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DNA protective activity of triterpenoids isolated from medicinal mushroom *Fomitopsis betulina*

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Abstract: Eleven 31-methylenlanostane triterpenoids, *i.e.*, seven 21- and four 26-oic acids, as well as a lupane triterpenoid betulin, isolated from the fruiting bodies of the mushroom *Fomitopsis betulina*, were tested for in vitro protective effect on chromosome aberrations in peripheral human lymphocytes using cytochalasin-B blocked micronucleus (CBMN) assay. Most of the tested compounds showed a beneficial effect by reducing DNA damage of human lymphocytes more effectively than amifostine, a radioprotective agent, used as a positive control. All the tested compounds decreased MN frequency in the concentration dependent manner, with the concentration of 2.0 μg mL^{-1} being the most effective – with increase of the concentration the activity slightly decreases. The structure–activity relationship (SAR) studies indicated that the lanostanes containing a conjugated 7,9 (11)-diene system exhibit lower activity than Δ^8-analogues. It was also demonstrated that the DNA protective activities within the Δ^8-lanostane-26-oic acid group are affected by the substitution in position 3 pattern. In the Δ^8 series the oxygenation at C-12 or 16 as well as 21- or 26-oic acid functionality proved beneficial for in vitro protective effect on chromosomal aberrations. Betulin exhibited the lowest protective activity, but it is still comparable to that of amifostine.

Keywords: lanostane triterpenoid derivatives; CBMN assey; micronucleus; *Fomitopsis betulina*.

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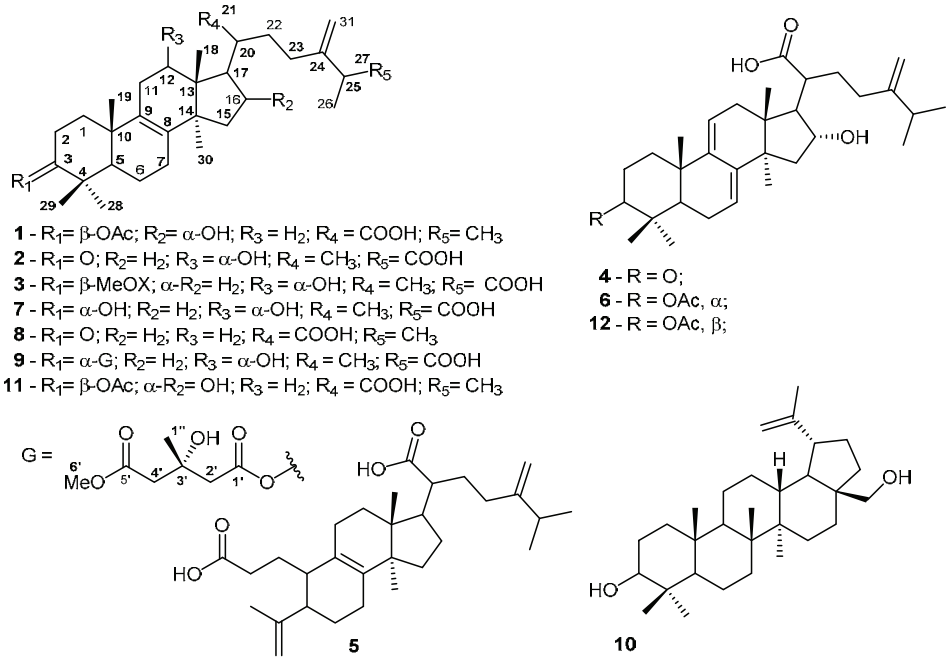
INTRODUCTION

