UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIENCIAS FARMACÊUTICAS

OBTENÇÃO DE DERIVADOS TRITERPENOS SEMISSINTÉTICOS COM ATIVIDADE ANTITUMORAL E ANTIVIRAL *IN VITRO* E *IN VIVO.*

ELENILSON FIGUEIREDO DA SILVA

Porto Alegre, 2016

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIENCIAS FARMACÊUTICAS

OBTENÇÃO DE DERIVADOS TRITERPENOS SEMISSINTÉTICOS COM ANTIVIDADE ANTITUMORAL E ANTIVIRAL *IN VITRO* E *INVIVO.*

Dissertação apresentada por **Elenilson Figueiredo da Silva** para obtenção do GRAU DE MESTRE em Ciências Farmacêuticas.

Orientadora: Prof. Dra. Simone Cristina Baggio Gnoatto

Porto Alegre, 2016

Dissertação apresentada ao programa de Pós-graduação em Ciências Farmacêuticas, em nível de mestrado acadêmico da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul e aprovada em 31 de maio de 2016, pela Banca Examinadora constituída por:

Prof. Dr. André Arigony Souto

Pontifícia Universidade Católica do Rio Grande do Sul

Profa. Dra. Bárbara Nery Porto

Pontifícia Universidade Católica do Rio Grande do Sul

Prof. Dr. Diogo Losch, de Oliveira

Universidade Federal do Rio Grande do Sul

CIP - Catalogação na Publicação

Figueiredo da silva, Elenilson
OBTENÇÃO DE DERIVADOS TRITERPENOS SEMISSINTÉTICOS COM ANTIVIDADE ANTITUMORAL E ANTIVIRAL IN VITRO E INVIVO. / Elenilson Figueiredo da silva. -- 2016.
110 f.
Orientador: Simone CB Gnoatto.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Programa de Pós-Graduação em Ciências Farmacêuticas, Porto Alegre, BR-RS, 2016.

1. ácido ursólico. 2. ácido betulinico. 3. atividade antiviral. 4. atividade antitumoral. 5. Síntese orgânica . I. CB Gnoatto, Simone, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Este trabalho foi desenvolvido no Laboratório de Fitoquímica e Síntese Orgânica do Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia da UFRGS, na cidade de Porto Alegre, RS com financiamento da CAPES. O autor recebeu bolsa de estudos da CAPES.

RESUMO

É crescente o número de publicações relatando o efeito biológico de derivados semissintéticos quanto à atividade antitumoral e antiviral. Essa busca de novas moléculas é justificada pela alta resistência de células tumorais aos fármacos atualmente disponíveis, bem como novos mecanismos de resistência e deficiência adquiridos ao longo do tratamento de patologias virais. Ácido ursólico (AU) e ácido betulínico (AB) vêm se destacando em pesquisas nos últimos anos, devido as suas atividades biológicas relatadas, dentre elas antitumoral e antiviral. Além dos diversos efeitos biológicos esses compostos apresentam como vantagem a fácil obtenção e purificação, fácil manipulação, alta estabilidade química e poucos efeitos citotóxicos. Baseado nesses relatos, este trabalho teve como objetivo o desenvolvimento de novos derivados semissintéticos de AU e AB contendo modificações nas posições C-3 e C-28, obtidos por duas rotas químicas diferentes, a fim de avaliar a potencial atividade antitumoral e antiviral. Os compostos obtidos com a primeira rota apresentam modificações na posição C-3 e foram avaliados frente a células tumorais K562 (Leucemia Mieloide Crônica Humana) *in vitro* e B16F10 (melanoma de camundongo) *in vitro* e *in vivo.* A segunda rota sintética deu origem a compostos contendo modificações nas posições C-3 e C-28, que foram avaliados *in vitro* frente ao virus RSV (Vírus Sincicial Respiratório) em células A549. Os resultados deram origem a três artigos científicos. O derivado ácido 3-aminoursólico **2c (artigo 1)** mostrou-se o mais ativo contra as células K562, além de induzir morte celular via apoptose e atuar sobre o ciclo celular dessas células. *In vivo* os derivados oxima do ácido betulínico **3** e ácido 3-aminobetulínico **4 (artigo 3)** mostraram-se extremamente ativos sendo capaz de inibir a proliferação celular metastática de B16F10 em camundongos. Todos os derivados foram ativos frente ao vírus RSV, sendo o candidato mais promissor o composto éster do ácido 3-*O*-acetil-28{(3-nitrofenil)-1,2,3-triazol}metiloxi-ursólico **3d (artigo 2)** por apresentar melhor resposta sobre a inibição da proliferação celular, além de se mostrar o derivado mais seletivo e menos citotóxicos.

Palavras-chaves. Ácido ursolico, ácido betulinico, tumor, atividade anti-SRV, triterpenos.

ABSTRACT

A growing number of publications reporting the biological effect of semisynthetic derivatives as antitumor and antiviral activity. This search for new molecules is justified by the high resistance of tumor cells to currently available drugs as well as new resistance mechanisms and disability acquired during the treatment of viral diseases. Ursolic acid **UA** and betulinic acid **BA**, has been highlighted in research in recent years because of its many reported biological activities, such antitumor and antiviral. In addition to the diverse biological effects of these compounds bring the advantage of easy obtaining and purification, easy handling, high chemical stability and few cytotoxicity. Based on these reports, this project aimed to the development of new semisynthetic derivatives with modifications in the C-3 and C-28 positions by two different chemical routes, in order to evaluate the antitumor and antiviral activity. Compounds obtained by the first route containing modifications at the C-3 position and were evaluated against tumor cells K562 (human chronic myelogenous leukemia) *in vitro* and B16F10 (mouse melanoma) *in vitro* and *in vivo*. The second synthetic route gave compounds containing modifications in positions C-3 and C-28, and evaluated *in vitro* against cells infected with RSV virus A549. From these routes, three scientific articles have been developed. Derivative 3-amine ursolic acid **2c (article 1)** showed the most activity against the K562 cells, and induce cell death via apoptosis and cell cycle act on these cells. *In vivo* derivatives 3-oxime-betulinic acid **3** and 3-amine betulinic acid **4** (article 3) proved to be extremely active and able to inhibit cell proliferation to metastatic B16F10. All derivatives were active against RSV virus, the most promising candidate compound 3-O-acetyl-28{(3-nitrophenyl)-1,2,3-triazol}methyloxy--ursolic ester **3d** by presenting better response on the inhibition of cell proliferation, and to show more selective and less cytotoxic derivative.

Keywords. Ursolic acid, betulinic acid, tumor, anti-RSV activity, triterpenes.

SUMÁRIO

I. INTRODUÇÃO

Ao longo dos séculos, produtos naturais vêm demonstrando ser uma fonte inspiradora na busca de moléculas com potenciais atividades biológicas, proporcionando alguns dos agentes terapêuticos mais promissores e eficazes contra o tratamento de diferentes patologias(1)(2)(3). Contudo, o uso direto de produtos naturais muitas vezes é limitado por diversos motivos que vão deste parâmetros farmacocinéticos inadequados à citoxicidade (4)(5)(6). Frente a esta realidade, a semissíntese surge, como uma ferramenta alternativa para a descoberta de novos fármacos, promovendo modificações estruturais, a fim de modelar as propriedades biológicas dos produtos naturais (7)(8)(9).

Exemplos clássicos de fármacos semissintéticos usados atualmente na terapia do câncer são o teniposídeo e o etoposídeo, derivados da podofilotoxina(10). A podofilotoxina, é uma substância tóxica de ocorrência natural, pertencente à classe das lignanas ariltetralínicas(11)(12). Modificações químicas na estrutura da podofilotoxina deram origem ao etoposídeo e teniposídeo, compostos, menos tóxicos e mais ativos que seu precursor natural, e permitiram a introdução desses na terapia do câncer(13)(14)(15) .

O processo de pesquisa e desenvolvimento de novos fármacos está diretamente relacionado à química medicinal e segundo a IUPAC envolve a invenção, a descoberta, o planejamento, a identificação, a preparação e a interpretação do mecanismo de ação molecular de compostos biologicamente ativos(16)(17)(18)(19). Tendo este conceito como meta, há décadas nosso grupo de pesquisa tem se dedicado ao estudo, planejamento e semissíntese de derivados visando à obtenção de compostos líderes e protótipos para diversas atividades farmacológicas, especialmente, antitumoral, antimalárica, antifúngica e mais recente antiviral (20)(21)(22)(23)(24)(25).

Triterpenos são produtos naturais amplamente distribuídos entre diversos organismos, incluindo bactérias, fungos, leveduras, plantas e mamíferos(26)(27). Sua estrutura básica é composta, por seis unidades de isopreno com a fórmula molecular $C_{30}H_{48}$, sendo sintetizados através da ciclização do esqualeno, e classificados em três

tipos principais com base em seu esqueleto estrutural: oleananos, ursanos e lupanos(28)(29)(30).

Mais de 20.000 triterpenos são conhecidos na natureza. Dentre estes o ácido ursólico (**AU, 1**) e o ácido betulínico (**AB, 2**) isômeros de cadeia, têm atraído a atenção da comunidade científica nas últimas décadas, devido a grande gama de atividade biológica que incluem; atividades antitumoral, antiviral, antibacteriana, antimalárica, dentre outras(31)(32)(33)(34)(35) .

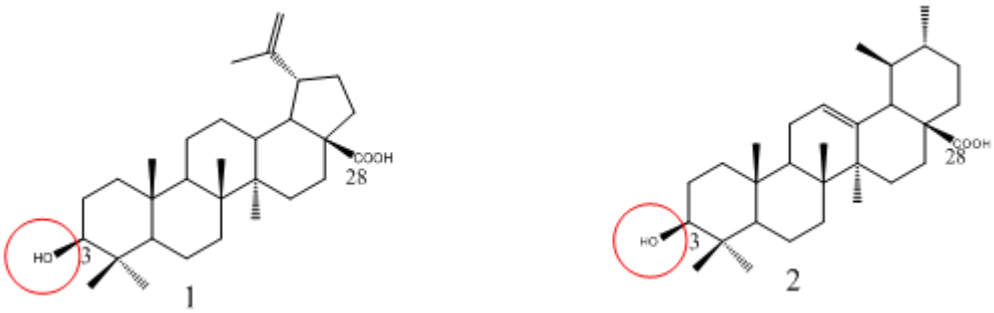


Figura 1. Estrutura química do ácido ursólico (1) e ácido betulínico (2)

Estudos têm demonstrado que as alterações nas posições C-3, C-12 e C-28, da estrutura triterpênica do **AU** e **AB** pode levar à produção de derivados semissintéticos mais ativos e mais seletivos que os correspondentes produtos de partida. Um exemplo clássico é o da síntese do bevirimat, um composto com atividade anti-HIV, que encontra-se no momento em estudos de fase clínica II. O bevirimat é um derivado do **AB**, obtido por uma reação de esterificação no C-3, pouco citotóxico e com uma acentuada atividade anti-HIV(36)(37) .

Com base na atividade destes compostos e na necessidade de novos fármacos eficazes com atividade antitumoral e antiviral, este trabalho descreve os esforços realizados para a semissíntese e avaliação biológica de novos derivados do **AU** e **AB**, visando obter compostos protótipos para estas atividades. O planejamento deste trabalho foi realizado de forma a estudar modificações nas posições C-3 e C-28 de ambos os triterpenos **AU** e **AB**, que foram então submetidos a ensaios de atividade antitumorais *in vitro* e *in vivo* e antiviral *in vitro*.

II. OBJETIVOS

- Objetivo geral.

Planejamento e semissintese de novos derivados do AB e AU visando à atividade antitumoral e antiviral.

- Objetivos específicos.

- ✓ Extração e purificação do AU e AB e desenvolvimento de rotas alternativas para a semissíntese de derivados do AB e AU.
- ✓ Avaliação da atividade antitumoral frente às linhagens celulares de K562 (Leucemia Mieloide Crônica Humana), B16F10 (melanoma de camundongos) e atividade citotóxica frente às células VERO, HEP, HEPG2 e linfócitos humanos.
- ✓ Ensaios de mecanismo de atividade dos compostos ativos via indução de apoptose e ação sobre o ciclo celular.
- ✓ Avaliação da atividade antitumoral *in vivo* de revidados do ácido betulinico.
- ✓ Avaliação da atividade antiviral frente ao vírus sincicial respiratório RSV.

Os resultados nesta dissertação de mestrado estão apresentados na forma de artigos científicos conforme segue:

1. Artigo **"Synthesis and antileukemic activity of an anti-proliferative and apoptotic ursolic acid derivative: a potential co-drug in combination with imatinib"** a ser submetido para o periódico **Drug Design, Development an Therapy.**

2. Artigo **"Synthesis of new nitro-1, 2, 3-triazoles-28- triterpenes derived through click chemistry reaction with potential anti-RSV activity"** a ser submetido para o periódico **Bioorganic** & **Medicinal Chemistry Letters.**

3. Artigo **"In vitro and in vivo anti-melanoma effects of betulinic acid derivatives induces apoptosis in B16F10 melanomas cell line"** a ser submetido para o periódico **British Journal of Dermatology.**

ARTIGO 1.

Synthesis and antileukemic activity of an ursolic acid derivative: a potential co-drug in combination with imatinib.

Elenilson F. Silva[1], Julia B. Willig[2], Cristiane B. de Oliveira[1], Grace Gosmann[1], Aline Rigon Zimmer[1], Diogo André Pilger[2], Andréia Buffon[2], Simone C. B. Gnoatto[1].

[1]Laboratory of Phytochemistry and Organic Synthesis, Graduate Program in Pharmaceutical Sciences, Federal University of Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, RS 90610-000, Brazil.

[2] Laboratory of Biochemical and Cytological Analysis, Faculty of Pharmacy, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Corresponding author: Simone C. B. Gnoatto, Federal University of Rio Grande do Sul, Faculty of Pharmacy, , Avenida Ipiranga, 2752 sala 708 Santana, Porto Alegre, RS, Brasil - CEP 90610000, e-mail simone.gnoatto@ufrgs.br.

Abstract

Betulinic (**1**) and ursolic acid (**2**) are naturally occurring aglycones, belonging to the class of pentacyclic triterpenes; these compounds have several known biological activity and antitumor activity is the most studied. In this paper we selected the C-3 position of the ursolic and betulinic acid scaffolds, in order to exploit the contribution of modifications in this position in the cytotoxic activity against K562 leukemia cell lines. Six new derivatives (**1a-2c**) was synthesized and evaluated. The derivative **2c** containing an amine group in the C-3 position of ursolic acid proved to be the most active with an IC_{50} of 21.9 µM and 5.2 µM after 24 and 48 h of treatment, respectively,

also this derivative showed inactive against mammalian cell lines (VERO, HepG2 and human lymphocytes cells). The **2c** derivative was still able to induce cell death by apoptosis in addition to cause cell cycle arrest. When associated with drug imatinib showed a synergistic effect..

Keywords. triterpenes, betulinic acid, ursolic acid, leukemia, K562 cells.

Introduction

Chronic Myeloid Leukemia (CML) is a disease of the hematopoietic stem cells associated with the abnormal formation of the Philadelphia chromosome (Ph), which results from a translocation between chromosomes 9 and 2[12]. Although the most reported incidence of CML is in older patients, this disease can affect all ages[3]. Only in 2012, more than 150,000 deaths resulting from CML were documented[4]. For decades, imatinib, a specific BCR-ABL tyrosine kinase inhibitor was considered one of the most successful specific antineoplastic agents, and is still the drug of choice in the treatment of CML[5]. Although the revolutionary discovery of imatinib has been a breakthrough in the treatment of CML, recent studies have shown that its effectiveness is greatly reduced by drug resistance response, thereby making up a limitation to the treatment of CML[6,7]. Therefore, in the recent years, there has been growing interest in developing new agents for the treatment of leukemia, or that, in combination with treatments already available, minimize the effects of drug resistance in CML[8]. For over 40 years, natural products or semi-synthetic molecules derived from natural products form an important part of our arsenal against cancer[9,10]. Betulinic acid (**1**) and ursolic acid (**2**) are naturally occurring aglycones, belonging to the class of pentacyclic triterpenes, which is an important class of molecules with a variety of known

therapeutic activities [11,12,13]. Among these activities there is the pronounced cytotoxicity of these compounds against a variety of tumor cell lines, such as neuroblastoma, melanoma, glioma, prostate cancer, lung cancer and leukemia[14,15,16,17,18,19,20]. Recent research shows that betulinic and ursolic acid act in different ways against tumor cells, such as, by induction of apoptosis, via caspase 3, cell cycle arrest, autophagic death, among others[21,22,23]. Moreover, investigations have shown that modifications in positions C-3 and C-28, can lead to production of semi-synthetic derivatives potentially more active and more selective than the corresponding starting products[24,25,26,27,28]. In this paper we selected the C-3 position of the ursolic and betulinic acid scaffolds, in order to exploit the contribution of changes in this position in the cytotoxic activity against K562 CML cell line. We also investigated the mechanism of action of the more active derivative obtained in this study, by cell death assays and the influence on the cell cycle, as well as the possible synergistic effect in combination with imatinib.

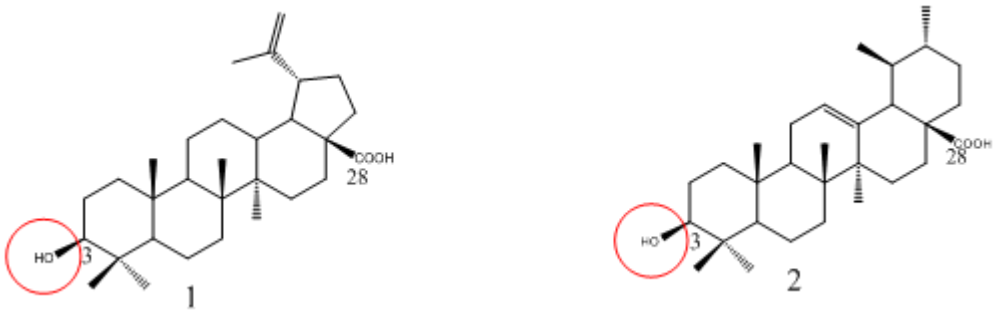


Figure 1. Structure of betulinic acid originated from lupane skeleton (**1**) and ursolic acid originated from ursan skeleton (**2**).

Methods and Materials

Plant materials

Ursolic acid (**1**) was obtained from *Malus domestica* shells (apple), and its corresponding isomer betulinic acid (**2**) was obtained from the bark of *Platanus acerifolia* L. (Maple), According to methodology described above[29]. The characterization of betulinic acid, ursolic acid and its purity was carried out by Thin Layer Chromatography (TLC), Nuclear Magnetic Resonance (NMR), mass spectrometry (MS) and infrared (IR).

Chemistry

All solvents used for reaction and further purification of the semisynthetic derivatives have previously been distilled. Column chromatography (CC) was carried out on silica gel (Merck, 60–230 mesh) using dichloromethane/ cyclohexane gradient eluent mixtures.

IR analysis was performed with a PerkinElmer FT-IR System Spectrum BX (Porto Alegre, Brazil). ^{1}H and ^{13}C NMR spectra were recorded with a Varian Inova 500 NMR spectrometer and mass spectrometer.

Synthesis

General synthetic route is depicted in Scheme 1.

3-keto-betulinic acid **(1a) and** ***3-keto-ursolic acid*** **(2a).**

To a solution of **1** or **2** (455 mg, 1.0 mmol) in acetone (6 mL) at 0 ° C was added Jones reagent (1.5 mL). The solution was stirred for an hour at room temperature, then was cooled to 0°C and added (10 mL) of isopropanol and kept under stirring at room temperature for 30 minutes. Then, the solution was filtered, and the filtrate was dried under reduced pressure. The product was purified on a silica gel column

chromatography (eluent cyclohexane/CH_2Cl_2). A white powder was obtained with 89% yield (**1a**) and 79% (**2a**).

3-oxime-betulinic acid **(1b) and** ***3-oxime-ursolic acid*** **(2b).**

To a solution of 3-keto-betulinic acid (**1a**) or 3-keto-ursolic acid (**2a**) (150mg, 0.3mmol) in ethanol and pyridine 2:1 was added under N_2 (g) hydroxylamine hydrochloride (70mg, 0.112 mmol) previously dissolved in ethanol. The reaction mixture was kept on stirring at room temperature for approximately 72 hours. Subsequently it was poured into cold H_2O and the precipitate formed was filtered, dried and stored. The product was purified on a silica gel column chromatography (eluent cyclohexane/CH_2Cl_2).

3-amine-betulinic acid **(1c) and** ***3-amine-ursolic acid*** **(2c).**

To a solution of 3-oxime-betulinic acid (**1b**) and 3-oxime-ursolic acid (**2b**) (200 mg, 0.4 mmol) in distilled THF (10 mL) at 0°C under N_2 (g) was added $LiAlH_4$ (351mg, 9,24mmol). The mixture was maintained at reflux for 6 hours .Then, the mixture was treated with aqueous 1N NaOH (2 mL) and stirred for another hour. Finally the solution was extracted with diethyl ether (3x 10 mL) the organic phase was separated and the product was purified on a silica gel column chromatography (eluent cyclohexane/CH_2Cl_2).

All derivatives were identified by IR analysis, ^1H and ^{13}C NMR spectra and by comparison with the previously synthesized[30].

Antitumor activity.

Cell Culture

K562 cells line (Human Chronic Myelogenous Leukemia) were obtained from Banco de Células do Rio de Janeiro (BCRJ, Rio de Janeiro, RJ, Brasil). Cells was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 0.5 U/mL penicillin/streptomycin at 37°C, in a 5% CO_2 atmosphere at 100% humidity.

In order to evaluate the biological effects obtained with the use of derivatives in blood cells, it was also evaluated lymphocytes from healthy donors. Human lymphocytes from venous blood of different donors were isolated by gradient centrifugation on Histopaque®. Cells was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 0.5 U/mL penicillin/streptomycin at 37°C, in a 5% CO_2 atmosphere at 100% humidity. All the consents, procedures and documents were approved for the Federal University of Rio Grande do Sul Ethical Committee under authorization number 666.655/2014.

VERO cells are not tumor cells and were chosen as controls in order to evaluate the effects of triterpene derivatives when exposed to non-tumor cells. We also use cell hepatocellular HEPG2 to investigate the possible toxic metabolites generated from the metabolism of the compounds tested by these cells. VERO (kidney from African green monkey cells) and HEPG2 cells (Kidney cells) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.5 U/mL penicillin/streptomycin at 37°C, in a 5% CO2 atmosphere at 100% humidity.

Treatments

Compounds **1-2c** were dissolved in dimethyl sulfoxide (DMSO) and culture medium. After reaching sub-confluence (70%–80% confluence), the cells were exposed to

compounds (10 to 100 µM) for 24 for **1-2c** derivatives and 48h for **2c** derivatives in DMEM. Cells treated with DMSO (0.5% final concentration) were used as a negative control.

Cell Assays.

Cell Counting

K-562 cells line and Human lymphocytes are nonadherent cells found in suspension in the culture medium, in order to evaluate the cytotoxicity of derivatives **1-2c**, K-562 cells line and Human lymphocytes ($1x10^4$ cells/well in 100 µL medium per well) were seeded in a 96 well plate. After 24 hours the cells were treated with the compounds **1-2c** (10 to 100 µM/100 µL per well). The experiments were performed in triplicate at 37°C and 5 % CO_2 for 48 hours. Analyses were conducted in FACSVerse™ (BD Biosciences) cytometer equipped with 488nm blue laser and FACSuite software. The results were expressed as % relative to control. We considered cytotoxic the samples that present a minim reduction of cells number of 50%. The cells morphology were previous verified for optical microscopy and the counting determined by flow cytometer FACSVerse™, with blue laser 488 nm and flow sensor (BD Biosciences, San Jose, CA, EUA) (BD FacSuite™ Software).

MTT Assay

The inhibition of cell proliferation by compounds in adherent cells VERO and HEPG2 was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. VERO and HEPG2 cells (1×10^4 cells/well in 190 µL medium per well) were seeded in a 96-well plate. After 24 h, the cells were treated with the compounds (**1**- **2c**) 10 to 100 µM. The optical density of each well was measured at 630 and 560

nm on Envision (PerkinElmer, Waltham, MA, USA) microplate reader. Four independent experiments were performed in triplicate for each test. The results were expressed as the percentage of cell viability where cells with no treatment were considered 100% viable.

Trypan blue

In order to inform the results obtained by cellular count in K-562 cells exposed to the derivative **2c** we evaluate the cytotoxicity of this derivative also by counting using Trypan blue. K-562 cells line (1×10^4 cells/well in 100 µL medium per well) were seeded in a 96-well plate. After 24 h, cells were treated with compound **2c** (10 to 100 µM in 100 µL medium per well). After a 48h the counting was performed using trypan blue and a hemocytometer. The results were expressed as a percentage of the control to obtain the corresponding IC_{50}.

Apoptosis Assay

K-562 cells line (4×10^4 cells/well in 300 µL medium per well) were seeded in a 24-well plate. After 48 h, cells were exposed to compound **2c** (25, 50 e 100 µM) for 48 h. After treatment, K-562 cells were double stained with FITC-conjugated annexin-V and PI using an Annexin-V Apoptosis Detection kit (QuatroG, Pesquisa e Desenvolvimento Ltda, Tecnopuc, Porto Alegre, RS, Brasil) according to the manufacturer's instructions. Cells were analyzed in FACSVerse™ cytometer (BD Biosciences) equipped with 488nm blue laser BD and FACSuite software. Triton X-100 (TX100) was used as a necrosis positive control and imatinib was used as an apoptosis positive control.

Cell cycle distribution analysis.

For cell cycle analysis, K-562 cells (1.2 x 10^5 cells /well in a 6 well plate), followed by treatments with the compound **2c**. After treatment, cells were harvested and fixed in cold ethanol 70% v/v in phosphate-buffered saline (PBS) for at least 2 h. Fixed cells were washed with PBS and marked with a solution containing PI 6 mM, Triton X-100 and RNAse for 30 min, in the dark, at room temperature. DNA content was analyzed through flow cytometer.

Evaluation of the synergistic effect of imatinib drug and 2c derivative.

For the evaluation of synergistic effect between the derivative **2c** and the drug imatinib, we used the analysis by isobolagram, previously described [31]. We used the concentrations of 0.1 to 0.6μM imatinib drug and maintained a fixed concentration of derivative **2c** 2.5μM. The interaction between the two compounds was calculated based on the following equation: index= $2c_c/2c_e$ + Imb_c/Imb_e where $2c_c$ and Imb_c is derived from the concentrations **2c** and imatinib, respectively, required for 50% inhibition of cell viability when used alone, and $2c_e$ and Imb_e are the concentrations of derivative **2c** and imatinib which produces an inhibition effect of cell viability when used in combination. According to this method, an interaction index of less than 1.0 indicates synergistic effect between two drugs. An interaction index of more than 1.0 indicates antagonism, and an index of 1.0 indicates additive effect.

Statistical analysis.

Data were presented as mean ± standard deviation of three individual experiments. Statistical analysis was performed by a one-way ANOVA analysis by means of the Prism statistical software package (GraphPad Software, Inc., La Jolla, CA, USA). *P,0.05 was considered statistically significant.

Results

Chemistry

Matches the product for semi-synthesis reactions, ursolic acid and betulinic acid, good yields were extracted with 2% and 3% respectively. Six new derivatives **1a-2c** with modifications at the C-3 position was synthesized **Figure 1**. Initially were obtained derivatives with a ketone function via an oxidation reaction using Jones reagent **1a** and **2a** with good yields, approximately 89%. From the derivatives **1a** and **2a** were obtained derivatives containing the oxime function **1b** and **2b** using hydroxylamine hydrochloride as a reaction promoter, reaching revenues of approximately 96%. Finally they were synthesized derivatives **1c** and **2c** in 68% and 62% yield by reduction reaction with lithium aluminum hydride using the oxime derivatives as a substrate.

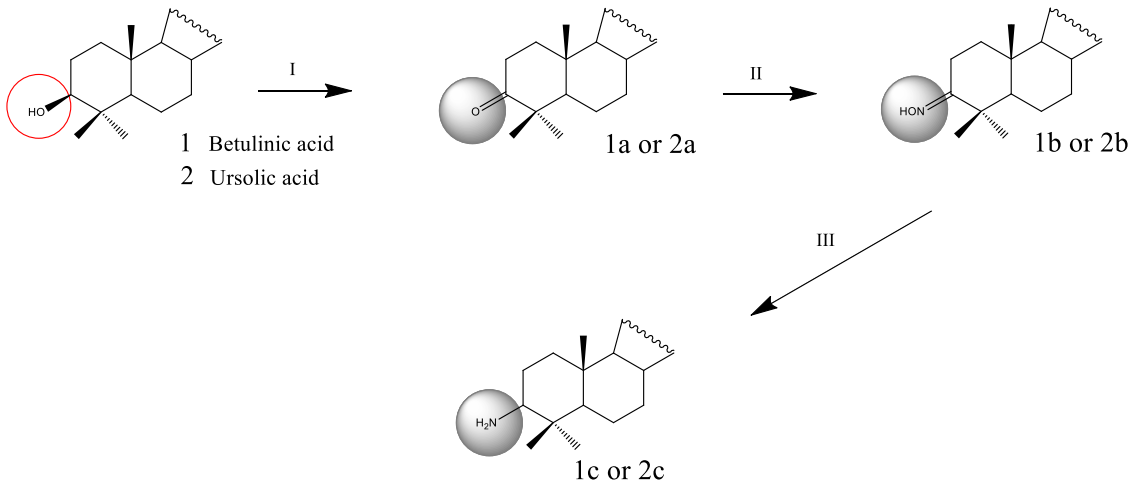


Figure 1: Synthetic Route of **1a-2c** compounds. **I** Jones reagent, 0 ° C, 1h acetone, isopropanol.**II** ethanol, pyridine, hydroxylamine hydrochloride, N_2(g), 72h, H_2O, 1h**. III**.

Antitumor activity

All derivatives were subjected to evaluation for their antitumor activity on K562 leukemia cells after 24 hours of treatment**.** We also evaluated the cytotoxic effect of these derivatives on lymphocytes, HEPG2 and VERO cells. The results obtained were expressed as IC_{50} (µM) for each derived **Table 1**.

