**Salvia Officinalis L. and Verbascum Phlomoides L.**

**Chemical, Antimicrobial, Antioxidant and Antitumor Investigations**

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Due to the fact that the therapeutic activity of the *Salvia officinalis* L. is well-known, we aim to evaluate the therapeutic properties of the *Verbascum phlomoides* L. In the present study, we conducted extractive solutions of the both plant species after having undergone lyophilization for a better preservation of the active extracted ingredients. Lyophilized extracts were evaluated in terms of chemical composition. The polyphenolic content of the extracts was obtained using HPLC method, while the total polyphenol content (expressed in gallic acid) and total flavonoids content (expressed in quercetin) were quantified using spectrophotometric methods. The studies carried out have shown the protective effect of plant extracts against free radicals – antioxidant properties assessed by various methods (DPPH method, FRAP method, TEAC method, SOD-like activity in vivo), the antimicrobial activity and cytotoxicity assays. After evaluating the therapeutic properties of freeze-dried extracts of the two plants, they will be associated in the formulation of a mouthwash with known therapeutic properties.

**Keywords:** phytochemical content, antioxidant capacity, antimicrobial activity, SOD-like activity, cytotoxicity

The results of the studies conducted so far have led us to focus our attention on *Verbascum* species. In order to achieve this, we performed a comparative study on the therapeutic activities of the *Salvia officinalis* L. and *Verbascum phlomoides* L. [1,2].

Mullein (great mullein), it is a plant widespread in the wild flora of Romania, but less used in traditional medicine and with less demonstrated effects.

Mullein is a herbaceous plant, biennial. In the first year, it forms a rosette of basal leaves and in the second year flowering stems. Type five flowers are yellow and grouped under bracts. It is commonly spread on fallow lands being deprived of pollutants and herbicides substances. It blooms in the summer between June and August and even in September. For this study, we used flowers without calyx (*Verbascum flos*) which we have gradually harvested depending on the degree of opening from June to August 2015, in lowlands and hills of western Romania.

The leaves of *Salvia officinalis* L. (*Salviae folium*) were harvested from May to June 2015 from crops untreated with herbicides substances.

Many disorders have been associated with reactive oxygen species (ROS) exposure, due to their skill to produce oxidative damages to DNA, proteins and lipid membranes, among other macromolecules. Despite the fact that almost all organisms are prepared against these injuries with antioxidant defenses and repairing systems, an imbalance between ROS generation and antioxidant systems may occur, leading to tissue damages. The impact of ROS on tissues are devastating regarding oxidative stress-induced and cell death [3-5].

Phenolic compounds exert multiple biological effects, including antioxidant and free radical-scavenging abilities. Their anti-radical property is directed toward hydroyx radical and superoxide anion, highly reactive species, especially the last one being one of the main ROS involved in the DNA damage processes [6-10].

Among the abundant data regarding ROS-induced cell death, especially in tumor cells, gallic acid is well-known as a natural antioxidant involved in both inhibitory and scavenging actions of ROS [11-14].

Previous studies have demonstrated that gallic acid exerts activity against several types of tumor cells, including leukemia [15], cervical cancer [16], lung cancer [17], colon cancer and breast cancer cell lines [18]. Particularly, the studies have shown that the antitumor activity seems to be related to the induction of apoptosis involving different signaling pathways. Apoptosis induced by gallic acid may be associated with oxidative stress derived from ROS [19], mitochondrial dysfunction and increase in intracellular Ca²⁺ levels. It also has been reported that the cell death promoted by gallic acid in different cell lines may be related with glutathione (GSH) depletion [20]. Interestingly, gallic acid has both pro-oxidant and antioxidant properties.

Quercetin has also an antioxidant and anticancer effect, being therefore reported as an efficient free radical scavenging. Therefore, it is capable of preventing cancer induced by oxidative stress [21]. Previous studies have...
already demonstrated that quercetine exerts activity against several types of tumor cells, including hepatic cancer [22], leukemia [23] and cervical cancer [24]. In the present study we obtained extractive solutions for both Salvia folium and Verbascum flos. Lyophilized extracts were evaluated in terms of chemical composition. Their antioxidant capacity and antimicrobial activity were investigated. Also, cytotoxicity assays were performed.