Lanostanes are a group of triterpenoids present in a large number of medicinal fungi such us *Ganoderma lucidum*,[1] *Gloeophyllum odoratum*[2] as well as other related fungi, *Poria cocos*,[3] *Laetiporus sulphureus*, *Inonotus obliquus*, *Antrodia camphorate*, *Daedalea dickinsii*, *Elfvingia applamata*,[4] *Fomitopsis pinicola*,[5] *Fomitopsis betulina*,[6] *etc*. Many lanostanes have shown great potential as anticancer agents owing to their cytotoxic and apoptotic effects on various cancer cell lines (Meth-A and LLC;[7] K562,[8] HepG2, Huh7 and Hep3B;[4] HL-60;[6] THP-1,[7] *etc.*). To the best of our knowledge, *in vitro* protective effect of lanostanes on chromosomal aberrations was only tested by Mata *et al.*[9] on some isolated from *Euphorbia conspicua*. Continuing our examination on wild growing *F. betulina* as a potential source of biologically active compounds,[6] we now report the evaluation of DNA protective activity of the lanostanes **1**–**12** (Scheme 1).



Scheme 1. Tested compounds **1**–**12**.

The cytokinesis-block micronucleus assay (CBMN) used in this study is a standard method for determining the safety of chemicals and pharmaceuticals. The result of the assay, measuring micronuclei (MN) in cultured human and/or mammalian cells, is specifically restricted to once-divided BN cells, which can express MN.[10,11] Binuclear cells are formed when the dividing cells are treated with cytochalasin-B (Cyt-B), a blocker of cytokinesis that strongly inhibit the

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ring assembling formation by actin filaments required for the end of cytokinesis.[10–12] The MN may originate from acentric chromosome fragments, chromosome loss or a budding process following exposure to γ-irradiation.[13] In their study, Ye *et al.*[14] showed that the MN originate from the multiple broken anaphase bridges, although whether this actually happens or not, in cytokinesis-blocked cells, remains unclear. The application of CBMN assay for the antioxidant evaluation of heterocyclic compounds is well known.[15–18] Hitherto, the antioxidant capacity of lanostanes is questionable and depends on the applied test.[19,20] A potential of the lanostanes as DNA protecting agents, as well as the possible mechanism of their action is discussed.

EXPERIMENTAL

Two aliquots of venous blood, 5 ml each were obtained using heparinized sterile vacutainers (Becton Dickinson, Bradford, MA). Six healthy non/smoking male volunteers who had not been exposed to chemicals, drugs or other substances gave their permission for using their blood in the experiment. A safety protocol concerning blood/born pathogen/biohazard was applied. The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002).[21] The blood samples were obtained at the Medical Unit in accordance with current Health and Ethical regulations in Serbia, Law on Health Care (2005).[22]

Lanostanes **1**–**16** were isolated and purified as described.[6] The culture lymphocytes were treated with three concentrations (1, 2, and 4 μg mL^{-1}) of tested compounds. One cell culture served as the control and the tested chemical was not added in it. Amifostine WR-2721 (Marligen-Biosciences, USA), 1 μg mL^{-1}, was used as a positive control and MMC (0.2 μg mL^{-1}, in phosphate buffer) as a negative control.

The treatment of binucleated (BN) cells with the examined compounds was carried out using the modified original procedure,[23-25] applied previously applied in our laboratory.[15,26] At least 1000 BN cells per sample were scored, registering MN according to the criteria of Countryman and Heddle[25] and Fenech and Morley.[23]

The effects of investigated complexes on cell proliferation were estimated by the cytokinesis-block proliferation index (*CBPI*), calculated as suggested by Surralles and others.[27,28] *CBPI* was calculated as:

$$CBPI = [(MI + 2MII + 3(MIII + MIV))]/N \quad (1)$$

where *M*I–*M*IV represent the number of cells with 1 to 4 nuclei, respectively, and *M* is the number of cells scored. For the analysis of MNi, only binucleated cells with well-preserved cytoplasm were scored (under a light microscope with a 40 and 100 magnification). The criteria for selection of BN cells and the identification of MNi given in the HUMAN project website (http://www.humn.org) were followed.[11] The number of BN cells with 1, 2, 3 or more MN were classified then. The data for each treatment were expressed as the frequency of MN per 1000 BN cells.

The statistical analysis was performed using Origin software package version 7.0. The statistical significance of difference between the data pairs was evaluated by analysis of variance (One-way ANOVA) followed by the Tukey test. Statistical difference was considered significant at $p < 0.01$.

