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RESEARCH ARTICLE

Determination of Antiviral Activity and Cytotoxicity of Selected Sage (*Salvia* L.) Species

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Summary

The objective of this study was to examine antiviral property and cytotoxicity of the extracts prepared from the selected Salvia species (Lamiaceae) growing in Turkey. The chloroform and methanol extracts from 14 Salvia species (S. albimaculata, S. aucheri var. canescens, S. candidissima subsp. occidentalis, S. ceratophylla, S. cryptantha, S. cyanescens, S. frigida, S. forskahlei, S. halophila, S. migrostegia, S. multicaulis, S. sclarea, S. syriaca, and S. verticillata subsp. amasiaca) were tested against Herpes simplex (type-1, HSV-1) and Parainfluenza (type-3, PI-3) using Madin-Darby Bovine Kidney and Vero cell lines. Cytotoxicity of the extracts was determined as maximum non-toxic concentrations (MNTCs). The chloroform extracts of S. cyanescens and S. microstegia were found to inhibit both HSV-1 and PI-3 effectively. Therefore, these species could proceed to further evaluation for their possible antiviral components.

Key Words: *Salvia, Lamiaceae, antiviral activity, cytotoxicity*

Seçilen Adaçayı (Salvia sp.) Türlerinin Antiviral Aktivitesi ve Sitotoksisitesinin Tayini

Özet

Bu çalışmanın amacı, Türkiye'de yetişen bazı Salvia türlerinin (Lamiaceae) antiviral özelliği ve sitotoksisitesini incelemektir. 14 Salvia türünün (S. albimaculata, S. aucheri var. canescens, S. candidissima subsp. occidentalis, S. ceratophylla, S. cryptantha, S. cyanescens, S. frigida, S. forskahlei, S. halophila, S. migrostegia, S. multicaulis, S. sclarea, S. syriaca ve S. verticillata subsp. amasiaca) kloroform ve methanol ekstreleri Herpes simplex (tip-1, HSV-1) ve Parainfluenza'ya (tip-3, PI-3) karşı Madin-Darby Bovine Kidney ve Vero hücre hatları kullanılarak test edilmiştir. Ekstrelerin sitotoksisitesi maksimum non-toksik konsantrasyon (MNTC) olarak tayin edilmiştir. S. cyanescens ve S. microstegia'nın kloroform ekstrelerinin hem HSV-1'i, hem de PI-3'ü etkili bir şekilde inhibe ettiği bulunmuştur. Dolayısıyla, bu türlerin muhtemel antiviral bileşikleri açısından daha ileri değerlendirmeye tabi tutulabilir.

Anahtar Kelimeler: *Salvia, Lamiaceae, antiviral aktivite, sitotoksisite*

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INTRODUCTION

Lamiaceae species have been used all over the world for seasoning and phytotherapeutical purposes, as food and beverage and also in perfumes. The genus *Salvia* (sage), the largest genus of Lamiaceae family, is comprised of approximately 900 species all over the world. *Salvia* is one of the plant genera having the longest history with folkloric utilization among culinary and medicinal herbs and has been reported to be used traditionally for antibacterial purposes in various parts of the world (1,2). In Turkey, this genus is represented by about 95 species, 46 of which are endemic (3). Among the species of *Salvia* growing in Turkey, *S. officinalis*, *S. triloba*, *S. dichroantha*, *S. multicaulis*, *S. tomentosa*, and *S. virgata* were recorded to be used as antiseptic and wound healing purposes in Anatolian folk medicine as herbal tea (4).

Although many studies have been reported about antibacterial or antifungal activities of *Salvia* species up to date, only a few studies described antiviral activity of the mentioned species. Taking the positive results that we have obtained from our previous reports on antiviral activity and cytotoxicity of various edible plants into account (5,6), in our ongoing extensive studies on Turkish *Salvia* species for their various biological activities (7-14), we have now aimed to screen antiviral activity of the non-polar (petroleum ether, chloroform) and polar (methanol) extracts of selected *Salvia* species. For this purpose; the chloroform ($CHCl_3$) and methanol (MeOH) extracts obtained from the aerial parts of fourteen *Salvia* species including *S. albimaculata* Hedge & Hub., *S. aucheri* Bentham var. *canescens* Boiss. & Heldr., *S. candidissima* Vahl. subsp. *occidentalis*, *S. ceratophylla* L., *S. cryptantha* Montbret & Bentham, *S. cyanescens* Boiss. & Bal., *S. frigida* Boiss., *S. forskahlei* L., *S. halophila* Hedge, *S. migrostegia* Boiss. & Bal., *S. multicaulis* Vahl., *S. sclarea* L., *S. syriaca* L., and *S. verticillata* (L.) subsp. *amasiaca* (Freyn & Bornm.) Bornm growing in Turkey were assessed against DNA virus *Herpes simplex* (HSV) and RNA virus *Parainfluenza* (PI-3) using Madin-Darby Bovine Kidney (MDBK) and Vero cell lines. Cytotoxicity of the extracts was determined as maximum non-toxic concentrations (MNTCs).

