Academy of Romanian Scientists *Volume 7, No. 2, 2018, pp. 99 – 111*
Annals Series on Biological Sciences ISSN 2285 – 4177

Grape Pomace and Red Clover Extracts Modulate the Proliferative Response of Murine Melanoma Cells

Brandusa G. DUMITRIU[1,4], Diana M. ENE[2,] Laura OLARIU[3,4], Natalia ROSOIU[4,6], Luiza M. CRĂCIUN[5,6], Abdi ADIL[6], Gina MANDA[7], Toma PAPACOCEA[8]

[1] *SC Biotehnos SA, 3-5 Gorunului Street, 075100-Otopeni, Ilfov, Romania*
[2] *Academy of Romanian Scientists, 54 Splaiul Independentei 050094, Bucharest, Romania.*
[3]*The Doctoral School of Applied Sciences, Ovidius University, Constanta, Romania.*
[4] *INCD Victor Babes Bucharest*
[5] *UMF Carol Davila Bucharest*

Coressponding author: Brandusa G. Dumitriu, *SC Biotehnos SA, 3-5 Gorunului Street, 075100-Otopeni, Ilfov, Romania (dbrandusa@biotehnos.com).*

Abstract

Our experimental design is focused on a cellular screening on murine melanoma standardized line B16-F10, in order to reveal the cytotoxic profile correlated with anti-proliferative and pro-apoptotic effects of two vegetal extracts, alone and associated. The grape pomace is the residue from the winemaking process, an important source of polyphenolic compounds, Trifolium pratense extract (red clover), rich in isoflavones (genisteine, daidzeine, biochanine, formononetin), had previously proved impact in the estrogenic modulation of different cancer types. It has been proved through flow cytometry and spectrophotometry methods that grape pomace and red clover extracts are apoptosis inducers and anti-proliferative agents in murine melanoma cells. Furthermore their combinations inhibit melanoma progression. Their combination could be an adjuvant solution in melanoma therapy, raising the anti-tumour efficacy. This research is a step forward to sustain the proper capitalisation of winery waste as well as the red clover as medicinal herb, including their use as a part of new developed delivering systems (ex. Nanosystems).

Keywords: grape pomace, Trifolium pratense, melanoma, proliferation, apoptosis.

Introduction

Melanoma is a highly invasive skin cancer, with an exponential mortality rate, its progression being often associated with oxidative stress. Free radicals trigger lipid peroxidation, oxidation of proteins, playing an important role in tumour development due to DNA strand braking, appearance of point mutations and aberrant DNA cross-linking, as well as changing in the proliferation potential [1]. Many therapeutically approaches for tumours are available, but the problem of multi-drug resistance leads to relatively low success rates of treatments. The safety and effectiveness against melanoma remain a question to be solved or at least improve through applied research, discovering new compounds or combinations that meet these demands. Developing in vitro tests of melanoma cell lines and murine melanoma models leads to identify mechanisms of action and characterised new compounds and extracts. Many anti-cancer drugs are derived from natural resources such as marine, microbial and botanical sources. Most important anti-melanoma effects of natural compounds include potentiating apoptosis, inhibiting cell proliferation and inhibiting metastasis [2]. We could mention the phytochemical pigments of natural products involved in the redox system; phytophenolics also prevent cancer cell proliferation and progression. *Nymphaea stellate* extract was toxic to B16 melanoma cells with IC50 = 814 μg/ml. The extract at 800 and 1,000 μg/ml demonstrated pro-oxidant activity related to the cell apoptosis. The low concentrations of the extract at 200 and 400 μg/ml showed the anti-oxidant function associated with the inhibitory effect of melanoma cell invasion [3]. The grape seed extract, rich in the bioflavonoids commonly known as procyanidins, inhibits growth and induces apoptotic death of human prostate cancer cells in culture and in nude mice. It causes mitochondrial damage leading to cytochrome c release in cytosol and activation of caspases resulting in PARP cleavage and execution of apoptotic death [4]. Soy isoflavones, genistein and daidzein, exhibiting estrogenic, anti-estrogenic and/or tyrosine kinase inhibitory activity, modify the dendritic morphology of B16 mouse melanoma cells [5]. Likewise, genistein inhibits in vitro the growth of a number of tumour cell lines, including B16 melanoma cells, and retards the growth of implanted tumours [6].