Experimental part
Materials and methods
Obtaining plant extracts
After harvesting, plant products were dried at the room temperature (20 - 25°C), away from light. Fluid extracts of Salviae folium and Verbascum flos were obtained by leaching, using as solvent a 70% hydroalcoholic mixture, according to [25]. Hydroalcoholic extracts were centrifuged and supernatants were evaporated to dryness under vacuum in a rotavapor. The dried extract was transferred to a vessel with 10 mL of distilled water and frozen at the temperature of -25°C. The lyophilized extracts were weighed and transferred to sample ampoules.

The determination of the phytochemical content of the lyophilizes
The total polyphenol content was determined using the Folin-Ciocalteu technique. 100 µL of each sample was mixed with 1750 µL of distilled water, 200 µL Folin-Ciocalteu reagent (1:10 dilution, v/v) and 1000 µL of 15% Na₂CO₃ solution, and the mixture was kept at the temperature of 25°C in the dark for 2 h. The total polyphenols content from the extracts was expressed as mg gallic acid equivalents (GAE)/100g using the following equation based on the calibration curve: y = 0.0017x + 0.0848, where x represents µmol absorbance determined at 595 nm and y was the absorbance recorded at 765 nm.

The total content of flavonoids was determined using a colorimetric method. We have used a mini spectro-photometer Shimadzu UV-Vis, the absorbance being determined at 510 nm and the results were expressed as mg quercetin equivalent (QE)/100g. The equation based on the calibration curve was: y = 1.9735x + 0.0261 (R² = 0.9928), where y was the absorbance recorded at 765 nm and x represents µmol gallic acid equivalent [26].

The total polyphenol content was determined using the Folin-Ciocalteu technique. 100 µL of each sample was mixed with 1750 µL of distilled water, 200 µL Folin-Ciocalteu reagent (1:10 dilution, v/v) and 1000 µL of 15% Na₂CO₃ solution, and the mixture was kept at the temperature of 25°C in the dark for 2 h. The total polyphenols content from the extracts was expressed as mg gallic acid equivalents (GAE)/100g using the following equation based on the calibration curve: y = 0.0017x + 0.0848, where x represents µmol absorbance determined at 595 nm and y was the absorbance recorded at 765 nm.

Identification of phenolic acids in the extract of Verbascum flos compared to the Salviae folium was performed using a Shimadzu SPD-M10A system equipped with a diode array detector (PDA). It was used a column Kinetex type 5 mm C18 100A (5/No:86996-11, B/No:5701-029). The mobile phase used for elution consisted of acetonitrile (A) and distilled water - formic acid (99:0.1) (v/v) (B). The gradient was performed as follows: min. 1% A, min. 25% 50% A, min. 35% 98% A. The flow rate used was 1.5 mL/min, and detection was performed at the wavelength of 360 nm.

The determination of the antioxidant capacity of the lyophilized extracts
For the evaluation of the antioxidant activity, we used several methods and the following reagents: 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), potassium persulfate, Folin-Ciocalteau’s reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,6-Tri (2-pyridyl) -s-triazine (FRAP). These reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid and sodium carbonate were purchased from Fluka (Switzerland). All reagents were chemically pure.

DPPH method
The radical scavenging activity of the lyophilized extracts using 2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH) was determined using the Brand-Williams et al method 1995 [28]. The absorbance of samples was determined after 15 minutes compared to the control samples.

FRAP method (ferric reducing antioxidant power)
The evaluation of the antioxidant activity is based on reducing the tripyridyltriazine iron complex [Fe(III)-TPTZ] as a reducing agent at an acidic pH. The determination is spectrophotometric.

Trolox was used as a standard solution, the method was linear between 0 and 300 mg/mL, with a correlation coefficient of R² = 0.9956 and the antioxidant capacity of the extracts was calculated from the regression equation (y = 0.0017x + 0.0848), where y represents the absorbance determined at 595 nm and x represents µmol trolox equivalents (TE)/g [29,30]

TEAC (Trolox equivalent antioxidant capacity) method
This method is based on the ability of antioxidants to decrease the cation-radical life (ABTS⁺), a blue-green chromophore that absorbs at 734 nm, compared to Trolox. The results are expressed as µmol Trolox equivalents (TE)/g [31].

The antimicrobial activity
The antimicrobial activity assays of Salvia officinalis L. and Verbascum phlomoides L. were carried out for four reference strains and five clinical isolates: Staphylococcus aureus (ATCC 25923), Streptococcus pneumoniae (ATCC 49619), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Candida albicans (ATCC 90029), Methicillin-sensitive Staphylococcus aureus, Methicillin-resistant Staphylococcus epidermidis, Streptococcus pyogenes (Group A Beta hemolytic Streptococcus (GABHS), Streptococcus agalactiae (Group B Beta hemolytic Streptococcus (GBBHS)), and Group C Beta hemolytic Streptococcus (GCBHS) using the disk diffusion method [32].