Table 1: IC_{50} µM, after treatment with **1-2c** compounds for 24h using MTT assay and cell counting, against leukemia K562, VERO, lymphocytes and HEPG2 cells. All values are the means ± SE of at least triplicate cultures in three in independent experiments. ** p <0.05 for treatments **2c** compared with VERO, lymphocytes and HEPG2 cells.

Compound	K562[a]	VERO[b]	Lymphocytes[c]	HEPG2[d]
1	85.5 ± 1.5	99 ± 1.5	78.11 ± 1.1	>100 ±1.2
2	33.4 ± 0.4	86 ± 0.4	91.2 ± 2.5	87.2 ± 3.3
1a	74.3 ± 2.1	89 ± 2.1	78.4 ± 0.9	86 ± 1.9
1b	55.2 ± 0.8	72 ± 0,8	84.15 ± 0.7	>100 ± 1.1
1c	19.5 ± 0.4	>100 ± 0.4	>100 ± 1.8	99.4 ± 0.9
2a	90.2 ± 0.9	90.2 ± 0.9	98.2 ± 0.5	>100 ± 1.6
2b	21.9 ± 0.6	>100 ± 0.9	>100 ±1.1	98.8 ± 2.1
2c	14.9 ± 0.7	>100 ± 0.7**	>100 ± 0.9**	97.9 ± 2.7**
Imatinib	nd	Nd	>100 ± 2.5	nd

[a,b,c,d,] IC_{50} (µM) for K562, VERO, Lymphocytes and HEPG2 cells, respectively.

nd: not determined.

The derivative **2c** containing an amino grouping, a proton donor, proved to be the most active of the derivatives with an IC_{50} of 14.9 µM. Derivatives containing groups ketones **1a** and **2a** at the C-3 position were considered inactive, with IC_{50} values of 74.3 and 90.2µM. These results suggest that modifications containing proton acceptor groups such as ketones functions in C-3 position may not be favorable for activity on K562 cells. The derivatives containing the oxime function **1b** and **2b** had IC_{50} of 55.2 and 21.9 µM, respectively. In general, it can be noted that the greater activity for ursano derivatives. Optimistic with the results obtained in cell viability assays for compost **2c**, we selected and submitted this to other biological assays in order to explorer, and understand the mechanisms by which this compound causes a decrease in cellular viability. Compound **2c** was then analyzed by 48 hours of treatment, for evaluation of time-dependent K562 leukemia, HEPG2, VERO and lymphocytes cells. We also assessed the effect on cell proliferation of lymphocytes exposed to imatinib, these results are shown in **Figure 2**. It is possible to realize a marked selectivity of compound **2c** demonstrating for leukemia cells an IC_{50} of 5.2µM, while for the other cell lines IC_{50} was greater than 50µM. We also assessed the cytotoxic effect of the drug imatinib in K562 treatment for 48 hours, demonstrating an IC_{50} of 0.8 µM (**Figure 5A**) .

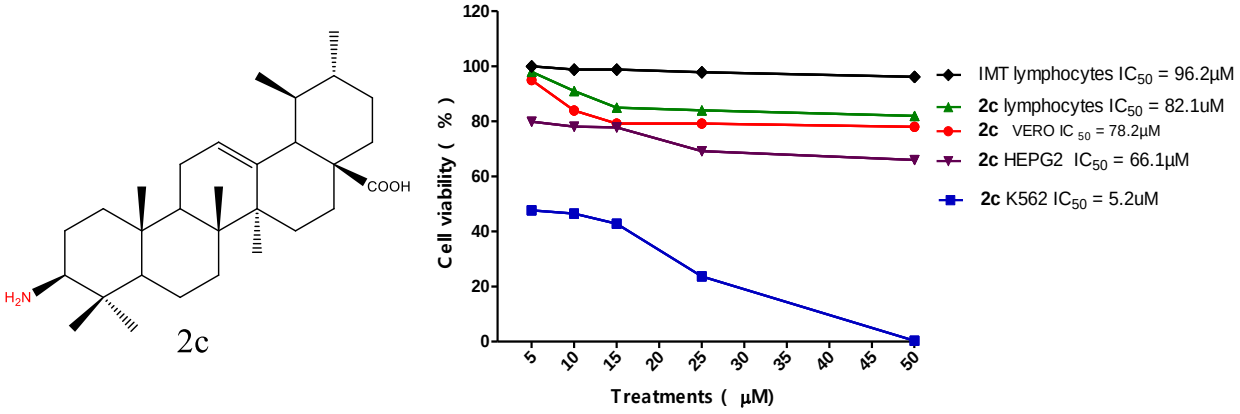


Figure 2. Cell viability assay after treatment with **2c** compound for 48h using MTT and cell counting assay. **A** chemical structure of the derivative **2c** containing an amine grouping in the C-3 position. **B** IC_{50} in µM of compound **2c** in lymphocytes, VERO, HEPG2 and K562 cells line and IC_{50} in µM of imatinibe drug in lymphocytes.

Induction of apoptosis and G2/M arrest and G1 arrest by 2c derivative.

Given the high activity and selectivity showed by derivative **2c**, we investigate the possible mechanism by which this derivative causes a decrease in cell viability K562 by two assays: Apoptosis using propidium iodide and annexin and action on the cell cycle.

The results obtained for the apoptosis assay are shown in **Figure 3.** We found that the compound **2c** induces apoptosis only in higher concentrations 50 to 100 µM, showing no death effect at concentrations of 5, 10, 15 and 25 µM, yet the test action on the cell cycle.

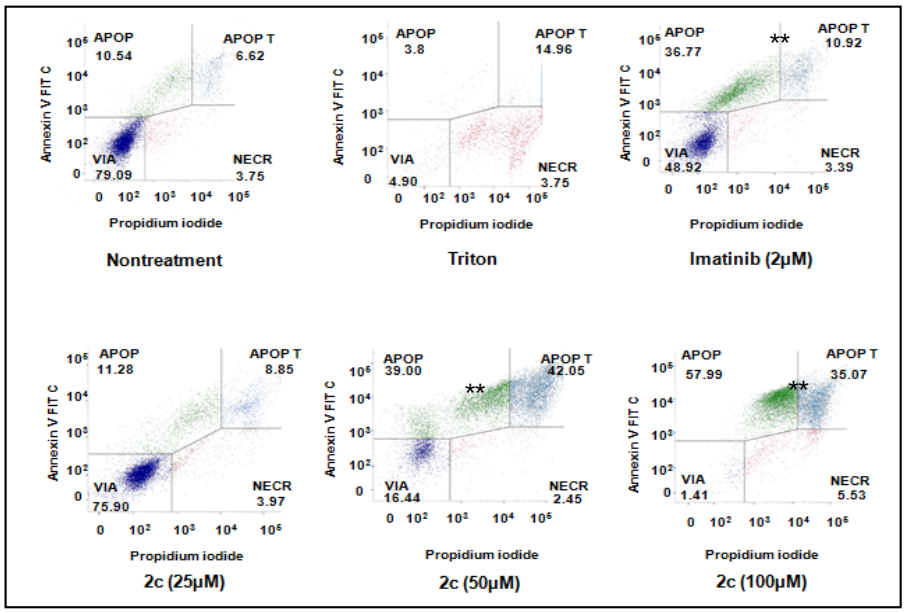


Figure 3. Apoptosis assay of K562 cells treated with **2c.** Flow cytometric analysis of apoptosis in K562 cells was performed by treatment with imatinibe (2µM) and **2c** (25, 50 or 100µM) for 48 h and by staining with annexin V–FITC and PI. ** p <0.05 for treatments compared with the Nontreatment control apoptosis of viable cells.

Table 2 showed an accumulation of cells in G1 phase and decreased the number of cells in the G2 phase in concentrations 12.5 and 7.5µM of **2c**. These results clearly show that the derivative **2c** is able to cause a perturbation in the cell cycle, leading to a decrease in cellular proliferation in K-562 cells .These results were confirmed by counting using trypan blue **Figure 4**, it is possible to realize a decrease of approximately 50% of the numerous of cells when exposed to compound **2c** in concentration of 10, 20 and 30 µM for 48 hours of treatment. However, no significant cellular death was observed compared with the control in these concentrations, only antiproliferative effect. Still in higher concentrations, 50 and 100 µM, cellular death is clearly observed in K562. **Table 2**. Effect of **2c** on cell cycle distribuition of leukemia K562 cells. The cells were harvested after 48h treatment with **2c** at concentrations of 7.5 e 12.5 µM. All values are the means ± SE of at least triplicate cultures in three independent experiments.** p <0.05 for treatments compared with the DMSO 1% .

Compound	**G1/G0[a]**	**S[b]**	**G2/M[c]**
Not treated	60.67%	7.5%	31.33%
DMSO control 1%	59.12%	8.69%	31.82%
2c at 7.5 µM	67.63%	6.60%	24.48% *
2c at 12.5 µM	73.95%	7.08 %	18.75 % **

a ,b, c cell cycle phases**,** G1/G0, S and G2/M**,** respectively.

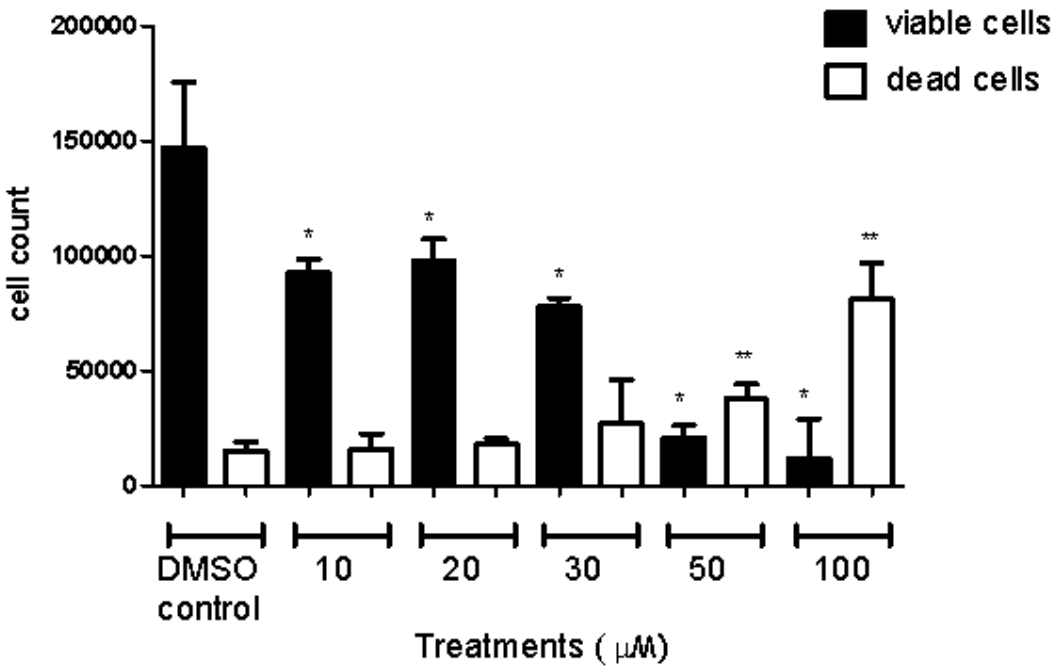


Figure 4. Count with trypan blue to **2c** derived after 48h treatment. The closed boxes represent the number of viable cells, the open boxes represent the number of dead cells. * p <0.05 for treatments compared with the DMSO control of viable cells and ** p <0.05 for treatments compared to DMSO control dead cells.

Synergistic effect test

Finally, we evaluated the synergistic effect of the association between derivative **2c** and the drug imatinib, **Figure 5**. We noted that according to isobolagram curve have a synergistic effect potentially leading to a decrease in drug IC_{50} imatinib 0.8 to 0.37μM when associated with 2.5μM compound **2c** (**Figure 5A and 5B**). The synergistic effect was confirmed from the construction of isobolagram curve, which shows a 0.95 correlation index which is worth a synergism parameter between drugs (**Figure 5C**).

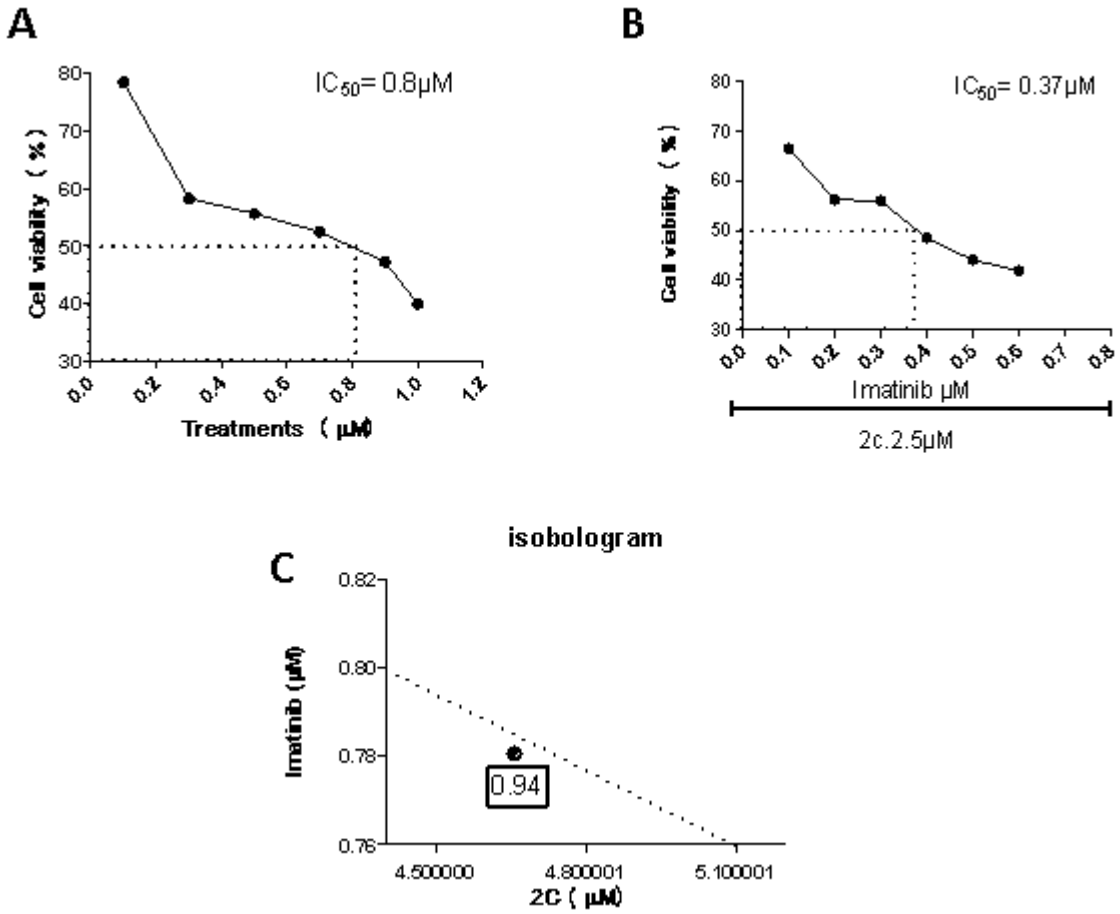


Figure 5: Effect of imatinib and/or **2c** derived from the decrease in cell viability K562 cells line. Cytotoxic effect was performed by cell counting and trypan blue and expressed in cell viability versus concentration (**A** and **B**). **A**. cytotoxic effect of the imatinib drug. **B 2c** compound to reduce cell viability by 50%. **C** analysis by isobolagram, demonstrating the level of interaction between the two compounds.

Discussion

The cause of chronic myeloid leukemia (CML) is a constitutively active BCR-ABL tyrosine kinase[32]. Approved in 2002 for the treatment of CML, imatinib, an tyrosine kinase inhibitor that works by competitive effect in ATP binding site, is currently considered the standard initial treatment for patients in chronic phase[33]. However, it is now known that about 20% to 30% of CML patients do not respond to imatinib or experience relapse after an initial response to the disease[34]. Several mutations in the BCR-ABL gene domain have been described in the past decades[35]. However, the

majority of changes occur in three distinct regions of the BCR-ABL gene: A-loop, the catalytic kinase domain or directly in ATP binding site[35]. These mutations result in amino acid substitutions, preventing imatinib binding to the ATP binding site and are responsible for the primary cause of acquired resistance to treatment with imatinib[36]. Comprehension of the imatinib resistance molecular mechanisms, contributed to the development of the second generation drugs, nilotinib and dasatinib, which were intended to break the imatinib resistance barrier in patients with LMC[37,38]. Nilotinib has a similar structure to imatinib and shares its binding mode and high specificity. However, dasatinib differs from imatinib as to its chemical structure, binding mode and pharmacological effects[38]. Despite the differences, dasatinib was found to be 100 times more active against the BCR-ABL gene when compared to imatinib, this effect is primarily attributable to their different mechanisms of action[39]. Although tolerant to most known mutations of BCR-ABL gene, both drugs were not able to overcome resistance conferred by mutations occurred in the T315I, that are considered pan-resistant mutations, still not having an effective treatment that circumvent acquired resistance by this mutation[40]. Current studies have shown that not only the resistance mechanism is a limiting to treatment with imatinib, were recently described studies showing that imatinib causes severe manifestations of cerebral edema and cutaneous and may lead to death in addition to many other side effects[41]. Yet studies show that skin reaction appears to be entirely dependent on the dose, meaning that is due more to the pharmacological effect of this medicine than a hypersensitivity reaction of the individual. This data has fueled many studies and research in the search for new forms of treatment of CML, or in the search for alternatives that added to the treatments already exist can contribute to the better treatment of patients[424344] Based on this, we dedicate this article to assess the potential derivatives activity of BA and UA against

CML. Numerous studies have reported the antitumor activity of UA, BA and derivatives against various tumor cell lines, among which strains of leukemia[45 46]. These studies show that BA as UA are able to decrease the viability of tumor cells *in vitro*, by various mechanisms such as induction of cell death by apoptosis and antiproliferative activity [47,48,49]. In recent decades a large number of articles have been published, showing the insertion of nitrogen containing functional groups in C-3 and C-28 position may promote the antitumor activity of triterpenes against several types of cancer *in vitro* and *in vivo*[50,51]. Similar chemical groups are found in the chemical structure of imatinib, nilotinib and desatinib[52,53,54] . From the observation of the chemical structure of these drugs can be identified nitrogen nuclei in common between them. The presence of this common group was included in this class of substances aiming to mimic the adenine ring of ATP substrate and facilitate the connection to the hinge region of the catalytic site[55]. Furthermore studies have shown that insertion other nitrogenous groups in chemical structure of imatinib plays an important role in inhibiting the BCR-ABL gene. Based on the potential activity of triterpenes against tumor cells added to low toxicity presented by these compounds we are dedicated to synthesis of derivatives containing nitrogen groups, in order to explore the contribution of these against CML cells. Our results demonstrate that ursano skeleton is more potent against K-562 compared with lupane and that modifications containing the amine function are important for the activity in this cell line as we presumed. Both starting materials, **UA** and **BA**, as well as its derivatives have shown little cytotoxic in Vero, HEPG2 and lymphocytes cells. In addition, the cell viability essay for 48h, clearly show the effect of time-dependent derivative **2c** in leukemia when compared to other cells. We have demonstrated that **2c** derivative induces cell death by apoptosis and possess anti-proliferative activity causing a disturbance in the K562 cell cycle. These results are similar to those

described in other studies that show that **UA** is able to induce apoptosis in K562 cells line[56] . However, there still reports in the literature about the activity in K562 cells for **BA**. The good results found in this work led to evaluate the effect of **2c** derivative in association with the drug imatinib. A large number of publications, particularly related to antitumor activity, have described synergism studies among drugs and triterpenic derivatives [57,58,59]. Furthermore, it is well known today that chemotherapy can be made by applying one or more chemotherapeutics[60]. Various combinations of drugs with imatinib are found in clinical phase studies, these studies show that the combination between a tyrosine kinase inhibitor and other drug can be a promising alternative[61,62,63,64,65]. Our results showed that when **2c** derivative is associated with imatinib it made possible to decrease the concentration of imatinib, while still maintaining the same effect of the drug on cell viability. One alternative that is currently being used in order to get a better response to treatment in the chronic phase is to increase the daily dose of imatinib for patients at this stage[66]. Despite obtained best results when treating many side effects are observed when the concentration is doubled[67]. Thus decrease the concentration and maintain the activity can be an excellent alternative. We show in this study that this is possible by the combination of two drugs. So far there are no studies that show a relationship between triterpenes and the BCR-ABL gene, but it is known the potential cell death induction by various routes as well as the antiproliferative activity of these compounds and their derivatives, against a variety of other tumor cells[68]. We believe that these pharmacological activities may be associated with the tyrosine kinase inhibitor effect of imatinib, which together potentiated the pharmacological effect featuring a synergistic effect. However, our results lead us to believe that the inclusion of a nitrogen group in the triterpenic

structure of **UA** might lead to a derivative that can inhibit BCR-ABL gene, increasing the pharmacological effect obtained by the combination of the two drugs.

Conclusion

In this study, eight derivatives were synthesized by a low-cost and easily applicable method, the derivative **2c** containing a amine group at the C-3 position of ursolic acid showed to be a potential alternative to discovery of new leukemia treatment being a selective compound in decreased cell viability K562, when compared to VERO cells, lymphocytes and HEPG2. Furthermore this compound induces cell death through apoptosis and late apoptosis, besides having anti-proliferative activity acting in the G0/G1 phases of the cell cycle. Even more this derivative showed a synergistic effect when combined with imatinib.

Acknowledgments

This work was supported by grants and financial support from Programa de Pós-graduação em Ciências Farmacêuticas UFRGS, Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPQ-Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil). The Laboratório Regional de Nanotecnologia (LRNANO) of Universidade Federal do Rio Grande do Sul UFRGS for the NMR analysis and Central analítica de instrumentação da Universidade de São Paulo USP for the HRMS analysis.

References.

1. Mendizabal AM, Younes N, Levine PH. Geographic and income variations in age at diagnosis and incidence of chronic myeloid leukemia. 2016:70–78. doi:10.1007/s12185-015-1893-y.

2. Results E. Chronic Myelogenous Leukemia. 2009;7(9):984–1023.

3. Howell DA, Warburton F, Ramirez A, Roman E, Smith AG, Forbes LJL. Risk factors and time to symptomatic presentation in leukaemia , lymphoma and myeloma. 2015:1114–1120. doi:10.1038/bjc.2015.311.

4. Mohammadi M, Cao Y, Glimelius I, Bottai M, Eloranta S, Smedby KE. The impact of comorbid disease history on all-cause and cancer-specific mortality in myeloid leukemia and myeloma – a Swedish population-based study. 2015:1–12. doi:10.1186/s12885-015-1857-x.

5. Leukemia CM, York N, Food US. Blood Forum The price of drugs for chronic myeloid leukemia (CML) is a re fl ection of the unsustainable prices of cancer drugs : from the perspective of a large group of CML experts. 2016;121(22):4439–4443. doi:10.1182/blood-2013-03-490003.Experts.

6. Lovera S, Morando M, Pucheta-martinez E, Martinez- JL, Saladino G, Gervasio FL. Towards a Molecular Understanding of the Link between Imatinib Resistance and Kinase Conformational Dynamics. 2015. doi:10.1371/journal.pcbi.1004578.

7. Sundar H, Radich J. Optimizing Patient Care In Chronic Phase Chronic Myelogenous Leukemia: A Multidisciplinary Approach. 2016;14(January).

8. Crawford S. Is it time for a new paradigm for systemic cancer treatment ? Lessons from a century of cancer chemotherapy. 2013;4(June):1–18. doi:10.3389/fphar.2013.00068.

9. Mondal S, Bandyopadhyay S, Ghosh MK, Mukhopadhyay S, Roy S. Natural Products : Promising Resources for Cancer Drug Discovery. 2012:49–75.

10. Demain AL, Vaishnav P. Minireview. 2011;4:687–699. doi:10.1111/j.1751-7915.2010.00221.x.

11. Activity A. Synthesis, Anti-Tumor and Anti-Angiogenic Activity Evaluations of Asiatic Acid Amino Acid Derivatives. 2015:7309–7324. doi:10.3390/molecules20047309.

12. Schuck C, Cruz LN, Moraes MS, Nakabashi M, Silva GNS. Investigation of antimalarial activity , cytotoxicity and action mechanism of piperazine derivatives of betulinic acid. 2015;20(1):29–39. doi:10.1111/tmi.12395.

13. Broniatowski M, Katarzyna H. Biochimica et Biophysica Acta Antagonistic effects of α -tocopherol and ursolic acid on model bacterial membranes. 2015;1848:2154–2162.

14. Navid MH, Laszczyk-lauer MN, Reichling J, Schnitzler P. Phytomedicine

Pentacyclic triterpenes in birch bark extract inhibit early step of herpes simplex virus type 1 replication. 2014;21:1273–1280.

15. Wiemann J, Heller L, Perl V, Kluge R, Str D. European Journal of Medicinal Chemistry Betulinic acid derived hydroxamates and betulin derived carbamates are interesting scaffolds for the synthesis of novel cytotoxic compounds. 2015;106:194–210.

16. Gao X, Deeb ÆD, Jiang ÆH, Liu Y, Dulchavsky ÆSA, Gautam SC. Synthetic triterpenoids inhibit growth and induce apoptosis in human glioblastoma and neuroblastoma cells through inhibition of prosurvival Akt , NF- j B and Notch1 signaling. 2007:147–157. doi:10.1007/s11060-007-9364-9.

17. Saha S, Ghosh M, Dutta SK. A potent tumoricidal co-drug “Bet-CA” - an ester derivative of betulinic acid and dichloroacetate selectively and synergistically kills cancer cells. 2015:1–10. doi:10.1038/srep07762.

18. Bache M, Bernhardt S, Passin S, Wichmann H. Betulinic Acid Derivatives NVX-207 and B10 for Treatment of Glioblastoma — An in Vitro Study of Cytotoxicity and Radiosensitization. 2014;(October):19777–19790. doi:10.3390/ijms151119777.

19. Meng Y, Lin Z, Ge N, Zhang D, Huang J, Kong F. Ursolic Acid Induces Apoptosis of Prostate Cancer Cells via the PI3K / Akt / mTOR Pathway. 2015;43(7):1471–1486. doi:10.1142/S0192415X15500834.

20. Kim S, Ryu HG, Lee J, et al. Ursolic acid exerts anti-cancer activity by suppressing vaccinia- related kinase 1-mediated damage repair in lung cancer cells. 2015;(May):1–12. doi:10.1038/srep14570.

21. Li G, Zhou T, Liu L, et al. Ezrin dephosphorylation / downregulation contributes to ursolic acid-mediated cell death in human leukemia cells. 2013;(February). doi:10.1038/bcj.2013.7.

22. Ali-seyed M, Jantan I. Betulinic Acid : Recent Advances in Chemical Modifications , Effective Delivery , and Molecular Mechanisms of a Promising Anticancer Therapy. 2016:517–536. doi:10.1111/cbdd.12682.

23. Fulda S, Kroemer G. Targeting mitochondrial apoptosis by betulinic acid in human cancers. 2009;14(September). doi:10.1016/j.drudis.2009.05.015.

24. Weng H, Tan Z, Hu Y, et al. Ursolic acid induces cell cycle arrest and apoptosis of gallbladder carcinoma cells. 2014:1–10.

25. Fulda S. Review Betulinic acid : A natural product with anticancer activity. 2009:140–146. doi:10.1002/mnfr.200700491.

26. Potze L, Mullauer FB, Colak S, Kessler JH, Medema JP. Betulinic acid-induced mitochondria-dependent cell death is counterbalanced by an autophagic salvage response. 2014. doi:10.1038/cddis.2014.139.

27. Cui H, He Y, Wang J, et al. European Journal of Medicinal Chemistry Synthesis of heterocycle-modi fi ed betulinic acid derivatives as antitumor agents.

2015;95:240–248.

28. Marquardt V, Heider E, Vigante B, et al. as TGF b / Smad inhibitors. 2015;95:249–266.

29. Nakabashi M, Sonnet P, Gosmann G. Synthesis and Antiplasmodial Activity of Betulinic Acid and Ursolic Acid Analogues. 2012:12003–12014. doi:10.3390/molecules171012003.

30. Dalla-vechia L, Dassonville-klimpt A, Grellier P, Sonnet P, Gosmann G, Gnoatto SCB. The Beckmann Rearrangement Applied to Ursolic Acid with Antimalarial Activity in Medicinal Chemistry Studies. 2012:92–95.

31. Sawada N, Kataoka K, Kondo K, et al. Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice. 2004;(February):1672–1678. doi:10.1038/sj.bjc.6601746.

32. Gathmann I, Sc M, Kantarjian H, et al. Five-Year Follow-up of Patients Receiving Imatinib for Chronic Myeloid Leukemia. 2006.

33. Manuscript A, Options T. NIH Public Access. 2014;13(5):515–529. doi:10.1016/j.clml.2013.03.018.Resistance.

34. Wieczorek A, Uharek L. Management of Chronic Myeloid Leukemia Patients Resistant to Tyrosine Kinase Inhibitors Treatment. 2015;10:49–54. doi:10.4137/BMI.S22431.TYPE.

35. Manuscript A, Downregulation CB. NIH Public Access. 2015;20(1):151–163. doi:10.1158/1078-0432.CCR-13-1063.Gambogic.

36. Melo J V, Chuah C. Resistance to imatinib mesylate in chronic myeloid leukaemia. 2007;249:121–132. doi:10.1016/j.canlet.2006.07.010.

37. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding Imatinib Resistance with a Novel ABL Kinase Inhibitor. 2004;305(July):399–402.

38. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107 , a selective inhibitor of native and mutant Bcr-Abl. 2005;7(February):129–141. doi:10.1016/j.ccr.2005.01.007.

39. Dunn EF, Iida M, Myers RA, et al. HHS Public Access. 2011;30(5):561–574. doi:10.1038/onc.2010.430.Dasatinib.

40. Article CME. CME ARTICLE CME Information : Chronic Myeloid Leukemia : 2016 update. 2016;91(2). doi:10.1002/ajh.24275.

41. Ebnöether M, Stentoft J, Ford J, Buhl L, Gratwohl A. Cerebral oedema as a possible complication of treatment with imatinib For personal use . Only reproduce with permission from The Lancet Publishing Group . 2002;359:1751–1752.