B16 melanoma is one of the very few pigmented melanoma lines available for use in mice, although recently transgenic models have been developed in which the reliable incidence of melanoma allows the establishment of new lines. It was successfully used to study skin tumour mechanisms relevant for humans, due to its relevance and good predictability [7, 8, 9].

Our experimental design is focused on a cellular screening on murine melanoma standardized line B16-F10, in order to reveal the cytotoxic profile correlated with anti-proliferative and pro-apoptotic effects of two vegetal extracts, alone and associated. The grape pomace is the residue from the winemaking process (shells, seeds, etc.) and is an important source of polyphenolic compounds (hydroxycinnamic acid and hydroxybenzoic acid derivatives, flavonoids, anthocyanins, tannins, proanthocyanidins). Its antioxidant and anti-inflammatory effects in stimulated normal cells) were proved [10], interesting aspects waiting to be discovered on grape marc impact in tumour development, particular on melanoma cells. Its potency could be increased in association with another extracts/ active complexes acting synergistically. From the multitude of natural bioactive compounds, we chose in this study the Trifolium pratense extract (red clover), rich in isoflavones (genisteine, daidzeine, biochanine, formononetin), with previously proved impact in estrogenic modulation of different cancer types [11, 12].

Materials and methods

Materials

Natural compounds:

Trifolium pratense extract – ET, containing the following isoflavones: *Daidzeine=1,125mg%mL;Genisteine=3,98mg%mL;Biochanine=12,65mg%ml; Formononetin=22.63mg%ml.* A 1:5 dilution of ET extract was used for the combinations with TES extract and a 1:10 dilution for simple extract.

Grape pomace extract – TES, containing important amounts of polyphenolic compounds, was prepared as follows: the crushed and dried grape marc powder was solubilised in water at a concentration of 100 mg/ml, then centrifuged for 15 minutes at 3500 rpm. The supernatant obtained was used in experiments as such or combined with clover extract thus:

Combination A: ET:TES= 1:9
Combination B: ET:TES= 1:4
Combination C: ET:TES= 1:3
Positive control: Methotrexate 3μM

Cell cultures:

B16-F10 (ATCC® CRL-6475™) Cells were cultured in DMEM with 10% foetal bovine serum, 1% antibiotic/antimicotic solution, under standard culture conditions (37°C, 95% humidified air and 5% CO_2), harvested 24h before treatment, 48 h with tested substances. Sub-confluent cells (70-80%) were split 1:3 to 1:10, seeding 1-3x10 000 cells / cm^2 using 0.25% trypsin or trypsin/EDTA.

Chemicals and reagents:

- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) kits - for the determination of metabolic activity (MTS) and
- CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega - for the release of lactate dehydrogenase (LDH) in extracellular medium.
- Cell Trace CFSE Cell Proliferation Kit (Invitrogen)
- Cycle TEST PLUS DNA Reagent (BD PHARMINGEN)
- ANNEXIN V - FITC apoptosis detection Kit (BD PHARMINGEN)

Equipment:

Flow cytometer FACS CANTO II with DIVA 6.1 and FCS Express software; Plate Reader-Berthold Technologies.

Methods

a) Evaluation of the cytotoxic effect

The cytotoxic effect was evaluated on cells exposed to increasing concentrations of the extracts and combinations of extracts by the correlation between decreased cell viability (MTS test) and enhancing lactate dehydrogenase activity in the culture medium as a toxicity marker (LDH assay).

Reduction of MTS (tetrazolium salt), cell viability marker: - evaluation of oxidative metabolism as response of cells to external factors (compounds) that may affect their life in culture. The conversion of MTS to water-soluble formazan measured as absorbance at 490 nm occurs under the action of dehydrogenases found in metabolically active cells [13, 14].

Release of lactate-dehydrogenase in cell medium, cytotoxicity marker: Lactate dehydrogenase (LDH) is a cytosolic enzyme present in all cells which is extracellular released when cell membrane integrity is affected by environmental

toxical compounds. The amount of LDH in the cell culture supernatant is measured by a test in which two coupled enzymatic reactions catalyzed by LDH and diaphorase are carried out which result in the conversion of a tetrazolium salt into a red formazan compound [15].

b) Apoptosis evaluation through phosphatidil serine translocation / propidium iodide double staining: In apoptotic cells, phosphatidil serine (PS) - normally found on the internal part of the membrane - is externalised. Annexin V-FITC bounds phosphatidil serine from the membrane of cells which began the apoptotic process. Propidium iodide stains the cellular DNA in cells where the cell membrane has been totally compromised (late apoptosis). Annexin V-FITC is detected through *flow cytometry* as green fluorescence; PI is detected as red fluorescence.

