MIKOTOKSINI - POTENCIJALNA TERAPEUTSKA ULOGA

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Mycotoxins - a potential therapeutic role

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Kratak sadržaj: Mikotoksini su sekundarni metaboliti plijesni. Imaju štetne učinke na usjeve te zdravlje domaćih životinja, ali i čovjeka. No, nisu sve plijesni i njihovi sekundarni metaboliti toksični. Pseurotin A i pseurotin D, sekundarni metaboliti koji su prvi puta izolirani iz *Pseudeurotium ovalis* STOLK, privlače interese znanstvenika zbog svoje strukture i različitih bioloških aktivnosti. Cilj ovog rada bio je istražiti utjecaj Pseurotina A i pseurotina D na limfoma stanice. Stanična linija, MEC-1, dobivena iz B stanica kronične leukemije tretirana je različitim koncentracijama PsA i PsD. Za procjenu utjecaja pseurotina na vijabilnost i citotoksičnost stanica korištena su oba oblika. No na procjenu utjecaja pseurotina na stanični ciklus i apoptozu, korišten je samo PsD. Rezultati pokazuju kako PsA nema utjecaja na B stanice kronične leukemije. PsD je utjecao na proliferaciju, metabolizam i citotoksičnost stanica u koncentracijama od 20 μM, 25 μM i 50 μM. Nakon 24, PsD je značajno inhibirao stanični ciklus i smanjio ekspresiju ciklina. No, tijekom 24, 48 i 72 h u koncentracijama od 1μM, 5 μM, 10 μM and 25 μM, PsD nije utjecao na apopotozu stanica.

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MYCOTOXINS - POTENCIAL THERAPEUTIC ROLE

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Supervisor: Mirna Velki, Ph.D., Assistant Professor
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Short abstract: Mycotoxins are toxic secondary metabolites of moulds. Mycotoxins have adverse effects on crops, domestic animals and humans, causing many diseases. However, some type of moulds and their secondary metabolites are not toxic. Pseurotin A and Pseurotin D, secondary metabolites first isolated from the cultures of *Pseudeurotium ovalis* STOLK, have different structures and diverse bioactivity. Because of these characteristics an interest in these compounds has significantly increased. The main goal of the present research was to investigate the effects of natural pseurotins on human lymphoma cell line. For this purpose, MEC-1 cell line, derived from human chronic B cell leukemia, was treated with different concentrations of PsA and PsD. To investigate the effects on viability and cytotoxicity of lymphoma cells, both PsA and PsD were used. In order to investigate the effects on cell cycle and cell death (apoptosis) of lymphoma cells, only PsD was used. The results show that none of the concentrations of PsA significantly affected cell proliferation, cellular metabolism and cytotoxicity. Contrary to that, PsD affected the tested cells in concentrations of 20 μM, 25 μM and 50 μM. After 24 h, PsD significantly inhibited the cell cycle and decreased expression of cyclins, but did not affect apoptosis of cell line in used concentrations (1μM, 5 μM, 10 μM and 25 μM) after 24, 48 and 72 h.

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1. INTRODUCTION

1.1. Fungi

Fungi are single-celled or multicellular eukaryotic organism that are heterotrophs. The kingdom Fungi includes the mushrooms, mildews, smuts, rusts, yeasts and moulds. Fungi is ubiquitous, one of the most widely distributed organisms on Earth. It can be found in the air and the soil, in rivers, lakes and even seas, on and within animals and plants, in food and clothing, and in the human body (Hardin et al., 2003).

Mould is multicellular fungi that grows as a mat of intertwined multicellular filaments known as hyphae. The moulds might live as symbionts, parasites or as saprophytes on a substrate. They cause biodegradation of natural materials so they play a significant role in the antibiotic preparations, production of certain beverages and food and in production of important industrial chemicals such as alcohols and enzymes (Bhat at al., 2010). On the other hand, certain moulds can produce mycotoxins and cause harmful illness of humans and animals.

1.2. Definition of mycotoxins

The name mycotoxin is a derived from two words: “mykes”, the Greek word for fungus, and “toxicum”, the Latin word meaning “poison”. Toxins are usually defined as a substance that are produced by living cells or organisms, such a micro-organism, an animal or a plant species, that are harmful to another organism (Turner et al., 2009). Mycotoxins are toxic secondary metabolites of filamentous fungi, or precisely the moulds. Molecular weight of mycotoxins is low, from about 200 to 500 (Whitlow et.al., 2010). They are not necessary for fungal growth. Some moulds produce only one mycotoxin; however, most mycotoxins are produced by more than one fungal species (Bräse et al., 2009). Mycotoxins have adverse effects to crops, domestic animals and man causing many diseases (Sweeney and Dobson, 1998). However, some type of moulds and their secondary metabolites are not toxic (Hussein and Brasel, 2001).

1.2.1. Historical aspect

It is considered, throughout the history, that mycotoxins had been causing some human and animal diseases. They had been associated with diseases in the writings of the

Dead Sea Scrolls and they are even mentioned as the cause of the last of the *Ten Plagues of Egypt* (Marr and Malloy, 1996). In the Middle Ages, ergot alkaloids caused a disease called ergotism, known as *St. Antony's Fire*. It caused gangrenous and convulsive effect.

During the late 1800s and early 1900s, discovery of fermentation increased interest in fungi. After Alexander Fleming's discovery of penicillin, and noticing the antibiotic's curative effect for some diseases, the antibiotic industry rapidly developed (Richard, 2007).

In the 1930s, in Russia, a devastating disease attacked both horses and humans. Thousands of horses died. During the 1940s and 1950s, another lethal disease appeared in Russia. Alimentary toxic aleukia (ATA) was caused by mould contamination of harvested grains. This disease has hemorrhagic, necrotizing and central nervous system effects, resulting often in death. (Richard, 2003). Later, it was proved that trichothecenes caused both diseases. Similar diseases occurred in the United States, mouldy corn toxicosis and Stachybotryotoxicosis (Forgacs and Carll 1962). At the same time, in New Zealand, sporodesmin caused another disease called facial eczema which attacked sheep (Richard, 2008).

Modern mycotoxicology, the study of mycotoxins, really began in 1960s with the outbreak of Turkey-X disease in the U.K. (Whitlow et al., 2010). A large number of turkeys, involving ducklings and pheasants, were dying of unknown disease. At the end it was realized that the problem was contaminated Brazilian groundnut meal and aflatoxin, the responsible agent that had caused disease, was found (Richard, 2008). Since then, research and interest in mycotoxins have been constantly increasing.

1.2.2. Types of mycotoxins

More than 400 types of mycotoxins have been discovered (Bhat at al., 2010).

The classification of mycotoxins is quite complicated, because of their extreme diversity of chemical structures, biological activity, biosynthetic origins and their production of a large number of fungal species (Zain, 2011).

Classification of mycotoxins is often performed according to the research area. Biochemists will classify mycotoxins according to their biosynthetic origins. Cell biologists classify mycotoxins according to the generic groups such as allergens, mutagens, teratogens and carcinogens. Organic chemists classify mycotoxins according to their chemical

structures and clinicians classify them according to their effects on the organ, like immunotoxins, neurotoxins, nephrotoxins. And for physician the classification is based on the illnesses caused by mycotoxins, such as ergotism or stachybotrytoxicosis (Bennett and Klich, 2003).

According to their biosynthetic category, mycotoxins are classified into terpenes (monoterpenes, sesquiterpenes diterpenes), polyketides (tetra-, penta-, hexa-hepta-, octa-, nona- and deca-), tetronic/tetramic acids, peptides, C_6C_3 products, diketopiperazines (simple and modified) (Table 1.) (Steyn,1980).

Table 1. Classification of mycotoxins according to biosynthetic category.

