Original Research

Marrubium vulgare ethanolic extract induces proliferation block, apoptosis, and cytoprotective autophagy in cancer cells *in vitro*

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Abstract: *Marrubium vulgare* is a European medicinal plant with numerous beneficial effects on human health. The aim of the study was to isolate the plant ethanolic extract (MVE) and to investigate its anti-melanoma and anti-glioma effects. MVE was prepared by the modified pharmacopoeial percolation method and characterized by UHPLC–LTQ OrbiTrap MS. MVE dose-dependently reduced viability of melanoma (B16) and glioma (U251) cells, but not peripheral blood mononuclear cells. It arrested cell cycle in S+G2/M phase, which was associated with the activation of MAP kinase p38 and up-regulation of antiproliferative genes *p53*, *p21* and *p27*. MVE induced oxidative stress, while antioxidants abrogated its antitumor effect. Furthermore, MVE induced mitochondrial depolarization, activation of caspase-9 and -3, Parp cleavage, phosphatidylserine exposure and DNA fragmentation. The mitochondrial apoptotic pathway was associated with the up-regulation of proapoptotic genes *Pten*, *Bak1*, *Apaf1*, and *Puma* and down-regulation of antiapoptotic genes *survivin* and *Xiap*. MVE also stimulated the expression of autophagy-related genes *Atg5*, *Atg7*, *Atg12*, *Beclin-1*, *Gabarab* and *Sqstm1*, as well as LC3-I conversion to the autophagosome associated LC3-II, while autophagy inhibitors exacerbated its cytotoxicity. Finally, the most abundant phenolic components of MVE, ferulic, p-hydroxybenzoic, caffeic and chlorogenic acids, did not exert a profound effect on viability of tumor cells, suggesting that other components individually or in concert are the mediators of the extracts' cytotoxicity. By demonstrating the ability of MVE to inhibit proliferation, induce apoptosis and cytoprotective autophagy, our results suggest that MVE, alone or combined with autophagy inhibitors, could be a good candidate for anti-melanoma and anti-glioma therapy.

Key words: *Marrubium vulgare*, melanoma, glioma, proliferation, apoptosis, autophagy.

Introduction

Malignant melanomas and gliomas are the most common skin and brain tumors, respectively, that share hallmark features such as notorious aggressiveness, high metastatic potential, and most importantly high refractoriness to the current therapies, thus causing poor prognosis and high mortality rates among patients (1, 2).

Nature is an inexhaustible source of different drugs and about 70 % of anti-cancer drugs are of natural origin (3). One of the plants with healing properties readily exploited in complementary and alternative medicine is *Marrubium vulgare* L. (white horehound, ocajnica) (4). Flowers and leaves of this plant are traditionally used for treatment of various ailments in the form of infusions or tinctures (5). *M. vulgare* belongs to the *Marrubium* genus (fam. Lamiacea), comprised of about forty species indigenous primarily to Europe, but also to northern Africa and Asia (4). The most important components of *M. vulgare*, as determined by phytochemical analysis, are polyphenols, steroids, phenylpropanoids, flavonoids and diterpenes (6). Numerous studies thus far have demonstrated that *M. vulgare* has potent biological effects such as gastroprotective, analgesic, cardioprotective, anti-diabetic, antimicrobial and anti-oxidative (6-11).

Although antitumor activity of methanolic extracts derived from *M. crassidens* and *M. persicum* and essential oil derived from *M. vulgare* (10-12) against different tumor cell lines have been demonstrated *in vitro*, anticancer activity of *M. vulgare* derived ethanolic extract (MVE)*, to the best of our knowledge, has not been investigated thus far. We here show that MVE induces proliferative arrest, cytoprotective autophagy and apoptosis in melanoma and glioblastoma cells *in vitro*.

Materials and Methods

Plant material

The aerial parts of *Marrubium vulgare* in the flowering stage were collected in July 2014 (Aleksinac, Serbia). Voucher specimen number 17113, was deposited in the Herbarium at the Institute of Botany and Botanical Garden "Jevremovac", at the Faculty of Biology, University of Belgrade, Serbia. The identification was provided by dr Snezana Vukojcic (Deputy Head of the Institute of Botany and Botanical Garden Jevremovac, University of Belgrade). The ethanolic extract was

prepared by the modified pharmacopoeial percolation method (triple percolation and drug to extract ratio of 1: 2) (Ph. Jug. IV; Ph Eur 7.0). 70 % (V/V) ethanol-aqueous solution was used as an extraction solvent. After evaporation the extract contained 4.44 % of dry matter.

