

ASSESSMENT OF OLIVE LEAVES EXTRACT – CYTOTOXICITY IN VITRO AND ANGIOGENESIS IN OVO

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Abstract

In this study, it was determined the composition of the olive leaves ethanolic extract by LC-MS, the *in vitro* cytotoxicity on A375 human melanoma cells was established, by means of the MTT assay (number of viable cells), the Annexin V-FITC assay (number of apoptotic cells) and *in vivo* irritant and toxic potential by chorioallantoic membrane assay. The extract contains a number of polyphenols, among which luteolin and rutin are the predominant compounds and triterpenic acids with a significant ursolic acid content. At the highest concentration tested, 100 µg/mL, it was noted an important cytotoxic effect on human melanoma cells, it influences the development of growing capillaries, it induces narrowed calibre vessels, but no signs of toxicity on HET-CAM were recorded, while 24 h after treatment, the extract expressed a stronger effect, upon both tumour cell growth and the vascularization of A375 melanoma cells on *in vivo* test.

Rezumat

S-a determinat compoziția extractului etanolic din frunze de măslin prin LC-MS, citotoxicitate *in vitro* pe celule A375, de melanom uman, prin testul MTT (numărul de celule viabile), prin testul Annexin V-FITC (numărul de celule apoptotice) și potențial iritant și toxic *in vivo* prin testarea pe membrană corioalantoică. Extractul conține o varietate de polifenoli, dintre care luteolina și rutinul sunt compușii predominanți și acizii triterpenici, cu un conținut semnificativ de acid ursolic. La cea mai mare concentrație testată, 100 µg/mL, s-a observat un efect citotoxic important asupra celulelor melanomului uman, influențarea dezvoltării capilarelor în creștere, inducerea vaselor de calibru îngust, dar nu s-au înregistrat semne de toxicitate pe HET-CAM, în timp ce, 24 de ore după tratament, extractul a exprimat un efect mai puternic atât în ceea ce privește creșterea celulelor tumorale, cât și vascularizarea celulelor de melanom A375 la testul *in vivo*.

Keywords: olive leaves, polyphenols, triterpenes, cytotoxicity, angiogenesis, melanoma

Introduction

The extract of olive leaves is marketed globally and is considered a natural medicine, which has a number of benefits on maintaining human health. It is rich in different classes of different compounds, which are thought to produce the therapeutic effects of the extract [23]. A major problem regarding the products of natural origin is the one related to the variation of the chemical composition due in particular to the origin, which emphasizes the composition taking into account also the nutrition of the plant, the way of extraction, the form of storage, etc. [10, 11, 20]. Oleanolic, betulinic and ursolic acids are members of the class of triterpenic acids, secondary metabolites of plants, mainly presents in bark and leaves and

medicinally the most important compounds from their class [1]. These are currently intensively studied due to the biological effects that they prove and which makes them possible candidates in pharmaceutical and nutraceutical applications. Several studies have highlighted antimicrobial, antitumour, antiallergic, antiinflammatory, hepatoprotective and other activities [18]. Other plant extracts, such as *Viscum album*, revealed the presence of important terpenoids and the antiproliferative activity was manifested through cytotoxic effects on liver and breast cancer cell lines [22]. The chorioallantoic membrane (CAM) is an extra-embryonic membrane, rich vascularized and easily accessible, which is why the CAM assay is commonly used to study angiogenesis, proangiogenic and anti-

angiogenic effects of natural or synthetic compounds [15]. In addition, immune and nervous systems are not fully developed at the embryonic developmental stages that the experiment is being conducted. CAM can also be used to incorporate tumour tissue to study different aspects of angiogenesis and metastasis which are characteristic of malignant diseases. Due to the large and rich vascularized surface, CAM can be used to determine the potential irritant effect of some substance, as an alternative to the classic Draize rabbit eye assay. Among the advantages of the Hen's Egg Chorioallantoic Membrane Test (HET-CAM), there are mentioned: the possibility of testing a wide range of substances, low time consuming, low costs and good reproducibility [3]. HET-CAM is a quantitative method of a substance irritant effect and involves determining the irritability score that presumes the continuous observation of the CAM after application of the test compound, usually over a period of 300 seconds [6].

The aim of this experiment was to determine the cytotoxicity of olive leaves ethanolic extract on human melanoma cells and to establish the irritant and toxic potential by employing the *in vivo* chorioallantoic membrane assay using the HET-CAM method.

