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ABSTRACT

**SYNTHESIS AND BIOLOGICAL EVALUATION OF BETULINIC ACID'S
FATTY ESTERS AND THEIR RESPECTIVE LIPOSOMAL
FORMULATIONS**

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1. INTRODUCTION

Throughout history several natural compounds have served as treatment for a wide range of maladies, including infectious diseases and different types of cancer. Due to their chemical versatility, natural derivatives offer many advantages compared to their synthetic analogues. Cancer, in all of its forms and manifestations, represents one of the most frequent causes of death worldwide. In spite of the wide range of antineoplastic agents available, their numerous side-effects and lack of selectivity towards cancerous cells prompted many researchers to develop anticancer drugs by modifying the main structures of different natural compounds that already possessed anticancer effects.

Pentacyclic triterpenes are secondary metabolites often found in the plant kingdom around the globe and can be classified according to their core structure in lupane, oleanane and ursane derivatives (Figure 1).

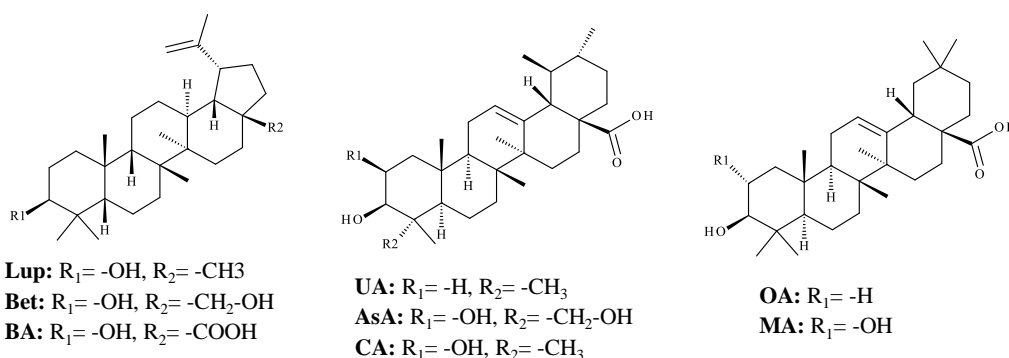


Figure 1. The classification of pentacyclic triterpenes based on their core structures; Lup (lupeol), Bet (betulin), BA (betulinic acid), UA (ursolic acid), AsA (asiatic acid), CA (corosolic acid), OA (oleanolic acid), MA (maslinic acid).

Betulinic acid (3β , hydroxy-lup-20(29)-en-28-oic acid; BA) is a lupane-type pentacyclic triterpene, widely distributed in many plant species, but mostly found in the birch bark. In terms of pharmacological efficacy, betulinic acid exerts a plethora of biological effects, being a strong anticancer, antiviral, antifungal, cardioprotective, renoprotective and neuroprotective agent.

Despite its tremendous pharmacological potential, BA's effects are hampered by its low bioavailability. To solve this issue, many strategies have been implemented, the main ones being: chemical derivatizations, cyclodextrin complexation and inclusion in different types of nanoformulations. Amongst the different types of chemical derivatizations, the esterification with fatty acids has emerged as an interesting approach to developing compounds with much improved pharmacological properties. Even though fatty acids were demonstrated as potent

antiviral, antifungal and anticancer agents, their highly lipophilic structures hamper their biological effects. Hence, to overcome this setback, a strategy of including them in liposomal formulations can be employed.

Liposomes (Figure 2) have been widely developed in the last decades due to their wide range of advantages such as their ability to incorporate small quantities of active compounds, as well as incorporate different types of highly lipophilic compounds and deliver them to a targeted site. However, the main disadvantage of employing conventional liposomes resides in their interactions with the mononuclear phagocyte system, causing the elimination of the active drug in the plasma.

