



## Terpene Esters from Natural Products: Synthesis and Evaluation of Cytotoxic Activity

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### ABSTRACT

Natural steroids and triterpenes such as  $\beta$ -sitosterol, stigmasterol, lupeol, ursolic and betulinic acids were transformed into its hexanoic and oleic esters, to evaluate the influence of chemical modification towards the cytotoxic activities against tumor cells. The derivatives were evaluated against five tumor cell lines [OVCAR-8 (ovarian carcinoma); SF-295 (glioblastoma); HCT-116 (colon adenocarcinoma); HL-60 (leukemia); and PC-3 (prostate carcinoma)] and the results showed only betulinic acid hexyl ester exhibits cytotoxic potential activity.

**Key words:** antitumor activity, chemical modification, derivative synthesis, natural products esters.

### INTRODUCTION

Cancer is a generic term used for a large group of diseases that can affect any part of the body, which is also described as malignant tumors and neoplasms. According World Health Organization, it accounted to 8.2 million deaths worldwide in 2012 (13% of all deaths) with 14.1 million new cases (WHO 2016). Main risk in cancer treatment is multidrug resistance, when cell lose their sensitivity to chemotherapeutics. Due this, actions

against cancer are focused on developing and reinforcing cancer control programs and also to the search alternative treatments.

The therapeutic use of active natural compounds or its derivatives as anticancer agents represents nowadays a highly-investigated research field. Triterpenic acids exhibit unique and important biological and pharmacological activities, including anti-inflammatory, antimicrobial, antiviral, cytotoxic, and cardiovascular effects (Silva et al. 2012). The total synthesis of this class of natural products is still not an easy strategy to obtain

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multigram quantities for *in vivo* biological screening and evaluated assays. For example, commercial betulinic acid is prepared by chemical selective oxidation of the alcohol derivative (betulin) and it is also isolated in good yields together the isomers ursolic and oleanolic acids from a restricted group of plants. *Eriope blanchetii*, a shrub belonging to the Lamiaceae family, is one of them (David et al. 2001).

Important classes of natural compounds or its derivatives are esters (Azimova 2013). This class of compounds shows variety of structural types leading to a wide spectrum of biological activity, including aliphatic and aromatic moieties. Although its biological role in plants has not been fully studied, a plethora of biological activities have been appointed, such as antibacterial, antifungal, estrogenic, antiestrogenic, inhibition of testosterone secretion, immunological, anti-inflammatory, cytotoxic, and ionophoretic properties, relaxant effect, vasodilatory effect, antagonist of calcium, etc. Derivative esters of natural products can be obtained from natural sources (Carvalho et al. 2001), but its use as lead compounds for medicinal chemistry gives an idea of the potential of this approach. As selected example of lead compound derivated from betulinic acid spawned the drug Bevirimat<sup>®</sup> (**2** and **1**, respectively, Fig. 1), which has been showed to be specific inhibitors of HIV-1 entry (Qian et al. 2010). According this strategy, this article described our work in syntheses of specific acyl derivatives of some natural products, and evaluation of their antiproliferative activities against some tumor cell lines.

## EXPERIMENTAL

### GENERAL EXPERIMENTAL PROCEDURES

DIC, DMAP, MTT, and stigmasterol were purchased from Sigma-Aldrich and used without further purification.  $\beta$ -Sitosterol was used as a commercial mixture with stigmasterol (70:30,

respectively, MP Biomedicals). Betulinic and ursolic acids were previously isolated from *Eriope blanchetti* (Lamiaceae). Lupeol was obtained from the hexane extract of the roots of *Bowdichia virgilioides* (Fabaceae) by silica gel conventional column chromatography. Dichlorometane was refluxed with  $\text{CaH}_2$  and distilled prior to use. All reactions were performed under argon atmosphere. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F254 (250  $\mu\text{m}$  thickness). Visualization was accomplished using UV light and potassium permanganate solution. Column chromatography was performed on silica gel 60-230 mesh. The melting points were uncorrected and determined on a MQAPF-302 apparatus. IR spectra were measured using a Shimadzu IR-Affinity 1 spectrophotometer. Nuclear magnetic resonance spectra were recorded on Varian (Inova-500) 500 MHz spectrometer in deuterated solvents.

### ISOLATION OF LUPEOL 4 FROM ROOTS OF BOWDICHIA VIRGILIOIDES (FABACEAE)

Dried roots of *B. virgilioides* (480 g) was powdered and submitted to maceration with 2 L of MeOH. The MeOH extract (40.7 g) was partitioned between hexane:MeOH/ $\text{H}_2\text{O}$  (5%) and the soluble fraction of hexane obtained (5.02 g) was submitted to a silica gel 60 CC employing mixtures of hexane:EtOAc. The fractions eluted with 90% hexane (430 mg) was reactive in Libermann-Buchard reagent and purified in a Sephadex LH-20 column eluted with DCM:MeOH (1:1). This procedure permitted to obtain 153 mg of lupeol **4**. This triterpene was identified by mp, IR and NMR spectra, comparing with the literature data (Mahato and Kundu 1994).