Biosynthetic category		**Mycotoxins**
Terpenes	Mono-	Viridicatumotoxin
	Sesqui-	Trichothecenes
	Di-	Paspaline
Polyketides	Tetra-	Patulin, penicilin acid, chlorfavonin
	Penta-	Citrinin, ochratoxins
	Hexa-	Maltoryzine
	Hepta-	Viriditoxin, cxtochalasins, rugulosin
	Octa-	Ergochromes
	Nona-	Zearalenone, viridicatumtoxin
	Deca-	Aflatoxin, austocystins, erythroskyrine
Tetronic/tetramic acids		Tenuazonic acid, cyclopiazonic acid, cytochalasins
Peptides		Tentoxin, ergotamine, tryptoquivaline
C6 C3 products		Chlorflavonin, xanthocillin, terphenyllin
Diketopiperazines	Simple	Aspergillic acid, echinulins
	Modified	Brevianamides, sporidesmins, fumitremorgens, oxaline

Most common and toxicologically significant mycotoxins are produced by moulds from the genera *Aspergillus, Fusarium and Penicillium* (Sweeney, 1998) (Table 2). Mycotoxins of greatest agroeconomic and public health significance are aflatoxins (AF), ochratoxins (OTA), fumonisins (F), trichothecenes, patulin, zearalenone (ZEN), and ergot alkaloids.

Table 2. Classification of mycotoxin producing fungi.

Classes of fungi	Fungi species	Mycotoxins
Aspergillus	*A. flavus* *A. parasiticus* *A. nominus*	Aflatoxin
	A. ochraceus	Ochratoxin A (OTA)
	A. versicolor	Sterigmatocystin
	A. flavus	Cyclopiazonic acid (CPA)
	A.clavatus	Patulin
Fusarium	*F. equiseti* *F. graminearum* *F. moniliforme* *F. sporotrichioides* *F. culmorum* *F. sporotrichioides* *F.sambucinum*	Trichothecenes
	F. moniliforme *F.proliferatum* *F. napiforme* *F. nygamai*	Fumonisins
	F. graminearum *F. sporotrichioides* *F. culmorum*	Zearalenone
Penicillium	*P. verrucosum*	Ochratoxin A (OTA)
	P.spp.	Cyclopiazonic acid (CPA)
	P. expansum *P. citrinum*	Patulin
	P. expansum *P. verrucosum*	Citrinin

1.2.3. Exposure to mycotoxins

Mycotoxins contamination of food and feed is a serious global problem. According to the Food and Agriculture Organization (FAO), approximately 25% of the worldwide crops can be contaminated by mycotoxins, leading to significant economic losses (FAO, 2002).

Mycotoxins can colonize crops causing contamination at the field, in the pre-harvest stage (*Fusarium*) or in the post-harvest stage, during storage, transport, processing and feeding (*Aspergillus* and *Penicillium*) (Patriarca and Fernández Pinto, 2017). Mycotoxins can enter the animal and human food chains through indirect or direct way (Figure 1). The direct way occurs when the product, food or feed, becomes infected by a mould and mycotoxins. Indirect way, on the other hand, occurs when any ingredient has been earlier contaminated by a mould and even though the mould has been eliminated during the processing, the mycotoxins remain in the final product (da Rocha et al., 2014). Mycotoxins are potential threat to the human health mainly through eating contaminated plant products, as well as through products derived from contaminated food, such as eggs, milk, cheese and meat (Smith et al., 1995).

Mycotoxins have tendency to be persistent and they are extremely difficult to remove from the food and feed chain (Luo et al., 2018).

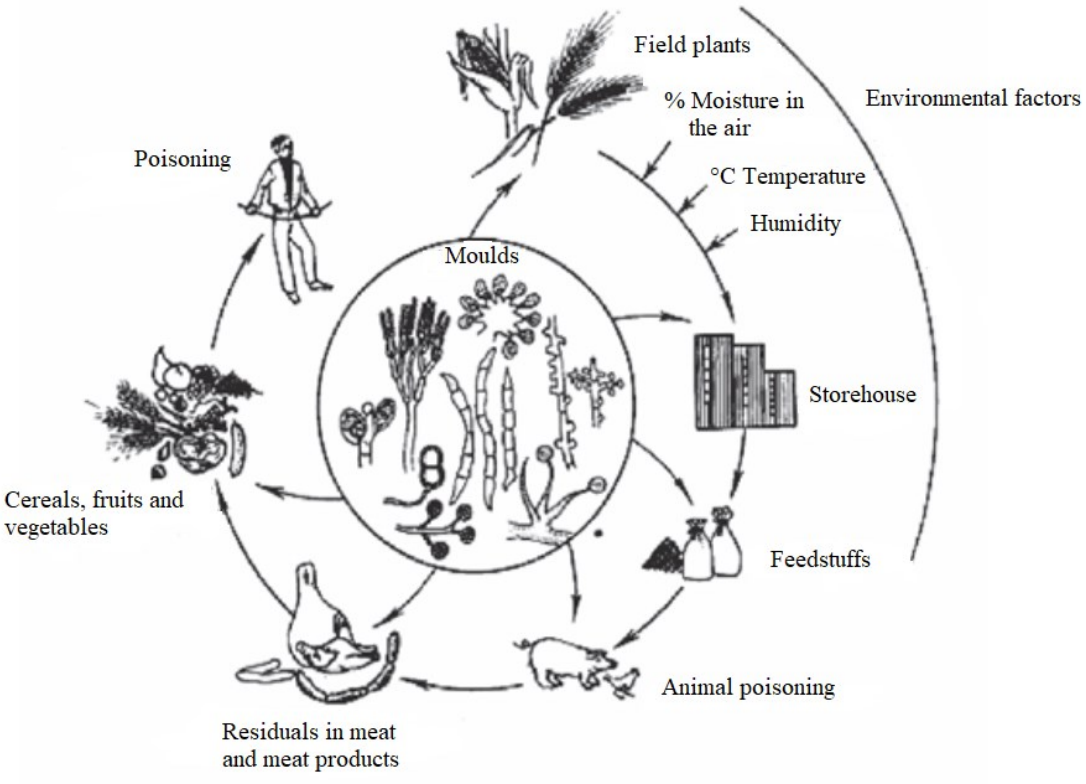

Figure 1. Mycotoxins in food and feed chain (Web 1).

Fungal growth and production of mycotoxins mostly depend on environmental factors such as relative humidity, activity water, pH, temperature, nutrient availability and substrate structure, microbial competition, presence of insects and application of fungicides and pesticides (Anfossi et al., 2016; Hameed et al., 2013). Temperature and humidity play the main rule. Generally, the most dangerous mycotoxins are found in the crops in tropical and subtropical areas with high temperature and humidity (Nešić et al. 2015; Reddy et al., 2010). Normally, fungi grow between 10 and 40 °C, water activity levels above 0.7, a pH range of 4 to 8 and moisture content >13-15% (Whitlow et al., 2010).

Food group mainly affected by mycotoxins are cereals, dried fruit, nuts, cocoa, coffee, spices, dried peas, oil seeds, beans and fruit, particularly apples. Mycotoxins may also be found in wine and beer resulting from the use of contaminated barley, grapes, and cereals in their production (Turner et al., 2009). Ochratoxins are found mainly in cereals, but significant levels of contamination may also occur in wine, beer, grape juice, spices, coffee and dried fruits. Aflatoxins are found in peanuts and maize, as well as in dried fruits and tree nuts (Miličević et al., 2008).

In animal feedstuffs, mycotoxins reduce feed nutrient levels and production. Contaminated feedstuffs can cause health damage and occurrence of serious disease in animals (Laan et al., 2006).

1.2.4. Impact of mycotoxins on human health

Human health risks are associated with direct intake of mycotoxins from contaminated food or indirect consummation with animal-origin products such as milk, cheese, eggs, meat, derived from animal fed with contaminated feed (Capriotti et al., 2012). Also, human exposure to air and dust containing toxins can also present a potential threat to human health (Jarvis, 2002).

Mycotoxins show a large variety of toxic effects in animals and humans, such as immunosuppression, teratogenic, mutagenic, carcinogenic or genotoxic effects (Zhu et al., 2015). The impact of mycotoxins depends on different factors, such as, body weight, species and age of the individual, levels of ingestion and the toxicity of the compound ingested, as well as exposure time, individual physiological condition, the presence of other mycotoxins, and action mechanism of the compound (Richard, 2007; Hussein and Brasel, 2001). Generally considered, populations in developed countries are less exposed to mycotoxins

than those in developing countries because of well-organised system for monitoring of food quality and safety (Bhat et al., 2010).

