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Novel betulin derivatives inhibit IFN-γ and modulates COX-2 expression

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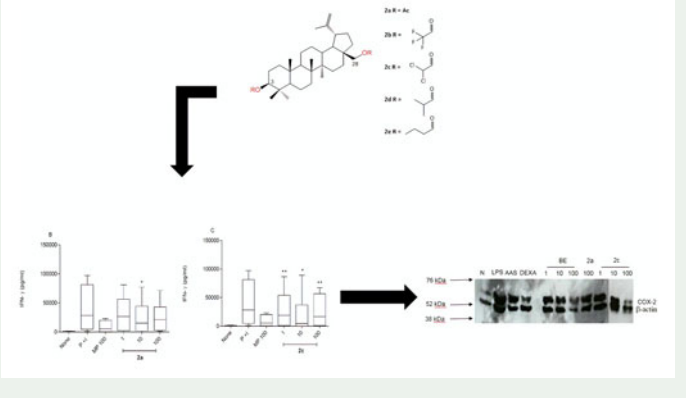
ABSTRACT

Betulin (**BE**) is a pentacyclic triterpenes, obtained from natural sources and with several biological activities described, such as anti-tumoral and anti-inflammatory activities. The **BE** esterification at hydroxyl group (C-3 and C-28) resulted in five new ester derivatives with different numbers of carbons or halogens (chlorine and fluorine). Among these **BE** derivatives, two (**2a** e **2c**) were able to significantly decrease IFN-g (*p = 0.0391; **p = 0.0156) and **2c** modulated the expression of COX-2 better than Dexamethasone (**DEXA**). Regarding to cytotoxic assay, the best results were obtained for **BE** without modifications, with emphasis on tumoral cell lines Raji and MCF-7. The derivatives **2a** and **2c** showed immunomodulation activity (for the cytokines IFN-g). The presence of chorine in **BE** seems to be important for the ability of modulate COX-2 expression, since the ester chloride derivative **2c** at 100 μM is more powerful inhibitor of COX-2 than **DEXA**.



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1. Introduction

The compounds of natural origin have been widely studied and used in current therapy due to their great chemical diversity in which can cover a wide range of diseases (Yogeeswari and Sriram 2005; Yadav et al. 2010). Betulin (**BE**) (lup-20(29)-ene-3β,28-diol) is a pentacyclic triterpene, found in abundance in nature. It can be extracted from different plant sources, such as from the bark of *Betula papyrifera* (Betulaceae) popularly known as white oak (white birch) and it can also be obtained by conversion of betulinic acid **BA** (Laszczyk 2009). A wide spectrum of biological and pharmacological activities have been reported for **BE** and its derivatives, including antitumor, antibacterial, anti-inflammatory, immunomodulation and antiviral (HIV) activities (Boryczka et al. 2013; Ding et al. 2013; Bebenek et al. 2015; Drąg-Zalesińska et al. 2015; Laavola et al. 2016).

The literature shows some studies where modified triterpenes, as **BE**, have had their action improved. Such as the study described by Zhao et al. (2016) that evaluated the renal septic effect of **BE** in mice. They observed that **BE** inhibited the nuclear factor kappa-B (NF-kB) signal, reduced serum creatinine and urea levels, and decreased secretion of proinflammatory cytokines (TNF-γ, IL-1β and IL-6) in septic mice. In the same year, Drąg-Zalesińska et al. (2015) evaluated the cytotoxic properties of five new **BE** derivatives against squamous cell carcinoma. They found that the highest cytotoxicity activity of these derivatives occurred in the two compounds containing a side chain of lysine ($IC_{50} = 7\ \mu M$) and ornithine ($IC_{50} = 10\ \mu M$).

In this sense, exploring the anti-inflamtory, immunomodulation and cytoxicity activity of **BE** and its derivatives is potential way to obtain a new medicine. In addition, the investigation of these activities together is based on Rudolf Virchow's research (1856), since he indicated that inflammation is closely linked to cancer, and showed for the first time, the inflammation as a predisposing factor for several cancers. Nowadays, the data suggest that at least one in seven malignant tumors is a result of inflammatory processes. Inflammatory cells such as macrophages and fibroblasts, transcription factors, enzymes such as cyclooxygenase-2 (COX-2) and cytokines such as IFN-γ, IL-6 and IL-17A are components present in the leucocyte infiltrate of the tumor microenvironment. The COX-2, a central molecule of the inflammatory process, is also involved in carcinogenesis (Xiong, Liu, and Yi 2014; Yang et al. 2015; Khan 2015). Previous studies have shown that this enzyme is found in high concentrations in several solid tumors, performing the function of promoting angiogenesis, tumor invasion, metastasis, as well as resistance to apoptosis (Harizi 2015; Qu and Liu 2015). For instance, there are some selected forms of chronic inflammation that predispose cancer, such as infection by *Helicobacter pilori* (Porta et al. 2009).