Standardized microbial inoculums (equivalent to a 0.5 McFarland standard) were plated onto suitable culture media (Mueller-Hinton Agar (Oxoid), Escherichia coli and Pseudomonas aeruginosa isolates; Mueller Hinton 2 agar + 5% sheep blood (BioMerieux) for streptococcal strains and Sabouraud Gentamicin Chloramphenicol 2 agar (BioMerieux) for Candida albicans). The sterile filter paper discs of 6 mm diameter (HiMedia Laboratories) impregnated with 20 µL of each plant extract where placed onto inoculated plates. Standard disks of Penicillin (10 U; Oxoid), Vancomycin (30 µg; Oxoid), Cefotaxin (30 µg; Oxoid), Ofloxacine (5 µg; Oxoid) and Fluconazole (25 µg; BD BBL) were used as positive controls and filter papers disks imbued with distilled water (20 µL) as negative controls. After overnight incubation at 37°C for bacteria and 24 h at 37°C plus 24 h at 25°C for Candida albicans, inhibition zone diameters were measured in millimeters. Each test was run for three times and means values were selected [33].

In vivo SOD-like activity
The protective effect of the plant extracts against free radicals produced by oxidative agents had been determined.

The SOD-like activities of the plant extracts where evaluated using a strain of S. cerevisiae Δaso1 (ATCC96687), which has the ability to delete/insert the
SOD gene encoding the synthesis of Cu,Zn SOD. The characteristics of S. Cerevisiae ∆sod1 (ATCC96687) are: MAT aura 3-52 trp1-289 his3-A1 leu 2-3 leu 2-112 sod1: URA3). The Cu,Zn SOD is the main SOD in the cell and it is localized in the cytoplasm. Yeast cells were grown in YPD reach medium (1% yeast extract, 2% peptone and 2% glycerol). The used culture medium does not contain glucose, it contains glycerol instead, because in its presence the levures can breath. This is determinant, as free radicals are going to be generated during the breath processus taking place in the mitochondria.

Solid media contained 1.5 % agar. Cell density from cultures grown overnight was determined by cell counting in a Nebauer hematimetre. 106 cells were resuspended in 15 ml of melted solid YPD media kept at 45°C. Solutions of the liophilizated extracts in a mixture of DMSO:EtOH (1:4) at increasing concentrations (30, 50, 70 µg, table 5) were added to the growth medium. Cell suspensions were poured into Petri dishes and allowed to solidify at room temperature. Paper disks measuring 6 mm in diameter (Antibiotica test Blättchen) containing 5 µL of a 5mM menadione solution in ethanol or 5 µL of 17.5% H2O2 have been used. The diameters of clear zones around the disks, measured after 3 days of incubation at 28°C, where taken as a quantitative estimate of the protective action.

Cell toxicity assay

Cell culture

The B16-F10 metastatic murine melanoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA). B16-F10 cells were grown in DMEM, supplemented with 10% fetal bovine serum (Hyclone), 2 mM glutamine (Sigma-Aldrich), 1% penicillin and streptomycin, 0.1 % amphotericin. The cells were cultured at 37°C in an atmosphere of 5% CO2 and 95% relative humidity.

Cytotoxicity assays

B16-F10 cells (8 x 103 cells/ well) were seeded on 96-well plate and cultured in DMEM containing 10 % FBS for 24 h. The medium was then replaced with complete medium containing or not plant extract at various concentrations (0, 15, 25, 35, 45, 60, 80, 100, µg/mL). A stock solution of plant extract was prepared containing 0.3 % DMSO. The treatment was applied for 24 h at 37°C and 5% CO2. The number of viable cells was determined with the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation reagent. Three PBS washing steps were followed by 1h of incubation with MTT solution (0.5 mg/mL) in DMEM without phenol red. The formazan particles were solubilized with DMSO. The absorbance was read at 550 nm, respectively at 630 nm (for background) with the microplate plate reader HT BioTek Synergy (BioTek Instruments, USA). The results were expressed as survival percent with respect to an untreated control [34,35].

