Synthesis of Betulinic Acid Dipeptide Derivates with Potential Biological Activity

DORIN MANCIULA[1]*, CARMEN ROBA[1], IOAN OPREAN[2], RADU MISCA-BARBU[3]

[1] Babes-Bolyai University, Cluj-Napoca, Faculty of Environmental Science and Engineering, 30 Fântânele Str. 400294,Cluj-Napoca, Romania

[2] ,Babes-Bolyai University, Cluj-Napoca, Raluca Ripan" Institute for Research in Chemistry, 30 Fântânele Str.,400294, Cluj-Napoca, Romania

[3] Babes-Bolyai University, Cluj-Napoca, Faculty of Chemistry and Chemical Engineering, 11 Arany János Str., 400028, Cluj-Napoca, Romania

Betulinic acid is a natural product that can be found in abundance in the outer bark of the white birch tree (Betula pendula Roth, Betulaceae). The experimental study was intended to extract betulinic acid from the white birch tree leaves and its derivatization to obtain dipeptide compounds with potential biologic activity. For this purpose, three dipeptides were used: alanyl-glycine, glycyl-leucine and leucyl-glycine. The biological activity of betulinoil- glycyl-leucine has been tested on several cancer cells lines, HeLa, MCF-7, A431 and A2780.

Keywords: betulinic acid, dipeptides, biological activity

Betulinic acid, ($C_{30}H_{48}O_3$), (fig.1) is a natural product, a pentacyclic triterpene that can be found in abundance in the outer bark of the white birch tree (Betula pendula, Betula costata, Betula ermanii), [1], or in other several plants species as in the leaves of Syzigium claviflorum, Vatica cinerea King or Aerva javanica, (Amaranthaceae fam.), Ziziphus mauritiana Lam. (Fam. Rhamnaceae), in the bark of Platanus acerifolius or in the Inonotus obliquus mushrooms, [2 - 6].

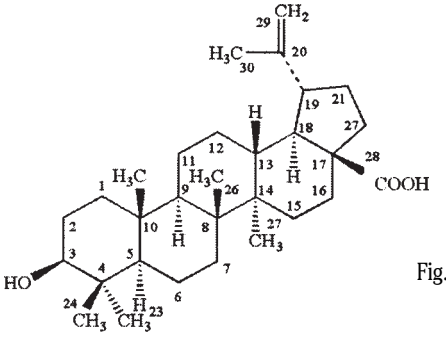

Fig. 1. Betulinic acid

Betulinic acid has been reported to exert numerous medical and biological actions, including anti-cancer, anti-tumor, [7], anti-inflammatory, [8], anti-microbial, [9], anti-ischemic, [10] anti-malarial, [11], anti-HIV, [12], anti-angiogenic, [13], anti-leukemic, anti-lymphoma, [14] and lowering the cholesterol level, [4]. The capacity of betulinic acid to induce apoptosis in melanoma and other cell types, [14, 15], as well as its favorable therapeutically index, of a low toxicity to normal cells, impart it with a great attractiveness among the potential antitumor agents.

Betulinic acid is reported to have three positions: C_3, C_{20} and C_{28} in which chemical changes can be determined towards obtaining derivates with increased antitumor activity. Betulinic acid derivates benefit at their turn from important active biologic features, from this point of view, the triterpenes lying among the most promising antitumor and anti-HIV agents, [16]. Within the research undertaken up to now in the field of antitumor agents, several structural changes and derivatizations of the betulinic acid have been studied, [17, 18], proving that through a simple change in the parent structure, an important number of potential derivatives that improves significantly the selective toxicity profile, [14], or induce a general toxic effect on the leukemic cells could be generated, [14].

Experimental part

Betulinic acid was extracted from dried up leaves of white birch tree (Betula pendula Roth, Betulaceae) through maceration, for 6 days, at 22 °C, using as solvent an ethanol-water solution. The leaves were gathered in the area around Băisoara and Ciucea, Cluj County, sorted and oven-dried at 65 °C for 3 days. The dried vegetal material was then grinded and in the end, the heterogeneous granular mixture was separated into granulometric classes. The extraction evolution was surveyed through refractometry, the influence of various parameters upon the extraction of the betulinic acid from the vegetal mass being analyzed. Within the study, there were used several concentration values of the ethanol-water solution, 30, 50, 60, 70, 80 and 95 %, various granulometric fractions, + 1250, - 1250, + 125, + 90 and - 90 μm and different liquid-solid shares, 10 : 1; 20 : 1; 30 : 1 and 40 : 1, (mL : g).