In this context, this article evaluated the immunomodulation, anti-inflammatory and cytotoxicity activity of the five new semisynthetic **BE** derivatives. In this purpose, we assessed the ability of these novel derivatives in modulating inflammatory cytokines IFN-γ, IL-6 and IL-17A and inhibiting COX-2 expression. After, we evaluated the cytotoxicity of the novel derivatives in four tumoral cell lines and one non-tumoral cell.

2. Results and discussion

This article reports for the first time the synthesis of five new derivatives **BE** with substituents in C-3 and C-28. In this present research, we evaluated the immunomodulation, anti-inflammatory and cytotoxicity activities of **BE** and its derivatives. We sought to evaluate the immunomodulation activity by using cytokines IFN-γ, IL-6 and IL-17A and anti-inflammatory potential through the inhibition of COX-2. We also analyzed its anti-tumoral activity against the Hep-G2 (well-differentiated hepatocellular carcinomas), Raji (Burkitts lymphoma), MCF-7 (breast cancer) and DU-145 (Prostate cancer).

Previous researches have showed the immunomodulation and anti-inflamatory activities for **BE** and derivatives, such as the formyl and pyrazol groups added to **BE** generating derivatives capable of reducing the expression of IL-6 and COX-2 enzyme in a significant manner (Laavola et al. 2016). However, it is only one of the few studies in the literature that have evaluated the anti-inflammatory effect of **BE** and its derivatives, as also few studies that related **BE** derivatives with modulating activity of some cytokines (Bernard et al. 2001; Alakurtti et al. 2006; Zhang et al. 2015; Laavola et al. 2016; Zhao et al. 2016). None studies were found in the literature that evaluated the modulating ability of the IFN-γ and IL-17A cytokines by **BE** and its derivatives. Therefore, our starting point was to evaluate the ability of the **BE** and its new derivatives in reducing the production of pro-inflammatory cytokines IFN-γ, IL-6 and IL-17A.

According to our results, the changes performedmade to obtain derivatives **2a** and **2c** made them able to significantly decrease IFN-γ and **2c** showed anti-inflammatory activity better than its precursor, **BE**, and also **DEXA** (Figure S11). It seems that the presence of substituents *O*-acetyl (**2a**) and the *O*-dichloroacyl (**2c**) at C-3 and C-28 positions make **BE** able to reduce the production of IFN-γ and COX-2. For the molecule inflammatory COX-2 assay, we added **BE** at concentrations of 1, 10 and 100 μM to compare the activity of derivatives with its precursor. Since the derivative **2c** at concentration of 100 μM was a better modulator of COX-2 than its precursor, **BE** and the drug already used in clinical practice, **DEXA**, it indicated that the presence of chorine is important to increase the anti-inflammatory activity of the **BE** (Figure S12). There is no previous studies about synthesized derivatives from **BE** using these substituents, which makes the synthesis and activities of our novel derivatives unprecedented.

After evaluating the immunomodulation and anti-inflammatory activities of the novel **BE** derivatives, we decided to investigate the cytotoxic action of these derivatives facing tumor cell lines. Studies have shown that the synthesis of derivatives from **BE** precursor has generated more cytotoxic and selective compounds for neoplastic cells (Grivennikov, Greten and Karin 2010; Csuk et al. 2010; Urban et al. 2012; Siewert et al. 2014).

Our results of the cytotoxicity assay to **BE** derivatives showed the best result to **2d**, that was more activity against Raji and Hep-G2 strains, specially on Hep-G2 ($IC_{50} = 60.47$ μM) and all derivatives were not cytotoxic to the PBMC, non-tumoral cell. **BE** without modification was more activity than its derivatives, especially on tumoral cells Raji and MCF-7 ($IC_{50} = 45$ μM). Doxorubicin, a drug used in clinical practice, was used as a negative control (Table S1). Since the modifications of **BE** did not improve the activity on tumor cells, new modifications should be proposed in order to reach this aim.

