EVALUATION OF ANTIVIRAL ACTIVITY OF FRACTIONATED EXTRACTS OF SAGE Salvia officinalis L. (LAMIAEAE)

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Abstract — In the present study, we examined cytotoxicity and extracellular and intracellular antiviral activity of fractionated extracts of wild and cultivated sage Salvia officinalis L. (Lamiaceae) in vitro using the WISH-VSV model system. Extracts were obtained by fractionating depigmented ethanol extracts of sage plants with supercritical CO2 at different pressures. Cytotoxicity was determined by examining cellular morphology in situ with the aid of a colorimetric micromethod and by cell staining with trypan blue. The fraction of distilled cultivated sage obtained at CO2 pressure of 300 bars and temperature of 60°C (149/3) was the most cytotoxic, with CTD10 44 µg/ml. That of non-distilled cultivated sage obtained at CO2 pressure of 500 bars and temperature of 100°C (144/5) was the least toxic (CTD10 199 µg/ml). Moreover, 144/5 had an antiviral effect at the intracellular level: when added 5 hours before VSV infection, it caused 100% reduction of CPE at concentrations of 99.5 and 199.0 µg/ml; when added after virus penetration had occurred, the same concentrations caused 35 and 60% reduction, respectively. The obtained results indicate that antiviral activity of 144/5 involves inhibition of the early steps of the virus infective cycle without a direct virucidal effect.

Abbreviations: WISH – human amnion epithelial cells, VSV – vesicular stomatitis virus, HSV - herpes simplex virus, CPE – cytopathic effect, IS – selectivity index, TCID50 – tissue culture infective dose, CTD10 – 10% cytotoxic concentrations.

Key words: Salvia officinalis, CO2 extracts, antiviral activity, WISH-VSV model.

INTRODUCTION

Salvia is an important genus consisting of about 900 species in the family Lamiaceae. Many species of Salvia, including Salvia officinalis L., have been used as traditional herbal medicine worldwide. In our traditional medicine, sage is used for many ailments, including inflammation of the mouth and throat (Tucakov, 1996). Extracts of different Salvia species have been examined for a number of biological activities so far, and their antimicrobial, anti-inflammatory, antioxidant, antimutagenic, cancer-preventive, spasmolytic, and cholinergic binding properties and involved mechanisms have been partially described (Djarmati et al., 1991; Cuvelier et al., 1994; Simić et al., 1997; Craig, 1999; Baricevic and Bartol, 2000; Mitić et al., 2001; Zupko et al., 2001; Capasso et al., 2004; Ren et al., 2004; Vujosević and Blagojević, 2004). It has even been reported that S. officinalis extracts are effective in the management of Alzheimer’s disease (Akhondzadeh et al., 2003).

Tada et al. (1994) observed a potent antiviral activity against VSV (vesicular stomatitis virus) in crude extracts of S. officinalis and isolated the antiviral diterpenes safficinolid and sageon. Saffinolid caused showed reduction of VSV, while sageon exhibited virus inactivation activity against VSV and HSV-1 (herpes simplex virus type 1). Anti-HSV-1 activity of methanol-water extracts of S. coccinia has also been reported (Rajbhandari et al., 2001). Sivropoulou et al. (1997) reported a virucidal effect of essential oil of S. fruticosa and its main monoterpenoids -thujone, 1,8-cineole, and camphor - against HSV-1. All three compounds are usually
present in essential oils of *S. officinalis* (Wichtl, 1994). In addition, the monoterpenoid isoborneol is a potent inhibitor of HSV-1 (Khan et al., 2005). In a double-blind, comparative, randomized trial, Saller et al. (2001) observed that a combined topical preparation with rhubarb and sage extracts was as effective as topical aciclovir cream in the treatment of labial herpes.

