Valley International Journals
Open Access Journal

International Journal Of Medical Science And Clinical Inventions
Volume3 issue 11 2016 page no. 2372-2378 e-ISSN: 2348-991X p-ISSN: 2454-9576
AvailableOnlineAt:http://valleyinternational.net/index.php/our-jou/ijmsci

Betulinic acid 2,4-dinitrophenylhydrazone derivatives induce caspases activation in A549 lung cancer cells

Leopoldo Clemente Baratto[a,d], Thomas Müller[c], Brás Heleno de Oliveira[d], Reinhard Paschke[b] and Kranthi Vanchanagiri[b*]

[A]Faculdade de Farmácia, Departamento de Produtos Naturais e Alimentos, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, RJ, Brazil.

[B]Biozentrum, Martin-Luther University, Weinbergweg, 06120, Halle (Saale), Germany.

[C]Department of Internal Medicine IV, Department of Oncology/Hematology, , Martin-Luther University, Ernst-Grube-Straβe, 06120, Halle (Saale), Germany.

[D]Laboratório de Produtos Naturais e Biotransformação, Departamento de Química, Universidade Federal do Paraná, Centro Politécnico, 81531-970, CP 19081, Curitiba, PR, Brazil.

***Corresponding author:** Kranthi Vanchanagiri - Biozentrum, Martin-Luther-Universität, Weinbergweg 22, 06120, Halle (Saale), Germany.

Abstract:** As previously reported, a series of Betulinic acid (1) derivatives containing 2,4-dinitrophenylhydrazone (2,4-DNPH) moiety was synthesized and evaluated for their anticancer potential. Aldehyde 2 was the most cytotoxic compound (IC_{50} 1.76-2.51 µM), while 3 was the most selective compound (5-7 times) towards cancer cells. 2,4-DNPH derivatives were able to arrest G0/G1 phase of the cell cycle of A549 cell line. In this report, Caspases-3, -8 and -9 activation was assayed in order to contribute to the elucidation of the mechanism of action of these Betulinic acid 2,4-DNPH derivatives. A549 lung cancer cells were treated with IC_{80} concentrations of compounds 1, 2 and 3 during 24, 48 and 72 h. Cells ($1.0x10^6$ cell/mL) were incubated with caspase-3, -8 and -9 inhibitors and analyzed by flow cytometry. Compound 1 activated around 90% caspases-3 and -8 after 24 h treatment. After 48 h, all caspases were practically 100% activated. Compounds 2 and 3 did not activate significantly any caspases after 24 h. Compound 2 was able to activate significantly caspases (ca. 50 %) only after 72 h. 3 substantially activated caspases-3 and -8 after 48 h, but after 72 h activation was up to 75%. 2,4-DNPH moiety plays an important role for the cytotoxicity of these molecules and also for the activation of caspases and apoptosis induction. By another hand, the introduction of 2,4-DNPH moiety in 1 altered significantly the kinetics of the molecules, since the mechanism of action of the derivatives was slower than the precursor.

Keywords: natural products, pentacyclic triterpenes, A549 cell line, cell death, apoptosis, caspases

I. Introduction

The biological properties of pentacyclic triterpenes (PT) have attracted great interest (Connolly & Hill, 2001; Cichewicz & Kouzi, 2004). One important example is the lupane-type PT betulinic acid (3*β*-hydroxylup-20(29)-en-28-oic acid, **1**). It is one of the most largely spread triterpenes in the Plant Kingdom (Frighetto et al., 2005), sometimes reaching concentrations higher than 2% (Galgon et al., 1999; Jäger et al. 2009).