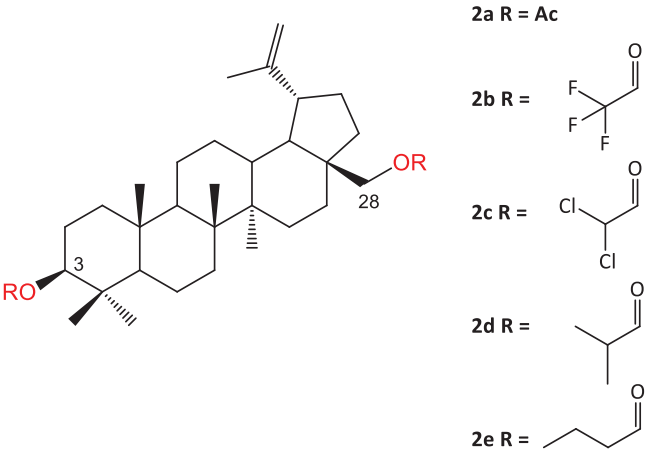

Figure 1. Chemical structure of Betulin (**BE**) and substituents that gave the derivatives.

3. Experimental

3.1. Semisynthesis of the BE derivatives

BE was purchased from Sigma – Aldrich (Missouri, EUA). For obtaining of the new **BE** derivatives, it was followed the same reaction scheme used for the obtaining of betulinic acid derivatives described by Silva and collaborators (2013) [26]. Briefly, the commercial anhydride (1.1 mmol, 5 Eq) and DMAP (0.22 mmol, 1 Eq) were added to **BE** (0.22 mmol) in CH_2Cl_2 (2 mL) and react for one hour. The crude residue was purified in column chromatography to give the expected pure compounds. Column chromatography was carried out using silica gel 60 (Merck). Analytical thin layer chromatography was performed on silica gel 60 F254 plates (Merck) and spots visualized by spraying with anisaldehyde/sulphuric. Then, two parts of the **BE** were modified due to the presence of two hydroxyl at C-3 and C-28 with the same reactivity. For the structure characterization the High-resolution mass spectra (HR-EI-MS) were obtained on a Micromass-Waters Q-TOF Ultima spectrometer and Infrared spectra were recorded on a PerkinElmer FTIR BX spectrometer. Also, ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AC 400 spectrometer operating at 400 and 75 MHz, respectively, using tetramethylsilane as the internal standard and chloroform-*d*. The chemical shifts (δ) were expressed in parts per million (ppm). The purity of the **BE** derivatives was also verified by melting points with a Kofler bench. The structure of **BE** and their derivatives are disposed in Figure 1.

3.1.1. 3,28-O-acetylbetulin (2a)

The compound **2a** was prepared by using acetic anhydride. White powder, yield = 90%.

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.03 (s, 15 H, CH_3-23; CH_3-24; CH_3-25; CH_3-26 and CH_3-27); 1.06 (m, 1 H CH-13); 1.09 (t, 1 H, CH-18); 1.12 (t, 2 H, CH_2-1); 1.24 (t, 2 H, CH_2-16); 1.36 (t, 2 H, CH_2-15); 1.39 (m, 4 H, CH_2-2 and CH_2-21); 1.43 (t, 4 H, CH_2-11 and CH_2-22); 1.50 (t, 2 H, CH_2-6); 1.51 (m, 2 H, CH_2-12); 1.56 (t, 2 H, CH_2-7); 1.62 (t, 1 H, CH-5); 1.79 (br s, 3 H, CH_3-30); 1.84 (m, 2 H, CH_2-2); 1.86 (t, 1 H, CH-9); 2.07 (s, 6 H, CH_3-32

and CH_3-34); 2.44 (ddd, 1 H, CH-19); 4.24 (dd, 2 H, CH_2-28); 4.46 (dd, 1 H, CH-3); 4.58 (s, 1 H, CH-29b); 4.68 (s, 1 H, CH-29a).^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 16.15 (C-25); 16.48 (C-26 and C-27); 18.15 (C-6); 19.09 (C-34); 20.78 (C-32); 21.06 (C-30); 21.32 (C-11); 25.12 (C-2); 26.90 (C-12); 23.68 (C-23 and C-24); 27.93 (C-15); 29.55 (C-21); 29.71 (C-16 and C-22); 35.53 (C-7); 37.03 (C-10); 37.53 (C-13); 37.77 (C-4); 38.36 (C-1); 40.86 (C-8); 42.66 (C-14); 48.74 (C-19); 46.28 (C-18); 47.69 (C-17); 50.25 (C-9); 55.35 (C-5); 62.78 (C-28); 80.89 (C-3); 109.88 (C-29); 150.09 (C-20); 170.99 (C-31); 171.60 (C-33).

HRMS (ESI-MS, m/z); $[M + Na]^+$ calculated for $C_{34}H_{54}O_4Na$: 549.7793; found: 549.2620. Melting point: 210–214 °C.