In the present study, we examined the cytotoxicity and extracellular and intracellular antiviral activity of fractionated extracts of wild and cultivated sage *S. officinalis* L. *in vitro* using the WISH-VSV model system. Extracts were obtained by fractionating depigmented ethanol extracts of sage plants with supercritical CO$_2$ at different pressures. Supercritical CO$_2$ extraction was used to allow isolation of higher terpenoids from wild and cultivated sage that were not known to be present in this plant (Djarmati et al., 1991, 1993, 1994).

**MATERIALS AND METHODS**

**Cell culture and viruses**

For detection of the antiviral and cytotoxic effect of extracts, we used WISH human amnion epithelial cells (Cell Line Catalog, 5th Ed., 1997). Cells were cultivated at 37°C with 5% CO$_2$ in Eagle’s MEM medium (Gibco) supplemented with 10% fetal calf serum (FCS), 100 i.u./ml penicillin, and 100 µg/ml streptomycin. The VSV (vesicular stomatitis virus) Indiana serotype was used to induce a cytopathic effect (CPE). Virus dilutions were performed in Eagle’s MEM medium supplemented with 2% FCS.

**Plant extracts**

Plant extracts were obtained from R. M. Jankov, Faculty of Chemistry, University of Belgrade. Two types of sage were extracted. Wild sage originated from the vicinity of Trpanj, Pelješac Peninsula, Dalmatia (Croatia). The D-70 variety of sage, bred for cultivation in continental climates by the Institute for Hop, Sorghum, and Medicinal Plants, Bački Petrovac, Vojvodina (Serbia) was grown in the experimental field of the Institute. Plants were collected during the flowering period (May – June, 1990) and the voucher specimens deposited in the herbarium of the Institute.

The preparation of ethanol extracts and method of CO$_2$ reextraction are described in detail by Djarmati et al. (1991, 1993, 1994). Briefly, dried aerial parts of the plants were subjected to three rounds of extraction with 96% ethanol, and the combined filtrates were evaporated to about 10% dry matter. The resulting total extracts of wild sage (152), cultivated sage (144), and steam-distilled cultivated sage (149) were passed through a column with active carbon to remove pigments and some polymerized products, and fractionated by supercritical CO$_2$ extraction at different CO$_2$ pressure for 6 h. The 144 extract was fractionated at 60°C at 200, 300, and 400 bars, and at 100°C at 500 bars, while the 152 and 149 extracts were fractionated at 60°C at 200 and 300 bars, respectively. Fractions were freeze-dried and stored at -20°C. The total yield of CO$_2$ re-extracts obtained from dried plant material was 52.5 g (0.31%), 84.76 g (0.88%), and 110.48 g (1.175%) for the 152, 144, and 149 extracts, respectively.

Sage extracts were first dissolved in 96% ethanol (1% of total volume) and then in Eagle’s MEM medium supplemented with antibiotics and 2% FCS (up to 100% of total volume). The solution was cleared by three rounds of centrifugation at 3000 rpm for 10 minutes before two-fold serial dilutions were made in Eagle’s MEM medium. For each experiment, fresh extract solutions were made and tested for sterility.

**Cytotoxicity of sage extracts**

WISH cells (density 4x10$^5$ cells/well) were seeded in 96-well microtiter plates and cultivated for 24 h at 37°C in 5% CO$_2$. Two-fold serial dilutions of plant extracts were added to the confluent cell monolayer and incubated for 24 h. After overnight incubation, cytotoxicity was determined by examining cellular morphology *in situ* (Fresney, 1983) with the aid of a colorimetric micromethod (Pestka et al., 1981) and by cell staining with trypan blue (Doyle et al., 1995). Blue-stained cells were scored as non-viable, unstained cells as viable. The maximum non-toxic concentration of the extract (CTD$_{10}$) was
defined as the concentration causing lethality of 10% of the cell population, i.e., resulting in cell survival of 90% with no apparent changes in cell morphology.