Statistics

All the experiments were conducted in triplicates and data are displayed as mean ± SEM. Two-way analysis of variance (two-way ANOVA) followed by the Bonferroni post-test was performed for the multiple comparisons for normally distributed samples with homogenous variance. Statistical significant differences were set at p < 0.05. The IC50 values representing the concentration required to inhibit 50% of cell proliferation were calculated from the dose response curve using non-linear regression. Statistical values and figures were generated using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

Results and discussions

The determination of the phytochemical content of the liophilizes

The total polyphenolic content was expressed in gallic acid equivalents (mg GAE/100g DW) and the total flavonoid content was expressed in quercetin equivalents (mg QE/100g DW). The results obtained by the spectrophotometric method are shown in table 1. HPLC method was used for the separation and identification of phenolic acids and flavonoids. The

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total polyphenol content (mg GAE/100 g DW)</th>
<th>Total flavonoid content (mg QE/100 g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verbascum flos</td>
<td>471.3±0.13</td>
<td>5.36±0.32</td>
</tr>
<tr>
<td>Salviae folium</td>
<td>1001.5±0.06</td>
<td>28.74±0.02</td>
</tr>
</tbody>
</table>

* Each value is the mean ±SD of three independent measurements.
GAE - gallic acid equivalent; QE - quercetin equivalent; DW - dry weight.

<table>
<thead>
<tr>
<th>Gallic acid (mg/kg DW)</th>
<th>Epicatechin (mg/kg DW)</th>
<th>Rutin (mg/kg DW)</th>
<th>p-Coumaric acid (mg/kg DW)</th>
<th>Luteolin (mg/kg DW)</th>
<th>Quercetin (mg/kg DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verbascum</td>
<td>56.026</td>
<td>497.956</td>
<td>313.275</td>
<td>262.616</td>
<td>1045.10</td>
</tr>
<tr>
<td>Phlomoides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salviae</td>
<td>119.071</td>
<td>1058.382</td>
<td>665.799</td>
<td>558.133</td>
<td>1875.206</td>
</tr>
</tbody>
</table>

* DW - dry weight

Table 1
POLYPHENOLIC TOTAL CONTENT OF THE EXTRACTS, USING SPECTROPHOTOMETRIC METHOD

Table 2
POLYPHENOLIC CONTENT OF THE EXTRACTS USING HPLC METHOD
As shown in table 1 and table 2, both studied plants are rich in polyphenols and flavonoids, which determined us to evaluate their therapeutic properties.

The determination of the antioxidant capacity of the lyophilized extracts

The results of the first studies on antioxidant activity undertaken for the two lyophilized extracts of Salvia officinalis L. and Verbascum L. phlomoides are presented in table 3.

In assessing the potential antioxidant, we noticed that, according to the methods used, between the two plants there are no significant differences, but associated, their action will be much improved, which is necessary in a medicinal product.

The antimicrobial activity

There are numerous studies on the antimicrobial activity of different Verbascum species and Salvia officinalis extracts, and sometimes with divergent results. In the table 4 is presented the antimicrobial activity of Salvia officinalis and Verbascum phlomoides extracts.

In our study, both Salvia officinalis and Verbascum phlomoides extracts shown antibacterial effect on Gram positive bacteria. These findings are in accordance with most of the studies [36,37]. In contrast, in a study of Nascimento et al. [38], Salvia officinalis extract did not show any antimicrobial activity.

Our extracts were not active on Escherichia coli, Pseudomonas aeruginosa and Candida albicans and similar results have been reported [38]. Other studies demonstrate Verbascum species and Salvia officinalis extracts exhibit growth inhibition on Gram negative rods [39].

The resistance of this yeast to Verbascum and sage extracts has been documented in the literature and the results of our research support this, although other studies show the contrary [40]. These differences can be explained by variation in plant composition according to geographical area, the technique of preparing the extract, or differences concerning the bacterial strains.

Verbascum phlomoides extract is more efficient than Salvia officinalis on all Gram positive strains, except Streptococcus pyogenes, although the lower flavonoid and polyphenol content. This suggests intervention of other compounds (saponins) in providing the antibacterial effect.

