Turk J Biol
32 (2008) 181-192

Bioactivities of the Various Extracts and Essential Oils of *Salvia limbata* C.A.Mey. and *Salvia sclarea* L.

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Received: 05.06.2008

Abstract: The present study evaluated the *in vitro* antimicrobial, antioxidant, and antiviral activities of the essential oil and various extracts of *Salvia limbata* C.A.Mey. and *Salvia sclarea* L., as well as the content of its essential oil. The activities of essential oils were tested against a wide range of human-, plant-, and food-borne microorganisms. A total of 55 microbial organisms belonging to 35 bacteria and 19 fungi and yeast species were tested using disk diffusion, micro dilution, and micro-well dilution. The essential oils were found to possess antimicrobial activity against some of the tested fungi, while no activity was observed against the bacteria. The chemical compositions of the plant hydrodistilled essential oils were analyzed by means of GC-MS. The main compounds of *Salvia* species oils were spathulenol (29.30%) and germacrene D (24.72%). Other important components were sclareoloxide (14.08%) and β-caryophyllene (16.24%). The results showed that the compositions of the essential oils of these *Salvia* species varied significantly. Antioxidant activity was measured by 2 methods, namely scavenging of free radical DPPH and the inhibition of linoleic acid oxidation. In the β-carotene/linoleic acid system, values were 85.1% ± 1 and 76.6% ± 1, respectively, being comparable with that of the synthetic antioxidant BHT (96% ± 1). In antiviral activity assays, the MeOH extracts from *Salvia* species had a significant anti-influenza virus effect and limited antiherpetic activity.

Key Words: Lamiaceae, *Salvia limbata*, *Salvia sclarea*, antimicrobial activity, antioxidant activity, antiviral activity

Salvia limbata C.A.Mey. ve *Salvia sclarea* L. Bitkilerinin Uçucu Yağ ve Çeşitli Özütlerinin Biyoaktiviteleri

Özet: Bu çalışma, *Salvia limbata* C.A.Mey. ve *Salvia sclarea* L. bitkilerinin çeşitli özütleri ve uçucu yağının *in vitro* antimikrobiyal, antioksidan, antiviral aktivitelerinin ve uçucu yağ içeriğinin araştırılması için tasarlanmıştır. Uçucu yağ aktivitesi, insan, bitki ve gıda kaynaklı geniş bir mikroorganizma grubuna karşı test edilmiştir. 35 bakteri, 19 mantar ve maya türlerinden oluşan toplam 55 mikroorganizma disk difüzyon metodu, mikro-dilüsyon ve mikro-well dilüsyon yöntemleri kullanılarak test edilmiştir. Uçucu yağlar test edilen mantarlardan bazılarına karşı antimikrobiyal aktiviteye sahip iken bakterilerden hiçbirine karşı aktivite gözlenmemiştir. Hidrodistilasyon ile elde edilen uçucu yağların kimyasal bileşenleri GC-MS ile analiz edilmiştir. *Salvia* türlerinin yağının temel bileşenleri spathulenol (% 29,30) ve germacrene D (% 24,72) olarak belirlenmiştir. Diğer önemli bileşenler ise sırası ile sclareoloxide (% 14,08) ve β-caryophyllene (% 16,24) olarak belirlenmiştir. Sonuçlar bu *Salvia* türlerinin bitkisel yağ bileşenlerinde önemli derecede farklılıklar olduğunu göstermiştir. Antioksidan aktivite, serbest radikal giderici DPPH ve linoleik asit oksidasyonu olmak üzere iki metot kullanılarak çalışılmıştır. Kontrol olarak kullanılan sentetik antioksidan BHT (% 96 ± 1)'e karşı, β-karoten/linoleik asit sistem seviyeleri sırası ile % 85,1 ± 1 ve % 76,6 ± 1, olarak tespit edilmiştir. Antiviral aktivite çalışmalarında *Salvia* türlerinin MeOH özütleri önemli anti-influenza virus etkisi ve sınırlı antiherpetik etki göstermiştir.

Anahtar Sözcükler: Lamiaceae, *Salvia limbata*, *Salvia sclarea*, antimikrobiyal aktivite, antioksidan aktivite, antiviral aktivite

Introduction

Salvia consists of 900 species in the family Lamiaceae and this genus is represented in the Turkish flora by 88 species and 93 taxa, of which 45 are endemic (1). *Salvia* species are well known in folk medicine and have been used for culinary purposes. Some species of this genus are of economic importance as flavoring agents in perfumery and cosmetology. Despite the large medicinal potential of the plants in Turkey, knowledge of this area and studies on these plants are not sufficient (2).