During the experimental study, both the qualitative and quantitative analysis of the betulinic acid in the extract were performed through reversed phase high performance liquid chromatography, (RP-HPLC), the method providing increased selectivity and sensitivity to identify and quantify the compound. The chromatographic separation was carried out on a column C_{18}, (250 × 4.6 mm, 5μm), the used mobile phase being represented by a mixture of acetonitrile and ultra pure water at a ratio of 90 : 10 (v : v) and a flow rate of 0.8 mL / min. Detection was reached at a wave length of 210 nm.

The dipeptide derivatives were obtained by condensing the betulinic acid with alanyl-glycine, glycyl-leucine and leucyl-glycine as showed in figure2. The synthesis of the betulinic derivatives occurred over a series of reactions developed on four successive stages, among which the first two, corresponding to the obtaining of the intermediary

* *email: dorin.manciula@ubbcluj.ro; Tel. 0264-307.030*

products, (1a) and (2a), are similar in the research methodology to those corresponding to the obtaining of the betulinic derivatives, (4), (6) and (8).

The first reaction was the treatment of betulinic acid with acetic anhydride resulting in monoacetyl betulinic acid, (1a), to which, during the second stage, thionyl chloride was added and the monoacetyl betulinic acid chloride was obtained, (2a). A quantity of 50 mg betulinic acid was dissolved in dichloromethane and then was stirred up with 0.15 mL acetic anhydride for 6 - 8 h at room temperature, with the obtaining of the monoacetyl betulinic acid, (1a). To this compound, 1 mL of thionyl chloride is added and after 12 h at room temperature, the redundant thionyl chloride was removed through low pressure distillation, at 75 °C, the residue being then recrystallized from hexane under heating, up to a constant melting point. After a drying up session of 2 h at 110 °C, the acetyl betulinic acid chloride was obtained, (2a). By condensing the compound (2a) with alanyl-glycine in the third phase, the intermediary product, 3-acetoxy-betulinoil-alanyl-glycine was obtained (3), then after removing the acetoxy group by treatment with NaOH in the fourth phase, the synthesis of the final product, betulinoil-alanyl-glycine, (4), was achieved. A quantity of 0.89 mmol of alanyl-glycine dissolved in dichloromethane was used in the synthesis process, which was then mixed with 0.1 mL of triethyl-amine. The resulted solution was poured under stirring over the monoacetyl betulinic acid chloride, (2a), kept over night at room temperature, then diluted with dichloromethane and washed with water and concentrated saline solution. After filtration and drying on anhydrous sodium sulfate (Na_2SO_4), the residue is then concentrated under vacuum in a rotary evaporator, and the 3-acetoxy-betulinoil-alanyl-glycine, (3) was obtained. The compound (3) is then dissolved within a mixture of tetrahydrofuran (THF) / methanol 50 %, 2 mL, and treated with a mixture of

