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RESEARCH ARTICLE

Characterization of the phenolic composition and antimicrobial activities of Turkish medicinal plants

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Abstract

In this paper, antibacterial and antimycobacterial activity of five Labiatae plant methanol extracts, commonly used for treating cold, stomachache, and sore throat, *Salvia fruticosa* Mill., *Salvia tomentosa* Mill., *Sideritis albiflora* Hub.-Mor. (endemic), *Sideritis leptoclada* O. Schwarz & P.H. Davis, (endemic), and *Origanum onites* L., were investigated, and their phenolic compounds were determined by HPLC. Antibacterial activity was analyzed against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae*. Antimycobacterial activity was assayed against *Mycobacterium tuberculosis*. The best antibacterial activity (MIC 640 µg/mL) was shown against *S. typhimurium* and *E. aerogenes* by *S. fruticosa*; *E. coli*, and *S. typhimurium*, *E. aerogenes* by *S. tomentosa*; *S. typhimurium*, and *E. aerogenes* by *S. leptoclada* and *S. typhimurium*, *E. aerogenes* and *S. epidermidis* by *O. onites*, respectively. The best antimycobacterial activity (MIC 196 µg/mL) was shown by *S. tomentosa*. *S. fruticosa* (MIC 392 µg/mL) and *O. onites* (MIC 784 µg/mL) showed moderate activity against *M. tuberculosis*. *S. albiflora*, with low level rosmarinic acid and carvacrol content, showed inhibition against bacteria except *K. pneumoniae*, *B. cereus* and *M. tuberculosis*. The correlation between *in vitro* activity and ethnobotanical usage was evaluated.

Keywords: Antibacterial; antimycobacterial; methanol extract; *Salvia*; *Sideritis*; *Origanum*

Introduction

The genus *Salvia* L. (Lamiaceae) is widely distributed with 90 species growing in Turkey, and has economic and medicinal importance (Davis, 1982; Baytop, 1984; Baser, 1995; Baydar et al., 2004; Hamzaoglu et al., 2005; Topcu et al., 2007). *Salvia* species are known to have diterpenoids (Ulubelen & Topcu, 1984; Ulubelen et al., 1997), triterpenoids (Pedreros et al., 1990; Sokovic et al., 2002; Ulubelen et al., 2001, 2002; Mehmood et al., 2006) essential oils (Tepe et al., 2004; Tabanca et al., 2006), and flavonoids (Ulubelen & Topcu, 1984). Bagci et al. (2004) studied fatty acid composition of some *Salvia* species. Kamatou et al. (2005) researched some *Salvia* species for *in vitro* pharmacological activities and chemical constituents.

Studies on methanol extracts of *Salvia* species were found to be quite interesting and provided valuable

results about plants properties. Tepe (2007) showed that rosmarinic acid and derivatives were likely responsible for antioxidant activities of some *Salvia* species. Fiore et al. (2006) examined the *in vitro* antiproliferative activity of the methanol crude extracts of six *Salvia* species and found potential antitumor agents. Eidi et al. (2005) studied the hypoglycemic effect of sage leaves (*Salvia officinalis* L.) and found that while the essential oil of sage did not change serum glucose, the methanol extract significantly decreased serum glucose in diabetic rats.

Sideritis L. (Lamiaceae) is represented in Turkey by 52 taxa belonging to 45 species of which 34 are endemic. Dried inflorescences of *Sideritis* species are used as a popular herbal tea in Turkey and Greece (Davis, 1982; Duman, et al., 1995; Tabanca et al., 2001; Baser, 2002). It is used for treatment of colds, stomachache, and sore

throat (Sezik & Ezer, 1983; Baser et al., 1986). Some *Sideritis* species have been investigated for non-volatile constituents and *ent*-kaurane diterpenes were isolated (Baser et al., 1996; Topcu et al., 1999, 2002). The main constituents of most *Sideritis* growing in Turkey were essential oils (α - or β -pinene or both) (Baser, 1995; Ezer et al., 1996; Tabanca et al., 2001).

The genus *Origanum* L. (Lamiaceae) is represented in Turkey by 23 species or 32 taxa, 21 being endemic (Davis, 1982; Duman et al. 1995; Baser, 2002). It is also antispasmodic (Baser, 1995, 2002; Satil et al., 2006a) and antibacterial (Lambert et al., 2001). Tumen et al. (1995) found the major essential oils of *Origanum* were terpenepheneol, carvacrol, *p*-cymene, linalool, and γ -terpene.