Mycotoxicosis are diseases caused by mycotoxins (Nelson et al., 1993). Mycotoxins can cause acute and chronic effects in both humans and animals. Acute toxicity generally has a rapid onset and an obvious toxic response. Chronic toxicity is characterized with a low dose exposure over a long time period, often resulting in cancers and other generally irreversible effects (Williams et al., 2015). The main health issues are related to chronic exposure, causing immune suppression, cancer, kidney and liver damage (Bennet and Klich, 2003). Some mycotoxins are neurotoxins and can cause brain damage or death (Pitt, 2000), whereas some of them can reduce immunity by inhibiting protein synthesis and cell proliferation (Bhat et al., 2010). The symptoms of mycotoxicoses are very diverse. The general symptoms of mycotoxicoses in humans are diarrhea, vomiting, and other associated gastro-intestinal problems (Bhat et al., 2010). Others mycotoxicoses may produce several effects including immunosuppression, skin necrosis and leucopoenia (Pitt, 2000).

1.2.5. Regulation of mycotoxins in foods and feeds

The mycotoxin contamination of food and feed is a serious global threat to animals or humans, especially in developing countries, and can lead to many health issues (Stoev, 2013a). In order to protect public health and to ensure fair trade at international level, regulatory control system was developed. The moulds and mycotoxins occurrence can be decreased by the application of various preventative measures before and after harvest such as timely harvesting, management of insect infestation, clean up appropriate drying and storage practices, crop rotation, creating of plant cultures resistant to fungi infestation, etc. (Bata et al., 2001; Doko et al., 1995). Professionals from different scientific fields such as biochemistry, medicine, agriculture, food science and technology, environmental health science, veterinary, can help improving these control and safety activities. An integrated approach to food safety, of Hazard Analysis Critical Control Point (HACCP) has shown as a good way in resolving these problems. HACCP system includes specific and systematic strategies for quality, control, prevention, good manufacturing practise at all stages of production, from the field up to the final consumer (Steov, 2015).

It has been necessary to set the limits for mycotoxins. The main reason is to protect the consumer from the harmful effects of these compounds and to preserve the quality of food (Bhat et al., 2009). Maximum permissible limits have been set for some mycotoxins by the

National or International organisations in EU or in some particular countries all over the world as World Health Organisation (WHO), EU Scientific Committee for Food (SCF), Joint FAO/WHO Expert Committee on Food Additives (JECFA), and some others. In the late 1960s, the first limits were set for the aflatoxins. By the end of 2003, approximately 100 countries have developed specific limits for mycotoxins in food, and number is increasing (Steov, 2015). Most significant mycotoxins are aflatoxins B1, B2, G1 and G2; aflatoxin M1, fumonisins B1, B2 and B3; patulin, ochratoxin A, zearalenone, deoxynivalenol, T-2 and HT-2 toxins. According to the FAO/WHO expert committee recommendations (1990) the tolerance limit for Aflatoxin M1 it is 0.05 μg/kg milk products, for AFB1 is 5 μg/kg food products and for AF B1+G1+B2+G2 is 15 μg/kg, as for example in raw peanuts (Bhat at el., 2009). In 2003, 37 countries enforced tolerance limits for OTA in cereals, and 29 of them have set the limit at 5 μg/kg (FAO, 2004). JECFA established a provisional maximum tolerable daily intake for ZEN to be 0.5 μg/kg of body weight (JECFA, 2000). Most of the countries have set the same level for patulin in food (50 μg/kg) which makes patulin one of the most regulated mycotoxins in the world. In European Union, most of the countries have accepted the limit for deoxynivalenol (750 μg/kg) in wheat and other cereals. The number of countries regulating fumonisins is too small to draw meaningful conclusions about generally agreed limits, but currently limit in most of the countries is 1000 μg/kg in maize. (FAO, 2004).

1.3. Spirocyclic family

A spirocyclic compound contain at least two rings which are linked by only one heavy atom belonging to both rings (Carreira and Fessard, 2014). Spiro containing systems introduce more structural novelty for patentability and have greater three-dimensionality than flat aromatic compounds (Zheng et al., 2014). A large number of spirocyclic compounds are found in many natural products. Because of their physical properties, wide range of biological activities and unique structure, a significant interest in these compounds has increased. Spirocyclic compounds are used for agricultural chemicals, medicines and perfumes (Jin et al., 2009). Despite development of many synthetic methodologies for constructing functionalized spirocycles, it is still a challenge because of their selective construction and stereochemistry (Du et al., 2002).

1.3.1. Spirocyclic PKS - NRPS based fungal metabolites

There is more than twenty-five spirocyclic polyketide synthase-nonribosomal peptide synthetase based fungal natural products. The components of this family contain common 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione core (Figure 2). They were isolated from fungi from *Pseudeurotium*, *Aspergillus*, *Neosartorya* and *Penicillium* genus (Jo and Han, 2018).

All spirocyclins of this family contain a spirocyclic γ-lactam moiety with stereogenic α, β, and γ centres, which two of them are tetrasubstituted chiral centres. They all have similar structure. Hypothesis is that substituent A (Figure 2) of most spirocyclics is derived from a reduction or oxidation of the conjugated diene moiety present in azaspirene (Yamada et al., 2017). The type and the oxidation state of substituent B provides diversity. Substituent C, methyl, is common to all fungal metabolites of this family, expect berkeleyamide D (Yamamoto et al., 2016). Finally, substituent D provides complete stereochemical variations.