UHPLC–LTQ OrbiTrap MS

Phenolic standards were prepared as previously described (13). Chromatographic separations were performed using a ultra-high-performance liquid chromatography (UHPLC) system consisted of quaternary Accela 600 pump and Accela Autosampler (ThermoFisher Scientific, Bremen, Germany). The analytical column used for separations was a Syncronis C18 Column (50 × 2.1 mm, 1.7 μm). The mobile phase consisted of (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic acid. Acetonitrile and formic acid (both of them MS grade) were purchased from Merck (Darmstadt, Germany). A linear gradient program at flow rate of 0.300 mL/min was used: 0.0-1.0 min 5 % (B), 1.0-12.0 min from 5 % to 95 % (B), 12.0-12.2 min from 95 % to 5 % (B), then 5 % (B) for 3 min. The injection volume was 5 μL. The UHPLC system was coupled to a linear ion trap-orbitrap hybrid mass spectrometer (LTQ OrbiTrap MS) equipped with heated-electrospray ionization probe (HESI-II, ThermoFisher Scientific, Bremen, Germany). The mass spectrometer was operated in negative mode. Parameters of the ion source were as follows: source voltage 4.5 kV, capillary voltage - 35 V, tube lens voltage - 100 V, capillary temperature 300 ºC, sheath and auxiliary gas flow (N_2) 32 and 7 (arbitrary units). MS spectra were acquired by full range acquisition covering 50-2000 *m/z*. Data dependant scan was performed by deploying the collision-induced dissociation for fragmentation study. The normalized collision energy of the CID cell was set at 35 eV. Phenolic compounds were quantified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation, and characteristic retention time. Xcalibur software (version 2.1) was used for instrument control, data acquisition and data analysis. Quantification was done according to the exact mass search method (± 5 ppm) by comparing the retention times and exact masses of available standards.

Cell culture

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. The human glioblastoma cell line U251 and murine melanoma cell line B16 were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of the Clinical Centre of Serbia and the Ethical Committee of the School of Medicine, University of Belgrade. Each volunteer provided a written consent for participation in the study after being informed about all the details of the study. Blood draws were conducted with syringes containing 10 % (v/v) of 3.8 % sodium citrate as an anticoagulant. PBMC were isolated by density gradient centrifugation using LymphoPrep (Axis Shield, Oslo, Norway) and immediately

used for experiments. Cells cultures were maintained and plated for experiments exactly as previously described (5, 14). After resting for 24 h, cells were treated with MVE ferulic (PubChem CID: 445858), p-hydoxybenzoic (PubChem CID: 135), caffeic (PubChem CID:689043) or chlorogenic (PubChem CID:1794427) in the presence or absence of bafilomycin A1 (PubChem CID: 6436223), wortmannin (PubChem CID:312145) (Tocris Bioscience, Bristol, UK), vitamin E (PubChem CID: 14985) or vitamin C (PubChem CID: 54670067), as described in Results and Figure legends.

Determination of cell viability

Crystal violet (CV), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and lactate dehydrogenase (LDH) release assays were used to assess cell number, mitochondrial dehydrogenase activity and cell membrane integrity, respectively, as markers of cell viability (14, 15). The results were presented as % of the control viability (CV, MTT) arbitrarily set to 100 %, or as % cytotoxicity (LDH), using Triton X-100-lysed untreated cells as a positive control.

Immunoblotting

Immunoblot detection of microtubule-associated protein 1 light chain (LC3 I) conversion to LC3 II, caspase-3 and Parp cleavage, β-actin expression levels and phosphorylation status of p38 in cell lysates were performed as previously described (14), using appropriate primary antibodies and peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody (all from Cell Signaling Technology, Cambridge, MA).

Flow cytometric analysis of apoptosis and cell cycle

Cell cycle was analyzed using DNA binding dye propidium iodide (PI), while apoptotic cell death was analyzed after double staining with Annexin V-fluorescein isothiocyanate (FITC) and PI by flow cytometry exactly as previously described (14). Activation of caspases or caspase-9 was measured by flow cytometry after labeling cells with a cell-permeable, FITC-conjugated pan-caspase inhibitor (ApoStat; R&D Systems, Minneapolis, MN) or FITC-conjugated caspase-9 inhibitor (FITC-LEHD-FMK; BioVision, Milpitas, CA), according to the manufacturer's instructions. The results of caspase activation were expressed and presented relative to the signal intensity of untreated control arbitrarily set to 1.

