in vivo *30*: 633-638 (2016)

In Vivo Biological Evaluation of Polyurethane Nanostructures with Ursolic and Oleanolic Acids on Chemically-induced Skin Carcinogenesis

CAMELIA OPREAN[1], FLORIN BORCAN[2], IOANA PAVEL[3], ALIS DEMA[4], CORINA DANCIU[3], CODRUTA SOICA[1], CRISTINA DEHELEAN[5], ANDREEA NICU[6], ANAMARIA ARDELEAN[6], MIRABELA CRISTEA[7], ALEXANDRA IVAN[8], CALIN TATU[8] and FLORINA BOJIN[8]

Departments of [1]Pharmaceutical Chemistry, [2]Analitical Chemistry, [3]Pharmacognosy and [5]Toxicology, Faculty of Pharmacy, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania; Departments of [4]Morphopathology and [8]Functional Sciences, Faculty of Medicine, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania; [6]Student at Faculty of Pharmacy, Victor Babeş University of Medicine and Pharmacy, Timisoara, Romania; [7]"Pius Brinzeu" Timişoara County Emergency Clinical Hospital, Timisoara, Romania

Abstract. *Background/Aim: Oleanolic and ursolic acids (OA and UA) are two pentacyclic triterpenes, ubiquitously spread in plants, previously known for their chemopreventive capacity on different types of cancer. The major pharmacological disadvantage of these phytocompounds is their poor water solubility, which often limits their applicability. Materials and Methods: Using the interfacial polycondensation combined with spontaneous emulsification technique, polyurethane nanostructures (PU) were synthetized in order to improve this problem. In order to test the in vivo chemopreventive potential of the two pure compounds, as well as the encapsulated compounds in PU used as drug carriers, a chemically-induced skin carcinogenesis model was constructed. Results: UA and OA have a moderate chemopreventive activity against tumors induced by 7,12-dimethylbenzantracene (DMBA) and 12-O-tetradecanoilphorbol-13-acetate (TPA) application. Incorporation of active agents in PU did not lead to increased chemopreventive effect. Conclusion: PU is not a suitable formulation of UA and OA.*

Oleanolic (OA) and ursolic (UA) acids are pentacyclic triterpenes, ubiquitously spread in plants such as *Rosmarinus officinalis* L., *Lavandula latifolia* B., *Salvia officinalis* L., *Salvia lavandulifoliae* Vahl., *Vinca minor* Sm., *Thymus vulgaris* Sm., *Olea europaea* L., *Aralia chinensis* L., *Arctostaphyllos uva-ursi* L., *Calluna vulgaris* Salisb. (1-4). Ursolic acid's capacity to stimulate collagen production in fibroblasts and ceramide production in epidermal keratinocytes has been previously reported (5). Moreover, the two triterpenic acids have been studied for their antitumor activity, both *in vitro* and *in vivo* (6). Since the incidence of skin cancer is continuously increasing, prophylaxis of this pathology has become a major target for the medicine of the XXI century. Natural compounds from different vegetal products used in food or cosmetic industry are exploited nowadays for their chemopreventive potential (7). The major disadvantage of UA and OA is their poor water solubility that often limits their applicability (8, 9).

Nanotechnology is a novel and multidisciplinary domain with an important contribution to many other scientific fields; the main role of this new modern approach is to employ atoms and molecules in order to build nanoscale materials with artificial intelligence, biocompatible structures, unconventional energy resources, nanorobots and nanocarriers for medicine, *etc*. (10). The drug delivery systems based on structures, such as nanocapsules, nanorods, nanofibers, nanotubes or dendrimers, with controlled release exhibit the following advantages: they can (i) modify the physical and chemical properties of loaded active substance (*i.e*. solubility), (ii) protect the drug against UV exposure, gastric acid and, most importantly, (iii) release the active substance so as to ensure a constant level of drug in the blood stream (11).

The present study aims to test the *in vivo* chemopreventive potential of the two pure compounds, as well as the

Correspondence to: Corina Danciu, Department of Pharmacognosy, Faculty of Pharmacy, Victor Babeş University of Medicine and Pharmacy, 2nd Eftimie Murgu Sq., Timişoara 300041, Romania. Tel: +40 744648855, Fax: +40 256226134, e-mail: corina.danciu@umft.ro

Key Words: Oleanolic acid, ursolic acid, polyurethane nanostructures, DMBA, TPA.

encapsulated compounds in polyurethane nanostructures (PU), used as drug carriers in chemically-induced skin carcinogenesis.