DOI:10.18535/ijmsci/v3i11.5

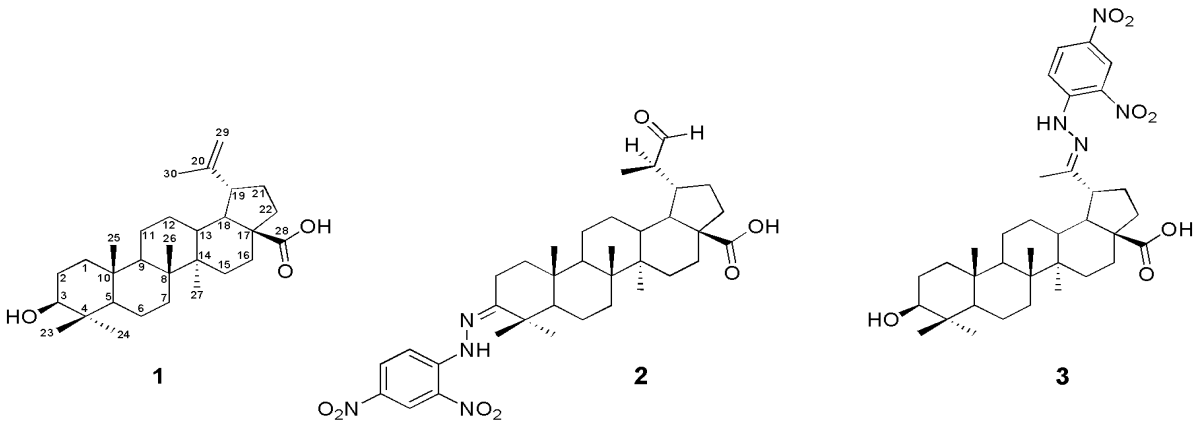

Figure 1. Structures of betulinic acid (**1**), 3-[(2,4-dinitrophenyl)hydrazono]-(20*R*)-29-oxolupan-28-oic acid (**2**) and 3-hydroxy-20-[(2,4-dinitrophenyl)hydrazono]-29-norlupan-28-oic acid (**3**).

Betulinic acid has a number of pharmacological activities, including anti-inflammatory, antibacterial, antimalarial, antischistosomal, analgesic, anti-HIV and antitumor properties (Fujioka et al., 1994; Bringmann et al., 1997; Cichewicz & Kouzi 2004; Yogeeswari & Sriram 2005; Nyasse et al. 2009; Domínguez-Carmona et al., 2010; Spivak et al. 2014). From the beginning of 1990 the anticancer potential of **1** started to be investigated (Pisha et al., 1995; Dzubak et al., 2006) and it was observed that it possessed a large spectrum of activities against cancer cells, such as melanoma, ovary, breast, lung, thyroid, colon, etc., acting in micromolar concentrations with high selectivity (Zuco et al., 2002; Rzeski et al. 2006; Kessler et al., 2007; Kommera et al., 2010a,b; Kommera et al., 2011). **1** is able to alter the mithocondrial membrane permeability, losing its membrane potential and releasing pro-apoptotic factors, triggering a cascade of events that will activate caspases and DNA fragmentation (Fulda, 2009; Fulda & Kroemer, 2009; Fulda, 2010).

Apoptosis is an energy-dependent controlled process and generally involves the activation of caspases, a group of cysteine proteases, which trigger a complex cascade of events that carry the cell to death. Apoptotic cells share as main features cell shortening, loss of intercellular adhesion, membrane blebbings, apoptotic bodies, and DNA laddering (Elmore, 2007). Apoptosis is subclassified in two different types, intrinsic and extrinsic ways. By intrinsic way permeability of the outer mitochondrial membrane is altered, leading to the loss of mitochondrial membrane potential, releasing cytochrome c and other pro-apoptotic proteins. Cytochrome c induces the formation of an apoptosome with initiator pro-caspase-9. The active form of caspase-9 cleaves and activates executor caspases-3 and -7, triggering DNA fragmentation process. By another hand, in the extrinsic way extracellular ligands (Fas, TNFα, TRAIL) bind to their respective death receptor located on plasma membrane. Intracellular death domains of these receptors recruit adaptor proteins (i.e. FADD, TRADD, FLIP) and initiator pro-caspase-8 forming the death-inducing signaling complex (DISC). Caspase-8 is activated inside DISC and then it propagates apoptosis directly by cleavage and activation of executor caspases such as caspase-3 (Indran et al., 2011). Additionally, intrinsic and extrinsic ways are cross-linked, since caspase-8 may trigger activation of caspase-9 by cleaving protein Bid to tBid, that induces the releasing of cytochrome c by mitochondria and formation of apoptosome (Nagata, 2000).