3.1.2. 3,28-O-trifluoracylbetulin (2b)

The compound **2 b** was prepared by using trifluoroacetic anhydride. White powder, yield 80%.

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.83 (t, 2 H, CH_2-16); 0.99 (m, 1 H, CH-13); 1.06 (s, 15 H, CH_3-23, CH_3-24, CH_3-25, CH_3-26 and CH_3-27); 1.08 (t, 1 H, CH-18); 1.15 (t, 2 H, CH_2-1); 1.30 (m, 2 H, CH_2-12); 1.33 (t, 2 H, CH_2-15); 1.42 (m, 2 H, CH_2-21); 1.43 (t, 4 H, CH_2-11 and CH_2-22); 1.45 (t, 2 H, CH_2-6); 1.62 (t, 1 H, CH-5); 1.64 (t, 2 H, CH_2-7); 1.78 (br s, 3 H, CH_3-30); 1.80 (m, 2 H, CH_2-2); 1.84 (t, 1 H, CH-9); 1.99 (ddd, 1 H, CH-19); 4.15 (dd, 2 H, CH_2-28); 4.58 (dd, 1 H, CH-3); 4.62 (s, 1 H, CH-29b); 4.71 (s, 1 H, CH-29a). ^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 15.98 (C-26); 16.14 (C-27); 16.24 (C-25); 18.07 (C-6 and C-30); 19.08 (C-11); 20.77 (C-23 and C-24); 23.25 (C-2); 25.04 (C-12); 26.92 (C-15); 27.77 (C-21); 29.33 (C-16); 34.00 (C-22); 34.21 (C-7); 37.04 (C-10); 37.74 (C-13); 38.07 (C-4); 38.20 (C-1); 40.88 (C-8); 42.74 (C-14); 46.61 (C-18); 47.53 (C-17); 48.76 (C-19); 50.19 (C-9); 55.23 (C-5); 66.84 (C-28); 86.23 (C-3); 110.30 (C-29); 116.05 (C-32); 116.11 (C-34) 149.50 (C-20); 157.74 (C-31); 158.16 (C-33).

HRMS (ESI-MS, m/z); $[M\text{-}H + 2K]^+$ calculated for $C_{34}H_{47}F_6O_4K_2$: 711.2636; found: 711.3934 . Melting point: 175–176 °C.

3.1.3. 3,28-O-dichloroacylbetulin (2c)

The compound **2c** was prepared by using chloroacetic anhydride. White powder, yield, 85%.

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.99 (s, 15 H, CH_3-23, CH_3-24, CH_3-25, CH_3-26 and CH_3-27); 1.06 (m, 3 H, CH-13 and CH_2-16); 1.06 (t, 1 H, CH-18); 1.13 (t, 2 H, CH_2-1); 1.26 (m, 2 H, CH_2-21); 1.30 (t, 2 H, CH_2-15); 1.34 (m, 2 H, CH_2-11); 1.42 (t, 2 H, CH_2-7); 1.45 (m, 4 H, CH_2-2 and CH_2-12); 1.51 (t, 2 H, CH_2-6); 1.64 (t, 1 H, CH-5); 1.74 (t, 2 H, CH_2-22); 1.79 (br s, 3 H, CH_3-30); 1.81 (t, 1 H, CH-9); 2.00 (ddd, 1 H, CH-19); 4.07 (dd, 2 H, CH_2-28); 4.48 (dd, 1 H, CH-3); 4.61 (s, 1 H, CH-29b); 4.70 (s, 1 H, CH-29a); 5.92 (s, 1 H, CH-34); 5.96 (s, 1 H, CH-32). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 15.96 (C-25); 16.11 (C-26); 16.32 (C-27); 18.01 (C-6); 19.08 (C-30); 20.74 (C-11); 23.19 (C-23 and C-24); 25.06 (C-2); 26.95 (C-12); 27.80 (C-15); 29.41 (C-21); 29.48 (C-16); 33.98 (C-7 and C-22); 34.29 (C-10); 37.01 (C-13); 37.66 (C-4); 38.21 (C-1); 40.84 (C-8); 42.70 (C-14); 46.75 (C-18); 47.66 (C-17); 48.80 (C-19); 50.17 (C-9); 55.28 (C-5); 64.43 (C-34); 64.78 (C-32); 66.16 (C-28); 80.90 (C-3); 109.08 (C-29); 149.70 (C-20); 164.30 (C-31); 164.96 (C-33).

HRMS (ESI-MS, m/z); $[M + H + 2Na]^+$ calculated for $C_{34}H_{51}Cl_4O_4Na_2$: 711.5579; Found: 711.4124. Melting point: 166–170 °C.