Materials and Methods

Chemicals. 7,12-dimethylbenzantracene (DMBA) and 12-O-tetradecanoilphorbol-13-acetate (TPA) were purchased from Sigma Aldrich (Steinheim,Germany). OA and UA were purchased from Fluka (Sigma Aldrich). Isophorone diisocyanate (IPDI), Polyethylene glycol M=200 (PEG), acetone, Span®85 and Tween®20 were purchased from Merck (Hohenbrunn, Germany). 1,4-butanediol (BD) was purchased from Carl Roth GmbH (Karksruhe, Germany) and ethylene glycol (EG) from Lach-Ner, s.r.o. (Neratovice, Czech R.).

Synthesis of polyurethane drug delivery system. A multi-step procedure based of interfacial polycondensation combined with spontaneous emulsification was used for the synthesis of the polyurethane drug delivery system. This procedure was previously described by Danciu *et al*. (12): an organic phase was injected into an aqueous phase under magnetic stirring (500 rpm). The components of the organic phase were 1.5 ml IPDI, 1.5 ml Span®85 and 15 ml acetone heated at 30˚C, while the components of the aqueous phase were as follows: 0.8 ml EG, 0.8 ml BD, 0.3 ml PEG and 1.5 ml Tween®20 mixed with 15 ml distilled water and heated at 30˚C. In order to ensure the reaction completion, the mixture of the two phases was heated at 40˚C for four hours. The products were repeatedly washed with acetone/water mixture (1:2, v/v). The water and acetone removal was assured by maintaining the obtained particles in thin layers in Petri dishes at 80˚C for 12 h. The two triterpenic acids were added (in organic phase) separately in the two experiments, respectively, in a concentration of 57 μM.

Animal studies. Twelve-week-old SKH1 nude females were purchased from Charles River (Budapest, Hungary). The tumor initiator, 200 μl DMBA solution (0.025% in acetone), was topically applied on the dorsal skin area for two weeks (1 application/week). Starting with the third week, mice were divided in 5 groups (4 mice/group) and 200 μl of the tumor promoter (15 nM TPA solution in acetone) was applied for 29 weeks (2 times/week). Thirty minutes before the TPA application, mice were treated with the tested compounds as follows:

Group 1: Control, without treatment.
Group 2: 200 μl UA solution (10 μM in acetone).
Group 3: 200 μl polyurethane nanostructures incorporating ursolic acid (PU-UA) (10 μM in acetone).
Group 4: 200 μl OA solution (10 μM in acetone).
Group 5: 200 μl polyutethane nanostructures incorporating oleanolic acid (PU-OA) (10 μM in acetone).

Final solutions of the tested substances were obtained by successive dilutions in acetone of the stock solution (1 mM in ethanol).

Non-invasive skin measurements. Twice weekly, before the application of tested substances, non-invasive measurements of the melanin (M) and erythema (E) index were conducted using a Mexameter®MX 18 probe, Multiprobe Adapter System (MPA5) from Courage-Khazaka (Koln, Germany). For the melanin and erythema spectrophotometric measurements, 2 wavelengths were used, respectively: 660 and 880 nm for melanin and 560 and 660 nm for erythema (13, 14). Mice were periodically measured in order to record weight changes.

Histological analyses. At the end of the experiment, skin and other tissue samples were collected and histologically analyzed. Samples were fixed in 10% formalin solution, embedded in paraffin and cut at 3-5 μ. Finally, the tissue sections were stained using the conventional hematoxylin-eosin (HE) method and microscopically examined. The microscopic evaluation was performed using a Leica DM750 microscope (Leica, Bucharest, Romania) and image acquisition was obtained using Leica ICC50HD camera and Leica DMD108 microscope (Leica).

Compliance with ethics requirements. The experiment was approved by the Ethical Committee of the "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania. The work protocol followed the rules of National Institute of Animal Health. Throughout the experiment, animals were maintained under standard conditions of 12-h light-dark cycle, food and water *ad libitum*, temperature 24±1˚C and humidity above 55%. At the end of the experiment, animals were sacrificed by ketamine anesthesia and cervical dislocation.