Some of the phenolic compounds of plants belonging to this genus have also shown excellent antimicrobial activity as well as scavenging activity of active oxygen types such as superoxide anion radicals, hydroxyl radicals, and singlet oxygen (3), inhibiting lipid peroxidation (4), and consequently the corresponding extracts have been widely used to stabilize fat and fat-containing foods (5). Determination of the essential oil and particularly polyphenolic constituents of the *Salvia* species is the main interest of many research groups. Polyphenolics of *Salvia* species are well reviewed by Lu and Foo (6). The presence of phenolic acids, mainly caffeic acid and its metabolites and flavonoids, in many *Salvia* species is discussed in detail.

In order to prolong the storage stability of foods, synthetic antioxidants are mainly used in industrial processing. However, according to toxicologists and nutritionists, some synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that are used in food processing have side effects. According to Ames (7) and Baardseth (8), these substances can have carcinogenic effects in living organisms. Therefore, governmental authorities and consumers are concerned about the safety of their food and about the potential effects of synthetic additives on their health (9). In addition, investigation of other species for culinary purposes may alter the usage of natural resources.

The aim of the present study was to investigate the antioxidant potential of the methanol extracts of *Salvia limbata* and *S. sclarea.* When antioxidant properties are supported by antimicrobial preservation, natural resources are more valuable. While the essential oils of many *Salvia* species possess antimicrobial (10) and antifungal (11,12) properties among many tested organisms, no report was found in the literature for the investigated species.

Materials and Methods

Collection of plant material

Salvia limbata (Erzurum) and *Salvia sclarea* (Uzundere, Tortum, Erzurum) plants were collected at the flowering stage from Eastern Anatolia, Turkey. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, Meryem Şengül, at the Department of Biology, Atatürk University, Erzurum, Turkey. Collected plant materials were dried in the shade, and the leaves were separated from the stem and ground in a grinder with a mesh 2 mm in diameter. The voucher specimen has been deposited at the Herbarium of the Department of Biology, Atatürk University (ATA HERB 9784, ATA HERB 9776 respectively).

Preparation of the extracts

The dried and powdered leaves of plant (500 g) were extracted successively with 1 l of methanol using a Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent (13). The aqueous extracts were filtered using Whatman filter paper (no. 1) and then concentrated in vacuo at 40 ºC using a rotary evaporator. The residues obtained were stored in a freezer at -80 ºC until further tests.

Isolation of the essential oils

The air-dried and ground aerial parts of plants collected were submitted for 3 h to water-distillation using a Clevenger-type apparatus (yields 1.53% v/w for *S. limbata* and 2.31% v/w for *S. sclarea*). The obtained essential oil was dried over anhydrous sodium sulfate and, after filtration, stored at +4 °C until tested and analyzed.

GC-MS analysis conditions

The analysis of the essential oil was performed using a Hewlett Packard 5890 II GC, equipped with a HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 µm) and a HP 5972 mass selective detector. For GC-MS detection an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas, at a flow rate of 1 ml/min. Injector and MS transfer line temperatures were set at 220 ºC and 290 ºC, respectively. Column temperature was initially kept at 50 ºC for 3 min, then gradually increased to 150 ºC at a 3 ºC/min rate, held for 10 min, and finally raised to 250 ºC at 10 ºC/min. Diluted samples (1/100 in acetone, v/v) of 1.0 µl were injected manually and in the splitless mode. The components were identified based on the comparison of their relative

retention times and mass spectra with those of standards, NBS75K library data of the GC-MS system, and literature data (14). The results were also confirmed by comparison of the elution order of the compound with their relative retention indices on non-polar phases reported in the literature (14).

Antimicrobial activity

Microbial Strains

The extracts and the essential oil of *S. limbata* and *S. sclarea* were individually tested against a panel of microorganisms including a total of 55 microbial cultures belonging to 35 bacteria and 19 fungi and a yeast species. The microorganisms used in this study are listed in Tables 1 and 2. They were provided by the Department of Clinical Microbiology, Faculty of Medicine, and Plant Diagnostic Laboratory, Faculty of Agriculture, Atatürk University, Erzurum, Turkey. Identities of the microorganisms used in this study were confirmed by the Microbial Identification System in the Biotechnology Application and Research Center at Atatürk University.