The aim of this study was to determine major phenolic compounds of *Salvia fruticosa* Mill, *S. tomentosa* Mill, *Sideritis albiflora* Hub.-Mor. (endemic), *S. leptoclada* O. Schwarz & P.H. Davis, (endemic), and *Origanum onites* L. methanol extracts by HPLC, and to determine their antibacterial and antimycobacterial activity.

Materials and methods

Plant materials

Aerial parts (Herba in flowering stage) of plants were collected in June 2005 from different parts of Turkey. The plants were identified by F. Satil in Balıkesir University. Voucher specimens were deposited in the herbarium of Balıkesir University, Department of Biology. Locality, altitude, collection time and herbarium number of species are given in Table 1.

Preparation of extracts

The air-dried plants at room temperature of *S. fruticosa* (140 g), *S. tomentosa* (84 g), *Sideritis albiflora* (135 g), *S. leptoclada* (117 g), and *O. onites* (135 g) were extracted with 1L of methanol (98%) at room temperature during ten days according to Seshadri (1962). The methanol extracts were filtered with filter paper, concentrated by using a rotary evaporator and dried *in vacuo* at 40°C. The total yield quantities were 1.30, 1.98, 1.21, 1.45 and 2.17 g, respectively. All stocks were stored at -20°C.

HPLC conditions

HPLC was performed with a Shimadzu HPLC device using phenolic compound preparation techniques (Caponio et al., 1999). The detector was a DAD detector (max=278 nm) and the auto sampler was an SIL-10ADvp. The system controller was an SCL-10Avp, the pump was an LC-10ADvp and the degasser was a DGU-14A. The column oven was a CTO-10Avp and the column was Agilent Zorbax EclipseXDB-C18 (250×4.60 mm) 5 μ m. Mobile phases were A) 3% acetic acid, and B) methanol, and flow speed was 0.8 mL/minute. Column temperature was 30°C and injection volume was 20 μ L.

Microorganisms and inoculum

A total of eight Gram-positive and Gram-negative bacteria were used for antibacterial activity studies. Gram-positive bacteria: *Staphylococcus aureus* (6538-P), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (CCM 99). Gram-negative bacteria: *Escherichia coli* (ATCC 11230), *Salmonella typhimurium* (CCM 583) *Enterobacter aerogenes* (CIP 6069) and *Klebsiella pneumoniae* (CCM 2318) were used for antibacterial testing.

The extracts were tested against the reference strain *Mycobacterium tuberculosis* H37Ra (ATCC 25177) for inhibitory activity in duplicate. Inoculum was prepared both from solid media and from a positive BACTEC *Mycobacteria* growth indicator tube (MGIT) according to the "inoculation procedure for susceptibility" recommended by the manufacturer Becton Dickinson (2004).

From solid media cultures less than 15 days old, a suspension was prepared in Middlebrook 7H9 broth. The turbidity of the suspension did not exceed 1.0 McFarland standard. The suspension was vortexed for 1–2 minutes, allowed to precipitate larger particles, and held for 20 min. The supernatant was transferred to an empty, sterile tube, and held for 15 min. After transferring to a new sterile tube, the suspension was adjusted to a 0.5 McFarland turbidity standard by visual comparison. One mL of the adjusted suspension was diluted in 4 mL of sterile saline.

Positive BACTEC MGIT tubes were used from the first day it became positive (day 1 positive) up to and including the fifth day (day 5 positive). After five days they were subcultured. Tubes from day 1 and day 2 were used for the inoculation procedure and susceptibility test.

Table 1. Herbarium data of plants.

Genus species authority (Labiatae)	Locality	Altitude (m)	Collection time	Herbarium number
<i>Salvia fruticosa</i>	Marmara adası, Balıkesir	150	02.06.2005	FS 1423
<i>Salvia tomentosa</i>	Yayla-Kazdağı, Balıkesir	850	01.06.2005	FS 1422
<i>Sideritis albiflora</i>	Muğla, Tıllalı Dağı, Yenice Köyü	150	07.09.2005	FS1468
<i>Sideritis leptoclada</i>	Muğla, Göktepe, Karlık çayı	300	07.09.2005	FS1469
<i>Origanum onites</i>	Balıkesir, Yaşyer Köyü	300	06.08.2006	FS1468

The tubes between 3 and 5 days were diluted 1 to 4 in saline; this diluted suspension was used for inoculation procedures.