42. Redmond KL, Lawler M, Schaeybroeck S Van. Overcoming Resistance to Targeted Therapies in Cancer. 2015;42(6):896–908.

43. Ahmed W, Etten RA Van, Medical T, Family C, Cancer C. HHS Public Access. 2015. doi:10.1182/asheducation-2013.1.189.Alternative.

44. Kantarjian HM, Hochhaus A, Saglio G, et al. Nilotinib versus imatinib for the treatment of patients with newly diagnosed chronic phase , Philadelphia chromosome-positive , chronic myeloid leukaemia : 24-month minimum follow-up of the phase 3 randomised ENESTnd trial. 2011;12(September):841–851. doi:10.1016/S1470-2045(11)70201-7.

45. Uto T, Sakamoto A, Tung NH, Fujiki T, Kishihara K. Anti-Proliferative Activities and Apoptosis Induction by Triterpenes Derived from Eriobotrya japonica in Human Leukemia Cell Lines. 2013:4106–4120. doi:10.3390/ijms14024106.

46. Gao N, Cheng S, Budhraja A, Gao Z, Chen J. Ursolic acid induces apoptosis in human leukaemia cells and exhibits anti-leukaemic activity in nude mice through the PKB pathway. 2012. doi:10.1111/j.1476-5381.2011.01684.x.

47. Periasamy G, Teketelew G, Gebrelibanos M, et al. Betulinic acid and its derivatives as anti-cancer agent : A review. 2014;6(3):47–58.

48. Dutta D, Chakraborty B, Sarkar A, Chowdhury C, Das P. A potent betulinic acid analogue ascertains an antagonistic mechanism between autophagy and proteasomal degradation pathway in HT-29 cells. 2016:1–19. doi:10.1186/s12885-016-2055-1.

49. Dar BA, Lone AM, Shah WA, Qurishi MA. European Journal of Medicinal Chemistry Synthesis and screening of ursolic acid-benzylidine derivatives as potential anti-cancer agents. 2016;111:26–32.

50. Shao J, Dai Y, Xue J, Wang J, Lin F, Guo Y. European Journal of Medicinal Chemistry In vitro and in vivo anticancer activity evaluation of ursolic acid derivatives. 2011;46:2652–2661. doi:10.1016/j.ejmech.2011.03.050.

51. Šaman D, Havlíc L, Bildziukevich U, et al. Polyamine derivatives of betulinic acid and b -sitosterol : A comparative investigation. 2015;100:27–35.

52. Li Y, Wang J, Pan C, Meng F, Chu X, Ding Y. Bioorganic & Medicinal Chemistry Letters Syntheses and biological evaluation of 1 , 2 , 3-triazole and. 2016;26:1419–1427.

53. Sang F, Ding Y, Wang J, et al. Structure − Activity Relationship Study of Rakicidins: Overcoming Chronic Myeloid Leukemia Resistance to Imatinib with 4 - Methylester-Rakicidin A. 2016. doi:10.1021/acs.jmedchem.5b01841.

54. Shukla S, Kouanda A, Silverton L, Talele TT, Ambudkar S V. Pharmacophore Modeling of Nilotinib as an Inhibitor of ATP-Binding Cassette Drug Transporters and BCR-ABL Kinase Using a Three- Dimensional Quantitative Structure − Activity Relationship Approach. 2014.

55. Kimura S, Ando T, Kojima K. Ever-advancing chronic myeloid leukemia treatment. 2014:3–9. doi:10.1007/s10147-013-0641-7.

56. Wu B, Wang X, Chi Z, et al. Ursolic Acid-induced Apoptosis in K562 Cells

Involving Upregulation of PTEN Gene Expression and Inactivation of the PI3K / Akt Pathway. 2012;35(3):543–548. doi:10.1007/s12272-012-0318-1.

57. Wang Y, Liu J, Dou Y. Sequential treatment with betulinic acid followed by 5-fluorouracil shows synergistic cytotoxic activity in ovarian cancer cells. 2015;8(1):252–259.

58. Wang J, Jiang Z, Xiang L, et al. No Title. 2014:1–12. doi:10.1038/srep05006.

59. Bar-zeev M, Assaraf YG, Livney YD. β-casein nanovehicles for oral delivery of chemotherapeutic Drug combinations overcoming P-glycoprotein-mediated multidrug resistance in human gastric cancer cells. 7(17).

60. Ganten TM, Woerns MA, Siveke J, et al. Dose Escalation of the HDAC Inhibitor Resminostat in Combination Treatment with Sorafenib in Patients with Hepatocellular Carcinoma (HCC) - The Phase I / II SHELTER study. 2012:111.

61. Yamaguchi SI, Ueki A, Sugihara E, et al. Synergistic antiproliferative effect of imatinib and adriamycin in platelet-derived growth factor. 2015;106(7). doi:10.1111/cas.12686.

62. Burchert A, Mu MC. J OURNAL OF C LINICAL O NCOLOGY Sustained Molecular Response With Interferon Alfa Maintenance After Induction Therapy With Imatinib Plus Interferon Alfa in Patients With Chronic Myeloid Leukemia. 2016;28(8). doi:10.1200/JCO.2009.25.5075.

63. Jahnke W, Cowan-jacob SW, Li AG, et al. Targeting Bcr – Abl by combining allosteric with ATP-binding-site inhibitors. 2010;463(January). doi:10.1038/nature08675.

64. Hantschel O. Ditorials & erspectives. 2012;97(2):157–159. doi:10.3324/haematol.2012.061812.

65. Bernard R, Getachew R, Kamato D, Thach L, Osman N. Evaluation of the potential synergism of imatinib-related poly kinase inhibitors using growth factor stimulated proteoglycan synthesis as a model response. 2016;68:368–378. doi:10.1111/jphp.12530.

66. Kantarjian H, Talpaz M, Brien SO, et al. Plenary paper High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome – positive chronic phase chronic myeloid leukemia. 2016;103(8):2873–2879. doi:10.1182/blood-2003-11-3800.Supported.

67. Zonder JA, Pemberton P, Brandt H, Mohamed AN, Schiffer CA. The Effect of Dose Increase of Imatinib Mesylate in Patients with Chronic or Accelerated Phase Chronic Myelogenous Leukemia with Inadequate Hematologic or Cytogenetic Response to Initial Treatment. 2003;9(June):2092–2097.

68. Medema JP. Betulinic acid induces cytochrome c release and apoptosis in a Bax / Bak-independent , permeability transition pore dependent fashion. 2009:191–202. doi:10.1007/s10495-008-0290-x.

ARTIGO 2

Synthesis and anti-RSV activity of new nitro-1,2,3-triazole-28-triterpene derivatives

Elenilson Figueiredo da Silva[a], Krist Helen Antunes Fernandes[b], Artur Stramari de Vargas[a], Denise Diedrich[a], Grace Gosmann[a], Ana Paula Duarte de Souza[b], Simone C.B. Gnoatto[a].

[a] Laboratório de Fitoquímica e Síntese Orgânica, Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, RS 90610-000, Brazil.

[b] Laboratório de Imunologia Clinica e Experimental, Centro Infant, Instituto de pesquisas biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) ,Porto Alegre Brasil.

Corresponding author: Simone C. B. Gnoatto, Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Departamento de Produção de Matéria Prima, Avenida Ipiranga, 2752 sala 708 Santana, Porto Alegre, RS Brasil - CEP 90610000, e-mail simone.gnoatto@ufrgs.br.

Abstract.

Respiratory syncytial virus (RSV) is an important etiological agent of the respiratory tract, affecting mainly children and elderly people. New nitro-triazoles derived from ursolic and betulinic acid have been synthesized and tested against RSV. The derivative **3d** has shown to be the most active and selective of the series, with an EC_{50} of 0.0053 nM and an IT of 11160.37. The intermediate reaction derivatives were also

evaluated for their antiviral activity, being it possible to establish a structure-activity relation for these compounds. The results have shown that an insertion of an ortho-nitro-triazol at the C-28 position of ursolic acid increases the antiviral effect on RSV.

Keywords. Anti-SRV, click chemistry, triterpene, ursolic acid, betulinic acid**.**

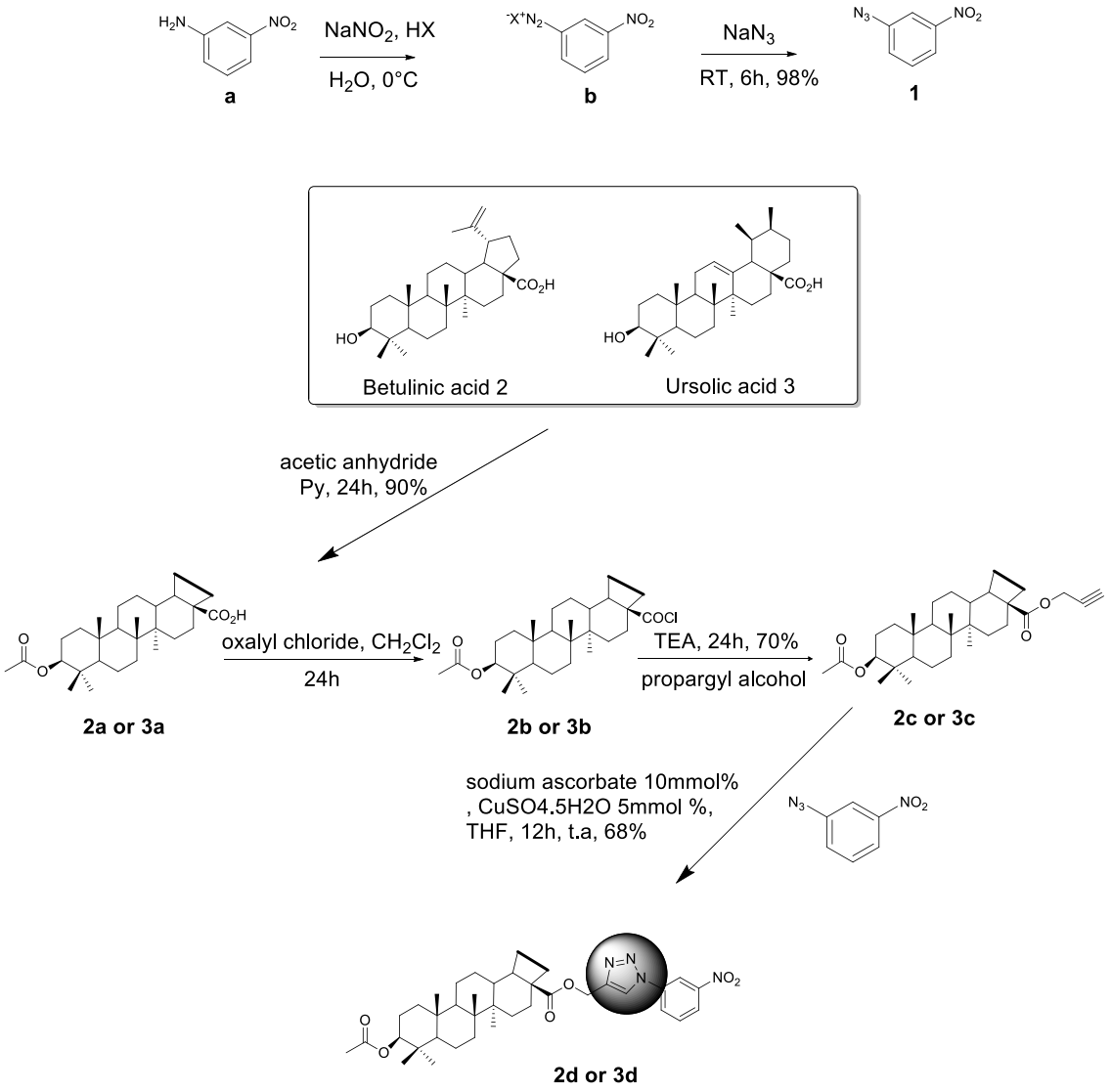
Respiratory syncytial virus (RSV) is a enveloped RNA-type virus first isolated in 1955, and since then characterized as an important disease of the respiratory tract, affecting mostly children and the elderly[1,2]. After over 50 years of its discovery, no vaccine or effective drugs were developed, reinforcing the urgency for new alternatives of treatment[3]. Triterpenes are natural products with various reported biological activities, as for example, antitumor, antibacterial, antifungal and antimalarial[4-8]. Betulinic acid **2** and ursolic acid **3** are widely studied pentacyclic triterpenes, not only for their therapeutic activities but also for their low toxicity[9,10]. Another factor that drives the medicinal chemistry studies of these compounds is their occurrence, since they are found in many species of plants around the world[11, 12]. Numerous studies have reported the potential antiviral activity of modified triterpenes[13] and in the last decade there was an increase in the studies about the structure-activity relationship of these compounds[14]. This is partially due to the discovery of bevirimat, a potential candidate for AIDS treatment, which is by now in clinical phase II studies[15]. Bevirimat is a betulinic acid derivative with an esterification at C-3 position that resulted in a more selective and more potent derivative against HIV[16]. Starting with the studies of bevirimat's anti-HIV mechanisms, several other studies have been published with derivatives having structural modifications in the C-3 and C-28 of betulinic and ursolic acid to determine the biological effect of these modifications to the anti-HIV activity[17 18].

The click-chemistry reaction has been widely used in recent years in the synthesis of many compounds possessing biological activity[19]. In addition to the numerous biological activities obtained in the click chemistry synthesis, it is noteworthy that this type of reaction is very accessible, with high yields and low cost, which makes it a great alternative in medicinal chemistry studies[20,21]. Taking in account the potential activity of pentacyclic triterpenes and easy handling of the click chemistry reactions, in this study we focused on the synthesis of new nitro-1,2,3-triazol-28-triterpene derivatives of betulinic acid and ursolic acid in order to explore the antiviral activity of these derivatives against respiratory syncytial virus (RSV) as well as to establish preliminary medicinal chemistry studies based on the structure activity relationship of these derivatives.

The click chemistry reaction type is also known as the reaction of 1,3-dipolar cycloaddition between a terminal alkyne and an organic azide catalyzed by copper (Cu) with regiospecific formation of 1,2,3-triazol-1,4-dissubstituted (Azide-Alkyne Cycloadition - AAC)[22]. The use of monovalent Cu(I) and divalentCu (II) copper as catalysts on the optimization of click chemistry reactions has been widely studied in the last 10 years[23-25]. One alternative that has various click chemistry methodologies is the use of $Cu(SO_4)_2$ in the presence of a weak reductant such as sodium ascorbate. This methodology provides the advantage of low cost and readily available reagents, as well as a source of stable Cu(II) salts, thus contributing to the reactional steps[26, 27]. For the AAC reaction it was first necessary to synthesize ortho-nitro-azides (**1**), starting from ortho-nitroaniline (**Scheme 1**). The reaction was conducted in two steps; in the first step intermediate **b** was obtained, using sodium nitrite in acidic medium, while the second step gave the ortho-nitro-azide **1** derivative in 98% yield, **Scheme 1**.

Ursolic acid and betulinic acid were extracted and purified as previously described [28]. Then the triterpenes were subjected to an acetylation reaction obtaining the acetylated derivatives **2a** and **3a** with 98% and 89% yields, respectively. These derivatives were then subjected to acyl chloride formation, to obtain the intermediate **2b** and **3b** which formation was monitored by IR spectroscopy. These acyl chloride derivatives were used as substrates for obtaining alkyne esters of ursolic and betulinic acid via an esterification reaction using propargyl alcohol . The products esterified at position C-28 were purified by silica gel column chromatography and obtained as a white crystalline powder with high yields, 98% and 96% respectively (**Scheme 1**). After obtaining the azide (**1**) and triterpenoid derived alkynes **2c** and **3c**, the triazole derivatives **2d** and 3d were obtainedas previously described [29]. Some conditions were tested by varying the concentration of copper (II) salt and sodium ascorbate to assess the best reactional yield (**Table 1**).

All the synthesized compounds and starting materials, betulinic acid and ursolic acid, were identified by ^{1}H and ^{13}C NMR and mass spectrometer data**.** The synthetic methods and chemical identification are available in the supplementary material.



Scheme 1. Synthesis of the triazol triterpene derivatives **2d** and **3d**.

Table 1. Variation of the amount of copper (II) salt and sodium ascorbate in order to obtain derivatives **2d** and **3d using as solvent H_2O/THF 1:1 and 12 h reaction.**

Entry	$CuSO_4.5H_2O$ (mmol%)	Sodium ascorbate	% Yield	
			2d	**3d**
1	5	10mmol%	68%	59%
2	2.5	10mmol%	39%	32%
3	0.5	10mmol%	30%	26%
4	2.0	5 mmol%	18%	12%
5	1	2 mmol%	28%	23%

The best condition found for the synthesis of triazol derivatives was using 5 mmol% of $CuSO_4.2H_2O$ and 10 mmol% of sodium ascorbate (Entry 1, Table 1). According to Table 1**,** it was possible to observe that variations in the concentration of both, copper salt (II) (Entry 1 x 2 x 3) and sodium ascorbate (Entry 4 and 5 x 1, 2 and 3) used, have influence on the yield of the reaction (Entry 1 x 2 x 3, for example). Another important data from Table 1 show that the reaction yields are higher for betulinic acid when compared to its isomer, ursolic acid.

In order to start medicinal chemistry studies, all compounds were evaluated *in vitro* for their antiviral activity against RSV by cell viability assay based on MTT and SRB method, for 96 hours. We also assessed the cytotoxic effect of these compounds in A549 cells uninfected by RSV, using the same methodology and treatment time used in the anti-RSV assay, in order to establish a selectivity parameter of the derivatives against the virus. Data from MTT and SRB experiments were analyzed using the software Graphpad Prism 6 and the values expressed in EC_{50} (µM) for the antiviral activity and IC_{50} (uM) for cytotoxic activity. The therapeutic index (TI) was calculated

according with previously described methodology[30], and expresses a ratio of the IC_{50} / EC_{50}, **Table 2.**

The experimental procedure antiviral and cytotoxic activity can be found in the supplementary material.

Table 2. Antiviral activity *in vitro* of the compounds 2-3abc against RSV.

Compound	IC_{50} (µM) A549	EC_{50} (µM) A549+RSV	TI
2	17.77	5.3	3.352
3	26.7	13.7	1.94
2a	53	44.4	1.20
2c	88.8	0.58	153.1
2d	42.7	0.314	135.98
3a	133	14.29	9.3
3c	67.2	36.2	1.85
3d	59.15	0.0053	11160.37

TI= IC_{50}/EC_{50}

All compounds tested were active against the RSV in micromolar concentration. The results show that betulinic acid (**2**), proved to be more active and selective for the RSV when compared to the ursolic acid (**3**), with EC_{50} = 5.3 µM and 13.7 µM, respectively. Similar results were described to betulinic acid against RSV[31], however, studies describing the anti-RSV activity of triterpenes are still scarce. The derivatives **2a** and **3a**, acetylated in the position C-3, showed EC_{50} of 44.4 and 14.29 µM, respectively.

These results show that protection of the hydroxyl group at position C-3 in both triterpenes may not be important for anti-SRV activity. Moreover, derivatives **2a** and **3a** have shown to be more cytotoxic when compared to betulinic and ursolic acid (IC_{50} = 53 and 133 µM). The insertion of an alkyne ester on the C-28 position favored anti-RSV activity when added to the betulinic acid, 2c with an EC_{50}-of 0.58 µM. However, when the same group was inserted to the ursolic acid , **3c**, it resulted in a decrease of anti-RSV activity with a EC_{50} of 36.2 µM and a low therapeutic index of 1.85. The presence of a nitro-triazol ring in the C-28 position of both **2d** and **3d** triterpenes increased the antiviral activity, resulting an EC_{50} of 0.314 and 0.00053 µM, respectively. These data show that the insertion of a nitro-triazole ring is important for anti-RSV activity. Furthermore, the presence of a nitro-triazol ring is more efficient when added to the backbone of ursolic acid being capable of increasing its therapeutic index to 11160.37. Finally, we can establish a preliminary structure activity relationship of triterpene ursolic acid derivatives against RSV, shown in **figure 1.**

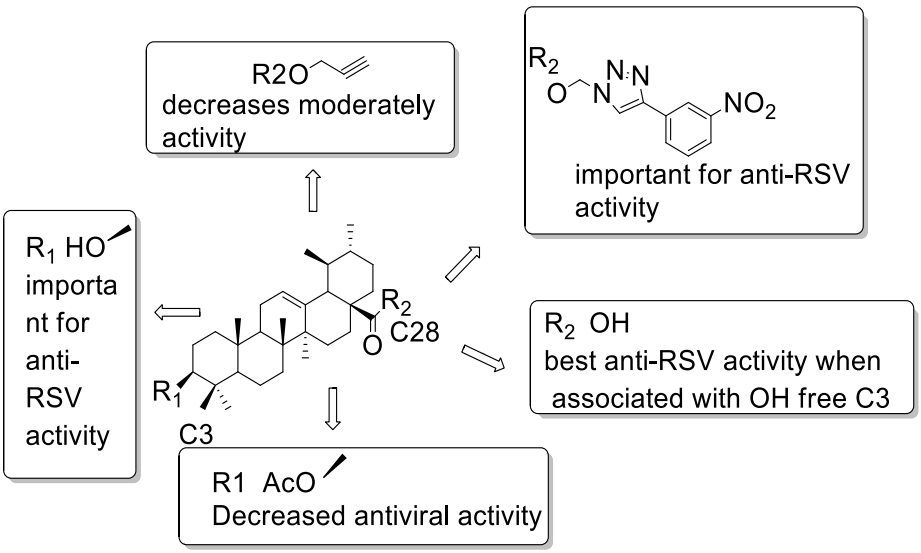


Figure 1. Relationship between structure activity for ursolic acid derivatives

In this study we have synthesized new nitro-triazol derivatives of ursolic and betulinic acids in good yields and by an easily applicable method, the click chemistry reaction. At the end of this work it was possible to establish studies of structure-activity relationship and optimize better conditions for the organic synthesis by click chemistry applied to pentacyclic triterpenoids. Our results demonstrated that the insertion of substituted triazoles at triterpenoid structure proved to be a potential alternative against the RSV, considering that the triazol derivative has shown to be active in low concentrations. However, our study provides only preliminary results, further testing will be conducted in order to clarify the mechanism of action of these derivatives against RSV.

Acknowledgements

This work was supported by grants and financial support from Programa de Pós-graduação em Ciências Farmacêuticas UFRGS, Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPQ-Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil). The Laboratório Regional de Nanotecnologia (LRNANO) da Universidade Federal do Rio Grande do Sul UFRGS for the NMR analysis and Central analítica de instrumentação da Universidade de São Paulo USP for the HRMS analysis.

References

1. Malley, A.; Shiigi, S.; Benjamini, E.; Werner, L.; Leung, C.; Kwang, H.; Axthelm, M.; Hallick, L. *Adv Exp Med Biol* **1989,** *251*, 169.
2. Bui The, H.; Nguyen Phuoc, L.; Le Phi, H.; Nguyen Thien, L.; Nguyen Hoang, A.; Tran Diem, N.; Mai Van, H.; Nguyen Thi Huyen, T.; Rafidinarivo, H. F.; Nguyen Ky, A.; Hawkes, D.; Nguyen Tien, H.; Hirayama, K. *Plos One* **2015,** *10*.
3. Tracy J Ruckwardt
4. Kong, L.; Li, S.; Liao, Q.; Zhang, Y.; Sun, R.; Zhu, X.; Zhang, Q.; Wang, J.; Wu, X.; Fang, X.; Zhu, Y. *Antiviral Research* **2013,** *98*, 44.
5. Wang, X.; Zhang, F.; Yang, L.; Mei, Y.; Long, H.; Zhang, X.; Zhang, J.; Qimuge, S.; Su, X. *Journal of Biomedicine and Biotechnology* **2011**, 1.
6. Cunha, L. C. S.; Andrade e Silva, M. L.; Furtado, N. A. J. C.; Vinholis, A. H. C.; Martins, C. H. G.; da Silva Filho, A. A.; Cunha, W. R. *Zeitschrift Fur Naturforschung Section C-a Journal of Biosciences* **2007,** *62*, 668.
7. da Silva, G. N. S.; Maria, N. R. G.; Schuck, D. C.; Cruz, L. N.; de Moraes, M. S.; Nakabashi, M.; Graebin, C.; Gosmann, G.; Garcia, C. R. S.; Gnoatto, S. C. B. *Malaria Journal* **2013,** *12*.
8. Innocente, A.; Casanova, B. B.; Klein, F.; Lana, A. D.; Pereira, D.; Muniz, M. N.; Sonnet, P.; Gosmann, G.; Fuentefria, A. M.; Gnoatto, S. C. B. *Chemical Biology & Drug Design* **2014,** *83*, 344.
9. Kashyap, D.; Tuli, H. S.; Sharma, A. K. *Life Sciences* **2016,** *146*, 201.
10. 고은이; 문애리. *Journal of Cancer Prevention* **2015,** *20*, 223.
11. Gossan, D.; Alabdul Magid, A.; Yao-Kouassi, P.; Josse, J.; Gangloff, S.; Morjani, H.; Voutquenne-Nazabadioko, L. *Fitoterapia* **2016,** *110*, 89.
12. Zhang, T.; Gao, S.; Hou, J.; Zhou, Y.; Zhou, J.; XG, W.; N, Q.; JC, C.; HQ, D.; JB., F. *Zhongguo Zhong Yao Za Zhi* **2015,** *12*, 119.
13. Han, W.; Renyang, X.; Yongying, S.; Longlong, S.; Pingxuan, J.; Zibo, F.; HanXingyu, W.; Xiaoshu, Z.; Fei, Y.; Yongmin, Z.; Liangren, Z.; Lihe, Z.; Demin, Z.; Sulong, X. *European Journal of Medicinal Chemistry* **2016,** *110*, 376.
14. Dan, W.; Wuxun, L.; Feng, L. *Acta Pharm Sin B* **2015,** *6*, 493.
15. Lee, K.-H. *Journal of Natural Products* **2010,** *73*, 500.
16. Coric, P.; Turcaud, S.; Souquet, F.; Briant, L.; Gay, B.; Royer, J.; Chazal, N.; Bouaziz, S. *European Journal of Medicinal Chemistry* **2013,** *62*, 453.
17. Gnoatto, S. C. B.; Dassonville-Klimpt, A.; Da Nascimento, S.; Galera, P.; Boumediene, K.; Gosmann, G.; Sonnet, P.; Moslemi, S. *European Journal of Medicinal Chemistry* **2008,** *43*, 1865.
18. Ma, C. M.; Cai, S. Q.; Cui, J. R.; Wang, R. Q.; Tu, P. F.; Hattori, M.; Daneshtalab, M. *European Journal of Medicinal Chemistry* **2005,** *40*, 582.
19. Agalave, S. G.; Maujan, S. R.; Pore, V. S. *Chemistry-an Asian Journal* **2011,** *6*, 2696.
20. Pasini, D. *Molecules* **2013,** *18*, 9512.
21. Yi, S.; Xiaosong, C.; Haifeng, G. *Nanoscale* **2016,** *8*, 4864.
22. Hein, J. E.; Fokin, V. V. *Chemical Society Reviews* **2010,** *39*, 1302.
23. Ganesh, V.; Sudhir, V. S.; Kundu, T.; Chandrasekaran, S. *Chemistry-an Asian Journal* **2011,** *6*, 2670.
24. Arseneault, M.; Wafer, C.; Morin, J.-F. *Molecules* **2015,** *20*, 9263.
25. Hein, C. D.; Liu, X.-M.; Wang, D. *Pharmaceutical Research* **2008,** *25*, 2216.
26. Tron, G. C.; Pirali, T.; Billington, R. A.; Canonico, P. L.; Sorba, G.; Genazzani, A. A. *Medicinal Research Reviews* **2008,** *28*, 278.
27. Cernat, A.; Tertis, M.; Cristea, C.; Sandulescu, R. *International Journal of Electrochemical Science* **2015,** *10*, 6324.
28. Innocente, A. M.; Silva, G. N. S.; Cruz, L. N.; Moraes, M. S.; Nakabashi, M.; Sonnet, P.; Gosmann, G.; Garcia, C. R. S.; Gnoatto, S. C. B. *Molecules* **2012,** *17*, 12003.

29. Yamada, Y. M. A.; Sarkar, S. M.; Uozumi, Y. *Journal of the American Chemical Society* **2012,** *134*, 9285.
30. Kashiwada, Y.; Sekiya, M.; Ikeshiro, Y.; Fujioka, T.; Kilgore, N. R.; Wild, C. T.; Allaway, G. P.; Lee, K. H. *Bioorganic & Medicinal Chemistry Letters* **2004,** *14*, 5851.
31. Li, Y.; Jiang, R.; Ooi, L. S. M.; But, P. P. H.; Ooi, V. E. C. *Phytotherapy Research* **2007,** *21*, 466.

Supplementary data

Synthesis and anti-RSV activity of new nitro-1, 2, 3-triazol-28-triterpene derivatives

Elenilson Figueiredo da Silva[a], Krist Helen Antunes Fernandes, Artur Stramari de Vargas[a], Denise Diedrich[a], Grace Gosmann[a], Ana Paula Duarte de Souza[b], Simone C.B. Gnoatto[a].

[a] Laboratório de Fitoquímica e Síntese Orgânica, Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, RS 90610-000, Brazil.

[b] Laboratório de Imunologia Clinica e Experimental, Centro Infant, Instituto de pesquisas biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) ,Porto Alegre Brasil.

Corresponding author: Simone C. B. Gnoatto, Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Departamento de Produção de Matéria Prima, Avenida Ipiranga, 2752 sala 708 Santana, Porto Alegre, RS Brasil - CEP 90610000, e-mail simone.gnoatto@ufrgs.br. Tel(55)5133085516, fax(55)5133085313.

Synthesis and characterization of the compounds 1-3a-d.

All of the solvents were dried and distilled prior to use. Column chromatography (CC) was carried out on silica gel (Merck, Sao Paulo, Brazil, 60 –230 mesh) using gradient eluent mixtures (cyclohexane/CH_2Cl_2). ^1H and ^{13}C NMR spectra were recorded with a Varian Inova 500 NMR spectrometer. Chemical shifts are shown in parts per million (δ) with tetramethylsilane (TMS) as a reference.