Disk diffusion assay

The dried plant extracts were dissolved in the same solvent (methanol) to a final concentration of 30 mg/ml and sterilized by filtration by 0.22 µm Millipore filters. Antimicrobial tests were then carried out by disk diffusion (16) using 100 µl of suspension containing 10^8 CFU/ml of bacteria, 10^6 CFU/ml of yeast, and 10^4 spore/ml of fungi spread on Muller-Hinton agar (MHA), Sabouraud dextrose agar (SDA), and potato dextrose agar (PDA) medium, respectively. The disks (6 mm in diameter) were impregnated with 10 µl of essential oil or the 30 mg/ml extracts (300 µg/disk) placed on the inoculated agar. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. Ofloxacin (10 µg/disk), sulbactam (30 µg) + cefoperazona (75 µg) (105 µg/disk) and/or netilmicin (30 µg/disk) were used as positive reference standards to determine the sensitivity of each strain/isolate of microbial species tested. The inoculated plates were incubated at 37 ºC for 24 h for clinical bacterial strains, 48 h for yeast, and 72 h for fungi isolates. Plant-associated microorganisms were incubated at 27 ºC. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated twice (15).

Micro-well dilution assay

The minimal inhibition concentration (MIC) values were studied for the bacterial strains that were sensitive to the essential oil and/or extracts in the disk diffusion assay. The inocula of the bacterial strains were prepared from 12 h broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. The essential oils of *S. limbata* and *S. sclarea* dissolved in 10% dimethylsulfoxide (DMSO) were first diluted to the highest concentration (500 µg/ml) to be tested, and then serial 2-fold dilutions were made in order to obtain a concentration range from 7.8 to 500 µg/ml in 10 ml sterile test tubes containing nutrient broth. MIC values of *S. limbata* and *S. sclarea* essential oil against bacterial strains were determined based on a micro-well dilution method (15).

The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µl of inoculum. A 100 µl aliquot from the stock solutions of plants' essential oil initially prepared at the concentration of 500 µg/ml was added to the first wells. Then 100 µl from their serial dilutions were transferred into 6 consecutive wells. The last well, containing 195 µl of nutrient broth without compound and 5 µl of the inoculum on each strip, was used as a negative control. The final volume in each well was 200 µl. Maxipime at the concentration range of 500-7.8 µg/ml was prepared in nutrient broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer. The contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth in each medium was determined by reading the respective absorbance (Abs) at 600 nm using the ELx800 universal microplate reader (BioTek Instruments, Winooski, VT, USA) and confirmed by plating 5 µl samples from clear wells on nutrient agar medium. The essential oil tested in this study was screened twice against each organism. The MIC values were defined as the lowest concentration required inhibiting the growth of microorganisms.

MIC agar dilution assay

MIC values for the fungal isolates were determined by the agar dilution method as described previously (16). The essential oils of *S. limbata* and *S. sclarea* were added aseptically to sterile molted PDA medium containing Tween 20 (Sigma 0.5% v/v) in appropriate volumes to produce the concentration range of 7.8-500 µg/ml. The

Table 1. Antibacterial activities of the essential oils of *Salvia limbata* and *Salvia sclarea* against the bacterial strains tested.