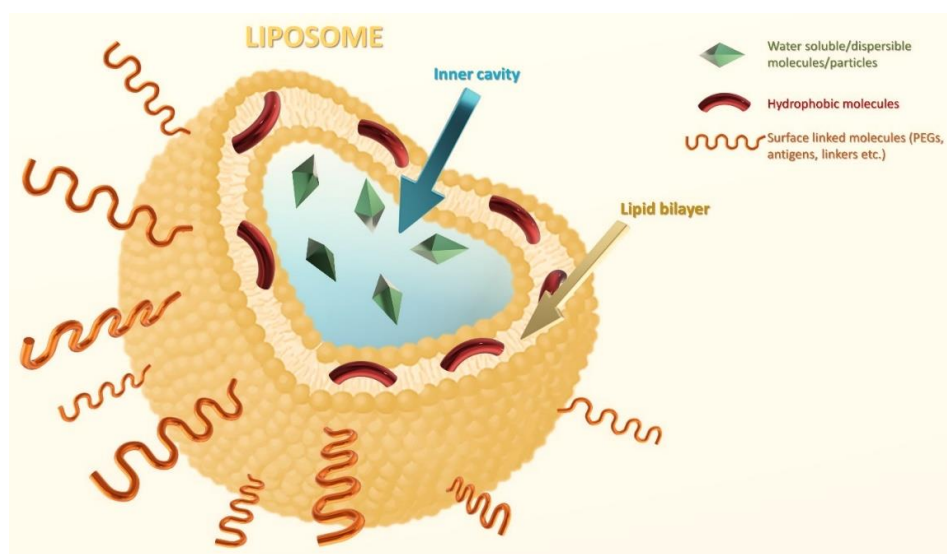


Figure 2. The schematic representation of a liposomal structure

This setback is eliminated by modifying the surfaces of conventional liposomes with natural polymers, like polyethylene glycol (PEG). Such surface modifications lead to much improved stability and help them evade the mononuclear phagocyte system, without inducing any inflammatory response.

2. AIM AND OUTLINE

The aim of this thesis is the synthesis and physicochemical evaluation of betulinic acid's fatty esters and their surface-modified liposomes as well as their *in silico*, *in ovo* and *in vitro* evaluation against melanoma, breast adenocarcinoma, colon adenocarcinoma and non-small lung cancer cells.

The first part of the thesis provides information about the recent specialized literature on pentacyclic triterpenes, betulinic acid's pharmacokinetic and pharmacodynamic profile and several types of liposomal formulations. Later on, different types of betulinic acid's liposomal formulations already tested *in vitro* and *in vivo* are described.

My personal contribution consists of the synthesis of betulinic acid fatty esters obtained with butyric, stearic and palmitic acids and their incorporation in PEGylated liposomes. The identity of the newly synthesized fatty esters was determined using FTIR and NMR analysis. The fatty esters were then incorporated in PEGylated liposomes; the respective liposomes were physico-chemically characterized in terms of stability, size and drug loading efficacy. Consequently, an *in silico* study was performed in order to determine the theoretical binding affinity of the fatty esters to the anti-apoptotic proteins Bcl-2, Bcl-XL and NF- κ B for assessing their theoretical *in vitro* results.

The next step was the *in vitro* assessment of the betulinic acid's fatty esters and their respective liposomal formulations against HaCaT human keratinocytes, A375 melanoma cells, MCF-7 breast adenocarcinoma, HT-29 colon adenocarcinoma and NCI-H460 small-cell lung cancer cells. I evaluated the anticancer potential of each fatty ester compared to the parent compound. Thus, I determined their cytotoxic profile against non-cancerous cells and whether their inclusion in liposomal formulations led to improved cytotoxic effects. Furthermore, I proposed a molecular mechanism responsible for their cytotoxic effects by determining the relative fold expression of pro-apoptotic and anti-apoptotic genes and their modulatory effect on caspase activity.

Finally, an *in ovo* assay was performed to evaluate the irritative potential of the fatty esters and their respective liposomes, compared to the parent compound. The aim was to determine whether these derivatives would be suitable for local and mucosal applications.

3. RESULTS

3.1. SYNTHESIS, PHYSICOCHEMICAL AND IN SILICO ASSESSMENT OF BETULINIC ACID FATTY ESTERS AND THEIR RESPECTIVE LIPOSOMAL FORMULATIONS

All three esters were obtained in high yields (>65%); the reaction conditions for obtaining the fatty esters are depicted in Figure 3.

