.1
M. luteus NRRL B–4375	8±0.1	9±0.1	-	11±0.2
B. subtilis ATCC 6633	15±0.1	9±0.1	-	-
S. aureus ATCC 25923	10±0.2	-	10±0.1	11±0.2
S. mutans CNCTC 8/77	-	-	-	-
S. capitis MU 27	11±0.1	10±0.2	-	9±0.2
Staphyl. sp. MU 28	-	8±0.2	11±0.1	12±0.1
S. epidermidis MU 30	-	-	-	-
S. xylosus MU 34	8±0.1	8±0.2	10±0.2	11±0.2
S. xylosus MU 35	9±0.2	8±0.1	10±0.2	11±0.1
S. xylosus MU 37	9±0.2	8±0.1	10±0.1	11±0.2
S. aureus MU 38	10±0.1	8±0.2	9±0.1	10±0.2
S. aureus MU 40	10±0.2	7±0.2	10±0.2	11±0.3
S. xylosus MU 42	9±0.1	8±0.1	11±0.1	12±0.1
S. lentus MU 43	-	-	9±0.1	10±0.3
S. aureus MU 46	11±0.1	7±0.1	11±0.1	11±0.1
C. albicans ATCC 10239	-	-	-	-
C. tropicalis RSKK 665	-	-	-	-

*Values represent average ± standard deviation for three replicates.
(-) : No activity.

These extracts demonstrated various antimicrobial activities on tested bacteria. The two different doses of all extracts have not effects on *S. mutans, S. epidermidis* MU 30 and yeasts. The inhibition zones of bacterial strains, sensitive to the 0.4 and 3.6 µg extracts of *S. sandrasicus*, were in the range of 7-15 and 10-18 mm, respectively. As it can be seen from Table 2 and 3, when the doses of extracts were increased by 9-fold, the antimicrobial activities of extracts were also increased. However, the increase on antimicrobial activities were not as much as the increase in doses of extracts.

The ethanol, hexane, chloroform and ethyl acetate extracts demonstrated antibacterial activities on *Staphylococcus* strains which shows resistance to various antibiotics. All the extracts had no effect on *S. epidermidis* MU 30. The hexane extract inhibited the growth of all multi-resistant *Staphylococcus*

Table 3: Antimicrobial activity of *S. sandrasicus* extracts (3.6 µg/disc).

Strains	Inhibition zone (mm) of the plant extracts (3.6 µg/disc)*			
	Hexane	Chloro-form	Ethanol	Ethyl acetate
E. aerogenes RSKK 720	17±0.1	18±0.2	15±0.2	13±0.2
P. aeruginosa ATCC 27853	16±0.1	14±0.1	14±0.1	13±0.1
E. coli ATCC 25922	17±0.1	13±0.1	15±0.1	15±0.1
M. luteus NRRL B–4375	12±0.2	13±0.2	-	14±0.2
B. subtilis ATCC 6633	18±0.2	12±0.1	-	-
S. aureus ATCC 25923	15±0.1	-	14±0.1	14±0.2
S. mutans CNCTC 8/77	-	-	-	-
S. capitis MU 27	16±0.1	14±0.1	-	13±0.2
Staphyl. sp. MU 28	-	11±0.2	16±0.3	16±0.1
S. epidermidis MU 30	-	-	-	-
S. xylosus MU 34	13±0.1	12±0.2	16±0.2	17±0.2
S. xylosus MU 35	14±0.2	12±0.1	16±0.1	16±0.2
S. xylosus MU 37	13±0.1	12±0.1	14±0.1	14±0.1
S. aureus MU 38	14±0.1	11±0.2	13±0.1	14±0.2
S. aureus MU 40	14±0.1	10±0.2	14±0.3	15±0.1
S. xylosus MU 42	13±0.2	12±0.1	14±0.1	14±0.1
S. lentus MU 43	-	-	13±0.1	13±0.1
S. aureus MU 46	15±0.1	10±0.3	15±0.1	15±0.1
C. albicans ATCC 10239	-	-	-	-
C. tropicalis RSKK 665	-	-	-	-

*Values represent average ± standard deviation for three replicates.
(-) : No activity

strains, except *Staphylococcus* sp. MU 28, *S. epidermidis* MU 30 and *S. lentus* MU 43. The chloroform extract exhibited antibacterial activity on multi-resistant *Staphylococcus* strains, except *S. epidermidis* MU 30 and *S. lentus* MU 43. The ethanol extract exhibited antibacterial activity on multi-resistant *Staphylococcus* strains, except *S. capitis* MU 27 and *S. epidermidis* MU 30. The 0.4 µg doses of the ethyl acetate extracts inhibited the growth of all *Staphylococcus* strains, except *S. epidermidis* MU 30. The four extracts of the *S. sandrasicus* had antimicrobial activity on the *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *E. aerogenes* RSKK 720. The chloroform and ethanol extracts showed antibacterial activity on *E. aerogenes* (cf. [5a]).