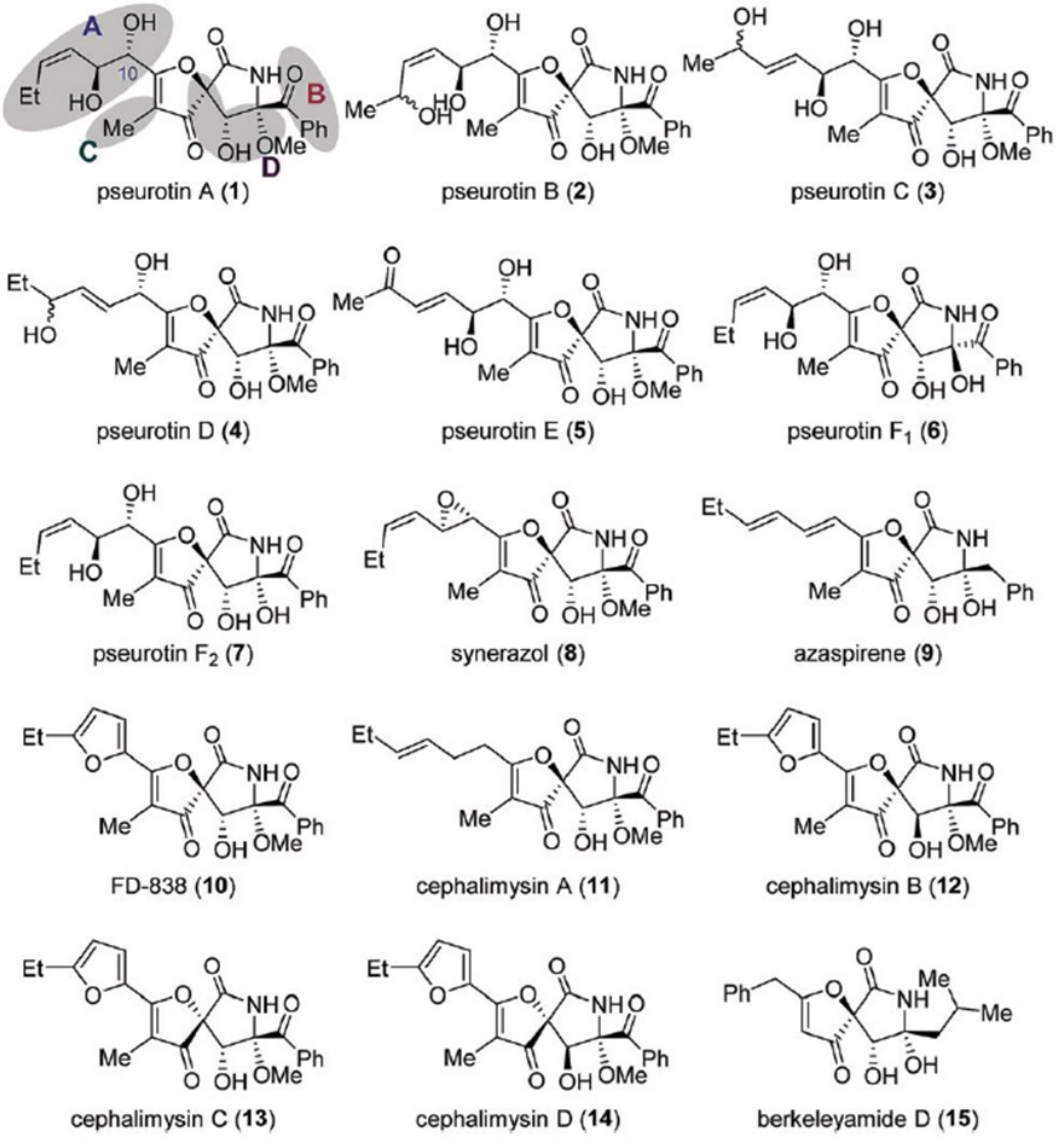

Figure 2. Spirocyclins containing 1-oxa-7-azaspiro [4.4] non-2-ene-4,6-dione core (Jo and Han, 2018).

1.4. The pseurotin family

Pseurotin family contains pseurotin A, B, C, D, E, F_1 and F_2 (Jo and Han, 2018). Bloch et al. (1976, 1981) have isolated pseurotins A-E from *Pseudeurotium ovalis* STOLK. Pseurotins F_1 and F_2 were isolated from *Aspergillus fumigatus* DSM 6598 (Wink et al., 1993).

1.4.1. Pseurotin A

Pseurotin A (PsA) (Figure 3) is a fungal secondary metabolite, first isolated from the cultures of *Pseudeurotium ovalis* STOLK in 1976 (Bloch et al., 1976). It was discovered that PsA has several biological activities, such as inhibition of monoamine oxidase (Maebayashi et al., 1985). In 1993. Wink et al. have discovered that PsA has apomorphine antagonistic activity, and same year, Wenke et al. have discovered that PsA is an inhibitor of chitin synthase. Komagata et al. (1996) have found that PsA induce the cell differentiation. PsA exhibited cytotoxicity with an IC_{50} value of 12 μg/mL in A2780 human ovarian carcinoma cell. It was reported that PsA is inhibitor of immunoglobulin E (IgE) production in vitro (IC_{50} = 3.6 μM) (Ishikawa et al, 2009). Also, PsA exhibited cytotoxicity against the human leukemia, HL-60 cell line (IC_{50} = 67 μM) (Wang et al, 2010).

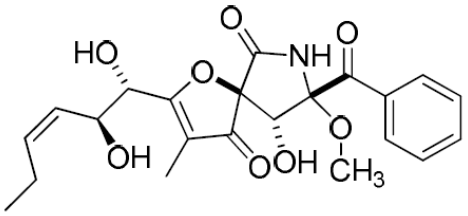

Figure 3. Structure of pseurotin A (Martinez-Luis et al., 2012).

1.4.2. Pseurotin D

Pseurotin D (PsD) (Figure 4) is a fungal secondary metabolite, first isolated from the cultures of *Pseudeurotium ovalis* STOLK in 1976 (Bloch et al., 1976). It was discovered that PsD has apomorphine antagonistic activity (Wink et al., 1993) and also anticancer activity (Martinez-Luis et al., 2012).

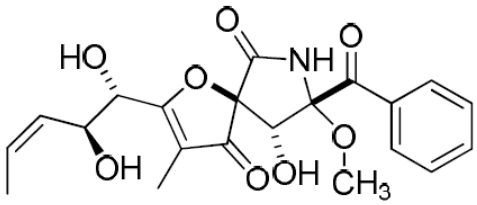

Figure 4. Structure of pseurotin D (Martinez-Luis et al., 2012).

1.5. Lymphoma

There are not many studies on effect of pseurotins on human cells. After initial investigations which outcome indicated that pseurotin itself, and its analogues, could positively affect immunological system in humans, these metabolites gained more attention from researches. Main object of interest was to find out do they have potential to be used as medicine or in prevention of disease, it this case, lymphoma.

The lymphomas include a large number of heterogeneous malignancies that originate in lymphocytes. Harris et al. (1994, 1999) proposed classification of lymphomas consist of Hodgkin's disease, B-cell neoplasms, and T/NK-cell tumors. Non-Hodgkin's lymphoma (NHL) are more common, while Hodgkin's disease represents only about 12% of all lymphomas (Fisher and Fisher, 2004).

1.5.1. Non- Hodgkin's lymphoma

Lymphocytes mature in bone narrow, from hematopoietic stem cells. After differentiation they have a specific phenotype with unique cell surface receptors and a distinct morphology. Lymphoma tumor cells are a malignant form of these precursor lymphocytes. The transformation of these cells represents a multi-step process which leads to the clonal expansion of malignant B- or T-cells, and the formation of leukemic or solid or solid malignant (Fisher and Fisher, 2004). In up to 90% of NHL cases, chromosomal translocations, the genetic hallmark of lymphoid malignancies, has been confirmed. These translocations may lead to oncogene activation or tumor suppressor gene inactivation (Offit et al., 1991). Incorporating the exogenous genes in cells, oncogenic viruses can also provide a potential dangerous for genetic lesions in NHL (Gaidano and Dalla-Favera, 1997). 60-75% of NHL are present in lymphoid tissues, such as bone narrow, lymph node and spleen, but NHL can occur in almost any tissue. NHL can affect skin, bones, brain, eyes, liver, kidney, spleen and digestive tract. NHL affects mostly men, whites and older people (Evens and Blum 2015). Because of the diversity of lymphoma subtypes (Table 3) it is a challenge to investigate this heterogenic and complex disease (Küppers, 2005).

Table 3. Human mature B-cell lymphomas.

Lymphoma	Frequency among lymphomas (%)
B-cell chronic lymphocytic leukemia (B-CLL)	7
Mantle-cell lymphoma	5
B-cell prolymphocytic leukemia	<1
Follicular lymphoma	20
Hairy-cell leukaemia	<1
MALT lymphoma	7
Nodal marginal-zone lymphoma	2
Splenic marginal-zone lymphoma	1
Burkitt's lymphoma	2
Diffuse large B-cell lymphoma	30-40
Primary mediastinal B-cell lymphoma	2
Post-transplant lymphoma	<1
Primary effusion lymphoma	<0.5
Lymphoplasmacytic lymphoma	1
Multiple myeloma	10
Classical Hodgkin's lyphoma	10
Lymphocyte-predominant Hodgkin's lyphoma	0.5

1.5.2. Chronic Lymphocytic Leukemia

B-chronic lymphocytic leukemia (CLL) is characterized by the clonal proliferation and accumulation of neoplastic B lymphocytes in the bone marrow, lymph nodes, blood and spleen (Rozman and Montserrat, 1995). Collins et al. (1989) have described it as a neoplastic disease characterized by a maturation arrest of relatively immature lymphocytes. The phenotype of these cells indicates that they are "frozen" at an early stage of maturation (McConkey et al., 1991). In Western countries, it is the most common leukemia in adults. In most series, more men than women are affected, and median age of patients is 65 years. Only 10 to 15 % are under 50 years old. The clinical course of disease is very variable. Some

patients die from the disease within a few months or years after the diagnosis, while others can live for 20 years or more (Rozman and Montserrat, 1995).

B lymphocytes mature in the bone marrow. In normal, healthy cells, in this process immunoglobulin variable (V) gene segments creates the code for an immunoglobulin molecule that serves as the B-cell receptor for antigen. When antigen reached the B-cell receptor, mature B cell is activated and ready to participate in immune response. CD 38 is a surface molecule that supports B-cell interactions and differentiation and the zeta-chain–associated protein 70 (ZAP-70) has similar activity for T lymphocytes (Chiorazzi et al., 2005).

The monoclonal population of B cells in CLL has showed differences in the mutation status of V genes (Oscier et al., 1997), expression of ZAP-70 (Crespo et al., 2003) and CD38 (Jelinek et al., 2001) and profiling of the expression of genes genome wide (Klein et al., 2001). Because of these mutations and differences in gene expression, CLL is such a heterogenic disorder (Chiorazzi et al., 2005).