Mass spectra were recorded using with an UltrOTOF (Bruker Daltonics, Sao Paulo, Brazil) mass spectrometer. Melting points were determined using a Koffler instrument.

***3-nitro azide* (1).**

This compound is obtained by *3*-nitroaniline as a yellow crystalline powder in 98% yield. The reaction was conducted by previously described methodology.[1-2.]

Betulinic acid (2) and ursolic acid (3).

Betulinic acid (**2**) was obtained from the bark of *Platanus acerifolia* L. (Maple), and its corresponding isomer ursolic acid (**3**) was obtained from *Malus domestica* peel (apple), as previously described [3].

***3-O-acetylbetulinic acid* (2a) and *3-O-acetylursolic acid* (3a).**

Compound **2a** was prepared from betulinic acid **2** with 100% yield. The **3a** compound was prepared from ursolic acid **3** with 98% yields as previously described[4-5].

3-O-acetyl-28-propargyl betulinic ester (**2c**)**.** To 3-*O*-acetylbetulinic acid **2a**, (0.25 g, 0.504 mmol) previously dissolved in 5 mL of dry dichloromethane, under N_2, oxalyl chloride was added (0.19 g, 1.5 mmol) under constant agitation for 24 h to complete the acyl chloride formation. After this, triethylamine (0.1515 g, 1.5 mmol) and propargyl alcohol (0.08409 g, 1.5 mmol) were added and kept under stirring for a further 24h.

Then, dichloromethane was removed under reduced pressure. Water (10 mL) was added to the crude product and extracted with dichloromethane (3 x 50 mL). The product was purified by column chromatography (cyclohexane/ dichloromethane) yielding 70% of a white crystalline powder **2c**, .

^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) d 0.68 (1H, m, H- 5), 0.74 (3H, s, H-25), 0.81 (3H, s, H-24), 0.89 and 1.62 (1H each, m, H-1), 0.90 (3H, s, H-26), 0.96 (6H, s, H-27 and H-23), 1.12 and 2.43 (1H each, m, H-11), 1.21 and 1.99 (1H each, m, H-12), 1.25 and 1.90 (1H each, m, H-15), 1.36 and 1.54 (1H each, m, H-6), 1.37 and 2.18 (1H each, m, H-21), 1.38 (1H, m, H-9), 1.39 and 1.43 (1H each, m, H- 7), 2.20 (3H, s, $\underline{H_3C}COO$), 1.44 and 2.22 (1H each, m, H-22), 1.48 and 2.22 (1H each, m, H- 16), 1.62 (1H, m, H-18), 1.68 (3H, s, H-30), 1.78 (2H, m, H-2), 2.42 (1H, m, H-13), 2.43 (1H, s, -$OCH_2C\underline{CH}$), 2.48 (1H, m, H-19), 4.45 (1H, dd, H-3), 4.60 and 4.63 (1H each, s, H-29), 4.72 (2H, m, -$O\underline{CH_2}$-). ^{13}C NMR (100 MHz, $CDCl_3$): 14.55, 15.99, 16.18, 16.46, 18.16, 19.33, 20.85, 21.31, 23.68, 25.47, 27.92, 29.56, 30.48, 31.92, 34.24, 36.77, 37.09, 37.77, 38.29, 38.39, 40.80, 42.37, 46.85, 49.47, 50.46, 51.31, 55.41, 56.37, 74.31, 78.12, 80.91, 109.68, 150.37, 170.98, 175.15. HRMS m/z calcd. for [M+H] $C_{35}H_{52}O_4$ 537.3899 found 537.3934.

***3-O-acetyl- 28-propargyl ursolic ester* (3c).**

This compound was prepared from 3-O-acetylursolic acid as described for **2c**. It was obtained a white crystalline powder **3c**, with 70% yield.

^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) 0.83 (1H, s, H-25), 0.86 (1H, H-5), 0.92 (3H, s, H-24), 0.93 (3H, s, H-23), 0.94 (3H, d, H-30), 0.95 (1H, H-1), 1.05 (3H, d, H-29), 1.09

(3H, s, H-26), 1.12 (3H, s H-27), 1.32 (1H, H-19), 1.39 (4H, m, H-2 and H-26), 1.59 (6H, m, H-1, H-7, H-9, H-15, H-16 and H-21), 1.68 (1H, dt, H-6), 1.72 (1H, H-16), 1.91 (2H, H-22), 2.04 (1H, dt, H-15), 2.03 (3H, s,$\underline{H_3C}$COO), 2.13 (1H, d, H-18),2.51(1H, s, -OCH$_2$CCH) 4.45 (1H, dd, H-3), 4.68 (2H, m, O$\underline{CH_2}$),5.26 (1H, tl,H-12). ^{13} CNMR (125 MZH $CDCl_3$): 15.53, 16.72, 17.00, 17.17, 18.19, 21.14, 21.29, 23.29, 23.44, 23.59, 24.17, 27.98, 28.06, 30.60, 32.97, 36.41, 36.84, 37.66, 38.31, 38.9, 39.04, 39.41, 42.07, 47.47, 48.15, 51.55, 52.80, 55.28, 74.35, 78.10, 80.90, 125.74, 137.49, 170.98, 176.62. HRMS m/z calcd. for [M+H] $C_{35}H_{52}O_4$ 537.3899 found 537.3929.

***3-O-acetyl-28{(3-nitrophenyl)-1,2,3-triazol}methyloxy-betulinic ester* (2d).**

To 3-nitro azide **1** (0.015706 g, 0.0957 mmol) dissolved in THF (5 ml) was added **2c** (0.050 g, 0.0975 mmol) and distilled water (2.5 mL) .After 10 minutes under constant stirring, it was added sodium ascorbate (0.8963 g, 10 mol%) and Cu(SO_4).5H_2O (0.1947 g, 5 mol%) in 2.5 ml of iced H_2O. The reaction was kept on stirring at room temperature for 24 hours. Then extracted with water/dichloromethane (3 x 50 ml) The crude product was purified by column chromatography (cyclohexane/dichloromethane) obtaining a white crystalline powder **2d**, with 68% yield.

^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) d 0.69 (1H, m, H- 5), 0.77 (3H, s, H-25), 0.81 (3H, s, H-24), 0.89 and 1.62 (1H each, m, H-1), 0.90 (3H, s, H-26), 0.96 (6H, s, H-27 and H-23), 1.18 and 2.46 (1H each, m, H-11), 1.21 and 1.99 (1H each, m, H-12), 1.25 and 1.90 (1H each, m, H-15), 1.36 and 1.54 (1H each, m, H-6), 1.37 and 2.18 (1H each, m, H-21), 1.38 (1H, m, H-9), 1.39 and 1.43 (1H each, m, H- 7), 2.22 (3H, s, $\underline{H_3C}$COO), 1.44 and 2.22 (1H each, m, H-22), 1.48 and 2.22 (1H each, m, H- 16), 1.62 (1H, m, H-18), 1.68 (3H, s, H-30), 1.78 (2H, m, H-2), 2.42 (1H, m, H-13), 2.43 (1H, s, -O$\underline{CH_2}$CCH-), 2.48 (1H, m, H-19), 4.45 (1H, dd, H-3), 4.59 and 4.71 (1H each, s, H-29), 7.26 (1H,s,

NCH), 8.15 (1H,d, Ar-H), 8.20 (1H, s ,Ar-H),8.32 (1H, d ,Ar-H), 8.58 (1H, t, Ar-H). ^{13}C NMR (125 MZH $CDCl_3$):15.63, 15.96, 16.38, 18.02, 19.25, 20.81, 20.99, 21.25, 23.60, 25.40, 27.84, 29.65, 30.53, 31.90, 34.07, 36.80, 37.00, 38.31, 38.36, 40.64, 42.28, 46.97, 49.46, 50.32, 55.32, 56.79, 59.78, 80.82, 82.7, 109.75, 115.33, 121.43, 123.29, 125.93, 130.95, 137.58, 144.65, 148.95, 150.21, 171.08, 176.16. HRMS m/z calcd. for [M+H] $C_{41}H_{56}N_4O_6$ 701.4233 found 701.4293

3-O-acetyl-28{(3-nitrophenyl)-1,2,3-triazol}methyloxy--ursolic ester **(3d).**

This compound was prepared as described for **2d**. It was obtained a white powder **3d**, with 59% yield.

^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) 0.85 (1H, s, H-25), 0.88 (1H, H-5), 0.92 (3H, s, H-24), 0.93 (3H, s, H-23), 0.94 (3H, d, H-30), 0.95 (1H, H-1), 1.05 (3H, d, H-29), 1.09 (3H, s, H-26), 1.12 (3H, s H-27), 1.32 (1H, H-19), 1.39 (4H, m, H-2 and H-26), 1.59 (6H, m, H-1, H-7, H-9, H-15, H-16 and H-21), 1.68 (1H, dt, H-6), 1.78 (1H, H-16), 1.96 (2H, H-22), 2.04 (1H, dt, H-15), 2.25 (3H, s,H_3CCOO), 2.13 (1H, d, H-18), 4.45 (1H, dd, H-3), 5.28 (1H, tl,H-12), 7.25 (1H, s, NCH),7.7 (1H, t, Ar-H), 8.1 (1H, d, Ar-H), 8.3(1H, d, Ar-H), 8.58 (1H, S, Ar-H)^{13} CNMR (125 MZH $CDCl_3$): 15.29, 16.65, 16.77, 16.99, 18.12, 21.13, 21.28, 23.20, 23.49, 24.13, 27.92, 28.02, 29.68, 30.57, 30. 64, 32.84, 36.77, 37.61, 38.25, 38.79, 39.05, 39.51, 42.03, 47.39, 48.18, 52.87, 55.19, 80.84, 82.33, 109.91, 115.29, 122.52, 123.27, 125.63, 125.85, 139.95, 137.93, 144.53, 148.7, 170.89, 177.63. HRMS m/z calcd. for [M+H] $C_{41}H_{56}N_4O_6$ 701.4233 found 701.4287.

Biological assays

Cell Culture

A549 cells were kindly donated by Professor Fernando Spilki, Feevale Brazil. Cells were cultured in DMEM (Cultilab) supplemented with 10% fetal bovine serum (FBS) (Cultilab) and 0.5 U/mL penicillin/streptomycin at 37 °C, in a 5% CO_2 atmosphere at 100% humidity.

Cultivation of RSV virus.

RSV A strain (line A2) was provided by Fernando Polack, Fundación Infant, Argentina. The virus was grown in Vero cells. Viral plaque forming units (PFU) were identified using an RSV F protein-specific antibody (Millipore, Billerica, MA).

Treatments

Compounds **2-3c** were dissolved in 0.5% of dimethyl sulfoxide (DMSO) and culture medium. After reaching sub-confluence (70%–80% confluence), the cells were exposed to compounds at concentrations of 0.01 to 50 µM for antiviral activity and 2 to 100 µM for testing cytotoxicity for 96 h in DMEM 2% of FBS. Cells treated with DMSO (0.5% final concentration) were used as a negative control.

Cytotoxicity assay (MTT).

The inhibition of cell proliferation by compounds was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. A549 cells (5 x 10^3 cells/well in 190 µL medium per well) were seeded in a 96-well plate. After 24 h, the cells were treated with the compounds **2- 3c** and incubated for 96 h**.** The optical density of each well was measured at 630 and 560 nm on Envision (PerkinElmer, Waltham,

MA, USA) microplate reader. Four independent experiments were performed in triplicate for each test. The results were expressed as the percentage of cell viability where cells with no treatment were considered 100% viable.

Sulforhodamine B assay for % growth inhibition.

The results obtained by MTT assay were confirmed using SRB assays. This assay is based on SRB dye ability to bind to cells previously basic protein attached to the culture plate with trichloroacetic acid (TCA).SRB is a dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions A549 cells (5 x 10^3 cells/well in 190 µL medium per well) were seeded in a 96-well plate. After 24 h, the cells were treated with the compounds 2- 3c and incubates for 96 h. The cells were fixed by adding 100 uL / well of 50% trichloroacetic acid for 60 min The plates were washed five times in tap water and stained with 100 ul / well of SRB reagent (0.4% w / v SRB in 1% acetic acid) for 30 min. The plates were washed five times in 1% acetic acid to remove unbound SRB and allowed to dry overnight. SRB was solubilized with 100 ml per well of 96 well 10 mM Tris base plates with shaking for 5 min and the optical density of each well was measured at 630 and 560 nm on Envision (PerkinElmer, Waltham, MA, USA) microplate reader. Four independent experiments were performed in triplicate for each test. The results were determined by plotting the concentration vs. graph of growth inhibition age%.

Statistical analysis.

Data were presented as mean ± standard deviation of three individual experiments. Statistical analysis was performed by a one-way ANOVA analysis by means of the Prism statistical software package (GraphPad Software, Inc., La Jolla, CA, USA). *P,0.05 was considered statistically significant.

References.

1. Iniyavan, P.; Balaji, G. L.; Sarveswari, S.; Vijayakumar, V. *Tetrahedron Letters* **2015,** *56*, 5002.

2. Kumar, S.; Pathania, A. S.; Satti, N. K.; Dutt, P.; Sharma, N.; Mallik, F. A.; Ali, A. *European Journal of Medicinal Chemistry* **2015,** *92*, 236.

3. Innocente, A. M.; Silva, G. N. S.; Cruz, L. N.; Moraes, M. S.; Nakabashi, M.; Sonnet, P.; Gosmann, G.; Garcia, C. R. S.; Gnoatto, S. C. B. *Molecules* **2012,** *17*, 12003.

4. Innocente, A.; Casanova, B. B.; Klein, F.; Lana, A. D.; Pereira, D.; Muniz, M. N.; Sonnet, P.; Gosmann, G.; Fuentefria, A. M.; Gnoatto, S. C. B. *Chemical Biology & Drug Design* **2014,** *83*, 344.

5. Gnoatto, S. C. B.; Susplugas, S.; Vechia, L. D.; Ferreira, T. B.; Dassonville-Klimpt, A.; Zimmer, K. R.; Demailly, C.; Da Nascimento, S.; Guillon, J.; Grellier, P.; Verli, H.; Gosmann, G.; Sonnet, P. *Bioorganic & Medicinal Chemistry* **2008,** *16*, 771.

ARTIGO 3

In vitro and *in vivo* anti-melanoma Effects of betulinic acid derivatives

Elenilson Figueiredo da Silva[1], Jheini Lis Antunes Fernandes[2], Krist Helen Antunes Fernandes[2], Laura Trevisan Corrêa[2], Cristiane Bernardes de Oliveira[2], Grace Gosmann[1] Ana Paula Duarte de Souza,[1] Rafael Fernades Zanin[2] and Simone Cristina Baggio Gnoatto[1].

[1] Laboratório de Fitoquímica e Síntese Orgânica, Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, RS 90610-000, Brazil.

[2] Laboratório de Imunologia Clinica e Experimental, Centro Infant, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) ,Porto Alegre Brazil.

Corresponding author: Simone C. B. Gnoatto, Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Departamento de Produção de Matéria Prima, Avenida Ipiranga, 2752 sala 708 Santana, Porto Alegre, RS Brazil - CEP 90610000, e-mail simone.gnoatto@ufrgs.br.

Abstract

Malignant melanoma is the most aggressive form of cancer with a high propensity to formation of metastasis. Betulinic acid (**1**), a triterpene present in many plant species has several biological and pharmacological activities *in vitro*, including antitumor activity. In this article we are dedicated to semi-synthesis of betulinic acid derivatives

in order to produce active molecules against malignant melanomas. Derivatives were obtained by modifications at the C-3 OH position and subjected to cell viability assay against B16F10 melanoma cells, non-tumoral Vero cells and Hep cells. We also evaluated the mechanism of cell death caused by these derivatives. Finally, derivatives were subjected to *in vivo* tests against tumors injected in mice. All derivatives showed active in a dose dependent manner but derivatives **3** and **4** had higher *in vitro* induction of apoptosis. *In vivo* studies showed that derivatives **3** and **4** were able to reduce the tumor proved to be promising molecules for the treatment of malignant melanomas.

Introduction.

Malignant melanoma is the most aggressive form of cancer with a high propensity to metastasis formation (1). Epidemiological studies have shown an increase in the number of new cases of melanoma in the past decades (2)(3). The regions with the highest rate of new cases are concentrated in the United States, Europe and Australia where this disease is characterized as an epidemia (4).This increase is mainly due to the intense sun exposure and severe sunburn that are particularly harmful in childhood (5). Diagnosed in early stages where the tumor thickness is less than 1mm, the likelihood of cure is about 70 percent. However, patients with late diagnosis, wherein the tumor thickness is greater than 1mm have very low survival rates (6)(7). In those cases where the clinical diagnosis is presented with multiple metastases, surgical operations are generally not performed, and the patients are mainly treated by chemotherapy(8)(9) .

The major drugs used to treat melanoma include cisplatin, carboplatin, vincristine, paclitaxel and temozolamide(10)(11) . However, all these drugs have pronounced cytotoxic effects, often making it not a good alternative to treatment. Moreover,

malignant melanoma is considered to be a chemotherapy-refractory tumor and commonly used anticancer drugs do not appear to modify the prognosis of metastatic phase(12). Studies showed high mortality rate among patients with melanoma is related to its resistance to therapy in stages III and IV of the disease(13). Considering the increase in the incidence of new cases of malignant melanomas and limitations on treatment with currently available drugs, several studies have focused on the search for new treatment alternatives(14)(15)(16). Natural products (NP) and NP-derived products with antitumor activities are gaining importance in anticancer drug research field(17). Betulinic acid (**1**), a triterpene present in many plant species has several biological and pharmacological activities, among them, *in vitro* antitumor activity (18)(19)(20)(21). It is well known and described the effect of betulinic acid on the inhibition of cell viability at different tumor cell lines, including malignant melanomas(22)(23)(24). Morever, studies show that betulinic acid is capable of inducing selective death by apoptosis, autophagy and senescence as well as antiproliferative effects acting at different phases of the cell cycle(25)(26)(27)(28). These pharmacological effects coupled with low cytotoxicity contributed to the publication of several studies reporting antitumor activity of a series of semisynthetic derivatives of betulinic acid. In this article we are dedicated to the semi-synthesis of betulinic acid derivatives in order to produce active molecules against malignant melanomas. Novel derivatives were obtained containing modifications at the C-3 OH position of betulinic acid and subjected to cell viability assay against malignant melanoma cells B16F10, VERO, and HEP cells. We also investigated the possible mechanism of cell death involved in the activity of active derivatives. Finally active derivatives were tested *in vivo* in tumor implanted in mice.

Materials and methods

Plant materials

Betulinic acid (**1**) was obtained from barks of *Platanus acerifolia* L. (Maple), according to previous methodology(29). The characterization of **1**, its purity was carried out by Thin Layer Chromatography (TLC), Nuclear Magnetic Resonance (NMR), mass spectrometry (MS) and infrared spectroscopy (IR).

Synthetic and chemical aspect

The synthetic route is presented in **Scheme 1**. The full details of the chemical and structural elucidation of the betulinic acid derivatives were described previously(30). The solvents used in the extraction, isolation and purification of **1** as well as in the synthesis reactions of derivatives **2-4**, were previously distilled in inert atmosphere N_2. The monitoring of reactions and formation of products was performed using thin layer chromatography (TLC) Merck, and infrared spectroscopy. Column chromatography (CC) was carried out on silica gel (Merck, 60–230 mesh) using dichloromethane/ cyclohexane gradient eluent mixtures.

In vitro assay

Cell lines

B16F10 (melanoma cells), VERO (kidney cells of adult African green monkey, non-tumor cells) and HEP2 (Laryngeal tumor cells) were obtained from Banco de Células do Rio de Janeiro (BCRJ, Rio de Janeiro, RJ, Brasil). Cells was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS)

and 0.5 U/mL penicillin/streptomycin at 37 °C, in a 5% CO_2 atmosphere at 100% humidity.

Determination of cell viability (MTT assay).

In order to assess cell viability after treatment with betulinic acid and derivatives, the MTT method was used as a screening for 24 hours. In addition to cell viability assays,in B16F10, cytotoxicity assays were also performed using as control non-tumor VERO cells and Hep cells, with the aim of evaluating the selectivity of the compounds when comparing with B16F10 cells. VERO, B16F10 and HEP cells (10^3 cells/well in 200 µL medium per well) were seeded in a 96-well plate. After 24h, the cells were treated with the compounds **1**-**4**, dissolved in 1% DMSO. The optical density of each well was measured at 630 and 560 nm on Envision (PerkinElmer, Waltham, MA, USA) microplates reader. Four independent experiments were performed in triplicate for each test. The results were expressed as the percentage of cell viability where cells with no treatment were considered 100% viable.

Sulforhodamine B assay for % growth inhibition (SRB assay).

The results obtained by MTT assay were confirmed using SRB assays. SRB is a dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions B16F10, VERO and HEP cells (5 x 10^3 cells/well in 200 µL medium per well) were seeded in a 96-well plate. After 24 h, the cells were treated with the compounds **1-4** and incubate for 24 h. The cells were fixed by adding 100 µL / well of 50% trichloroacetic acid for 60 min. The plates were washed five times in tap water and stained with 100 µl / well of SRB reagent (0.4% w / v SRB in 1% acetic acid) for 30 min. After, the plates were washed five times in 1% acetic acid to remove unbound SRB and allowed to dry overnight. SRB was solubilized

with 100 mL per well of 96 well 10 mM Tris base plates with shaking for 5 min and the optical density of each well was measured at 630 and 560 nm on Envision (PerkinElmer, Waltham, MA, USA) microplate reader. Four independent experiments were performed in triplicate for each test. The results were determined by plotting the concentration vs. growth inhibition age%.

Staining with annexin V-FITC.

B16F10 cells line (4×10^4 cells/well in 300 µL medium per well) were seeded in a 24-well plate. After 48 h, cells were exposed to compound **2-4** for 24h in concentrations of 5, 10 e 25µM or cisplatin 50µM. After treatment, B16F10 cells were double stained with FITC-conjugated annexin-V and PI using an Annexin-V Apoptosis Detection kit (QuatroG, Pesquisa e Desenvolvimento Ltda, Tecnopuc, Porto Alegre, RS, Brasil) according to the manufacturer's instructions. Cells were analyzed in FACSVerse™ cytometer equipped with 488nm blue laser BD and FACSuite software. Triton X-100 (TX100) was used as a necrosis positive control and cisplatin was used as an apoptosis positive control.

***In vivo* assay.**

Treatments

The betulinic acid derivatives **2**-**4** were selected for *in vivo* testing and previously prepared in saline containing 6% of DMSO.

Effects of BA derivatives and on tumor development in C57BL/6 mice injected with B16F10 melanoma cells.

A blind experiment was conducted and animal groups were divided into 4, identified by G1, G2, G3 and G4 (4 mice per group) and treated intratumoral. Twelve to nine-week-

old male C57BL/6 were purchased from Cembe (Centro de Modelos Biológicos e Experimentais – PUCRS). Mice were implanted subcutaneously in the thigh with $5x10^5$ B16F10 cells, after anesthesia with 50 mg/kg of ketamine and 20 mg/kg of xylazine. (AB1, 2, 3 and 4 – 1.5 mg/Kg, 80µL) or vehicle (80µL) were injected intratumorally on days 6, 8 and 10th after B10F10 injection **figure 2A**. Tumor growth was evaluated using a digital caliper from days 2, 4 and 6, in PBS 1x. Tumor volume was calculated by the modified ellipsoid formula 0.52 (Length X $Width^2$). On the 12th day after tumor injection, mice were euthanized. This study had the consent of Ethics Committee on Animal Research (CEUA-PUCRS) (protocol 14/00394).

Data analysis.

Data were presented as mean ± standard deviation of three individual experiments. Statistical analysis was performed by a one-way ANOVA analysis by means of the Prism statistical software package (GraphPad Software, Inc., La Jolla, CA, USA). ***P*,0.05 was considered statistically significant.

Results

Chemistry

Derivatives **2-4** were synthesized using easily applicable methods and with good yields (Scheme 1). Compound **2** was obtained in 89% yield from betulinic acid **1** oxidation in C-3 position; oxidized product was used as starting material for the synthesis of derivative **3** with 98% yield. From the reduction of the oxime function, derivative **4** containing an amine function in position C-3 was obtained with 68% yield.

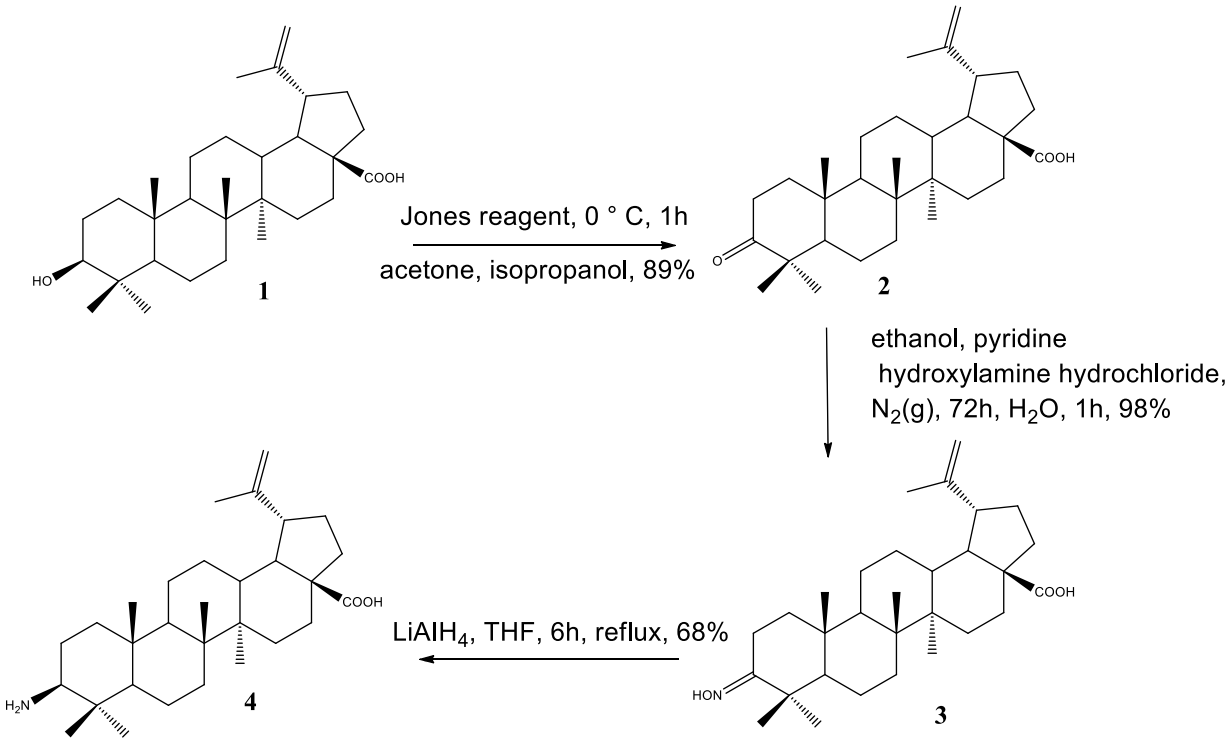


Figure 1: Synthetic Route of **2-4** compounds.

Inhibition in cell viability by BA and derivatives 2-4

Cell viability *in vitro* assays based on the MTT and SRB method was showed that betulinic acid and derivatives **2**-**4** were able to decrease B16F10 cell viability in a 24h treatment. Results were compared with the 1% DMSO control and expressed in IC_{50} (µM). Betulinic acid showed an IC_{50} of 84.1µM as derivatives **2**, **3** and **4** had IC_{50} of 22.5, 3.2 and 12.4 µM respectively, **table 1**.

Table 1: IC_{50} in µM, after treatment with **1-4** compounds for 24h using MTT and SRB assay, against B16F10, VERO, and HEP cells. All values are the means ± SE of at least triplicate cultures in three in independent experiments. ** p <0.05 for treatments **1-4**, compared with DMSO 1% control.

Compounds	B16F10[a]	VERO[b]	HEP[c]
DMSO 1%	99.2 ± 0.6	100 ± 2.2	99 ± 0.8
1	84.11 ± 0.2	99. ± 1.5	>100 ± 0.3
2	22.5 ± 1.1	89 ± 2.1	59.6 ± 0.4
3	3.2 ± 1.8	72 ± 0.8	86.3 ± 0.1
4	12.42 ± 1.3	>100 ± 0.4	97.2 ± 0.6

[a,b,c,] IC_{50} (µM) for B16F10, VERO and HEP cells, respectively.

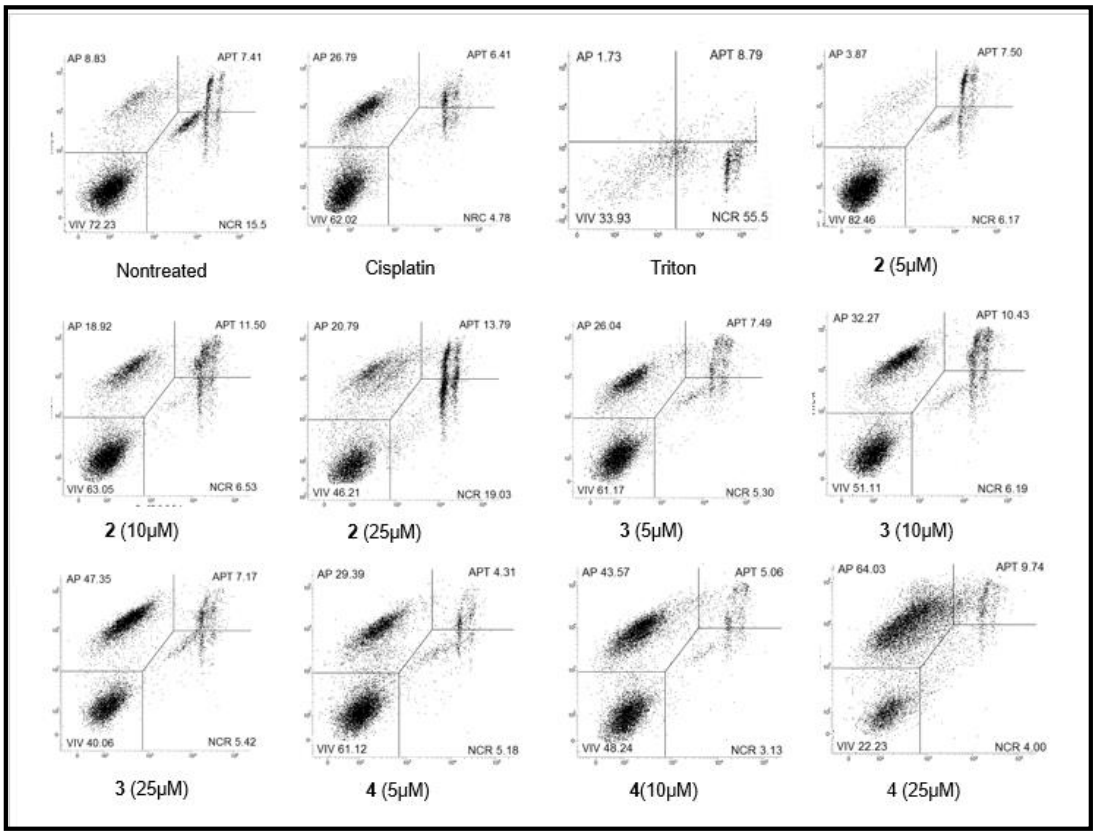
All derivatives were more active against B16F10 than the corresponding starting material and derivative **3** was the most active of the series. In addition, the results show that both betulinic acid and its derivatives **2-4** showed low cytotoxicity in VERO and HEP cells, being considered inactive when tested after 24 hours of treatment (Table 1). Similar results were found and described in other studies that show that betulinic acid is capable of inhibiting cell viability in B16F10, showing a marked selectivity(31). Our results showed that chemical modifications in the C-3 position of betulinic acid can lead to synthesis of a more active and more selective derivatives.