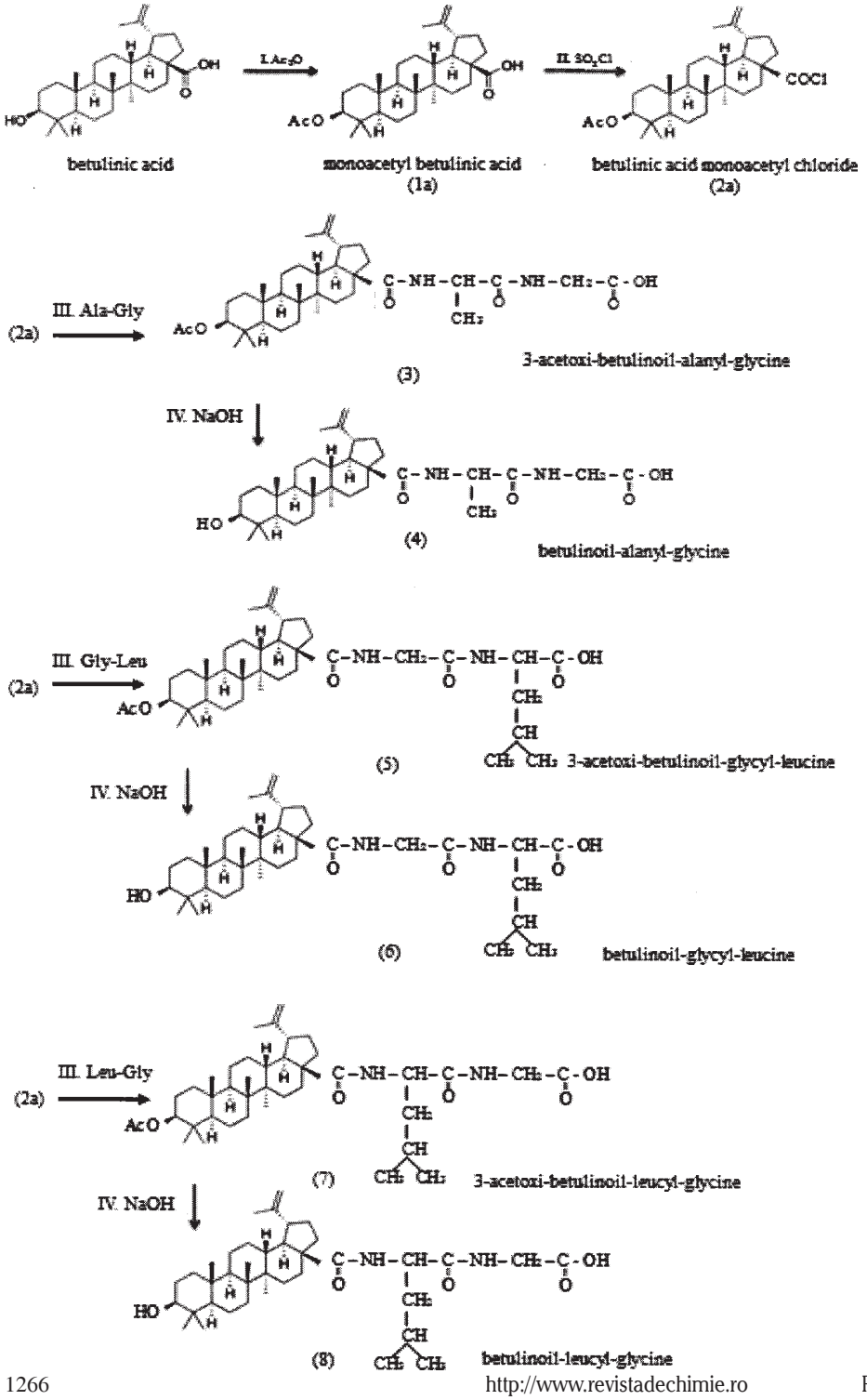

Fig. 2. Scheme of the synthesis of betulinic derivatives

COMPOUND	IDENTIFICATION DATA	
Bet-Ala-Gly (4)	m / z = 71, C_3H_5ON; 74, $C_2H_4O_2N$; 145, $C_5H_9N_2O_3$; 439, $C_{30}H_{47}O_2$; 510, $C_{33}H_{52}O_3N$;	υ_{C-H} = 3018 cm^{-1}, υ_{NH} = 3389 cm^{-1}; $\upsilon_{C=O}$ = 1622 cm^{-1}; δ_{N-H} = 1525 cm^{-1};
	Melting point: 190 - 192 °C; Yield: 9,75 %	
Bet-Gly-Leu (6)	m / z = 57, C_2H_3ON; 130, $C_6H_{12}O_2N$; 187, $C_8H_{15}O_3N_2$; 439, $C_{30}H_{47}O_2$; 496, $C_{32}H_{50}O_3N$;	υ_{C-H} = 3018 cm^{-1}, υ_{NH} = 3325 cm^{-1}; $\upsilon_{C=O}$ = 1621 cm^{-1}; δ_{N-H} = 1512 cm^{-1};
	Melting point: 180 - 184 °C; Yield: 18,22 %	
Bet-Leu-Gly (8)	m / z = 74, $C_2H_4O_2N$; 113, $C_6H_{11}ON$; 187, $C_8H_{15}O_3N_2$; 439, $C_{30}H_{47}O_2$; 552, $C_{36}H_{58}O_3N$;	υ_{C-H} = 3018 cm^{-1}, υ_{NH} = 3290 cm^{-1}; $\upsilon_{C=O}$ = 1620 cm^{-1}; δ_{N-H} = 1520 cm^{-1};
	Melting point: 195 - 198 °C; Yield: 41,88 %	