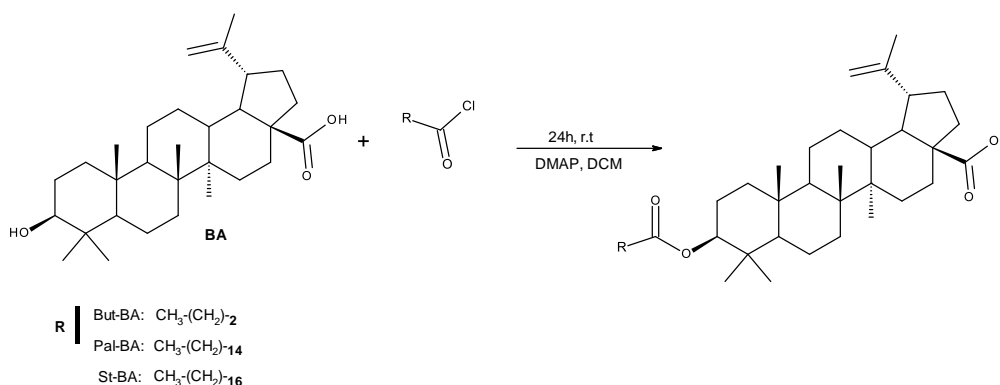


Figure 3. Synthesis of BA's fatty esters derivatives; BA: betulinic acid, But-BA: 3-O-butyryl-betulinic acid, Pal-BA: 3-O-palmitoyl-betulinic acid, St-BA: 3-O-stearoyl-betulinic acid; DCM: dichloromethane, DMAP: 4-dimethylaminopyridine.

The identity of the three formed esters was validated through FTIR spectroscopy (Figure 4) and, more accurately, was once again confirmed by employing the NMR spectroscopy.

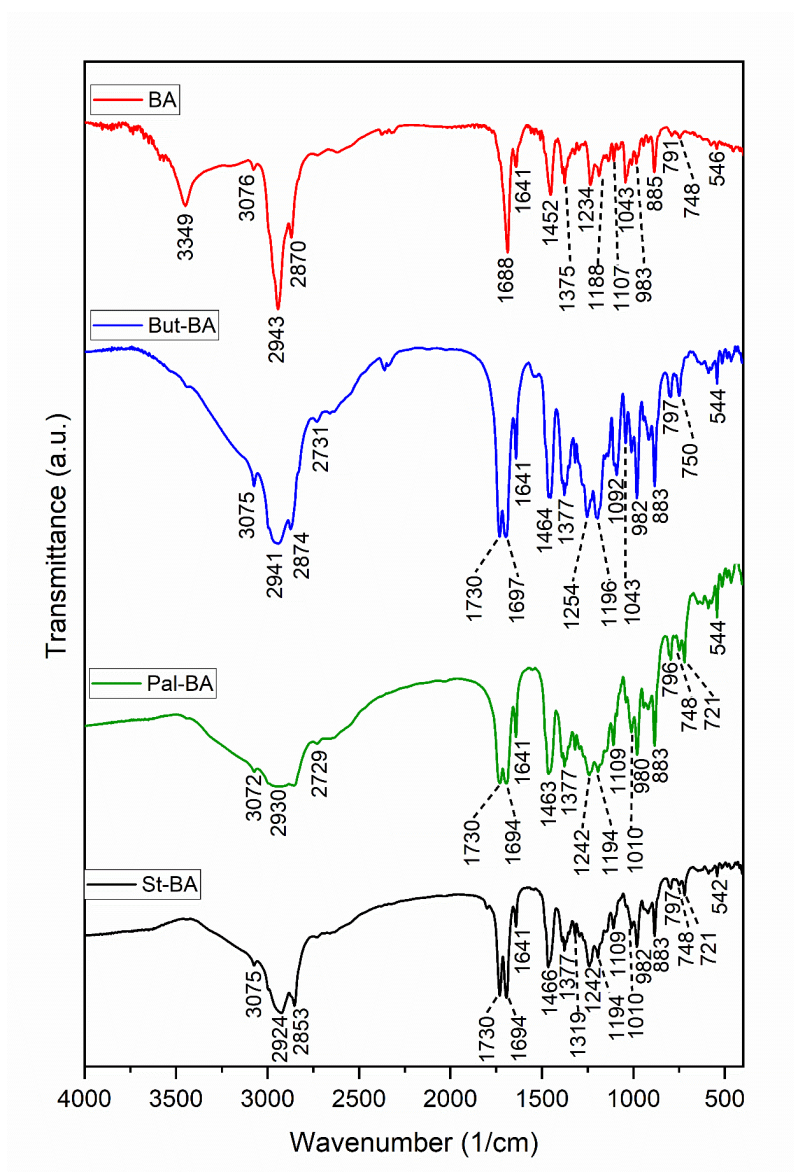


Figure 4. FTIR spectra of BA, But-BA, Pal-BA and St-BA

I employed the lipid film hydration technique to obtain bare liposomes and BA's fatty esters loaded liposomes. I used transmission electronic microscopy (TEM), scanning electron microscopy (SEM) and dynamic light scattering (DLS) to analyse the obtained liposomes. The SEM and TEM analysis revealed stable, spherical liposomes of various sizes. The DLS measurements were assessed daily for a week. The formulations proved stable within the tested period, demonstrating no significant changes in dispersity index values and hydrodynamic size.