To treat patients with CLL, glucocorticoid hormones are commonly used. They reduce the number of peripheral lymphocyte (McConkey et al., 1991). The current strategy is to intensify treatment with combination of chemo-radiotherapy, stem cell transplantation and chemotherapy. In last couple of years, attention has been focused on substances that affect the JAK / STAT signal pathway, which is important in cell proliferation (Nairismägi et al., 2018). In recent time, mycotoxins, secondary metabolites, are an important source of inspiration for the development of organic drugs which will provide the effective treatment. Many of these bioactive substances, have already become valuable pharmaceuticals or are being tested.

1.6. Research aims

Pseurotins are usually not considered to be acutely toxic but are supposed to affect human and farm animal health. The effect of pseurotin on human cells is poorly researched. The main goal of the present research was to investigate the effects of natural pseurotins on human lymphoma cell line and bring new knowledge about their potential therapeutic use. For this purpose, MEC-1 cell line, derived from human chronic B cell leukemia, was treated with different concentrations of PsA and PsD. To investigate the effects on viability and cytotoxicity of lymphoma cells, both PsA and PsD were used. In order to investigate the effects on cell cycle and cell death (apoptosis) of lymphoma cells, only PsD was used.

2. MATERIALS AND METHODS

2.1. Cell line

Cell line used for this experiment was MEC-1 derived from human chronic B cell leukemia. MEC-1 cells were maintained and cultivated on RPMI 1640 medium (HyClone, USA). Media was supplemented with 2.05 mM L-glutamine, 10% fetal bovine serum (FBS) and 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma, USA). Cells were maintained at 37 °C in an atmosphere consisting of 5 % CO_2 and 95 % air. The cells were grown in cell culture flasks of 25 or 75 cm^2 (Techno Plastic Products AG, Switzerland). MEC-1 cells were passaged every 2-3 days at the concentration of 0.5-2.0 x 10^6 cells/ml.

2.2. Exposure of cells to pseurotin A and pseurotin D

The cells were cultured in 24 well cell culture plates (Techno Plastic Products AG, Switzerland) at concentration of 0.2 x 10^6 cells/well and exposed to different concentrations (1 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM and 50 μM) of pseurotin A (PsA) and pseurotin D (PsD) in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) with a total volume of 500 μl/well. DMSO was not toxic for MEC-1 cells in concentrations used as proven by lactate dehydrogenase (LDH) tests (data not shown). Wells without PsA or PsD served as controls. Each treatment was performed in triplicates.

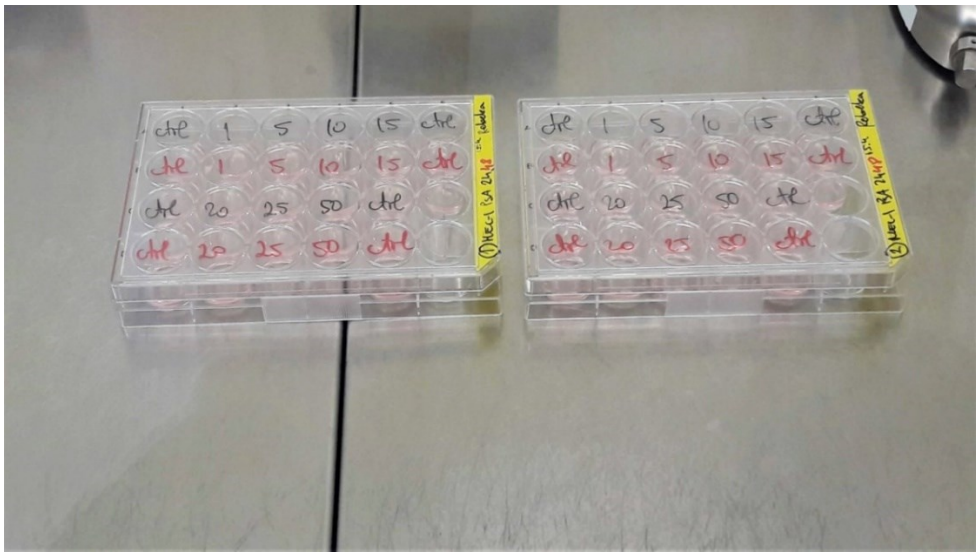

Figure 5. Cell culture plate**s** (photo taken by: Rebeka Bošnjaković)

2.3. The effect of pseurotins on viability and cytotoxicity of lymphoma cell

After exposure for 24, 48, 72 and 96 h, the effect of PsA and PsD on viability and cytotoxicity of lymphoma cell were measured by applying several methods (Figure 6).

2.3.1. Proliferation of cells

For cell counting CASY model TT cell counter and analyser (Roche Innovatis, Germany) was used. Cell suspensions were diluted in an Hemasol (Hemax, Czech Republic).

In CASY cuvette 10 ml filtered Hemasol and 20 µl of cell suspension was added. Before measurement, lid was secured and sample was mixed by tilting the cuvette three times. These data were collected and processed.

2.3.2. Cellular metabolism

Cellular metabolism activity was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (American Type Culture Collection, USA).

After transferring cell suspensions in plastic tubes (2 ml, Eppendorf) and centrifugation at 250 g for 3 min (Eppendorf centrifuge 5417R), all medium was removed. 10 µl of dissolved MTT in phosphate-buffered saline (PBS), pH 7.2, at 2.5 mg/ml was added in cells with 290 µl of (1X) Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA). DMEM was supplemented with 4.5 g/L D-glucose, L-glutamine and pyruvate. The samples were incubated at 37 ºC for three hours.

After incubation, samples were centrifugated at 250 g for 3 min (Eppendorf centrifuge 5417R). The medium was removed, 100 µl of 10% Triton X100 in 0.1 M HCl were ad.ded and samples were placed on Bio RS-24 Mini-Rotator (Biosan, Latvia) for 15 min at room temperature. Samples were transferred to microtiter plate and absorbance was measured at 570 nm on Sunrise Tecan microplate reader (Tecan, Mannedorf, Switzerland). Each measurement was repeated three times. Results were calculated as (100*X)/control; where X is the average reading of a single treatment group.

2.3.3. Lactate dehydrogenase detection

Cytotoxicity Detection Kit ^{PLUS} Lactate dehydrogenase assay (Roche, Germany) was used for measurement of LDH activity released from the cytosol of damaged cells.

After collection of cell suspensions from cell culture plate and transferring in plastic tubes (2 ml, Eppendorf), 15 µl of Lysis solution was added to two samples and incubated at 37 ºC for 1 h. These two samples represent the positive control. The remaining samples were centrifuged at 250 g for 3 min (Eppendorf centrifuge 5417R) and 480 µl of supernatants was kept in a dark at room temperature. In the meantime, working solution was made from catalyst and dye solution in the ratio 1:45.

For measuring absorbance, in each well of microtiter plate 80 µl of sample and 80 µl of working solution were added. Absorbance was measured at 490 nm on Sunrise Tecan microplate reader (Tecan, Mannedorf, Switzerland). Each measurement was repeated three times (independent experiments). The percentage of released LDH in medium was compared to positive controls with the maximum release of LDH.