Antibacterial activity test

Stock solutions of all extracts were prepared in 10% dimethylsulphoxide (DMSO). Determination of minimal inhibitory concentration (MIC) by the microdilution method were performed according to the National Committee of Clinical Laboratory Standard guidelines (NCCLS, 2000) and Koo et al. (2000). Sterile 96-well microplates were used for the assay (0.2 mL volume, Fisher Scientific). Samples were diluted to twice the desired initial test concentration with Trypton soya broth-soybean casein digest medium USP, (TSB), (Oxoid, Code: CM0129); samples that were difficult to dissolve were sonicated. All wells were filled with TSB (80 μ L). Test sample (80 μ L) was added to the first well and serial two-fold dilutions were made down to the desired minimum concentration. Serial dilutions are performed so extract concentrations in the range of 10240-10245 μ g/mL were obtained. Day-old cultures of bacteria grown on Tryptone soy agar (TSA) (Oxoid, Code: CM0131) agar plates were suspended in TSB until turbidity was equal to a 0.5 McFarland Standard (Koneman et al., 1997). Gentamycin (Oxoid) was used as a positive control. Serial dilutions were performed so that gentamycin concentrations in the range of 128-0.06 μ g/mL were obtained. The plates were inoculated with the bacterial suspension (10 μ L per well) and incubated at 37°C overnight. All tests were done in triplicate in three different experiments.

Antimycobacterial activity test

MGIT mycobacteria growth indicator tubes containing 4 mL of modified Middlebrook 7H9 broth base were used. Each test tube included a fluorescence-quenching-based oxygen sensor embedded in silicone in the bottom of the tubes. The fluorescent compound is sensitive to the presence of dissolved oxygen in the broth. The initial concentration of dissolved oxygen quenches the fluorescent emission from the compound. After actively respiring, the microorganisms consume the oxygen and allow the fluorescence to be observed using a 365 nm UV transilluminator (Palaci et al., 1996; Reisner et al., 1995; Walters & Hanna, 1996; Becton Dickinson, 2002).

Assays were performed by instructions of the MGIT manual fluorometric susceptibility test procedure recommended by the manufacturer Becton Dickinson. OADC enrichment (0.5 mL), and a mixture of oleic acid, albumin, dextrose and catalase were added to each tube. Compound was added in a volume of 0.1 mL per MGIT tube.

The final concentration of the extracts adopted to evaluate antimycobacterial activity was included from 1.5 to 0.012 mg/mL. An uninoculated MGIT tube was used as a negative control. The positive control tube contained only organisms and OADC but not the plant extract. Any suspicious growth of other bacteria was checked using blood agar for each test. The vials were incubated at 37°C and MIC was determined to be the lowest dilution giving negative by MicroMGIT fluorescence reader within 2 days when the controls turned positive. Tubes were read daily starting on the second day of incubation using a MicroMGIT fluorescence reader which has a long wave UV light (Becton Dickinson, 2002).

Results and discussion

Five methanol extracts prepared from the aerial parts of plants were screened against Gram-positive bacteria *S. aureus* (6538-P), *S. epidermidis* (ATCC 12228), *E. faecalis* (ATCC 29212), *B. cereus* (CCM 99), and Gram-negative bacteria *E. coli* (ATCC 11230), *S. typhimurium* (CCM 583) *E. aerogenes* (CIP 6069) and *K. pneumoniae* (CCM 2318) (Tables 2 and 3). Gram- bacteria and Gram+ bacteria tested for susceptibility with gentamycin.

On the other hand, methanol extracts obtained from the aerial parts of plants were also tested against *M. tuberculosis* H37Ra (ATCC 25177) for antimycobacterial activity using the MGIT manual fluorometric susceptibility test. Standard drugs were streptomycin, rifampin, ethambutol, and isoniazid. *M. tuberculosis* was found sensitive to standard drugs (Table 2).

S. tomentosa displayed the best activity (MIC 640 μ g/ml) against *E. coli*, *S. typhimurium*, and *E. aerogenes*. Other bacteria (*K. pneumoni*, *B. cereus*, *E. faecalis*, *S. aureus*, and *S. epidermidis*) were inhibited between MIC 1280-10240 μ g/mL (Table 3).

S. leptoclada and *O. onites* showed the best activity on Gram- bacteria with MIC 640 μ g/mL against *S. typhimurium* and *E. aerogenes*. *O. onites* also inhibited *S. aureus* (MIC 640 μ g/mL). Although *S. leptoclada* didn't show any significant activity, *O. onites* exhibited activity (MIC 784 μ g/mL) against *M. tuberculosis*. *S. albiflora*, with low level rosmarinic acid and carvacrol content, showed particular inhibition against bacteria, except *K. pneumoniae*, *B. cereus*, and *M. tuberculosis*.