Due to certain chemical modulations leading to increased affinity for anti-apoptotic protein targets, I conducted a two-way molecular docking study using two distinct functions (Vina and Glide) to determine the BA's fatty esters modulatory effect on the anti-apoptotic proteins Bcl-2, Bcl-XL and NF- κ B. The obtained scores are depicted in Table 1. Both anti-

apoptotic proteins and docking scores revealed that the most promising results were generated for long-chain fatty acids, in this case Pal-BA, compared to the parent compound, BA, or But-BA.

Table 1. The docking scores of BA, But-BA, Pal-BA and St-BA against Bcl-2 and Bcl-XL using Vina and Glide docking programs.

Ligand	Bcl-2 (PDB ID: 4LVT)		Bcl-XL (PDB ID: 2YXJ)		NF- κ B (PDB ID: 1NFK)	
	Vina docking score	Glide docking score	Vina docking score	Glide docking score	Vina docking score	Glide docking score
BA	-7,7	-4,3	-8,5	-4,9	-8	-3.3
But-BA	-7,8	-3,8	-8,4	-3,9	-7.6	-4.1
Pal-BA	-8,5	-6,4	-9,1	-6,5	-6	-5.1
St-BA	-7,6	-6,1	-8,7	-6,7	-5.1	-4.7

3.2. BIOLOGICAL ANTI-MELANOMA ASSESSMENT OF BETULINIC ACID FATTY ESTERS AND THEIR LIPOSOMAL FORMULATIONS

Biological *in vitro* assessment of the newly obtained BA fatty esters and their liposomal formulations was the focus of the second study, which was conducted on HaCaT human keratinocytes and A375 human melanoma cells. The biological tests conducted on the tested derivatives assessed their effects on cell viability and morphology, their anti-migratory potential, as well as their effect on the fold-gene expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein BAX. Cell viability results indicated selective cytotoxic properties towards melanoma cells. Both the fatty esters and their liposomal formulations were able to inhibit A375 cells proliferation in a time- and dose-dependent manner, while not significantly affecting the viability of HaCaT cells (Table 2).

Table 2. The calculated IC₅₀ values (μ M) of 5-FU, BA, BA-Lip, Pal-BA, Pal-BA-Lip, St-BA, St-BA-Lip, But-BA and But-BA-Lip on HaCaT and A375 cell lines 48h post-stimulation.

Compounds	HaCaT	A375
5-FU	40.14 \pm 1.2	22.61 \pm 0.82
BA	>100	65.9 \pm 1.07
BA-Lip	>100	59.01 \pm 0.45
Pal-BA	>100	85.58 \pm 1.32
Pal-BA-Lip	>100	67.59 \pm 0.33
St-BA	>100	75.75 \pm 0.75
St-BA-Lip	>100	60.11 \pm 1.56
But-BA	>100	60.77 \pm 0.29
But-BA-Lip	>100	50.71 \pm 0.67

It was noted that all liposomes exerted stronger cytotoxic effects compared to their respective fatty esters, But-BA-Lip demonstrating the most potent cytotoxic effects against A375 melanoma cells.

Furthermore, the pro-apoptotic effects of the newly synthesized compounds were disclosed by Hoechst and beta-actin staining. Apoptotic hallmarks such as nuclear condensation, shrinkage and fragmentations were observed in both BA fatty esters and their respective liposomes in A375 cells, while no sign of apoptosis was remarked in HaCaT cells. These results can be corroborated with the anti-migratory Scratch assay, where the compounds inhibited cancer cell migration and didn't significantly affect the keratinocytes' migration. The pro-apoptotic effect of these derivatives was confirmed by assessing the gene expressions of BAX and Bcl-2 proteins (Figure 5). The results showed that all compounds up-regulated the expression of pro-apoptotic BAX gene and down-regulated the expression of the anti-apoptotic Bcl-2 gene.