In our previous studies, a series of novel betulinic acid 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives was designed and synthesized showing potent anticancer properties. **1** and 2,4-DNPH derivatives were assayed by cytotoxicity, selectivity,

DOI:10.18535/ijmsci/v3i11.5

cell cycle arrest, annexin V and DNA laddering tests. The results showed that some of these derivatives were more cytotoxic and selective towards different cancer cell lines than original compound **1** (IC_{50}= 8.75-14.8 µM), for example compounds **2** (IC_{50}= 1.76-2.51 µM) and **3** (IC_{50}= 7.52-16.9 µM). Compound **2** was able to arrest cell cycle in G0/G1 phase, as well it induced apoptosis as seen in annexin V and DNA laddering assays (Baratto et al., 2013) (Fig. 1).

In order to complement the previous results, in this report we looked for the mechanism of action of apoptosis induction in A549 lung cancer cell line of these new 2,4-DNPH derivatives, contributing to the understanding about the cytotoxic potential and their structure-activity relationship (SAR).

II. Methods

A. General

Betulinic acid **1** was isolated from *Platanus orientalis* outer barks (Draeger et al., 2001) collected from specimens growing in Curitiba-PR, Brazil. Compounds 3-[(2,4-dinitrophenyl)hydrazono]-(20*R*)-29-oxolupan-28-oic acid (**2**) and 3-hydroxy-20-[(2,4-dinitrophenyl)hydrazono]-29-norlupan-28-oic acid (**3**) were synthesized modifying positions C-3 and C-20 of betulinic acid. Synthesis procedures were previously reported (Baratto et al., 2013). Flow cytometry assays were done using FACS Attune Acoustic Focusing Cytometer (Applied Biosystems®).

B. Biological evaluation

B.1. Cell line

A549 lung cancer cell line was maintained as monolayer in RPMI-1640 supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin (100x), at 37ºC, in a humidified atmosphere with 5% CO_2.

B.2. Preparation of test compounds

Stock solutions of test compounds were prepared in DMSO (20 mM) and then diluted with nutrient RPMI-1640 medium. The DMSO concentration was kept below 0.5% which was non-toxic to the cells.

B.3. Apoptosis test-Caspase-3, -8 and -9 activation

$8.0x10^5$ A549 cells were treated with IC_{80} concentrations of compounds **1**, **2** and **3** during 24, 48 and 72 h. Cells were washed twice with 1 mL PBS (with Ca^{2+}/Mg^{2+}). The concentration of the samples was adjusted to $1.0x10^6$ cell/mL and 300 µL were transferred to eppendorf tubes with 1 µL caspase-3, -8 and -9 inhibitors (PromoKine, Germany). The samples were maintained at 37ºC, 5% CO_2, for 1 h. The tubes were centrifuged and the cells washed twice with wash buffer (PromoKine, Germany). The cells were resuspended in 300 µL wash buffer and directly analyzed by flow cytometry recording $1.0x10^4$ events. Staurosporin (0.5 µM) was used as positive control.

III. Results-Caspase induction assay

1 activated around 90% caspases-3 and -8 after 24 h treatment, while caspase-9 was activated more than 50%. After 48 h, all caspases were practically 100% activated. After 24 h, **2** and **3** did not activate significantly any caspases. Compound **2** was not able to activate caspases analyzed after 48 h; a significant activation (ca. 50 %) was observed after 72 h. **3** substantially activated caspases-3 and -8 after 48 h, and after 72 h the activation was up to 75% (Fig. 2, Table 1).

The results showed that all caspases were activated during the experiment period, with higher magnitude of caspases-8 and -3.

IV. Discussion

As previously reported, Betulinic acid 2,4-DNPH derivatives have great cytotoxic potential, with higher selectivity against selected cancer cell lines. These derivatives were able to arrest cell cycle at G0/G1 phase and induce apoptosis towards A549 lung cancer cell line. Interestingly, kinetics of the mechanism of action of