3.1.4. 3,28-O-isobutylbetulin (2d)

The compound **2d** was prepared by using isobutyric anhydride. White powder, yield = 93%.

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.96 (t, 2 H, CH_2-16); 1.02 (s, 15 H, CH_3-23, CH_3-24, CH_3-25, CH_3-26 and CH_3-27); 1.06 (t, 1 H, CH-18); 1.13 (m, 3 H, CH_2-1 and CH-13); 1.17 (d, 12 H, CH_3-33, CH_3-34, CH_3-37 and CH_3-38); 1.38 (t, 4 H, CH_2-15 and CH_2-21); 1.41 (m, 4 H, CH_2-2 and CH_2-11); 1.49 (m, 4 H, CH_2-6 and CH_2-12); 1.57 (t, 2 H, CH_2-7); 1.60 (t, 1 H, CH-5); 1.67 (t, 2 H, CH_2-22); 1.79 (br s, 3 H, CH_3-30); 2.42 (m, 1 H, CH-36); 2.53 (m, 1 H, CH-32); 1.82 (t, 1 H, CH-9); 2.03 (ddd, 1 H, CH-19); 2.26 (t, 2 H, CH_2-36); 2.38 (t, 2 H, CH_2-32); 4.26 (dd, 2 H, CH_2-28); 4.44 (dd, 1 H, CH-3); 4.57 (s, 1 H, CH-29b); 4.67 (s, 1 H, CH-29a). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 16.05 (C-25); 16.07 (C-26); 16.59 (C-27); 19.12 (C-33, C-34, C-37 and C-38); 19.25 (C-6); 20.82 (C-11 and C-30); 23.68 (C-23 and C-24); 25.18 (C-2); 26.95 (C-12); 27.96 (C-15); 29.61 (C-21); 29.82 (C-16); 34.12 (C-22); 34.26 (C-36); 34.49 (C-32); 34.57 (C-7); 37.10 (C-10); 37.57 (C-13); 37.97 (C-1 and C-4); 38.36 (C-8); 40.91 (C-14); 42.73 (C-18); 46.57 (C-17); 48.83 (C-19); 50.29 (C-9); 55.39 (C-5); 62.42 (C-28); 80.35 (C-3); 109.88 (C-29); 150.19 (C-20); 176.82 (C-35); 177.52 (C-31).

HRMS (ESI-MS, m/z); $[M\text{-}2H + K]^+$ calculated para $C_{38}H_{60}O_4K$: 619.9789; found: 619.3745. Melting point: 110–112 °C.

3.1.5. 3,28-O-butanoylbetulin (2e)

The compound **2e** was prepared by using butyric anhydride. White powder, yield = 64%.

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 0.96 (t, 6 H, CH_3-34 and CH_3-38); 0.99 (s, 15 H, CH_3-23, CH_3-24, CH_3-25, CH_3-26 and CH_3-27); 1.02 (t, 1 H, CH-18); 1.15 (m, 1 H CH-13); 1.36 (t, 2 H, CH_2-16); 1.41 (m, 2 H, CH_2-12); 1.49 (t, 2 H, CH_2-6); 1.52 (t, 2 H, CH_2-1); 1.60 (t, 2H; CH_2-7); 1.61 (t, 1 H, CH-5); 1.63 (t, 2 H, CH_2-15); 1.64 (m, 2 H, CH_2-21); 1.67 (t, 2 H, CH_2-11); 1.70 (t, 2 H, CH_2-22); 1.74 (m, 2 H, CH_2-37); 1.77 (m, 2 H, CH_2-33); 1.78 (br s, 3 H, CH_3-30); 1.84 (m, 2 H, CH_2-2); 1.98 (t, 1 H, CH-9); 2.21 (ddd, 1 H, CH-19); 2.26 (t, 2 H, CH_2-36); 2.38 (t, 2 H, CH_2-32); 4.40 (dd, 3 H, CH-3 and CH_2-28); 4.52 (s, 1 H, CH-29b); 4.61 (s, 1 H, CH-29a). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 13.71 (C-38); 14.72 (C-35); 16.54 (C-25); 18.14 (C-26); 18.53 (C-27); 18.62 (C-33 and C-37); 19.10 (C-6); 20.77 (C-11 and C-30); 23.76 (C-23 and C-24); 25.13 (C-2); 27.93 (C-12); 29.55 (C-15); 29.76 (C-16 and C-21); 34.08 (C-22); 34.56 (C-36); 36.40 (C-32); 36.75 (C-7); 37.03 (C-13); 37.53 (C-10); 37.80 (C-4); 38.34 (C-1); 40.86 (C-8); 42.67 (C-14); 46.37 (C-18); 47.70 (C-17); 48.74 (C-19); 50.24 (C-9); 55.34 (C-5); 62.35 (C-28); 80.55 (C-3); 109.83 (C-29); 150.15 (20); 173.54 (C-31); 174.16 (C-35).