Measurement of ROS and mitochondrial depolarization

Intracellular production of ROS was determined by measuring the intensity of green fluorescence emitted by the redox-sensitive dye dihydrorhodamine 123 (DHR, Invitrogen, Paisley, UK), while mitochondrial depolarization was measured using DePsipher (Trevigen Inc., Gaithersburg, MD), exactly as previously described (14). The increase in green/red fluorescence ratio reflects the mitochondrial depolarization and the results were presented relative to the signal intensity of untreated control arbitrarily set to 1.

Real-time RT-PCR

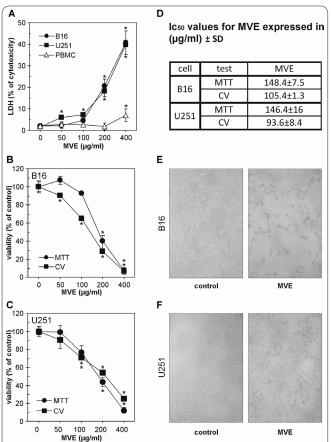RNA isolation, reverse transcription, quantitative

Figure 1. MVE exerts anti-melanoma and anti-glioma effect *in vitro*. (A-D) B16 (A, B), U251 cells (A, C) or PBMCs (A) were incubated with different concentrations of MVE for 24 h. (A) Cell cytotoxicity was determined using LDH assay. (B-D) Cell viability was determined using MTT and CV tests (B, C) and these results were used for IC_{50} calculation (D). B16 (E) and U251 (F) cells were treated with MVE (120 µg/ml) for 24 h and cell morphology was examined by inverted microscopy (20 × magnification). The results are presented as mean ± SD values of triplicates from a representative of three experiments (A, B, C) or as mean ± SD values from three independent experiments (D) (*p < 0.05 compared to untreated cells).

real-time (qRT)-PCR and data analysis were performed exactly as previously described (15) using the following TaqMan primers/probes for: mouse cyclin-dependent kinase inhibitor 1 - Cdkn1a (*p21^Cip1*; Mm00432448_m1), cyclin-dependent kinase inhibitor 1B - Cdkn1b (*p27^Kip1*; Mm00438168_m1), apoptotic peptidase activating factor 1 (*Apaf1*; 01223702_m1), p53-upregulated modulator of apoptosis (*Puma*; Mm00519268_m1), phosphatase and tensin homolog (*Pten*; Mm00477208_m1), Bcl-2 homologous antagonist killer 1 (*Bak1*; Mm00432045_m1), B-cell lymphoma 2 interacting mediator of cell death - Bcl2l11 (*Bim*; Mm00437796_m1), baculoviral IAP repeat-containing 5 - Birc5 (*survivin 40*, Mm00599749_m1), transformation related protein 53 - Trp53 (*p53*; Mm01731287_m1), X-linked inhibitor of apoptosis protein (*Xiap*; Mm00776505_m1), *Atg5* (Mm00504340_m1), *Atg7* (Mm00512209_m1), *Atg12* (Mm00503201_m1), *Beclin-1* (Mm01265461_m1), gamma-aminobutyric acid receptor-associated protein (*Gabarap*; Mm00490678_m1), *Atg4b* (Mm00558047_m1), sequestosome 1 (*Sqstm1*; Mm00448091_m1) and 18S ribosomal RNA - *Rn18S* (Mm03928990_g1) and β2-microglobulin (Mm00437762_m1) as house-keeping genes (all from Applied Biosystems, Cheshire, UK).

Statistical analysis

The statistical significance of the differences was analyzed by using t-test (two independent samples) or ANOVA (multiple comparisons).

Results

MVE exerts anti-melanoma and antiglioma effect *in vitro*

Anti-melanoma and antiglioma effects of MVE were investigated *in vitro*. MVE exerted strong cytotoxic effect on B16 melanoma and U251 glioma cells in a dose-dependent manner, while PBMCs isolated from healthy volunteers were almost insensitive, as demonstrated by LDH test (Fig. 1A). Accordingly, results from MTT and CV tests showed that MVE decreased the viability of both tumor cell lines (Fig. 1B, C), with IC_{50} values approximately 120 µg/ml (Fig. 1D) (used in further experiments). In agreement with the results of viability tests, MVE decreased the number and size of both B16 (Fig. 1E) and U251 (Fig. 1F) cells, concomitantly causing cell rounding and size decrease. Importantly, similar results were observed with MVE and extract dissolved in DMSO (results not shown). Collectively, these observations suggest that MVE exerts antitumor effect *in vitro*.