Test microorganisms	*Salvia limbata*		*Salvia sclarea*		Antibiotics[a]	
	DD[b]	MIC[c]	DD[b]	MIC[c]	*DD*[a]	MIC[a] (max)
Acinetobacter baumanii-A8	10	250	N.R.[d]	N.R.	18 (OFX)	31.25
Acinetobacter lwoffi-F1	N.R.	N.R.	N.R.	N.R.	24 (OFX)	62.50
Bacillus macerans-A199	20	31.25	N.R.	N.R.	19 (OFX)	15.62
Bacillus megaterium-A59	N.R.	N.R.	N.R.	N.R.	9 (SCF)	15.62
Bacillus subtilis-ATCC-6633	N.R.	N.R.	N.R.	N.R.	28 (OFX)	62.50
Bacillus subtilis-A57	22	31.25	N.R.	N.R.	28 (OFX)	125
Brucella abortus-A77	N.R.	N.R.	N.R.	N.R.	12 (SCF)	62.50
Burkholdria cepacia-A225	N.R.	N.R.	N.R.	N.R.	22 (SCF)	125
Clavibacter michiganense-A227	N.R.	N.R.	N.R.	N.R.	25 (SCF)	16.62
Cedecea davisae-F2	N.R.	N.R.	N.R.	N.R.	14 (OFX)	62.50
Enterobacter cloacae-A135	N.R.	N.R.	N.R.	N.R.	20 (NET)	31.25
Enterococcus faecalis-ATCC-29122	N.R.	N.R.	N.R.	N.R.	18 (SCF)	31.25
Escherichia coli-A1	N.R.	N.R.	N.R.	N.R.	N.R. (OFX)	62.50
Klebsiella pneumonia-F3	N.R.	N.R.	N.R.	N.R.	12 (OFX)	125
Klebsiella pneumoniae-A137	N.R.	N.R.	N.R.	N.R.	12 (OFX)	125
Morgonella morganii-F4	N.R.	N.R.	N.R.	N.R.	14 (OFX)	125
Proteus vulgaris-A161	8	250	N.R.	N.R.	12 (OFX)	125
Proteus vulgaris-KUKEM1329	N.R.	N.R.	N.R.	N.R.	13 (OFX)	125
Pseudomonas aeruginosa-ATCC9027	N.R.	N.R.	N.R.	N.R.	22 (NET)	31.25
Pseudomonas aeruginosa-ATCC27859	N.R.	N.R.	N.R.	N.R.	22 (NET)	15.62
Pseudomonas aeruginosa-F5	N.R.	N.R.	N.R.	N.R.	18 (NET)	125
Pseudomonas pseudoalkaligene-F6	N.R.	N.R.	N.R.	N.R.	18 (NET)	125
Pseudomonas syringae pv. *tomato*-A35	N.R.	N.R.	N.R.	N.R.	24 (OFX)	125
Salmonella cholerasuis arizonae-F7	N.R.	N.R.	N.R.	N.R.	14 (NET)	250
Salmonella enteritidis-ATCC-13076	N.R.	N.R.	N.R.	N.R.	27 (SCF)	62.50
Serratia plymuthica-F8	N.R.	N.R.	N.R.	N.R.	16 (NET)	125
Shigella sonnei-F9	N.R.	N.R.	N.R.	N.R.	24 (NET)	31.25
Staphylococcus aureus-A215	9	250	N.R.	N.R.	22 (SCF)	31.25
Staphylococcus aureus-ATCC-29213	8	250	N.R.	N.R.	22 (SCF)	62.50
Staphylococcus epidermis-A233	N.R.	N.R.	N.R.	N.R.	N.R. (SCF)	15.62
Staphylococcus hominis-F10	N.R.	N.R.	N.R.	N.R.	N.R. (SCF)	15.62
Streptococcus pyogenes-ATCC-176	N.R.	N.R.	N.R.	N.R.	10 (OFX)	62.50
Streptococcus pyogenes-KUKEM-676	N.R.	N.R.	N.R.	N.R.	13 (OFX)	31.25
Xanthomonas campestris-A235	N.R.	N.R.	N.R.	N.R.	20 (SCF)	31.25
Yersinia enterocolitica-F11	N.R.	N.R.	N.R.	N.R.	16 (OFX)	62.50

[a]DD = OFX = Ofloxacin (10 µg/disk); SCF = sulbactam (30 µg) + cefoperazona (75 µg) (105 µg/disk) and NET = Netilmicin, (30 µg/disk) were used as positive reference standards antibiotic disks (Oxoid);

[a] MIC = Maxipine (µg/ml) was used as reference antibiotic in micro well dilution assay (Sigma).

[b] Inhibition zone in diameter (mm) around the disks impregnated with 300 µg/disk of methanol extract.

[c] Minimal inhibitory concentrations (µg/ml)

[d] N.R. No result or inhibition zone in diameter < 7 mm

resulting PDA solutions were immediately poured into petri plates after vortexing. The plates were spot inoculated with 5 µl (10^4 spore/ml) of each fungus isolate. Amphotericin B (Sigma A 4888) was used as a reference antifungal drug. The inoculated plates were incubated at 27 ºC and 37 ºC for 72 h for plant and clinical fungi isolates, respectively. At the end of the incubation period, the plates were evaluated for the presence or absence of growth. MIC values were determined as the lowest concentration of the essential oil where absence of growth was recorded. Each test was repeated at least twice.

Table 2. Anticandidal and antifungal activities of the essential oils of *Salvia limbata* and *Salvia sclarea* against the yeast and fungi isolates.