### GENERAL PROCEDURE FOR THE SYNTHESIS OF COMPOUNDS **2A**, **B-6A**, **B** (REACTIONS WERE PERFORMED IN A 2 ML VIAL)

To a solution of appropriate substrate (0.02 mmol) in dichloromethane was added a solution

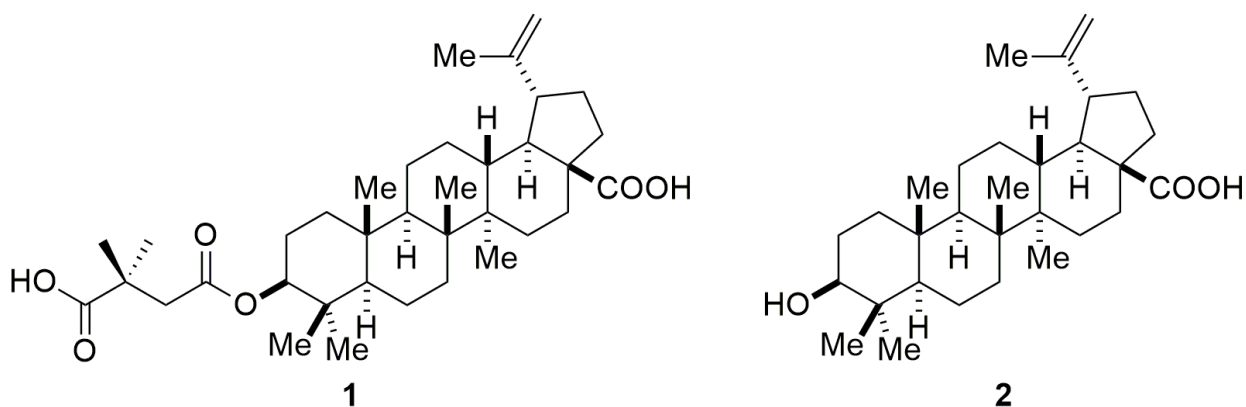


Figure 1 - Betulinic acid and its derivative Bevirimat<sup>®</sup> (2 and 1, respectively).

of DMAP (4.9 mg, 0.04 mmol) and DIC (5.1 mg, 0.04 mmol) in 0.5 mL of dichloromethane via cannula. Thereafter a solution of appropriate acid (0.04 mmol) in dichloromethane (0.5 mL) at room temperature was added. After stirring for 24-48 hours at the same temperature, the reaction was stopped by diluting with dichloromethane, followed by filtration of solids and concentrated in a vacuum. Purification by column chromatography in silica-gel eluted with ethyl acetate:hexane furnished the desired product.

#### Betulinyl hexanoate (2a)

Mass obtained: 10.0 mg, 94% yield; rf 0.66 in EtOAc:hexane (20:80);  $[\alpha]_D^{25} +3.0$  (*c* 1.0, CHCl<sub>3</sub>); FTIR (KBr)  $\nu / \text{cm}^{-1}$  3300, 2856, 1728, 1458, 1296; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  5.30 (s, 1H), 4.74 (s, 1H), 4.62 (s, 1H), 4.48 (dd, 1H, *J* 10.9, 5.5 Hz, 1H), 3.03 – 2.96 (m, 1H), 2.40-2.18 (m, 5H), 2.02 – 1.94 (m, 2H), 1.70 (s, 3H), 1.67 – 1.58 (m, 6H), 1.56 – 1.48 (m, 2H), 1.48- 1.26 (m, 14H), 1.23-1.19 (m, 2H), 0.98 (s, 3H), 0.95 (s, 3H), 0.90 (t, 3H, *J* 11.0 Hz), 0.87 – 0.82 (m, 9H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  181.87, 173.48, 149.96, 109.87, 80.31, 58.41, 56.37, 55.56, 50.56, 49.35, 38.44, 38.41, 37.86, 37.24, 37.10, 34.78, 34.32, 32.16, 31.20, 31.06, 30.54, 29.69, 27.96, 25.49, 24.79, 24.31,

23.74, 22.28, 20.89, 18.25, 16.52, 16.03, 14.66, 13.86, 13.82, 13.80.

#### Betulinyl oleate (2b)

Mass obtained: 11.5 mg, 80% yield; rf 0.55 in EtOAc:hexane (20:80);  $[\alpha]_D^{25} +14.0$  (*c* 1.0, CHCl<sub>3</sub>); FTIR (KBr)  $\nu / \text{cm}^{-1}$  2999, 1732, 1643 1452; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  5.38 – 5.31 (m, 2H), 4.75 (s, 1H), 4.62 (s, 1H), 4.48 (dd, 1H, *J* 10.8, 5.5 Hz), 3.07 – 2.93 (m, 1H), 2.33 – 2.26 (m, 2H), 2.08 – 1.91 (m, 4H), 1.70 (s, 3H), 1.68 – 1.54 (m, 4H), 1.53 – 1.37 (m, 6H), 1.36 – 1.22 (m, 36H), 0.98 (s, 3H), 0.95 (s, 3H), 0.89 (t, 3H, *J* 6.9 Hz), 0.86 (s, 3H), 0.84 (s, 3H), 0.83 (s, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  173.62, 150.32, 129.97, 129.73, 109.72, 80.59, 77.22, 76.97, 56.32, 55.46, 50.45, 49.31, 46.92, 42.45, 40.74, 38.41, 37.85, 37.15, 37.02, 34.83, 34.29, 32.16, 31.89, 30.57, 29.75, 29.70, 29.67, 29.50, 29.31, 29.29, 29.14, 29.09, 27.97, 27.21, 27.15, 25.49, 25.14, 23.74, 22.66, 20.88, 19.34, 18.18, 16.53, 16.15, 16.03, 14.67, 14.06.