**Antiviral activity**

The antiviral effect of extracts was monitored through reduction of the cytopathic effect (CPE) caused by them in the VSV-WISH model system. A colorimetric micromethod and visual determination were used for detection of antiviral activity. As zero CPE, we adopted the color intensity of the cell culture control (cells + medium); 100% CPE was the color intensity of the virus infectivity control (cells + virus). The value of CPE reduction was calculated as percent compared to the virus infectivity control. Controls of cell survival (cells + extract) were also included. Experiments were performed in the range of non-toxic concentrations of the extracts.

The antiviral activity of plant extracts was quantitatively expressed as the selectivity index (IS), defined as the ratio of the maximum to minimum non-cytotoxic concentrations giving 100% reduction of CPE. Extracts exhibit antiviral activity if IS≥2 (Abou-Karam and Shier, 1990). As a positive control of intracellular antiviral activity and a negative control of extracellular antiviral activity, we used human interferon-α (Torlak, Belgrade, Serbia).

**Antiviral effect at the extracellular level**

A high concentration of virus particles (10⁵ TCID₅₀/ml) and the highest non-toxic concentration of extracts were mixed and incubated for 5 h at 37°C in 5% CO₂. At the end of the incubation period, a two-fold dilution of the virus-extract mixture was applied to the on cell monolayer. After incubation for 24-36 h at 37°C in 5% CO₂, the presence of 100% CPE in virus infectivity control cultures was confirmed and the cultures fixed and stained.

**Antiviral effect on the virus infective cycle following penetration**

A virus suspension (100 TCID₅₀/ml) was preincubated with cell culture. After 90 min, cell cultures were rinsed with PBS and two-fold serial dilutions of extracts were added to cell monolayers. After the incubation for 24-36 h at 37°C in 5% CO₂, the presence of 100% CPE in virus infectivity control cultures was confirmed and the cultures fixed and stained.

**RESULTS**

The sage extracts used in this study were ethanol extracts of wild (152) and cultivated plants (144) fractionated by supercritical CO₂ at different pressures (200, 300, 400, and 500 bars) to yield extracts designated 152/2, 144/2, 144/3, 144/4, and 144/5. Extract 149/3, obtained from cultivated sage steam-distilled prior to ethanolic extraction to remove volatile terpenoids, was also included in the study. The experiments were performed in 1994/95.

**Cytotoxicity of sage extracts**

Examination of the cytotoxicity of sage extracts was performed in the range of concentrations up to 10,000 µg/ml. The maximum non-cytotoxic concentrations (CTD₁₀) were read individually from the obtained survival curves.

According to the results of this experiment (Table 1), the fraction of wild sage obtained at 200 bars (152/2) and fractions of cultivated sage obtained at 200, 300, and 400 bars (144/2, 144/3, and 144/4) had a similar range of cytotoxicity (CTD₁₀ 84-93 µg/ml), while the fraction of cultivated sage obtained at 500 bars (144/5) was non-toxic up to 199 µg/ml. The fraction of distilled sage obtained at 300 bars (149/3) was the most cytotoxic, with CTD₁₀ of 44 µg/ml.

Increased toxicity of all fractions was accompanied by two types of changes in cell morphology. At concentrations up to 625 µg/ml, cells became round,
nuclei were more prominent, and the cells were found to float in the medium. At higher concentrations, there was an additional type of change in cell morphology, e.g., asteroid cells appeared which remained adhered to the substrate and were not stained with trypan blue. With increase in concentrations of the extracts, the latter type of cells was more frequent; this gave U-shaped survival curves (not shown). Attempts to sub-cultivate asteroid cells failed, suggesting their physiological death.

**Extracellular antiviral effect of sage extracts**

The effect of sage extracts on viral infectivity was monitored in a direct preincubation test (Weber et al., 1992). As a negative control of extracellular antiviral activity, we used interferon-α (IFN-α), which is known to act at the intracellular level (Canfell and Strander, 1966). The results obtained in this test (100% CPE) showed that neither extracts nor IFN-α had any extracellular antiviral effect.