MATERIALS and METHODS

Plant materials

Collection sites and herbarium numbers of the respective Turkish *Salvia* species used in this study are given in Table 1. Voucher specimens of all studied species are preserved at the Herbarium of Faculty of Pharmacy of Ankara University, Ankara, Turkey. The species were identified by Prof. Dr. Hayri

Table 1. Collection sites, dates and herbarium numbers of Turkish *Salvia* species used

***Salvia* species**	**Collection site**	**Collection date**	**Herbarium number**
S. albimaculata Hedge & Hub.	Balkusan-Ermenek, Konya	June, 2005	AEF 23520
S. aucheri Bentham var. *canescens* Boiss. & Heldr.	Ermenek-Gülnar, Konya	June, 2005	AEF 23525
S. candidissima Vahl. subsp. *occidentalis*	Ermenek, Konya	June, 2005	AEF 23522
S. ceratophylla L.	Ergani-Maden, Diyarbakir	June, 2005	AEF 23559
S. cryptantha Montbret & Bentham	Beynam Forest, Ankara	June, 2005	AEF 23614
S. cyanescens Boiss. & Bal.	Beynam Forest, Ankara	June, 2005	AEF 23620
S. frigida Boiss.	Ermenek town, Konya	June, 2005	AEF 23528
S. halophila Hedge	Karakulluk town, Konya	June, 2005	AEF 23649
S. migrostegia Boiss & Bal.	Ermenek-Tekecati, Konya	June, 2005	AEF 23523
S. multicaulis Vahl.	Ergani-Maden, Diyarbakir	June, 2005	AEF23561
S. sclarea L.	Bozkir, Konya	June, 2005	AEF 23521
S. syriaca L.	Hadim-Bozkir, Konya	June, 2005	AEF 23530
S. verticillata (L). subsp. amasiaca (Freyn & Bornm.) Bornm.	Cubuk-Karagöl National Park, Ankara	July, 2005	AEF 23552

Duman from Department of Biology, Faculty of Art and Science, Gazi University, Ankara, Turkey. The voucher specimens are deposited at the Herbarium of Faculty of Pharmacy, Ankara University, Ankara, Turkey, except for *S. forskahlei*, which was identified by Dr. Salih Terzioglu of Department of Forest Botanic, Faculty of Forestry, Karadeniz Technical University, Trabzon, Turkey. The voucher specimen of *S. forskahlei* is preserved at the Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Preparation of the extracts

The *Salvia* species used in this study were weighed accurately about 6.0 g and successively extracted with $CHCl_3$ (Merck, 250 ml) and then MeOH (Merck, 250 ml). After filtration, the organic phases were independently concentrated *in vacuo* by evaporating to dryness. The crude extracts obtained were immediately stored at -20ºC until the experiments were started and employed in antiviral and cytotoxicity assays. % Yields of the $CHCl_3$ and MeOH extracts (w/w), respectively, are as follows: *S. albimaculata* (4.56 and 13.45%), *S. aucheri* var. *canescens* (5.44 and 17.80%), *S. candidissima* subsp. *occidentalis* (4.98 and 15.67%), *S. ceratophylla* (3.29 and 19.21%), *S. cryptantha* (5.01 and 17.68%), *S. cyanescens* (5.09 and 12.37%), *S. frigida* (4.99 and 15.88%), *S. forskahlei* (5.77 and 13.99%), *S. halophila* (4.76 and 16.01%), *S. migrostegia* (4.55 and 19.02%), *S. multicaulis* (3.76 and 14.45%), *S. sclarea* (4.56 and 16.98%), *S. syriaca* (6.03 and 13.91%), and *S. verticillata* subsp. *amasiaca* (4.07 and 15.44%).