Statistical analysis. One-way analysis of variance (ANOVA) test was used to determine the statistical significance between the groups. The results are presented as mean±standard deviation (SD).

Results

Using the interfacial polycondensation technique combined with spontaneous emulsification, polyurethane structures encapsulating UA (PU-UA) and OA (PU-OA) with nanoscale dimensions were synthesized. The final structures were confirmed by physicochemical analysis using scanning electron microscopy (SEM), X-ray diffraction and differential scanning calorimetry (DSC) assays (data not shown). Along with the pure UA and OA, the newly synthesized nanostructures with the two compounds were used on DMBA- and TPA-chemically-induced skin carcinogenesis model in order to test their chemopreventive potential.

Following DMBA and TPA application, all animal groups developed papilloma. Figure 1 shows the papilloma's incidence/group. From this perspective, one can conclude that pure UA and PU-OA decreased tumor incidence in comparison with the untreated group ($p>0.05$).

In terms of the number of tumors/mouse, one can notice (Figure 2) a decrease of tumor number in groups 2, 3 and 5 using the control group as reference ($p>0.05$). Group 4 exhibited an increase in papillomas; variability was observed within the same group in terms of tumor volume.

Figure 3 presents the macroscopic aspect of the skin at the end of the experiment.

Changes in skin pigmentation, increase of blood supply around a lesion or modification of skin pigmentation in an existing lesion are indicators of the development of a malignant process. Melanin index evolution in skin pathology

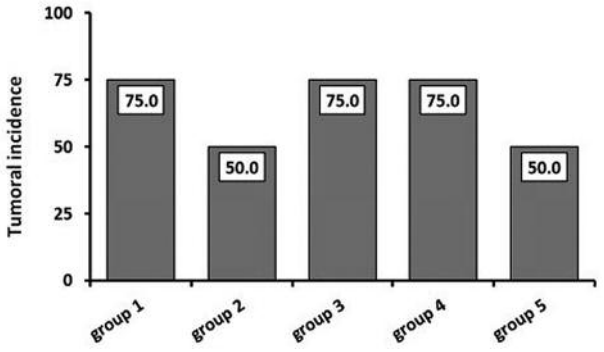

Figure 1. *Tumor incidence/group. Group 1, Without treatment; Group 2, UA; Group 3, PU-UA; Group 4, OA; Group 5, PU-OA.*

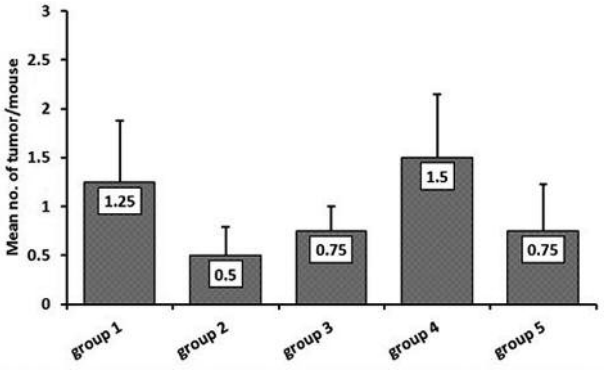

Figure 2. *Number of tumors/mouse. Group 1, Without treatment; Group 2, UA; Group 3, PU-UA; Group 4, OA; Group 5, PU-OA.*

is a parameter of pathology development and also of treatment efficacy (15). Skin pigmentation (melanin) and erythema (hemoglobin) were measured weekly. Figures 4 and 5 show melanin and erythema index values during the experiment. The evolution of melanin index was similar in all groups with only small modifications in group 2. At the end of the experiment, the melanin index was slightly decreased in the control group (119.44±10.47 arbitrary units), while the highest values were observed in the case of groups 2 and 3 (144.61±22.79 and 146.33±21.85, respectively).

The differences regarding the erythema index were not statistically significant (n.s.) (Figure 5). Erythema represents the redness developed at skin level as a result of capillary dilation and congestion. All five groups had a similar evolution with increased values in the first weeks probably due to the irritative effect of DMBA (16).