Table 1
CHARACTERIZATION OF THE SYNTHESIS COMPOUNDS (4), (6) AND (8)

Cell type	Betulinic acid			Bet-Gly-Leu (6)	
	IC_{50} μM	% antiproliferation effect		% antiproliferation effect	
		10 μM ± DSM	30 μM ± DSM	10 μM ± DSM	30 μM ± DSM
MCF-7	143 [23] MTT 72 h 50 [24] MTS 72h	52.92 ± 1.39	95.30 ± 0.97	48.45 ± 0.73	92.50 ± 0.17
A431	12.60 [25] SRB 96h	81.52 ± 0.88	98.80 ± 0.36	58.13 ± 0.54	96.09 ± 0.38
HeLa	26.0 [26] MTT 72 h	67.17 ± 0.92	98.79 ± 0.35	52.71 ± 1.09	94.99 ± 0,34
A2780	1.8 [27] MTT 72 h	-	-	44.00 ± 0.95	86.97 ± 0.59

Table 2
ANTIPROLIFERATION EFFECT OF BETULINIC ACID, [23 - 27], AND ITS DERIVATIVE BETULINOIL-GLYCYL-LEUCINE, (6)

0.160 g NaOH, (1 mL, 4 M) and 1 mL water under a ratio of NaOH : H_2O = 1 : 1. After stirring the solution for 5 h at room temperature, the product was neutralized with 1 mL HCl 1 N up to precipitation then the obtained precipitate was washed with water and dried under vacuum, resulting in a quantity of 51.10 mg of betulinoil-alanyl-glycine, (4), with a synthesis yield of 9.75%. In a similar manner, 78.85 mg of betulinoil-glycyl-leucine, (6), with a synthesis yield of 18.22% and 182.12 mg of betulinoil-leucyl-glycine, (8), with a synthesis yield of 41.88% were obtained. The compounds (4), (6) and (8) were characterized by two analytical methods, mass spectrometry, (GC-MS), by electron impact ionization, (EI) and infrared spectroscopy, (IR). The results describing the compounds characterization are presented in table 1.

The antiproliferative effects of betulinic acid and its condensed analogue with glycyl-leucine, (6), were measured for the following cell lines: HeLa (cervical adenocarcinoma); MCF-7 (human breast adenocarcinoma); A431 (squamos cell carcinoma) and A2780 (human ovarian carcinoma). The MTT assay protocol for cell proliferation was used during research, [19, 20].

Measurement of the antiproliferative effects

Within the undertaken experiment, the antiproliferation effects were expressed as percentages. The simultaneous development of several experiments allowed also the calculation of the mean standard deviation, (MSD). For the betulinic acid, the IC_{50} (μg / mL) values mentioned in previous studies were also pointed out for comparison purposes. As a general rule, the previously mentioned values were given for the same protocol type, MTT, protocol that was also used in the experiments, but were considered also values from the MTS protocol, closely related to MTT and based on the reduction reaction of tetrazole in formazan, and SRB protocol, a colorimetric test with sulforhodamine B, [22]. For laboratory testing of the biological effects associated to the derivative (6), solutions with concentration values of 10 and 30 (μM / l) were used. The results of experiments are shown in table 2.