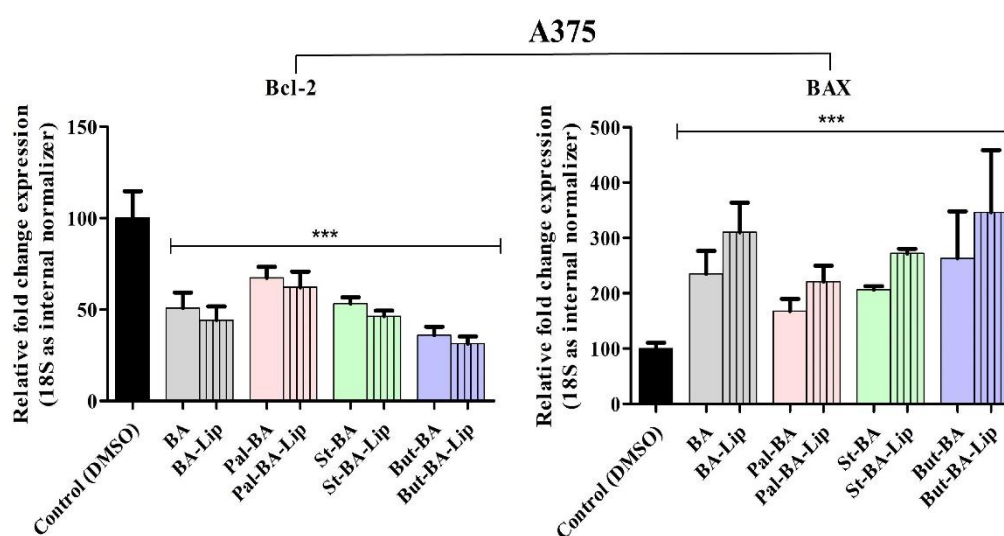


Figure 5. The relative fold change in Bcl-2 and BAX mRNA expression in A375 cells after treatment with BA, BA-Lip, Pal-BA, St-BA, But-BA, Pal-BA-Lip, St-BA-Lip and But-BA-Lip (10 μ M). The expressions were normalized to 18S and the control was DMSO. The data represents mean values and standard deviations of three independent experiments. *** $p < 0.001$, represent statistically significant differences in relationship with DMSO-stimulated cells, as determined by one-way ANOVA with Dunnett's post-test.

3.3. BIOLOGICAL EVALUATION OF BETULINIC ACID FATTY ESTERS AND THEIR LIPOSOMAL FORMULATIONS AGAINST BREAST, COLON AND LUNG CANCER

In light of the promising anti-melanoma results obtained for both BA fatty esters and their respective liposomal formulations, the third study focused on exploring their anticancer potential against MCF-7 human breast adenocarcinoma, HT-29 human colorectal adenocarcinoma and NCI-H460 non-small lung cell adenocarcinoma. The biological tests conducted in this study focused on the compounds' effects on cell viability, cell morphology, their effect on caspase-3/-7 activation; it also assessed their irritative potential on the chorioallantoic membrane. The viability evaluation revealed that, both the fatty esters and their respective liposomes exerted cytotoxic effects against all tested cancer cells in a time- and dose-dependent manner. Moreover, it was noted that the liposomes exhibited stronger anticancer effects compared to their respective unincorporated fatty esters (Table 3).

Table 3. The calculated IC₅₀ values (μM) of 5-FU, BA, BA-Lip, Pal-BA, Pal-BA-Lip, St-BA, St-BA-Lip, But-BA and But-BA-Lip on MCF-7, Ht-29 and NCI-H460 cell lines 48h post-stimulation.

Compound	MCF-7	HT-29	NCI-H460
5-FU	30.79 ± 0.34	82.53 ± 0.77	62.89 ± 0.28
BA	54.97 ± 0.87	91.16 ± 0.93	39.48 ± 0.74
BA-Lip	54.89 ± 0.26	59.04 ± 1.44	35.42 ± 0.91
Pal-BA	>100	>100	52.37 ± 0.35
Pal-BA-Lip	>100	70.06 ± 0.54	41.72 ± 0.94
St-BA	>100	>100	61.17 ± 1.48
St-BA-Lip	>100	>100	43.35 ± 1.06
But-BA	63.17 ± 0.28	77.72 ± 0.63	50.26 ± 0.53
But-BA-Lip	48.88 ± 1.32	30.57 ± 1.02	30.74 ± 1.16

The morphological assessment of the cells treated with BA's fatty esters and their liposomal formulations revealed specific apoptotic hallmarks after staining with F-actin and Hoechst reagent. The cytoskeletons were disorganized, while the nuclei underwent specific apoptotic modifications such as shrinkage, fragmentations and chromatin condensation. The CellEvent caspase-3/-7 detection staining was employed for determining whether the treatment with BA's derivatives was able to modulate the caspase activity; the results showed that all compounds activated the caspase activity, leading to apoptosis in cancer cells.

