Lupeol, a pentacylcic triterpene that reduces the lesions and irritability on murine skin and is effective on in vitro tumor models

Sorina Ciurlea¹, Daniela Ionescu¹, Lavinia Craciun (Redes)², Codruta Soica¹, Camelia Peev¹, Iuliana Popovici³

¹University of Medicine and Pharmacy “Victor Babes” Timisoara, Faculty of Pharmacy, Eftimie Murgu Square nr.2, RO-300041, Timisoara, Romania
²S.C. Olimpia Farm s.r.l. Lipova, University of Medicine and Pharmacy “Gr.T.Popă” Iasi, Faculty of Pharmacy, Iasi, Romania
³University of Medicine and Pharmacy “Gr.T.Popă” Iasi, Faculty of Pharmacy, Iasi, Romania

Received: 30 August 2010; Accepted: 25 November 2010

Abstract
Melanoma and lupeol are two aspect that could have a common knowledge because of the activity of pentacyclic triterpene in the mentioned pathology. It has also been shown that lupeol induces differentiation of mouse melanoma cells. The aim of present study was an evaluation of topical active compound lupeol on an experimental chemical melanoma and an in vitro evaluation on sensitive cells such as HeLa (cervix carcinoma) and A431 (skin carcinoma). The methods used specific cells and animals like mice C57BL/6J. The measurements and evaluation included histopathological aspect and erythema evaluation using a Mexameter MX18. The main results indicated an important antitumor activity of lupeol both in vivo and in vitro.

Keywords: lupeol, melanoma cell, tumor models

1. Introduction
Melanoma, a malignant neoplasm of melanocytes, is recognized as one of the most aggressive cancers with relatively high propensity for metastasis. The incidence of melanoma continues to increase despite public health initiatives [1,2]. Lup-20(29)-en-3β-ol is known as lupeol, clerodol, fagarsterol and lupenol and is a lupan-type triterpene present in plant kingdom. It is present in plants like mango pulp, carrot root, cucumber, soybean and melon seeds uva ursi, aloe plants and a lot of medicinal plants, etc. It has been studied for more then a century and in the last years it is analysed for the biological properties.

The last period of publications included lupeol in more then 54/year generally attributable to anticancerous activity (review super). Lupeol [Lup-20(29)-en-3h-ol], a triterpene found in fruits such as olive, mango, strawberry, grapes, and figs; in many vegetables; and in several medicinal plants [3]. Lupeol is found as an active constituent of various medicinal plants used by native people in the treatment of various skin ailments in North America, Japan, China, Latin America, and Caribbean islands. Lupeol possesses strong antioxidant, anti-inflammatory, antiarthritic, antimutagenic, and antimalarial activity in vitro and in vivo systems;

Corresponding author: e-mail: sorinaciurlea@yahoo.com
acts as a potent inhibitor of protein kinases and serine proteases; and inhibits the activity of DNA topoisomerase II, a known target for anticancer chemotherapy [4,5,6]. It has also been shown that lupeol induces differentiation of mouse melanoma cells [7]. Lupeol exhibits a significant antitumor-promoting activity in a two-stage model of mouse skin carcinogenesis [8]. Lupeol was observed to significantly inhibit the activity of ornithine decarboxylase, protein which is a well known biomarker of tumor promotion. The data suggested the chemopreventive potential of Lupeol against the development of skin cancer [14].

The aim of present study is the in vivo and in vitro analysis of lupeol dissolved with hidroxipropil gamma cyclodextrin in 1:1 ratio. In vivo studies are developed on a murine model on C57BL/6J mice and in vitro on A431 and HeLa cells.

2. Materials and methods

Lupeol was purchased from Sigma Aldrich (Taufkirchen, Germany). Components of ointment: 2-propanol, isopropyl myristate and PEG 4000 (Ph.Eur. 6), diethylene glycol monoethyl ether (Transcutol®) and caprylocaproyl macrogolglycerides (Labrasol®) were form Gattefossé (France). The carcinogens were 7,12-dimethylbenzanthracene (DMBA) as tumor initiator and 12-O- tetradecanoylphorbol-13-acetate (TPA) as tumor promoter. Both substances were prepared as solutions in acetone as is mentioned in the literature [39,40] and the quantity of applied substance was 200 nmol in 100 µl acetone and 5 nmol TPA in 100 µl acetone. The protocol of application was correlated with a reproducible one also mentioned in previous studies [41].

