Revisiting the Effect of 3 Sesquiterpenoids From *Conocephalum conicum* (Snake Liverwort) on Rat Spleen Lymphocyte Viability and Membrane Functioning

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Abstract

Previously 3 sesquiterpenoids from *Conocephalum conicum* (L.) Dum. (Conocephalaceae) were found to modulate lymphocyte response to different stimuli, suggesting their immunomodulatory potential. Herein we evaluated the impact of low concentrations of these sesquiterpenoids on rat splenocyte viability and membrane permeability, as well as lactate dehydrogenase (LDH) activity, in order to, possibly, shed light on their mechanism of action. After a 24 h incubation of splenocytes with the sesquiterpenoids (from $10^{-8}$ to $10^{-6}$ M), MTT and trypan blue (TB) assays, as well as histochemical staining for LDH, were performed. The tested compounds were shown not to reduce the ability of cells to metabolize MTT; however, cell membrane permeability to TB was altered, suggesting that a certain percentage of cells were dead. Histochemical staining for LDH presence revealed that only 2, out of the 3 sesquiterpenoids, decreased the staining intensity, indicating either LDH leakage or its inhibition. In conclusion, having in mind the already proven modulatory potential of the tested sesquiterpenoids, the present results suggest that through the changes in the cell membrane function and leakage/inhibition of LDH in unaltered immune cells, some of the tested compounds could be considered promising candidates for further research as anticancer agents.

Keywords

liverwort, sesquiterpenoids, spleen lymphocytes, cell membrane

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Introduction

The cell membrane represents a necessary unit that is associated with cell life. It enables communication of the cell, molecule transport, and some metabolic features. The main molecules of the membrane are amphiphilic, encompassing polar, hydrophilic, and apolar, hydrophobic, counterparts.¹ Under various physiological and pathological conditions, the cell membrane is exposed to stress which could arise from proteins, toxins, and mechanical activity, all forcing the cell to adapt to new conditions to survive.² Certain cell death mechanisms encompass cell membrane rupture, causing an inflammatory reaction and immunomodulatory effects.³ During this stage, intracellular content is released into the surroundings, for example, extracellular fluid, plasma, and cell culture medium. One of the markers of damaged cell/cell membranes is lactate dehydrogenase (LDH), which has been used to monitor such events, even in clinical practice.³

   The trypan blue (TB) exclusion assay represents one of the first methods used to determine cell viability.⁴ This dye, with a molecular weight of ca. 1000-Da, cannot cross the cell membrane. In that sense, TB only enters cells with disrupted membranes and imparts a blue color to such cells.⁶ It is well known that cell membrane integrity is one of the criteria for making the distinction between dead and living cells.⁶ Thus, TB has been widely used as a vital dye, that is, it selectively penetrates the cytoplasm of dead cells, and is a very frequent assay for evaluating cytotoxicity.⁶

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Metastatic tumor cells are known to possess higher fluidity of the plasma membrane compared to non-metastatic cells. In that sense, restoring contact inhibition between cells through decreased membrane fluidity is effectuated by increasing rigidity at cell junctions. Hence, molecules possessing these features may cause slower cell division due to reduced membrane fluidity. Ferreira et al. described that the already known NSAIDs increase the fluidity of mouse splenocyte membranes in a concentration-dependent manner.

Recently, we isolated 3 new sesquiterpenoids, compounds 1-3 (Figure 1), from Conocephalum conicum (L) Dum., Conocephalaceae, (Snake liverwort) and in vitro screened them for immunomodulatory activities. The sesquiterpenoids were shown to be cytotoxic to non-stimulated rat splenocytes to a certain extent, while 2 and 3 were immunosuppressive toward ConA-stimulated splenocytes, while not exerting cytotoxicity at the same concentrations. As these activities were all determined using the MTT assay, revealing the reducing capability of live cells, usually connected with the functioning of mitochondria, they may be subject to interpretation that is other than altered cell viability. This motivated us to reevaluate the effect of the 3 liverwort compounds on rat splenocytes in assays that reveal the functioning of the cell membrane. Thus, in this study, we investigated the possible impact of low concentrations of 3 sesquiterpenoids, isolated from C. conicum, on rat splenocyte viability and membrane permeability, and LDH activity as well, and compared the results with those obtained from the MTT assays.