DOI:10.18535/ijmsci/v3i11.5

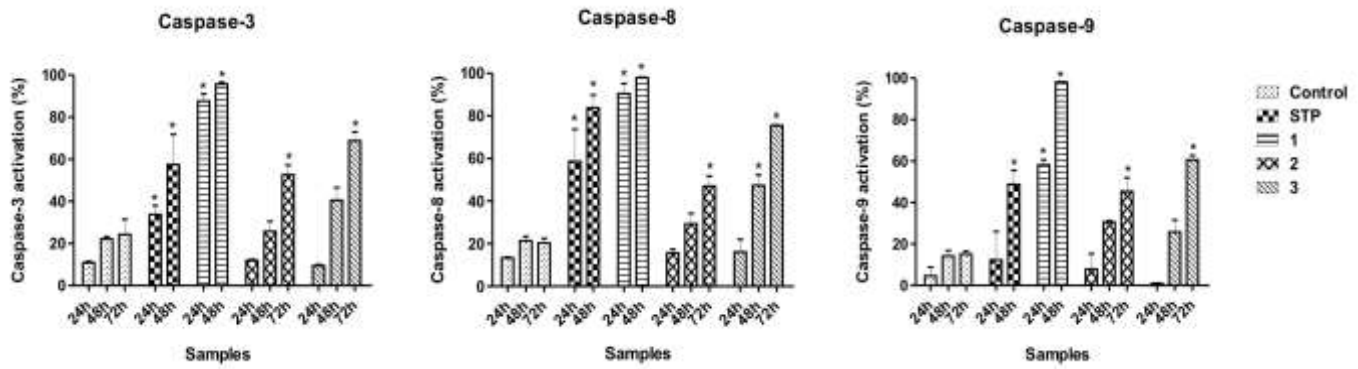

Figure 2. Caspase-3, -8 and -9 activation (%) of A549 cells by **1**, **2** and **3** (IC_{80}) and positive control staurosporin (STP) (0.5 µM). Experiment was conducted during 24, 48 and 72 h by flow cytometry and results were compared to an untreated control. Results are the average±SD of two independent experiments. [*] $p<0.05$.

Table 1. Caspase-3, -8 and -9 activation (%) of A549 cells after treatment with IC_{80} doses of **1** and its derivatives **2** and **3**, and positive control staurosporin (STP) (0.5 µM), during 24, 48 and 72 h by flow cytometry. Results were compared to an untreated control and represent the average±SD of two independent experiments. [*] $p<0.05$.

Caspases	Control	STP	1	2	3
3 – 24h	10.8±0.71	33.8±4.28	87.6±3.44	11.9±0.48	9.44±0.56
3 – 48h	22.1±1.13	57.5±14.3	95.7±0.87	25.7±4.81	40.6±5.98
3 – 72h	24.3±7.23	-	-	52.6±4.57	68.8±4.12
8 – 24h	13.3±0.42	58.6±15.2	90.5±4.74	15.6±1.77	16.1±5.89
8 – 48h	21.4±2.09	83.8±6.06	98.0±0.22	29.3±4.90	47.4±4.87
8 – 72h	20.3±2.20	-	-	47.0±4.77	75.5±0.25
9 – 24h	4.63±4.24	12.3±13.7	58.1±2.48	7.87±7.39	0.95±0.16
9 – 48h	14.2±2.68	48.8±6.73	98.1±0.23	30.6±0.58	25.8±5.78
9 – 72h	15.1±1.36	-	-	45.4±6.55	60.6±2.18

The following caspase inhibitors were used: FITC-DEVD-FMK (caspase-3); FITC-IETD-FMK (caspase-8); Red-LEHD-FMK (caspase-9).

these new betulinic acid derivatives were slower when compared to **1**, evidencing that structural features changed the biological potency (Baratto et al., 2013). Considering these results, it was inferred that the mode of action of **1** and its 2,4-DNPH derivatives **2** and **3** against cancer cells is by caspase-dependent apoptosis induction. However, the different kinetics are clear. The 2,4-DNPH derivatives induced programmed cell death after 48 h, while apoptotic effects of **1** started soon after 24 h treatment. These observations show that structural differences between precursor and derivatives are responsible for such a variable mechanism of action and probably the compounds act differently over plasma membrane.