Table 4
ANTIMICROBIAL ACTIVITY OF SALVIA OFFICINALIS AND VERBASCUM PHLOMOIDES EXTRACTS ASSAYED BY DISK DIFFUSION METHOD

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Salvia officinalis 100 mg/ml</th>
<th>Verbascum phlomoides 100 mg/ml</th>
<th>Penicillin 10 U</th>
<th>Vancomycin 30 µg</th>
<th>Cefoxitin 30 µg</th>
<th>Ofloxacine 5 µg</th>
<th>Flucanazole 25 µg</th>
<th>Distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus ATCC 25923</td>
<td>12.66</td>
<td>15.66</td>
<td>30</td>
<td>18</td>
<td>26.33</td>
<td>27</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>S. pneumoniae ATCC 49619</td>
<td>8.66</td>
<td>10.33</td>
<td>26</td>
<td>22</td>
<td>NT</td>
<td>18</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>NA</td>
<td>NA</td>
<td>NT</td>
<td>NT</td>
<td>25</td>
<td>30.66</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 27853</td>
<td>NA</td>
<td>NA</td>
<td>NT</td>
<td>NT</td>
<td>19</td>
<td>NT</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Candida albicans ATCC 50029</td>
<td>NA</td>
<td>NA</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>32</td>
<td>NA</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>12</td>
<td>14</td>
<td>6</td>
<td>NT</td>
<td>24.33</td>
<td>19.66</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>12</td>
<td>12.66</td>
<td>6</td>
<td>NT</td>
<td>12</td>
<td>13.33</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>10</td>
<td>8</td>
<td>34</td>
<td>19</td>
<td>NT</td>
<td>20.66</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>20.33</td>
<td>NT</td>
<td>21</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>Group G Beta hemolylve Streptococcus</td>
<td>8</td>
<td>8</td>
<td>33</td>
<td>19.33</td>
<td>NT</td>
<td>18</td>
<td>NT</td>
<td>NA</td>
</tr>
</tbody>
</table>

NT = not tested; *NA = not active
The oxidative stress is produced by two oxidative agents: menadione which toxicity is due to the superoxide radicals production and H₂O₂ which toxicity is registered due to OH• radicals. It will be considered that the extract has a SOD-like activity if a decrease of the diameter of the inhibition zone is registered versus the control zone. The efficacy will then be evaluated by comparison of the diameter of the inhibition area for the extract and control.

Figure 1 shows the results obtained from the two plant extracts. In the presence of the plant extracts at 30, 50 and 70 µM a significant reduction of the inhibition area is observed when the oxidative stress is produced by both menadione and H₂O₂. The diameter of the inhibition area for both, Salvia officinalis L. and Verbascum phlomoides L. plant extracts in different concentrations, using menadione and H₂O₂ are given in table 5.

The reduction of the inhibition area is between 35-40% for the Salvia officinalis L. plant extract and 25-28% for the Verbascum phlomoides L. plant extract against oxidative stress generated by menadione. The protective activities of extracts do not seem to be dependent on extract concentration.

The protection of the extracts against free radicals generated by H₂O₂ is lower than in the case of free radicals generated by menadione. Salvia officinalis L. extracts produce a reduction of the inhibition diameter about 22-27% while Verbascum phlomoides L. extracts only between 17-20%. Nor in this case the protective action produced by the extracts does not depend on extract concentration.

As a conclusion, Salvia officinalis L. extracts register a higher SOD-like activity compared with Verbascum phlomoides L.

The current study clearly suggests that both plant extracts are able to protect efficiently against superoxide anions and they could be considered as promising effective agents against toxicity of superoxide anion, improving significantly the growth of Δsod1 strain. They supply the Cu₂Zn₂SOD deficiency of the mutant. For this reason they are potential therapeutic agents in the prevention and treatment of diseases mediated by free radicals.

Cell toxicity assay

The in vitro cytotoxicity of plant extracts was tested on B16F10 melanoma cell lines. The response was quantified using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Both extracts exhibit cytotoxicity on B16F10 melanoma cells lines. At small concentration of plant extract there are no significant differences between the two plant extracts but at higher concentrations (60; 80; 100 µg/mL) Salvia officinalis L. showed a superior inhibitory effect (FIG. 2). This pattern is clearly supported by the IC₅₀ values (table 6).

As a conclusion, Salvia officinalis L. plant extracts register a higher cytotoxicity compared with Verbascum phlomoides L.

Conclusions

Both plant extracts may be considered as potential therapeutic agents, higher SOD-like activity and cytotoxicity being registered for the Salvia officinalis L. extract compared to the Verbascum phlomoides L. This may be considered a premise in recommending the Salvia officinalis L. extracts as a potential therapeutic agent of choice. Salvia officinalis and Verbascum phlomoides extracts exhibit antibacterial activity on gram-positive, but not on gram negative bacteria or on Candida albicans. Regarding the antibacterial activity, Verbascum phlomoides extract is more efficient than Salvia officinalis.

The carried out analyses demonstrate the therapeutic potential of both sage leaves and Verbascum flos and that a mixture of the two extracts in the formula of a mouthwash will be beneficial to prevent diseases of the oral cavity.