**Results and Discussion**

Compounds 1, 2, and 3 were assayed for their effect on spleen lymphocyte viability in different in vitro models using 2 dyes: MTT and TB, which mirrored the functioning of different cell structures, that is, mitochondria and cell membrane, respectively. Compounds 2 and 3 at 2 tested concentrations (10^{-7} and 10^{-8} M) exerted a significant reduction in cell viability based on the findings of the MTT assay (Figure 2). Additionally, according to the TB assay, compound 1 at 10^{-8} and 10^{-6} M, and 3 at 10^{-7} M caused death of c.a. 25% to 50% of rat spleen lymphocytes. Dexamethasone (Dex), a known cytotoxic compound (at 10^{-6} M), reduced cell viability by 50% (Figure 2). On the other hand, after a 24 h treatment with Dex at the same concentration, c.a. 75% of splenocytes were viable based on the TB assay (Figure 3). The application of 2 changed cell membrane permeability detected by the TB assay only at the concentration of 10^{-6} M (Figure 3).

Our previous study revealed that the investigated compounds 1, 2, and 3 expressed cytotoxicity towards rat splenocytes only in either high concentrations (100 μM) or after prolonged incubation (48 h). Also, these compounds were found to inhibit cell proliferation in the presence of T and B cell mitogens. In the mentioned study, we employed the MTT assay, which is considered to be one of the most sensitive methods for determining cell viability since it relies on cell mitochondrial enzymes to perform the reduction of MTT to blue formazan crystals. In the present study, we found that lower concentrations of the tested compounds only slightly affected the cell’s ability to metabolize MTT (Figure 2). These findings indicate that in the tested concentrations compounds 1, 2, and 3 are not cytotoxic. The standard drug, Dex, used in this study was found to reduce rat spleen cell ability to metabolize MTT, which is in accordance with our previous studies. It is worth mentioning that Marchantia convoluta leaf extracts are found to inhibit liver and lung cancer cell proliferation using the MTT assay. However, the reported results are coming from cancer cells which are different from the non-altered splenocytes, which potentially indicates that the tested compounds might be less toxic to non-cancer cells.

The results of the TB assay indicate that 1, and 2, in the highest tested concentration, could potentially cause either cell death or increase cell permeability (Figure 3), while compound 3 influenced cell membrane permeability at 10^{-7} M (Figure 3). Our previous study showed that compounds isolated from another species of liverwort differently influence cell viability, and the results of the TB assay overlapped with those of the MTT assay. Other investigators found that 14 h after an in vitro treatment with Dex (10^{-6} M) 50% of viable mouse splenocytes were detected using TB staining. In our study, Dex at the same concentration, incubated with rat spleen lymphocytes for 24 h, caused the death of c.a. 25% of cells stained with TB,

![Figure 1. Structures of the tested sesquiterpenoids isolated from C. conicum: (Z,Z,4E)-lepidotoa-1(10),4-dien-14-ol (1), rel-(1(10)Z,4E,5E,7R)-germacra-1(10),6 diene-11,14-diol (2), and rel-(1(10)Z,4E,5E,7R)-humula-1(10),5-diene-7,14-diol (3).](image-url)
which is lower than in the reported study, while in one of our previous studies\textsuperscript{11} the results overlapped with the reported ones for mouse splenocytes.

When one compares the results from the MTT and TB assays, a clear discrepancy in the effects exerted by the test compounds could be seen (Figures 2 and 3), which was not the case for Dex. One of the possible explanations could be that compounds 1-3 increase the membrane permeability, especially true for the most lipophilic compound, 1, yielding false-positive results, which suggests a cytotoxic potential for these compounds. On the other hand, an increase in the cell membrane permeability could be a beneficial property of a molecule, since these types of substances could play a role in cancer cell elimination by host-mediated mechanisms.\textsuperscript{14} However, phenylalanine, an amino acid that possesses various physiological roles, could increase the cell membrane permeability, which could further be connected with symptoms of the disease such as phenylketonuria.\textsuperscript{15}

Compared to the appearance of RPMI-treated cells, stained for LDH, the intensity of otherwise treated splenocytes was scored (Figure 4A). The application of 1 caused a concentration-dependent decrease in the staining intensity, suggesting either leakage of LDH or a decrease in its activity (Table 1). Based on the findings of LDH-staining intensity, only 2 did not modify the amount of LDH (Table 1 and Figure 4B). When the cells were exposed to 3 for 24 h, a marked decrease in LDH-staining intensity was observed in cells treated at concentrations of 1 and 0.1 µM (Table 1 and Figure 4C).