MVE induces apoptosis and inhibits proliferation of B16 and U251 cells

We next investigated which type of cell death was induced by MVE. FACS analysis demonstrated that plant extract caused an increase in the percentage of both early (Annexin⁺PI⁻) and late apoptotic cells (Annexin⁺PI⁺) in B16 (Fig. 2A) and U251 cells (data not shown). Also, cell cycle analysis showed that MVE caused DNA fragmentation in the time dependent manner (Fig. 2B, C), as well as a significant increase in the number of cells in S+G2/M phase in both tumor cell lines (Fig. 2B, D). Therefore, these results suggest that the antimelanoma and anti-glioma activity of MVE is mediated by both proliferation arrest and induction of apoptosis.

MVE-induced apoptosis is associated with activation of caspases, up-regulation of proapoptotic and down-regulation of antiapoptotic genes

We further dissected molecular mechanisms responsible for antiproliferative/proapoptotic activity of MVE. Employing flow cytometry based techniques using fluorescently labelled pan-caspase inhibitor or caspase-9 specific inhibitor, we found that MVE, indeed, caused the activation of caspases (Fig. 3A, B) more specifically, the activation of the initiator caspase-9 (Fig. 3C, D) in both B16 and U251cells in a time dependent manner, as shown by the shift of the corresponding fluorescence peaks compared to control. MVE induced apoptosis was also associated with the activation of effector caspase-3 and cleavage of its substrate Parp (Fig. 3E, F). In addition, the plant extract caused an increase in the p38 MAP kinase activity (Fig. 3G), up-regulated the expression of proapoptotic genes (*Apaf1*, *p53*, *Bak1* and *Puma*) and cell cycle inhibitors (*p21* and *p27*) and down-regulated the expression of antiapoptotic genes *survivin* and *Xiap* (Fig. 3H). Therefore, MVE probably inhibits tumor cell proliferation through *p53*, *p21* and *p27* mediated cell
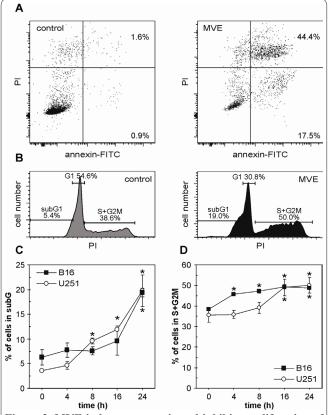
Figure 2. MVE induces apoptosis and inhibits proliferation of B16 and U251 cells. B16 (A-D) or U251 cells (C, D) were incubated in the presence of 120 µg/ml MVE for 24 h (A, B) or indicated time periods (C, D). The cells were stained with Annexin-FITC/ PI (A) or PI (B-D) and the flow cytometric analyses of phosphatidylserine externalization and membrane damage (A) or cell cycle (B-D) were performed. The dot plots (A) or histograms (B) from a representative of three independent experiments are presented, and the results presented in (C, D) are mean ± SD values from three independent experiments (*p < 0.05 compared to untreated cells).

cycle arrest, and concomitantly activating caspase dependent apoptosis through the stimulation of proapoptotic genes *Apaf1*, *Bak1* and *Puma*, as well as through the repression of prosurvival genes *Xiap* and *survivin*. Collectively, MVE-induced apoptosis is associated with activation of caspases, up-regulation of proapoptotic and down-regulation of antiapoptotic genes.

MVE induces oxidative stress and mitochondrial depolarization

We next investigated if MVE induced oxidative stress and mitochondrial depolarization which were usually associated with the apoptotic cell death and proliferative block (16). MVE indeed induced strong and rapid mitochondrial depolarization in glioma cells reaching its maximum after 8 h and afterwards gradually declining (Fig. 4B); whereas in melanoma cells mitochondrial depolarization was steadily increasing over 16 h (Fig. 4A, B). This was accompanied by a significant time-dependent increase of ROS production in both cell lines (Fig. 4C, D), while antioxidants vitamin E and vitamin C partly rescued tumor cells from MVE induced cytotoxic/antiproliferative effect (Fig. 4E, F). Altogether, these results showed that the antitumor effect of MVE is mediated by oxidative stress and mitochondrial damage.