Materials and Methods

Reagents

Solvents, methanol and ethanol, acetic acid, ammonium acetate were acquired from Merck (Darmstadt, Germany) while standard polyphenolic compounds and standard pentacyclic triterpenes were purchased from Sigma-Aldrich (Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit from Roche (Germany), specific cell culture reagents (medium, antibiotic mixture, foetal bovine serum, etc.) from American Type Culture Collection (ATCC). Olive leaves were collected from Ksamil (Southern Albania), certified at the Pharmaceutical Botany Department, Faculty of Pharmacy, Timișoara, Romania, and then dried (at room temperature, away from light, for 10 days) and ground.

Extract preparation

The ethanolic extract of olive leaves was obtained by maceration, as described by Biris *et al.* [4]. Briefly, 10 g of dried material (d.m.) were extracted with 40 mL ethanol 95% (room temperature, 250 rpm, 72 h) in an orbital shaker (Incubator Environmental Shaker ES-20/60), then filtered, the solvent was removed with a rotary-evaporator, freeze-dried and stored at 4°C, protected from light, until further tests.

LC-MS analysis

The extract was subjected to LC-MS analysis for the quantification of polyphenols and triterpenes. A 6120 LC-MS system from Agilent, Santa Clara, USA was used. OpenLAB CDS ChemStation Workstation software was utilized in order obtains the LC-MS results.

Due to the quantification of two different classes of chemical compounds, two chromatographic methods were applied, namely: (1) for polyphenols - mobile phase 0.1% CH₃COOH and CH₃OH, gradient program (0 - 5 min. 5% CH₃OH; 5 - 38 minutes gradient 42% CH₃OH; 38 - 41 minutes 42% CH₃OH; 41 - 42 min. 5% CH₃OH), flow rate 1 mL/min, injection volume 10 µL, column temperature 40°C (Zorbax Eclipse Plus C18 column; 3.0 x 100 mm x 3.5 µ), UV detection at 330 and 370 nm, MS detection in the SIM detection mode, in the negative ion mode, capillary voltage 3500 V, dry gas flow 12 L/min at 350°C and (2) for triterpenes - mobile phase 1 mM NH₄CH₃CO₂ and CH₃OH (1:3 v/v), isocratic elution, flow rate 1 mL/min, injection volume 20 µL, column temperature 20°C (Ultrasphere ODS C18 column; 4.6 x 250 mm x 5 µ), UV detection at 200 and 210 nm, MS detection in the SIM mode, in the negative ion mode, capillary voltage 3500 V, dry gas flow 12 L/min at 350°C. Calibration curves were obtained for the quantification of polyphenols/triterpenes in the samples by the external standard method in the 0.05 - 2 µg/mL range for a six-point/seven-point plot for each compound.

Cytotoxicity *in vitro*

The human melanoma cell line A375 (ATCC® CRL-1619™) was acquired from ATCC and was cultured in Dulbecco's modified Eagle Medium (DMEM) with high glucose, Hepes and L-glutamine, supplemented with antibiotic mixture and foetal calf serum (FCS) as previously described [9, 13].

Cells viability was assessed by the microculture tetrazolium assay [2]. Briefly, 10,000 cells/well were seeded in culture plates (96-well) and treated with different concentrations of the tested extract - 0.1, 0.5, 10, 15 and 100 µg/mL, subsequent 10 µL of 5 mg/mL MTT solution was added and after 3 h contact period the precipitated crystals were dissolved in 100 µL of lysis solution and the absorbance was read at 570 nm, using a microplate reader (xMark Microplate Spectrophotometer, Bio-Rad, USA). The formula applied to obtain the percentage of cell viability was:

$$\text{Cell viability (\%)} = 100 - [(A_0 - A_s)/A_0 \times 100],$$

where: A₀ = absorbance of the blank sample and A_s = absorbance of the tested extract.

Apoptosis assay

Annexin V/PI apoptosis assay was applied in order to estimate the number of apoptotic cells in the presence of olive leaves ethanolic extract. Cells were examined by flow cytometry on FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The protocol was conducted according to the literature: 300,000 A375 human melanoma cells/well were seeded into plates (6-well, flow cytometry was performed with an Annexin V-FITC kit acquired from eBioscience, Vienna, Austria) and concentrations from 0.1 to 100 µg/mL of extract were added [21].