Induction of apoptosis in B16F10 cells by BA compounds 1-4.

Based on previous studies that show a high apoptotic potential for betulinic acid in various cell lines we evaluated the cell death mechanism of compounds **2-4** using annexin V-FITC kit. All derivatives were capable of inducing apoptosis in a dose dependent manner, and derivatives **3** and **4** caused greater induction of apoptosis in a smaller concentration. These results confirm the potential apoptotic power of betulinic acid and derivatives when exposed to cells of B16F10, **Figure 2**.

Figure 2. Apoptosis assay of B16F10 cells treated with **2-4** Flow cytometric analy

sis of apoptosis in B16F10 cells was performed by treatment with cisplatin (50µM) and **2-4 derivatives** (5, 10 or 25µM) for 24 hours and by staining with annexin V–FITC and PI. ** $p < 0.05$ for treatments compared with the nontreated control apoptosis of viable cells.



Antimetastatic effects of BA derivatives 2-4 in B16F10 melanoma in C57BL/6 mice.

In order to assess whether the effects found for **1** and derivatives may be reproduced *in vivo*, we performed an experiment with these derivatives in B16F10 *in vivo*, as described in Figure 3A. In order to provide greater reliability and accuracy results in our experiment was conducted blindly and revealed only after completion of the experiments. The groups were referred to as G1, G2, G3, and G4, corresponding to treatment with DMSO control, derivatives **2**, **3** and **4,** respectively. The data showed that the derivative **2** which contains a ketone in position C-3 showed no statistically significant difference when compared to the control group, and therefore is considered inactive. However, derivatives **3** and **4** showed significant reduction in growth of B16F10 tumors at a concentration of 1.5 mg / kg of animal, figure **3B** and **C.** These data are supported by previous studies showing that **1** was capable of causing B16F10 significant suppression at doses of 2.5mg / kg.

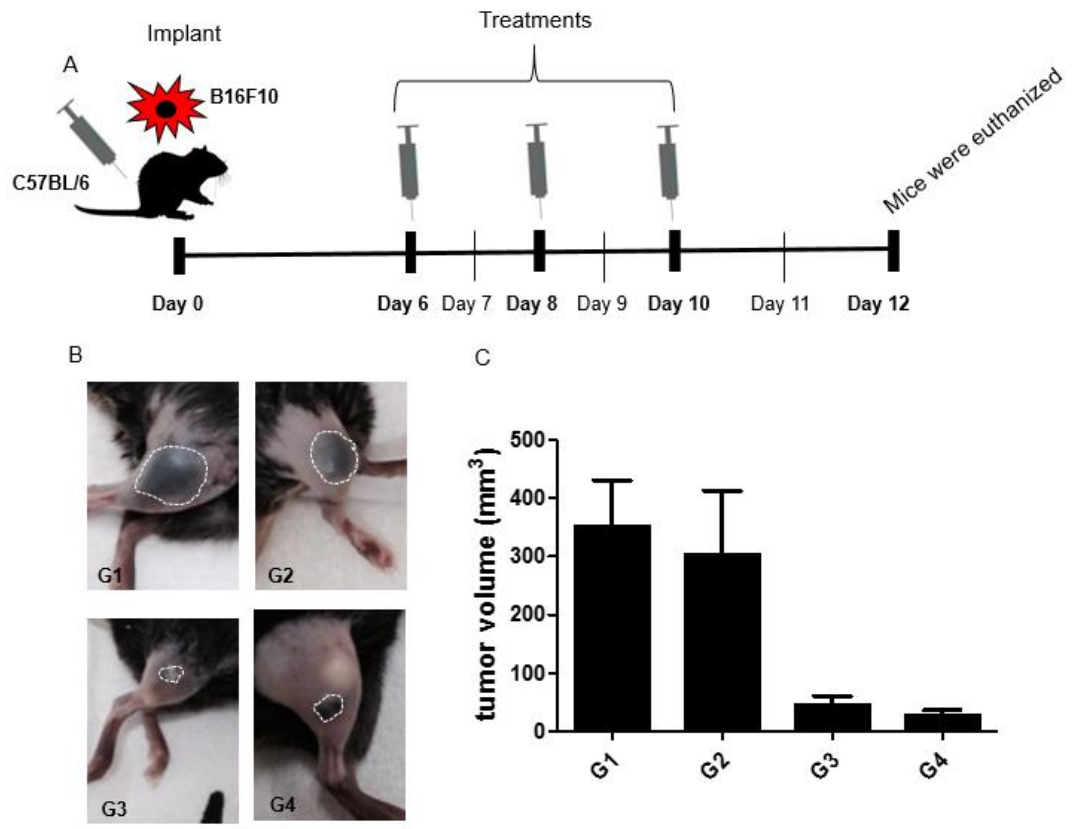


Figure 3. Experimental protocol and results for animal experiment with compounds **2**-4. **A**, Mice were implanted with 5x10^5 B16F10 cells in 0 day. On the sixth day, they started the treatment (**2-4** derivatives, 1.5mg / kg). On eleven day mice were euthanized. **B** treatment of tumors to the DMSO control (G1) and derivatives **2-4** (G2, G3 and G4 respectively). **C**, graph demonstrates the decrease in tumor volume (mm^3) after treatment with the derivative DMSO control (G1) and derivatives **2-4** (G2, G3, G4, respectively). ** p <0.05 for treatments compared with the DMSO control (G 1).

Discussion

Betulinic acid and synthetic derivatives have been reported by several studies *in vitro* for their potential anti-tumor activity against various cell lines(32)(33)(34). These studies show that betulinic acid can induce apoptosis in different concentrations, besides being a compound with low cytotoxicity and easy to obtain, which contributed

to a large number of publications on synthetic derivatives containing betulinic acid(35)(36)(37). However, studies reporting the antitumor activity *in vivo* of betulinic acid and derivatives are still restrict. We evaluate in this article the *in vitro* and *in vivo* effect of betulinic acid derivatives containing pharmacomodulations in the C-3 position. All derivatives showed to be active and selective *in vitro* when assessed against B16F10 having a high ability to induce dose dependent apoptosis. These results are contingent with the previously reported that betulinic acid induced apoptosis by different routes in a dose dependent manner in B16F10 melanoma cell line(38). Our *in vivo* results showed that the animals with B16F10 melanoma tumors which were treated with derivatives **3** and **4** showed significant reduction in tumor load, showing inhibition of growth. However, animals treated with derivative **2** showed no reduction in tumor volume to differentiate it from the control group. In addition, we observed that the inclusion of organic groups containing nitrogen atoms as in the case of derivatives **3** and **4**, may be important for antitumor activity, which explains the elevated *in vivo* activity of both derivatives. This hypothesis is reinforced by several chemical studies where amine and amide containing functions in betulinic acid structure is correlated with antitumor activity(39)(40)(41)(42)(43). Based on the results obtained from this study, we demonstrated the development of new drugs **3** and **4**, derivatives of betulinic acid with potential antitumor activity on B16F10 melanoma cell line. These promising derivatives were capable of reducing tumors implanted in mice at very low doses, 1.5 mg / kg, these results are similar to those obtained by another research group reporting the *in vivo* activity of betulinic acid derivatives against B16F10, the study showed a significant decrease in tumor volume when a dose of 2.5mg / kg was administered(43). We present here two drugs with similar effects to those already reported in the literature, however at lower doses and with a low-cost and easily applicable synthetic

methodology. In summary, the development of two new drugs was possible, compounds **3** and **4**, with activity against B16F10 *in vitro* through induction of apoptosis and activity in the prevention of B16F10 metastasis of experimental melanoma by direct suppression of cell growth *in vivo*. We believe that these derivatives may be active against other tumor types, and may prove to be a viable alternative in the search for new malignant melanoma treatments. New tests will also be carried out in order to explore the main pathways for induction of apoptosis by these derivatives, as well as to unravel the mechanism of action by which these compounds are capable of causing a decrease in the *in vivo* tumor volume.

Conclusion

In this paper we describe the semisynthesis of betulinic acid derivatives containing nitrogen function at C-3 position, these derivatives when evaluated *in vitro* and *in vivo* proved to be extremely active and less cytotoxic. We believe that these derivatives can be used in the treatment of human melanomas but further studies will be performed in order to explore the activity of these derivatives when exposed to human melanoma cells. Our efforts lead us to believe that different induction pathways can be correlated to the mechanism of action of these compounds, however this is still a challenge that we will achieve.

Acknowledgments

This work was supported by grants and financial support from Programa de Pós graduação em Ciências Farmacêuticas UFRGS, Nacional de Desenvolvimento Cientifico e Tecnológico (CNPQ-Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil). The Laboratório Regional de Nanotecnologia (LRNANO) da Universidade Federal do Rio Grande do Sul UFRGS for the NMR analysis and Central analítica de instrumentação da Universidade de São Paulo USP for the HRMS analysis.

Reference

1. Chaabane F, Mustapha N, Mokdad-Bzeouich I, Sassi A, Kilani-Jaziri S, Dijoux Franca M-G, et al. In vitro and in vivo anti-melanoma effects of Daphne gnidium aqueous extract via activation of the immune system. Tumor Biol [Internet]. 2015; Available from: http://link.springer.com/10.1007/s13277-015-4492-x

2. Whiteman DC, Green AC, Olsen CM. The growing burden of invasive melanoma: projections of incidence rates and numbers of new cases in six susceptible populations to 2031. J Invest Dermatol [Internet]. 2016; Available from: http://www.ncbi.nlm.nih.gov/pubmed/26902923

3. Erdei E, Torres SM. A new understanding in the epidemiology of melanoma. Expert Rev Anticancer Ther [Internet]. 2010;10(11):1811–23. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3074354&tool=pmcentrez&rendertype=abstract

4. Manuscript A, Epidemiology M. NIH Public Access. 2011;23(3).

5. Nogueira MA, Magalhães EG, Magalhães AF, Biloti DN, Laverde A, Pessine FBT, et al. A novel sunscreen agent having antimelanoma activity. Farmaco. 2003;58(11):1163–9.

6. Angelica MD, Fong Y. NIH Public Access. October. 2008;141(4):520–9.

7. White RR, Stanley WE, Johnson JL, Tyler DS, Seigler HF. Long-term survival in 2,505 patients with melanoma with regional lymph node metastasis. Ann Surg [Internet]. 2002;235(6):879–87. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1422519&tool=pmcentrez&rendertype=abstract

8. Staudt M, Lasithiotakis K, Leiter U, Meier F, Eigentler T, Bamberg M, et al. Determinants of survival in patients with brain metastases from cutaneous melanoma. Br J Cancer [Internet]. 2010;102(8):1213–8. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2856002&tool=pmcentrez&rendertype=abstract

9. Hao XS, Li Q, Chen H. Small bowel metastases of malignant melanoma: palliative effect of surgical resection. Jpn J Clin Oncol. 1999;29(9):442–4.

10. Legha SS, Ring S, Bedikian A, Plager C, Eton O, Buzaid AC, et al. Original article Treatment of metastatic melanoma with combined chemotherapy containing. 1996;(Cvd):827–35.

11. Chhabra A, Mukherjee V, Chowdhary M, Danckers M, Fridman D. Black pleural effusion: A unique presentation of metastatic melanoma. Case Rep Oncol. 2015;8:222–5.

12. Fuggetta MP, D'Atri S, Lanzilli G, Tricarico M, Cannavò E, Zambruno G, et al. In vitro antitumour activity of resveratrol in human melanoma cells sensitive or resistant to temozolomide. Melanoma Res. 2004;14(3):189–96.

13. Brożyna AA, Jóźwicki W, Roszkowski K, Filipiak J. Melanin content in melanoma metastases affects the outcome of radiotherapy. 2016;7(14):1–10.

14. Verdú JM, Batalla RJ, Martínez-casasnovas JA. Un Co R Re Ct Oo Fs Un Co R Re Oo Fs. 2013;(March):333–41.

15. Ascierto PA, Grimaldi AM, Anderson AC, Bifulco C, Cochran A, Garbe C, et al. Future perspectives in melanoma research: meeting report from the “Melanoma Bridge”, Napoli, December 5th-8th 2013. J Transl Med [Internet]. 2014;12:277. Available from: https://apps.webofknowledge.com/full_record.do?product=WOS&search_mode=GeneralSearch&qid=2&SID=Y1O6iYUJ7cMdcZLDOhp&page=1&doc=48

16. Menzies AM, Long G V. Recent advances in melanoma systemic therapy. BRAF inhibitors, CTLA4 antibodies and beyond. Eur J Cancer. 2013;49(15):3229–41.

17. Naithani R, Huma LC, Moriarty RM, McCormick DL, Mehta RG. Comprehensive review of cancer chemopreventive agents evaluated in experimental carcinogenesis models and clinical trials. Curr Med Chem [Internet]. 2008;15(11):1044–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18473802

18. Innocente AM, Silva GNS, Cruz LN, Moraes MS, Nakabashi M, Sonnet P, et al. Synthesis and antiplasmodial activity of betulinic acid and ursolic acid analogues. Molecules. 2012;17(10):12003–14.

19. Jin H, Pi J, Yang F, Wu C, Cheng X, Bai H, et al. Ursolic acid-loaded chitosan nanoparticles induce potent anti-angiogenesis in tumor. Appl Microbiol Biotechnol [Internet]. 2016; Available from: http://link.springer.com/10.1007/s00253-016-7360-8

20. Liu Y, Gao D, Zhang X, Liu Z, Dai K, Ji B, et al. Antitumor drug effect of betulinic acid mediated by polyethylene glycol modified liposomes. Mater Sci Eng C. 2016;64:124–32.

21. Vaishnav P, Demain AL. Unexpected applications of secondary metabolites. Biotechnol Adv. 2011;29(2):223–9.

22. Mahmoudi M, Rabe SZT, Balali-Mood M, Karimi G, Tabasi N, Riahi-Zanjani B. Ursolic acid induced apoptotic cell death following activation of caspases in isolated human melanoma cells. Cell Biol Int. 2015;39(2):230–6.

23. Lee YH, Wang E, Kumar N, Glickman RD. Ursolic acid differentially modulates apoptosis in skin melanoma and retinal pigment epithelial cells exposed to UV-VIS broadband radiation. Apoptosis. 2014;19(5):816–28.

24. Looi CY, Moharram B, Paydar M, Wong YL, Leong KH, Mohamad K, et al. Induction of apoptosis in melanoma A375 cells by a chloroform fraction of Centratherum anthelminticum (L.) seeds involves NF-kappaB, p53 and Bcl-2-controlled mitochondrial signaling pathways. BMC Complement Altern Med [Internet]. 2013;13(1):166. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3718627&tool=pmcentrez&rendertype=abstract

25. Yi J, Zhu R, Wu J, Wu J, Xia W, Zhu L, et al. Pharmacological Reports In vivo protective effect of betulinic acid on dexamethasone induced thymocyte apoptosis by reducing oxidative stress. Pharmacol Reports [Internet]. 2016;68(1):95–100. Available from: http://dx.doi.org/10.1016/j.pharep.2015.07.003

26. Khan I, Guru SK, Rath SK, Chinthakindi PK, Singh B, Koul S, et al. A novel triazole derivative of betulinic acid induces extrinsic and intrinsic apoptosis in human leukemia HL-60 cells. Eur J Med Chem [Internet]. 2016;108:104–16. Available from: http://www.sciencedirect.com/science/article/pii/S0223523415303548

27. Chuang W-L, Lin P-Y, Lin H-C, Chen Y-L. The Apoptotic Effect of Ursolic Acid on SK-Hep-1 Cells is Regulated by the PI3K/Akt, p38 and JNK MAPK Signaling Pathways. Molecules [Internet]. 2016;21(4):460. Available from: http://www.mdpi.com/1420-3049/21/4/460

28. Li Y, Lu X, Qi H, Li X, Xiao X, Gao J. Ursolic Acid Induces Apoptosis Through Mitochondrial Intrinsic Pathway and Suppression of ERK1/2 MAPK in HeLa Cells. J Pharmacol Sci [Internet]. 2014;125(2):202–10. Available from: http://jlc.jst.go.jp/DN/JST.JSTAGE/jphs/14017FP?lang=en&from=CrossRef&type=abstract

29. da Silva GNS, Maria NRG, Schuck DC, Cruz LN, de Moraes MS, Nakabashi M, et al. Two series of new semisynthetic triterpene derivatives: differences in anti-malarial activity, cytotoxicity and mechanism of action. Malar J [Internet]. 2013;12:89. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3616855&tool=pmcentrez&rendertype=abstract

30. Dalla-vechia L, Dassonville-klimpt A, Grellier P, Sonnet P, Gosmann G, Gnoatto SCB. The Beckmann Rearrangement Applied to Ursolic Acid with Antimalarial Activity in Medicinal Chemistry Studies. 2012;92–5.

31. Jin KS, Oh YN, Hyun SK, Kwon HJ, Kim BW. Betulinic acid isolated from Vitis amurensis root inhibits 3-isobutyl-1-methylxanthine induced melanogenesis via the regulation of MEK/ERK and PI3K/Akt pathways in B16F10 cells. Food Chem Toxicol. 2014;68:38–43.

32. Dutta D, Chakraborty B, Sarkar A, Chowdhury C, Das P. A potent betulinic acid analogue ascertains an antagonistic mechanism between autophagy and proteasomal degradation pathway in HT-29 cells. BMC Cancer [Internet]. 2016;16(1):23. Available from: http://www.biomedcentral.com/1471-2407/16/23

33. Yang L, Chen Y, He J, Yi S, Wen L, Zhao J, et al. Betulinic acid inhibits autophagic flux and induces apoptosis in human multiple myeloma cells in vitro. Acta Pharmacol Sin [Internet]. 2012;33(12):1542–8. Available from: http://dx.doi.org/10.1038/aps.2012.102

34. Cichewicz RH, Kouzi SA. Chemistry, Biological Activity, and Chemotherapeutic Potential of Betulinic Acid for the Prevention and Treatment of Cancer and HIV Infection. Med Res Rev. 2004;24(1):90–114.

35. Wiemann J, Heller L, Perl V, Kluge R, Ströhl D, Csuk R. Betulinic acid derived hydroxamates and betulin derived carbamates are interesting scaffolds for the synthesis of novel cytotoxic compounds. Eur J Med Chem [Internet]. 2015;106:194–210. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0223523415303251

36. Santos RC, Salvador JAR, Mar??n S, Cascante M, Moreira JN, Dinis TCP. Synthesis and structure-activity relationship study of novel cytotoxic carbamate and N-acylheterocyclic bearing derivatives of betulin and betulinic acid. Bioorganic Med Chem. 2010;18(12):4385–96.

37. Csuk R, Barthel A, Sczepek R, Siewert B, Schwarz S. Synthesis, encapsulation and antitumor activity of new betulin derivatives. Arch Pharm (Weinheim). 2011;344(1):37–49.

38. Sawada N, Kataoka K, Kondo K, Arimochi H, Fujino H, Takahashi Y, et al. Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice. Br J Cancer. 2004;90(8):1672–8.

39. Drąg-Zalesińska M, Wysocka T, Borska S, Drąg M, Poręba M, Choromańska A, et al. The new esters derivatives of betulin and betulinic acid in epidermoid squamous carcinoma treatment – In vitro studies. Biomed Pharmacother [Internet]. 2015;72:91–7. Available from: http://www.sciencedirect.com/science/article/pii/S0753332215000943

40. Bildziukevich U, Vida N, R??rov?? L, Kol???? M, ??aman D, Havl????ek L, et al. Polyamine derivatives of betulinic acid and ??-sitosterol: A comparative investigation. Steroids. 2015;100:27–35.

41. Csuk R, Nitsche C, Sczepek R, Schwarz S, Siewert B. Synthesis of antitumor-active betulinic acid-derived hydroxypropargylamines by copper-catalyzend mannich reactions. Arch Pharm (Weinheim). 2013;346(3):232–46.

42. Csuk R, Sczepek R, Siewert B, Nitsche C. Bioorganic & Medicinal Chemistry Cytotoxic betulin-derived hydroxypropargylamines trigger apoptosis. 2013;21:425–35.

43. Saha S, Ghosh M, Dutta SK. A potent tumoricidal co-drug “Bet-CA”--an ester derivative of betulinic acid and dichloroacetate selectively and synergistically kills cancer cells. Sci Rep [Internet]. 2015;5:7762. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4293591&tool=pmcentrez&rendertype=abstract

III. DISCUSSÃO

Segundo a organização mundial da saúde OMS, os novos casos de câncer, e principalmente os relacionados com a resistência a tratamentos quimioterápicos têm aumentado nos últimos anos. A estimativa da OMS é que em 2030, o número de novos casos atinja 27 milhões de pessoas e 17 milhões de óbitos[38].

Ainda segundo o Instituto nacional do câncer INCA, o câncer é a segunda maior causa de morte entre os Brasileiros causando em média 190 mil óbitos a cada ano[39]. Apesar do surgimento de novos e seletivos fármacos antitumorais nas últimas décadas, o tratamento quimioterápico do câncer continua a ser um desafio, justificado em partes pela alta resistência das células tumorais quando expostas ao agente quimioterápico[40,41,42]. Outro fator que limita o tratamento quimioterápico está correlacionado diretamente com os diversos efeitos colaterais dos fármacos antitumorais, sendo muitos deles ainda desconhecidos[43,44].

A principal característica de uma célula tumoral é o seu crescimento descontrolado que invade órgãos e tecidos[45,46]. Vários fatores têm sido associados com o processo oncogênico, como meio ambiente, estilo de vida, fatores do hospedeiro, agentes infecciosos e herança genética, que são na maioria das vezes geneticamente pré-determinados[47,48,49,50].

Entre esses fatores, os agentes infecciosos têm atraído à atenção da comunidade cientifica nos últimos 10 anos, estudos mostram a relação entre as infeções virais e o desenvolvimento de tumores[51,52,53] e que os vírus são os agentes mais comuns envolvidos na patogênese do câncer[54,55]. Ainda, a Agência Internacional para Pesquisa sobre o câncer estima que a cada cinco casos de câncer em todo o mundo, dois são causados por infecção, sendo a maioria de fonte viral[56].

Esses dados despertam a preocupação em se buscar alternativas e formas de tratamento para o câncer e para diversos tipos de infecção viral, de forma prevenir o desenvolvimento de tumores originados a partir de infeções virais.

Frente a essa realidade, os produtos naturais e semissintéticos estão entre os agentes antitumorais e antivirais mais utilizados no mundo, sendo os fármacos de

primeira escolha no tratamento de diversos tipos de câncer[57,58,59]. A semissíntese surge nesse contexto como uma ferramenta auxiliar, pois permite a obtenção de um fármaco mais ativo e mais seletivo a partir do produto natural bioativo[60,61]. Além disso, a partir dos estudos de química medicinal é possível o desenvolvimento de um fármaco direcionado a um alvo molecular de forma a melhorar a especificidade do produto natural[62,63,64]. Triterpenos representam uma importante classe de produtos naturais exibindo diversas atividades terapêuticas, dentre elas a atividade antitumoral e antiviral [65,66].

Há décadas nosso grupo de pesquisa tem se dedicado a obtenção de novos derivados triterpênicos objetivando a busca de novos protótipos para o tratamento de diferentes patologias[67,68,69,70]. Além das inúmeras atividades biológicas descritas pelos triterpenos, estes apresentam como vantagem a fácil obtenção, baixa instabilidade e fácil manipulação, características essas que contribuíram para o elevado números de publicações destes compostos bem como de seus derivados semissintéticos nos últimos anos[71,72,73,74]. Estimulados com os resultados obtidos por estudos anteriores do nosso grupo de pesquisa, nós nos dedicamos neste trabalho à obtenção de novos derivados semissintéticos dos triterpenos ácido ursólico e betulínico, com a finalidade de explorar a potencial atividade antitumoral e antiviral destes derivados. Baseados em estudos anteriores que demonstram grupamentos nitrogenados contribuem para a atividade biológica de triterpenos, nós propusemos a obtenção de derivados nitrogenados, com farmacomodulação nas posições C-3 e C-28 da estrutura triterpênica. Ainda os derivados obtidos foram testados contra câncer de pele (melanomas- B16F10), leucemia mielóide crônica (K562) e contra o vírus sincicial respiratório (RSV), foi avaliada também a citotoxicidade dos derivados frente a células não tumorais (VERO) e linfócitos humanos a fim de estabelecer parâmetros de seletividade, ainda com o objetivo de avaliar a toxicidade de possíveis metabólitos destes derivados, nós avaliamos a citotoxicidade em células HEPG2 e Hep.

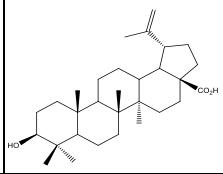
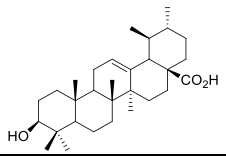
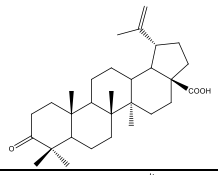
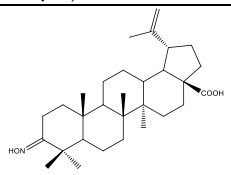
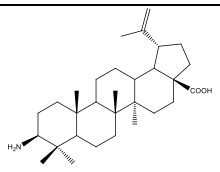
A partir dos ensaios de citotoxicidade, foi possível perceber que os derivados semissintéticos do ácido ursólico, bem como do ácido betulínico não apresentaram efeito significativo sobe a viabilidade celular em células de HEPG2, HEP e Vero

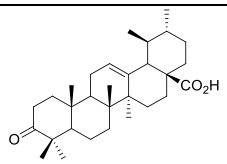
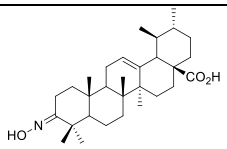
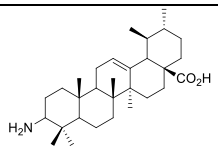
Os resultados dos estudos *in vitro* demonstraram que tanto os triterpenos ácidos ursólico e betulínico quanto os derivados semissintéticos nas concentrações

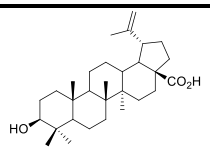
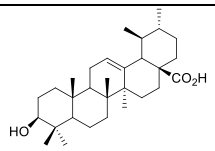
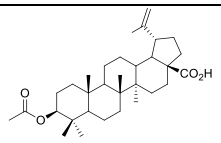
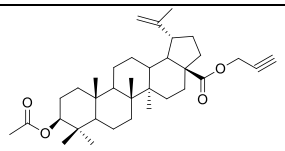
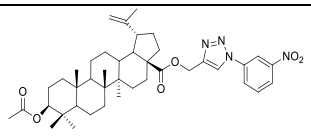
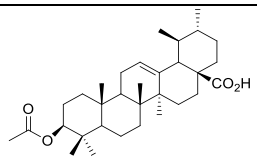
testadas foram capazes de provocar diminuição da viabilidade de células tumorais de melanoma (B16F10) e leucemia (K562.

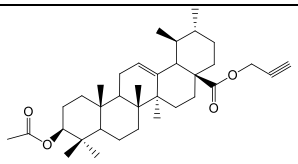
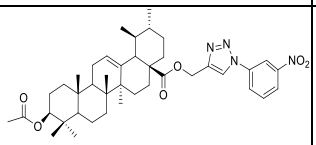
A **tabela 1** resume os resultados da atividade em células de melanoma e de leucemia. Resultados semelhantes foram descritos em estudos anteriores, que demonstram que ácido ursólico e ácido betulínico são capazes de inibir a viabilidade de células de melanomas (B16F10) e leucemia (K562), porém para os compostos obtidos neste trabalho ainda não há relatos desta atividade na literatura[75, 76].

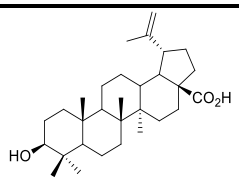
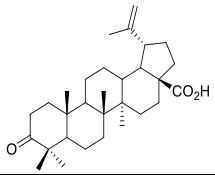
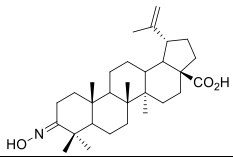
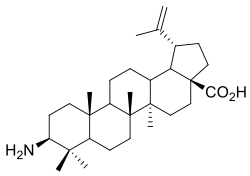
Tabela 1. Atividade antitumoral e antiviral *in vitro* dos ácidos ursólico, betulínico e derivados semissintéticos.