The bioactive hexane extract afforded 19 identifiable compounds by GC and GC-MS methods. Compounds

Table 4: Chemical compositions of *S. sandrasicus* hexane extract.

	Compound[a]	RI[a]	%	Methods
1	Thymol	923	2.7	a, b
2	Carvacrol	946	5.3	a, b
3	α-Caryophylene	1247	4.4	a, b
4	β-Caryophylene	1298	11.8	b
5	β-Ionone	1415	1.7	b
6	Dihydroactinidiolide	1559	4.8	b
7	Ledol	1603	1.4	a,b
8	Spathulenol	1636	12.5	a, b
9	Caryophyllene oxide	1647	11.6	a, b
10	Aromadendrene oxide 2	1698	13.3	b
11	Cubenol	1755	3.8	b
12	γ-Gurjenene epoxide	1761	1.6	a, b
13	*tau*-Cadinol	1784	3.8	a, b
14	δ-Cadinol	1793	3.6	a, b
15	Leden oxide	1801	4.0	b
16	α-Cadinol	1815	6.3	a, b
17	8-hydroxy-endo-cycloisolongifolene	1874	1.9	b
18	Hexahydrofarnesyl acetone	1957	4.3	b
19	Lupeol	2120	1.2	b
	TOTAL		100.00	

a: co-injection with authentic compounds,
b: MS,
[a]: In DB-5 fused silica capillary column.

were identified by comparison with reference substances, NIST 2002, Wiley and locally constructed libraries. The major compounds of hexane extract of this plant were found to be aromadendrene oxide 2 (13.3%), spathulenol (12.5%), β-caryophylene (11.8%), caryophyllene oxide (11.6%), α-cadinol (6.3%) (see Table 4). The hexane extract comprised 69.3% sesquiterpenoids. The antimicrobial activities of aromadendrene oxide 2, spathulenol and caryophyllene oxide are known [7-9]. These are thought to be responsible for the *S. sandrasicus* high antimicrobial activity.

In this study, it was observed that methicillin resistant *S. aureus* MU 38, MU 40 and oxacillin resistant *S. aureus* MU 46 were inhibited by all of the extracts. Oxacillin resistance in both community and hospital acquired strains of staphylococci has emerged as an important and growing resistance threat [10a-10c]. Oxacillin-resistant *Staphylococcus aureus* (ORSA) was first recognized in the United Kingdom in 1961 after the introduction of methicillin into clinical practice, it has now become a leading cause of nosocomial infections worldwide [10d]. MRSA is currently recognized as a major problem in hospitals and the broader community in the United States and throughout the world [11]. MRSA strains are often resistant to antimicrobials other than β-lactams, thereby limiting the range of therapeutic options and increasing the risk of treatment failure as well as the costs for antimicrobial therapy and hospitalization [12]. There is an established need to develop new

antimicrobial agents to combat these pathogens [5a,13]. In this study, the extracts demonstrated antibacterial activity on some of the CNS, *S. xylosus*, *S. lentus, S. capitis*.

Various extracts of *S. sandrasicus* have indicated strong activities against *S. aureus* and CNS, which are resistant to various antibiotics were used in this study. These extracts are possible alternative therapies for infections of the Multidrug-resistant organisms (MDROs) such as Methicillin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Enterococcus* (VRE), extended spectrum beta-lactamases (ESBL), and stably derepressed AmpC enzyme producers among *Enterobacteriaceae*, nonfermentative Gram-negative bacilli, principally *P. aeruginosa*, *Acinetobacter* spp. and *S. maltophilia.* Alternate therapies to accepted antibiotics can prove valuable. The doses of extracts used in this study were active at much lower concentrations than these accepted antibiotics. This *in vitro* study provides evidence that the plant studied is potentially a rich source of antibacterial agent against the multi-resistant bacteria tested.

Experimental

Plant material: Indigenous *S. sandrasicus* (an *Asteraceae)* were collected at the flowering stage from Mugla, Turkey. A voucher specimen was identified by Associated Professor Dr. Omer VAROL, Department of Biology, Faculty of Arts and Sciences, Mugla University, and deposited at the Herbarium of Department of Biology at the Mugla University in Turkey under number O.V. 4463.