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RESULTS AND DISCUSSION

The tested compounds were isolated in our previous research.[6] The identification was based on 1D and 2D NMR and HRESIMS spectra and their comparison with the literature data (for NMR data see Supplementary material to this paper, Tables S-I–S-IV). Tested compounds were: pachymic acid (**1**),[29] polyporenic acid G (**2**),[6] polyporenic acid F (**3**),[6] polyporenic acid C (**4**),[30] piptolinic acid B (**5**),[8] 3-*epi*-dehydropachymic acid (**6**),[8] polyporenic acid A (**7**),[31] fomefficinic acid A (**8**),[32] 12*α*-hydroxy-3*α*-(3′-hydroxy-4′-methoxycarbonyl-3′-methylbutyryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (**9**),[31] betulin (**10**),[32] 3*α*-(acetyloxy)-16*α*-hydroxy-24-methylene-lanost-8-en-21-oic acid (**11**)[33] and dehydropachymic acid (**12**).[34]

All the compounds tested displayed a beneficial effect on the control lymphocyte cells giving a significant decrease of the frequency of MN in comparison with the control cell cultures (Fig. 1). According to the cytokinesis-block proliferation index (*CBPI*), routinely used to determine cytotoxicity of examined compounds in the *in vitro* MN test,[23] the tested compounds did not show any statistically significant decrease. The comparable *CBPI* values for the investigated compounds and tests with positive or negative control suggested an inhibitory effect on the lymphocyte cell proliferation of tested compounds (Table S-V, Supplementary material). The treatment of lymphocyte cells culture was carried out with mitomicyn C (MMC), a clastogenic agent that has been used to study the susceptibility of cell to chromosomal damage and cytotoxic effects.[14] MMC is capable of inducing chromosome aberrations *in vitro*,[35] DNA damage and MN.[36]

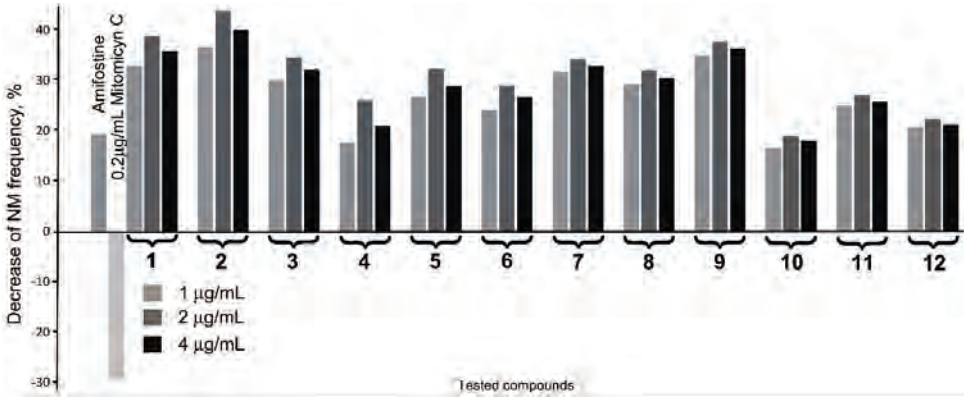

Fig. 1. Decrease of MN frequency in computation to the control (see Table S-V, Supplementary material), effected by lanostane triterpenes **1**-**12**.

During this experiment, we analyzed 126,000 BN cells. Our results provide the evidence of the protective effect of the all tested lanostanes on cytogenetic potential and the reduction of damage on human lymphocytes treated *in vitro*. It

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was also found that the tested compounds reduced the frequency of MN in concentration dependent manner, with the medium concentration of 2.0 μg mL^{-1} being the most effective (Fig. 1).

