Multitarget actives from *Verbascum* and *Ajuga* species on prevention and treatment of nonmelanoma skin cancer

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ABSTRACT: Nonmelanoma skin cancers, represented by basal and squamous cell carcinomas, are the cancer diseases with the highest frequency worldwide, associated mainly with ultraviolet radiation overexposure (chronic sun exposure). Because of the depletion of the ozone layer which filters the harmful UVB radiation, the incidence of nonmelanoma skin cancer has been increasing in the past decades, worldwide. The aim of this study was the development of suitable methods for bioassay-guided isolation of plants extractives having the capacity to act simultaneously on different pathways involved in the initiation, development and progression of nonmelanoma skin cancer and other adverse skin conditions.

1 INTRODUCTION

Nowadays, bioactive natural products are still an essential part of the medical and cosmetics and toiletries research field. Taking into account that the incidence of nonmelanoma skin cancer (NMSC) has been substantially increasing worldwide as a result of increased sun exposure and other environmental factors, a preventative approach using cosmetic or dermatologic formulations could yield better treatment results (1, 2). The available therapeutical treatments are non-satisfactory due to high costs and multiple side effects (3).

Complex mixtures of phytochemicals from plant extractives are expected to exert synergistic and/or complementary effects, which could be beneficial for prevention and treatment of NMSC (4, 5). The actual limits of prevention and treatment of nonmelanoma skin cancer justify recent studies aiming to identify new prophylactic and therapeutic agents. As the pathogenesis of nonmelanoma skin cancer is very complex, the development of multitarget agents is undoubtedly a valuable approach in the prevention and treatment of this disease (6, 7). Plant extracts are complex mixtures of phytochemicals. Appropriate fractionation procedures might yield extractive fractions containing phytochemicals that act synergistically and/or complementarily. Therefore, such extractive fractions might modulate multiple targets which cause or trigger the disease.

Several phytochemicals (genistein, lycopene, ingenol mebutate, paclitaxel, epigallocatechin-3-gallate, curcumin) and plant extractives (green tea polyphenol extract, grape seed proanthocyanidin extract, silymarin, phenolic fractions from *Lonicer caerulea* and *Vaccinium myrtillus* fruits) have already been studied regarding their ability to prevent, suppress or reverse processes involved in nonmelanoma skin cancer (4, 8, 9, 10). To the best of our
knowledge, no investigations have been done on species of *Verbascum* and *Ajuga*, which are rich in phenylethanoids and other polyphenols (11, 12).

Apart from other polyphenols, when exposed to UVA and UVB, verbascoside, a major phenylethanoid in the above mentioned species, have proved photo-stability and the capacity to interfere with different pathways leading to the development of nonmelanoma skin cancer (local inflammation, cell proliferation) (1, 2, 13).

2 METHODOLOGY

2.1 PLANT MATERIAL
The plant material was collected and identified by Nina Ciocirlan and Veaceslav Ghendov - Botanical Garden (Institute) of Academy of Sciences of Moldova in 2014. Voucher specimens are kept at the University of Medicine and Pharmacy “Grigore T. Popa” – Iasi, Faculty of Pharmacy, Romania.

2.2 EXTRACTION AND PURIFICATION PROCEDURE
The dried and powdered aerial parts of *Ajuga chia* Schreb. (Lamiaceae) and *Verbascum nigrum* L. (Scrophulariaceae) (100 g each) were extracted with methanol (1000 ml) for 48 hours, at room temperature, protected from light. (Ratio 1:10) The crude methanolic extracts (CME) were filtered under vacuum then the solvent was evaporated in a rotary vacuum evaporator at 40-45°C. The extracts were dried with a vacuum oven at 37°C. The dried crude methanolic extracts were kept at -20°C until further use.

Purification of extracts was done by liquid – liquid extraction: 15 g of dried crude methanolic extract of *A. chia* and were mixed with distilled water (1:20 ratio) in ultrasonic bath, until reaching a homogenous suspension; the aqueous phase was extracted with diethyl ether (C2H5)2O - 1:1, 4-5 times. The aqueous layer of each extract was concentrated in a rotary evaporator t°= 40-45°C and then lyophilized to obtain an ether - delipidized dried methanolic extract.