HRMS (ESI-MS, m/z); $[M + Na]^+$ calculated for $C_{38}H_{62}O_4Na$: 605.8563; Found: 605.3057. Melting point: 100–104 °C.

3.2. Enzyme-linked immunosorbent assays (ELISA)

From the culture supernatant at cytokines pro-inflammatory like interleukin IL-6, IL-17A and Interferon gamma (IFN-g) measured by using enzyme-linked immunosorbent assay (ELISA) kits (eBiosciences, USA, and BD Biosciences, USA) according to the

Table 1. Comparison of the IC_{50} values (μM) of **BE** and its new derivatives against tumor cell lines (Raji (Burkitts lymphoma), MCF-7 (breast cancer), Hep-G2 (well-differentiated hepatocellular carcinoma) and DU-145 (prostate cancer)) and non-tumor cell (peripheral blood mononuclear cell (PBMC)), determined by using MTT assay.

		MCF-7		HEP-G2		DU145		PBMC	
Compounds	RAJI IC_{50}	IS	IC_{50}	IS	IC_{50}	IS	IC_{50}	IS	IC_{50}
BE	45.78 ± 0.71	2.18	45.21 ± 31.40	2.21	72.23 ± 3.42	1.38	49.36 ± 13.43	2.02	>100
2a	85.10 ± 4.78	1.17	>100	—	88.71 ± 0.00	1.12	>100	—	>100
2b	92 ± 3.07	1.08	>100	—	85.86 ± 0.00	1.16	>100	—	>100
2c	89.45 ± 7.60	1.11	>100	—	89.27 ± 0.00	1.12	>100	—	>100
2d	95.53 ± 0.75	1.04	>100	—	60.47 ± 0.00	1.65	>100	—	>100
2e	96.75 ± 3.28	1.03	>100	—	86.09 ± 0.00	1.16	>100	—	>100
Doxorubicin	11.91 ± 9.15	8.39	2.71 ± 0.87	36.90	39.22 ± 14.48	2.54	6.88 ± 1.06	14.53	>100

manufacturers' instructions. The absorbance used the difference between 570 and 450 nm readings. The minimum limits of detection of the ELISA kits used in the experiment were 4.69 pg/mL for IL-6, 3.9 pg/mL for IL-17A, 31.25 pg/mL and 4.68 pg/mL for IFN-g.

3.3. Western blot analysis

Monocytes (U937) were plated contraction of 106 cells per ml and then stimulated with *Escherichia coli* lipopolysaccharide LPS 0111: B4 (10 mg/ml). For negative control were used non-selective COX, acetylsalicylic acid (ASA) dose of 10 mg/ml as well as COX-2 inhibitor, 10 μM **DEXA**. Test group, they were added to the novel **2a** and **2c** as well as **BE**. Analysis of protein expression was performed polyacrylamide gel electrophoresis (SDS-PAGE) 10%. Then the proteins contained in the gel were transferred to Polyvinylidene Fluoride Membranes (PVDF) (Amersham Hybond-P PVDF Membrane). Membranes incubated overnight with COX-2 and β-actin, used as normalizer. Both antibodies were diluted at concentrations of 1: 2000. The secondary antibody added to the membrane and incubated for 2 h in concentration 1:2000. The densitometry determined by ImageJ software. Final quantification given by the ratio between the expression of the protein of interest and β-actin, used as normalizer.

3.4. MTT assay

The cytotoxic activities of all the compounds were evaluated using the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) (Thermofischer, Massachusetts, EUA). The cells were plated in 96-well plates (1×10^4 cells/well) and incubated overnight. The cells were treated with various concentrations of new **BE** derivatives (1, 10 and 100 μM) for 72 h at 37 °C, in a humidified atmosphere with 5% CO_2. Control cells treated with 0.1% DMSO alone and 0.1μM Doxorubicin was included as a negative control. After, was added in cells incubated 30 μL MTT solution (5 mg/mL) for 3 h at 37 °C. Then was added the solution Sodium dodecyl sulfate (SDS 20%) for dissolution of the precipitate. The absorbance was measured at 570 nm using the ELISA plate reader after 24 h (Table 1).

3.5. Ethical approval

The ethics committee was approved by the research Ethics Committee Involving Human Beings of the Federal University of Pernambuco CEP-CCS-UFPE: 1.351.357.