			Linhagens celulares IC_{50} (µM)			
Composto ARTIGO 1	**Estrutra quimica**	**MM (g/mol)**	**K562**	**VERO**	**Linfócito**	**HEPG2**
1		**456,3603**	**85,5 ± 1,5**	**99 ± 1,5**	**78,11 ± 1,1**	**>100 ± 1,2**
2		**456,3603**	**33,4 ± 0,4**	**86 ± 0,4**	**91,2 ± 2,5**	**87,2 ± 3,3**
1a		**454,3447**	**74,3 ± 2,1**	**89 ± 2,1**	**78.4 ± 0,9**	**86 ± 1,9**
1b		**469,3556**	**55,2 ± 0,8**	**72 ± 0,8**	**84,15 ± 0,7**	**>100 ± 1,1**
1c		**455,3763**	**19,5 ± 0,4**	**>100 ± 0,4**	**>100 ± 1,8**	**99,4 ± 0,9**

2a		**454,3447**	**90,2 ± 0,9**	**90,2 ± 0,9**	**98,2 ± 0,5**	**>100 ± 1,6**
2b		**469,3556**	**21,9 ± 0,6**	**>100 ± 0,9**	**>100 ± 1,1**	**98,8 ± 2,1**
2c		**455,3763**	**14,9 ± 0,7**	**>100 ± 0,7**	**>100 ± 0,9**	**97,9 ± 2,7**

Composto ARTIGO 2	**Estrutra quimica**	**MM (g/mol)**	**A549 IC_{50}**	**A549 + RSV EC_{50}**
2		**456,3603**	**17,77**	**5,3**
3		**456,3603**	**26,7**	**13,7**
2a		**498,3709**	**53**	**44,4**
2c		**536,3866**	**88,8**	**0,58**
2d		**700,4200**	**42,7**	**0,314**
3a		**498,3709**	**133**	**14,29**

3c		**536,3866**	**67,2**	**36,2**
3d		**700,4200**	**59,15**	**0,0053**

Composto ARTIGO 3	**Estrutra quimica**	**MM (g/mol)**	**B16F10**	**VERO**	**HEP2**
1		**456,3603**	**84,11 ±0,2**	**99 ± 1,5**	**>100 ± 0,3**
2		**454,3447**	**22,5 ± 1,1**	**89 ± 2,1**	**59,6 ± 0,4**
3		**469,3556**	**3,2 ± 1,8**	**72 ± 0,8**	**86,3 ± 0,1**
4		**455,3763**	**12,42 ±1,3**	**>100 ± 0,4**	**97,2 ± Nosso0,6**

Artigo 1, a viabilidade celular foi avaliada pelo método de MTT, por 24 horas de tratamento, IC_{50} em µM. **Artigo 2**, a viabilidade celular foi avaliada utilizando os métodos de MTT e SRB, por 96 horas de tratamento, os resultados foram expressos em IC_{50} e EC_{50}(µM). **Artigo 3**, a viabilidade celular foi avaliada por métodos de MTT e SRB, após 24 de tratamento e os resultados foram expressos em IC_{50} (µM).

Nós investigamos também o possível mecanismo pelo qual os derivados mais ativos causam a diminuição na viabilidade celular, a fim de entender os possíveis mecanismos de ação envolvidos na atividade desses compostos. Todos os derivados

mostraram-se capazes de induzir apoptose em concentrações dependentes em células de melanoma e de leucemia.

Além de induzir apoptose o derivado **2c (artigo 1)** Mostrou-se capaz de provocar alterações no ciclo celular das células de leucemia K562 promovendo um acúmulo de células na fase G1/G0, resultando assim na diminuição da proliferação celular. Esses resultados nos levam a inferir que o derivado **2c** possui atividade apoptótica e antiproliferativa para as células de leucemia K562.

Nós avaliamos também o efeito tempo dependente deste derivado, submetendo ao ensaio de viabilidade celular por 48 horas de tratamento em células de leucemia (K562) e células de HEPG2, linfócitos humanos e VERO, avaliamos também o efeito do imatinibe quando exposto a células de linfócitos humanos por 48 horas de tratamento, **Figura 1**.

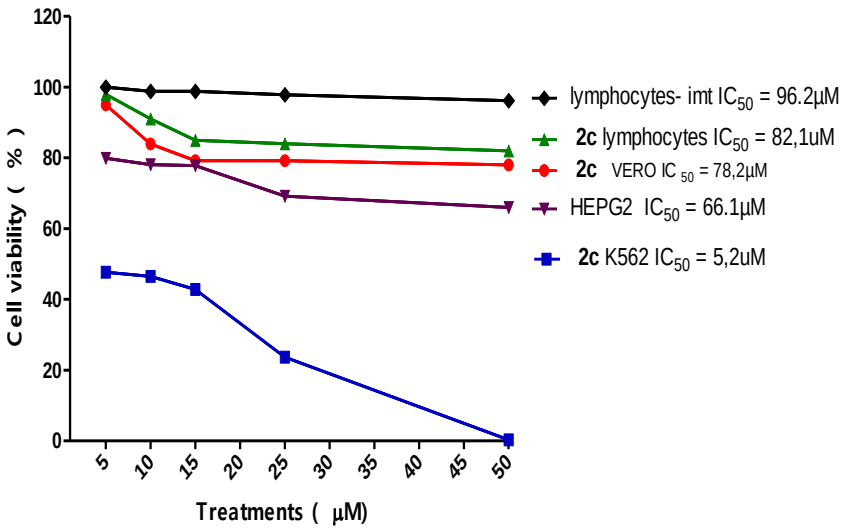


Figura 2. Ensaio de viabilidade celular em 48 horas de tratamento para o derivado **2c**. Os ensaios foram conduzidos baseados no método de MTT e contagem por citometria de fluxo**.**

De posse do bom resultado obtido *in vitro* com células de melanomas (B16F10), nós investigamos a atividade desses derivados frente a tumores *in vivo.* Os resultados mostram que os derivados **3** e **4 Figura 2,** (dose de 1,5mg/Kg) contendo grupamentos nitrogenados na posição C-3, apresentaram significativa redução da carga tumoral, mostrando-se capazes de inibir o crescimento tumoral e metástases.

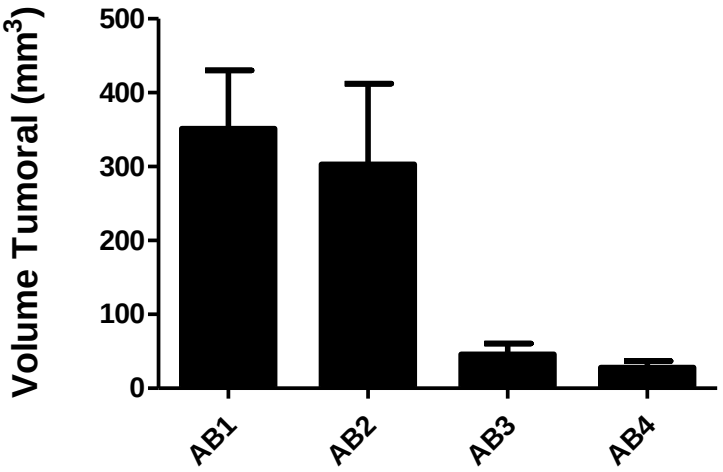


Figura 3. Atividade antitumoral *in vivo* dos derivados **2-4** (artigo 3), expressa em volume tumoral (mm3), em células de melanomas (B16F10). O experimento foi conduzido às cegas e os tratamentos identificados como AB1, AB2, AB3 e AB4, que correspondem aos tratamentos controle, derivado **2**, **3** e **4**, respectivamente.

Isolado pela primeira vez em 1955, o vírus sincicial respiratório (RSV), é conhecido por causar infecção aguda do trato respiratório principalmente em crianças, onde a infecção atinge sua forma mais severa, porém estudos mostram que essa doença pode acometer indivíduos de todas as idades[77].

O único fármaco aprovado para o tratamento de infeções pelo vírus RSV, disponível atualmente é a ribavirina e apesar de sua boa atividade antiviral, essa molécula mostrou-se altamente tóxica em crianças, o que inviabiliza sua utilização para o tratamento de infeções nestes pacientes [78].

Portanto, corroborando com dados de literatura de indicam que triterpenos com atividade antitumoral têm demonstrado atividade antiviral, nós observamos que os compostos **2d** e **3d (artigo 2)** foram ativos contra RSV nas concentrações testadas. Observamos que modificações na posição C-28 da estrutura do ácido ursólico e betulínico potencializou a, sendo possível a obtenção de derivados mais ativos e mais seletivos que os correspondentes produtos de partida.

Ainda foi possível ao final deste estudo estabelecer uma breve relação estrutura atividade destes novos derivados frente a atividade antiviral para RSV. Os resultados estão resumidos na **tabela 1**.

Finalmente, nossos resultados demonstram que a inserção de grupamentos doadores de hidrogênio nas posições C-3 e C-28 mostrou-se importante para a atividade antitumoral e antiviral.

Os resultados obtidos neste trabalho mostram que a semissintese é uma estratégia viável na busca de novos derivados triterpênicos com atividade antitumoral e antiviral. Ainda novos estudos serão realizados com o objetivo de estabelecer o completo mecanismo antitumoral e antiviral destes compostos.

IV. CONCLUSÃO

Ao final deste estudo foi possível a obtenção de três artigos científicos com estudos de atividade antiviral *in vitro* e antitumoral *in vitro* e *in vivo*. A extração e purificação bem como as reações de semissíntese foram bem sucedidas permitindo a obtenção de novos derivados com altos rendimentos reacionais além de ser utilizada uma metodologia de fácil aplicação e baixo custo.

O composto ácido 3-aminoursólico **2c (artigo 1)** mostrou-se o mais ativo contra as células K562, além de induzir morte celular via apoptose de provocar alterações no ciclo celular dessas células promovendo um acúmulo de células na fase G1/G0, resultando assim na diminuição da proliferação celular.

O compostos éster do ácido 3-*O*-acetil-28{(3-nitrofenil)-1,2,3-triazol}metiloxi-ursólico **3d (artigo 2)** apresentou atividade anti-RSV, mostrando-se este uma alternativa promissora para o desenvolvimento de novos tratamentos para este vírus.

Os derivados oxima do ácido betulínico **3** e ácido 3-aminobetulínico **4 (artigo 3)** mostraram-se extremamente ativos *In vivo* sendo capazes de inibir a proliferação celular metastática de B16F10 em camundongos

Esses estudos mostram claramente que triterpenos são uma importante classe de moléculas bioativas. Ainda, nossos resultados indicam que modificações na estrutura triterpênicas podem conduzir a obtenção de derivados mais ativos e mais seletivos que o correspondente produto de partida. Esses dados abrem portas para novas pesquisas abordando a atividade terapêutica desses compostos.

Referências

1 Mondal, S.; Bandyopadhyay, S.; et al. Natural Products : Promising Resources for Cancer Drug Discovery. p. 49–75, 2012.

2 Mishra, B.B.; Tiwari, V.K. European Journal of Medicinal Chemistry Natural products : An evolving role in future drug discovery. v. 46, 2011.

3 Lourenço, A.M.; Ferreira, L.M.; et al. Molecules of Natural Origin , Semi-synthesis and Synthesis with Anti-Inflammatory and Anticancer Utilities. p. 3979–4046, 2012.

4 Schnermann, M.J.; Shenvi, R.A. HHS Public Access. v. 32, n. 4, p. 543–577, 2016.

5 Aygün, A.; Pindur, U. Chemistry and Biology of New Marine Alkaloids from the Indole and Annelated Indole Series. p. 1113–1127, 2003.

6 Medica, P. New Polyindolenine Alkaloids from Calycodendron milnei. n. May 2016, 2006.

7 Wen, G.; Qu, X.; et al. Fitoterapia Recent advances in design , synthesis and bioactivity of paclitaxel-mimics. v. 110, p. 26–37, 2016.

8 Fu, Y.; Li, S.; et al. Medicinal Chemistry of Paclitaxel and its Analogues. p. 3966–3985, 2009.

9 Xue, M.; Long, B.H.; et al. Structure ± Activity Relationships Study at the 3 H - N Position of Paclitaxel . Part 2 : Synthesis and Biological Evaluation of 3 H - N - Thiourea- and 3 H - N -Thiocarbamate-Bearing Paclitaxel Analogues. v. 10, p. 1327–1331, 2000.

10 Liu, H.; Liao, J.; et al. A Highly E ffi cient Approach To Construct (epi) - Podophyllotoxin- 4 - O - glycosidic Linkages as well as Its Application in Concise Syntheses of Etoposide and Teniposide. p. 4–7, 2016.

11 Hagemeister, F.B. Treatment of relapsed aggressive lymphomas : regimens with and without high-dose therapy and stem cell rescue. v. 49, p. 13–20, 2002.

12 Effects, A.; Sinkule, J.A.; et al. Etoposide : A Semisynthetic Epipodophyllotoxin and Use as an Antineoplastic Agent.

13 The New England Journal of Medicine Downloaded from nejm.org at UFRGS on May 11, 2016. For personal use only. No other uses without permission. Copyright © 1991 Massachusetts Medical Society. All rights reserved. 1991.

14 Cersosimo, B.R.J.; Hong, W.K. Epirubicin : A Review of the Pharmacology , Clinical Activity , and Adverse Effects of an Adriamycin Analogue. 2016.

15 Nishikawa, K.; Shibasaki, C.; et al. DBA / 2 , C57BL / 6 (Charles River Japan Corp ., Atsugi , Japan), CDFl5 BDF1 ? ICR (Shizuoka Labora- tory Animal Center , Hamamatsu , Japan), C3H / He and BALB / c-nu / nu mice (Clea Japan Inc ., Tokyo , Japan) and Donryurats (Nippon Rat Co ., Urawa , Japan) were used in experiments at an age of 6 to 7 weeks . Each treatment group was composed of at least 6 animals . (5-FU), and cytosine arabinoside (Ara-Q) were also established by serial in vivo treatment with subtoxic dose of each drug . To compare the growth inhibitory activity of SGLand NKT-01 , we used 14 kinds of tumors shown Antitumor Experiments In Vitro

Growth rate was calculated from the cell number counted by Coulter Counter model ZBI before and. v. 44, n. 10,

16 Hsieh, P.; Hung, C.; et al. Current Prodrug Design for Drug Discovery. p. 2236–2250, 2009.

17 Jana, S.; Mandlekar, S.; et al. Prodrug Design to Improve Pharmacokinetic and Drug Delivery Properties : Challenges to the Discovery Scientists. p. 3874–3908, 2010.

18 Arpicco, S.; Dosio, F.; et al. Anticancer Prodrugs : An Overview of Major Strategies and Recent Devel- opments. p. 2346–2381, 2011.

19 Rautio, J.; Kumpulainen, H.; et al. Prodrugs : design and clinical applications. v. 7, n. march, p. 255–270, 2008.

20 Schuck, C.; Cruz, L.N.; et al. Investigation of antimalarial activity , cytotoxicity and action mechanism of piperazine derivatives of betulinic acid. v. 20, n. 1, p. 29–39, 2015.

21 Innocente, A.; Casanova, B.B.; et al. Synthesis of Isosteric Triterpenoid Derivatives and Antifungal Activity. p. 344–349, 2014.

22 Dalla-vechia, L.; Dassonville-klimpt, A.; et al. The Beckmann Rearrangement Applied to Ursolic Acid with Antimalarial Activity in Medicinal Chemistry Studies. p. 92–95, 2012.

23 Gnoatto, S.C.B.; Susplugas, S.; et al. Pharmacomodulation on the 3-acetylursolic acid skeleton : Design , synthesis , and biological evaluation of novel derivatives as antimalarial agents. v. 16, p. 771–782, 2008.

24 Noatto, S.G.; Uillon, J.G.; et al. X-ray Structure Analysis Online Crystal Structure of 3- O -Acetyloleanolic Acid. v. 24, p. 239–240, 2008.

25 Digital, B. Synthesis and Antiplasmodial Activity of Betulinic Acid and Ursolic Acid Analogues. p. 12003–12014, 2012.

26 Yang, Y.; Wang, Z.; et al. Chemical Constituents of Plants from the Genus Phlegmariurus. v. 11, n. 12, p. 269–274, 2016.

27 Imai, A.; Lankin, D.C.; et al. Cycloartane Triterpenes from the Aerial Parts of Actaea racemosa. 2016.

28 Jia, Z.; Koike, K.; et al. Saponarioside C , the First r - D -Galactose Containing Triterpenoid Saponin , and Five Related Compounds from Saponaria officinalis. p. 449–453, 1999.

29 Sánchez-medina, A.; Stevenson, P.C.; et al. Phytochemistry Triterpenoid saponins from a cytotoxic root extract of Sideroxylon foetidissimum subsp . gaumeri. v. 70, p. 765–772, 2009.

30 Xu, B.; Chu, F.; et al. A Series of New Ligustrazine-Triterpenes Derivatives as Anti-Tumor Agents : Design , Synthesis , and Biological Evaluation. p. 21035–21055, 2015.

31 Zaprutko, B.B.L. Recent advances in synthesis and biological activity of triterpenic acylated oximes. n. 6, p. 203–231, 2015.

32 Parra, A.; Martin-fonseca, S.; et al. European Journal of Medicinal Chemistry

Semi-synthesis of acylated triterpenes from olive-oil industry wastes for the development of anticancer and anti-HIV agents. v. 74, p. 278–301, 2014.

33 Planas, J.M.; Ruiz-gutierrez, V.; et al. Antiproliferative and apoptosis-inducing effects of maslinic and oleanolic acids , two pentacyclic triterpenes from olives , on HT-29 colon cancer cells. p. 36–43, 2008.

34 Reyes-zurita, F.J.; Rufino-palomares, E.E.; et al. Maslinic acid , a natural triterpene from Olea europaea L ., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway. v. 273, p. 44–54, 2009.

35 Reyes-zurita, F.J.; Pachón-peña, G.; et al. The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK- p53-dependent mechanism. 2011.

36 Nitz, T.J.; Wild, C.T.; et al. Alkyl Amine Bevirimat Derivatives Are Potent and Broadly Active HIV-1 Maturation Inhibitors. v. 60, n. 1, p. 190–197, 2016.

37 Coric, P.; Turcaud, S.; et al. European Journal of Medicinal Chemistry Synthesis and biological evaluation of a new derivative of bevirimat that targets the Gag CA-SP1 cleavage site. v. 62, 2013.

38 Ã, M.J.T.; Delancey, J.O.; et al. The global burden of cancer : priorities for prevention. v. 31, n. 1, p. 100–110, 2010.

39 Nacional, I.; Inca, D.C. **A situação do câncer no Brasil**.

40 Shea, M.; Costa, D.B.; et al. Management of advanced non-small cell lung cancers with known mutations or rearrangements : latest evidence and treatment approaches. p. 113–129, 2016.

41 Komninou, D.; Rigas, B. NIH Public Access. v. 71, n. 24, p. 7617–7627, 2012.

42 Gardini, A.C.; Tenti, E.; et al. Multicentric survey on dose reduction / interruption of cancer drug therapy in 12 . 472 patients : indicators of suspected adverse reactions. p. 1–6, 2016.

43 Salihah, N. Chemotherapy-induced nausea and vomiting : exploring patients ' subjective experience. p. 145–151, 2016.

44 Hilarius, D.L.; Kloeg, P.H.; et al. Chemotherapy-induced nausea and vomiting in daily clinical practice : a community hospital-based study. p. 107–117, 2012.

45 Manuscript, A. Treatment of Metastatic Melanoma : An Overview. v. 23, n. 6, p. 488–496, 2010.

46 Newbold, A.; Falkenberg, K.J.; et al. How do tumor cells respond to HDAC inhibition ? p. 1–15, 2016.

47 No Title. v. 5020, p. 1–42,

48 Khan, R.; Ibrahim, H.; et al. Bladder cancer in a young patient : Undiscovered risk factors. p. 3202–3204, 2016.

49 Lin, J.; Feng, N.; et al. Distinct Roles of Type I and Type III Interferons in Intestinal Immunity to Homologous and Heterologous Rotavirus Infections. p. 1–29, 2016.

50 Ramanan, V.; Trehan, K.; et al. Viral genome imaging of hepatitis C virus to probe heterogeneous viral infection and responses to antiviral therapies. v. 494, p. 236–247, 2016.

51 Steininger, C. Europe PMC Funders Group Relative seroprevalence of human herpes viruses in patients with chronic lymphocytic leukaemia. v. 39, n. 6, p. 497–506, 2013.

52 Walton, J.A.; Lydyard, P.M.; et al. Patients with B cell chronic lymphocytic leukaemia have an expanded population of CD4 + perforin expressing T cells enriched for human cytomegalovirus specificity and an effector-memory phenotype. p. 274–284, 2009.

53 Zhang, Q.X.; Li, S.L.; et al. Association between interleukin-21 gene polymorphisms (rs12508721) and HBV-related hepatocellular carcinoma. v. 86, n. 1, p. 1–8, 2016.

54 Abbadessa, G.; Bagella, L.; et al. Viral infections as a cause of cancer (Review). p. 1521–1528, 2007.

55 Liao, J.B. Viruses and Human Cancer. v. 79, p. 115–122, 2006.

56 **No Title**.

57 Aras, B.; Yerlikaya, A. Bortezomib and etoposide combinations exert synergistic effects on the human prostate cancer cell line PC-3. p. 3179–3184, 2016.

58 Rajitha, B.; Nagaraju, G.P.; et al. Novel Synthetic Curcumin Analogs as Potent Antiangiogenic Agents in Colorectal Cancer. n. April, p. 1–12, 2016.

59 Ji, D.; Li, Q.; et al. Thalidomide enhanced the efficacy of CHOP chemotherapy in the treatment of diffuse large B cell lymphoma : A phase II study. 2016.

60 Barnes, E.C.; Davis, R.A. Natural Product Reports The use of isolated natural products as sca ff olds for the generation of chemically diverse screening libraries for drug discovery. p. 372–381, 2016.

61 Yuan, L.; Zhang, C.; et al. Bioorganic & Medicinal Chemistry Letters The semi-synthesis of novel andrographolide analogues and anti-influenza virus activity evaluation of their derivatives. v. 26, p. 769–773, 2016.

62 Schwagerus, S.; Reimann, O.; et al. Semi-synthesis of a tag-free O -GlcNAcylated tau protein by sequential chemoselective ligation §. n. April, 2016.

63 Reimann, O.; Glanz, M.; et al. Bioorganic & Medicinal Chemistry Native chemical ligation between asparagine and valine : Application and limitations for the synthesis of tri-phosphorylated C-terminal tau. v. 23, p. 2890–2894, 2015.

64 Liu, L. **Protein Ligation and Total Synthesis I**.

65 Razani, B.; Fan, D. 1,2* ,. p. 1–38,

66 Swidorski, J.J.; Liu, Z.; et al. Bioorganic & Medicinal Chemistry Letters Inhibitors of HIV-1 maturation : Development of structure – activity relationship for C-28 amides based on C-3 benzoic acid-modified triterpenoids. v. 26, p. 1925–1930, 2016.

67 Ns, G.; Maria, N.R.G.; et al. Two series of new semisynthetic triterpene

derivatives : differences in anti-malarial activity , cytotoxicity and mechanism of action. p. 1–7, 2013.

68 Antitumoral, C.O.M.A.; Antioxidante, A.E.; et al. Revisão. v. 32, n. 5, p. 1245–1252, 2009.

69 Gnoatto, S.C.B.; Dassonville-klimpt, A.; et al. Evaluation of ursolic acid isolated from Ilex paraguariensis and derivatives on aromatase inhibition. v. 43, 2008.

70 Vechia, D.; Médicaments-parasitologie, F.P.P.F.; et al. Synthesis and Preliminary Evaluation of New Ursolic and Oleanolic Acids Derivatives as Antileishmanial Agents. p. 2008, 2008.

71 Rao, S.; Sastry, N.; et al. European Journal of Medicinal Chemistry Synthesis of new secretory phospholipase A 2 -inhibitory indole containing isoxazole derivatives as anti-in fl ammatory and anticancer agents. v. 112, p. 289–297, 2016.

72 Nataraju, A.; Gowda, C.D.R.; et al. Group IIA Secretory PLA 2 Inhibition by Ursolic Acid : A Potent Anti- Inflammatory Molecule. n. Atcc 9637, p. 801–809, 2007.

73 Liebscher, G.; Vanchangiri, K.; et al. Chemico-Biological Interactions In vitro anticancer activity of Betulinic acid and derivatives thereof on equine melanoma cell lines from grey horses and invivo safety assessment of the compound NVX-207 in two horses. v. 246, p. 20–29, 2016.

74 Bache, M.; Bernhardt, S.; et al. Betulinic Acid Derivatives NVX-207 and B10 for Treatment of Glioblastoma — An in Vitro Study of Cytotoxicity and Radiosensitization. n. October, p. 19777–19790, 2014.

75 Sawada, N.; Kataoka, K.; et al. Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice. n. February, p. 1672–1678, 2004.

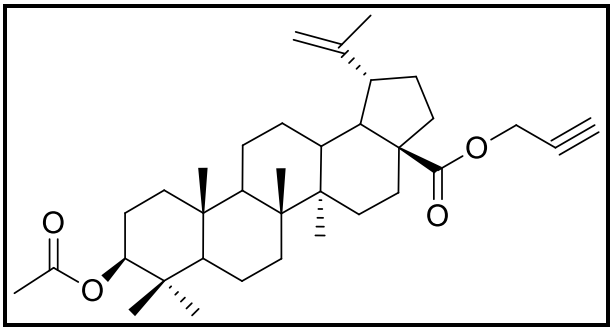
76 Wu, B.; Wang, X.; et al. Ursolic Acid-induced Apoptosis in K562 Cells Involving Upregulation of PTEN Gene Expression and Inactivation of the PI3K / Akt Pathway. v. 35, n. 3, p. 543–548, 2012.

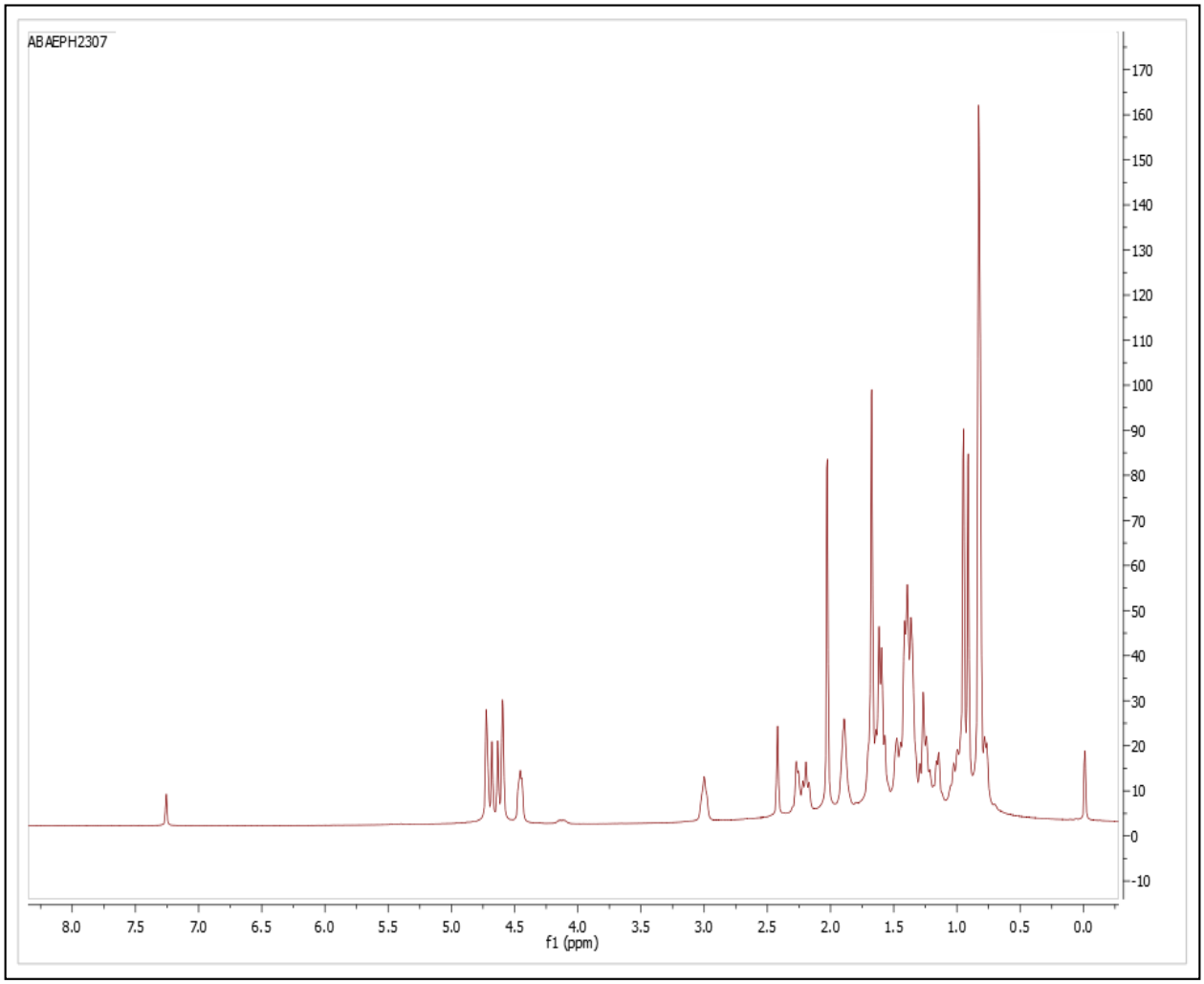
77 Anderson, L.J.; Editors, B.S.G. **Current Topics in Microbiology and Immunology Challenges and Opportunities for Respiratory Syncytial Virus Vaccines**.

78 Gut, A.; Riccetto, L.; et al. Respiratory Syncytial Virus (RSV) in Infants Hospitalized for Acute Lower Respiratory Tract Disease : Incidence and Associated Risks. v. 10, p. 357–361, 2006.