**Intracellular antiviral effect of sage extracts**

The antiviral assay was performed by incubating a WISH cell monolayer with non-toxic concentrations of plant extracts for 5 h before viral infection. In this set of experiments, a positive control consisted of IFN-α incubated with the WISH cell monolayer. The highest and the lowest concentrations of IFN-α which caused 100% reduction of CPE were 50 and 25 i.u./ml, respectively (Table 2). Sage fractions 144/2 and 149/3 had no intracellular antiviral effect, as CPE remained 100% at all tested concentrations (data not shown). Although sage fractions 144/3, 144/4, and 152/2 showed some reduction of CPE at sub-toxic concentrations, the obtained antiviral effect was not considered significant (Table 2). A clear antiviral effect was detected with fraction 144/5 of cultivated sage. This fraction showed complete inhibition of virus replication (100% reduction of CPE) at concentrations of 99.5 and 199 µg/ml (Table 2), and the effect was comparable with the effect of IFN-α. To quantify the intracellular antiviral activity of 144/5, IS was calculated from the results presented in Table 2; the obtained value of 2 confirmed antiviral activity of 144/5.

**Effect of sage extracts on phases of the viral infective cycle following penetration**

Confluent monolayer cultures of WISH cells were infected by VSV 90 min before the application of plant extracts. Many authors (VandenBerge et al., 1986; Zgorniak-Nowosielska et al., 1989; Tommasi et al., 1991) consider 60-90 min of incubation sufficient for adsorption and penetration of the virus to occur. The reduction of CPE by plant extracts in this experiment reflects their inhibitory effect on the phases of virus replication following penetration. Sage fractions 144/2, 149/3,
Table 2. Intracellular antiviral effect of sage extracts.

<table>
<thead>
<tr>
<th>Concentr. (i.u./ml)</th>
<th>0</th>
<th>0.4</th>
<th>0.8</th>
<th>1.5</th>
<th>3.1</th>
<th>6.2</th>
<th>12.5</th>
<th>25.0</th>
<th>50.0</th>
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<td>0</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>35</td>
<td>70</td>
<td>100</td>
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</table>

<table>
<thead>
<tr>
<th>Concentr. (µg/ml)</th>
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<th>0.7</th>
<th>1.4</th>
<th>2.6</th>
<th>5.2</th>
<th>11.1</th>
<th>21.0</th>
<th>42.0</th>
<th>84.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction of CPE (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
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Data are the means of three independent experiments with 10 microtitrations. Reduction of CPE (%) is calculated relative to virus infectivity control.

and 152/2 caused no reduction of CPE at any of the tested concentrations (data not shown), indicating no inhibitory potential on virus replication. The highest tested concentrations of sage fractions 144/3 and 144/4 slightly suppressed virus replication (Table 3), but according to the criterion for antiviral activity, i.e., 80% reduction of CPE or plaque number (Abou-Karam and Shier, 1990), they also have to be considered as having no antiviral potential. Fraction 144/5 at concentrations 49.7, 99.5, and 199 µg/ml showed reduction of CPE by 12, 35 and 60%, respectively (Table 3). In this experiment, 144/5 was less efficient in inhibition of VSV replication than IFN-α (90 and 100% reduction of CPE at concentrations of 25 and 50 i.u./ml, respectively, Table 3).

**DISCUSSION AND CONCLUSIONS**

According to Vandenberghe et al., (1986), when screening plant extracts for antiviral activity in vitro one is looking for non-specific action of antivi-
eral agents on infected cells. Antiviral effects such as a lower virus titer may result from virucidal action and/or non-specific effects on cells that limit viral infection. However, by varying experimental conditions, we can address questions concerning direct extracellular effects on virus particles, i.e., whether they involve chemical or physical damage to viruses resulting in the prevention of virus adsorption (Sydiskis et al., 1991) or direct intracellular (reversible or irreversible) selective action on some steps in virus biosynthesis (Leary et al., 2002).

