

Spectroscopic Data of 3-*O*- β -D-Glucopyranoside-betulinic Acid: An Anti-Cancer Agent

Hamisu Abdu¹, Faujan B. H. Ahmad², M. Basri¹, Intan Safinar Ismail¹ & M. B. Abdul Rahman¹

¹ Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, Malaysia

² Natural Product Laboratory, Faculty of Applied Sciences, Universiti Teknologi MARA, Malaysia

Correspondence: Faujan B. H. Ahmad, Natural Product Laboratory, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam 40450, Selangor, Malaysia. Tel: 60-123-091-844. E-mail: faujanahmad@gmail.com

Received: June 4, 2012 Accepted: June 19, 2012 Online Published: September 19, 2012

doi:10.5539/ijc.v4n5p28

URL: <http://dx.doi.org/10.5539/ijc.v4n5p28>

Abstract

3-*O*- β -D-Glucopyranoside-betulinic acid an anti-cancer agent compound, was prepared through enzymatic reaction of betulinic acid and glucose in organic solvent which gave 87.5% yield. Its spectroscopic data is now reported. Interestingly, 3-*O*- β -D-glucopyranoside-betulinic acid also showed strong activity against cultured Human breast cancer (MCF-7), Human T-promyelocytic leukemia (HL-60) and Mouse embryonic fibroblast cancer (3T3) with IC₅₀ values of 8.5, 8.4 and 2.75 μ g/ml respectively. However, it was found to have moderate activity against Human cervical carcinoma cancer (HeLa) cell line with IC₅₀ value of 12.0 μ g/ml.

Keywords: betulinic acid, glucosidation, 3-*O*- β -D-Glucopyranoside-betulinic acid, nuclear magnetic resonance, mass spectrometry

1. Introduction

Natural products have been used for combating human diseases for thousands of years since they exhibit biological properties which can be exploited for medical applications (Newman et al., 2003). Synthetic transformations of these natural compounds for the developing biologically active agents have become the basis of the actively advancing scientific direction of perfect organic synthesis and medical chemistry (Tolstikov et al., 2005). The greatest attention of researchers is attracted by native compounds with reliably established biological activity.

Betulinic acid (1) in particular, is a natural agent isolated from many plants and it was first described to induced apoptosis in neuro ectodermal tumour cells (Pisha et al., 1995). Betulinic acid belongs to the naturally occurring pentacyclic lupane-type triterpenoids which was reported to possesses various pharmacological activities which include anti-cancer, anti-HIV, anti-malarial, anti-inflammatory and anti-fungal (Recio et al., 2005; Yuan et al., 2008; Mukherjee et al., 1997; Hanne et al., 2004). However, the major hinderance for the future clinical development of betulinic acid and its analogs resides in its weak hydrosolubility in aqueous media like serum, blood and non-polar solvents like water used for bioassays (Gauthier et al., 2006).

One of the strategies to increase betulinic acid hydrosolubility is by the synthesis of its glycoside derivatives. Furthermore, the bioactivity of betulinic acid, in some cases can be improved upon the addition of sugar moiety at either C-3 or C-28 or both. Some natural and synthetic betulinic acid glycosides were also reported in the literature (Hamisu et al., 2012). Example, 3-*O*- β -D-glucopyranoside-betulinic acid (2) was synthesised by chemical reactions (Gauthier et al., 2006) and shown some bioactivities (Recio et al., 1995). Their synthesis however seems to be difficult in purification procedural. Thus, in connection with our continuous effort on the enzymatic reaction of betulinic acid we now report the preparation of 3-*O*- β -D-glucopyranoside-betulinic acid (2) by enzymatic glucosidation of betulinic acid (1) and glucose using Novozyme® 435 in organic solvent. Interestingly, it was observed that the reaction was clean and simple, and gave high yield of product. The evaluation of this compound toward some cancer cell lines was also reported herein. The structures of compound (1) and (2) were shown in Figure 1.