2.3 FRACTIONATION OF EXTRACTS
The delipidized extracts were fractionated by RP-C18 flash chromatography using a low pressure flash chromatography kit (Macherey-Nagel) with water, methanol 50%, methanol 100% in step gradients and finally eluted with isopropanol, for the most lipophilic compounds, to obtain different fractions (flow rate ≈ 10 ml/min) (Tab. I).
**Table I.** Fractionation of A. chia and V. nigrum extracts

<table>
<thead>
<tr>
<th></th>
<th>Delipidized methanolic extract</th>
<th>Verbascum nigrum L.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ajuga chia</strong></td>
<td><strong>Schreb.</strong></td>
<td></td>
</tr>
<tr>
<td>Eluent</td>
<td>Fractions</td>
<td>Eluent</td>
</tr>
<tr>
<td>Water</td>
<td>2 fractions</td>
<td>Water</td>
</tr>
<tr>
<td>Methanol 50%</td>
<td>3 fractions</td>
<td>Methanol 50%</td>
</tr>
<tr>
<td>Methanol 100%</td>
<td>3 fractions</td>
<td>Methanol 100%</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>1 fraction</td>
<td>Isopropanol</td>
</tr>
</tbody>
</table>

For fractionation of Verbascum nigrum extract the following materials were used: Polygoprep® 60-50 C18 (Macherey-Nagel, Düren, Germany) stationary phase, silanized glass fiber (Macherey-Nagel, Düren, Germany), Quarzsand (Macherey-Nagel, Düren, Germany), Dichlormethane (Honeywell, Seele GmBH, Germany), Ultrapure water (Sartorius Stedim Biotech filter), Methanol (LiChrosolv Reag. Ph. Eur., Merck, Darmstadt, Germany) and Isopropanol (Honeywell, Seele GmBH, Germany).

For the fractionation of Ajuga chia extract only the stationary phase was changed with a ZEOprep 60 C18 40-63 µm (Zoechem, Uetikon, Switzerland).

### 2.4 Thin Layer Chromatography Studies

Chromatographic analysis was performed using CAMAG automatic equipment (Camag Company, Switzerland). Standardized HPTLC (high performance thin layer chromatography) fingerprints were carried out for flavonoids, iridoids, phenolic acids and saponins, using the appropriate derivatization reagents. HPTLC glass plates coated with silica gel 60 F<sub>254</sub>, were used for sample application, development and subsequent derivatization. (Tab. II)

**Table II.** Thin layer chromatography screening systems for A. chia and V. nigrum extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fingerprint</th>
<th>Solvent system</th>
<th>Derivatization reagent(s)</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajuga chia + Verbascum nigrum CME and fractions</td>
<td>flavonoids</td>
<td>Formic acid, water, ethylmethylketone, ethyl acetate 10:10:30:50</td>
<td>I. NP&lt;sup&gt;1&lt;/sup&gt; reagent 5g/L II. PEG 400&lt;sup&gt;2&lt;/sup&gt; reagent 50g/L</td>
<td>UV 366nm</td>
</tr>
<tr>
<td>A. chia + V. nigrum CME and fractions</td>
<td>saponins, iridoids</td>
<td>chloroform, glacial acetic acid, methanol, water 64:32:12:8</td>
<td>Anisaldehyde-sulphuric acid reagent</td>
<td>VIS, UV 366nm</td>
</tr>
<tr>
<td>A. chia + V. nigrum CME</td>
<td>phenolic compounds, tannins</td>
<td>dichlormethane, methanol, water 70:34:4</td>
<td>Fast blue B salt</td>
<td>VIS</td>
</tr>
</tbody>
</table>

<sup>1</sup> NP reagent – natural products reagent (diphenylborinic acid aminoethylster) in ethyl acetate

<sup>2</sup> PEG reagent – polyethylene glycol 400 (macrogol) in dichlormethane
2.5  HPLC ANALYSIS

2.5.1  Chemicals
Acetonitrile and methanol of analytical grade were purchased from Merck, Darmstadt, Germany. Formic acid of analytical grade (99%) was purchased from Carlo Erba Reagents, Val de Reuil, France. Water was purified using a Sartorius Stedim Biotech filter.