Further, the irritative potential of BA's fatty esters and their respective liposomes was conducted using the HET-CAM assay (Figure 6). The results indicated that both BA's fatty esters and their respective liposomes may be considered biocompatible with mucosal tissues and a safe type of nanoformulations for local applications. No sign of toxicity was registered in terms of vascular lysis, hemorrhage and coagulability.

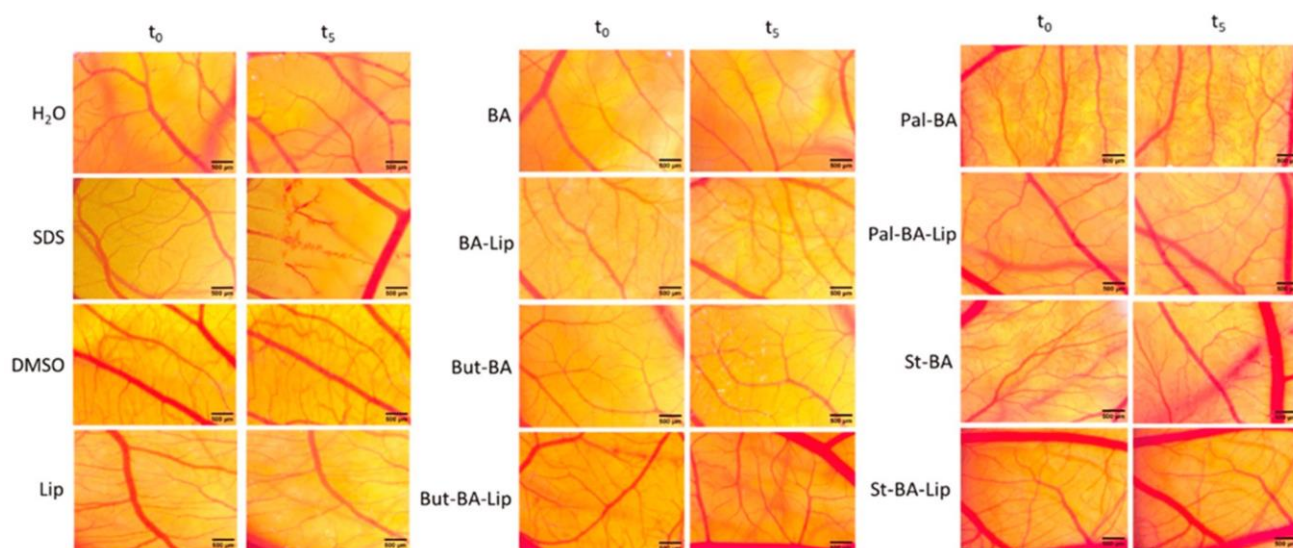


Figure 6. The irritation test based on the HET-CAM assay. The images of chorioallantoic membrane were taken before (T_0), 300s (T_5) after stimulation with 600 μL BA, BA-Lip, But-BA, But-BA-Lip, Pal-BA, Pal-BA-Lip, St-BA, St-BA-Lip (100 μM), distilled water as negative control and sodium dodecylsulfate (SDS) 0.5%. The scale bar was set at 500 μm .

4. CONCLUSION

All in all, the esterification of betulinic acid with butyric, stearic and palmitic acids represents a promising approach in discovering BA derivatives with improved anticancer effects, that can also be used *in vivo* due to their lack of toxic effects against keratinocytes cells and chorioallantoic membrane. Their pro-apoptotic potential can be enhanced by incorporating them in PEGylated liposomes.

These results aim to bridge the gap in specialized literature regarding these types of BA derivatizations by employing fatty acids. They also help elucidate the molecular mechanisms responsible for their pro-apoptotic effects against cancer cells. These studies may represent a ground work for developing BA derivatives with potent anticancer effects that could be used in further clinical trials.