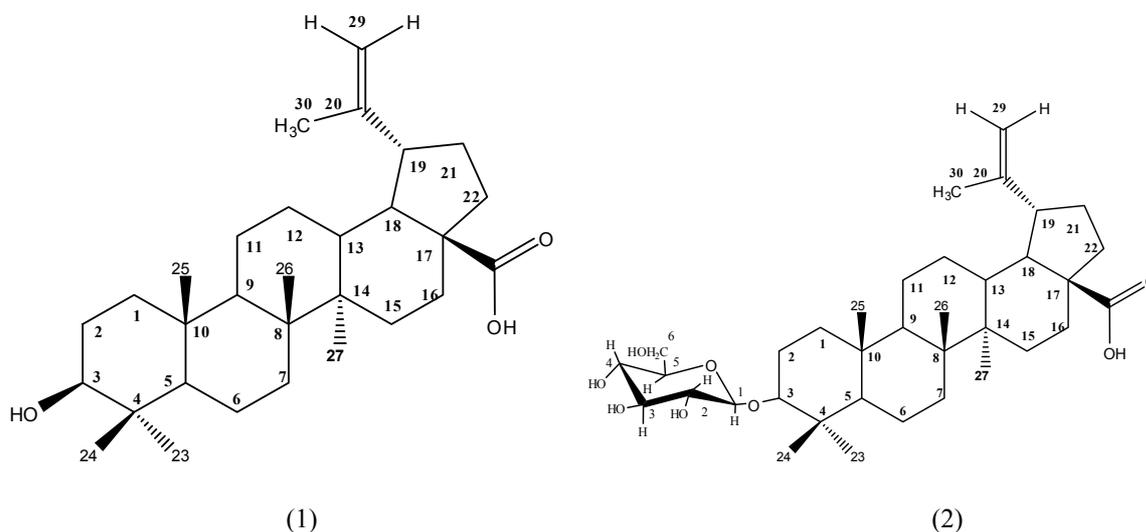


Figure 1. Structures of compound 1 and 2

2. Experiment and Materials

Betulinic acid was donated by Prof.(Dr) Faujan B.H.A, glucose was purchased from Merck, Germany, Novozyme® 435 was purchased from Novo Nordisk A/S (Denmark), *t*-butanol, hexane, ethylethanoate, Dimethylsulfoxide (DMSO) and Microculture Tetrazolium salt (MTT) were purchased from Merck, Germany. It is important to note that all chemicals used in this work were of analytical reagents grade. Nevertheless, they are also pure and distilled. The cancer cell lines HL-60, MCF-7, HeLa; and 3T3 were supplied by Institute of Bioscience (IBS), UPM and were purchased from American Type Cell Culture Collection (ATCC), USA.

3. Preparation of 3-*O*-β-D-Glucopyranoside-betulinic Acid (2)

This compound was prepared using betulinic acid (22.8mg, 0.5×10^{-1} mmol) and glucose (18.0mg, 1.0×10^{-1} mmol) dissolved in *t*-butanol (10 ml). Novozyme® 435 (180mg) was then added and the reaction mixture was incubated on a water bath shaker (Memmert WB 14, Germany) for 30 h, at 55.0 °C and 150 rpm. The progress of the reaction was monitored using thin layer chromatography (TLC) with hexane and ethylacetate as the eluent (8:2 v/v). The enzymes was then removed by filtration. Removal of the solvent under reduce pressure gave a yellowish solid. The product was then purified through celite flash column chromatography followed by crystallized from *t*-butanol to give the pure material of the desired product as light yellow crystal, 0.028g (87.5 %). It melted at of 235.5-238.2 °C (uncorrected) (literature 234-236 °C, Gauthier et al., 2006).

The NMR spectra of the compound was recorded with Varian Unity Inova 400 NMR spectrometer operating at a resonance frequency of 499.89 MHz for ¹H-NMR spectra and 125.71MHz for ¹³C-NMR spectra. The mass spectrum was recorded using Shimadzu, QP5050, Japan.

Cytotoxic activity of the product was evaluated against HL-60, Cancer cell line), MCF-7 Cancer cell lines (Human breast cancer), HeLa Cancer cell line (Human cervical carcinoma cancer) and 3T3 cell line (Mouse embryonic fibroblast cancer) respectively. All these cell lines were supplied by Institute BioScience (IBS) University Putra Malaysia, and were purchased from American Type Cell Culture Collection (ATCC), USA. Culture were maintained according to Harish et al. (2010) as monolayers in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere using 5% carbon(IV)oxide (CO₂).