The interaction of lupane-type pentacyclic triterpenes with phospholipids in cell membranes has been suggested as the most important step of their mechanisms of action (Broniatowski et al., 2012). **1** has a bola-amphiphile structure, i.e. it possesses two hydrophilic groups at both termini of the hydrophobic moiety. It can acquire two different ori-

DOI:10.18535/ijmsci/v3i11.5

entations at the air/water interface and in the membrane environment. In both orientations it can form hydrogen bonds with the membrane phospholipids. This behavior introduces additional disorder into the monolayer. On the other hand, in compounds like lupeol, which has only one orientation, the hydrogen bonds between its C-3 hydroxyl group and phospholipids stabilize the monolayer and decrease the migration of the components, giving a periodic ordination.

Other factors can also influence the passage of a substance through the cell membrane, such as lipophilicity, pH and pKa, molecular weight, stability, etc. More liposoluble substances have a higher partition coefficient (log P) and can cross the lipidic bilayers more easily. The optimal partition coefficient (log P_o) of the drugs is in general between 2 and 7; substances with log P_o higher than 7 could be retained in the lipidic membrane due to their high lipophilicity. Log P of **1** is 7.38, while **2** and **3** have, respectively, 9.13 and 8.77 log P values, accordingly determination by the software ACD/ChemSketch®. This shows that the 2,4-DNPH derivatives are more lipophilic than the precursor.

The 2,4-DNPH derivatives are more lipossoluble, have higher molecular weight and bulky functional groups when compared to **1**. It is likely that 2,4-DNPH derivatives have difficulties to cross the membrane since they have affinity for lipidic substances, contributing with a retention process in the membrane. Another possibility is related to the size and volume of the derivatives, because during the crossing of the membrane the compounds could disorganize the lipidic bilayer and the 2,4-dinitrophenylhydrazone moiety could act like a "hook", retaining the molecules. These evidences could justify the slower mechanism of action of these derivatives in comparison to **1**.

V. Conclusions

In summary, the apoptotic mechanism of action of betulinic acid 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives was evaluated. The biological results showed that the 2,4-DNPH moiety plays an important role for the cytotoxicity of these molecules and also for the activation of caspases and apoptosis induction. The structural features such hydroxyl at C-3 or aldehyde at C-20
changed the biological potential. Although the 2,4-DNPH derivatives are highly cytotoxic and plays a role in apoptosis cascade activation, it is noteworthy that the kinetics of their mechanism of action is slower than the precursor betulinic acid (**1**).

Declaration of Interest

The authors report no declarations of interest. This research was supported by bilateral cooperation between Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and Deutsche Akademische Austauschdienst (DAAD, Germany) that granted a PhD sandwich scholarship to L.C. Baratto.

References

Baratto LC, Porsani MV, Pimentel IC, Pereira-Netto AB, Paschke R, Oliveira BH. (2013). Preparation of betulinic acid derivatives by chemical and biotransformation methods and determination of cytotoxicity against selected cancer cell lines. Eur J Med Chem, 68, 121-31.

Bringmann G, Saeb W, Assi LA, Francois G, Sankara Narayanan AS, Peters K, et al. (1997). Betulinic acid: isolation from Triphyophyllum peltatum and Ancistrocladus heyneanus, antimalarial activity, and crystal structure of the benzyl ester. Planta Med, 63, 255-7.

Broniatowski M, Flasinski M, Wydro P. (2012). Investigation of the interactions of lupane type pentacyclic triterpenes with outer leaflet membrane phospholipids – Langmuir monolayer and synchrotron X-ray scattering study. J Colloid Interf Sci, 381, 116-24.

Cichewicz RH, Kouzi SA. (2004). Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of

DOI:10.18535/ijmsci/v3i11.5

cancer and HIV infection. Med Res Rev, 24, 90-114.

Connolly JD, Hill RA. (2001). Triterpenoids. Nat Prod Rep, 18, 131-47.

Domínguez-Carmona DB, Escalante-Erosa F, García-Sosa K, Ruiz-Pinell G, Gutierrez-Yapu D, Chan-Bacab MJ, et al. (2010). Antiprotozoal activity of betulinic acid derivatives. Phytomedicine, 17, 379-82.

Draeger B, Galgon T, Neubert R, Wohlrab W. (2001). US 6,175,035 B1, Method of producing betulinic acid.

Dzubak P, Hajduch M, Vydra D, Hustova A, Kvasnica M, Biedermann D, et al. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications. Nat Prod Rep, 23, 394-411.