#### Ursolyl hexanoate (3a)

Mass obtained: 10.0 mg, 90% yield; rf 0.70 in EtOAc:hexane (20:80);  $[\alpha]_D^{25} +8.0$  (*c* 1.0, CHCl<sub>3</sub>); FTIR (KBr)  $\nu / \text{cm}^{-1}$  3414, 2856, 1730, 1485, 1240; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  5.29 – 5.25 (m, 1H),

4.54 (dd, 1H,  $J$  10.4, 5.8 Hz), 2.32 (t, 2H,  $J$  7.3 Hz), 2.22 (d, 1H,  $J$  11.5 Hz), 2.07 (s, 1H), 2.03 (dd, 1H,  $J$  13.4, 4.3 Hz), 1.78 – 1.59 (m, 9H), 1.58 – 1.48 (m, 4H), 1.46 – 1.22 (m, 16H), 1.14 (s, 3H), 1.11 (s, 3H), 1.01 – 0.98 (m, 3H), 0.93 (t, 3H,  $J$  6.1 Hz), 0.91 – 0.85 (m, 6H), 0.82 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  182.33, 173.56, 137.97, 125.78, 80.59, 59.56, 55.36, 52.66, 47.96, 47.50, 41.99, 39.56, 39.05, 38.85, 38.32, 38.16, 37.74, 36.94, 36.72, 34.78, 32.91, 31.33, 31.21, 30.61, 29.65, 28.09, 28.02, 24.80, 24.13, 23.60, 23.56, 23.30, 22.26, 21.10, 18.19, 17.11, 16.97, 16.74, 15.49, 13.82.

#### *Ursolyl oleate (3b)*

Mass obtained: 7.0 mg, 90% yield; rf 0.65 in EtOAc:hexane (20:80);  $[\alpha]_{\text{D}}^{25} +19.0$  ( $c$  1.0,  $\text{CHCl}_3$ ); FTIR (KBr)  $\nu / \text{cm}^{-1}$  3338, 2926, 1710, 1619;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.36 – 5.32 (m, 2H), 5.26 – 5.23 (s, 1H), 4.50 (dd, 1H,  $J$  10.5, 5.7 Hz), 2.28 (td, 1H,  $J$  7.5, 3.3 Hz), 2.19 (d, 1H,  $J$  11.5 Hz), 2.04 – 1.98 (m, 4H), 1.94 – 1.82 (m, 3H), 1.76 – 1.58 (m, 6H), 1.55 – 1.45 (m, 4H), 1.43 (s, 3H), 1.38 – 1.20 (m, 32H), 1.14 (s, 6H), 1.12 (s, 3H), 0.97 (s, 3H), 0.93 (t, 3H,  $J$  6.1 Hz), 0.91 – 0.86 (m, 3H), 0.82 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  173.59, 137.96, 129.97, 129.73, 125.78, 80.58, 59.54, 55.33, 52.67, 47.94, 47.49, 41.99, 39.53, 39.05, 38.84, 38.30, 38.15, 37.73, 36.91, 36.71, 34.82, 34.38, 32.91, 31.89, 31.22, 30.61, 29.75, 29.67, 29.50, 29.31, 29.29, 29.14, 29.09, 28.10, 28.01, 27.21, 27.15, 25.14, 24.97, 24.15, 23.60, 23.54, 23.30, 22.65, 21.13, 18.19, 17.09, 16.99, 16.77, 15.51, 14.23, 14.06.

#### *Lupenyl hexanoate (4a)*

Mass obtained: 20.0 mg, 72% yield; rf 0.50 in EtOAc:hexane (20:80),  $[\alpha]_{\text{D}}^{25} +31.0$  ( $c$  1.0,  $\text{CHCl}_3$ ), lit. (Brum et al. 1998)  $[\alpha]_{\text{D}}^{25} +31.0$  ( $c$  1.0,  $\text{CHCl}_3$ ); (KBr)  $\nu / \text{cm}^{-1}$  2926, 1730, 1691, 1541;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  4.70 – 4.67 (m, 1H), 4.58 –

4.55 (m, 1H), 4.47 (dt, 1H,  $J$  16.8, 8.4 Hz), 2.36 (td, 1H,  $J$  11.0, 5.6 Hz), 2.28 (t, 2H,  $J$  7.6 Hz), 2.05 – 1.98 (m, 1H), 1.96 – 1.86 (m, 1H), 1.67 – 1.59 (m, 8H), 1.52 – 1.45 (m, 2H), 1.43 – 1.25 (m, 21H), 1.04 (s, 3H), 0.94 (s, 3H), 0.89 (t, 3H,  $J$  6.1 Hz), 0.86 (s, 3H), 0.84 (s, 3H), 0.84 (s, 3H), 0.79 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  173.59, 150.89, 109.31, 80.63, 55.44, 53.34, 50.39, 48.35, 48.01, 43.00, 42.86, 40.90, 40.01, 38.43, 38.11, 37.85, 37.13, 35.60, 34.80, 34.27, 31.90, 31.33, 29.88, 29.67, 29.44, 29.33, 29.24, 29.16, 27.97, 27.47, 25.17, 24.82, 23.76, 22.66, 22.28, 20.98, 19.29, 18.23, 18.00, 16.54, 16.15, 16.00, 14.53, 14.05, 13.85.

#### *Lupenyl oleate (4b)*

Mass obtained: 13.7 mg, 61% yield; rf 0.7 in EtOAc:hexane (20:80);  $[\alpha]_{\text{D}}^{25} +8.0$  ( $c$  1.0,  $\text{CHCl}_3$ ); FTIR (KBr)  $\nu / \text{cm}^{-1}$  3068, 2926, 1730, 1452, 1174;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.37 – 5.33 (m, 2H), 4.70 (d, 1H,  $J$  2.4 Hz), 4.58 (dd, 1H,  $J$  2.4, 1.4 Hz), 4.48 (dd, 1H,  $J$  11.0, 5.4 Hz), 2.43 – 2.35 (m, 1H), 2.31 – 2.27 (m, 2H), 2.06 – 1.98 (m, 3H), 1.97 – 1.87 (m, 2H), 1.70 (s, 3H), 1.68 – 1.55 (m, 8H), 1.52 – 1.45 (m, 3H), 1.44 – 1.15 (m, 38H), 1.04 (s, 3H), 0.95 (s, 3H), 0.89 (t, 3H,  $J$  6.5 Hz), 0.87 (s, 3H), 0.85 (s, 3H), 0.85 (s, 3H), 0.80 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  173.83, 151.12, 130.16, 129.94, 109.53, 80.81, 55.59, 50.54, 48.49, 48.20, 43.19, 43.03, 41.05, 40.19, 38.58, 38.25, 38.03, 37.29, 35.77, 35.03, 34.42, 32.12, 32.10, 30.03, 29.96, 29.89, 29.87, 29.71, 29.55, 29.52, 29.51, 29.35, 29.34, 29.30, 28.16, 27.64, 27.41, 27.35, 25.34, 25.31, 23.94, 22.87, 21.15, 19.48, 18.40, 18.19, 16.76, 16.36, 16.17, 14.71, 14.29.