2.5.2  HPLC-ESI-DAD-MS chromatographic conditions (analysis)
The chromatographic analysis was carried out on a Waters, Acquity UPLC System. This instrument was equipped with a Waters Acquity BEH C18 column (1.7 µm, 2.1×50mm). The mobile phase consisted in acidified water (0.1% formic acid, v/v) – solvent A and acetonitrile – solvent B. The injection volume for samples was 5µl and for the reference substances was 3µl. The UPLC gradient used for both analysis of *Ajuga chia* and *Verbascum nigrum* was: initial, 1.0% B; 22.50 min. 99.9% B; 23.50 min 99.9% B; 24.0 min 1.0% B, and the initial conditions were kept for 2 minutes for re-equilibration. Flow rate was set at 0.60 ml/min throughout the whole gradient. Flow rate from the UPLC system into the ESI-QDa detector was 60 µl/min and the makeup pump flow from the Isocratic Solvent Manager (ISM) into the QDa was 0.20 ml/min of ACN/H2O 50/50, 0.05% formic acid. Column temperature was maintained at 30.0°C. The parameters for the mass spectrometry analysis were established both in positive and negative modes, with a mass range from m/z from 210 to 700. The values for the ESI (electrospray ionization source) single-quadrupole MS were: capillary voltage, -0.8 and +0.8 kV, cone voltage, - 15 and +15 V.

The UV detection wavelength range was between 200 - 800 nm. The data was processed using an Empower 3 Software Build 3471 SPs.

2.6  CYTOTOXICITY STUDIES

2.6.1  Cell culture conditions
Immortalized HaCaT keratinocyte cell were grown in Dulbecco's Modified Eagle Medium - Nutrient Mixture F-12 (DMEM/F12) supplemented with glutamine 1% (GlutaMAX™) and fetal bovine serum 10% (FBS). Cells were cultured at 37°C in a 5% CO2 atmosphere and 95% humidity.

2.6.2  In-vitro cytotoxic activity assay
The cytotoxic evaluation of the crude methanolic extracts of *A. chia* and *V. nigrum* was performed, followed by the evaluation of the delipidized methanolic extracts.

The methanolic extracts of *Ajuga chia* and *Verbascum nigrum*, as well as the delipidized crude methanolic extract of *V. nigrum* and verbascoside as a pure compound were tested against HaCaT keratinocytes, in order to evaluate the cytotoxic selectivity of extracts, using the thiazolyl blue test (MTT) assay.
Test sample preparation: Stock solutions of each extract were prepared at a concentration of 100 mg/mL in dimethyl sulfoxide (DMSO). From the stock solutions, working solutions were prepared by dilution with cell culture medium at a final concentration of 100 µg/mL extract, and 0.1% DMSO and finally sterilized by filtration using 0.2 µm Millipore sterile filters. A serial two-fold dilution was then made from the initial working solution, to produce working solutions of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/ml extract, as well as solutions of 0.1% DMSO in complete culture were used as the vehicle control.

2.6.3 Cell line and Chemicals
The DMEM/F12 medium and GlutaMAX™ supplement were purchased from Gibco, Life Technologies. Fetal Bovine Serum (FBS) and Dulbecco’s Phosphate Buffered Saline (D’PBS) were purchased from Sigma Aldrich. The dimethyl sulfoxide was of analytical grade (Carlo Erba Reagents, Val de Reuil, France). The Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from AppliChem GmbH (Darmstadt, Germany). Verbascoside