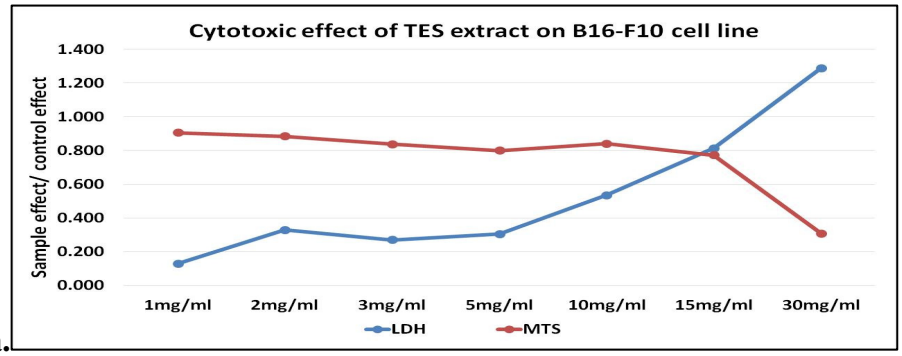
c) ***DNA staining for flow cytometry cell cycle analysis:*** Propidium iodide is stoichiometrically bound to melanoma nuclei; stained nuclei emit fluorescent light primarily at wavelengths between 580 and 650nm, detected by *Flow cytometry*.

d) ***Cell proliferation assay – flow cytometric analysis of cell division – by dye dilution*** (1µM CFSE - carboxy fluorescein diacetat succin imidil ester standing): Upon cell division, the dye is distributed equally between daughter cells, allowing the resolution of cycles of cell division by flow cytometry [16, 17]. Results are presented by calculating through FacsExpress software the proliferative index (PI).

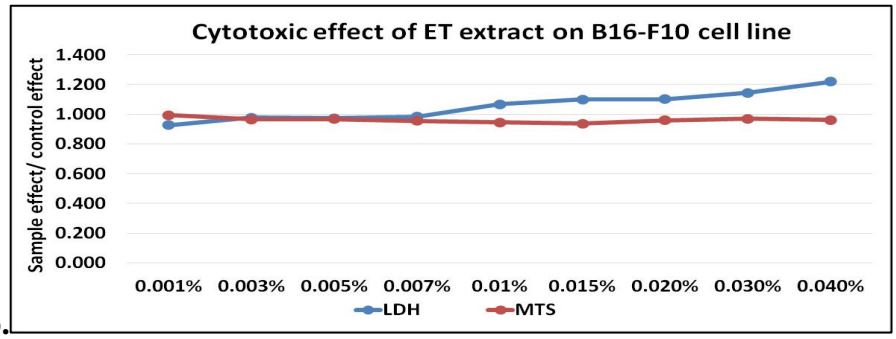
RESULTS AND DISCUSSIONS

Evaluation of the cytotoxic effect of ET, TES and combinations A,B,C on murine melanoma cell culture:

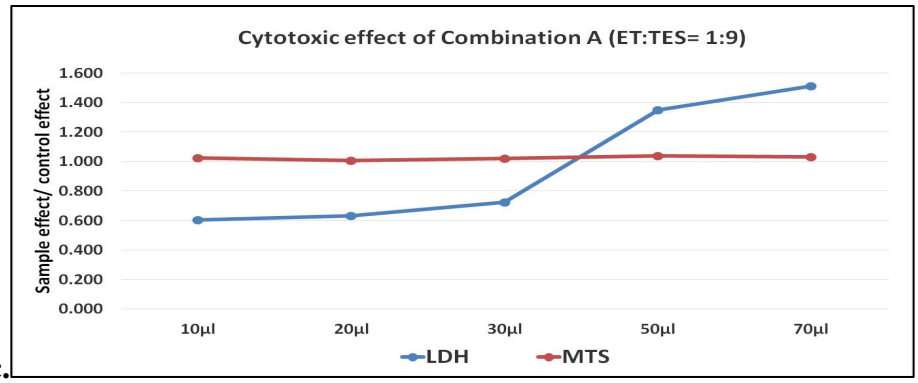
a.