#### *Stigmasteryl hexanoate (5a)*

Mass obtained: 29.9 mg, 90% yield; rf 0.7 in EtOAc:hexane (20:80);  $[\alpha]_{\text{D}}^{25} -37.0$  ( $c$  1.0,  $\text{CHCl}_3$ ), lit. (Kuksis and Beveridge 1960)  $[\alpha]_{\text{D}}^{25} -37.7$  ( $c$  1.0,  $\text{CHCl}_3$ ); FTIR (KBr)  $\nu / \text{cm}^{-1}$  2926, 1737, 1437,

1382;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.38 (d, 1H,  $J$  5.0 Hz), 5.17 (dd, 2H,  $J$  15.2, 8.6 Hz), 5.03 (dd, 1H,  $J$  15.2, 8.7 Hz), 4.62 (tdd, 1H,  $J$  11.1, 6.8, 4.2 Hz), 2.32 (d, 1H,  $J$  6.9 Hz), 2.27 (t, 2H,  $J$  7.5 Hz), 2.08 – 1.94 (m, 4H), 1.87-1.83 (m, 2H), 1.75 – 1.68 (m, 2H), 1.66 – 1.40 (m, 12H), 1.36 – 1.25 (m, 6H), 1.22 - 1.12 (m, 4H), 1.04 (s, 3H), 1.04 (d, 3H,  $J$  6.5Hz), 0.91 (t, 3H,  $J$  6.7Hz), 0.86 (d, 3H,  $J$  6.6 Hz), 0.82 (d, 3H,  $J$  7.4 Hz), 0.80 (d, 3H,  $J$  6.3 Hz), 0.71 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  212.77, 175.21, 153.53, 149.84, 134.67, 134.44, 134.20, 133.71, 129.38, 129.25, 129.10, 129.08, 84.55, 84.05, 83.00, 82.75, 82.57, 82.49, 81.33, 81.17, 77.89, 75.80, 74.33, 73.86, 35.43, 35.39, 35.09, 31.62, 31.61, 31.56, 31.54, 31.50, 31.45, 31.36, 31.32, 28.42, 27.02, 26.75, 26.65, 26.60, 24.20, 24.19, 24.01, 23.79, 19.84.

#### *Stigmasteryl oleate (5b)*

Mass obtained: 30.0 mg, 72% yield; rf 0.7 in EtOAc:hexane (20:80);  $[\alpha]_{\text{D}}^{25}$  -49.0 ( $c$  1.0,  $\text{CHCl}_3$ ), lit. (Kuksis and Beveridge 1960)  $[\alpha]_{\text{D}}^{25}$  -49.9 ( $c$  1.0,  $\text{CHCl}_3$ ); FTIR (KBr)  $\nu / \text{cm}^{-1}$  3551, 1783, 1487, 1183;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.38 – 5.32 (m, 3H), 5.16 (dd, 1H,  $J$  15.2, 8.7 Hz), 5.02 (dd, 1H,  $J$  15.1, 8.8 Hz), 4.64 – 4.55 (m, 1H), 2.31 (d, 2H,  $J$  7.1 Hz), 2.26 (t, 2H,  $J$  7.4 Hz), 2.10 – 1.93 (m, 6H), 1.89 – 1.81 (m, 2H), 1.78 – 1.68 (m, 1H), 1.64 – 1.39 (m, 8H), 1.36 - 1.22 (m, 28H), 1.20 – 1.12 (m, 4H), 1.02 (s, 3H), 1.02 (d, 3H,  $J$  6.5Hz), 0.88 (t, 3H,  $J$  6.7Hz), 0.85 (d, 3H,  $J$  6.6 Hz), 0.81 (d, 3H,  $J$  7.4 Hz), 0.80 (d, 3H,  $J$  6.3 Hz), 0.70 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  173.26, 139.71, 138.29, 129.97, 129.75, 129.28, 122.55, 73.67, 56.79, 55.95, 51.23, 50.06, 42.21, 40.48, 39.63, 38.16, 37.01, 36.61, 34.71, 31.90, 31.89, 31.88, 29.77, 29.68, 29.52, 29.32, 29.31, 29.15, 29.09, 28.89, 27.82, 27.21, 27.16, 25.40, 25.05, 24.35, 22.67, 21.21, 21.07, 21.02, 19.31, 18.98, 14.10, 12.23, 12.04.