Microscopic analysis of fragments of various tissues harvested from the sacrificed mice showed minor microscopic changes at the following levels: Liver: discrete stasis, hydropic swelling of hepatocytes (groups 1, 2, 5), discrete microvacuolar steatosis (groups 1, 2); Kidney: congestion of small vessels from the medullary compartment (all groups), discrete focal interstitial lymphocytic infiltration (all groups); Myocardium: hyperemia of interstitial vessels (all groups), lipomatosis (groups 1, 2, 4, 5). Lung: stasis, groups of collapsed alveoli, a case with a small hemorrhagic area and 2 cases with focal perivascular and peribronchial lymphocytic infiltrate (groups 2, 3, 4, 5).

In the skin, epidermal lesions were identified as follows: early squamous papilloma (groups 1, 2, 3, 4, 5), well-established exophytic squamous papilloma (groups 1, 2, 3, 4), keratoacanthoma (group 3), keratinized squamous cell carcinoma (group 3), nodular hyperplasia of the sebaceous glands (groups 1 and 4). Representative images of the skin microscopic examination are shown in Figure 6.

In case of groups 1, 2, 4 and 5, the induced tumors were diagnosed as squamous papilloma. Due to a mild induction with the tumor initiator DMBA (0.025%), the malignant progression into squamous carcinoma did not occur within the studied period. In case of group 3 (treated with PU-UA), malignant tumors were observed with the development of keratinized squamous cell carcinoma in 50% of cases.

Discussion

Altogether, the macroscopic and microscopic evaluation lead to the conclusion that UA offered a moderate skin protection: fewer mice with tumors and fewer tumors/group; microscopic examination did not show significant changes compared to the untreated group. The results are consistent with results from the literature; Tokuda *et al*. and Huang *et al*. have reported that topical application of UA in a model of chemical carcinogenesis led to a decreased number of mice with papilloma, as well as a decreased number of papillomas/mouse (17, 18). In the case of OA, no improvement was observed either in terms of macroscopic or microscopic evaluation. These data are inconsistent with those reported by Tokuna *et al*. who noticed a stronger inhibitory capacity on tumor formation for OA than for UA (18).

Regarding the use of nanotechnology in order to improve UA and OA bioactivities, there are data in literature reporting positive results. Mixture of the two acids in the formulation of a nanoemulsion proved to be beneficial for transdermal administration increasing the anti-inflammatory effect and demonstrated to be non-toxic to skin (19). Yang *et al*. prepared nanoparticles of UA with improved physicochemical properties (20), while pegylated liposomes with OA have shown superior stability and increased *in vitro* anti-tumor effect on HeLa cervical cancer cell lines (21).

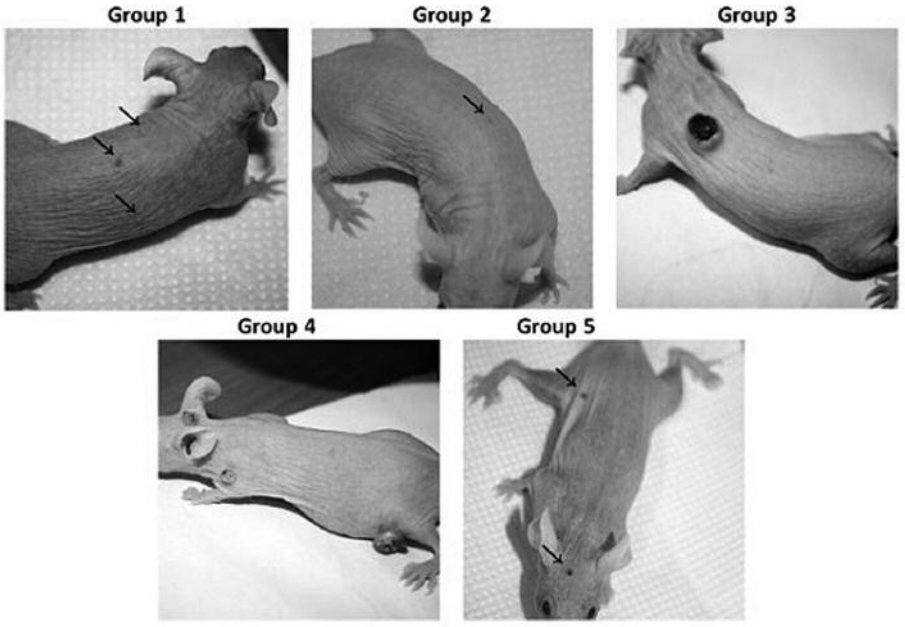

Figure 3. *Macroscopic images of SKH1 mice skin. Group 1, Without treatment; Group 2, UA; Group 3, PU-UA; Group 4, OA; Group 5, PU-OA.*