Test yeast and fungi	*Salvia limbata*		*Salvia sclarea*		Antibiotics[a]	
yeast	DD[b]	MIC[c]	DD[b]	MIC[c]	DD[a]	MIC[a] (Amp B)
Candida albicans-A117	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	31.25
Fungi						
Alternaria alternate	33	15.62	N.R.	N.R.	N.R. (NET)	31.25
Alternaria solani	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	31.25
Aspergillus flavus	46	15.62	N.R.	N.R.	N.R. (NET)	15.62
Aspergillus niger	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	15.62
Aspergillus variecolor	34	15.62	N.R.	N.R.	N.R. (NET)	15.62
Fusarium acuminatum	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	62.50
Fusarium oxysporum	24	31.25	N.R.	N.R.	N.R. (NET)	62.50
Fusarium solani	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	62.50
Fusarium tabacinum	35	15.62	N.R.	N.R.	N.R. (NET)	62.50
Microsporum canis	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	62.50
Moniliania fructicola	28	31.25	N.R.	N.R.	N.R. (NET)	15.62
Mortieraula alpine	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	62.50
Penicillum spp.	37	15.62	N.R.	N.R.	N.R. (NET)	31.25
Rhizopus spp.	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	125
Rhizoctonia solani	35	15.62	N.R.	N.R.	N.R. (NET)	31.25
Sclorotinia minor	35	15.62	N.R.	N.R.	N.R. (NET)	125
Sclorotinia sclerotiorum	35	15.62	N.R.	N.R.	N.R. (NET)	62.50
Trichophyton rubrum	N.R.	N.R.	N.R.	N.R.	N.R. (NET)	31.25
Trichophyton mentagrophytes	35	15.62	N.R.	N.R.	N.R. (NET)	15.62

[a] DD = NET = Netilmicin (30 µg/disk) was used as positive reference standards antibiotic disks (Oxoid);

[a] MIC = AmpB = Amphotericin B (µg/ml) was used as reference antibiotic in micro well dilution (Sigma).

[b] Inhibition zone in diameter (mm) around the disks impregnated with 300 µg/disk of methanol extract.

[c] Minimal inhibitory concentrations (µg/ml).

[d] N.R. No result or inhibition zone in diameter < 7 mm

Antioxidant activity

DPPH assay

The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple methanol solution of stable free radical DPPH. This spectrophotometric assay uses stable free radical diphenylpicrylhydrazyl (DPPH) as a reagent (17,18). Fifty microliters of various concentrations of the extracts in methanol was added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I %) was calculated as follows:

$$I\ \% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. The tests were carried out in triplicate.

β-Carotene-linoleic acid assay

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (19). A stock solution of β–carotene-linoleic acid mixture was prepared as

follows: 0.5 mg of β–carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 µl linoleic acid, and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 ml of distilled water saturated with oxygen (30 min 100 ml/min.) was added with vigorous shaking. Subsequently 2500 µl of this reaction mixture was dispersed to test tubes and 350 µl portions of the extracts prepared at 2 g/l concentrations were added and the emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with positive control BHT and a blank. After this incubation period absorbance of the mixtures was measured at 490 nm. Antioxidant capacities of the extracts were compared with those of BHT and blank.

Assay for total phenolics

Total phenolic contents of the aforementioned extracts of *Salvia* species were determined by the literature method involving Folin-Ciocalteu reagent and gallic acid as standard (20,21). A 0.1 ml aliquot of extract solution containing 1000 µg of extract was placed in a volumetric flask; then 46 ml of distilled water and 1 ml of Folin-Ciocalteu reagent were added, and the flask was shaken thoroughly. After 3 min, 3 ml of 2% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated with all standard gallic acid solutions (0-1000 g per 0.1 ml) and a standard curve was obtained from the equation given below:

Absorbance: 0.0012 × Gallic acid (µg) + 0.0033

Antiviral activity

Cells, media, and viruses

Madin-Darby canine kidney (MDCK) and Madin-Darby bovine kidney (MDBK) cells were passaged in Dulbecco's Eagle medium, supplemented with 5% fetal calf serum (FCS) and antibiotics (100 IU/ml benzyl penicillin and 100 µg/ml streptomycin). MDCK cells were cultivated at 37 ºC in the presence of 5% CO_2 until the formation of confluent monolayers. In the antiviral experiments 0.5% FCS was added. MDCK and MDBK cells were kindly provided by Mrs. I. Roeva, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia. Avian influenza virus A/chicken/Germany/27, strain Weybridge (H7N7) (A/Weybridge), and human influenza virus A/Aichi/2/68 (H3N2) (A/Aichi) were grown in 11-day-old fertile hen's eggs and allantoic fluids were used as virus inoculum. The virus infectious titers were over 10^6-10^7 $TCID_{50}$/ml (50% tissue culture infectious doses/ml) and the hemagglutination (HA) titers 1024-2048. Herpes simplex viruses type 1, strain DA (HSV-1) and type 2, strain Bja (HSV-2) were passaged in MDBK cells; their infectious titers were over $10^{9.5}$-10^{13} $TCID_{50}$/ml. The virus was from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences.