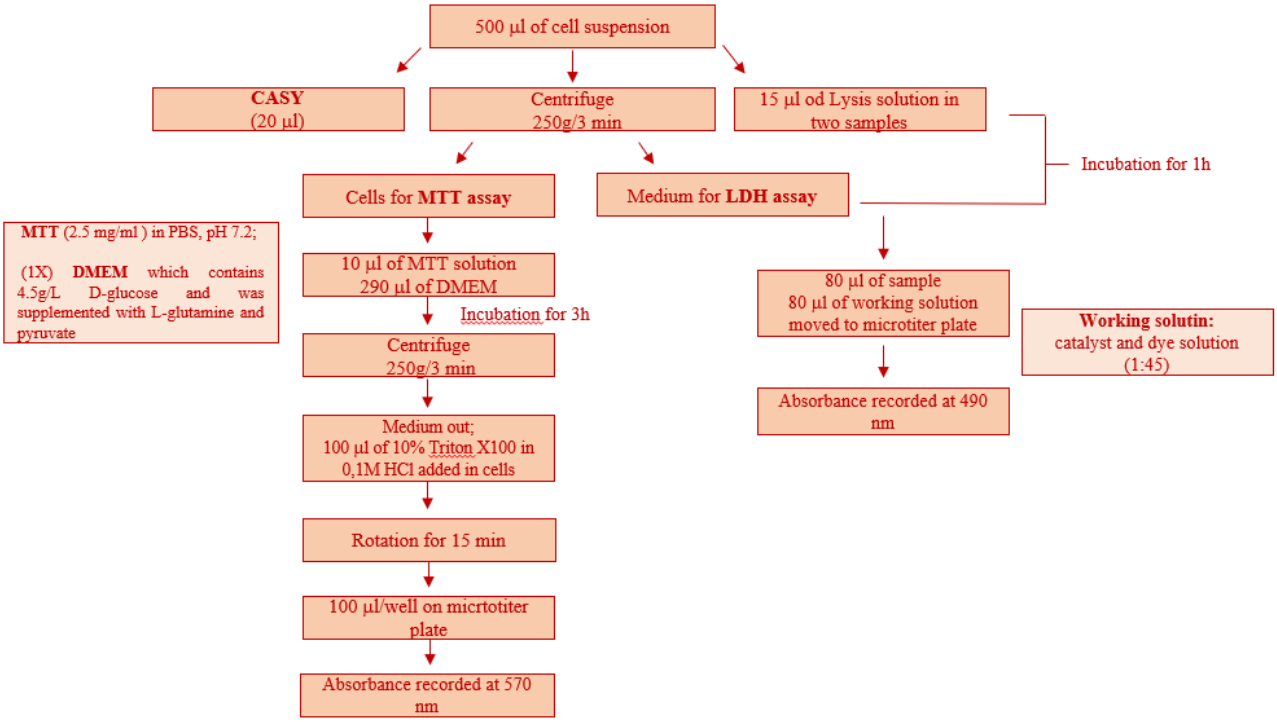

Figure 6. Summary of conducted methods.

2.4. The effect of pseurotins on cell cycle of lymphoma cell

For studying and understanding cell cycle related events it is useful and almost necessary to synchronise the cells because of different metabolic processes in different phases of cell cycle (Ashihara and Baserga, 1979).

Before exposure to PsD, cells were synchronised for 24 h, in the same, RPMI 1640 medium (HyClone, USA) but without FBS.

2.4.1. Detection of cell cycle

The cells were cultured in 24 well cell culture plates (Techno Plastic Products AG, Switzerland) at concentration of 0.2 x 10^6 cells/well and exposed to different concentrations (1μM, 5 μM, 10 μM and 25 μM) of PsD in DMSO (Sigma-Aldrich) with a total volume of 500 μl/well. Wells without PsD served as controls. Each treatment was performed in three independent experiments.

After exposure of 0, 12 and 24 h to PsD for detection of cell cycle progression and synthesizing DNA was used Click-iT® EdU Alexa Fluor ™ 647 Flow Cytometry Assay Kits (Invitrogen, USA).

2.4.1.1. EdU labelling

5 μl of 1 mM Edu (5-ethynyl-2'deoxyuridine) was added to cell suspension and incubated at 37 ºC for 2 h.

2.4.1.2. Cell permeabilization, fixation and nuclear isolation

The cells were transferred to Falcon polypropylene round-bottom tubes (5 ml, Corning, USA) and washed with 3 ml of 1% Bovine serum albumin (BSA) in PBS. Cells were pellet by centrifugation at 300 g for 5 min (Eppendorf centrifuge 5804 R) and supernatant was removed. In pellet was added 100 μl of Click-iT® fixative. Cells were incubated for 15 min at room temperature and protected from light. The cells were washed again with 3 ml of 1% BSA in PBS, pelleted by centrifugation at 300 g for 5 min (Eppendorf centrifuge 5804 R) and supernatant was removed. This time, in pellet 100 μl of 1X Click-iT® saponin-based permeabilization and wash reagent were added. Cells were incubated for 15 min at room temperature and protected from light. The Click-iT® reaction cocktail was prepared according to protocol and the number of samples. 490 μl of reaction cocktail was added to each tube and cells were incubated for 30 min at room temperature and protected from light. The cells were washed with 3 ml of 1X Click-iT® saponin-based permeabilization and wash

reagent, centrifugated at 300 g for 5 min (Eppendorf centrifuge 5804 R) and supernatant was removed.

2.4.1.3. DNA Staining

For staining DNA 300 µl of mixture which contains 1 mg/ml propidium iodide (PI) and 2 mg/ml RNase A in PBS was used. Cells were incubated for 30 min at room temperature and protected from light.

Cells were analysed by flow cytometer (FACSVerse™, Becton Dickinson Bioscience, Germany). The obtained data were analysed using Flowjo7.6.5 software (FlowJo, LLC; USA; www.flowjo.com).

2.4.2. Detection of cyclins expression

Cyclins A, B1, D1 and E were detected by using Western blot method.

The cells were cultured in 24 well cell culture plates (Techno Plastic Products AG, Switzerland) at concentration of 0.5 x 10^6 cells/well and espoused to different concentrations (1µM, 5 µM, 10 µM and 25 µM) of PsD in DMSO (Sigma-Aldrich) with a total volume of 500 µl/well. Before exposure to PsD, cells were synchronised for 24 h, in the same, RPMI 1640 medium (HyClone, USA) but without FBS. Wells without PsD served as controls. The exposure lasted for 0, 6, 8, 12 and 24 h.

2.4.2.1. Sample preparation

To prepare samples for western blot and running on a gel, cells needed to be lysed to release the proteins. Cells were transferred from cell culture plates to 2 ml tubes (Eppendorf) and placed on centrifugation at 500 g for 7 min (Eppendorf centrifuge 5417R). 90 µl mixture of 1 % Sodium Dodecyl Sulfate (SDS) lysis solution and phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich) was added in pellet. Samples were placed on thermo-shaker (TS – 100C, Biosan, Latvia) for 20 min at 100 ºC. Samples were used for proteins detection and the rest were stored in freezer for Western blot.

2.4.2.2. Detection of proteins

The samples were analysed for total protein content using the BCA protein assay kit (Pierce, USA). 10 µl of samples and 200 µl of working solution (50 reagent A:1 reagent B) was transferred to microtiter plate and incubated for 30 min at 37 ºC. After 10 min at room

temperature, absorbance was measured at 562 nm on Sunrise Tecan microplate reader (Tecan, Mannedorf, Switzerland). Amount of proteins was calculated based on the calibration curve with BSA. The specific enzymatic activity was calculated and expressed as relative activity in relation to the control activity.

2.4.2.3. Preparation of PAGE gel

Apparatus used for preparation of polyacrylamide gel consisted of two glass plates, casting frame, casting stand and comb (Bio-Rad, USA). Gel was prepared from separation gel (1.5 M Tris/HCl pH 8.8) and stacking gel (0.5 M Tris/HCl pH 6.8) with a final concentration of 10% (table).

Table 4. Preparation of gel

	Separation gel, 10%	**Stacking gel, 5 %**
Acrylamide-bis solution (30%)	3.33 ml	1.67 ml
Separating buffer 4x	2.50 ml	-
Staking gel buffer 4x	-	2.50 ml
Water demin. or aqua dest.	4.03 ml	5.79 ml
SDS, 10%	100 µl	100 µl
TEMED	22.5 µl	22.5 µl
APS, 10%	45 µl	45 µl

2.4.2.4. Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was applied for separation. Before applying to gel, 20 µl of 5x Laemlli sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris- HCl, pH 6.8) was added in samples and placed on thermo-shaker (TS – 100C, Biosan, Latvia) for 5 min at 100 ºC for denaturation.

Gel was transferred to western blot gel tank (Bio-Rad, Mini-protean Tetra Cell, USA) In first well was loaded 3 µl of protein ladder (Thermo Fisher Scientific USA, and 1.5 µl in the last well. In the rest wells was loaded 10 µl of samples. Running buffer (25 mM Tris, 190 mM glycine and 0.1% SDS) was added in a gel tank. The gel was run at 60 V for 10 min and then at 130 V for 1 h and 30 min. The power was turned off when the dye molecule reached the bottom of gel.