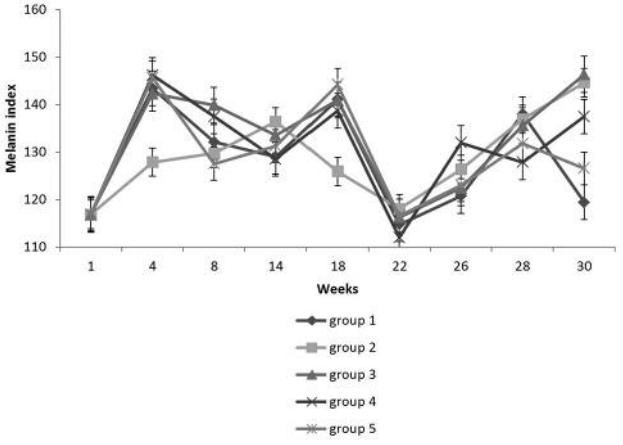

Figure 4. *Melanin index. Group 1, Without treatment; Group 2, UA; Group 3, PU-UA; Group 4, OA; Group 5, PU-OA.*

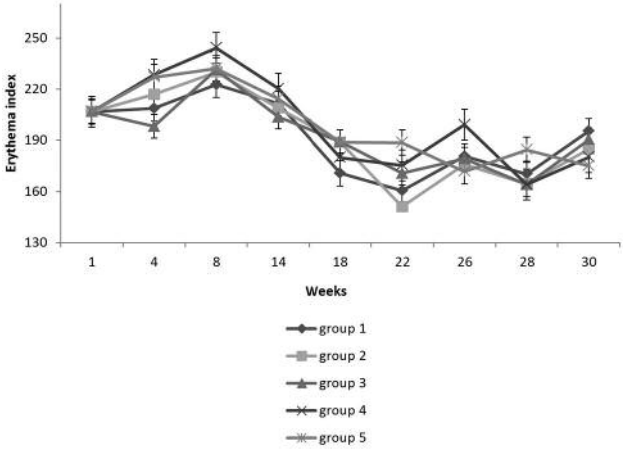

Figure 5. *Erythema index. Group 1, Without treatment; group 2, UA; group 3, PU-UA; group 4, OA; group 5, PU-OA.*

In the present study, after encapsulation of UA and OA inside polyurethane nanostructures, controversial results were noticed. Topical application of PU-UA favored the malignant transformation of benign tumors leading to the emergence of keratinized squamous cell carcinoma. Interestingly, the application of PU-OA did not lead to carcinoma but, rather, decreased the incidence of tumors in mice compared to the control group. The present results lead to the conclusion that polyurethane nanostructures, based on IPDI, are not a suitable formulation for the transdermic delivery of UA and OA. On the contrary, synthesizing particles of polyurethane by the same procedure, Borcan *et al*. obtained microstructures with good *in vitro* and *in vivo* toxicological profile, thus concluding that IPDI-based structures could be a successful