HET CAM assay

The evaluation of extracts biocompatibility and toxicity was assessed *in vivo* by the Hen's Egg Chorioallantoic Membrane Test (HET-CAM), applied in order to evaluate a possible irritant effect on the vascular plexus of the chorioallantoic membrane [12]. Summarily, the eggs (fertilized hen, *Gallus gallusdomesticus*, eggs) were incubated at 37°C, controlled humidity; on day 3 of incubation, 3 - 4 mL of albumen were extracted, on day 4 a window was cut and the eggs were stored in the incubator until day 9; three eggs were utilized for each tested concentration; 500 µL of control or test solution was applied and the changes produced at the CAM level were monitored by means of stereomicroscopy. Negative control was phosphate buffer solution (PBS) and positive control was 1% sodium dodecyl sulphate (SDS) in PBS.

The observation time of the reactions was 300 seconds, by using stereomicroscopy with a Discovery 8 Stereomicroscope Zeiss (Göttingen, Germany) and the time at which the occurrence of a particular reaction took place was noted in seconds, images being recorded with Axio CAM 105 colour, Zeiss and processed using AxioVision SE64. Rel. 4.9.1 Software, Zeiss). The onsets of the following negative events were monitored: haemorrhage – H (blood vessel bleeding), vascular lysis – L (disintegration of blood vessels), coagulation – C (intra- or extra-vascular protein denaturizing). The most extensively used evaluation is done by calculating an irritation score (IS), using the formula:

$$IS = 5 \times [(301 - \text{Sec H})/300] + 7 \times [(301 - \text{Sec L})/300] + 9 \times [(301 - \text{Sec C})/300],$$

where: IS – Irritation score, H – haemorrhage, L – vessel lysis, C – coagulation, Haemorrhage time (Sec H) – onset of haemorrhage reactions on CAM (in seconds), Lysis time (Sec L) – onset of vessel lysis

on CAM (in seconds), Coagulation time (Sec C) – onset of coagulation formation on CAM (in seconds). Irritation severity was evaluated by severity score (SS) as described in the literature [6].

The A375 cells were cultured according to the method described forwards: the cells were harvested from the culture plate, resuspended in culture medium in order to reach the final concentration of 100000/3 µL. On the day 10 of incubation, 3 µL of the A375 melanoma cell suspension were inoculated while control samples were inoculated with 3 µL of cell culture medium and the evaluation was done according to the method described previously in the literature [5].

Statistical analysis

In the current study GraphPad Prism 7 and Flowing Software Version 2.5.1 were utilized in order to obtain the data which were subsequently interpreted using one-way ANOVA. The establishment of statistical differences (control vs. exposed were marked with *; *** $p < 0.001$, **** $p < 0.0001$) was realized with Tukey's post-tests.

Results and Discussion*Polyphenols content*

The OLEM extract was subjected to LC-MS analysis for the purpose of identifying and quantifying major polyphenols and pentacyclic triterpenes. The polyphenols from the extract are presented in Table I (expressed as µg of phytocompound *per g* dry weight - d.w. lyophilized extract).

Data obtained showed that luteolin and rutin are the most abundant compounds from polyphenols class. The phytochemical characterization of OLEM extract regarding the screening and quantification of pentacyclic triterpenes revealed that ursolic and oleanolic acids are detected, quantified and listed in Table II.

Table I

Polyphenols from OLEM extract identified and quantified by LC-MS

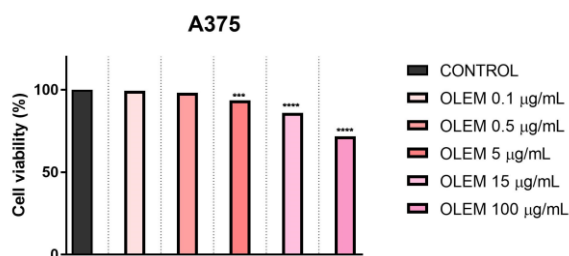
No.	Compound name	Rt (min)	[M-H] ⁺ (<i>m/z</i>)	OLEM (µg/g d.w.)
1.	Caftaric acid	1.92	311	10.66
2.	Gentisic acid	2.62	153	-
3.	Chlorogenic acid	6.38	353	-
4.	Caffeic acid	6.92	179	437.29
5.	p-coumaric acid	10.50	163	18.23
6.	Ferulic acid	13.88	193	108.86
7.	Sinapic acid	15.89	223	-
8.	Hyperoside	21.48	463	-
9.	Isoquercitrin	22.61	463	286.73
10.	Rutin	23.12	609	10386.40
11.	Myricetin	24.37	317	88.76
12.	Fisetin	25.71	285	7.42
13.	Quercitrin	26.22	447	207.62
14.	Quercetol	30.43	301	51.34
15.	Luteolin	32.82	285	12764.30
16.	Kaempferol	35.59	285	13.28
17.	Apigenin	36.82	269	436.27