The cytotoxic activity of the product was evaluated using calorimetric Microculture Tetrazolium salt assay (MTT). Exponentially the growing cells were plated in 96-well microplates (Coster Corning Inc.) at a density of 5×10^3 cells per well in 100μL of the culture medium and these were allowed to adhere for 72 h before treatment in order to prevent confluence (Harish et al., 2010). After 72 h of incubation, the fractions of the surviving cells were measured relative to the untreated cell population by MTT assay. A volume of 20ml of MTT salt (5mg/ml) in phosphate buffer solution was added to each microtiter well and incubated again for 3-4 h. 100μl of Dimethyl sulfoxide (DMSO) was then added to dissolve the remaining MTT formazan crystal by pipetting up and down 10-20 times. The plates was left at room temperature for 15-30 minutes. The optical density (OD) was measured on an ELIZA microplate reader at 570 nm and the percentage of cell viability was calculated using the equation:

$$\% \text{ viability} = (\text{OD sample} / \text{OD control}) \times 100\%$$

A plot of percentage cell viability against the concentration of the drug gives a measure of the cytotoxicity. The cytotoxic index used was IC_{50} , the drug concentration lethal to 50% of the tumor cells as calculated from the plate.

4. Results and Discussion

The product obtained after purification appeared to be a light yellow crystal (87.5%) with a melting point of 235.5-238.2 °C (literature 234-236 °C, Gauthier et al., 2006).

The $^1\text{H-NMR}$ spectra of 3-*O*- β -D-Glucopyranoside-betulinic acid indicates the presence of 6 methyl groups (each singlets) at δ 0.85, 1.03, 1.10, 1.08, 1.25 and 1.82 respectively (each 3H, assigned for 23- CH_3 , 24- CH_3 , 25- CH_3 , 26- CH_3 , 27- CH_3 and 30- CH_3 respectively). The signal at δ 3.48 (1H, dd) was due to the hydrogen proton assigned at C-3 position. The presence of two hydrogens at C-29 position was confirmed by the presence of proton signals at δ 4.80 (1H,s) and δ 4.97 (1H,s). The doublet proton signal at δ 4.58 (1H, d) was assigned as the proton attached to the carbon bearing at position C-3. The signal at δ 3.48 (1H, m) was due to the hydrogen at C-19 position. The $^{13}\text{C-NMR}$ of 3-*O*- β -D-Glucopyranoside-betulinic acid showed the presence of signal at δ 78.9, which was assigned to C-3 of the compound. The signal at 151.4 ppm and 110.1 ppm was due to the carbon double bond between C-20 and C-29 respectively. The $^{13}\text{C-NMR}$ of the compound also shows a carboxyl carbon signal at δ 178.9 ppm assigned as C-28 (COOH).

The structure of the expected product was further confirmed using HMBC, HSQC and COSY spectrum. The selected HMBC (Figure 1) shows that proton signal H-30 have a correlation with C-3, C-19, C-20, C-28 and C-29 respectively. Furthermore C-30 was correlated with H-29 proton signal. There is also a correlation between H-27 proton signal with C-3. The selected HSQC (Figure 2) indicated a correlation between C-29 and an olefinic hydrogens at δ 4.80 and δ 4.97, there is also a correlation between hydrogen signal H-19 with C-19. Another correlation exists between hydrogen signal H-3 and C-3. The $^1\text{H-}^1\text{H}$ COSY data indicated a correlations between H-19 and H-30, H-19 and H-29, H-29 and H-30. The $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, HMBC and COSY are summarised on Table 1.