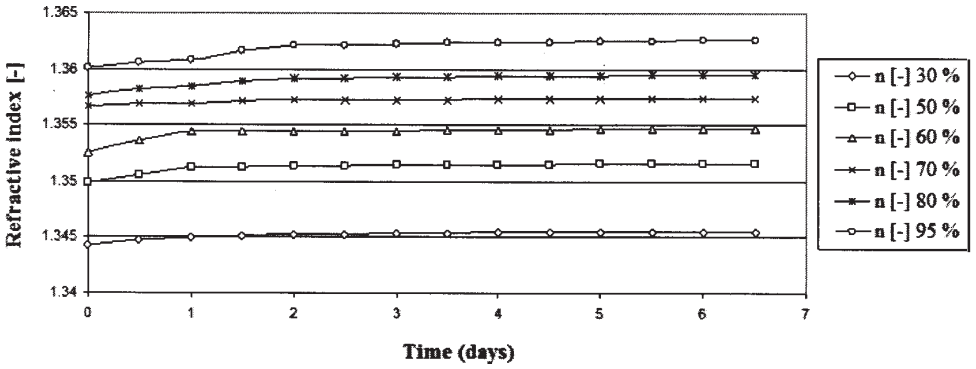

Fig. 3. Influence of solvent concentration upon the refractive index

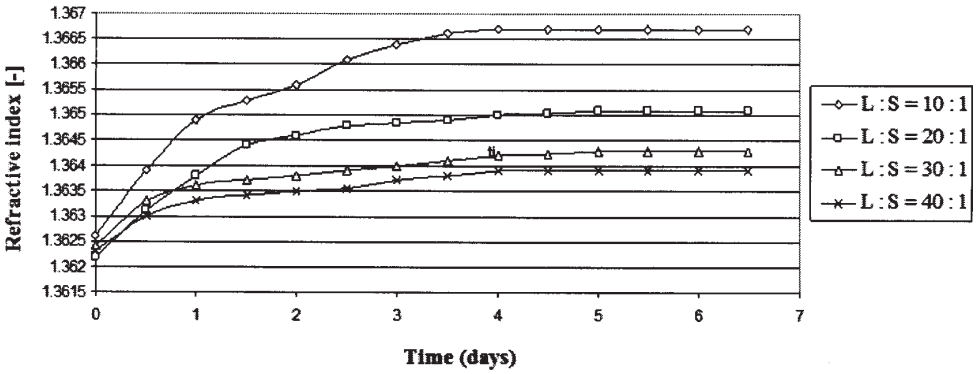

Fig. 4. Influence of the liquid-solid ratio upon the refractive index

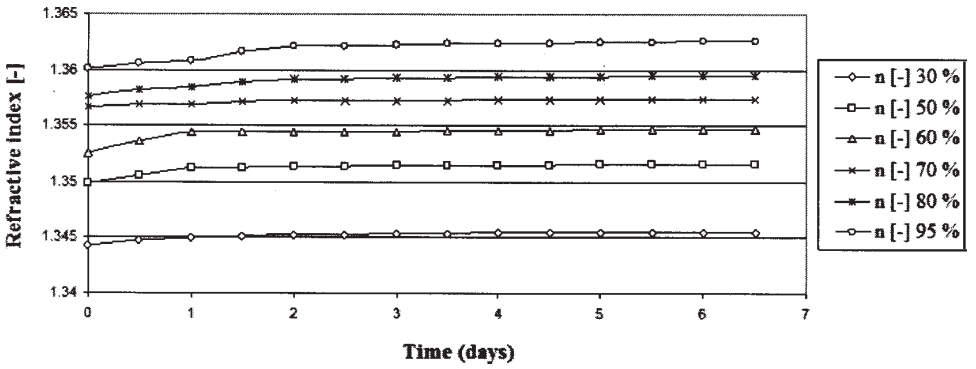

Fig. 5. Influence of the vegetal mass dimension upon the refractive index

Results and discussions

The study for the extraction of betulinic acid from the white birch tree by maceration has been undertaken by following the influence of several parameters upon the process. The following parameters were investigated: solvent concentration, liquid-solid ratio and dimension of the vegetal mass upon the separation process.

Influence of the solvent concentration

In order to investigate the influence of solvent concentration upon extraction, various concentrations of ethanol-water solution were tested, 30, 50, 60, 70, 80 and 95 %, for each used concentration the ratio L : S of 20 : 1, (mL : g) and the granulometric fraction - 1250 μm being kept constant. The variation of the refractive index is showed in figure 3. The experiment pointed out that the ethanol-water solution with a concentration of 95 % is the most appropriate for use in the extraction of betulinic acid from white birch tree leaves, in the given experimental conditions, the equilibrium being reached after approximately 6 days.

Influence of liquid-solid ratio

The following parameters were used in the research: L : S ratios of 10 : 1; 20 : 1; 30 : 1 and 40 : 1, (mL : g), an ethanol-water solvent concentration of 95 % and the particle size fraction - 1250μm. The variations of the refractive index are presented in figure 4. Extraction was registered at maximum speed when using the L : S ratio of 10 : 1, (mL : g), and the equilibrium was reached after approximately 4 days.