In our study, we found that the application of Dex caused a decrease in LDH staining intensity with splenocytes (Table 1), which could arise from either decrease in LDH activity through its inhibition or a decrease in the amount of the enzyme within the cell after its leakage. In a study conducted on human chondrocytes, treatment with Dex, at concentrations of 0.5 and 1 µM, did not alter intracellular LDH activity, nor caused an increase in its leakage into the medium during 48 h of incubation.\textsuperscript{16} On the other hand, a concentration of 0.5 µM Dex caused an increase in LDH leakage from the exposed hepatoma cells.\textsuperscript{17} These differences might arise from different cell types treated with Dex, which include different cell membrane fluidity, seen in normal versus tumor cells. Also, splenocytes are rather sensitive to the action of corticosteroids, meaning that they might be more prone to cell death/damage, which is associated with increased membrane permeability and leakage of LDH.

A similar viewpoint as the one suggested for Dex could be transferred to the effect of the sesquiterpenoids isolated from the \textit{C. conicum} extract. Through an increase in membrane permeability, LDH could easily leak causing a decrease in staining intensity (Table 1). This mechanism could potentially be proposed for 1, which led to an increase in TB accumulation and a decrease in LDH staining intensity (Figure 2 and Table 1). On the other hand, we could not suggest a similar mechanism for 3, which was the most potent one in altering LDH staining (Table 1), but without affecting the TB results (Figure 2), implying that this compound might inhibit LDH activity. The activity
Figure 3. The percentage of viable cells was estimated using the Trypan blue method after 24 h incubation with various concentrations of compounds 1, 2, and 3, as well as Dex at the concentration of $10^{-6}$ M. The data are given as mean ± SD, and the comparison was made using one-way ANOVA followed by Tukey’s post-hoc test. *$P < .05$, ***$P < .0001$ versus RPMI-treated cells.

Figure 4. Microscopic appearance (magnification $\times 1000$) of LDH stained cells obtained from the different treatments: (A) RPMI-treated cells, (B) cells treated with compound 2 at a concentration of 1 µM, (C) cells treated with compound 3 at a concentration of 0.1 µM, and (D) cells treated with Dex at a concentration of 1 µM.
of LDH is found to be associated with different stages of tumor development from tumorigenesis, to tumor progression, its growth, and, even, could be used as an indicator of disease prognosis. Thus, molecules that potentially inhibit LDH could be good candidates for anti-cancer drugs.

In the end, our results might be viewed as interesting from the point of these compounds being potential anticancer agents since they might possess membrane damaging properties, as well as LDH inhibitory activity, all of which are desirable features of these types of drugs. Also, we should bear in mind that the earlier results showed that marchantin A isolated from liverworts is a potent inducer of apoptosis in MCF-7 human breast cancer cells.

Materials and Methods

Chemistry

The tested compounds were isolated in a previous study. Their identity and purity were checked by 1H NMR and GC-MS, as described before, prior to the performed in vitro assays.

Drugs and Chemicals

Cell culture medium (RPMI 1640, containing 10% fetal calf serum, and antibiotic/antimycotic solution) was acquired from Sigma-Aldrich, St. Louis, USA, dexamethasone (Dex), used as the positive control (a steroid drug with anti-inflammatory and immunosuppressant effects), from Galenika (Beograd, Serbia), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and tetrasodium 3,3'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl]bis(azo)]bis[5-amino-4-hydroxynaphthalene-2,7-disulphonate] (trypan blue, TB), used for the estimation of cell viability, from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade or better.

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Animal Housing

Male Wistar rats (250-300 g), from the Vivarium of the Institute of Biomedical Research, Faculty of Medicine, University of Niš, maintained under standard laboratory conditions, were used in the present study. All experiments were performed following the Declaration of Helsinki and the European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/EU) and the related ethics regulations of the University of Niš, Serbia (323-07-06862/2016-05/2).