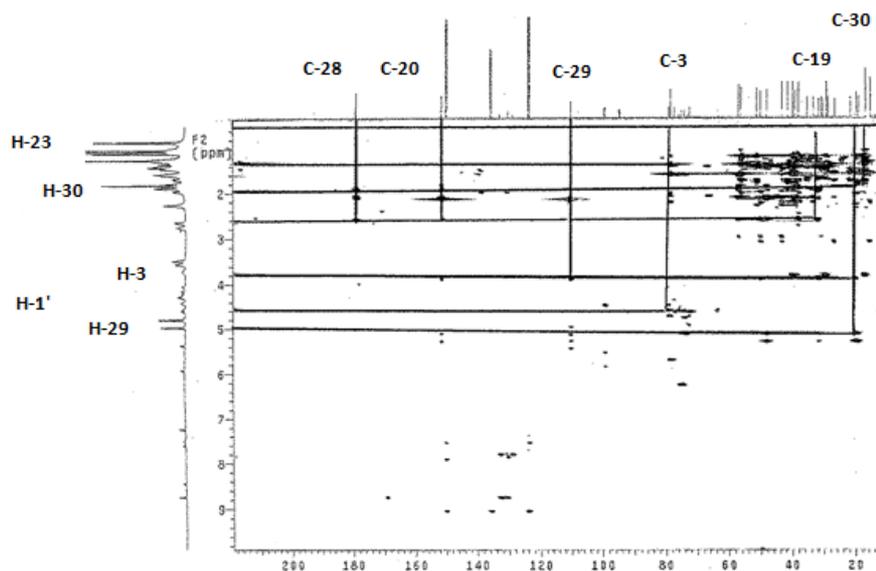


Figure 2. HMBC spectrum of 3-*O*- β -D-Glucopyranoside-betulinic acid

The mass spectral data of 3-*O*- β -D-Glucopyranoside-betulinic acid indicated a molecular ion m/z 618 (M^+) and fragments at m/z 189 as base peak.

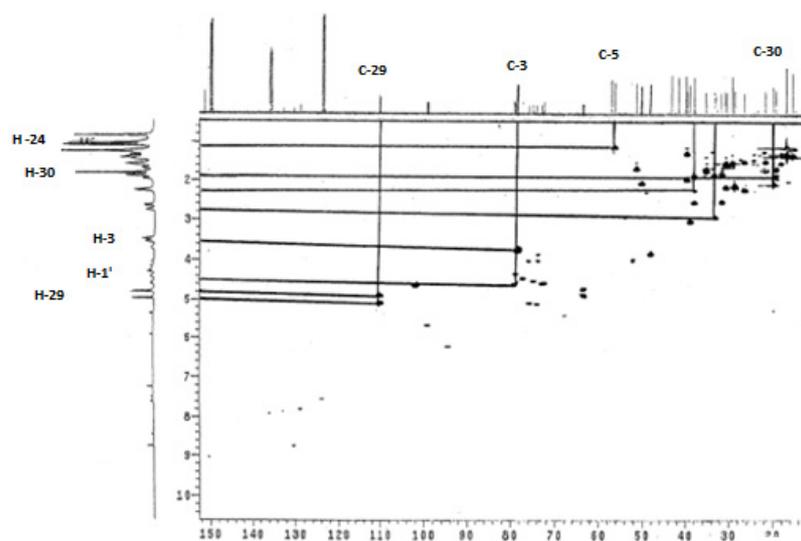


Figure 3. HSQC spectrum of 3-O- β -D-Glucopyranoside-betulinic acid

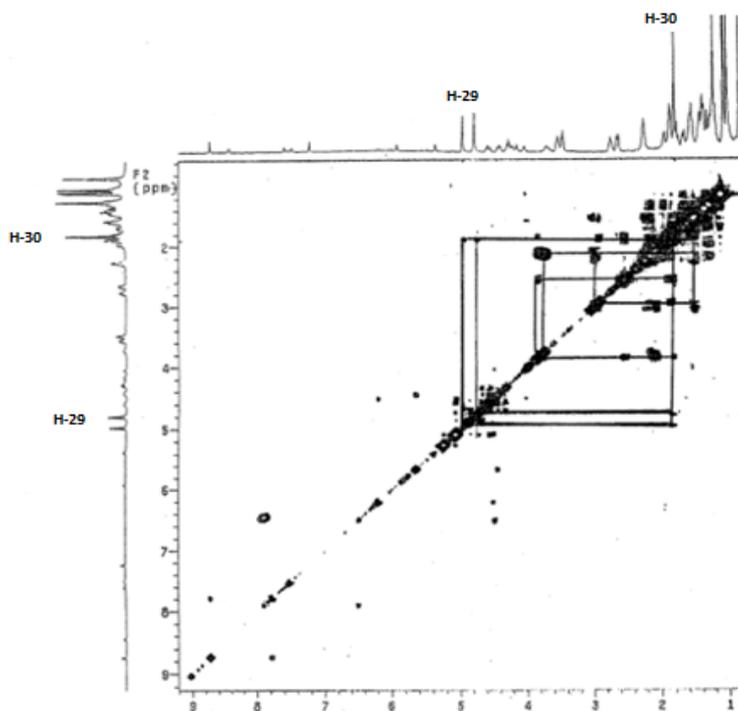


Figure 4. ^1H - ^1H COSY spectrum of 3-O- β -D-Glucopyranoside-betulinic acid

Table 1. ¹H NMR, ¹³C NMR, HMBC and ¹H-¹H COSY Data of the 3-*O*-β-D-glucopyranoside-betulinic acid