Plant methanol extracts showed promising results; *S. fruticosa* displayed the best activity on Gram- bacteria with MIC 640 μ g/mL against *S. typhimurium* and *E. aerogenes*. The rest of the bacteria exhibited activity between 1280-10240 μ g/mL (Table 3).

Methanol extracts obtained from the aerial parts of plants were investigated by HPLC analysis. Chemicals in the methanol extracts are given in Table 4. Chromatograms of phenolics in methanol extracts

Table 2. Susceptibility test results against *M. tuberculosis* H37Ra (ATCC 25177) obtained by the MGIT fluorometric manual method.

Plant no.	Extracts	Final concentration in MGIT tubes ($\mu\text{g/mL}$)
1	<i>Salvia fruticosa</i>	392
2	<i>Salvia tomentosa</i>	196
3	<i>Sideritis albiflora</i>	1568
4	<i>Sideritis leptoclada</i>	1568
5	<i>Origanum onites</i>	784
Standard drugs	Streptomycin	0.8
	Rifampin	1.0
	Ethambutol	3.5
	Isoniasid	0.1

Table 3. Antibacterial activity of methanol extracts of the plants as MIC ($\mu\text{g/mL}$).

Bacteria	<i>S. fruticosa</i>	<i>S. tomentosa</i>	<i>S. albiflora</i>	<i>S. leptoclada</i>	<i>O. onites</i>	Gentamycin
<i>Escherichia coli</i>	1280	640	1280	1280	1280	2
<i>Salmonella typhimurium</i>	640	640	1280	640	640	2
<i>Enterococcus faecalis</i>	2560	1280	2560	2560	2560	32
<i>Enterobacter aerogenes</i>	640	640	1280	640	640	2
<i>Staphylococcus aureus</i>	5120	5120	1280	1280	1280	4
<i>Staphylococcus epidermidis</i>	1280	1280	1280	1280	640	2
<i>Klebsiella pneumoniae</i>	10240	10240	>10240	>10240	10240	8
<i>Bacillus cereus</i>	10240	10240	>10240	>10240	10240	8

Table 4. Amount of compounds ($\mu\text{g/mL}$) presented in the methanol extracts of plants.

Compound ($\mu\text{g/mL}$)	<i>S. fruticosa</i>	<i>S. tomentosa</i>	<i>S. albiflora</i>	<i>S. leptoclada</i>	<i>O. onites</i>
Gallic acid	*	*	2.9	*	8.6
Catechin	45.0	1603.5	*	*	*
Caffeic acid	7538.6	355.5	1548.6	1911.1	446.7
Epicatechin	9.4	*	*	*	*
Vitexin	*	*	*	*	*
Rutin	*	866.9	*	350.3	486.2
Naringin	92.1	*	251.9	*	*
Hesperidin	2.2	10.7	*	153.2	*
Api-glu	249.9	*	249.1	*	570.7
Rosmarinic acid	5267.9	79202.3	6284.3	19641.2	28413.4
Eridictiol	*	56.4	*	*	764.1
Quercetin	28.7	441.5	*	*	460.0
Naringenin	*	*	*	51.3	452.8
Luteolin	335.6	51.3	202.2	117.5	778.7
Apigenin	1595.0	117.9	719.8	193.5	2003.1
Carvacrol	7127.5	444.1	8469.6	243.2	83684.6
Acacetin	*	*	*	*	*

*not determined.

compared to chromatograms of standards (Figures 1–6). Results were evaluated according to literature. The most prominent phenolic compounds of these plants determined by HPLC analyses were carvacrol and apigenin for *O. onites*; rosmarinic acid, rutin, and catechin for *S. tomentosa*; caffeic acid for *S. fruticosa*. Gallic acid, catechin, epicatechin, rutin, naringin, hesperidin, eridictiol, and quercetin were minor compounds (Figures 1–6, Table 2).

Botelho et al. (2007) and Kunle et al. (2003) reported that carvacrol showed potent antimicrobial activity. Satil et al. (2006b) reported that a solution of *S. tomentosa* is also used by pouring onto open cuts and is called “Tentürdiyot out” (Iodine herb), “Moşabla” or “Boş yaprak”. Other references about phenolics and their antimicrobial effects are given below. Our results are supported by antibacterial effects, especially on Gram (-) bacteria. *S. tomentosa* has an inhibitory effect on