Since MN expression is dependent on cell division, the quantification of cell proliferation and cell death is the best way to obtain reliable results is evaluation of cell kinetics and MN frequencies. Among the tested compounds at concentrations of 1, 2, and 4 μg mL^{-1}, compound **2** exhibits the most prominent effect of decreasing the ($p < 0.01$) frequency of MN by 37, 44 and 40 %, respectively, when compared with the control cell cultures. A somewhat smaller effect was shown for compounds **1**, **3** and **9** at the same concentrations, decreasing the ($p < 0.01$) frequency of MN by 33, 39, 36; 30, 35, 32; 35, 38 and 36 %, respectively, when compared with the control cell cultures (Table S-V, Supplementary material). The compounds **4**, **10** and **12** at all tested concentrations caused decrease of the MN frequency in the range similar to that of amifostine, a radioprotective compound. Owing to selective action of amifostine on healthy tissues and its anti-mutagenic and anti-carcinogenic properties, it was used as a drug on oncology patients, against the damaging effects of radiation and chemotherapy.[37] All the compounds tested showed the highest activity at the concentration of 2 μg mL^{-1} – with an increase of the concentration the activity slightly decreases, which was not the case with the previously studied jatrophanes and sesquiterpene lactones (Krstić *et al.*[17] and Cvetković *et al.*[18], respectively). The typical DNA protective activity of natural products is mostly attributed to their antioxidative potential.[15–18] The cytotoxic and antioxidant activity of nine lanostane-type triterpenes, isolated from *P. cocos*, were examined previously by Zhou *et al.*[20] They tested the ability of scavenging free oxygen radicals and inhibition of oxidation using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical test. In comparison to the vitamin E used as a positive control, the compounds tested did not show antioxidant activity with $p < 0.05$ that may be considered statistically significant. On the other hand, Sekiya *et al.*[19] carried out the free radical-induced lyses using 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH) of human red blood cells, in order to investigate anti-hemolytic activity of triterpenes isolated from Hoelen, sclederma of *P. cocos*. They found that lanostane triterpene carboxylic acids isolated from the methanol extract of Hoelen, *i.e.*, pachymic acid, polyporenic acid, 3-epidehydrotumulosic acid, 3*β*-hydroxylanosta-7,9(11), 24-trien--21-oic acid and 3-O-acetyl-16-*α*-hydroxytrametenolic acid, exhibited inhibitory activities against AAPH-induced lyses of red blood cells. Whereas in the first study,[20] stabile free radicals were used. In the second investigation free radicals were induced by AAPH thermal decomposition in aqueous conditions. Sekiya *et al.*[19] measured AAPH-induced hemolysis at 540 nm which, according to Nuruki *et al.*[38] was not the optimal wavelength, because AAPH oxidizes the oxygenated hemoglobin to methemoglobin and absorbance at 540 nm does not correctly ref-

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lect the amount of released hemoglobin by AAPH-induced hemolysis. Thus, the absorbance at 523 nm correctly reflects the amount of released hemoglobin regardless of the status of hemoglobin (isosbestic point).[38]

Generally, the compounds **4**, **6** and **12** containing a conjugated 7,9 (11)--diene system exhibited lower activity than the Δ^8-analogues **1**–**3**, **7**–**9** and **11**. At the same time, compared to Δ^8-lanostanes,[6] the conjugated 7,9 (11)-diene system has a positive effect on cytotoxicity to HL 60 cells.[6] It was also shown that the DNA protective activities within the Δ^8-lanostane-26-oic acid group (**2**, **3**, **7** and **9**) (Fig. 1) are affected by the substitution in position 3 pattern. Thus, 3-keto lanostane **2,** showed slightly higher activity than those bearing 3-hydroxy (**7**) or 3-*O*-acyl substituents (**3** and **9**). Moreover, in the Δ^8-lanostane-21-oic acid series (**1** and **11**), the DNA protection activity was dependent on the configuration at C-3. Lanostane **1** with the acetoxy group in 3β-position revealed significantly higher activity than the epimeric 3α-acetoxy lanostane **11** (Fig. 1). It should be noted that the compound **1** also exhibited higher cytotoxic activity in comparison with **11**.[6]

In the Δ^8 series the oxygenation at C-12 or 16 as well as 21- or 26-oic acid functionality were shown beneficial for *in vitro* protective effect on chromosomal aberrations. The smallest activity among the tested compounds was observed for compound **10**, botulin. This pentacyclic compound, lacking these functionalities, decreased the frequency of MN to the same extent as amifostine. Compound **5** has an open (3,4-*seco*) A ring and the rest of the skeleton is the same as in compound **8**. Both compounds have shown the same effect on NB cells so the scission of ring A showed no activity effect.