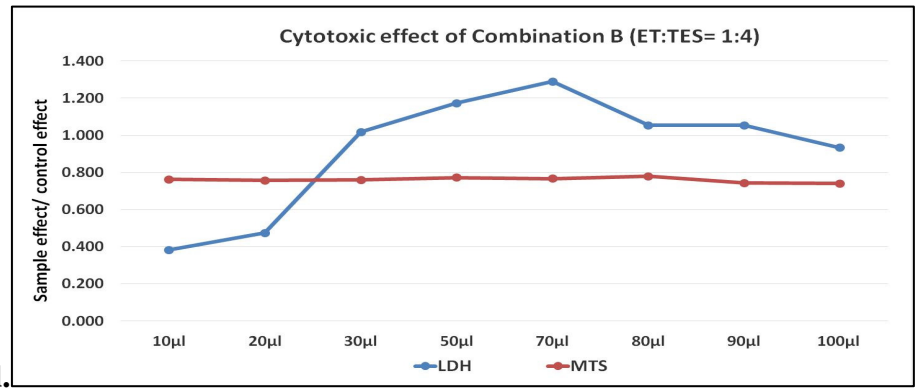
b.



c.



d.



e.

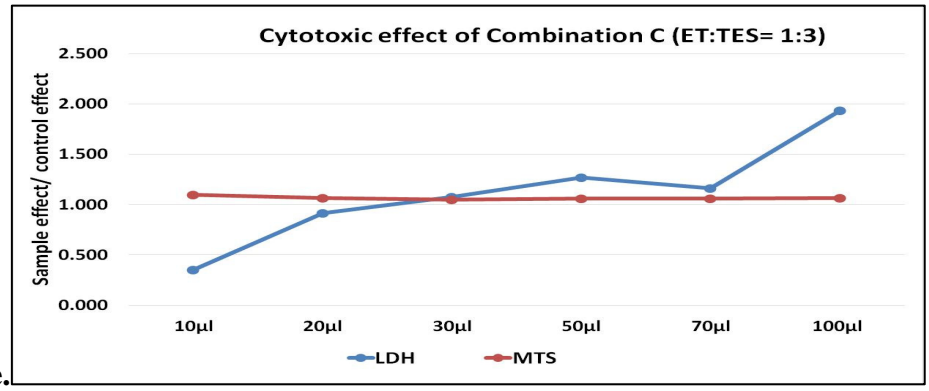


Fig. 4. Evaluation of the cytotoxic potential of TES (a.), ET (b.), combinations A (c.), B (d.), and C (e.) revealed by MTS and LDH assays on B16-F10 melanoma cell line. The cells were allowed to adhere for 24h (7000 cells / well) and treated for 48 hours with the test substance according to the protocol for the specific reagent kit (MTS / LDH). All concentrations of active principle were tested in triplicate.

In Fig. 1 the active dose for all tested samples can be extracted as follows: the TES extract shows a cytotoxicity threshold at a dose of 15 mg / ml, increasing the amount of lactate dehydrogenase released in the medium, while reduction of MTS decreases indicating a loss of cell membrane integrity and decreased cell viability. The ET complex shows a cytotoxic effect starting at a concentration of 0.001%. Under these doses, there is a superior ratio (sample / control effect > 1) of metabolic activity evaluated by MTS technique reported, suggesting an activation of cellular metabolism induced by extract components. In the case of cells treated with combination A, the cytotoxicity threshold is of 20 µl/ ml culture medium, while combinations B and C show a cytotoxic effect starting at a 20µl dose / ml medium.

Apoptotic process in murine melanoma B16-F10 cells.

Apoptosis plays a central role in the prevention of melanoma development as well as in the responsiveness to current treatment options for metastatic disease. Impaired apoptosis execution could result in treatment-induced selection for cell populations with increased stress tolerance, leading to the recurrence of tumours, an increased resistance to apoptosis being a hallmark feature of cancer [18]. We assessed the cellular impact of compounds on early and late apoptosis of melanoma cells (flow cytometry double staining with annexin/ Propidium iodide). The cells were adhered 24h, treated 48h with TES, ET and combinations (TES+ET). Positive control used was **Methotrexate 3µM**, an anti-proliferative agent in cancers and immune disorders. The results are presented in Table 1, highlighting the effective response in green and representative flow cytometry images in Fig. 2.