Influence of vegetal mass dimension

The influence of the following granulometric fractions has been monitored within the study: + 1250, - 1250, + 125, + 90 and - 90μm. The ethanol-water solvent concentration was kept at 95 % and the L : S ratio at 20 : 1 (mL : g). The variations of the refractive index are presented in figure 5. Extraction was carried out at maximum speed when using the granulometric fraction - 1250 μm, in the given experimental conditions, the equilibrium being reached after 6 days.

The experimental study led to the identification of optimal parameters of the extraction process, respectively the water-ethanol solution with a concentration of 95 %, ratio L : S of 10 : 1, (mL : g) and granulometric fraction - 1250 μm.

Qualitative analysis of betulinic acid was performed based on the retention time (Rt) of 13.73 min , and the quantitative analysis was carried out through the external standard method, by using the calibration curve. The standard solutions used for calibration, 50, 100, 250, 500 and 1000 μg / mL were prepared from the stock solution of 1000 μg / mL through successive dilution with methanol. The sensitivity of the method was expressed as the limit of detection (LOD) of 1.21μg / mL and the limit of quantification of 3.66 μg / mL. As a result of the extraction,

5.57 mg of betulinic acid were obtained, in a yield of 0.55 % on the vegetal material processed.

The synthesis of betulinic acid derivatives through "one-pot" procedure involved conducting a series of reactions which were carried out in four successive stages. The first two, respectively obtaining intermediate product (1a) and (2a), (fig. 2), are similar in methodology as obtaining of the three betulinic derivatives, (4), (6) and (8). In all syntheses, the same ratios and amount of substances were used. The best synthesis performance was obtained for compound (8), the lowest being obtained for compound (4). No explanation was found for the differences arising between the three values of efficiency in the synthesis of betulinic derivatives.

The measuring of antiproliferative effects was carried out for compound (6), and the analysis led to the conclusion that there are no dramatic differences between the activity of betulinic acid and its substituted derivate, in particular for cell lines, MCF-7 and HeLa. For A431 there is a slight tendency to decrease the antiproliferative effect, for lower concentration, 10 μM.

Conclusions

Experimentally, it has been stated that after a period of about 5 - 6 days, at 22°C, the extraction is completed and variations in the refractive index are insignificant. The highest extraction efficiency was attained in the case of ethanol-water solvent with 95 % concentration, to a ratio L : S of 10 : 1, (mL: g) and the granulometric fraction - 1250 μm. 5.57 mg betulinic acid was obtained as a result of optimal parameter extraction, in a yield of 0.55 %, amount determined through the liquid chromatography analysis, (HPLC).

Three betulinic derivatives were synthesized and were obtained 51.10 mg betulinoil-alanyl-glycine, (4), with a yield of 9.75 %, 78.85 mg betulinoil-glycyl-leucine, (6), with a yield of 18.22 %, and 181.12 mg betulinoil-leucyl-glycine (8) with a yield of 41.88 %.

The antiproliferative effects were tested for the compound (6), results showing that there are no major differences between the activity of betulinic acid and its substituted derivatives, in particular cell lines, MCF-7 and HeLa, only in the case of A431 where a vague trend of reduction in the antiproliferative character for the lower concentration, 10μM was observed. The results show that it is worth investing time and effort in further research to identify the dipeptide derivatives exhibiting antiproliferation effect possibly stronger than the basic compounds.

ACKNOWLEDGEMENTS: The authors would like to thank the Department of Oncotherapy from the University of Szeged, Hungary for their support in conducting the laboratory tests and measuring the antiproliferative effects.