Animals. C57BL/6J mice of eight weeks were purchased from Charles River (Sulzfeld, Germany). The work protocol followed all NIAH-National Institute of Animal Health rules: animals were maintained during the experiment in standard conditions: 12h light-dark cycle, food and water ad libidum, temperature 24 º C, humidity above 55%. The number of mice taken into study was 8.

For topical test was applied a long term application of DMBA and phorbol esters (croton oil) as was describe in Yafan Li et al. protocol in 2007, a double DMBA initiation in alternation with TPA.

Preparation of topical formulation. Only the one part of lupeol dissolves in the mixture of liquid components (2-propanol, Transcutol®, isopropyl myristate and Labrasol®). After mixing this suspension with the melted PEG 4000, it was stirred (2000 rpm) with a Heidolph Diax (Heidolph Instruments GmbH &Co.) mixer until cooling (solidification).

Table 1. Semisolid formulation and its composition for100g formulation (5% lupeol)

<table>
<thead>
<tr>
<th>Active agent</th>
<th>5g</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-propanol</td>
<td>37.5g</td>
</tr>
<tr>
<td>Transcutol®</td>
<td>9.3g</td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td>9.3g</td>
</tr>
<tr>
<td>Labrasol®</td>
<td>0.3g</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>38.8g</td>
</tr>
</tbody>
</table>

Histology. Tissue samples (skin) were fixed in 10% formalin solution and were embedded in paraffin and cut at 4 microns. Finally after deparaffinized the samples were stained with H&E (hematoxylin-eosin) and microscopically analysed. The biopsy for sample 1 was obtained in the day 34 after external application and for sample 2 after 20 days from external application of and 14 days of treatment.

MTT-assay. Antiproliferative effects of the test compound were measured in vitro on the following human cell lines: HeLa (cervix carcinoma) and A431 (skin epidermoid carcinoma), by using the MTT ([3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide]) assay [15]. Cancer cells (5000/well) were seeded onto a 96-well microplate and attached to the bottom of the well overnight. The process continues on the second day when 200 µL of new medium with the test substances was added. After an incubation time for 72 h, the living cells were assayed by the addition of 20 µL of 5 mg/mL MTT ([3-(4,5-dimethylthiazol-2 - yl) 2,5 - diphenyltetrazolium bromide]) solution. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a period of minimum 4 h contact. The medium was then removed, and the precipitated crystals were dissolved in 100 µL of dimethyl sulfoxid during a 60 min period of shaking. Finally, the reduced MTT was assayed at 545 nm, using a specific microplate reader; wells with untreated cells were utilized as controls samples. All in vitro experiments were carried out on microplates with a number of parallel wells. Stock solutions of the tested substances were prepared with contribution of DMSO and the highest DMSO concentration (0.3-0.5%) of the medium didn’t affect significant the cell
proliferation. The active compound was dissolved in water and DMSO (10 mM). Several dilutions were applied: 100, 300 and 500.

**Erythema measurement (hemoglobin).** The haemoglobin measurements were performed using a research device from Courage Khazaka, with a Mexameter MX18 (Courage&Khazaka Electronics, Cologne, Germany). The maximum units for Mexameter are 999 (interval 0-999) and the measurement is based on the absorption/reflexion. For the measurements, the mice were anesthetised with xylazine and ketamine. The time of measuring was continuous, for 20s. The protocol followed the observations on melanin evolution and haemoglobin status (pigmentation and erythema). The device was applied on most obvious affected areas and maintain on the skin for 20 seconds. The data were registered by the specific soft from the Mexameter MX18 device and then expressed as units. All data were processed as initial and final measurements values on the same area.

### 3. Results and Discussions

The external application of chemical tumor inductors determine apparition of important and visible damages (Figure 2).

**Figure 2.** (A) Macroscopic aspect of skin on C57BL/6J mouse after 34 days of external application of DMBA and croton oil (B) Skin aspect after treatment application

**Figure 3.** Dermal-epidermal tissue with intense (A) and reduce (B) collagenisations and hyperemiated capillary (HEx400)

The morphopathological evaluation confirmed the improvement of skin after a short period of permanent application of semisolid formulation.

Lupeol is an effective agent against some tumor cells such as skin carcinoma and is also very effective on cervix carcinoma cells.

**Figure 4.** Erythema units in 2 regions of mouse skin before and after treatment
Figure 5. *In vitro* antitumor activity of lupeol on A431 and HeLa cells at 3 different concentrations.