2.4.2.5. Transferring the protein from gel to the membrane

After protein separation by gel electrophoresis, proteins were transferred from gel to the membrane in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). Positively-charged nylon (PVDF) membrane required a pre-treatment: membrane was activated by soaking in methanol. The membrane and gel were sandwiched between paper and sponge and clamped tightly together. The sandwich cassette was placed in the transfer tank (Mini 2-D cell Bio-Rad, USA) and transfer was set at 330 mA for 2 h.

2.4.2.6. Antibody incubation

Cyclin A, B1, D1 and E have the similar molecular weight. Cyclin B1 Antibody (GNS1; sc- 245, Santa Cruz Biotechnology, USA) has the highest molecular weight (60 kDa), cyclin A Antibody (BF683; sc-239, Santa Cruz Biotechnology, USA) has molecular weight of 54 kDa, cyclin E Antibody (HE111; sc-248, Santa Cruz Biotechnology, USA) has molecular weight of 53 kDa and cyclin D1 Antibody (72-13G; sc- 450, Santa Cruz Biotechnology, USA) with the lowest molecular weight (37 kDa). All monoclonal antibodies were raised in mice. Vinculin (E1E9V; #13901, Cell Signaling Technology, Netherlands), used as housekeeping protein, is monoclonal antibody raised in a rabbit.

The membrane was blocked with non-fat milk (5% in TBST) for 1 h and placed on tube roller (R3005, tube roller, Benchmark) and incubated overnight in the primary antibodies cyclin A (1:200), cyclin B1 (1:200), cyclin D1 (1:200), cyclin E (1:200) and Vinculin (1:1000) in a cold room.

Membrane was rinsed three times in 15 ml Tris-buffered saline with Tween 20 (TBST) buffer (20 mM Tris, Ph 7.5, 150 mM NaCl, 0.1 % Tween 20). Secondary antibodies, anti-mouse IgG (1:2000) and anti-rabbit IgG (1:3000) (both Cell Signaling Technology, Netherlands), were added for 1 h on a room temperature. Membrane was again 3x rinsed in 15 ml of TBST.

2.4.2.7. Film developing

SuperSignal™ West Femto Chemiluminescent Substrate and SuperSignal™ West Pico PLUS Chemiluminescent Substrate were used as a chemiluminescent substrate (Thermo Fissher Scinetific, USA). Chemiluminescent substrate was applied to the blot. Chemiluminescent substrate was prepared by mixing weak and strong compounds. Weak compounds were SuperSignal West Pico PLUS Luminol/ Enhancer Solution and SuperSignal West Pico Plus Stable Peroxide Solution. Strong compounds were SuperSignal

West Femto Luminol/ Enhancer Solution and SuperSignal West Femto Stable Peroxide Buffer. Different ratios of these two compounds were used for these antibodies. Soaked in substrate, blot was wrapped in plastic for 2 min. Substrate was removed, a blot was placed on the film (medical X-ray film blue, Agfa, Belgium) and placed in the film cassette for several minutes. Film was soaked in the film developer until the band showed, then moved to water and fixator for several minutes (Figure 7). Films were hanged to dry, scanned with computer scanner and stored in sheet protectors. Optical density was analysed with Fiji program.

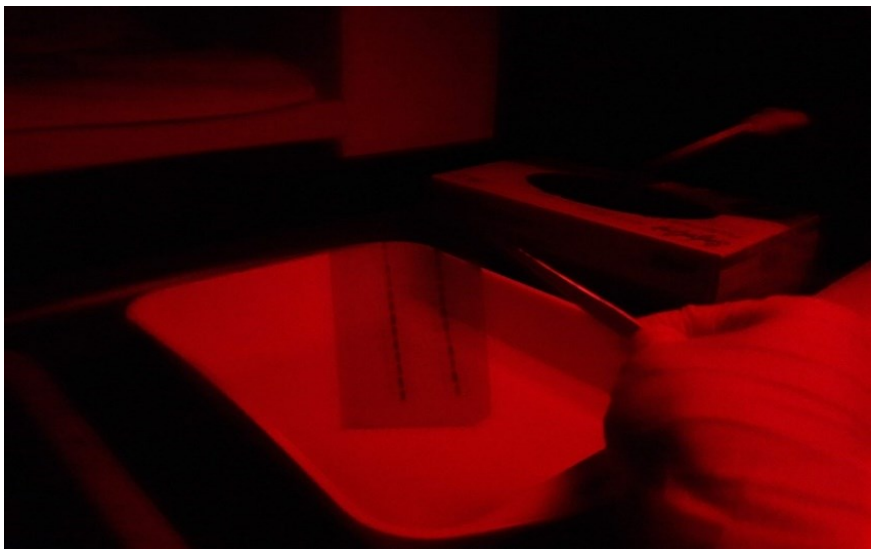

Figure 7. Film developing (photo taken by: Rebeka Bošnjaković)

2.5. The effect of pseurotins on apoptosis of lymphoma cell

Before exposure to PsD, cells were synchronised for 24 h, in the same, RPMI 1640 medium (HyClone, USA) but without FBS.

The cells were cultured in 24 well cell culture plates (Techno Plastic Products AG, Switzerland) at concentration of 0.2 x 10^6 cells/well and exposed to different concentrations (1μM, 5 μM, 10 μM and 25 μM) of PsD in DMSO (Sigma-Aldrich) with a total volume of 500 μl/well. Wells without PsD served as controls. Each treatment was performed in two independent experiments.

After exposure of 24, 48 and 72h to PsD for detection of apoptosis of lymphoma cells, Annexin V Apoptosis Kit-FITC (Apronex biotechnologies, Czech Republic) was used.

The cells were transferred to Falcon polypropylene round-bottom tubes (5 ml, Corning, USA), pellet by centrifugation at 250 g for 4 min (Eppendorf centrifuge 5804 R) and supernatant was removed. 10x Annexin V Binding Buffer was diluted in dist. H_2O (1:10). To that volume, Annexin V-FITC was added in ratio 1:150. 200 µl of this mixture was added in pellet. Samples were vortexed and incubated on ice, in the dark for 20 min. Immediately before analysis by flow cytometry (FACSVerse™, Becton Dickinson Bioscience, Germany), 2 µl of PI (diluted in dist.H_2O; 1:1) were added to each sample.

2.6. Statistical analysis

For statistical analysis, GraphPad Prism, version. 6.01 (GraphPad Software, Inc. San Diego, CA, USA) is used. The results from there/four independent experiments were converted to a % of control and expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; * $p<0.05$; # $p<0.01$ vs. control.

3. RESULTS

3.1. The effect of pseurotins on viability and cytotoxicity of lymphoma cell

3.1.1. Proliferation of cells

After exposure for 24, 48, 72 and 96 h to different concentrations (1 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM and 50 μM) of PsA and PsD, the effect of pseurotins on proliferation of cells was measured by cell counting. Wells without addition of PsA or PsD served as controls. The results showed that none of the concentrations of PsA significantly affected the number of cells and their proliferation during 96 h (Figure 8).