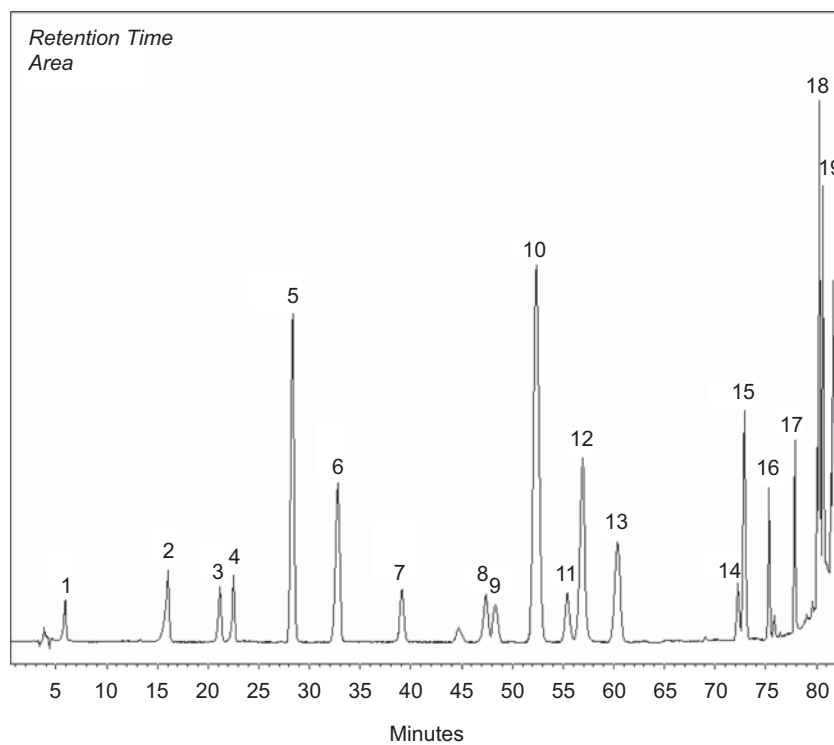


Figure 1. Chromatogram of standards: 1) gallic, 2) catechin, 3) caffeic, 4) epicatechin, 5) p-coumaric acid, 6) ferulic acid 7) vitexin, 8) rutin, 9) naringin, 10) hesperidin, 11) apigenin-glucoside, 12) rosmarinic acid, 13) eridictiol, 14) quercetin, 15) naringenin, 16) luteolin, 17) apigenin, 18) carvacrol, 19) acecetin.

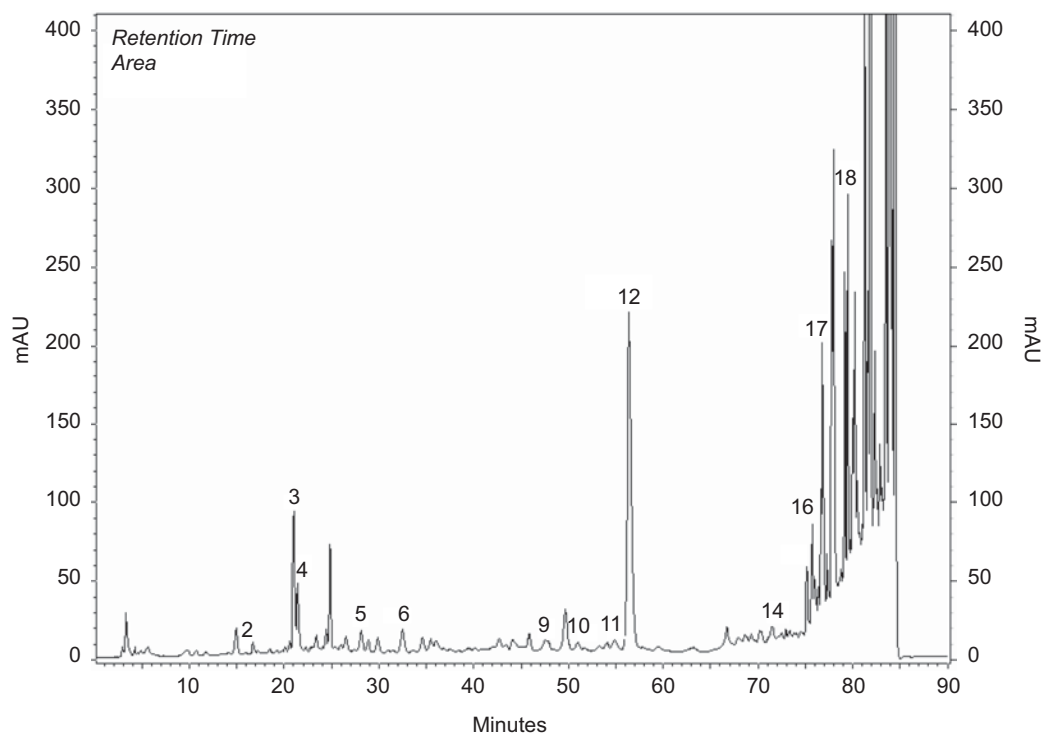


Figure 2. HPLC chromatogram of methanol extracts of *Salvia fruticosa*.