Carbon	¹³ C δ (ppm) in Pyridine	δ H (ppm) in Pyridine	HMBC	COSY	¹³ δ C (ppm) Lit. in(C ₅ D ₅ N)	H δ (ppm) Lit. in (C ₅ D ₅ N)
1	39.6				39.6	
2	28.4				26.8	
3	78.9	3.48 (dd)	H-23, H-24		88.8	3.41 (dd)
4	38.7				38.6	
5	56.0				55.9	
6	18.9				18.4	
7	34.9				34.7	
8	41.2				41.1	
9	51.0				50.8	
10	37.7				37.6	
11	21.3				21.2	
12	26.2				26.1	
13	39.4				39.0	
14	42.9				42.8	
15	30.4				30.1	
16	32.9				32.9	
17	56.7				56.6	
18	47.9				47.7	
19	48.8	3.80 (m)	H-29, H-30	H-30	49.7	
20	151.4				151.3	
21	30.1				31.2	
22	37.6				37.2	
23	28.8	0.85 (3H,s)			28.2	0.73 (3H, s)
24	16.5	1.03 (3H,s)			16.8	0.97 (3H, s)
25	16.5	1.10 (3H,s)			16.4	1.01 (3H, s)
26	16.5	1.08 (3H,s)			16.3	1.09 (3H, s)
27	15.0	1.25 (3H,s)			14.3	1.30 (3H, s)
28	178.9		H-30		178.9	
29	110.1	4.8, 4.87 (1H,s)	H-19, H-30	H-30	109.9	
30	19.6	1.82 (3H,s)			19.4	1.77 (3H, s)
C-1'	99.1	4.58 (d)			106.9	4.95 (d)
C-2'	78.2				75.8	
C-3'	77.0	4.25 (d)			78.8	4.26 (m)
C-4'	75.5				71.8	
C-5'	78.7				78.3	
C-6'	63.1				63.0	

Literature reported by Gauthier et al. (2006).

Bioactivity Studies

Based on IC_{50} values, compounds with $IC_{50} < 10 \mu\text{g/ml}$ were considered to be strongly active, those with IC_{50} ranging from 10-30 $\mu\text{g/ml}$ were considered to be moderately active while compounds with $IC_{50} > 30 \mu\text{g/ml}$ were considered as weakly active (Ahmad et al., 2010a; 2010b). Our bioactivity results conducted, betulinic acid (1) showed high activity against cultured human breast cancer (MCF-7), human T-promyelocytic leukaemia (HL-60), and human cervical carcinoma cancer (HeLa) cell lines with IC_{50} values 0.8, 4.4 and 4.8 $\mu\text{g/ml}$ respectively, however, betulinic acid was shown to be highly inactive against mouse embryonic fibroblast normal cell line (3T3) with IC_{50} value $> 30 \mu\text{g/ml}$. In contrast, 3-*O*- β -D-glucopyranoside betulinic acid still showed strong activity against cultured human breast cancer (MCF-7), human T-promyelocytic leukaemia (HL-60) and mouse embryonic fibroblast normal cell line (3T3) with IC_{50} values 8.5, 8.4 and 2.75 $\mu\text{g/ml}$ respectively, but it was found to have moderate activity against human cervical carcinoma cancer (HeLa) cell line with IC_{50} value 12.0 $\mu\text{g/ml}$. (Table 2). Since the introduction of glucose in betulinic acid reduced their cytotoxic activity, it may due to the fact that the glycoside is having higher molecular weight thus cannot pass through the cell membrane completely, although the introduction of sugar in betulinic acid molecule was expected to increase its hydrosolubility properties.

Table 2. Cytotoxicity activity of betulinic acid (1) and 3-*O*- β -D-glucopyranoside betulinic acid, BAG (2), against HeLa, 3T3, MCF-7 and HL-60 cell lines

Compounds	IC_{50} value ($\mu\text{g/ml}$)			
	HeLa	3T3	MCF-7	HL-60
BA(1)	4.8	>30	0.8	4.4
BAG (2)	12	2.75	8.5	8.4