Table II

Pentacyclic triterpenes from OLEM extract identified and quantified by LC-MS				
No.	Compound name	Rt (min)	[M-H ⁺] ⁺ (m/z)	OLEM (µg/g d.w.)
1.	Ursolic acid	50.88	455	31994.20
2.	Oleanolic acid	57.63	455	109.67

In vitro activity

Stimulation of human melanoma cells (A375) was performed with selected concentration of the extract (0.1, 0.5, 5, 15 and 100 µg/mL) for 24 h. OLEM exerted some minor effects at low concentrations on cells viability: ~ 99% viable cells at 0.1 µg/mL, ~ 98% viable cells at 0.5 µg/mL and ~ 93% viable cells at 5 µg/mL. As the concentration increases, the number of viable cells shows a decrease, and the results indicated ~ 86% viable cells at 15 µg/mL and ~ 71% viable cells at 100 µg/mL (Figure 1).

**Figure 1.**

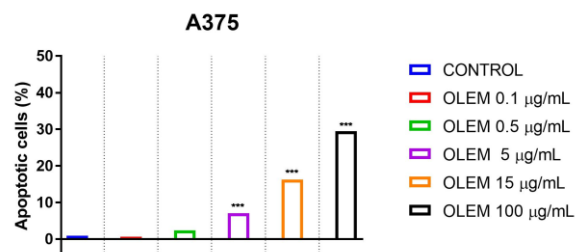
Percent of viable melanoma cells after stimulation with different concentrations of olive leaves ethanolic extract.

Statistical differences, control vs. exposed cells, were established by one-way ANOVA analysis, followed by Tukey's multiple comparisons post-test; *** p < 0.001 and **** p < 0.0001

The active compounds from olive leaves showed cytotoxic activity on different cell lines, such as leukaemia, colon cancer, breast cancer, especially HER2 positive ones [19]. Methanolic extract of olive leaves tested by Fu *et al.* on three breast carcinoma cell lines led to the obtaining values for IC₅₀ between 200 to 300 µg/mL for MCF-7 and SKBR3 cells while for JIMT-1 cells was > 400 µg/mL [7].

In Figure 2 are depicted the percentages of apoptotic cells in the presence of different concentration of

OLEM and as can be observed, at 100 µg/mL the highest number of dead cells was recorded.

**Figure 2.**

The percentage of apoptotic melanoma cells after treatment with different concentrations of olive leaves ethanolic extract

Statistical differences, control vs. exposed cells, were established by one-way ANOVA analysis, followed by Tukey's multiple comparisons post-test; *** p < 0.001

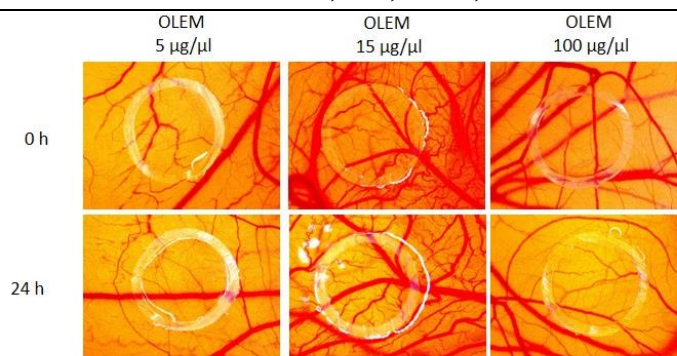
In ovo activity

The reaction induced by the tested extract (Table III) was classified according to Luepke, as follows: 0 - 0.9 non-irritant; 1 - 4.9 weak irritant; 5 - 8.9/9.9 moderate irritant; and 8.9/9.9 - 21 strong irritant as described in the literature [6, 8]. The effects induced by the tested extract at different concentrations, along with the controls (positive – SDS and negative – PBS) were registered and all three endpoints (haemorrhage, coagulation and lysis) were noticed only for positive control. The OLEM extract at 100 µg/mL showed late and limited signs of both haemorrhage and coagulation, and early though limited signs of vasodilatation which suggests the occurrence of a weak irritant effect at this concentration. The positive control which induced major vascular damage produces death within 60 min., the extract that induced a weak irritant effect produces death within the first 24 hours and the extracts that were non-irritant on the CAM does not affect the viability for more than 24 hours.