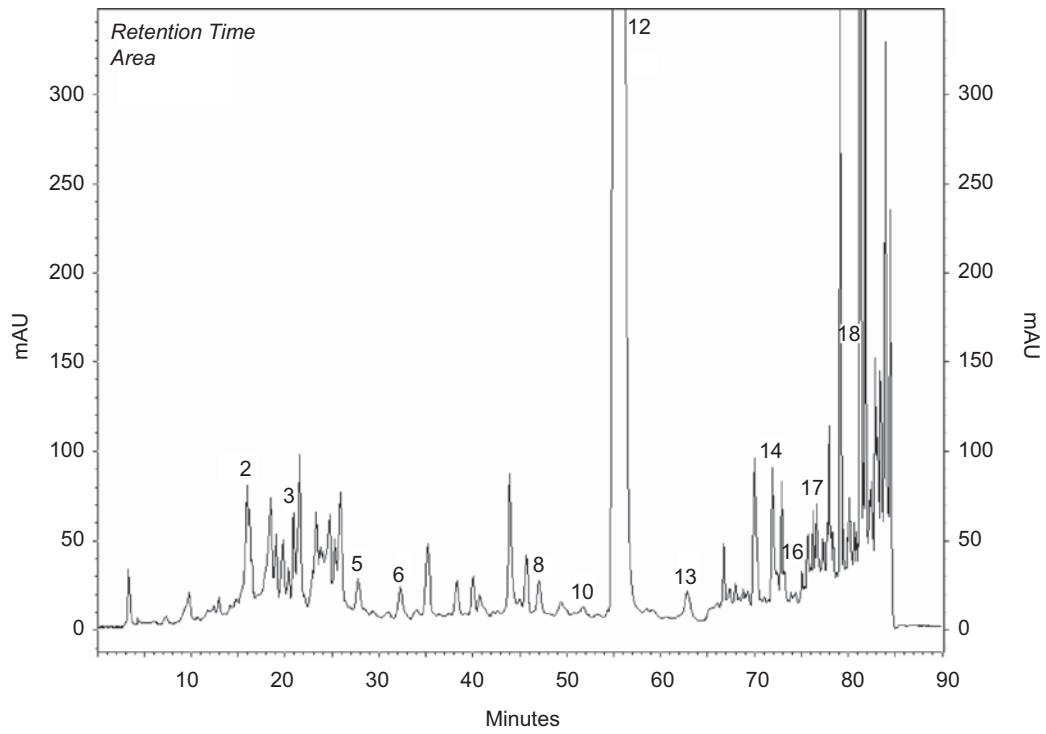


Figure 3. HPLC chromatogram of methanol extracts of *Salvia tomentosa*.