Culture of Rat Splenocytes

Dissected rat spleens, cut into fine pieces, were passed through mesh cell sieves under sterile conditions. After centrifugation, the obtained splenocyte suspensions were treated with isotonic NH₄Cl solution to remove red blood cells from them and washed with phosphate-buffered saline (PBS). Re-suspended cells in RPMI medium were adjusted to 2×10⁶ cells/mL density. These cells were further cultured in 96-well plates, in which the suspension was mixed with either RPMI (negative control) or compounds 1-3/Dex diluted in the same medium. Dexamethasone was used as the positive control at 10⁻⁶ M, while compounds 1-3 were assayed in their predetermined non-toxic concentrations ranging from 10⁻⁸ to 10⁻⁶ M. The plates were incubated for 24 h under an atmosphere of 95% air and 5% CO₂ (v/v) at 37 °C. All experiments were made in triplicate and repeated 4 times.

Viability Assays (MTT Assay and TB Assay)

Splenocytes were incubated with compounds 1-3 and Dex for 24 h and then both assays were performed, while cells cultured under the same conditions in RPMI medium served as the negative control. After plate centrifugation, fresh 100 µL of RPMI medium and MTT sterile solution (0.5 mg/mL) were added and further incubated for 4 h. A solution of HCl (0.04 M) in isopropanol was used to dissolve the formazan crystals. An automated microplate reader was used to measure the absorbance at 550 nm. The percent viability relative to the RPMI-cultured cells is presented as the mean ± standard deviation (SD).

TB viability assay was conducted according to an earlier described method, where the cells from each well were mixed with TB solution (0.4%, w/w) in PBS (pH 7.2-7.3). Bright (viable) and dark blue (dead) cells were counted in a hemocytometer chamber under 40X lens magnification. The percentage of viable cells per 100 counted cells was reported.

Lactate Dehydrogenase Staining on Splenocyte Sections/Smears

Histochemical LDH staining was performed by incubating the cells attached to slides for 5 min at 37 °C. The staining solution was 100 mM phosphate buffer (pH 7.45), containing 17%
polyvinyl alcohol, 3 mM NAD\(^+\), 150 mM sodium L-lactate, 0.32 mM 1-methoxyphenazine methosulphate, 5 mM Na\(_3\) and 5 mM nitro-blue-tetrazolium. Control reactions were performed with the same staining solution, with the omission of sodium L-lactate and NAD\(^+\).\(^{22}\) The positivity rate was represented based on LDH positive cells under 1000× lens magnification (100 cells counted). The intensity of staining was scored based on the slightly modified method described by Song et al.,\(^{23}\) and the staining intensity was scored as follows: 3 (strong intensity), 2 (moderate intensity), 1 (weak intensity), and 0 (negative).

Statistical Analysis
The obtained results were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA), followed by Tukey’s post-hoc test for multiple comparisons (Statistics software Graphpad Prism version 5.03), was used to determine statistically significant differences. Probability values (\(P\)) less than .05 were considered to be statistically significant.

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Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Dedication
The authors wish to dedicate this work to the occasion of the 80th birthday of Professor Yoshinori Asakawa.