Table III

The irritant potential of tested extract

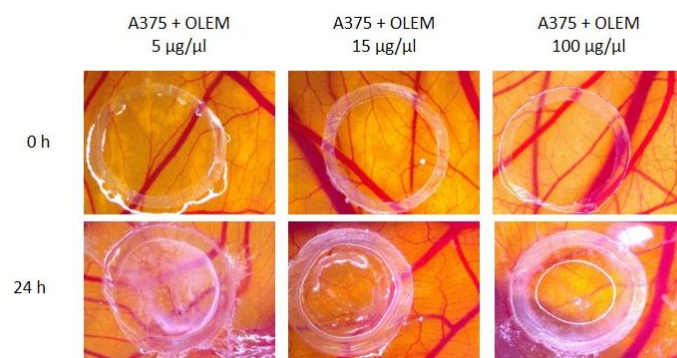
No.	Test extract and controls	Irritation score (mean)	Irritation severity (mean)	Classification of the effect
1	Negative control PBS	0	0	Non irritant
2	Positive control SDS	14.15	3	Strong irritant
3	OLEM 0.1 µg/mL	0	0	Non irritant
4	OLEM 0.5 µg/mL	0	0	Non irritant
5	OLEM 5 µg/mL	0	0	Non irritant
6	OLEM 15 µg/mL	0	0	Non irritant
7	OLEM 100 µg/mL	0.67	1	Weak irritant

**Figure 3.**

Irritant potential assessment of test compounds using HET-CAM assay before (0 h) and after the application (24 h)

OLEM ethanolic extract evaluated during rapid development stage of the vascular plexus, 24 hours after application, influenced the development of growing capillaries, inducing narrowed calibre vessels, but no signs of toxicity (Figure 3).

OLEM 15 µg/mL and OLEM 100 µg/mL 24 h after treatment expressed a stronger effect compared to OLEM 5 µg/mL, upon both tumour cell growth and the vascularization of A375 melanoma cells, with no spokes wheel angiogenic reaction towards the ring (Figure 4).

**Figure 4.**

Effects of OLEM on CAM assay, A375 melanoma cells with three doses of the test extract on day 13

The chorioallantoic membrane of chick embryo is a thin, highly vascularized respiratory extra-embryonic tissue especially applied in cancer and angiogenesis research. Benefitting from an ease of manipulation and assessment, low costs and good reproducibility, it also represents an optimal pre-screening assay to animal models in multiple biological studies, such as: biochemistry, virology, immunology and drug toxicity [14]. The production of a complete inflammatory reaction similar to that induced by irritants to conjunctival tissue makes this model useful in evaluating the potential tolerability and ocular irritation effects of various chemicals [17]. The irritation potential ranges from 0 to 21 and the tested extracts were classified according to this score. This method allows different type of compounds, such as surfactants, cosmetics, other ingredients, different chemical products, dental adhesives etc. to be evaluated as irritant and noxious ingredients [16]. The irritation score and the severity of this effect was evaluated in comparison with strong irritant SDS and the non-irritative PBS, was expressed as moderate and indicated some interference with the vascular function. The HET-

CAM is considered as a good predictive model for the irritation potential and biocompatibility, but still, further comparison is required through other *in vitro* and *in vivo* assays.

Conclusions

The present research was conducted in order to assess cytotoxicity of olive leaves ethanolic extract on human melanoma cells *in vitro* and to establish the irritant and toxic potential *in vivo* by HET-CAM method. The extract exerts some cytotoxic effects on melanoma cells at the highest concentration tested, 100 µg/mL, and *in vivo*, at 24 hours after treatment, expressed a stronger effect compared upon both tumour cell growth and the vascularization of A375 melanoma cells.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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