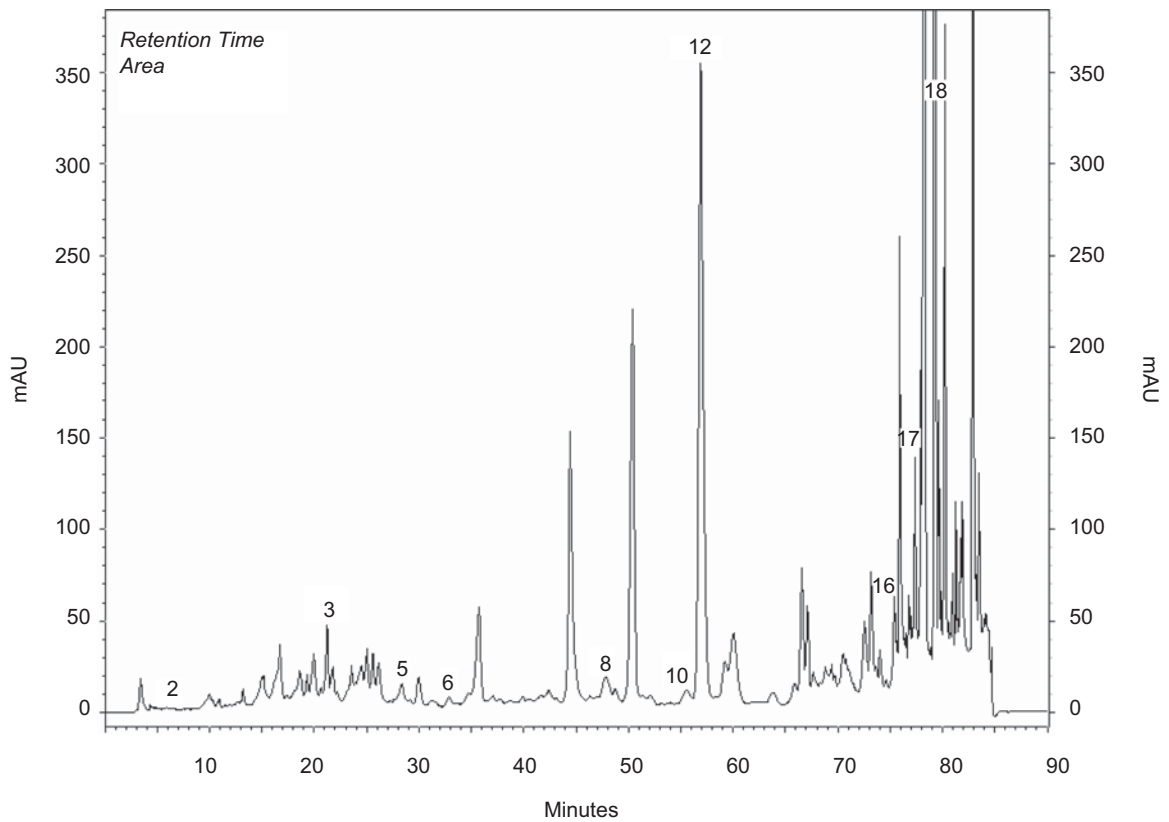


Figure 4. HPLC chromatogram of methanol extracts of *Sideritis albiflora*.

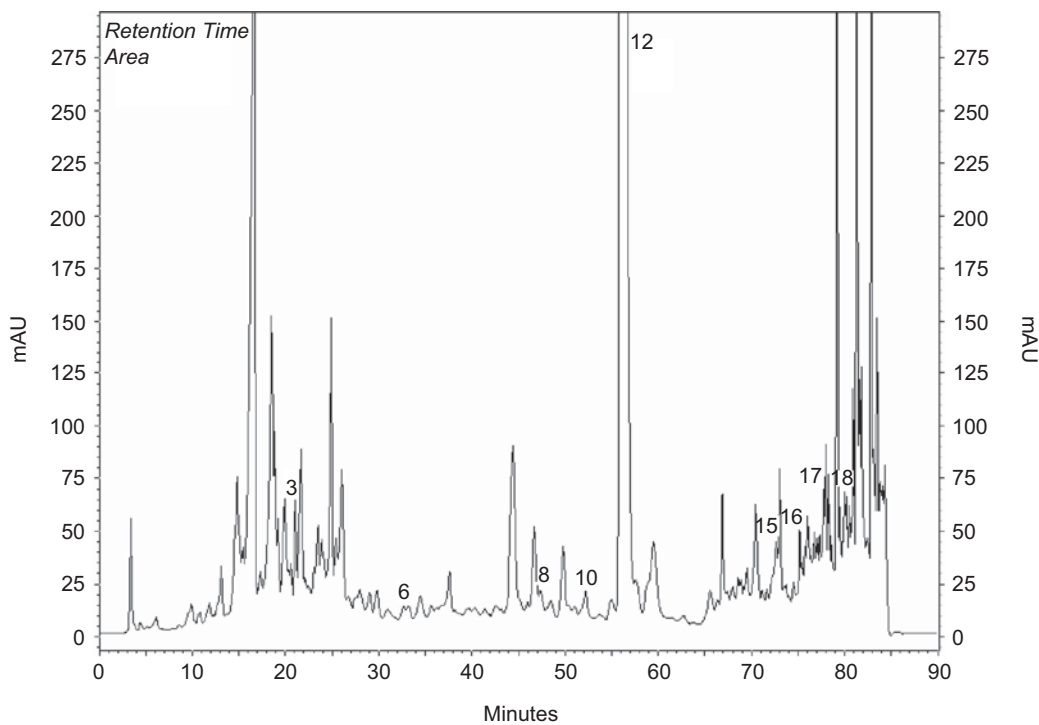


Figure 5. HPLC chromatogram of methanol extracts of *Sideritis leptoclada*.