#### *$\beta$ -Sitosteroyl hexanoate (6a)*

Mass obtained: 30.0 mg, 86% yield; rf 0.75 in EtOAc:hexane (20:80);  $[\alpha]_{\text{D}}^{25}$  -36.0 ( $c$  1.0,  $\text{CHCl}_3$ ), lit. (Kuksis and Beveridge 1960)  $[\alpha]_{\text{D}}^{25}$  -38.0 ( $c$  1.0,  $\text{CHCl}_3$ ); FTIR (KBr)  $\nu / \text{cm}^{-1}$  3300, 2824, 2854, 1724, 1616, 1512, 1508, 1450, 1381, 1172;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.30 (s, 1H), 4.70 – 4.58 (m, 1H), 2.32 (d, 2H,  $J$  7.0Hz), 2.27 (t, 2H,  $J$  7.6Hz), 2.08 – 1.94 (m, 4H), 1.90 - 1.80 (m, 4H), 1.70 – 1.45 (m, 10H), 1.40 – 1.25 (m, 9H), 1.25 - 1.08 (m, 4H), 1.04 – 1.02 (m, 6H), 0.94 - 0.88 (m, 6H), 0.88 – 0.78 (m, 6H), 0.68 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  173.48, 139.92, 138.49, 129.48, 122.75, 77.45, 77.19, 76.94, 73.86, 56.99, 56.90, 56.30, 56.25, 56.15, 51.43, 50.26, 50.24, 46.05, 42.51, 42.41, 40.68, 39.93, 39.84, 39.05, 38.36, 37.21, 36.81, 36.80, 36.35, 36.09, 34.88, 34.15, 33.91, 32.62, 32.11, 32.07, 31.50, 30.49, 29.37, 29.10, 28.44, 28.02, 26.31, 25.60, 24.94, 24.55, 24.49, 23.28, 22.51, 21.41, 21.27, 21.23, 20.40, 20.00, 19.51, 19.24, 19.18, 18.97, 18.90, 18.45, 15.57, 14.11, 12.43, 12.24, 12.18, 12.05.

#### *$\beta$ -Sitosteroyl oleate (6b)*

Mass obtained: 40.0 mg, 87% yield; rf 0.65 in EtOAc:hexane (20:80);  $[\alpha]_{\text{D}}^{25}$  -28.0 ( $c$  1.0,  $\text{CHCl}_3$ ), lit. (Kuksis and Beveridge 1960)  $[\alpha]_{\text{D}}^{25}$  -28.0 ( $c$  1.0,  $\text{CHCl}_3$ ); FTIR (KBr)  $\nu / \text{cm}^{-1}$  2927, 1735, 1485, 1248;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.42 – 5.29 (m, 3H), 4.63 – 4.55 (m, 1H), 2.31 (d, 2H,  $J$  7.1 Hz), 2.26 (t, 2H,  $J$  7.5 Hz), 2.10 – 1.92 (m, 8H), 1.90 – 1.80 (m, 4H), 1.66 – 1.42 (m, 11H), 1.38 – 1.24 (m, 26H), 1.20 – 1.10 (m, 4H), 1.04 – 1.01 (m, 6H), 0.94 - 0.78 (m, 12H), 0.68 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  173.11, 139.68, 138.29, 129.94, 129.71, 129.29, 122.54, 77.27, 77.02, 76.76, 73.63, 56.80, 56.71, 56.13, 56.08, 56.02, 55.97, 51.25, 50.09, 50.07, 45.86, 42.32, 42.21, 40.49, 39.76, 39.66, 39.10, 38.86, 38.18, 37.03, 36.61, 36.59, 36.21, 36.16, 35.90, 34.68, 33.96, 33.72, 32.41, 31.92, 31.89, 31.48, 30.62, 30.33, 29.78, 29.69,



29.54, 29.47, 29.33, 29.20, 29.17, 29.10, 28.90, 28.52, 28.25, 28.19, 27.83, 27.22, 27.17, 26.15.

#### TUMOR CELL CULTURE AND CYTOTOXIC ACTIVITY

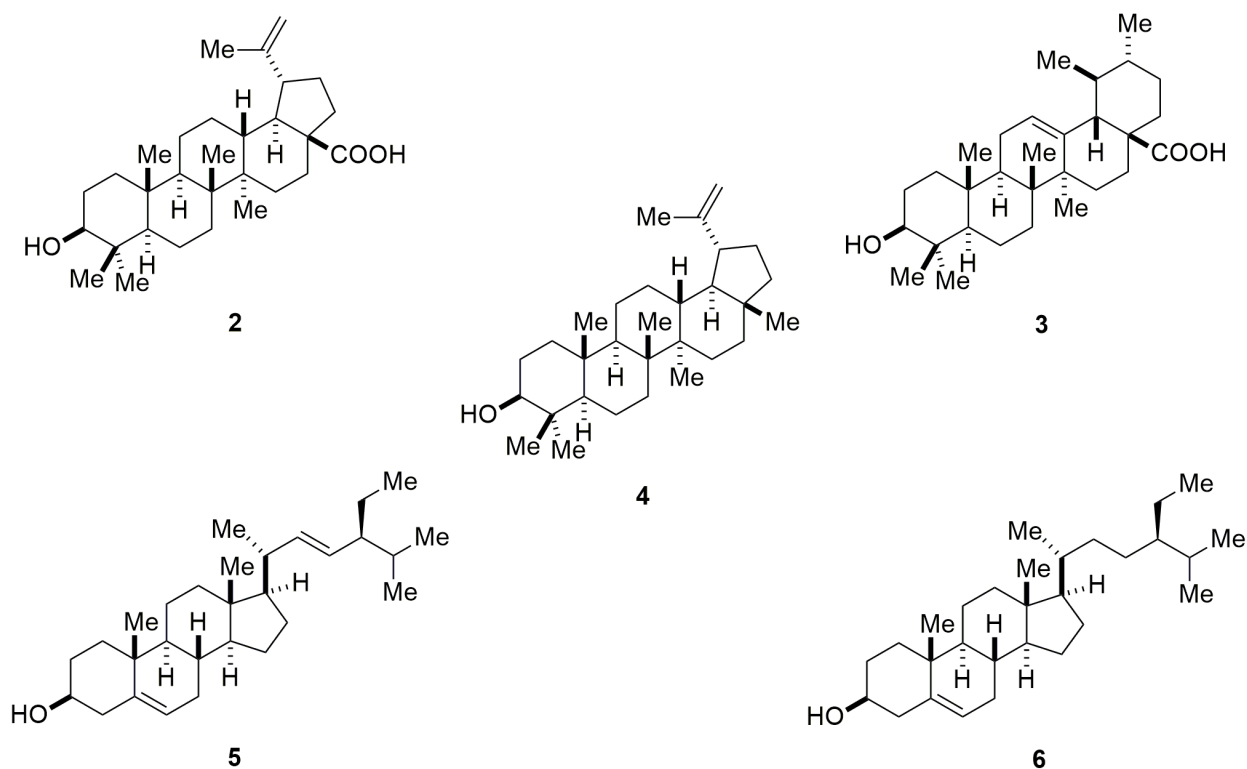
The tested tumor cell lines (leukemia HL-60, colon adenocarcinoma HCT-116, ovarian carcinoma OVCAR-8, prostate carcinoma PC-3 and glioblastoma SF-295) were kindly donated by the National Cancer Institute (Bethesda, MD, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL<sup>-1</sup> penicillin, and 100 µg mL<sup>-1</sup> streptomycin at 37°C with 5% CO<sub>2</sub>. The cytotoxicity of the compounds was initially tested against three solid human tumor cell lines: HCT-116 (colon adenocarcinoma), SF-295 (glioblastoma) and OVCAR-8 (ovarian carcinoma) to evaluate the cell growth inhibition using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay (Mossman 1983). Cells were plated in 96-well plates (0.1 x 10<sup>6</sup> cell mL<sup>-1</sup> for OVCAR-8 and SF-295 lines and at 0.7 x 10<sup>5</sup> cell mL<sup>-1</sup> for the HCT-116 line in 100 µL of medium) and compounds (25 µg mL<sup>-1</sup>) were dissolved in DMSO, added to each well using the HTS – high-throughput screening-biomek 3000-Beckman Coulter (Inc. Fullerton, California, USA). After 69 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg mL<sup>-1</sup>). Three hours later, the MTT formazan product was dissolved in 150 µL of DMSO and the absorbance was measured at 595 nm (DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, CA, USA).