Preliminary phytochemical examination of the extracts

An aliquot of each extract was spotted onto the silica gel plate (Silica gel coated TLC plates; Merck) with a developing solvent system of ethyl acetate:glacial acetic acid:formic acid:water (100:11:11:27). The spots were checked under a UV detector at 254 nm and 365 nm wavelengths. Plates were visualized under UV light and later sprayed with chromogenic agents including anis aldehyde-sulfuric acid (for terpenes and steroids), iron (III) chloride (for phenolic compounds including flavonoids) and Dragendorff (for alkaloids). Presence of flavonoids was further confirmed by spraying the plates with 5% $AlCl_3$ in ethanol. The results showed clear existence of phenolics and terpenes in the MeOH extracts, particularly.

Cell lines and growth conditions

Vero (African green monkey kidney) and Madin-Darby bovine kidney (MDBK) cell lines used in this study were obtained from the Department of Virology, Faculty of Veterinary, Ankara University, Turkey. The cell cultures were grown in Eagle's Minimal Essential Medium (EMEM) enriched with 10% fetal calf serum (FCS) (Biochrom, Germany), 100 mg/ml of streptomycin and 100 IU/ml of penicillin in a humidified atmosphere of 5% CO_2 at 37ºC, and harvested using trypsin solution (Bipco Life Technologies, UK).

Test viruses

In order to determine the antiviral activity of the extracts, *Herpes simplex virus* (type 1, HSV-1) and *Parainfluenza-3 virus* (PI-3) were employed. The test viruses were obtained from the Department of Virology, Faculty of Veterinary, Ankara University, Turkey.

Antiviral activity assays

Following placement of media (EMEM) into each well of the 96-well microplates (Greiner^R, Germany), stock solutions of the extracts were added into first row of microplates and two-fold dilutions of the extracts (512-0.25 µg/ml) were made by dispensing the solutions to the remaining wells. Two-fold dilutions of each material were obtained according to Log_2 on the microplates. Acyclovir (Biofarma Co.) and oseltamivir (Roche Co.) were used as references. Strains of HSV and PI-3 titers were calculated by the Frey and Liess method as tissue culture infecting dose ($TCID_{50}$) and were inoculated into all the wells (15). The sealed microplates were incubated in 5% CO_2 at 37°C for 2 h to detect the possible antiviral activities of the samples. After incubation, 50 µl of the cell suspension of 300.000 cells/ml, which were prepared in EMEM together with 5% fetal bovine serum, were put in each well and the plates were incubated in 5% CO_2 at 37°C for 48 h. After the end of this duration, the cells were evaluated

using cell culture microscope (× 400), comparing with treated-untreated control cultures as well as acyclovir and oseltamivir. Consequently, maximum cytopathogenic effect (CPE) concentrations as the indicator of antiviral activities of the extracts were determined (6).

Cytotoxicity

The maximum non-toxic concentration (MNTC) of each sample was determined by the method described previously based on cellular morphologic alteration (5,6). Several concentrations of each sample were placed in contact with confluent cell monolayer and incubated in 5% CO_2 at 37°C for 48 h. MNTCs were determined by comparing treated and controlling untreated cultures.

RESULTS AND DISCUSSION

Antiviral action of twenty-eight extracts belonging to fourteen *Salvia* species was evaluated against HSV and PI-3, the ubiquitous human pathogens. As tabulated in Table 2, the $CHCl_3$ extracts belonging to *S. albimaculata*, *S. cyanescens*, and *S. migrostegia* as well as the MeOH extracts of *S. halophila* and *S. sclarea* were active against HSV, while the $CHCl_3$ extracts of *S. cyanescens* and *S. migrostegia* and the MeOH extract of *S. ceratophylla* inhibited PI-3 in range of 32-1 μg/ml CPE inhibitory concentrations. In particular, the $CHCl_3$ extract belonging to *S. cyanescens* had a quite remarkable activity (16-1.0 μg/ml) against HSV with a better MNTC value (64 μg/ml) than that of acyclovir (16 μg/ml), while the same extract had also a moderate inhibition against PI-3 (16-1.0 μg/ml) as compared to oseltamivir (32- <0.25 μg/ml). Additionally, the $CHCl_3$ extract of *S. migrostegia* exerted a very similar inhibitory effect towards PI-3 to that of *S. cyanescens*, whereas its cytotoxic property was lower than that of *S. cyanescens* and as potent as oseltamivir.