In this study, we evaluated cytotoxicity and extracellular and intracellular antiviral activity of fractionated extracts of wild and cultivated sage containing terpenoids in the WISH-VSV model system. Common terpenoids in both plants are viridiflorol (a sesquiterpene) and the diterpenoids manool and rosmanol 9-ethyl ether. The diterpenoid 12-deoxy-carnosol is present only in wild sage, while the diterpenoids galdosol, carnosic acid 6-methyl ether, carnosic acid 6-methyl ether-γ-lactone, and triterpenoid oleanic acid are present only in cultivated sage. Fractions contain different proportions of terpenoids: manool and viridiflorol are predominant in the fractions obtained at 200 bars (152/2 and 144/2), carnosic acid 6-methyl ether and carnosic acid 6-methyl ether-γ-lactone in the fractions obtained at 300 bars (149/3 and 144/3), and oleanic acid in the fraction obtained at 500 bars (144/5). In addition, extracts 152 and 144 contain monoterpenoids from essential oils (Djarmati et al., 1993; 1994; Djordjević, 1994).

The results obtained in investigation of cytotoxicity showed that the cytotoxicity of extracts depended on the plant used and the method of extraction applied. The most toxic extract was 149/3 from distilled cultivated sage, indicating that higher terpenoids are cyotoxic principals of this extract. 149/3 contains a larger proportion of higher terpenoids than 144/3 obtained from non-distilled cultivated sage at the same CO$_2$ pressure (Djordjević, 1994), among which galdosol, rosmanol 9-ethyl ether, and carnosic acid were reported to have high cytostatic activity (Hayashi et al., 1987; Darias et al., 1990; Steiner et al., 2001).

It is interesting that all tested extracts induced two types of changes in cell morphology at cytotoxic concentrations. At concentrations up to 625 µg/ml, only spheroid cells floating in the medium were observed, indicating necrosis, but higher concentrations induced an increasing number of cells whose morphology was similar to apoptotic cells within the limits of optical microscopy (Doyle et al., 1995). This observation deserves further study because apoptosis-inducing plant extracts are potentially useful in chemoprevention.

Evaluation of extracellular antiviral activity showed that preincubation of VSV particles with extracts did not arrest their infectivity, suggesting that none of the extracts caused physical or chemical changes of the virions. Our results differ from the results of Sivropoulou et al. (1997), who found that the monoterpenoids thujone and camphor (present in extracts 152 and 144) are virucidal to HSV-1. Many reports indicate that antiviral agents possess different activity against different viruses (Zgorniak-Novosielksa et al., 1989; Weber et al., 1991; Shih-Bin et al., 2001; Leary et al., 2002).

The results of investigating the intracellular antiviral activity of sage extracts showed that sage fractions 144/2 and 149/3 did not reduce the CPE of VSV in the range of non-toxic concentrations. Although fractions of cultivated and wild sage 144/3, 144/4, and 152/2 at sub-toxic concentrations did show some reduction of the CPE of VSV in cell culture, meaning that they probably inhibit to some extent virus multiplication at intracellular level, this reduction was not sufficient to pronounce fractions 144/3, 144/4, and 152/2 antiviral. According to the classification of antiviral agents (Vanden Berghe et. al., 1986; Abou-Karam and Shier, 1990), antiviral activity exists if 100% reduction of CPE is observed at subsequent two-fold dilutions in the range of non-toxic concentrations of the extract. The reduction of CPE caused by 144/3, 144/4, and 152/2 at the highest tested concentrations was less than 100%, and at two-fold lower concentrations it was only about 10%.

On the contrary, fraction 144/5 caused 100%
reduction of CPE, both at the highest non-toxic concentration and at the next two-fold dilution. Since the number of infecting viral particles was constant, this indicates stable antiviral activity. Less than 100% reduction of CPE was also detected in the two next two-fold dilutions. The selectivity index (IS) was 2, which indicates minimal antiviral activity in the WISH-VSV model system.