The IC<sub>50</sub> value of active samples were determined by MTT assay using increasing concentrations (0.2 to 25 µg mL<sup>-1</sup>) against HCT-116, SF-295, HL-60 (leukemia) and PC-3 (prostate carcinoma) cells lines after 72 hours of incubation. Doxorubicin was used as the positive control. Control groups received the same amount of

DMSO. The IC<sub>50</sub> value and their 95% confidence intervals (CI 95%) were obtained by non-linear regression using the GraphPad Software 5.0.

#### RESULTS AND DISCUSSION

Our work began by choose of some representative natural products as lead compounds (**2-6**, Fig. 2). These findings led to betulinic acid (3β-hydroxy-lup-20-(29)-en-28-oic acid) (**2**) as one of natural compounds employed. The therapeutical use of this active natural compound as anticancer agent is a highly-investigated research field (Gheorghesou et al. 2014, Jonnalagadda et al. 2013), and became itself a natural candidate to further chemical modifications. Although the commercial betulinic acid can be easily prepared from common natural sources, its complete synthesis is not available. Other pentacyclic triterpenic acid that has recently attracted a rising attention for its multifunctional anticancer activity is ursolic acid (3β-hydroxy-12-urs-12-ene-28-oic acid) (**3**) (Chen et al. 2015, Wosniak et al. 2015). Inhibition (Kuttan et al. 2011) or cancer prevention (Shanmugam et al. 2013) are some of its properties. Lupeol (3β)-lup-20-(29)-en-3-ol (**4**) is another triterpene which has gained wide attraction as a therapeutic and chemopreventive agent for treatment of cancer (Saleem 2009), which was also used as template for esters transformations and isolated as an ester derivative as well (Brum et al. 1998). Besides these triperpenic structures, some phytosterols were also used as lead compounds in our study. It class of compounds have been proposed to offer protection against cardiovascular diseases and cancer (Bradford and Awad 2007). Among most common phytosterols, stigmasterol (3β,20R,22E,24S)-stigmasta-5,22-dien-3-ol (**5**) and β-sitosterol (3β,20R,24R)-stigmast-5-en-3-ol (**6**) were employed. While the former was already tested against cancer in natural form (Ali et al. 2015, Ghosh et al. 2011), the latter showed effect on tumor cells in ester form (Rathee et al. 2012).