MVE induces cytoprotective autophagy in tumor cells

Having ascertained that MVE induces apoptosis, we next investigated its ability to stimulate autophagy, a survival mechanism, which under certain conditions could also be a programmed cell death type II (17). Immunoblot analysis demonstrated an increased conversion of LC3-I isoform to the autophagosome-associated LC3-II, in both B16 (Fig. 5A) and U251 cells (data not shown), which was associated with a significant up-
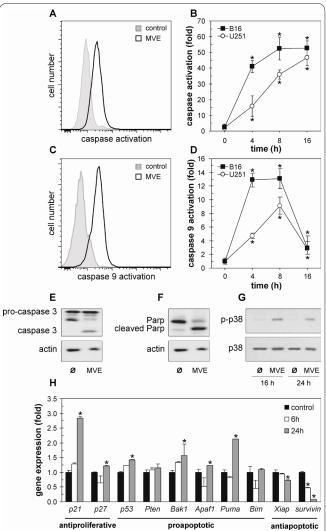

Figure 3. MVE induced apoptosis is associated with activation of caspases, up-regulation of proapoptotic and down-regulation of antiapoptotic genes. B16 (A-H) or U251 cells (B, D) were incubated in the absence or presence of 120 µg/ml MVE. After 8 h (A, C) or indicated time periods (B, D) the cells were stained with Apostat (A, B) or LEHD-FITC (C, D) and caspase (A, B) or caspase-9 activation (C, D) were determined by flow cytometry. Representative histogram plots of the indicated FITC-inhibitor staining (A, C) after 8 h of stimulation and the time courses of activation of the relevant caspases (B, D) are shown. The results are presented as a mean ± SD of fold increase in the amount of fluorescence relative to the untreated cells. After 24 h (E, F) or indicated time periods (G), cleavage of caspase-3 (E) and Parp (F) or the levels of phosphorylated/total p38 (G) were determined by immunoblotting and the blots from a representative of three independent experiments are presented. (H) After indicated time periods the expression of mRNA for antiproliferative, proapoptotic and antiapoptotic genes was analyzed by qRT-PCR. The results are mean ± SD values of triplicate measurements from a representative of three experiments (H) (*p < 0.05 compared to untreated cells).
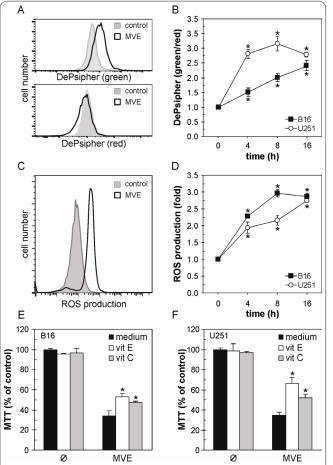
Figure 4. MVE induces oxidative stress and mitochondrial depolarization. B16 (A-E) or U251 cells (B, D, F) were incubated with 120 µg/ml MVE alone (A-D), or MVE in combination with vitamin E (50 µM) or vitamin C (50 µM) (E, F). After 8 h (A, C) or indicated time periods (B, D) cells were stained with DePsipher (A, B) or DHR (C, D), and mitochondrial depolarization (A, B) and ROS production (C, D) were examined by flow cytometry. (E, F) After 24 h cell viability was determined by MTT test. The results are mean ± SD values of three independent experiments (B, D) or triplicate measurements from a representative of three experiments (E, F) (*p < 0.05 compared to untreated cells).

Figure 5. MVE induces cytoprotective autophagy in tumor cells. B16 (A-C) or U251 cells (D) were incubated with 120 µg/ml MVE alone (A, B), or in combination with bafilomycin A1 (BAF, 20 nM) and wortmannin (WM, 200 nM) (C, D). After indicated time periods (A, B) or 24 h (C, D), the intracellular levels of LC3-II were determined by immunoblotting (A), the expression of Atg genes was analyzed by qRT-PCR (B), while cell viability was determined by LDH test (C, D). The representative blots from three independent experiments are shown (A). The results are mean ± SD values of three independent experiments (A) or triplicate measurements from a representative of three experiments (B-D) (*p < 0.05 compared to untreated cells).

regulation of autophagy-related (ATG) genes *Beclin-1, Atg5*, *Atg7*, *Atg12, Sqstm1* and *Gabarap* (17) (Fig. 5B). Since autophagy inhibitors bafilomycin A1 and wortmannin, applied at nontoxic concentrations, potentiated MVE toxicity (Fig. 5C, D), we suggest that MVE induces cytoprotective autophagy in both cell lines.