Cellular toxicity: Confluent MDCK and MDBK cell monolayers in 96-well plastic plates were incubated with 4-fold dilutions of the samples in growth medium and were observed microscopically for changes in cell morphology and viability at 24, 48, and 72 h of incubation. The cytopathic effect (CPE) was scored under an inverted microscope (score 0 = 0% CPE, score 1 = 0%-25% CPE, score 2 = 25%-50% CPE, score 3 = 50%-75% CPE, score 4 = 75%-100% CPE). The dilution causing 50% CPE (TC_{50}) with respect to cell control (intact cells) was estimated.

Antiviral assays: The methods used are described in Serkedjieva and Hay (22). In short:

1. Cytopathic effect (CPE) reduction assay. The antiviral effect was studied in multicycle experiments of viral growth. The virus-induced cytopathic effect (CPE) was used as a measure for viral reproduction. Quadruplicate confluent monolayers in 96-well plates were overlaid with 2 × drug-containing medium (0.1 ml) and an equal volume of virus suspension (100 $TCID_{50}$/ml). The virus-induced CPE was scored after 48-72 h of incubation at 37 ºC as described above. The concentration reducing CPE by 50% (EC_{50}) with respect to virus control (virus infected, mock-treated cells) was estimated from graphic plots.

The selectivity index (SI) was determined by the ratio TC_{50}/EC_{50}. SI ≥ 4 was considered to stand for a significant selective inhibition.

2. The 50% end point titration technique (EPTT) was performed according to Vanden Berghe et al. (23) Quadruplicate monolayers in 96-well microtiter plates were infected with 0.05 ml serial 10-fold dilutions of the virus suspension, and then serial 2-fold dilutions of the preparation in a tissue culture medium (0.05 ml) were added. The cultures were incubated at 37 ºC and

examined microscopically daily for CPE. CPE was scored as described above. The antiviral activity was determined by the difference between the virus titers of control and treated viruses (δ $\log_{10}TCID_{50}$/ml). The significance of the difference was estimated by Student's t test. The 90% effective concentration (EC_{90}) was determined from graphic plots as the dose that caused reduction of 1 log $TCID_{90}$/ml of viral infectious titer.

The selective anti-influenza drug Rimantadine hydrochloride (Hoffman - La Roche Inc., Nutley, NJ, USA) and the antiherpetic drug BVDU ((E)-5-(2-bromovinyl)-2'-deoxyuridine, Sigma-Aldrich Chemie GmbH, Deisenhofer, Germany) were used as positive controls.

Results and Discussion

Chemical composition of the essential oil

Air-dried herbal parts of the plants were subjected to hydrodistillation using a Clevenger apparatus and yellow essential oil was obtained. The composition of *S. limbata* and *S. sclarea* essential oil were analyzed by employing GC-MS, leading to a comparison of the relative retention times and the mass spectra of oil components with those of authentic samples and mass spectra from the data library. Spathulenol (29.30%) and germacrene D (24.72%) were the main components of the oils. Other important compounds were sclareoloxide (14.08%) and β–caryophyllene (16.24%) (Tables 3 and 4).

Antimicrobial Activity

The antimicrobial activities of *S. limbata* and *S. sclarea* essential oils and extracts assayed against a wide range of human-, plant-, and food-borne microorganisms and their potency were qualitatively and quantitatively assessed by evaluating the presence of inhibition zones, zone diameter, and MIC values. The results are given in Tables 1 and 2. Since the essential oils from *Salvia* species have not been evaluated in terms of antimicrobial activity against a large number of microorganisms to date, this is the first study to provide data that essential oil of *Salvia* species plants evaluated against a wide range of microorganisms possess potential antibacterial, antifungal, and anticandidal activities that are comparable to those of standard drugs. The results showed that the essential oil of *S. limbata* has a stronger and broader spectrum of antifungal activity as compared to the other *Salvia* species tested. The minimal inhibition zones and MIC values for bacterial strains that were sensitive to the essential oil of *S. limbata* were 24-46 mm and 15.62-62.50 µl/ml, respectively (Table 1). According to the results given in Table 1, the essential oil of *S. limbata* has weak antibacterial activity against 6 of the bacteria species tested.

Antioxidant Activity

Methanol extracts were subjected to screening for their possible antioxidant activity by employing 2 complementary test systems, namely DPPH radical and beta carotene/linoleic acid.

Free radical scavenging activities of the extracts were measured in DPPH assay and the reaction followed a concentration dependent pattern.