There have been only a few reports on antiviral activity of *Salvia* species up to date. For instance; *S. migrostegia* growing in Lebanon, which displayed a marked antiviral activity against HSV and PI-3 in our study, was reported to contain a high amount of caryophyllene oxide (16). In another study carried out on *S. fruticosa* essential oil, its three main components (1,8-cineole, thujone, and camphor) displayed cytotoxic activity against African Green Monkey kidney (Vero) cells and high levels of virucidal activity against HSV (17). In another previous study, the main components of the essential oil of *S. syriaca* collected from Jordan were shown to contain thymol, α-pinene, and isobornyl acetate (15.5%, 12.6%, and 12.0%, respectively) as the major constituents, which might be similar to that of our sample of *S. syriaca*, since both of the countries (Jordan and Turkey) are affected by the Mediterranean climate (18). In another study, isoborneol, a monoterpene widely encountered in essential oils, was reported to be a potent inhibitor of HSV-1 that supports high antimicrobial effect of *S. syriaca* (19). On the other hand, rosmarinic acid, broadly found in *Salvia* species and the rest of Lamiaceae family, has been stated to possess antibacterial and antiviral activities (20). In a recent study performed by our group on phenolic acid analysis (rosmarinic, gallic, caffeic, and chlorogenic acids) on Turkish *Salvia* species using a new validated method by reversed-phase HPLC (21), phenolic acid quantities in the species examined have been found to be similar to some extent and phenolics could be speculated to be accountable for antiviral effect of the *Salvia* extracts studied.

Many phenolics including flavonoids have been known to possess antiviral activity against various virus types (22). Consequently, phenolics might be speculated to be the major contributor to notable antiviral activity of *Salvia* species.

In conclusion, our results indicated that the extracts of *S. cyanescens*, *S. albimaculata*, *S. migrostegia*, *S. halophila*, and *S. sclarea* may deserve a further analysis from the viewpoint of chemical identification of their active constituents. To the best of our knowledge, this is the first report describing antiviral activity and cytotoxicity of above-mentioned *Salvia* species.

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Table 2. Antiviral activity and cytotoxicity results of the extracts of the *Salvia* species

Vero Cells (μg/mL)				**MDBK Cells (μg/mL)**		
***Salvia* extracts**	**MNTCa (μg/mL)**	**CPEb Inhibitory Concentration**		**MNTC (μg/mL)**	**CPE Inhibitory Concentration**	
		HSV-1			**PI-3**	
		Maximum	**Minimum**		**Maximum**	**Minimum**
$CHCl_3$ extracts						
S. aucheri var. *canescens*	64	-c	-	16	-	-
S. albimaculata	64	16	8	16	-	-
S. candidissima sp. *occidentalis*	64	-	-	16	-	-
S. ceratophylla	128	-	-	16	-	-
S. cyanescens	64	16	1	16	16	1
S. crypthantha	64	-	-	64	-	-
S. frigida	64	-	-	8	-	-
S. halophila	32	-	-	16	-	-
S. migrostegia	64	64	32	32	16	2
S. multicaulis	64	-	-	16	-	-
S. sclarea	64	-	-	32	-	-
S. syriaca	64	-	-	64	-	-
S. verticillata ssp. *amasiaca*	64	-	-	64	-	-
MeOH extracts						
S. aucheri var. *canescens*	64	-	-	64	-	-
S. albimaculata	64	-	-	64	-	-
S. candidissima subsp. *occidentalis*	64	-	-	128	-	-
S. ceratophylla	64	-	-	64	32	16
S. cyanescens	64	-	-	64	-	-
S. crypthantha	64	-	-	64	-	-
S. frigida	64	-	-	128	-	-
S. halophila	64	32	16	128	-	-
S. migrostegia	64	-	-	128	-	-
S. multicaulis	64	-	-	128	-	-
S. sclarea	64	32	16	64	-	-
S. syriaca	64	-	-	64	-	-
S. verticillata subsp. amasiaca	64	-	-	64	-	-
References						
Acyclovir	16	16	<0.25	-	-	-
Oseltamivir	-	-	-	32	32	<0.25

a Maximum non-toxic concentration
b Cytopathogenic effect
c No activity observed

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