Table 1: *Apoptosis induction in melanoma cell line B16-F10 by TES and ET alone and associated*

	Live cells (%Parent)	Early apoptosis (%Parent)	Late apoptosis (%Parent)	Necrosis (%Parent)	% Total apoptosis	%variation apoptosis
Control cells	**79.1**	**8.8**	**10.8**	**1.3**	**19.6**	**100**
Solvent control	**78.6**	**7.4**	**12.4**	**1.7**	**19.8**	**100**
ET 0.02%	**67.8**	**12.4**	**17**	**2.8**	**29.4**	**148.48**
ET 0.01%	**67.9**	**15.4**	**14.8**	**1.9**	**30.2**	**152.53**
ET 0.006%	**69.8**	**10.6**	**17.4**	**2.2**	**28**	**141.41**
TES 2mg/ml	**75.2**	**7.2**	**14.7**	**2.9**	**21.9**	**111.73**
TES 4mg/ml	**66.8**	**16.7**	**15.4**	**1.1**	**32.1**	**163.78**
TES 8mg/ml	**67.3**	**16.9**	**14.1**	**1.7**	**31**	**158.16**
Combination A 0.2%	**69.5**	**14.4**	**14.3**	**1.8**	**28.7**	**146.43**
Combination A 0.1%	**69.2**	**15.6**	**13.7**	**1.4**	**29.3**	**149.49**
Combination B 0.2%	**73.9**	**12.9**	**11.8**	**1.5**	**24.7**	**126.02**
Combination B 0.1%	**71.3**	**13.8**	**13.8**	**1.1**	**27.6**	**140.82**
Combination C 0.2%	**70.2**	**14.1**	**13.8**	**1.9**	**27.9**	**142.35**
Combination C 0.1%	**67.3**	**18.9**	**12.6**	**1.2**	**31.5**	**160.71**
Methotrexate 3µM	**60.9**	**38.1**	**1**	**0**	**39.1**	**199.49**

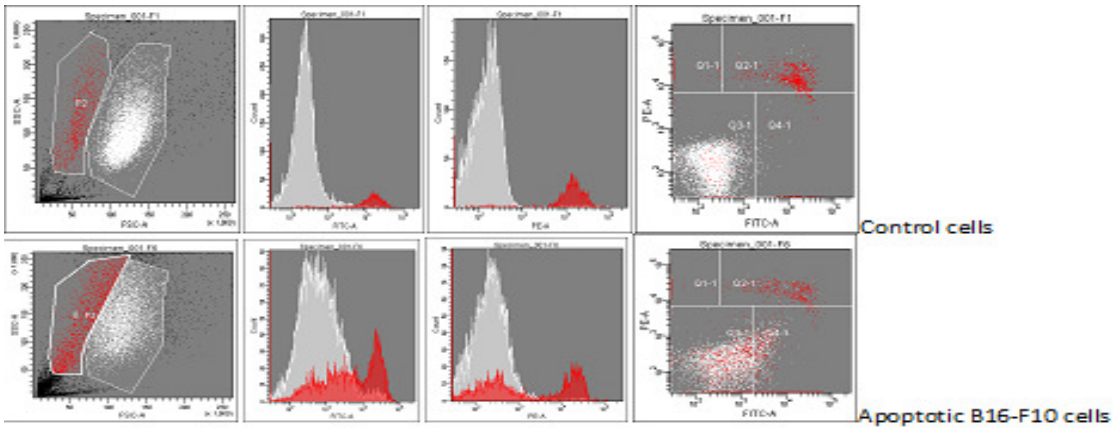


Fig. 1: *B16-F10 cells early (FITC- positive) / late apoptosis (FITC and PE-positive) detection through flow cytometry*

ET (0.02% - 0.006%) and TES (4-8mg/ml) induce apoptosis in murine melanoma cells, the total apoptotic cells rising with approximate 50% compared with the control cells. The two extracts combination also acts as apoptosis inductors (40% - 60% compared with the control), without a significant enhancing effect.

Proliferative status (cell cycle sequentiation and succession of proliferative generations) in murine melanoma B16-F10 cells.

The cell cycle of proliferation encompasses a sequential series of biochemical events leading to DNA replication and cell division [19]. After mitosis completion, the cell enters the G_1-phase, the DNA replication took place during the S-phase; after genetic material duplication the cell enters the G_2-phase that ends the process required for division. At mitosis the cell divides into two daughter cells which possibly re-enter the G_1-phase for reinitiating another cell cycle. Chemotherapeutic action on melanoma cells are mainly based on anti-proliferative effects, usually expressed by cell cycle arrest in one of multiplication phases: S and G2/M.