Activities of methanol extracts could be attributed to the presence of several types of compounds belonging to different classes such as polar thermo labile and/or thermo stable phenolics. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lypophilic compounds (24).

Antioxidants minimize the oxidation of lipid components in cell membranes or inhibit the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation that are known to be carcinogenic (25). The presence of antioxidant constituent polyphenols particularly polyphenolic acids in *Salvia* species was reported previously (6,26-31). Therefore, total phenolic constituents of these *Salvia* species were determined as the gallic acid equivalent and are given in Table 5.

Antiviral Activity

The dichloromethane (DCM) extract from *Salvia sclarea* and the MeOH extract from *Salvia limbata* possessed a pronounced anti-influenza virus activity towards both tested strains; the hexane extracts from both plants showed a limited inhibitory effect (Table 6). The MeOH extract from *Salvia sclarea* inhibited significantly the replication of HSV-1 and marginally the replication of HSV-2 (Table 7).

To our knowledge there are no literature data on the antiviral properties of the investigated *Salvia* species. Scarce reports are available on the antiviral activity of *Salvia officinalis*: aqueous extracts inhibited the replication of HSV-1 and HSV-2 (32), and Saller et al. (33) reported

Table 3. Chemical compositions of *Salvia limbata* essential oil (%).

	Compounds[a]	Rt.[b] (min)	RI[c]	% Composition FID
1	α-Pinene	8.090	845	1.4
2	Camphene	8.634	866	0.1
3	Sabinene	9.594	900	0.7
4	β–Pinene	9.706	904	2.2
5	*p*-Cymene	11.753	966	0.4
6	1,8-Cineole	11.993	972	3.2
7	Cis-Sabinene hydrate	13.665	1014	0.2
8	Trans-Sabinene hydrate	15.096	1047	0.2
9	α-Campholenal	16.352	1072	0.1
10	Trans-Pinocarveol	16.991	1085	0.4
11	Trans-Verbenol	17.271	1090	0.4
12	Borneol	18.271	1108	2.0
13	Terpinen-4-ol	18.799	1117	0.6
14	α-terpineol	19.487	1129	0.7
15	Myrtenal + Myrtenol	19.655	1132	0.4
16	Verbenone	20.326	1143	0.1
17	Bornyl acetate	23.805	1194	1.4
18	δ-Elemene	26.132	1224	0.3
19	α-Terpinyl acetate	26.716	1231	0.2
20	α-Ylangene	27.900	1245	0.3
21	β–Bourbonene	28.308	1249	0.6
22	β–Elemene	28.636	1253	0.6
23	β–Caryophyllene	29.843	1266	0.8
24	β–Copaene	30.267	1271	0.2
25	Aromadendrene	30.691	1275	0.1
26	Dehydro-Aromadendrane	31.251	1281	1.2
27	Allo-aromadendrane	31.643	1285	0.7
28	γ-Murolene	32.338	1292	0.2
29	Germacrene D	32.546	1294	1.5
30	Bicyclogermacrene	33.210	1301	2.3
31	γ-Cadinene	33.922	1308	0.3
32	δ-Cadinene	34.298	1311	0.5
33	α-Calacorene	35.169	1319	0.3
34	Spathulenol	36.937	1335	29.3
35	Salvial-4(14)-en-1-one	37.321	1339	0.7
36	$C_{15}H_{24}O$	37.513	1340	0.4
37	$C_{15}H_{24}O$	38.024	1345	1.0
38	Isospathulenol	39.096	1354	2.0
39	β–Eudesmol	39.648	1358	7.4
40	Drimenol	45.438	1402	0.1
41	6,10,14-trimethyl-2-Pentadecanone	46.574	1410	0.7
42	Sclareol-oxide	48.261	1421	14.8
	Total			81.0

[a] Tentatively identified compounds,

[b] Retention time (in minutes),

[c] Retention index on HP5-MS column in reference to n-alkanes (C_8H_{18} – $C_{20}H_{42}$).

Table 4. Chemical compositions of *Salvia sclarea* essential oil (%).