It is recognized that most human malignancies develop from the progeny of a single cell through sequential steps as a result of biochemical, physiological, and molecular alterations [16].

The prognosis of melanoma patients remains poor and is uninfluenced by any treatment intervention yet applied in large randomized studies [14,15].

Carcinogenesis in mouse skin and other animal tumor bioassay systems and, possibly, in humans is a stepwise process of at least three distinct stages: initiation, promotion, and progression [17,18]. For more than 50 years, mouse skin has been used as a conventional model to study the mechanism of carcinogenesis and modulation of sequential steps involved in this process. The mouse skin model, which provides a conceptual framework to study the carcinogenesis process, has also been used extensively to assess whether chemical and/or physical agents carry a carcinogenic hazard to humans and to evaluate the cancer-chemopreventive effects of different agents and define the mechanism involved with their protective effects [16,17,18].

Several natural agents with high anticancer efficacy and no or acceptable toxicity to normal tissues are suggested as possible candidates for use by melanoma patients [19-24].

In the current study, we provide evidence that lupeol could ameliorate the inefficiency of melanoma cells and inhibit the melanoma growth during *in vivo* studies developed on a murine model on C57BL/6J mice and in vitro on A431 and HeLa cells.

Lupeol, a triterpene, is one such polyphenolic agent found in various edible plants [25]. It is a well documented fruit-, vegetable-, and bark-based natural product found in olives, figs, mangoes, and other fruits and medicinal herbs [30-32].

It has been shown that Lupeol provides a strong antioxidant protection against benzoyle peroxide-induced toxicity in Swiss albino mouse skin [26]. Lupeol has been demonstrating to significantly reduce the PGE2 production and inhibit the production of TNFα and interleukin-1β in vitro [29]. The topical application of TPA to mouse skin or its treatment in certain epidermal cells is known to result in a number of biochemical alterations, changes in cellular functions and histological changes leading to skin tumor promotion. Existing data may clearly demonstrate that preapplication of Lupeol before TPA treatment affords a significant inhibition of TPA-induced skin edema and hyperplasia [27]. Previously, Lupeol has been reported to provide protection against croton oil-induced edema in mouse ear and was reported to have more efficacy than indomethacin [28].

Lupeol produced a moderate but specific anti-cancer activity against androgen-sensitive prostate cancer cells [34], B16 2F2 melanoma cells (inhibition of the migration of malignant melanoma cells by disassembling the actin cytoskeleton) [35,36] and pancreatic adenocarcinoma cells (inhibition of the Ras signaling pathway), [3] and possesses anti-tumour-promoting effects in a mouse skin tumourigenesis model (modulates NF-κB and PI3 K/Akt pathways and inhibits skin cancer in CD-1 mice), [8-12] and is cytoprotective against free radical toxicity [33]. The potential use of lupeol as a preventative anti-cancer component is an important aspect of the above-referenced studies. Lupeol and its derivatives have been suggested to be of use for the prevention and treatment of skin disorders, skin cancer, prostate cancer and pancreatic cancer.

Cancer chemoprevention is increasingly being realized as an important area for cancer research, which, in addition to providing a practical approach of identifying potentially useful inhibitors of cancer development, also affords excellent opportunities to study the mechanisms of carcinogenesis [37,38].

4. Conclusion

The mouse skin model of multistage carcinogenesis has been a useful experimental framework to study basic mechanisms associated with the carcinogenesis and defining newer chemopreventive agents.
Therefore, pharmacologic agents and therapeutic strategies interfering with disrupted apoptosis regulation could improve the therapeutic arsenal against melanoma in the future.

Our data clearly demonstrate that Lupeol could be a potent antitumor-promoting agent because it inhibits TPA-induced tumor promotion in an in vivo animal model. One might envision the use of chemopreventive agents such as Lupeol in an emollient or patch for chemoprevention or treatment of skin cancer.

In addition, because Lupeol exerts multiple effects on biomarkers associated with carcinogenesis, it could be tested for the cancer chemoprevention of other organs.

Acknowledgements:
This work was supported by CNCSIS-UEFISCSU, project PNII-IDEI code 1257/2007 (contract 212).

References
8. Saleem M., Afaq F., Adhami V.M., Mukhtar H., Lupeol modulates NF-κB and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice, Oncogene, 2004, 23(30), 5203-5214