The above results indicate that the mechanism of action, as DNA protective agents, is not based on the antioxidant potential of tested compounds. Some other biochemical process, regulated by enzymes, should be considered as the mechanism of their action. Zhang *et al.*[39] tested inhibitory effects of triterpene inotodiol, the constituent of the fungus *I. obliquus*, commonly known as "chaga" on HeLa cells migration, invasion and induction of apoptosis. The results of Western blot analysis demonstrated that inotodiol could activate p53 and p21 proteins expression. The p 53 protein is crucial in the regulation of the cell cycle and, depending on the level of DNA damage, stops the G1 phase and activates repair mechanisms, increasing the expression of p21, or conduct cell into apoptosis. Protein p21, the cyclin-dependent kinase inhibitor located downstream of p53 gene, is closely related to tumor inhibition.[440]

We believe that tested lanostanes have similar regulatory mechanism on cell cycle regulating the p53 exertion however, this needs further investigation.

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CONCLUSION

This paper presents a study of the influence of twelve triterpenes on cytogenetic potential and reduction of damage on human lymphocytes treated *in vitro*.

According to the cytokinesis-block proliferation index (*CBPI*), all the compounds tested exerted a significant decrease of the MN frequency in comparison with the control cell cultures. At the same time, the tested compounds did not show statistically significant cytotoxicity. The highest DNA protective activity was at concentration of 2 μg mL^{-1}, whereas at the higher concentration (4 μg mL^{-1}), the activity slightly decreased. The best results were obtained with polyporenic acid G (**2**), Δ^8-lanostane-26-oic acid with 3-keto group. The oxygenation at C-12 or 16 as well as 21- or 26-oic acid functionality were beneficial for *in vitro* protective effect on chromosomal aberrations. The comparison of our results with the literature data indicated the tested lanostanes effects on cell cycle regulating the p53 exertion which require further investigation.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/index, or from the corresponding author on request.

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ИЗВОД

ДНК ПРОТЕКТИВНА АКТИВНОСТ ТРИТЕРПЕНА ИЗОЛОВАНИХ ИЗ МЕДИЦИНСКЕ ГЉИВЕ *Fomitopsis betulina*

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Једанаест 31-метиленланостанских тритерпеноида, то јест седам 21- и четири 26-ланостанских киселина, као и лупански тритерпеноид бетулин изолованих из плодоносних тела гљиве *Fomitopsis betulina* тестирани су *in vitro* на заштитни ефекат на аберације хромозома у периферним хуманим лимфоцитима. Примењен је тест мерења учесталости микронуклеуса индукованих применом инхибитора цитокинезе, цитохалазина Б. Испитивања су показала да већина тестираних једињења показује значајан протективни ефекат на ДНК хуманих лимфоцита, већи него комерцијални радиопротективни агенс аминофостин. У опсегу концентрација 1, 2 и 4 μg mL^{-1} сва испитивана једињења су смањивала учесталост микронуклеуса (MN), при чему је најефиканија била концентрација од 2,0 μg mL^{-1}. Са повећањем концентрације (4 μg mL^{-1}) активност се благо смањује. Студије односа структуре и активности (SAR) показале су да ланостани који садрже конјуговани 7,9-(11)-диенски систем имају нижу активност од 8-аналога. Такође је пока-

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зано да на заштитне активности ДНК унутар групе 8-ланостан-26-киселина утиче супституција у положају 3. У 8-серији присуство кисеоничних функција на C-12 или C-16, као и C-21 или C-26 карбоксилних група повећава *in vitro* протективни ефекат на на ДНК хуманих лимфоцита. Међу испитиваним једињењима тритерпен бетулин (лупанска серија) је показао најмањи протективни ефекат сличан са протективним ефектом амифостина.

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