	Compounds[a]	Rt.[B] (min)	RI[C]	% Composition FID
1	Myrcene	12.80	993	0.11
2	Linalool	18.32	1109	1.23
3	α-Terpineol	23.13	1184	1.63
4	Nerol	24.34	1201	0.31
5	Linallyl acetate	25.35	1214	5.52
6	Bornyl acetate	27.08	1235	0.02
7	α-Elemene	28.94	1257	1.75
8	α-Cubebene	29.62	1264	0.17
9	Neryl acetate (1303)	30.34	1272	1.11
10	α-Copaene	30.97	1278	3.78
11	Acetate (1352)	31.24	1281	2.17
12	Geranyl -elemene	31.53	1284	2.17
13	β–Caryophyllene	32.99	1299	16.24
14	α-Humulene	34.48	1313	1.33
15	Germacrene-D (1594)	35.69	1324	24.72
16	Bicyclogermacrene	36.23	1329	9.63
17	γ-Cadinene	37.04	1336	1.46
18	Spathulenol	40.23	1363	1.90
19	Caryophyllene oxide	40.48	1365	1.85
20	β–eudesmol	45.33	1401	1.12
	Total			94,02

[a] Tentatively identified compounds,
[B] Retention time (in minutes),
[C] Retention index on HP5-MS column in reference to n-alkanes (C8H18 – C20H42).

Table 5. Antioxidative potentials of the essential oils and various extracts from *Salvia limbata* and *Salvia sclarea* and positive control (BHT) in DPPH and β-carotene/linoleic acid assays.

Sample	DPPH assay (µg/ml)	Inhibition ratio of linoleic acid oxidation (% ± 1)	Amounts of total phenolics *
Salvia limbata			
MeOH	47.50 ± 1.30	85.1	4.1
Hexane	N.A. [1]	20.2	2.1
DCM	N.A	71	3.8
Essential oil	N.A.	44	-
Salvia sclarea			
MeOH	69.30 ± 1.50	76.6	4.9
Hexane	N.A	31	2.7
DCM	N.A	60.9	2.8
Essential oil	N.A.	46.6	-
BHT (positive control)	19.80 ± 0.52	96	-

* As gallic acid equivalent (%, w/w)
[1] Not active

Table 6. Toxicity to MDCK cells and anti-influenza virus effects of various extracts and the essential oils obtained from *Salvia sclarea* and *Salvia limbata*.

Sample	MDCK	A/Weybridge			A/Aichi		
	TC_{50} [a] (mg/ml)	EC_{50} [b] (mg/ml)	SI [c]	EC_{90} [d] (mg/ml)	EC_{50} [b] (mg/ml)	SI [c]	EC_{90} [d] (mg/ml)
Salvia limbata							
MeOH	1.5	0.13	12	0.35	0.13	12	0.125
Hexane	2.5	0.62	4		$>TC_{50}$		
DCM	0.06	$>TC_{50}$					
Essential oil	N.T	N.T.					
Salvia sclarea							
MeOH	2.5	$>TC_{50}$					
Hexane	0.02	0.004	5		$>TC_{50}$		
DCM	0.6	0.06	10	0.1	0.06	10	0.09
Essential oil	0.003	$>TC_{50}$					
Rimantadine hydrochloride	>0.03	0.4 µg/ml	>80		0.1 µg/ml	>300	

[a] 50% toxic concentration, the dose required to cause visible changes in 50% of intact cultures
[b] 50% effective concentration, the dose that caused 50% reduction of virus-induced CPE
[c] Selectivity index
[d] 90% effective concentration, the dose that caused reduction of infectious virus yield 1 lg $TCID_{50}$

Table 7. Toxicity to MDBK cells and antiherpetic effects of various extracts and the essential oils obtained from *Salvia sclarea* and *Salvia limbata*.

Sample	MDBK	HSV-1			HSV-2		
	TC_{50} [a] mg/ml	EC_{50} [b] mg/ml	SI [c]	EC_{90} [d] mg/ml	EC_{50} [b] mg/ml	SI [c]	EC_{90} [d] mg/ml
Salvia limbata							
MeOH	2.5	$>TC_{50}$					
Hexane	2.0	$=TC_{50}$	1				
DCM	0.08	$>TC_{50}$					
Essential oil	N.T	N.T.					
Salvia sclarea							
MeOH	2.5	0.12	20.8	0.5	0.5	5	0.5
Hexane	0.07	0.05	1.4				
DCM	0.15	$>TC_{50}$					
Essential oil	0.003	$>TC_{50}$					
BVDU	>100 µg/ml	1 µg/ml	>100	1 µg/ml	2 µg/ml	>50	2 µg/ml

[a, b, c & d] as in Table 6

the topical treatment of herpes labialis by a combined herbal preparation containing sage extracts. A high level of virucidal activity against HSV-1 was found for the essential oil of *Salvia fruticosa* (34).

Conclusions

These results suggest that *Salvia limbata* essential oil can be used as an antifungal agent in new drugs for the therapy of infectious diseases in humans and plant diseases.

Acknowledgment

This study was supported by a grant from the Research Funds appropriated to Atatürk University.

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