Obtaining of the crude extracts: The air dried and powdered aerial parts of *S. sandrasicus* (200 g) were extracted successively with hexane, chloroform, ethyl acetate and ethanol in a Soxhlet apparatus until the last portion of the extract became colorless. Solvents from the extracts were removed *in vacuo* using rotary evaporation. Each residue was re-dissolved at 20 mg/mL and 180 mg/mL with hexane, chloroform, ethanol and ethyl acetate, respectively. Crude extracts were maintained at +4ºC prior to use. Crude extracts were investigated for antimicrobial activity. Identity of the compounds from the bioactive hexane extracts was determined using GC and GC/MS.

Microorganisms and condition for cultivation: Multi-resistant staphylococci (*S. aureus* MU 38, MU 40, MU 46, *Staphylococcus xylosus* MU 34, MU 35, MU 37, MU 42, *Staphylococcus capitis* MU 27, *Staphylococcus epidermidis* MU 30, *Staphylococcus lentus* MU 43, *Staphylococcus* sp. MU 28) and *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* CNCTC 8/77, *Micrococcus luteus* NRRL B-4375, *Enterobacter aerogenes* RSKK 720, *Candida albicans* ATCC 10239, *Candida tropicalis* RSKK 665 were used as test microorganisms. The strains (MU coded) were obtained from Mugla University Culture Collection. The antibiotic resistance patterns of Staphylococci determined according to the recommendations NCCLS [14].

The above mentioned bacteria, except *S. mutans,* were cultured in Nutrient Broth (NB) (Difco) *S. mutans* were cultured in Brain Heart Infusion Broth (BHIB) (Difco), *C. albicans* and *C. tropicalis* cultured in Sabouraud Dextrose Broth (SDB) (Difco). *P. aeruginosa* and the fungi were incubated at 30±0.1°C 18-24 h and 24-48 h, respectively. Other bacteria strains were incubated at 37±0.1°C 24-48 h. Inocula were prepared by adjusting the turbidity of the medium to match the 0.5 Mcfarland Standard Dilutions of this suspension in 0.1 % peptone (w/v) solution in sterile water inoculated on NB, BHIB, SDB to check the viability of the preparation. The cultures of microorganisms were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Disc diffusion assay: The antibacterial activity was evaluated using accepted disc diffusion methodology [15a-15c] using bacterial cell suspension whose concentration was equilibrated to a 0.5 McFarland standard. A 100 µL of each bacterial suspension was spread on a Mueller–Hinton agar plate. Twenty-five µL of each extract containing 0.4 µg and 3.6 µg crude extract were injected in discs of 6 mm in diameter (Schleicher & Schuell). The discs were allowed to dry and then placed on the inoculated agar. The plates were incubated at appropriate temperature and time for microorganisms. Discs of hexane, chloroform, ethanol, ethyl acetate were used as controls. After the prerequisite incubation time a zone of inhibition was measured. The experiment was performed in triplicate and the average values are presented.

Column chromatography (CC): Firstly, CC was performed to highlight the structure of the active extract. For CC, silica-gel 60 (70-230 mesh) as adsorbent in a column with 2x80 cm measurements

was used and mobile phases were respectively 95:5, 90:10 and 85:15 hexane: acetone systems. The fractions were purified by TLC and subjected to GC and GC-MS analysis.

Gas chromatography (GC): GC analyses of the extract was performed using a Shimadzu GC-17 AAF, V3, 230V LV series gas chromatograph equipped with a FID and a DB-5 fused silica capillary column (30 m x 0.32 id., film thickness 0.25 µm); the initial oven temperature was held at 100°C for 5 min, then programmed to 240°C at 3°C/min and held at this temperature for 30 min; injector temperature and detector temperature was 250 and 270°C, respectively; carrier gas was He at a flow rate of 1.4 mL/min; sample size, 1.0 µL; split ratio, 50:1. The percentage composition of the hexane extract was determined with Class-GC 10 computer program.

Gas Chromatography – Mass Spectrometry (GC-MS): 50 mg/mL solution of the bioactive hexane extract was prepared for GC and GC-MS analyses. The analysis of the extract was performed using a Varian Saturn 2100, (E.I Quadrupole) equipped with a DB-5 MS fused silica capillary column (30 m x 0.32 mm id., film thickness 0.25 µm). For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium at a flow rate of 1.7 mL/min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. The oven temperature was held at 100°C for 5 min, then increased up to 240°C with 3°C/min increases and held at this temperature for 25 min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 mL were injected manually in the splitless mode. The relative percentage of the extract constituents was expressed as percentages by peak area normalization.

Identification of components of the extract was computer matching of mass spectra with those of standards (NIST 2002, Wiley library data of GC–MS systems and a locally customized library of 320 spectra), as well as by comparison with the fragmentation patterns of the mass spectra with those reported in the literature [15d] and, whenever possible, by co-injection with authentic compounds.

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