B10-F16 melanoma cells were seeded and grown for 24h before the incubation for another 48h-period with extracts and combinations of extracts. After harvesting, the cells were stained for cell cycle analysis through flow-

cytometry. For successive proliferative generation quantification, cells were stained with CFSE before the seeding on plate, were treated and analyzed after 48h incubation with active substances. The results are shown in Table 2.

Both extracts, **ET (0.02% - 0.006%) and TES (4-8 mg/ml)** decrease the proliferation index of murine melanoma and the percent of cells in S-phase (DNA replication), ET being more active than TES. All the complementary methods used describe the same effect: a rehabilitation of proliferative status, previously augmented by malignisation process. Two essential mithosis phases (S+G2/M) are slowed down, leading to less dividing generations of cells. The combinations TES+ET preserve the anti-proliferative actions of both components, with a special mention for combination B.

Table 2: *Proliferative status of melanoma cell line B16-F10 modulated by TES and ET alone and associated*

% of cell cycle phases distribution	%G0/G1	%S	%G2/M	%S+ %G2/M	% decreasing S+G2/M	Proliferative Index (PI)	% decreasing PI
Control cells	69.58	23.23	7.18	30.41	0	3.23	0.00
Solvent control	68.63	26.9	4.48	31.38	0	3.49	0.00
ET 0.02%	73.54	21.89	4.57	26.46	15.68	2.62	24.93
ET 0.01%	76.98	20.45	2.57	23.02	26.64	2.26	35.24
ET 0.006%	77.19	20.07	2.74	22.81	27.31	1.75	49.86
TES 2mg/ml	69.59	24.01	6.04	30.05	1.18	3.03	6.19
TES 4mg/ml	73.11	25.9	0.99	26.89	11.58	2.57	20.43
TES 8mg/ml	73.87	20.03	6.1	26.13	14.07	2.18	32.51
Combination A 0.2%	75.27	17.74	7	24.74	18.65	1.89	41.49
Combination A 0.1%	77.13	18.43	4.4	22.83	24.93	2.47	23.53
Combination B 0.2%	75.71	18.81	5.48	24.29	20.12	1.96	39.32
Combination B 0.1%	78.7	17.61	3.69	21.3	29.96	1.71	47.06
Combination C 0.2%	74.37	19.47	6.16	25.63	15.72	1.77	45.20
Combination C 0.1%	73.96	17.12	8.92	26.04	14.37	1.86	42.41
Methotrexate 3µM	77.31	21.09	1.61	22.7	25.35	1.3	59.75

Conclusions

Grape pomace and red clover extracts are apoptosis inducers and anti-proliferative agents in murine melanoma cells. Additionally, their combinations inhibit melanoma progression. The concerted action on apoptosis and cell cycle arrest / proliferation inhibition is sustained by the complexity of active principles (isoflavones in ET, anthocyanins in TES, etc) from both vegetal extracts.

Together with previously demonstrated actions (ET as estrogenic modulator, TES as an antioxidant and anti-inflammatory agent), their combination could be an adjuvant solution in melanoma therapy, rising the anti-tumour efficacy.

This research is a step forward to sustain the proper capitalisation of winery waste as well as the red clover as medicinal herb, including their use as a part of new developed delivering systems (e.g. Nanosystems). Future studies had to explore key factors supporting mechanisms or signalling pathways related to proliferative status regulation.

Acknowledgment

The study was performed as part of the AOSR project: "*Capitalization in industry of residues from winemaking as food additives and antioxidants*" and of the 55/2016 project: *"Advanced theranostic approach in cancer combining photodynamic therapy and nanoparticles"*, as part of M-Era.Net Topic 5 "Tailoring of bioactive material surfaces for health applications"

References

[1] Papacocea T., Papacocea R., Bădărău A., Ion A.D., Buraga I., Gaman L., Papacocea A., Oxidative Stress and Antioxidants in Brain Tumors, Therapeutics, Pharmacology and Clinical Toxicology, 2011, 15, 234-239.

[2] Chinembiri T.N., du Plessis L.H., Gerber M., Hamman J.H., du Plessis J., Review of Natural Compounds for Potential Skin Cancer Treatment, Molecules, 2014, 19(8),11679-721.