Anexos

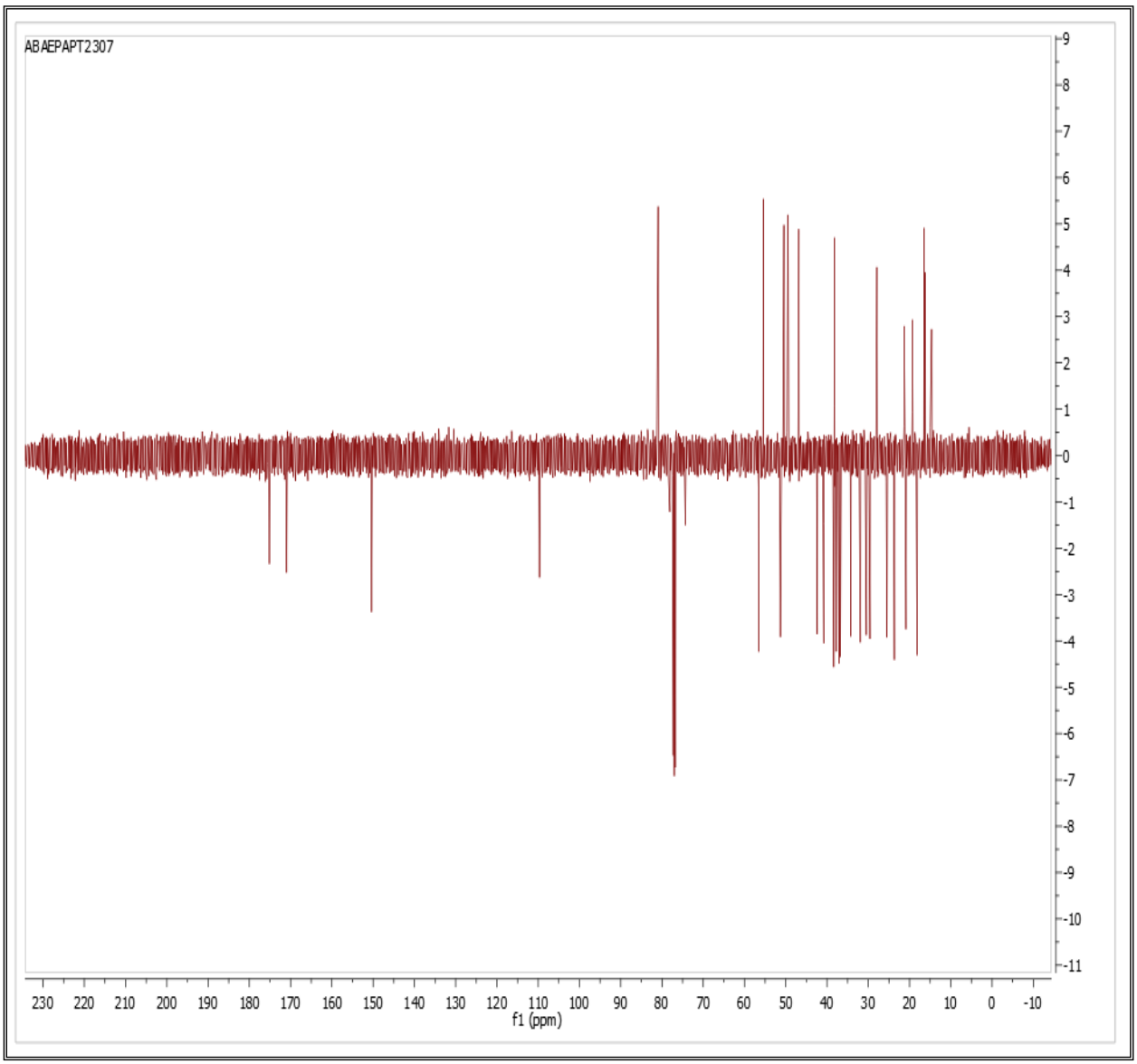
Anexo 1 Espectros de ressonância magnética nuclear de ^{1}H e ^{13}C (APT).



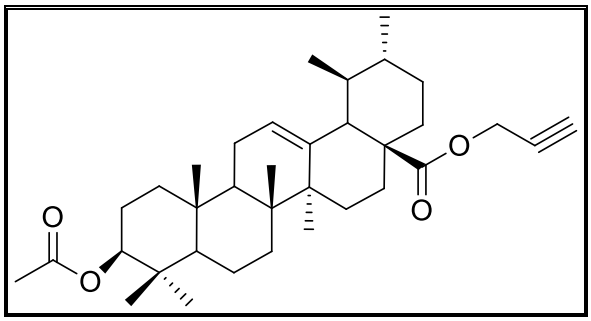


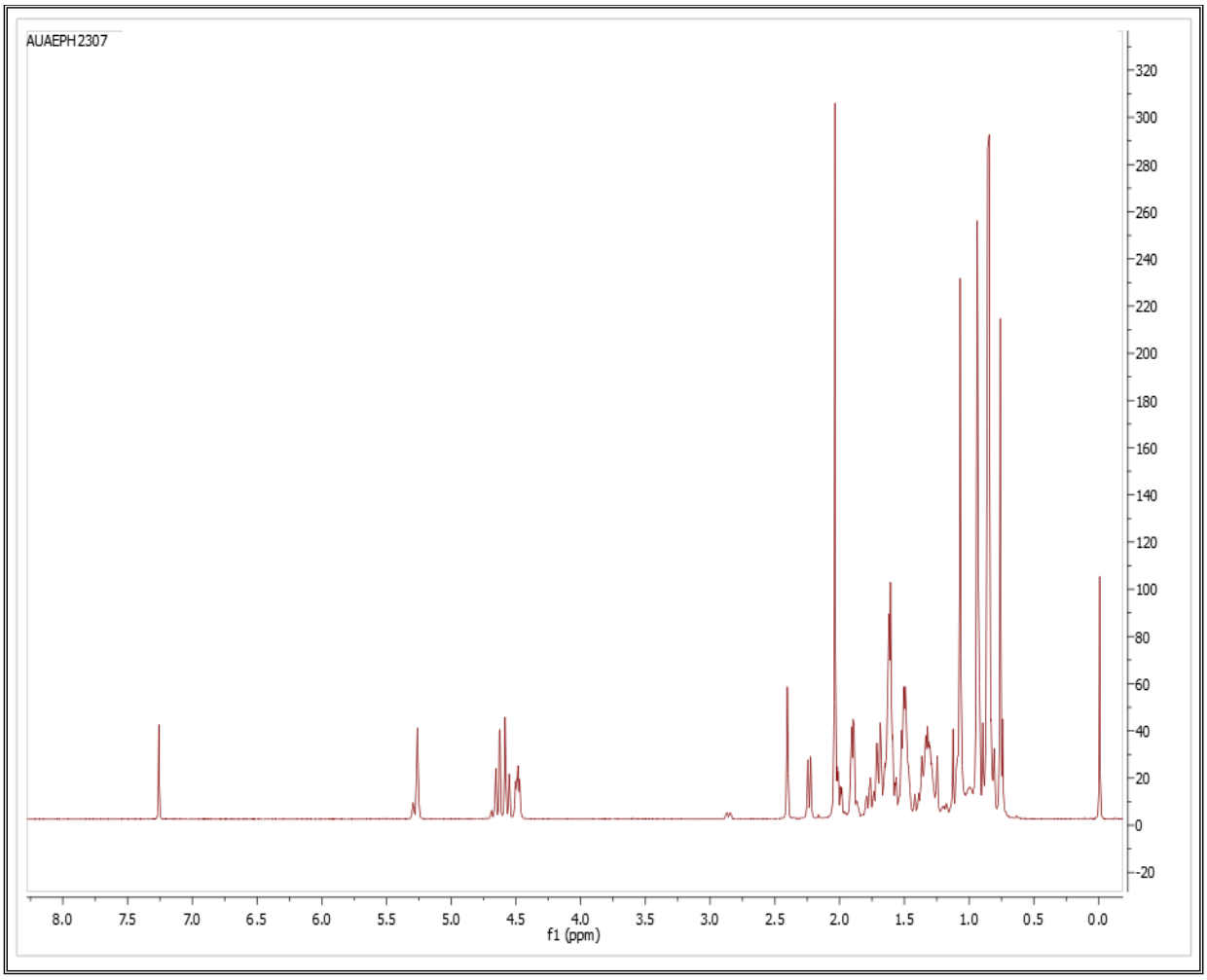
^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) d 0.68 (1H, m, H- 5), 0.74 (3H, s, H-25), 0.81 (3H, s, H-24), 0.89 and 1.62 (1H each, m, H-1), 0.90 (3H, s, H-26), 0.96 (6H, s, H-27 and

H-23), 1.12 and 2.43 (1H each, m, H-11), 1.21 and 1.99 (1H each, m, H-12), 1.25 and 1.90 (1H each, m, H-15), 1.36 and 1.54 (1H each, m, H-6), 1.37 and 2.18 (1H each, m, H-21), 1.38 (1H, m, H-9), 1.39 and 1.43 (1H each, m, H- 7), 2.20 (3H, s, $\underline{H_3C}COO$), 1.44 and 2.22 (1H each, m, H-22), 1.48 and 2.22 (1H each, m, H- 16), 1.62 (1H, m, H-18), 1.68 (3H, s, H-30), 1.78 (2H, m, H-2), 2.42 (1H, m, H-13), 2.43 (1H, s, -$OCH_2C\underline{C}H$), 2.48 (1H, m, H-19), 4.45 (1H, dd, H-3), 4.60 and 4.63 (1H each, s, H-29), 4.72 (2H, m, -$O\underline{C}H_2$-).

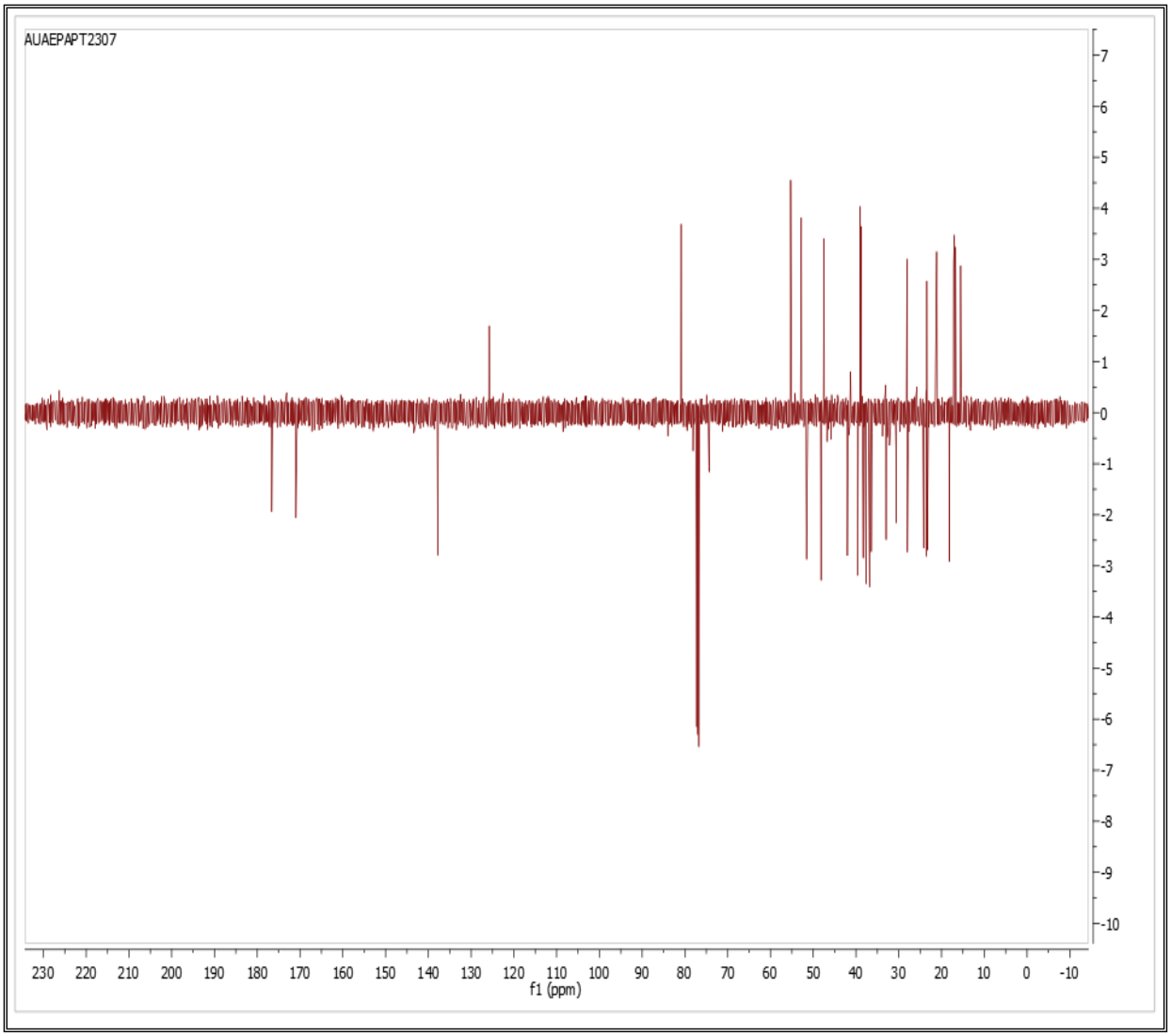


^{13}C NMR (100 MHz, $CDCl_3$): 14.55, 15.99, 16.18, 16.46, 18.16, 19.33, 20.85, 21.31, 23.68, 25.47, 27.92, 29.56, 30.48, 31.92, 34.24, 36.77, 37.09, 37.77, 38.29, 38.39, 40.80, 42.37, 46.85, 49.47, 50.46, 51.31, 55.41, 56.37, 74.31, 78.12, 80.91, 109.68, 150.37, 170.98, 175.15.

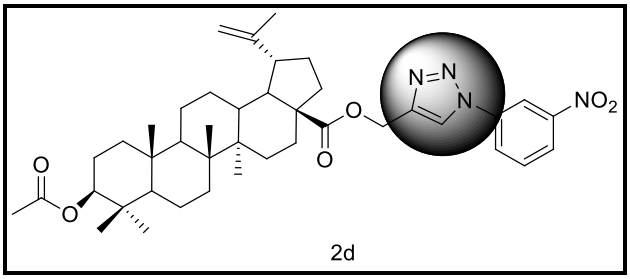




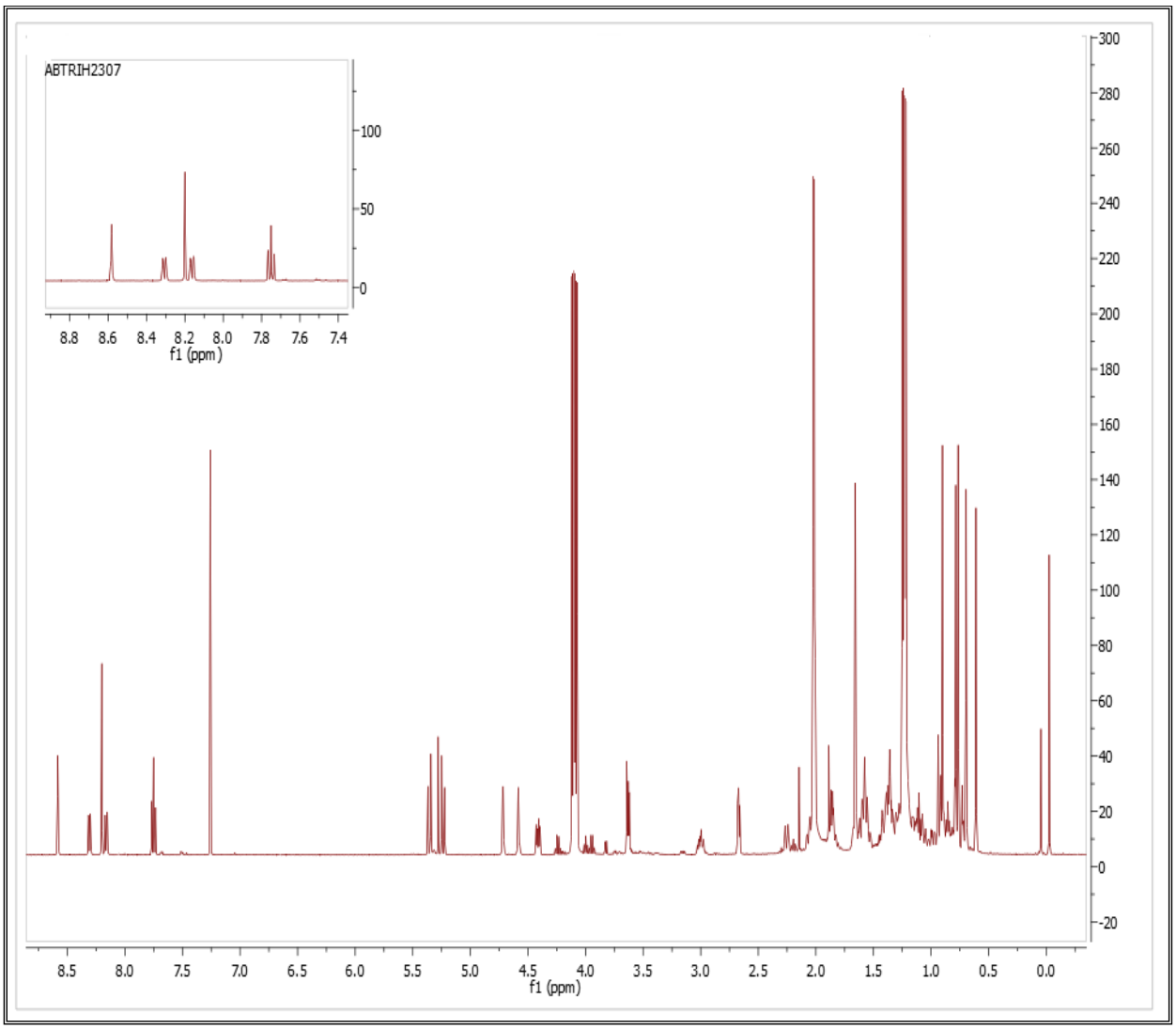
^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) 0.83 (1H, s, H-25), 0.86 (1H, H-5), 0.92 (3H, s, H-24), 0.93 (3H, s, H-23), 0.94 (3H, d, H-30), 0.95 (1H, H-1), 1.05 (3H, d, H-29), 1.09 (3H, s, H-26), 1.12 (3H, s H-27), 1.32 (1H, H-19), 1.39 (4H, m, H-2 and H-26), 1.59 (6H, m, H-1, H-7, H-9, H-15, H-16 and H-21), 1.68 (1H, dt, H-6), 1.72 (1H, H-16), 1.91 (2H, H-22), 2.04 (1H, dt, H-15), 2.03 (3H, s,$\underline{H_3C}COO$), 2.13 (1H, d, H-18),2.51(1H, s, -OCH_2CCH) 4.45 (1H, dd, H-3), 4.68 (2H, m, $O\underline{CH_2}$),5.26 (1H, tl,H-12).



^{13} CNMR (125 MZH $CDCl_3$): 15.53, 16.72, 17.00, 17.17, 18.19, 21.14, 21.29, 23.29, 23.44, 23.59, 24.17, 27.98, 28.06, 30.60, 32.97, 36.41, 36.84, 37.66, 38.31, 38.9, 39.04, 39.41, 42.07, 47.47, 48.15, 51.55, 52.80, 55.28, 74.35, 78.10, 80.90, 125.74, 137.49, 170.98, 176.62.

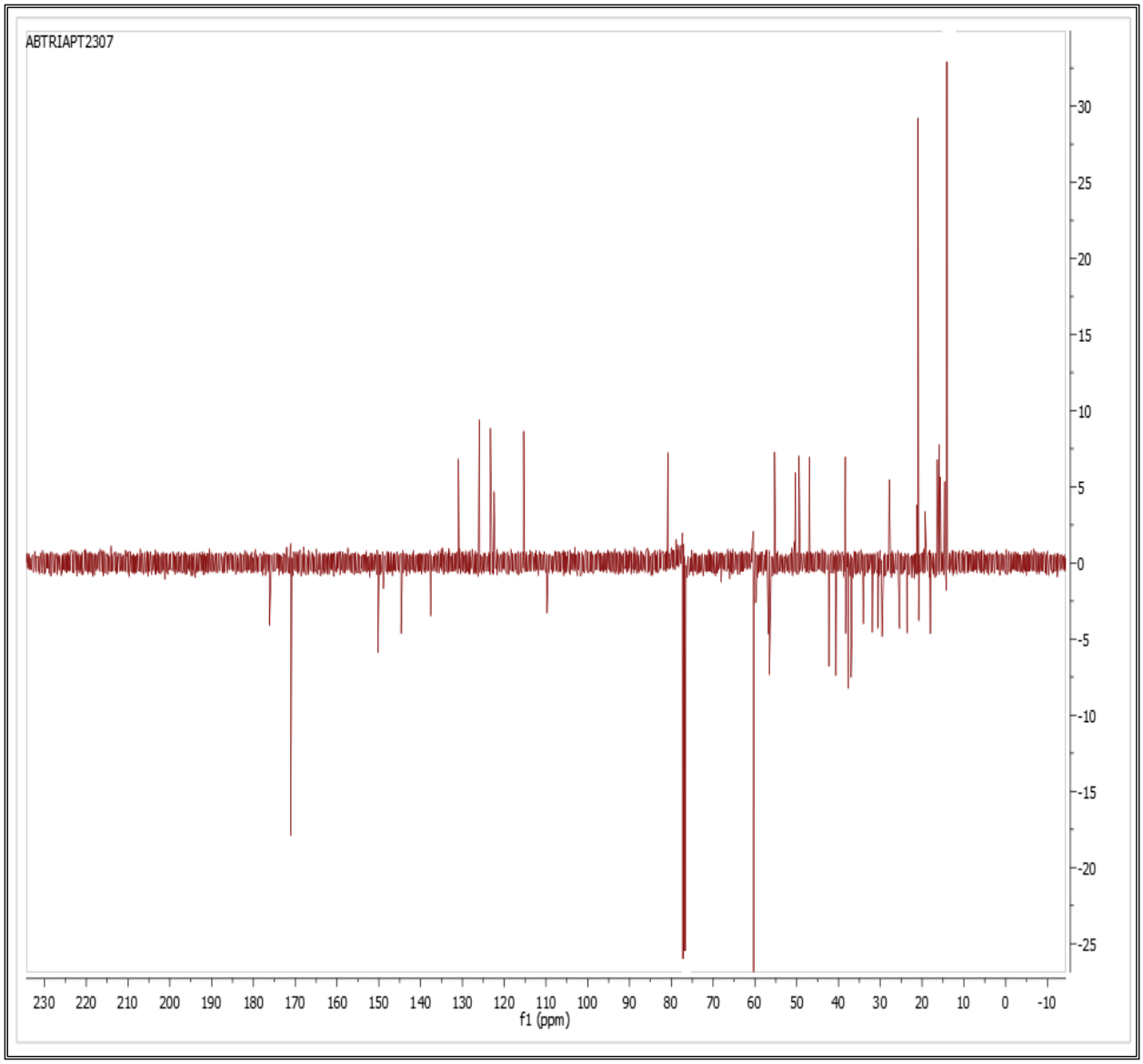


2d

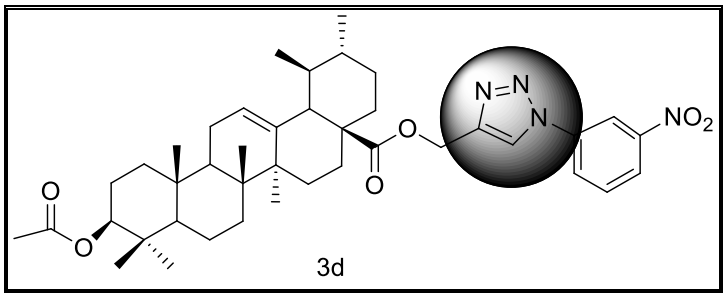


^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) d 0.69 (1H, m, H- 5), 0.77 (3H, s, H-25), 0.81 (3H, s, H-24), 0.89 and 1.62 (1H each, m, H-1), 0.90 (3H, s, H-26), 0.96 (6H, s, H-27 and H-23), 1.18 and 2.46 (1H each, m, H-11), 1.21 and 1.99 (1H each, m, H-12), 1.25 and 1.90 (1H each, m, H-15), 1.36 and 1.54 (1H each, m, H-6), 1.37 and 2.18 (1H each, m, H-21), 1.38 (1H, m, H-9), 1.39 and 1.43 (1H each, m, H- 7), 2.22 (3H, s, $\underline{H_3C}COO$), 1.44 and 2.22 (1H each, m, H-22), 1.48 and 2.22 (1H each, m, H- 16), 1.62 (1H, m, H-18), 1.68 (3H, s, H-30), 1.78 (2H, m, H-2), 2.42 (1H, m, H-13), 2.43 (1H, s, -O$\underline{CH_2}$CCH-

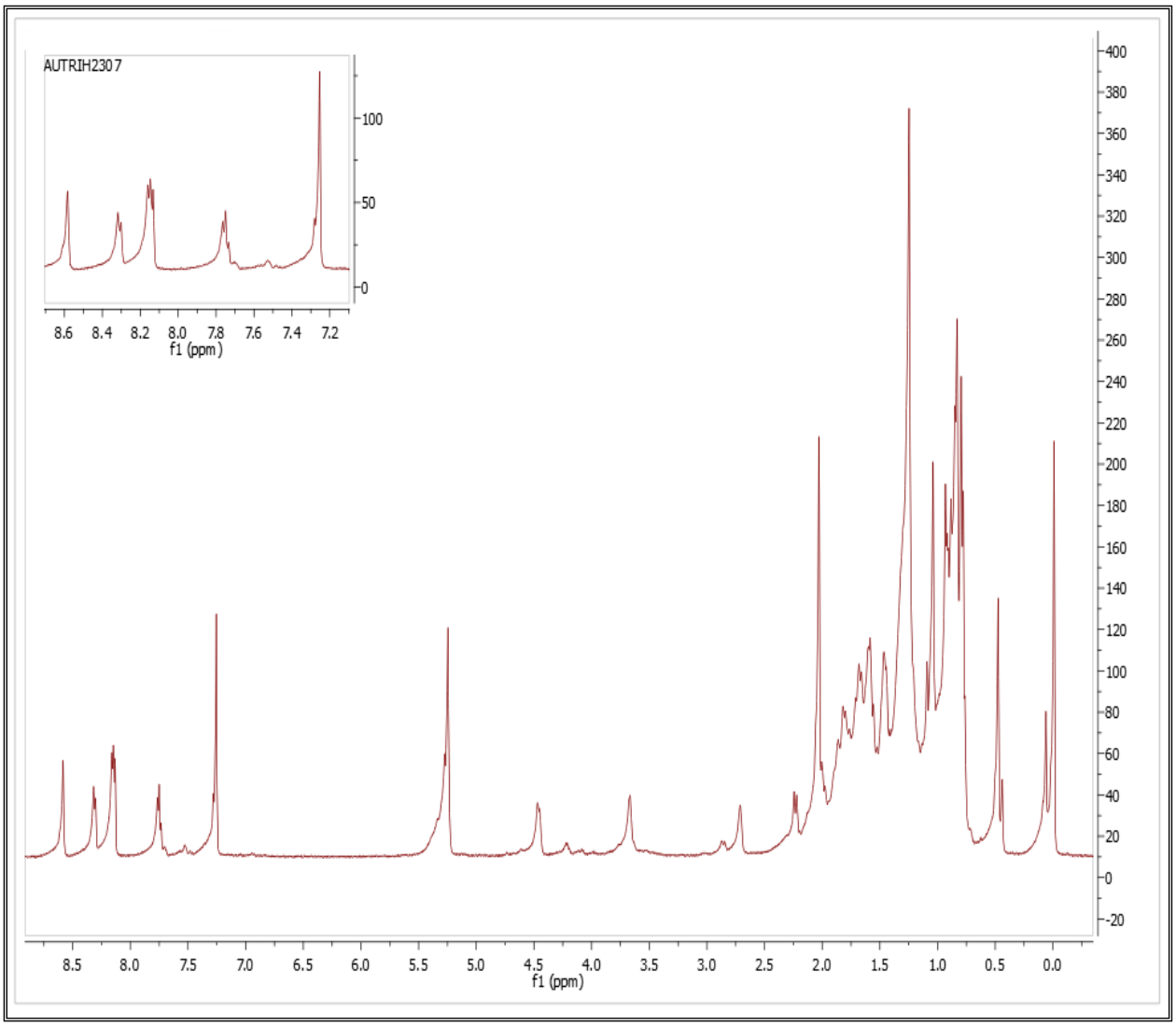
), 2.48 (1H, m, H-19), 4.45 (1H, dd, H-3), 4.59 and 4.71 (1H each, s, H-29), 7.26 (1H,s, NCH), 8.15 (1H,d, Ar-H), 8.20 (1H, s ,Ar-H),8.32 (1H, d ,Ar-H), 8.58 (1H, t, Ar-H).