The most abundant phenolic components of MVE, are not responsible for its antitumor effect

Finally, we investigated the composition of the plant extract. Mass spectrometry analysis showed that MVE contains numerous phenolic compounds, among which ferulic, p-hydroxybenzoic, caffeic and chlorogenic acids were the most copious (Fig. 6A, B). However, these phenolic components on its own, even at higher doses than present in the extract, only slightly affected the viability of both U215 (Fig. 6C and D) and B16 cells (data not shown). Therefore, the most abundant phenolic components of MVE, are not the main mediators of the extract cytotoxicity.
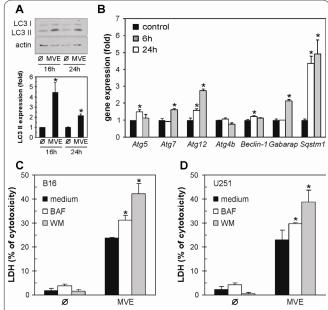
Discussion

The present study has shown strong cytotoxic effects of *M. vulgare* ethanolic extract on cancer cell lines, B16 melanoma and U251 glioma, while exerting minimal effects on primary immune cells (PBMCs). Although several studies revealed the ability of methanolic extracts or essential oils derived from plants belonging to the *Marrubium* genus to exert antitumor effects against different tumor cells (10-12), the molecular mechanisms of this activity have not been investigated thus far. We here demonstrated that mechanisms driving anticancer effects in both tumor cell lines were similar and involved induction of oxidative stress, accompanied by mitochondrial depolarization and caspases activation, finally leading to the execution of the intrinsic apoptotic program. In addition, MVE blocked the progression through the cell cycle causing an S+G2/M phase arrest and induced cytoprotective autophagy. These effects were associated with the modulation of expression of key regulators of apoptosis, cell cycle and autophagy.

Dysregulated ROS production in association with the mitochondrial membrane damage may cause apoptosis, cell cycle arrest and autophagy (16). MVE, indeed, induced massive oxidative stress, while its proapoptotic/antiproliferative effect was prevented by antioxidants. Interestingly, *M. vulgare* methanolic extract exhibited strong antioxidative effect in lipid peroxidation assay (9) and isoproterenol-induced acute myocardial infarction (8). This discrepancy could be explained by the differences in composition of methanolic and ethanolic extract of *M. vulgare*, as well as different experimen-
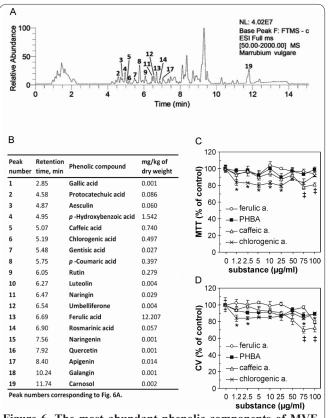
Figure 6. The most abundant phenolic components of MVE, are not responsible for the MVE antitumor effect. Analysis of phenolic content of MVE was performed using UHPLC–LTQ OrbiTrap MS and phenolic standards. The most prominent components are shown in HPLC profile (A) and summarized in the table (B). U251 cells were incubated with different concentrations of ferulic, p-hydroxybenzoic (PHBA), caffeic or chlorogenic acids and cell viability was determined after 24 h by MTT (C) and CV (D) tests. (C, D) The results are mean ± SD of triplicate measurements from a representative of three experiments (*$p < 0.05$ for chlorogenic, ‡ $p < 0.05$ for caffeic acid compared to untreated cells).

Peak number	Retention time, min	Phenolic compound	mg/kg of dry weight
1	2.85	Gallic acid	0.001
2	4.58	Protocatechuic acid	0.086
3	4.87	Aesculin	0.060
4	4.95	p-Hydroxybenzoic acid	1.542
5	5.07	Caffeic acid	0.740
6	5.19	Chlorogenic acid	0.497
7	5.48	Gentisic acid	0.027
8	5.75	p-Coumaric acid	0.397
9	6.05	Rutin	0.279
10	6.27	Luteolin	0.004
11	6.47	Naringin	0.029
12	6.54	Umbelliferone	0.004
13	6.69	Ferulic acid	12.207
14	6.90	Rosmarinic acid	0.057
15	7.56	Naringenin	0.001
16	7.92	Quercetin	0.001
17	8.40	Apigenin	0.014
18	10.24	Galangin	0.001
19	11.74	Carnosol	0.002

Peak numbers corresponding to Fig. 6A.

tal settings applied here and in the two aforementioned studies (8, 9).