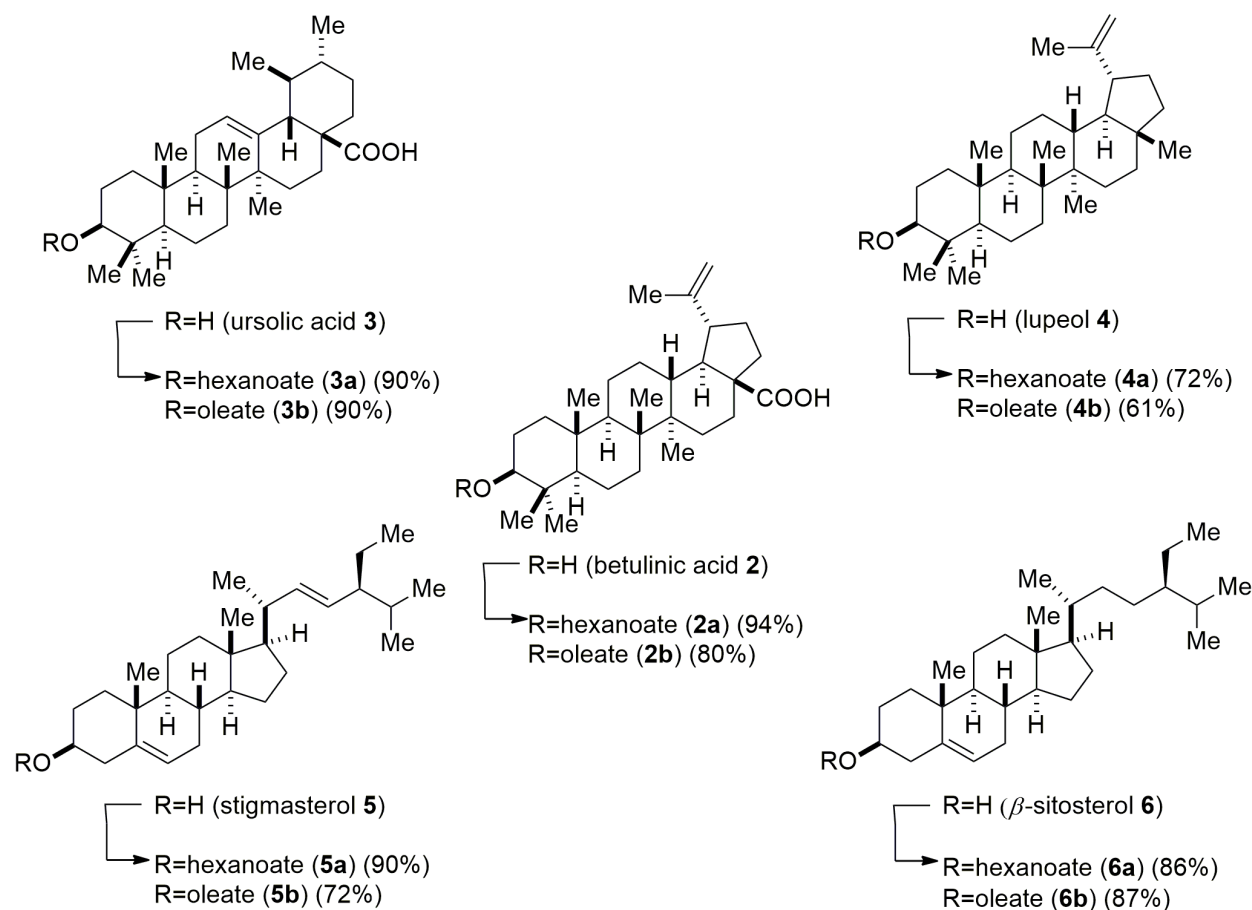
**Figure 2** - Structures of betulinic and ursolic acids (**2** and **3**, respectively), lupeol (**4**), stigmasterol (**5**) and  $\beta$ -sitosterol (**6**).

Despite the esters derivatives of phytosterols (Kuksis and Beveridge 1960), and of oleic ester of betulinic acid (Nakagawa-Goto et al. 2009) and lupeol (Chakraborty and Rangari 2011) had already been synthesized, no evaluation of their biological activity against the cancer cells were performed.

The synthesis of hexanoic and oleic ester derivatives from natural terpenes were accomplished by use of Steglich methodology (Neises and Steglich 1978). Treatment of a dichloromethane solution of natural substrate, catalyst (DMAP: 4-*N,N*-dimethylaminopyridine) and diisopropyl carbodiimide (DIC) with a solution of acid at room temperature led to respective esters (Scheme 1) in moderate to good yields (61-95%) after 24-48h under removal of solid by filtration, concentration and purification by column chromatography in silica-gel (Pilli et al. 2000).

The synthesis was corroborated as described to esters derivatives of betulinic acid **2**. The IR

spectra of the derivative showed absorption band at  $1728\text{ cm}^{-1}$ , characteristic to C=O of ester, and maintenance of acid absorption band between  $3600\text{-}2600\text{ cm}^{-1}$  were indicative of esterification. In the  $^1\text{H}$  NMR spectra the H-3 upshielded signal (from  $\delta_{\text{H}}$  3.45 in **2** (Chichewicz and Kouzi 2004) to  $\delta_{\text{H}}$  4.48 in **2a**) was indicative of the esterification at C-3, and upshielding effect on C-3 in  $^{13}\text{C}$  NMR (from  $\delta_{\text{C}}$  78.1 in **2** to  $\delta_{\text{C}}$  80.3 in **2a**) as well. The presence of side chain was corroborated by appearance of characteristic signal in  $^1\text{H}$  NMR spectra (triplet at  $\delta_{\text{H}}$  0.90 due terminal  $\text{CH}_3$ ) and  $^{13}\text{C}$  NMR as well (signal at  $\delta_{\text{C}}$  173.5 due carbonyl ester, besides total of 36 signals for **2a**). The IR spectra of oleic derivative **2b** showed a new band at  $1732\text{ cm}^{-1}$  indicating esterification at C-3. Upshielding of H-3 signal from  $\delta_{\text{H}}$  3.45 in **2** to  $\delta_{\text{H}}$  4.48 in **2b** in the  $^1\text{H}$  NMR corroborated with the presence of an ester bearing C-3. Same effect was observed to C-3 in  $^{13}\text{C}$  NMR: upshielding C3 signal from  $\delta_{\text{C}}$



**Scheme 1** - Condition to syntheses of esters: DMAP (2 eq.), DIC (2 eq.) in  $\text{CH}_2\text{Cl}_2$ , then acid (2 eq.) in  $\text{CH}_2\text{Cl}_2$ , 24-48h.