Elmore S. (2007). Apoptosis: a review of programmed cell death. Toxicol Pathol, 35, 495-516.
Frighetto N, Welendorf RM, Silva AMP, Nakamura MJ, Siani AC. (2005). Purification of betulinic acid from Eugenia florida (Myrtaceae) by High-speed Counter-current Chromatography. Phytochem Analysis, 16, 411-4.

Fujioka T, Kashiwada Y, Kilkuskie RE, Cosentino LM, Ballas LM, Jiang JB, et al. (1994). Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from Syzigium claviflorum, and the anti-HIV activity of structurally related triterpenoids. J Nat Prod, 57, 243-7.

Fulda S. (2009). Betulinic acid: a natural product with anticancer activity. Mol Nutr Food Res, 53, 140-6.

Fulda S. (2010). Modulation of apoptosis by natural products for cancer therapy. Planta Med, 76, 1075-9.

Fulda S, Kroemer G. (2009). Targeting mitochondrial apoptosis by betulinic acid in human cancers. Drug Discov Today, 14, 885-90.

Galgon T, Höke D, Dräger B. (1999). Identification and quantification of betulinic acid. Phytochem Analysis, 10, 187-90.

Gauthier C, Legault J, Lebrun M, Dufour P, Pichette A. (2006). Glycosidation of lupane-type triterpenoids as potent in vitro cytotoxic agents. Bioorgan Med Chem, 14, 6713-25.

Indran IR, Tufo G, Pervaiz S, Brenner C. (2011). Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochim Biophys Acta, 1807, 6, 735-745.

Jäger S, Trojan H, Kopp T, Laszczyk MN, Scheffler A. (2009). Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent extracts. Molecules, 14, 2016-31.

Kessler JH, Mullauer FB, de Roo GM, Medema JP. (2007). Broad in vitro efficacy of plant-derived betulinic acid against cell lines derived from the most prevalent human cancer types. Cancer Lett, 251, 132-45.

Kommera H, Kaluderovic GN, Dittrich S, Kalbitz J, Dräger B, Mueller T, et al. (2010a). Carbamate derivatives of betulinic acid and betulin with selective cytotoxic activity. Bioorgan Med Chem Lett, 20, 3409-12.

Kommera H, Kaluderovic GN, Kalbitz J, Dräger B, Paschke R. (2010b). Small structural changes of pentacyclic lupane type triterpenoid derivatives lead to significant differences in their anticancer properties. Eur J Med Chem, 45, 3346-53.

Kommera H, Kaluderovic GN, Kalbitz J, Paschke R. (2011). Lupane triterpenoids - betulin and betulinic acid derivatives induce apoptosis in tumor cells. Invest New Drugs, 29, 266-72.

Nagata S. (2000). Apoptotic DNA fragmentation. Exp Cell Res, 256, 12-18.

Nyasse B, Nono JJ, Nganso Y, Ngantchou I, Schneider B. (2009). Uapaca genus (Euphorbia-

DOI:10.18535/ijmsci/v3i11.5

ceae), a good source of betulinic acid. Fitoterapia, 80, 32-4.

Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, et al. (1995). Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med, 1, 1046-51.

Rzeski W, Stepulak A, Szymanski M, Sifringer M, Kaczor J, Wejksza K, et al. (2006). Betulinic acid decreases expression of bcl-2 and cyclin D1, inhibits proliferation, migration and induces apoptosis in cancer cells. N - S Arch Pharmacol, 374, 11-20.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer I, 82, 1107-12.

Spivak AY, Keiser J, Vargas M, Gubaidullin RR, Nedopekina DA, Shakurova ER, et al. (2014). Synthesis and activity of new triphenylphosphonium derivatives of betulin and betulinic acid against Schistosoma mansoni in vitro and in vivo. Bioorgan Med Chem, 22, 6297-6304.

Yogeeswari P, Sriram D. (2005). Betulinic acid and its derivatives: a review on their biological properties. Curr Med Chem, 12, 657-66.

Zuco V, Supino R, Righetti SC, Cleris L, Marchesi E, Gambacorti-Passerini C, et al. (2002). Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. Cancer Lett, 175, 17-25.

DOI:10.18535/ijmsci/v3i11.5