MVE-induced oxidative stress was associated with the strong activation of MAP kinase p38. While p38 can have proapoptotic, cytoprotective, antiproliferative (18) and autophagy promoting effects (19), its exact role in antitumor activity of the plant extract remains to be determined. However, in agreement with the previously shown ability of p38 to induce p53 gene expression (20), MVE treatment increased *p53* mRNA levels. Apart from its proapoptotic activity, p53 in cooperation p21 and p27 is found to arrest cell cycle in S+G2M phase (21). Accordingly, MVE induced S+G2M phase arrest indeed was associated with the increased expression of *p53*, *p21* and *p27* genes.

Furthermore, p53 target genes and members of Bcl2 protein family *Bak1* and *Puma*, are important for the mitochondrial pore opening (22, 23). The subsequent release of cytochrome c from permeabilized mitochondria into the cytosol triggers activation of caspase-3 via formation of the cytochrome c/Apaf-1/caspase 9-containing apoptosome (24). Accordingly, we here demonstrated that MVE treatment up-regulated *Bak1* and *Puma* expression and presumably activated apoptosis via mitochondrial pathway, since it caused the activation of caspase-9 and its target caspase-3, as well as the

cleavage of Parp, a target of caspase-3 involved in DNA repair (25). Finally, these findings were associated with the decreased expression of caspase-9 inhibitors *Xiap* and *survivin* (26). Survivin down-regulation should have therapeutic importance, since its expression is found to be associated with the drug-resistant melanoma phenotype (27) and it is inversely correlated with the survival rate of glioma patients (28).

This study has shown autophagy promoting activity of MVE. In addition to the increased expression of *Beclin-1*, *Atg5*, *Atg7*, *Atg12*, involved in autophagy induction, and conversion of LC3, necessary for autophagosome formation, MVE also stimulated expression of *Sqstm1* and *Gabarap*, which serve to link protein cargo to the autophagic machinery (29). Apart from its cytotoxic effect, autophagy may also be a protective mechanism under stress conditions (30). Accordingly, in our study, both wortmannin, inhibitor of autophagosomes formation and bafilomycin A1, which blocks autophagosome/lysosome fusion and lysosomal acidification (17), increased the cytotoxicity of MVE, implying the protective role of autophagy. These results implicate that antimelanoma and antiglioma activity of MVE could be potentiated by the simultaneous application of autophagy inhibitors.

The most prominent phenolic component of MVE identified in this study is ferulic acid which has been recognized as a potential treatment for many disorders including Alzheimer's disease, cardiovascular diseases, diabetes mellitus and cancer (31, 32). Even though phenolic phytochemicals are known anti-oxidants which remove the excess of H_2O_2 necessary for thriving of cancer cells, they may also induce oxidative stress that is lethal to the cancer cells (33). In line with that, ferulic acid was shown to exert strong antitumor activity against breast (34) and liver cancer cells (35), however, extreme concentration of this phenolic phytochemical was necessary to inhibit growth of melanoma (36) and glioma cells (37). Similarly, we here presented that when used alone, ferulic, p-hydroxybenzoic, caffeic and chlorogenic acids had only a moderate effect on the survival of glioma U251 and B16 melanoma cells. Hence, it seems that either their combination or other extract components not investigated in this study mediate observed cytotoxicity. It is well known that different extract compounds can modulate unrelated signaling pathways and thus produce a synergistic effect (38). Therefore, although the complexity of natural products is a major obstacle for their broad acceptance in Western medicine, our results support the idea that this complexity could actually be a great advantage. Finally, it is important to underline that immune cells were virtually insensitive to cytotoxic effect of MVE.

By demonstrating the ability of *Marrubium vulgare* ethanolic extract to inhibit proliferation and to induce apoptosis and cytoprotective autophagy, our results suggest that this extract, alone or combined with autophagy inhibitors, could be a valuable candidate for therapy of the highly resistant and refractory tumors such as melanoma and glioblastoma.

Acknowledgements

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