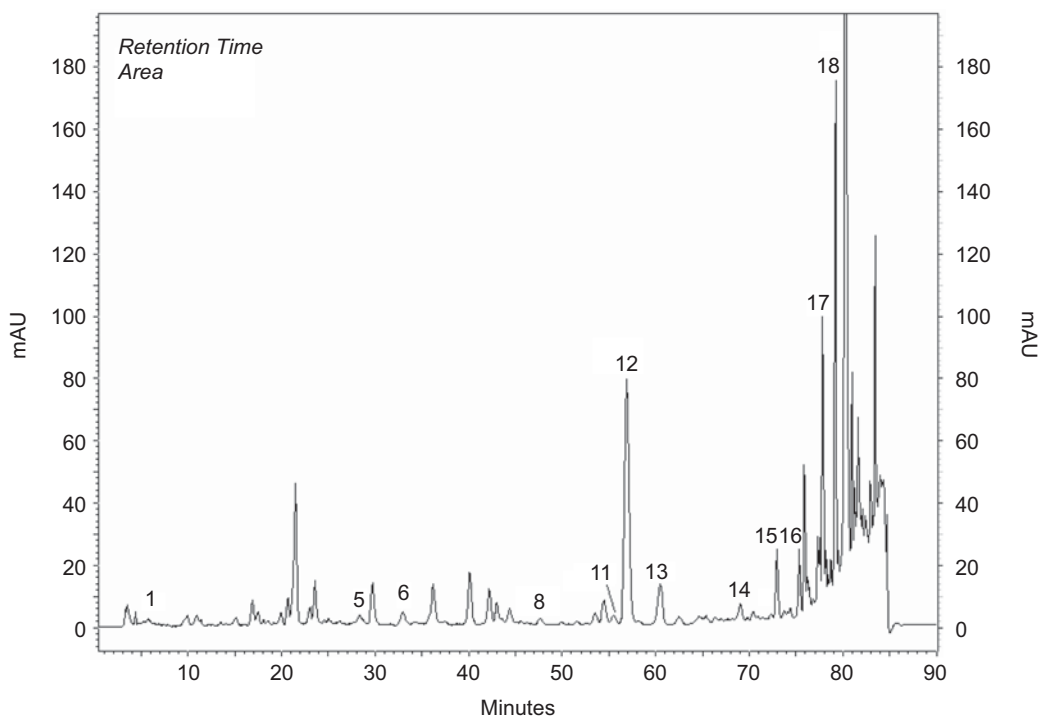


Figure 6. HPLC chromatogram of methanol extracts of *Origanum onites*.

M. tuberculosis (MIC 196 µg/mL). According to HPLC analyses, we found that the amount of rosmarinic acid was quite high. There is a good correlation between rosmarinic acid and the MIC value. It is our opinion

that this activity may come from rosmarinic acid compounds. Therefore, it might be a useful antimicrobial agent, especially on Gram- bacteria and *M. tuberculosis* for commercial use.

S. fruticosus tea, called “Adaçayı”, or “Elmaçayı”, is commonly used to cure colds and stomachache (Kirimer et al., 1999; Sezik et al., 2001). It has shown similar effects on Gram- bacteria. Although its content of rosmarinic acid is lower than that of *S. tomentosus*, we identified a significant amount of carvacrol in the methanol extract. Sharififar et al. (2007) and Sokmen et al. (2004) showed that carvacrol has antibacterial activity, especially on Gram- bacteria.

While all extracts except *O. onites* showed efficacy on Gram+ bacteria, *O. onites* was the only species exhibiting significant activity (MIC 640 µg/mL) against *S. epidermidis*, but not *S. aureus*. According to some reports in the literature, luteolin is active against Gram+ bacteria. The luteolin content might be responsible for this activity against *S. epidermidis* (Ramesh et al., 2002; Sousa et al., 2006; Obied et al., 2007).

As to usage of plants, the genus *Salvia* L., *Sideritis* L., and *Origanum* L., have a wide range of spreading, many species, and economic and medicinal importance in Turkey (Davis, 1982; Baytop, 1984; Baser, 1995; Hamzaoglu, 2005; Topcu et al., 2007). *Sideritis* L. species, also known by different local names and traditional names, are widely used as herbal tea in Turkey. *Sideritis* is used as an antispasmodic (Ezer et al., 1992), antibacterial (Ezer et al., 1994) and as a folk medicine to cure cold (Baser, 1995; Kirimer et al., 1999). *Origanum*, known as “kekik”, is used after distillation to obtain a special medicinal oil (kekik oil) and water (kekik water). It is also sold commercially. It is used for ethnic medicine for colds and respiratory tract infections (Baser, 1995). It is also used as a preservative for dried figs by soaking in boiled kekik water (Tumen, 1989).

Therefore, it can be explained why these plants have common usage as a remedy to cure some ailments. These effective phenolics could be responsible for antimicrobial activity. This is the first report describing the antimicrobial and antibacterial activity of these plants.

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