3.6. Obtaining and isolation of peripheral blood lymphocytes (PBMC)

Eight healthy volunteers were selected to evaluate the anti-inflammatory activity of new **BE** derivatives. Blood samples collected at Immunomodulation and New Therapeutic Approaches Laboratory (LINAT-UFPE). Informed written consent obtained. The exclusion criterion was to show no other systemic diseases that could have potentially altered their inflammatory profile functions. PBMCs were isolated from fresh peripheral blood collected in sodium heparin tubes. Briefly, whole blood was laid over one layer of Ficoll–Paque Plus (density gradient of 1.077 g/L, GE Healthcare, Little Chalfont, UK) and centrifuged at 400 *g* for 45 min, cells were washed once in PBS (pH 7.4) (Ebioscience, San Diego, CA, USA).

3.7. Culture of peripheral blood lymphocytes (PBMC)

PBMCs isolated from healthy individuals were plated in 12-well plates (1×10^6/1000 μL cells/well) in RPMI-1640 (Gibco, Massachusetts, EUA) with L-glutamine containing 10% heat inactivated fetal bovine serum (FBS) (Lonza, Valais, Switzerland), 10 nM HEPES (Gibco, Massachusetts, EUA) and 1% penicillin/streptomycin (Gibco, Massachusetts, EUA)**,** at 37 °C, in a humidified atmosphere with 5% CO_2 for 48 h. Under two conditions: unstimulated cells (None) and stimulated cells with Phorbol 12-myristate 13-acetate (2 μL) and Ionomycin (5 μL) (P + I), (Ebioscience, San Diego, CA, USA), and cells with methylprednisolone in 100 μg/mL (MP) (Sigma-Aldrich, Missouri, EUA). They were also added **BE** derivatives at concentrations (1, 10 and 100 μM). After 48 h supernatants were collected and stored at 20 degrees for later measurement of cytokines.

3.8. Statistical analysis

Statistical analysis performed using GraphPad Prism version 6 program. The data evaluated using the normal test and variance test. Data presented as median maximum and minimum, and standard deviation Wilcoxon test was used and $p < 0.05$ was considered significant. IC_{50} measured by Origin 8 software.

4. Conclusion

The strategy of adding esters (with different numbers of carbons or halogens) at C-3 and C-28 positions in **BE**, to generate derivatives with immunomodulation and anti-inflammatory potential was conquered. After analyzing the five derivatives semisynthesized, two of them (**2a** and **2c**) showed modulatory effect of pro-inflammatory cytokine IFN-g. In addition, **BE** and **2a** and **2c** were capable of modulating the expression of a protein, which is a key component of the inflammatory process, the COX-2. The derivative **2c** at 100 μM is a more powerful inhibitor of COX-2 than drugs used in

clinical practice (**DEXA**), indicating that chloride might be important for improving the anti-inflammatory activity of **BE**. However, for the anti-tumor evaluation, the new **BE** derivatives did not show promissory cytotoxic activity against tumor cell lines, the better result was for **BE** without modification. Then, other modifications must be performed in order to improve the anti-tumor activity, but the good result obtained against non-tumor cell must be reproduced.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Alakurtti S, Mäkelä T, Koskimies S, Yli-Kauhaluoma J. 2006. Pharmacological properties of the ubiquitous natural product betulin. Eur J Pharm Sci. 29(1):1–13. doi:10.1016/j.ejps.2006.04.006.

Ancrile B, Lim KH, Counter CM. 2007. Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes Dev. 21(14):1714–1719. doi:10.1101/gad.1549407.

Bebenek E, Chodurek E, Orchel A, Dzierzewicz Z, Boryczka S. 2015. Antiproliferative activity of novel acetylenic derivatives of betulin against G-361 human melanoma cells. Acta Pol Pharm - Drug Res. 72:699–703.

Bernard P, Scior T, Didier B, Hibert M, Berthon JY. 2001. Ethnopharmacology and bioinformatic combination for leads discovery: application to phospholipase A2 inhibitors. Phytochemistry. 58(6):865–874. doi:10.1016/S0031-9422(01)00312-0.

Boryczka S, Bębenek E, Wietrzyk J, Kempińska K, Jastrzębska M, Kusz J, Nowak M. 2013. Synthesis, structure and cytotoxic activity of new acetylenic derivatives of betulin. Molecules. 18(4):4526–4543. doi:10.3390/molecules18044526.

Csuk R, Barthel A, Kluge R, Ströhl D. 2010. Synthesis, cytotoxicity and liposome preparation of 28-acetylenic betulin derivatives. Bioorganic Med Chem. 18(20):7252–7259. doi:10.1016/j.bmc.2010.08.023.