References

1. KAUR R., KAPOOR K., KAUR H., J. Nat. Prod. Plant Resour., **1**, no. 1, 2011, p. 119;
2. JAGADEESH S., KRUPADANAM D., SRIMANNARAYANA G., J. Agric. Food Chem., **46**, 1998, p. 2797;
3. CHATTERJE C., PEZZUTO J., KOUZI S., J. Nat. Prod., **62**, 1999, p. 761;
4. CÂNŢA S., PÂNZARU A, LEOPOLD N., KIEFER W., Talanta, **57**, 4, 2002, p. 625;
5. CHIEN Q., HUNG N., SANTARSIERO B., MESECAR A., CUONG N., SOEJARTO D., PEZZUTO J., FONG H., TAN C., J. Nat. Prod., **67**, 2004, p. 994;
6. GAO Y., XU H., LU Z.; XU Z., Se Pu, **27**, 6, 2009, p. 745;
7. RZESKI W., STEPULAK A., SZYMA SKI M., JUSZCZAK M., GRABARSKA A., SIFRINGER M., KACZOR J., KANDEFER-SZERSZE M., Basic Clin. Pharmacol. Toxicol., **105**, 6, 2009, p. 425;
8. CHI-REI WU, YOU-CHENG H., JIN-CHERNG L., LI-WEI L., YUNG-TA L., HUI C., Molecules, **16**, 2011, p. 1;
9. KUETE V., KAMGA J., SANDJO L., NGAMENI B., POUMALE H., AMBASSA P., NGADJUI T., Adv. Exp. Med. Biol., **11**, 6, 2011, p. 2;
10. DEMIRAL E., KARDAS E., ÖZGÜL S., YAG CI T., BILGIN H., SEHIRLI O., ERCAN F., SENER G., Phytother. Res., **24**, 2010, p. 325;
11. DE-SANTOS M., OLIVEIRA J., KRETTLI A., ZALIS M., MAIA G., SETTE I., CAMARA C., FILHO M., HARLEY A., DOS-SANTOS R., Parasitol. Res., **105**, 1, 2009, p. 275,
12. JIN-E YI, MRUKOWICZ B., LI-YUN Y., HUI Y., J. Vet. Sci., **11**, 4, 2010, p. 305;
13. MUKHERJEE R., JAGGI M., RAJENDRAN P., SIDDIQUI M., SRIVASTAVA S., VARDHANA A., BURMAN A., Bioorg. Med. Chem. Lett., **14**, 9, 2004, p. 2181;
14. FAUJAN N., Cytotoxic effects of betulinic acid and its derivatives on human myeloid leukemia (hl-60) cell line, Master thesis, University of Putra, Malaysia, 2008, p.52-105;
15. CIURLEA S., DEHELEAN C., BERKO D., CSANYI E., HADARUGA D., GANTA S., AMIJI M., J. Agroalim. Proc. Technol., **16**, 4, 2010, p. 420;
16. HUANG L., HO P., LEE K., CHEN C., Bioorg. Med. Chem. Lett., **14**, 7, 2006, p. 2279;
17. GHAFARRI M., AHMAD F., BASRI M., RAHMAN A., J. Appl. Sci., **10**, 4, 2010, p. 337;
18. DEHAEN W., MASHENTSEVA A., SEITEMBETOV T., Molecules, **16**, 2011, p. 2443;
19. CSOMÓS P., ZUPKÓ I., RÉTHY B., FODOR L., FALKAY G., BERNÁTH G., Bioorg. Med. Chem. Lett., **16**, 2006, p. 6273;
20. RÉTHY B., CSUPOR-LÖFFLER B., ZUPKÓ I., HAJDÚ Z., MÁTHÉ I., HOHMANN J., TAMÁS-RÉDEI T., FALKAY G., Phytotherapy Res., **21**, 2007, p. 1200;
21. CORY A., OWEN T., BARLTROP J., CORY J., Cancer Commun., **3**, 1991, p. 207;
22. SKEHAN P., STORENG R., SCUDIERO D., MONKS A., MCMAHON J., VISTICA D., WARREN J., BOKESCH H., KENNEY S., BOYD M., J. Natl. Cancer Inst., **82**, 1990, p. 1107;
23. URBAN M., SAREK J., KLINOT J., KORINKOVAAND G., FLAJDUCH M., J. Nat. Prod., **67**, 2004, p. 1100;
24. KOOHANG A., MAJEWSKI N., SZOTEK E., MAR A., EIZNHAMER D., FLAVIN M., XU Z., Bioorg. Med. Chem. Lett., **19**, 2009, p. 2168;
25. KOMMERA H., KALUDEROVIC G., DITTRICH S., KALBITZ J., DRAGER B., MUELLER T., PASCHKE R., Bioorg. Med. Chem. Lett., **20**, 2010, p. 3409;
26. SANTOS R., SALVADOR J., MARIN S., CASCANTE M., Bioorg. Med. Chem. Lett., **17**, 2009, p. 6241;
27. ZUCO V., SUPINO R., RIGHETTI S., Cancer Lett., **175**, 1, 2002, p. 17

Manuscript received: 12.07.2013