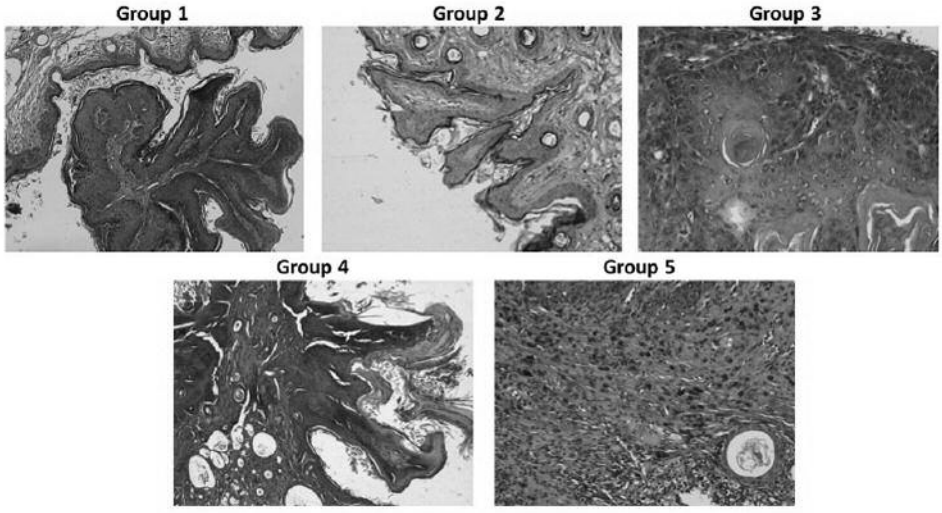

Figure 6. *Microscopic changes in the skin. Group 1, well-established squamous papilloma (in 75% of the mice) (HE ×100); Group 2, early squamous papilloma (in 50% of the mice) (HE ×200); Group 3, keratinized squamous cell carcinoma (in 50% of the mice) (HE ×400); Group 4, marked hyperkeratotic squamous papilloma (in 75% of the mice) (HE ×100); Group 5, inflammatory reaction with many mast cells in the upper dermis of a focal inverted papilloma (in 50% of the mice) (HE ×100).*

candidate as a transdermal drug delivery system (22). However, the present results are consistent with other findings obtained by our group using, *i.e.*, genistein as active agent incorporated into polyurethane microstructures. After encapsulation, genistein lost its *in vitro* anti-proliferative and antibacterial activity proving that polyurethane microstructures were an unsuitable partner for genistein (12). Moreover, our group has previously tested the *in vitro* anti-proliferative activity of the encapsulated UA and OA on melanoma cells where a lack of improvement was observed. A possible explanation could be the strong entrapment of the active agent inside the polymeric nanostructures that probably fails to exert its pharmacological activity (23).

Using the experimental model of skin carcinogenesis induced by DMBA and TPA, the study showed a moderate chemopreventive activity of UA against DMBA- and TPA-induced tumors.

Conclusion

In an attempt to obtain formulations with improved biopharmaceutical properties, polyurethane nanocapsules incorporating OA and UA were synthesized. However, biological analysis proved that that the encapsulation inside PU caused loss of UA's chemopreventive effect. Furthermore, treatment with PU-UA led to the formation of keratinized squamous cell carcinoma. In case of OA and PU-OA, no significant changes were observed compared to the control group.

Acknowledgements

Dr. Cristina Dehelean, Dr. Corina Danciu, Dr. Florina Bojin and Dr. Ioana Pavel were supported by the UMFT grant Parteneriate în cercetarea fundamentală inovativă-PIII-C2-PCFI-2015/2016, acronym FLAVOFORM.

References

1 Jäger S, Holger Trojan H, Thomas Kopp T, Laszczyk M and Scheffler A: Pentacyclic Triterpene Distribution in Various Plants - Rich Sources for a New Group of Multi-Potent Plant Extracts. Molecules *14*: 2016-2031, 2009.

2 Martin R, Fausten G and Bischoff F: Screening verschiedener Arten auf ihren Gehalt an den Triterpenen Ursol- und Oleanolsaure (Screening of different species on their content of the triterpenes ursolic and oleanolic acid). Z Arznei-Gewurzpfla, *14(1)*: 37-43, 2009.

3 Cijo-George V, Kumar N, Suresh P and Kumar A: Oleanolic acid inhibits cell growth and induces apoptosis in A375 melanoma cells. Biomedicine and Preventive Nutrition *4*: 95-99, 2014.

4 Shanmugam M, Dai X, Kumar A, Tan B, Sethi G and Bishayee A: Oleanolic acid and its synthetic derivatives for the prevention and therapy of cancer: Preclinical and clinical evidence. Cancer Lett *346*: 206-216, 2014.

5 Yarosh D, Both D and Brown D: Liposomal ursolic acid (merotaine) increases ceramides and collagen in human skin. Horm Res *54*: 318-321, 2000.

6 Meng Q, Roubin R and Hanrahan J: Ethnopharmacological and bioactivity guided investigation of five TCM anticancer herbs. J Ethnopharmacol *148*: 229-238, 2013.