Ding W, Sun M, Luo S, Xu T, Cao Y, Yan X, Wang Y. 2013. A 3D QSAR study of betulinic acid derivatives as anti-tumor agents using topomer CoMFA: model building studies and experimental verification. Molecules. 18(9):10228–10241. doi:10.3390/molecules180910228.

Drąg-Zalesińska M, Wysocka T, Borska S, Drąg M, Poręba M, Choromańska A, Kulbacka J, Saczko J. 2015. The new esters derivatives of betulin and betulinic acid in epidermoid squamous carcinoma treatment: in vitro studies. Biomed Pharmacother. 72:91–97. doi:10.1016/j.biopha.2015.04.003.

Grivennikov SI, Greten FR, Karin M. 2010. Immunity, inflammation, and cancer. Cell. 140(6): 883–899. doi:10.1016/j.cell.2010.01.025.

Harizi H. 2015. Epigenetic regulations of inflammatory cyclooxygenase-derived prostanoids: molecular basis and pathophysiological consequences. Mediators Inflamm. 2015:841097. doi: 10.1155/2015/841097.

Khan N. 2015. Investigation of cyclooxygenase and signaling pathways involved in human platelet aggregation mediated by synergistic interaction of various agonists. Drug Des Devel Ther. 9:3497–506.

Laavola M, Haavikko R, Hämäläinen M, Leppänen T, Nieminen R, Alakurtti S, Moreira VM, Yli-Kauhaluoma J, Moilanen E. 2016. Betulin derivatives effectively suppress inflammation in vitro and in vivo. J Nat Prod. 79(2):274–280. doi:10.1021/acs.jnatprod.5b00709.

Laszczyk MN. 2009. Pentacyclic triterpenes of the lupane, oleanane and ursane group as tools in cancer therapy. Planta Med. 75(15):1549–1560. doi:10.1055/s-0029-1186102.

Porta C, Larghi P, Rimoldi M, Grazia Totaro M, Allavena P, Mantovani A, Sica A. 2009. Cellular and molecular pathways linking inflammation and cancer. Immunobiology. 214(9–10): 761–777. doi:10.1016/j.imbio.2009.06.014.

Qu L, Liu B. 2015. Cyclooxygeanse-2 promotes metastasis in osteosarcoma. Cancer Cell Int. 15: 69. doi:10.1186/s12935-015-0220-2.

Siewert B, Wiemann J, Köwitsch A, Csuk R. 2014. The chemical and biological potential of C ring modified triterpenoids. Eur J Med Chem. 72:84–101. doi:10.1016/j.ejmech.2013.11.025.

Urban M, Vlk M, Dzubak P, Hajduch M, Sarek J. 2012. Cytotoxic heterocyclic triterpenoids derived from betulin and betulinic acid. Bioorganic Med Chem. 20(11):3666–3674. doi:10.1016/j.bmc.2012.03.066.

Xiong SL, Liu X, Yi GH. 2014. High-density lipoprotein induces cyclooxygenase-2 expression and prostaglandin I-2 release in endothelial cells through sphingosine kinase-2. Mol Cell Biochem. 389(1–2):197–207. doi:10.1007/s11010-013-1941-y.

Yadav VR, Prasad S, Sung B, Kannappan R, Aggarwal BB. 2010. Targeting inflammatory pathways by triterpenoids for prevention and treatment of cancer. Toxins (Basel). 2(10):2428–2466. doi: 10.3390/toxins2102428.

Yang S-J, Liu M-C, Xiang H-M, Zhao Q, Xue W, Yang S. 2015. Synthesis and in vitro antitumor evaluation of betulin acid ester derivatives as novel apoptosis inducers. Eur J Med Chem. 102: 249–255. doi:10.1016/j.ejmech.2015.08.004.

Yogeeswari P, Sriram D. 2005. Betulinic acid and its derivatives: a review on their biological properties. CMC. 12(6):657–666. doi:10.2174/0929867053202214.

Zhang S-Y, Zhao Q-F, Fang N-N, Yu J-G. 2015. Betulin inhibits pro-inflammatory cytokines expression through activation STAT3 signaling pathway in human cardiac cells. Eur Rev Med Pharmacol Sci. 19:455–460.

Zhao H, Zheng Q, Hu X, Shen H, Li F. 2016. Betulin attenuates kidney injury in septic rats through inhibiting TLR4/NF-κB signaling pathway. Life Sci. 144:185–193. doi:10.1016/j.lfs.2015.12.003.