Investigation of the antiviral effect of sage extracts against phases of virus replication following penetration confirmed that sage fractions 144/2, 149/3, and 152/2 had no antiviral potential, while fractions 144/3 and 144/4 disturbed virus replication to a slight extent (20 and 5% inhibition of CPE). Extract 144/5 at the maximum non-toxic concentration reduced CPE by 60%, indicating some inhibition of virus replication. However, the absence of 100% reduction of CPE at non-toxic concentrations indicates that the effect of extract 144/5 is not stable and selective for phases of virus replication following penetration. Comparing the antiviral activity of IFN-α and fraction 144/5, we can conclude that extract 144/5 possessed antiviral activity similar to that of interferon at the intracellular level. However, due to weaker activity of 144/5 in comparison with interferon against virus replication following penetration, we can only speculate about the "interferon-like" activity of 144/5.

Taken together, the obtained results indicate that the mechanism of antiviral activity of extract 144/5 involves inhibition of the early steps of the virus infective cycle without a direct virucidal effect. This mechanism has been proposed for the antiviral effect of Spirulina maxima extract against HSV-2 (herpes simplex virus type 2) (Hernandez-Corona et al., 2002). The reduction of CPE by 144/5 after adsorption and penetration of VSV had occurred probably involved some non-specific action on host cells affecting virus biosynthesis and limiting viral infection. At the moment we can only speculate that triterpenoid oleanic acid, which is predominant in 144/5, might be responsible for the antiviral activity of this fraction. Antiviral activity of triterpenoids such as maslinic and ursolic acid against many viruses has been reported (Jassim and Naji, 2003).

Study of the antiviral effects of plant extracts is aimed at developing new strategies in the treatment of different viral infections. Many traditional medicinal plants have been reported to possess strong antiviral activity, and some of them have already been used to treat animals and people against different viral infections (Jassim and Naji, 2003). From our results with sage it is clear that cultivated sage, especially fraction 144/5, deserves further investigation to evaluate its antiviral potential and active principals against different viruses. Regardless of negative results obtained in the WISH-VSV model system, it is still of interest to investigate the antiviral effect of cultivated sage in other model systems. This is especially true for fractions 144/3 and 144/4, because in our study the reduction of CPE was above zero. The possibility of cultivating sage variety D-70 on an industrial scale and obtaining large quantities of CO₂ fractions should facilitate these investigations and lead to future application of antivirals from this plant.

In conclusion, the terpenoid fraction of the D-70 variety of cultivated sage, obtained at CO₂ pressure of 500 bars and temperature of 100°C was the least toxic to WISH cells. It also showed the lowest antiviral activity against the VSV virus. The antiviral activity of this fraction is intracellular, stable, and more pronounced against the whole VSV infective cycle than at the phases of virus replication following penetration.

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ANTIVIRAL ACTIVITY OF SALVIA OFFICINALIS 429


У раду је испитивана антивирална активност различито фракционисаних екстраката дивље и гајене жалфије Salvia officinalis L. (Lamiaceae) у in vitro условима користећи WISH-VSV модел систем. Екстракти су добијени фракционисањем депигментисаног етанолног биљног екстракта под различitim притиском CO2. Цитотоксичност је одељивана праћењем ћелитeнске морфологије in situ, а и бојењем ћелитeнска са тринап плавим. Фракциjа гајене жалфије добијена на CO2 притиску од 300 бара и температури од 60°C (149/3) је показала највећу цитотоксичност (CTD10, 34 µg/ml). Фракциjа не-дестилисана гаjеne жалфије добијена на CO2 притиску од 500 бара и температури од 100°C (144/5) је показала наймању цитотоксичност (CTD10, 199 µg/ml). Такођe, фракциjа 144/5 је показала антивиралну активност коja сe остваруji кроз инхибициjу раних ступника вирулентне инфекциjе без директног вируцидалног ефекта.