5. Conclusion

In conclusion, 3-*O*- β -D-glucopyranoside-betulinic acid was prepared and characterized using spectroscopic data. The anticancer activity was evaluated against cancer cell lines. It was shown that betulinic acid (1) showed high activity against MCF-7, HL-60, and HeLa cell lines with IC_{50} values 0.8, 4.4 and 4.8 $\mu\text{g/ml}$ respectively. Interestingly, 3-*O*- β -D-glucopyranoside-betulinic acid also showed strong activity against cultured MCF-7, HL-60 and 3T3 with IC_{50} values 8.5, 8.4 and 2.75 $\mu\text{g/ml}$, respectively, and moderate activity against HeLa cell line with IC_{50} value 12.0 $\mu\text{g/ml}$. In general, the glucopyranoside-betulinic acid showed less activity against tested cell lines as compared to betulinic acid itself. It may be due to the higher molecular weight of the glucopyranoside-betulinic acid as compared to betulinic acid.

References

- Ahmad, F. B. H., Ghaffari Moghaddam, M., Basri, M., & Abdul Rahman, M. B. (2010a). Anticancer Activity of 3-*O*-Acylated Betulinic Acid Derivative obtained by Enzymatic Synthesis. *Bioscience Biotechnology and Biochemistry*, 74(5), 1025-1029. <http://dx.doi.org/10.1271/bbb.90917>
- Ahmad, F. B. H., Moghaddam, M. G., Basri, M., & Abdul Rahman, M. B. (2010b). Enzymatic Synthesis of Betulinic Acid ester as anticancer agent: Optimization study. *Biocatalysis and Biotransformation*, 28(3), 192-200. <http://dx.doi.org/10.3109/10242421003753795>
- Gauthier, C., Legault, J., Lebrun, M., Dufour, P., & Pichette. (2006). A. Glycosidation of lupane- type triterpenoids as potent in vitro cytotoxic agents. *Biorganic med. Chem.*, 14, 6713-6725.
- Hamisu Abdu, Faujan B. H., Ahmad, & Ismail, I. S. (2012). Betulinic Acid Glycosides: A Review. *Oriental J of Chemistry*.
- Hanne, L. Z., Henrik, F., Majid, S., Mehrnoush, T., Mahaboubeh, D., Tehrai, K. B., ... Jerzy, W.J. (2004). Erythrocyte membrane modifying agents and the inhibition of plasmodium palcifrum growth. Structure activity relationships for betulinic acid analogues. *Biorganic and medicinal chemistry*, 12, 119-127.
- Harish, K., Goran, N. K., Martin, B., Jutta, K., Petra, F., Simone, F., ... Reinhard, P. (2010). In vitro anti-cancer studies of α - and β -D-glucopyranose betulin anomers. *Chemico-Biological interactions*, 185, 128-136.
- Mukherjee, P. K., Saha, K., Das, J., Pal, M., & Saha, B. P. (1997). Studies on the anti-inflammatory activity of Rhizomes of *Nelumbo nucifera*. *Planta medica*, 6, 367- 369. <http://dx.doi.org/10.1055/s-2006-957705>

- Newman, D. J., Cragg, G. M., & Snader, K. M. (2003). Natural products as sources of new drugs over the periods 1981-2001. *J. Nat. prod.*, *66*, 1022-1037. <http://dx.doi.org/10.1021/np0300961>
- Pisha, E., Chai, H., Lee, I. S., Chagwedera, T. E., Farnsworth, N. R., Cordell, G. A., ... Brown, A. D. (1995). Discovery of betulinic acid as a selective inhibitor of human melanoma that function by induction of apoptosis. *Nat. Med.*, *1*, 1046-1051. <http://dx.doi.org/10.1038/nm1095-1046>
- Recio, M. C., Geiner, R. M., Manez, S., Guheo, J., Julien, H. R., Hostettmann, K., & Rios, J. L. (1995). Investigations on the steroidal anti inflammatory activity of triterpenoids from *Diospyros leucomelas*. *Planta medica*, *18*, 9-14. <http://dx.doi.org/10.1055/s-2006-957988>
- Tolstikov, G. A., Flekhter, O. B., Shultz, E. E., & Baltina, L. A. (2005). Betulin and its derivatives. Chemistry and Biological activity. *Chemistry for sustainable development*, *13*, 1-29.
- Yuan, J. Q., Yang, X. Z., Miao, J. H., Tang, C. P., Ke, C. Q., Zhang, J. B., ... Yang, Y. (2008). New triterpene glucosides from the roots of *Rosa laevigata* michx. *Molecules*, *13*, 2229-2237. <http://dx.doi.org/10.3390/molecules13092229>