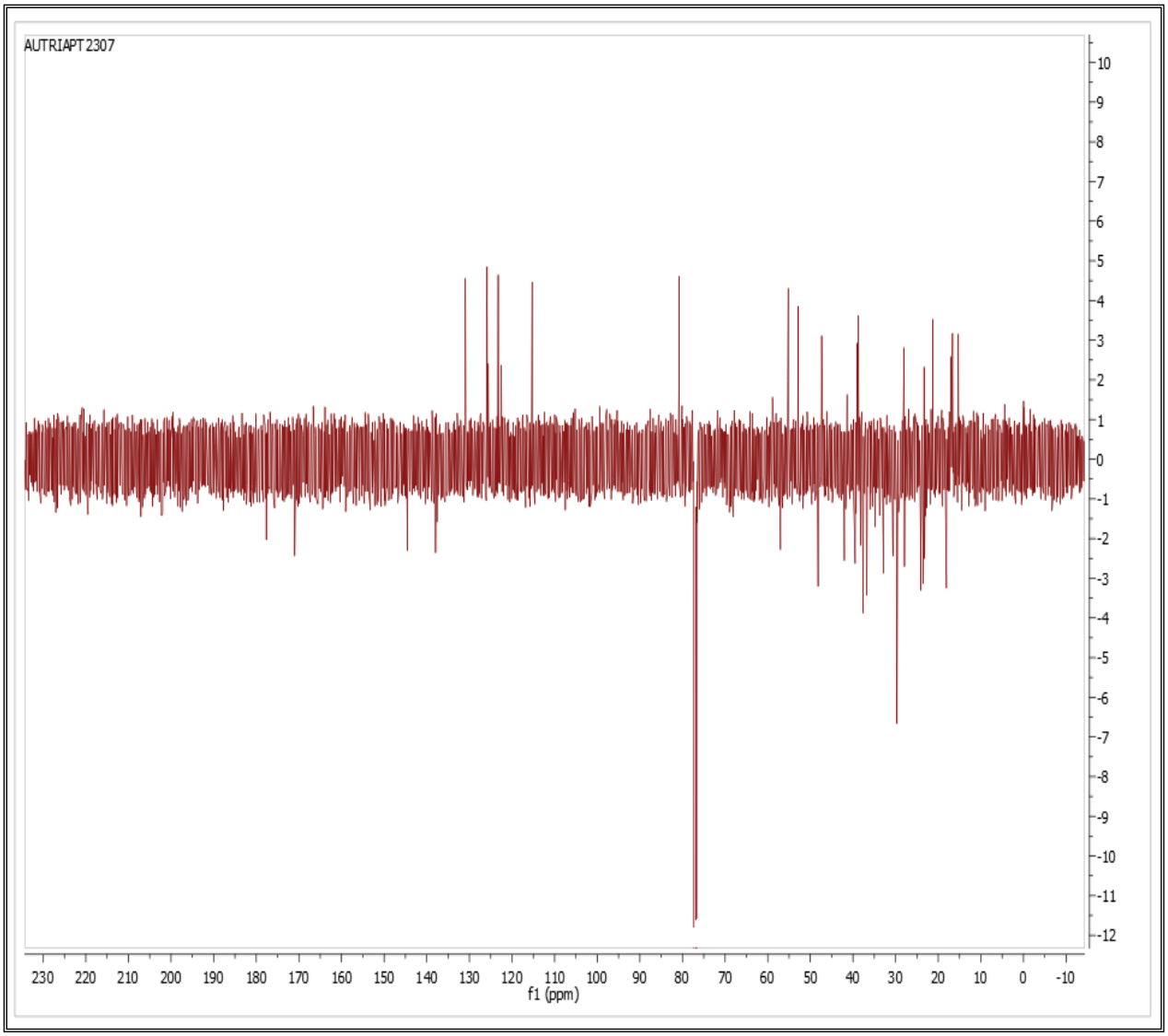
^{13}C NMR (125 MZH $CDCl_3$):15.63, 15.96, 16.38, 18.02, 19.25, 20.81, 20.99, 21.25, 23.60, 25.40, 27.84, 29.65, 30.53, 31.90, 34.07, 36.80, 37.00, 38.31, 38.36, 40.64, 42.28, 46.97, 49.46, 50.32, 55.32, 56.79, 59.78, 80.82, 82.7, 109.75, 115.33, 121.43, 123.29, 125.93, 130.95, 137.58, 144.65, 148.95, 150.21, 171.08, 176.16.



3d

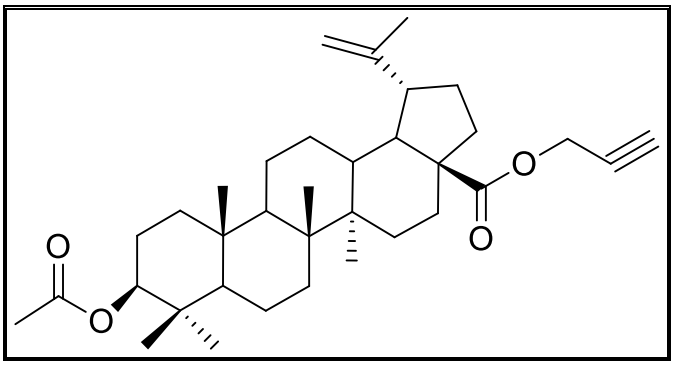


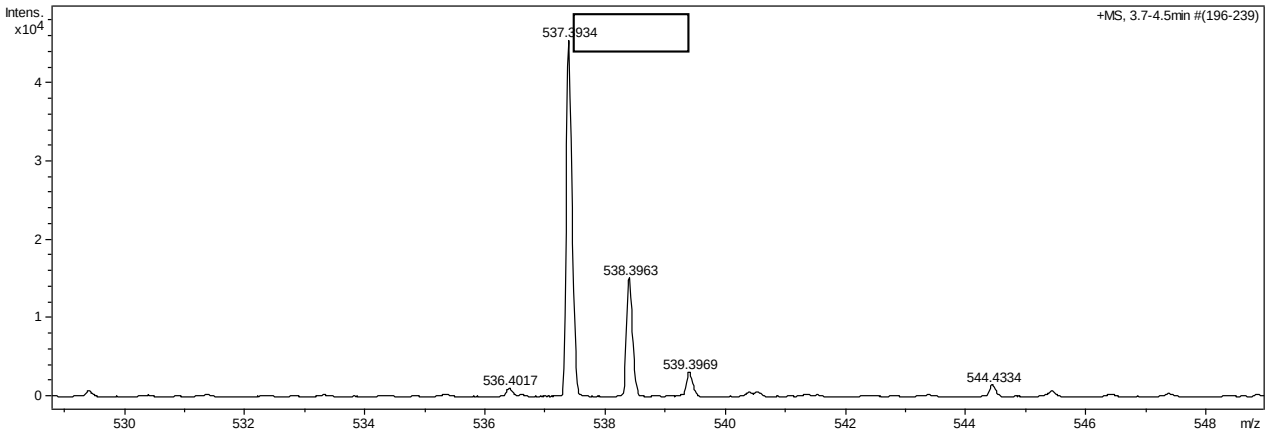
^{1}H NMR (500MHz, $CDCl_3$): δ (ppm) 0.85 (1H, s, H-25), 0.88 (1H, H-5), 0.92 (3H, s, H-24), 0.93 (3H, s, H-23), 0.94 (3H, d, H-30), 0.95 (1H, H-1), 1.05 (3H, d, H-29), 1.09 (3H, s, H-26), 1.12 (3H, s H-27), 1.32 (1H, H-19), 1.39 (4H, m, H-2 and H-26), 1.59 (6H, m, H-1, H-7, H-9, H-15, H-16 and H-21), 1.68 (1H, dt, H-6), 1.78 (1H, H-16), 1.96 (2H, H-22), 2.04 (1H, dt, H-15), 2.25 (3H, s,H_3CCOO), 2.13 (1H, d, H-18), 4.45 (1H, dd, H-3), 5.28 (1H, tl,H-12), 7.25 (1H, s, NCH),7.7 (1H, t, Ar-H), 8.1 (1H, d, Ar-H), 8.3(1H, d, Ar-H), 8.58 (1H, S, Ar-H)

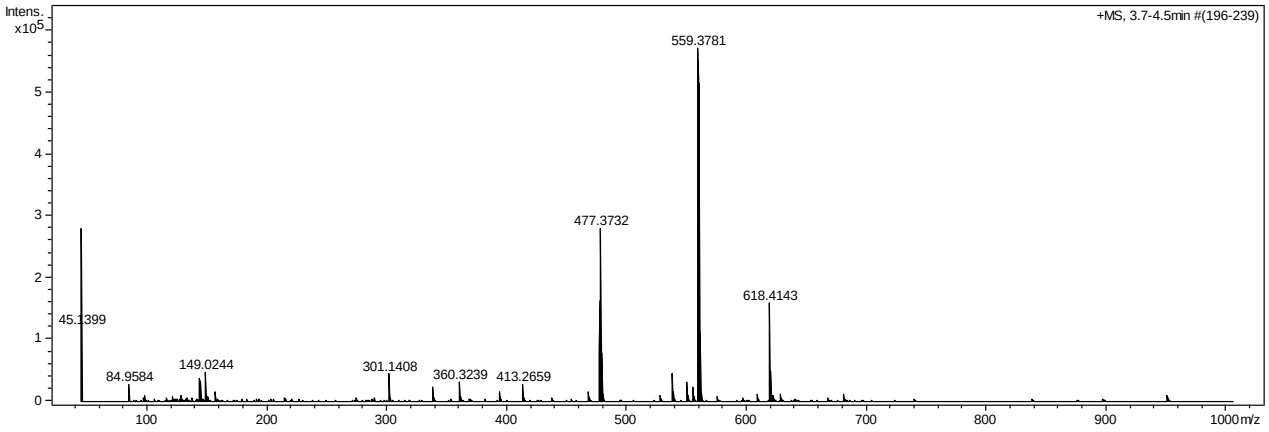


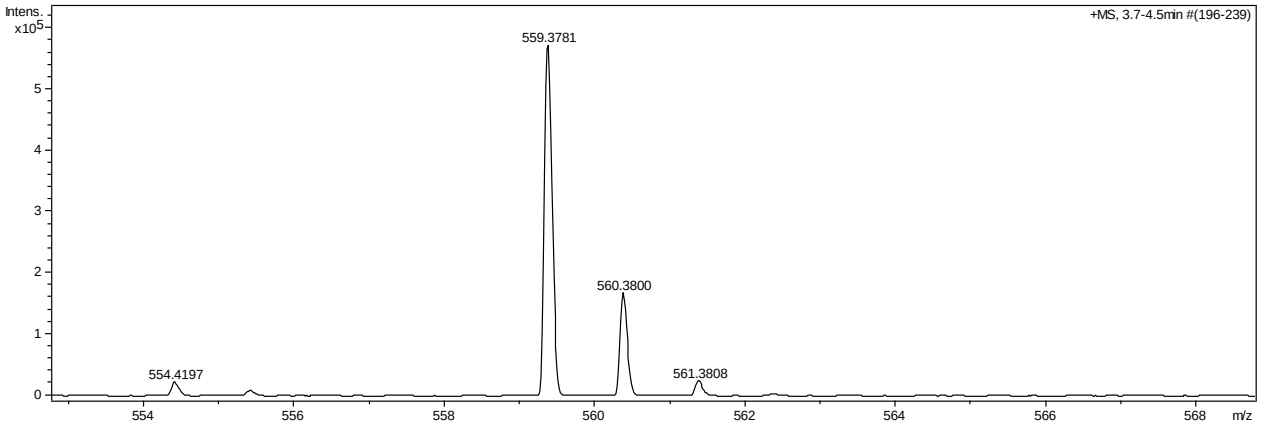
^{13} CNMR (125 MZH $CDCl_3$): 15.29, 16.65, 16.77, 16.99, 18.12, 21.13, 21.28, 23.20, 23.49, 24.13, 27.92, 28.02, 29.68, 30.57, 30. 64, 32.84, 36.77, 37.61, 38.25, 38.79, 39.05, 39.51, 42.03, 47.39, 48.18, 52.87, 55.19, 80.84, 82.33, 109.91, 115.29, 122.52, 123.27, 125.63, 125.85, 139.95, 137.93, 144.53, 148.7, 170.89, 177.63.

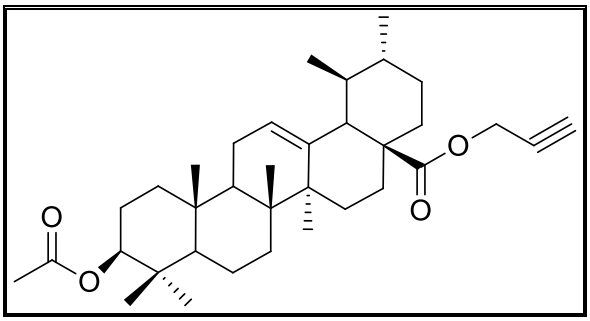
Anexo 2. Espectros de Massas (**amostras dissolvidas em CH_2Cl_2 e diluída em MeOH 0.1% ácido fórmico).**

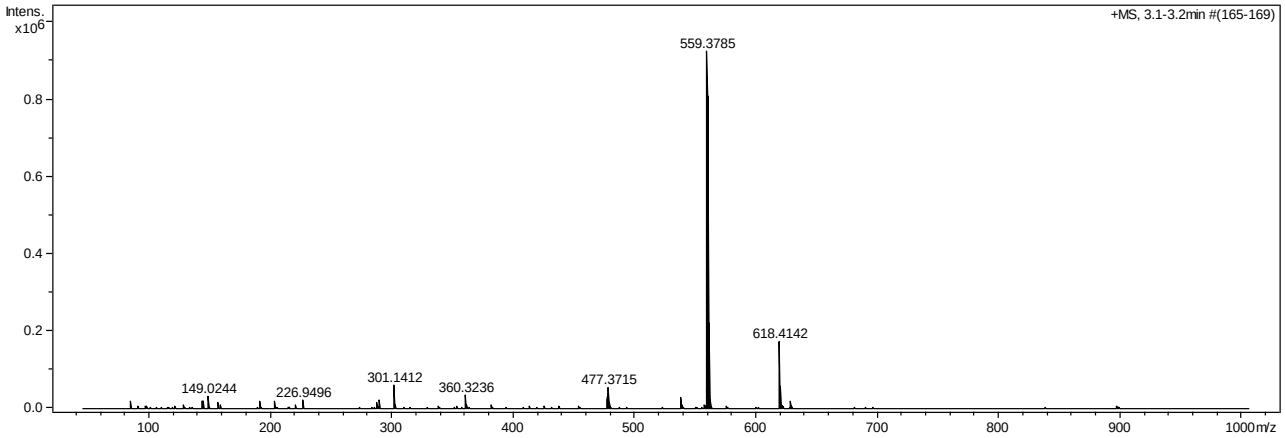

O
O
O
O

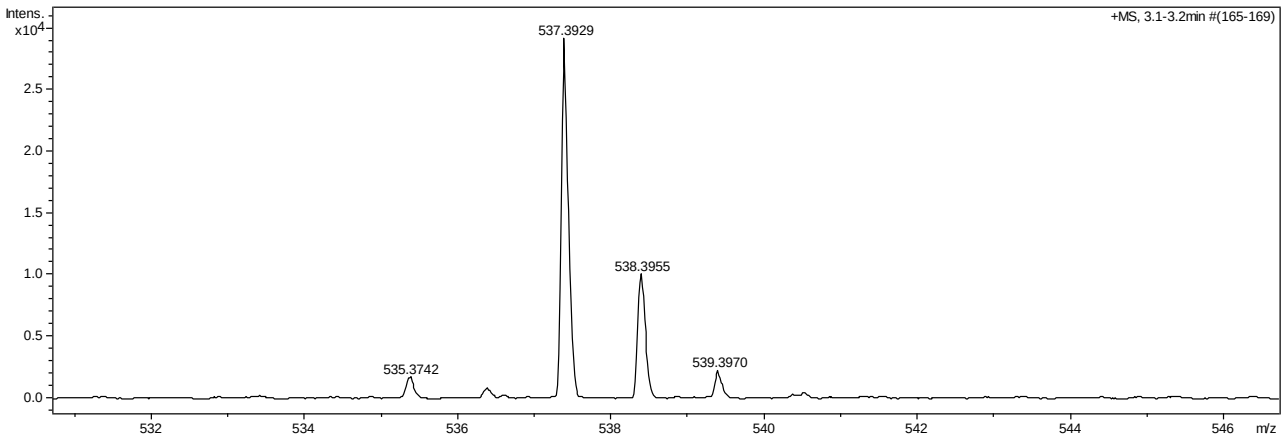

Intens.
x10^4
+MS, 3.7-4.5min #(196-239)
537.3934
538.3963
539.3969
536.4017
544.4334
m/z

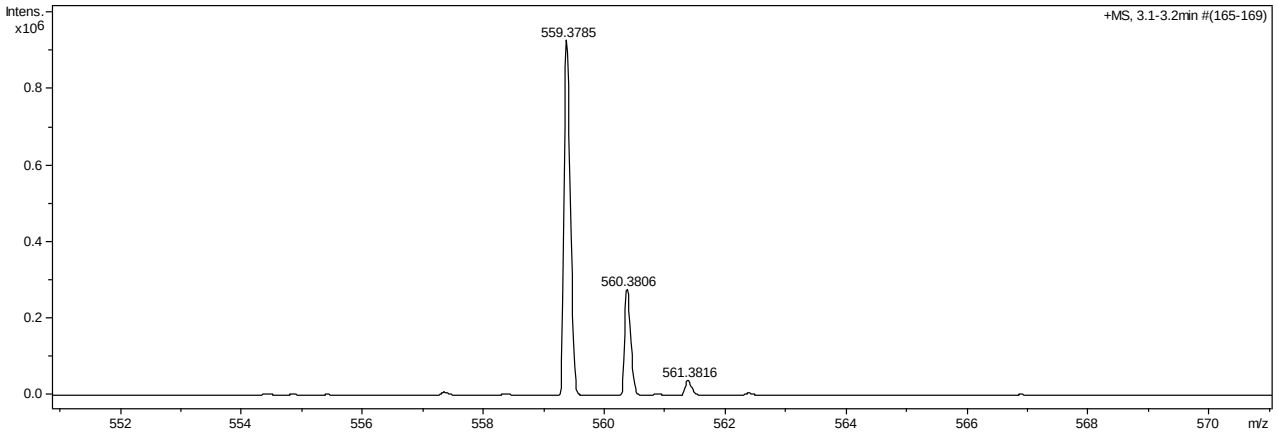

Intens.
x10^5
+MS, 3.7-4.5min #(196-239)
559.3781
477.3732
618.4143
45.1399
84.9584
149.0244
301.1408
360.3239
413.2659
m/z

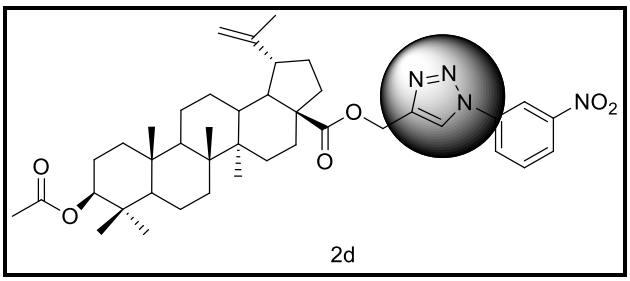

Intens.
x10^5
+MS, 3.7-4.5min #(196-239)
559.3781
560.3800
554.4197
561.3808
m/z


O
O
O
O

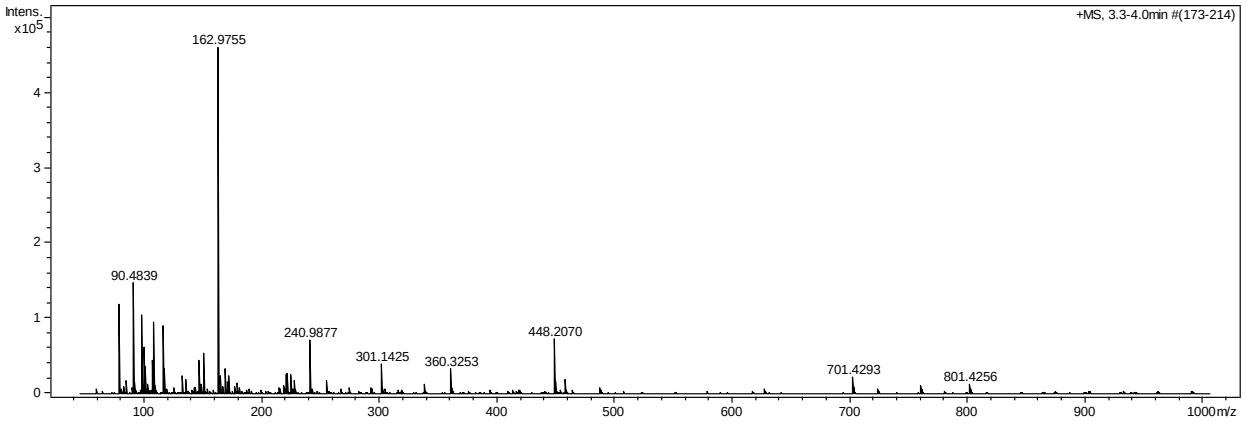

Intens.
x10^6
+MS, 3.1-3.2min #(165-169)
559.3785
618.4142
149.0244
226.9496
301.1412
360.3236
477.3715
m/z

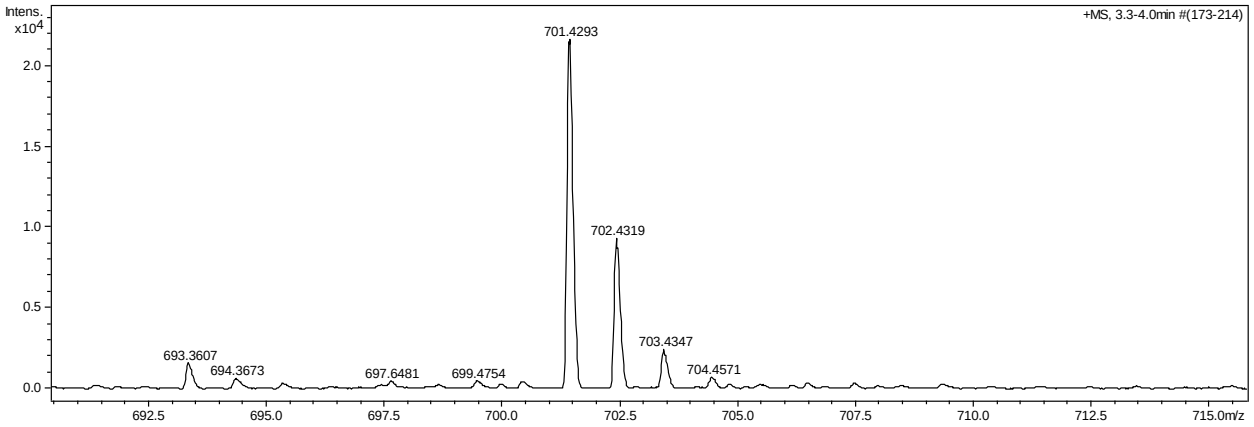

Intens.
x10^4
+MS, 3.1-3.2min #(165-169)
537.3929
538.3955
535.3742
539.3970
m/z

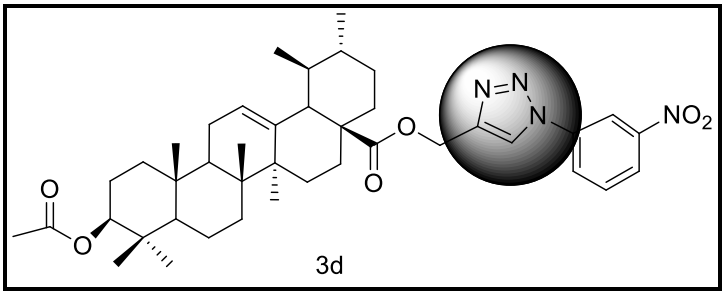

Intens.
x10^6
+MS, 3.1-3.2min #(165-169)
559.3785
560.3806
561.3816
m/z


N=N
N
NO2
O
O
O
O

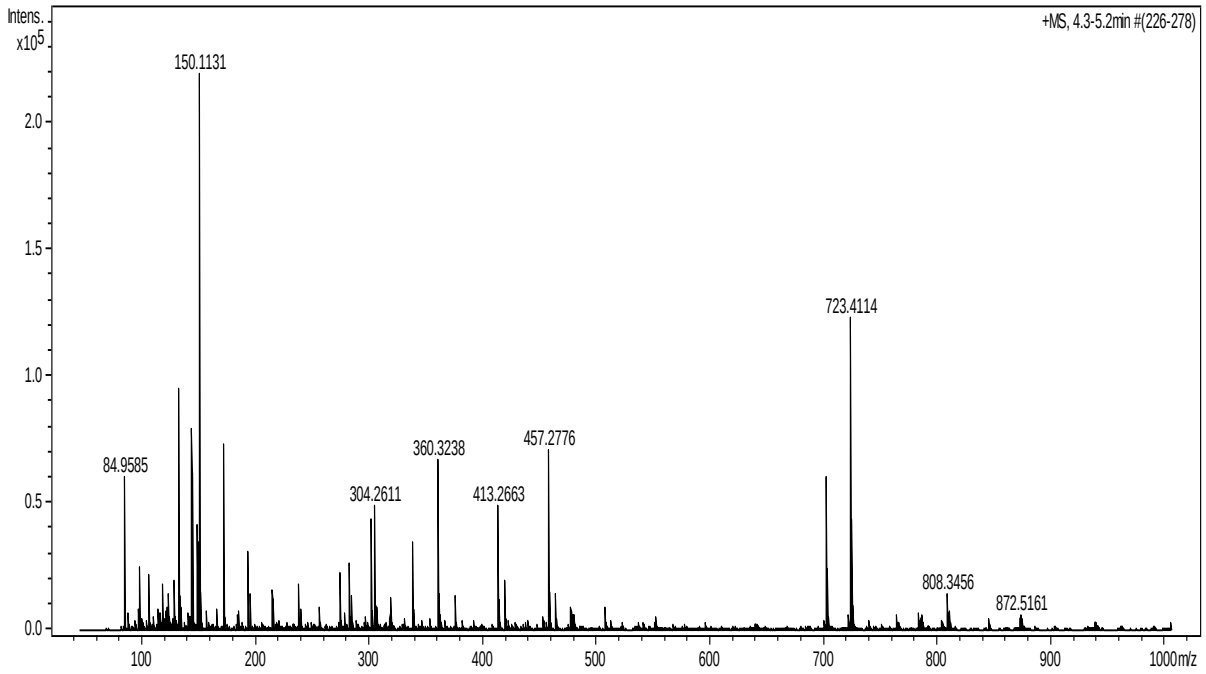
2d

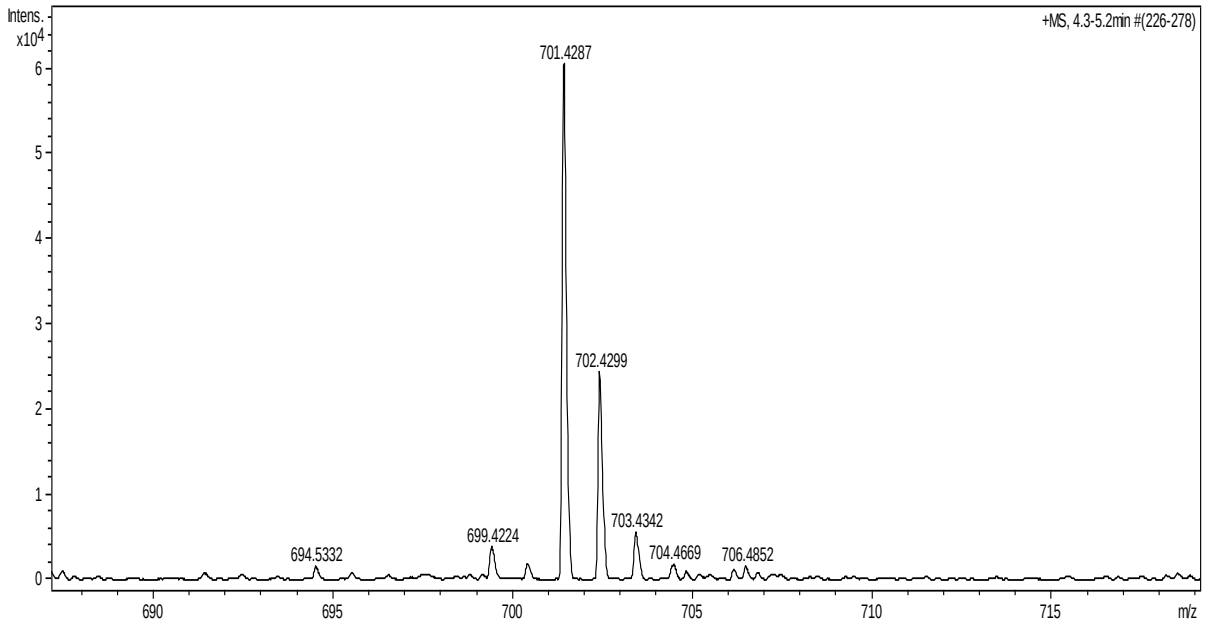

Intens.
x10^5
+MS, 3.3-4.0min #(173-214)
162.9755
90.4839
240.9877
301.1425
360.3253
448.2070
701.4293
801.4256
m/z

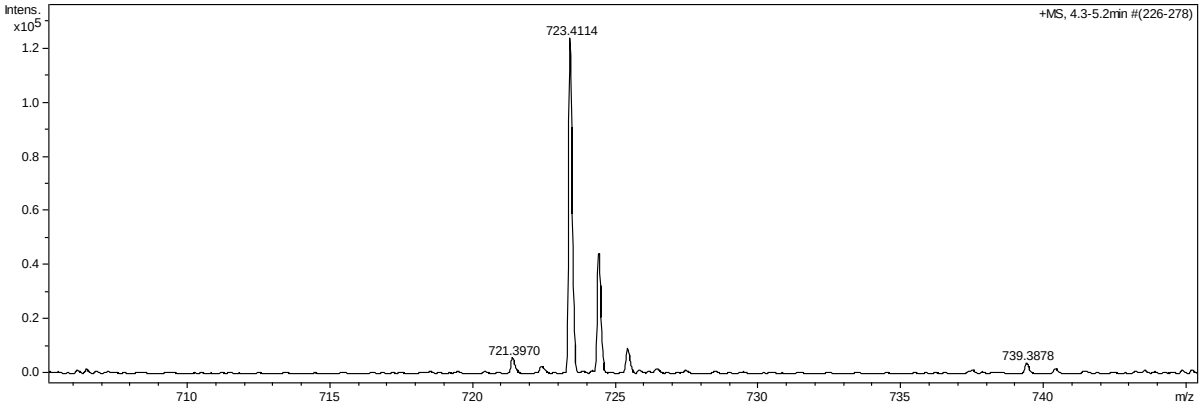

Intens.
x10^4
+MS, 3.3-4.0min #(173-214)
701.4293
702.4319
703.4347
693.3607
694.3673
697.6481
699.4754
704.4571
m/z


N=N
N
NO2
O
O
O
O

3d


Intens.
x10^5
+MS, 4.3-5.2min #(226-278)
150.1131
723.4114
84.9585
360.3238
457.2776
304.2611
413.2663
808.3456
872.5161
m/z


Intens.
x10^4
+MS, 4.3-5.2min #(226-278)
701.4287
702.4299
703.4342
699.4224
694.5332
704.4669
706.4852
m/z


Intens.
x10^5
+MS, 4.3-5.2min #(226-278)
723.4114
721.3970
739.3878
1.2
1.0
0.8
0.6
0.4
0.2
0.0
710
715
720
725
730
735
740
m/z