[3] Aimvijarn P., Palipoch S., Okada S., Suwannalert P., Thai Water Lily Extract Induces B16 Melanoma Cell Apoptosis and Inhibits Cellular Invasion

Through the Role of Cellular Oxidants, Asian Pac J Cancer Prev, 2018, 19 (1), 149-153.

[4] Agarwal C., Singh R.P., Agarwal R., Grape Seed Extract Induces Apoptotic Death of Human Prostate Carcinoma DU145 Cells via Caspases Activation Accompanied by Dissipation of Mitochondrial Membrane Potential and Cytochrome c Release, Carcinogenesis, 2002, 23(11), 1869-76.

[5] Miyazaki K., Novel Approach for Evaluation of Estrogenic and Anti-estrogenic Activities of Genistein and Daidzein Using B16 Melanoma Cells and Dendricity Assay, Pigment Cell Res., 2004, 17(4), 407-12.

[6] Record I.R., Broadbent J.L., King R.A., Dreosti I.E., Head R.J., Tonkin A.L., Genistein inhibits Growth of B16 Melanoma Cells in Vivo and in Vitro and Promotes Differentiation in Vitro, Int J Cancer., 1997, 72(5), 860-4.

[7] Overwijk W.W., Restifo N.P., B16 as a Mouse Model for Human Melanoma, Curr Protoc Immunol., 2001, Chapter 20:Unit 20.1.

[8] Chin L., Pomerantz J., Polsky D., Jacobson M., Cohen C., Cordon-Cardo C., Horner J.W., DePinho R.A., Cooperative Effects of INK4a and Ras in Melanoma Susceptibility in Vivo, Genes Dev., 1997, 11, 2822–2834.

[9] Zhu H., Reuhl K., Zhang X., Botha R., Ryan K., Wei J., Chen S., Development of Heritable Melanoma in Transgenic Mice, J. Invest. Dermatol., 1998, 110, 247–252.

[10] Crăciun L.M., Dumitriu B.G., Ene D.M., Adil A., Olariu L., Rosoiu N., Valorification of Grape Marc by Obtaining Bioactive Complexes Tested through in Vitro Experimental Models, Academy of Romanian Scientists, Annals Series on Biological Sciences, 2017, 6(2), 5-9.

[11] Lin V.C., Ding H.Y., Tsai P.C., Wu J.Y., Lu Y.H., Chang T.S., In Vitro and in Vivo Melanogenesis Inhibition by Biochanin A from Trifolium Pratense, Biosci Biotechnol Biochem., 201, 75(5), 914-8.

[12] Montes de Oca M.K.., Pearlman R.L., McClees S.F., Strickland R., Afaq F., Phytochemicals for the Prevention of Photocarcinogenesis, Photochemistry and Photobiology, 2017, 93(4), 956-974.

[13] Barltrop J.A. et al. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimenthylthiazoly)-3-(4-sulfophenyl) Tetrazolium, Inner Salt (MTS) and Related Analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium Bromide (MTT) Reducing to Purple Water-soluble Formazans as Cell-viability Indicators, Bioorg. Med. Chem. Lett., 1991, 1, 611–4.

[14] Riss T.L., Moravec R.A., Comparison of MTT, XTT, and a Novel Tetrazolium Compound for MTS for in Vitro Proliferation and Chemosensitivity Assays. Mol. Biol. Cell (Suppl.), 1992, 3, 184a.

[15] Fotakis G., Timbrell J.A., In Vitro Cytotoxicity Assays: Comparison of LDH, Neutral Red, MTT and Protein Assay in Hepatoma Cell Lines Following Exposure to Cadmium Chloride, Toxicology Letters, 2006, 160, 171-177. 10.

[16] Lyons A.B., Analysing Cell Division in Vivo and in Vitro Using Flow Cytometric Measurement of CFSE Dye Dilution, Journal of Immunological Methods, 2000, 243 (1-2), 147-154.

[17] Lyons A.B., Doherty K.V., Flow Cytometric Analysis of Cell Division by Dye Dilution, Curr Protocols Cytom., 2004, pp. 9.11.1–9.11.10.

[18] (Charles E.M, Rehm M., Key Regulators of Apoptosis Execution as Biomarker Candidates in Melanoma, Molecular & Cellular Oncology, 2014, 1.3.

[19] Quatresooz P., Piérard G.E., Malignant Melanoma: from Cell Kinetics to Micrometastases, American Journal of Clinical Dermatology, 2011, 12(2), 77–86.