2.6.4 Cytotoxicity assay
HaCaT cells were plated in 96-well plates (10⁴ cells/well), allowed to grow for 24 hours in the incubator (37°C, 95%O₂, 5% CO₂) and then treated with extract or pure compound in the given concentrations for another 24 hours. The culture medium was used as blank and the negative control were the untreated cells. A DMSO solution at 0.1% was tested also, as vehicle control. At the end of the incubation time, the extracts were removed and fresh medium with 0.5mg/mL MTT was added to all wells and incubated for 4 hours. After incubation time, the formazan crystals were dissolved in DMSO and absorbance was recorded at 570 nm (test) and 640 nm (background).

\[
\% \text{ Cell viability} = \frac{\text{Absorbance of cell with treatment}}{\text{Absorbance of cell without treatment}} \times 100
\]

3 RESULTS

3.1 PHYTOCHEMICAL FINGERPRINT
The primary phytochemical screening of the methanolic extracts of Ajuga chia and Verbascum nigrum revealed the existence of flavonoids, phenolic acids, iridoids, saponins, as well as other unknown compounds.
To our knowledge, Ajuga chia has not yet been fully phytochemically characterized. The flavonoid profile of A. chia methanolic extract alongside a series of reference substances, showed the presence of luteoline-7-O-glucoside, apigenin and also the phenylethanoid glycoside – verbascoside. The presence of these substances was then confirmed by the UHPLC-MS data aquired by comparison of the retention time, UV spectrum and MS data with those of the reference substances.
For *Verbascum nigrum*, the presence of verbascoside and harpagoside was confirmed by both HPTLC and UHPLC analysis. Based on the UV spectra and mass spectrometry data, a quercetin-glucuronide and possible aucubin derivatives are identified, as well as several flavonoids.

3.2 Cytotoxicity Assay

The preliminary results of the tests performed in duplicate have shown no cytotoxic effect against the normal human HaCaT cell line for neither of the extracts tested so far (Fig. 3, 4, 5, 6). According to the statistical analysis of the data obtained the standard deviation didn’t exceed a value of 10%. Low cytotoxicity on normal human keratinocytes may be a possible indicator of reduced adverse effects.
Fig. 3. HaCaT keratinocytes viability (%) at different concentrations of crude methanolic extract of *Ajuga chia*

As seen in figure 3, the cell viability was maintained above 90% after treatment with all applied concentrations of the crude methanolic extract of *Ajuga chia*, for 24 hours.

Fig. 4. HaCaT keratinocytes viability (%) at different concentrations of crude methanolic extract of *Verbascum nigrum*
Fig. 5. HaCaT keratinocytes viability (%) at different concentrations of verbascoside

Fig. 6. HaCaT keratinocytes viability (%) at different concentrations of delipidized methanolic extract of Verbascum nigrum
4 DISCUSSION

The limits and side effects of conventional therapy of nonmelanoma skin cancer, which includes among others surgery, cryo- radio- and chemotherapy, support the search for discovery of new prophylactic and therapeutic agents from natural origin, that could offer the possibility of non-invasive, multitarget treatment options, with less side-effects.

The present study represents the basis for characterization and the evaluation of *Ajuga chia* and *Verbascum nigrum* as suitable for chemoprevention and or treatment of nonmelanoma skin cancer, taking into consideration the different antioxidant, cytotoxic and enzyme inhibitory studies achieved on species belonging to the same species, *Ajuga sp.* and respectively *Verbascum sp.*, corroborated with the available ethnopharmacological knowledge of both species.

5 CONCLUSION AND PERSPECTIVES

For the first time, we report a partial characterization of *Ajuga chia* Schreb. (Lamiaceae). More sophisticated phytochemical analysis and in vitro studies are required in the future, in order to complete the knowledge on potential natural actives for cosmetic applications and exclude the presence of adverse effective or toxic constituents.

To the extent of our knowledge, the *Verbascum* and *Ajuga* species chosen for this study, have not been researched on the possibility to prevent and/or treat nonmelanoma skin cancer. The promising screening results suggest the continuation of the research on cosmetic or dermatological actives for sun protection formulations from this natural source.

6 ACKNOWLEDGEMENTS

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7 REFERENCES