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References
1. Lombard J. Once upon a time the cell membranes: 175 years of cell boundary research. Biol Direct. 2014;9:32. doi:10.1186/s13062-014-0032-7.
2. Espiritu RA. Repairing plasma membrane damage in regulated necrotic cell death. Mol Biol Rep. 2021;48(3):2751-2759. doi:10.1007/s11033-021-06252-w.
3. Krishnamurthy K, Medina AM, Howard L. The utility of elevated serum lactate dehydrogenase in current clinical practice. Lab Med. 2021;52(2):e17-e22. doi:10.1093/labmed/lmaa059
4. Louis KS, Siegel AC. Cell viability analysis using trypan blue: manual and automated methods. In: Stoddart MJ, ed. Mammalian cell viability: Methods and protocols. Vol 740. Springer; 2011:7-12.
5. Altman SA, Randers L, Rao G. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. Biotechnol Prog. 1993;9(6):671-674. doi:10.1021/bp00024a017
6. Avelar-Freitas BA, Almeida VG, Pinto MCX, et al. Trypan blue exclusion assay by flow cytometry. Braz J Med Biol Res. 2014;47(4):307-315. doi:10.1590/1414-431X201443437
7. Clarke R, van den Berg HW, Murphy RF. Reduction of the membrane fluidity of human breast cancer cells by tamoxifen and 17 beta-estradiol. J Natl Cancer Inst. 1990;82(21):1702-1705. doi:10.1093/jnci/82.21.1702
8. Wiseman H, Smith C, Halliwell B, Cannon M, Arnstein HRV, Lennard MS. Droloxetine (3-hydroxytamoxifen) has membrane antioxidant ability; potential relevance to its mechanism of thera-peutic action in breast cancer. Cancer Lett. 1992;66(1):61-68. doi:10.1016/0304-3835(92)90281-Y
9. Ferreira H, Lúcio M, Lima JLFC, Anabela Cordeiro-da-Silva A, Tavares J, Reis S. Effect of anti-inflammatory drugs on splenocyte membrane fluidity. Anal Biochem. 2005;339(1):144-149. doi:10.1016/j.ab.2004.12.023
10. Radulović NS, Filipović SI, Neić MS, et al. Immunomodulatory constituents of Conocephalum conicum (Snake Liverwort) and the relationship of isolepidotozenes to germacrane and humulane. J Nat Prod. 2020;83(12):3554-3563. doi:10.1021/acs.jnatprod.0c00585
11. Radulović NS, Filipović SI, Zlatković DB, et al. Immunomodulatory pinguisane-type sesquiterpenes from the liverwort Porella cordana (Porellacée): the “new old” furanopenuguisanol and its oxidation product exert mutually different effects on rat splenocytes. RJC Adv. 2016;48:41847-41860. doi:10.1039/C6RA04308A
12. Xiao JB, Chen XQ, Zhang YW, Jiang XY, Xu M. Cytotoxicity of Marchantia convoluta leaf extracts to human liver and lung cancer cells. Braz J Med Biol Res. 2006;39(6):731-738. doi:10.1590/s1980-07852006000500005
13. Nair-Menon J, Campbell G, Blake C. Toxic effects of octylphenol on cultured rat and marine splenocytes. Toxicol Appl Pharmacol. 1996;139(2):437-444. doi:10.1006/tapp.1996.0185
14. Geurink PP, van der Linden WA, Mirabella AC, et al. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. J Med Chem. 2013;56(3):1262-1275. doi:10.1021/jm3016987
15. Perkins R, Vaida V. Phenylalanine increases membrane permeability. J Am Chem Soc. 2017;139(41):14388-14391. doi:10.1021/jacs.7b09219
16. Li Q, Chen H, Li Z, Zhang F, Chen L. Glucocorticoid caused lactic acid accumulation and damage in human chondrocytes via...
ROS-mediated inhibition of Monocarboxylate Transporter 4. *Bone*. 2022;155:116299. doi:10.1016/j.bone.2021.116299

17. Rajaraman G, Wang GQ, Yan J, Jiang P, Gong Y, Burczynski FJ. Role of cytosolic liver fatty acid binding protein in hepatocellular oxidative stress: effect of dexamethasone and clofibrate treatment. *Mol Cell Biochem*. 2007;295(1–2):27-34. doi:10.1007/s11010-006-9268-6

18. Fiume L, Manerba M, Vettraino M, Di Stefano G. Inhibition of lactate dehydrogenase activity as an approach to cancer therapy. *Future Med Chem*. 2014;6(4):429-445. doi:10.4155/fmc.13.206

19. Jendrossek V, Handrick R. Membrane targeted anticancer drugs: potent inducers of apoptosis and putative radiosensitisers. *Curr Med Chem Anticancer Agents*. 2003;3(5):343-353. doi:10.2174/1568011033482341

20. Huang WJ, Wu CL, Lin CW, et al. Marchantin A, a cyclic bis(bi-benzyl ether), isolated from the liverwort *Marchantia emarginata* subsp. *tsunae* induces apoptosis in human MCF-7 breast cancer cells. *Cancer Lett*. 2010;291(1):108-119. doi:10.1016/j.canlet.2009.10.006

21. Stojanović NM, Randjelović PJ, Mladenović MZ, et al. Toxic essential oils, part VI: acute oral toxicity of lemon balm (*Melissa officinalis* L.) essential oil in BALB/c mice. *Food Chem Toxicol*. 2019;133:110794. doi:10.1016/j.fct.2019.110794

22. Van Noorden CJF, Frederiks WM, Aronson DC, et al. Changes in the acinar distribution of some enzymes involved in carbohydrate metabolism in rat liver parenchyma after experimentally induced cholestasis. *Virchows Arch*. 1987;52:501-511. doi:10.1007/BF02889989

23. Song K, Yu X, Lv T, et al. Expression and prognostic value of lactate dehydrogenase-A and -D subunits in human uterine myoma and uterine sarcoma. *Medicine (Baltimore)*. 2018;97:14(e0268). doi:10.1097/MD.00000000000010268