7 Laszczyk M: Pentacyclic Triterpenes of the Lupane, Oleanane and Ursane Group as Tools in Cancer Therapy. Planta Med *75*: 1549-1560, 2009.
8 Yang L, Sun Z, Zu Y, Zhao C, Sun X, Zhang Z and Zhang L: Physicochemical properties and oral bioavailability of ursolic acid nanoparticles using supercritical anti-solvent (SAS) process. Food Chem *132(1)*: 319-325, 2012.
9 Chen H, Gao Y, Wang Y, Zhou X, Zheng Y and Zhou J: Evolution in medicinal chemistry of ursolic acid derivatives as anticancer agents. Eur J Med Chem *92*: 648-655, 2015.
10 Stamatin I: Nanomateriale aplicaţii în biosenzori, surse de energie, medicină şi biologie, Elemente de nanotehnologie. Universitatea Bucureşti, 2008.
11 Heghes A, Soica CM, Ardelean S, Ambrus R, Muntean D, Găluscan A, Dragos D, Ionescu D and Borcan F: Influence of emulsifiers on the characteristics of polyurethane structures used as drug carrier. Chem Cent J *7(1)*: 66, 2013.
12 Danciu C, Borcan F, Soica C, Zupko I, Csanyi E, Amburs R, Muntean D, Sass C, Antal D, Toma C and Dehelean C: Polyurethane Microstructures-a Good or Bad *in vitro* Partner for the Isoflavone Genistein? Nat Prod Comm *10(6)*: 951-954, 2015.
13 Cerga O, Borcan F, Ambrus R and Popovici I: Syntheses of new cyclodextrin complexes with oleanolic and ursolic acids. J Agroaliment Process Technol *17*: 405-409, 2011.
14 Dehelean CA, Feflea S, Gheorgheosu D, Ganta S, Cimpean AM, Muntean D and Amiji MM: Anti-angiogenic and anti-cancer evaluation of betulin nanoemulsion in chicken chorioallantoic membrane and skin carcinoma in balb/c mice. J Biomed Nanotechnol *9*: 577-589, 2013.
15 Hojjatoleslami SA, Claridge E and Moncrieff M: Accuracy of the skin model in quantifying blood and epidermal Melanin. www.researchgate.net, 2000.
16 Dwivedi C, Maydew ER, Hora JJ, Ramaeker DM and Guan X: Chemopreventive effects of various concentrations of α-santolol on skin cancer development in CD-1 mice. Eur J Cancer Prev *14*: 473-476, 2005.
17 Huang M, Ho C, Wang Z, Ferrato T, Lou YR, Stauber K, Ma W, Georgiadis C, Laskin JD and Conney AH: Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. Cancer Res *54*: 701-708, 1994.
18 Tokuda H, Ohigashi H, Koshimizu K and Ito Y: Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 120-tetradecanoylphorbol-13-acetate. Cancer Lett *33*: 279-328, 1986.
19 Alvarado HL, Abrego G, Souto EB, Garduño-Ramirez ML, Clares B, García ML and Calpena AC: Nanoemulsions for dermal controlled release of oleanolic and ursolic acids: *In vitro*, ex vivo and *in vivo* characterization. Coll Surf B *130*: 40-47, 2015.
20 Yang L, Sun Z, Zu Y, Zhao C, Sun X and L Z: Physicochemical properties and oral bioavailability of ursolic acid nanoparticles using supercritical anti-solvent (SAS) process. Food Chem *132(1)*: 319-325, 2012.
21 Gao D, Tang S and Tong Q: Oleanolic acid liposomes with polyethylene glycol modification: promising antitumor drug delivery. Int J Nanomed *7*: 3517-3526, 2012.
22 Borcan F, Soica C, Ganta S, Amiji MM, Dehelean CA and Munteanu MF: Synthesis and preliminary *in vivo* evaluations of polyurethane microstructures for transdermal drug delivery. Chem Cent J *6*: 87, 2012.
23 Oprean C, Borcan F, Cristea M, Bojin F, Ivan A, Mioc M, Trandafirescu C, Soica C and Paunescu V: Polyurethane nanostructures incorporating ursolic and oleanolic acids: *in vitro* antiproliferative evaluation. Fiziologia – Physiology *25.1(85)*: 59-44, 2015.

Received April 23, 2016
Revised May 30, 2016
Accepted May 31, 2016