78.1 in **2** to  $\delta_c$  80.6 in **2b**. Side chain was assured by characteristics oleic signals in  $^1\text{H}$  NMR spectra (olefinic signals at  $\delta_H$  5.38-5.31, vicinal carbonyl  $\text{CH}_2$   $\delta_H$  2.33-2.26, allylic protons at  $\delta_H$  2.08-1.91 and others signals of the aliphatic chain). In  $^{13}\text{C}$  NMR spectra new olefinic signals at  $\delta_c$  130.0 and 129.7 and all others chains signals at  $\delta_c$  20-40 region and  $\delta_c$  14.1 and 14.7 for terminal methyl showed esterification was accomplished in **2b**. The compounds **3a,b-6a,b** had their syntheses corroborated as similar as described to **2a,b** (see Figures S1-S40-Supplementary Material).

The ester derivatives **2a,b-6a,b** have their cytotoxic effect evaluated using the MTT assay after 72h incubation (Mossman 1983). Initially, the esters derivatives were tested at concentration

of  $25 \mu\text{g mL}^{-1}$  against three solid human tumor cell lines: HCT-116 (colon adenocarcinoma), SF-295 (glioblastoma) and OVCAR-8 (ovarian carcinoma), and only the hexanoate derivative of betulinic acid **2a** was found to be active, inhibiting tumor cell growth over than 70% (see Table SI - Supplementary Material). Additionally, compound **2a** and its parent triterpene betulinic acid **2** were tested at increasing concentrations (0.2 to  $25 \mu\text{g mL}^{-1}$ ) for  $\text{IC}_{50}$  calculation using HCT-116, SF-295, HL-60 (leukemia) and PC-3 (prostate carcinoma) cells (Table I. Results for all compounds are shown in Table SII). Betulinic acid **6** was more active than its hexanoate derivative **2a**, exhibiting  $\text{IC}_{50}$  values ranging from  $0.84 \mu\text{g mL}^{-1}$  ( $1.84 \mu\text{M}$ ) in HL-60 cells to  $6.10 \mu\text{g mL}^{-1}$  ( $13.36 \mu\text{M}$ ) in SF-295 cells.



**TABLE I**  
**Cytotoxic activity of betulinic acid 2 and its hexanoate derivative 2a against HCT-116 (colon adenocarcinoma), SF-295 (glioblastoma), HL-60 (leukemia) and PC-3 (prostate carcinoma) cells using MTT assay after 72 h incubation. Doxorubicin was used as positive control.**

Compound	HL-60	HCT-116	SF-295	PC-3
	IC <sub>50</sub> (95% confidence interval) $\mu\text{g mL}^{-1}$ (mM)			
<b>2</b>	0.84 (1.84) (0.74-0.97)	2.05 (4.49) (1.73-2.42)	6.10 (13.36) (4.89-7.62)	1.78 (3.9) (1.42-2.23)
<b>2a</b>	10.86 (19.57) (8.54-13.81)	9.50 (17.12) (8.31-10.88)	17.43 (31.41) (12.91-23.54)	14.17 (25.54) (10.31-19.47)
Doxorubicin	0.02 (0.03) (0.01-0.02)	0.12 (0.21) (0.09-0.17)	0.24 (0.41) (0.2-0.27)	0.26 (0.44) (0.18-0.29)

IC<sub>50</sub> values and its 95% confidence interval were obtained by non-linear regression from two independent experiments performed in duplicate.

The hexanoate derivative **2a** exhibited IC<sub>50</sub> values ranging from 9.50  $\mu\text{g mL}^{-1}$  (17.12  $\mu\text{M}$ ) in HCT-116 cells to 17.43  $\mu\text{g mL}^{-1}$  (31.41  $\mu\text{M}$ ) in SF-295 cells (Table I). The *in vitro* antitumor cytotoxicity of betulinic acid has been extensively studied against several human cancer cell lines including neuroblastoma, glioblastoma, melanoma, leukemia as well as several carcinomas, like head and neck, colon, breast, lung, prostate, renal cell, ovarian and cervix carcinoma, with IC<sub>50</sub> values range 1.1 to 16  $\mu\text{g mL}^{-1}$  depending of tested cell line (Zuco et al. 2002, Hata et al. 2003, Fulda et al. 1997, 1999, Thurnher et al. 2003, Ehrhardt et al. 2004, Manoj et al. 2010). The cytotoxic potential of betulinic acid **2** isolated from *Mimosa caesalpiniiifolia* showed inhibition of cell proliferation over than 86.5% against HCT-116, SF-295 and OVCAR-8 (Monção et al. 2015). Therefore, our results confirm previous findings in the literature.

In conclusion, this article described the synthesis of a series of novel hexanoate and oleate esters from some natural products (stigmaterol,  $\beta$ -sitosterol, lupeol, and betulinic and ursolic acids), and a cytotoxic panel cells against human ovarian carcinoma OVCAR-8, human glioblastoma SF-295, and human colon carcinoma HCT-116 tumor cell line using an *in vitro* cytotoxicity assay was performed. Among these compounds, only hexanoic betulinic acid derivative displayed

moderate cytotoxic activity and it was efficacious against all tumor cell lines employed (SF-295, HCT-116, PC-3 and HL-60), but it was indeed consistently less active than betulinic acid itself. These preliminary results showed that the synthesis of new derivatives is required to improve anticancer *in vitro* anticancer activity of the tested compounds.

#### ACKNOWLEDGMENTS

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#### SUPPLEMENTARY MATERIAL

**Table SI** - Percentual inhibition of cell growth in three human tumor cell lines. Data are presented as media  $\pm$  standard deviation.

**Table SII** - Cytotoxic activity of triterpenic esters derivatives **6a,b-10a,b** against HCT-116 (colon adenocarcinoma), SF-295 (glioblastoma), HL-60 (leukemia) and PC-3 (prostate carcinoma) cells using MTT assay after 72 h incubation. Doxorubicin was used as positive control.

**Figures S1-S40** - describing characterization data (IR, <sup>1</sup>H and <sup>13</sup>C NMR spectra) of compounds **2a,b-6a,b**.