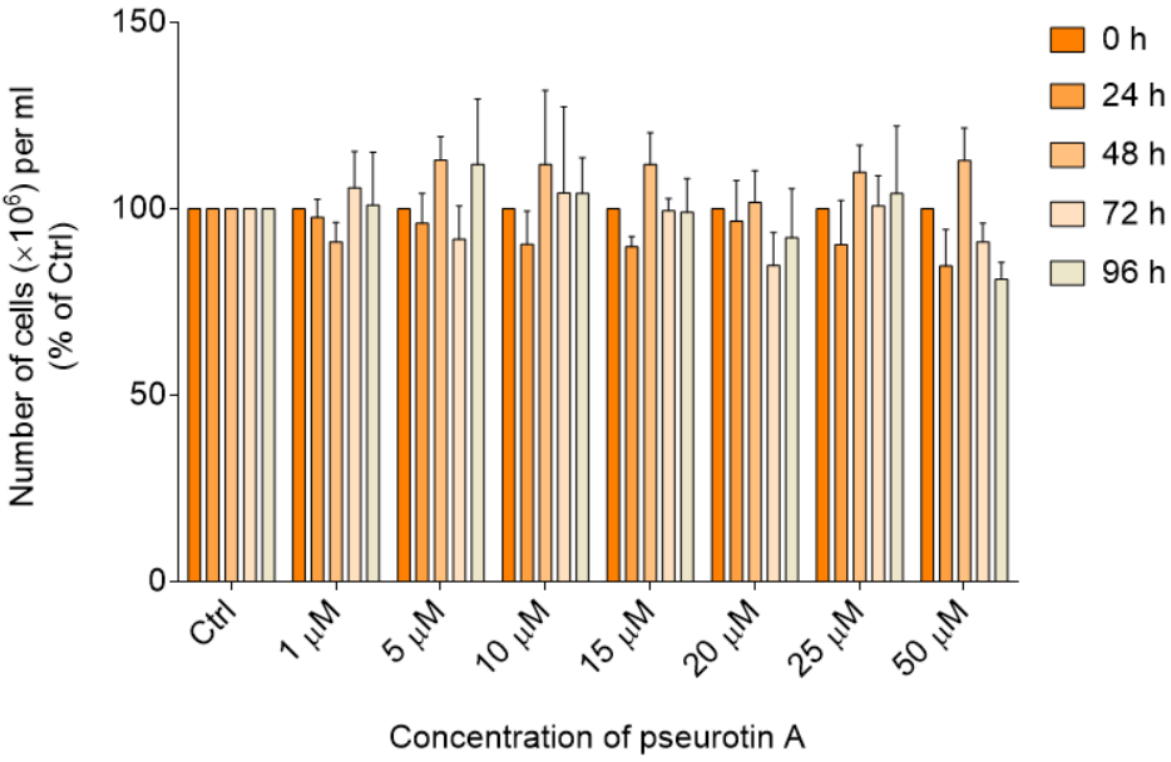

Figure 8. Effect of pseurotin A on proliferation of cells. Pseurotin A did not affect the proliferation of cells during 96h. The results from four independent experiments were converted to a % of control and expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; * $p<0.05$; # $p<0.01$ vs. control.

On the other hand, PsD affected proliferation of MEC-1 cell line. PsD was able to significantly decrease the number of cells (Figure 9). Results showed that the highest

concentration of PsD (50 µM) decreased the number of cells at all time points, 25 µM of PsD after 72 and 96 h and 20 µM of PsD after 96 h.

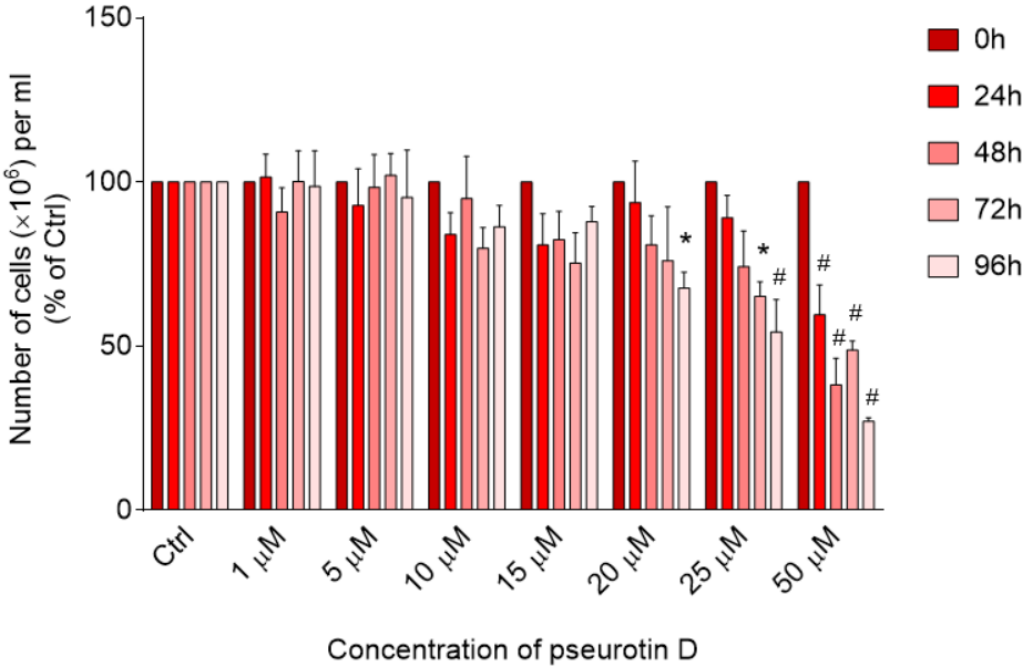

Figure 9. Effect of pseurotin D on proliferation of cells. Pseurotin D decreased the number of cells. The highest concentration of PsD has the strongest effect. The results from four independent experiments were converted to a % of control and expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; * $p<0.05$; # $p<0.01$ vs. control.

3.1.2. Cellular metabolism

After exposure for 24, 48, 72 and 96 h to different concentrations (1 µM, 5 µM, 10 µM, 15 µM, 20 µM, 25 µM and 50 µM) of PsA and PsD, the effect of pseurotins on metabolic activity of cells was measured by using the MTT assay (American Type Culture Collection, USA). The measured absorbance was compared to the control group. Results showed that PsA did not affect the metabolic activity of cells during 96 h (Figure 10). There were no statistically significant differences between the groups comparing to the control group.

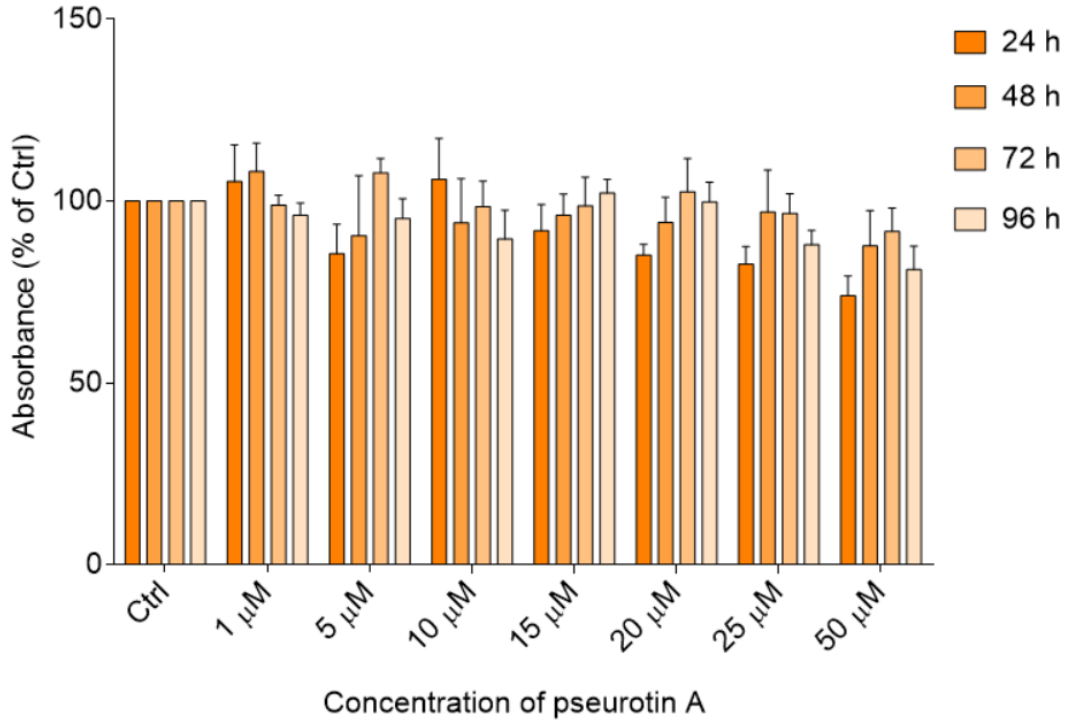

Figure 10. Effects of pseurotin A on metabolic activity of cells. Pseurotin A did not affect the metabolic activity of cells during 96 h. The results from four independent experiments were converted to a % of control and expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; * $p<0.05$; # $p<0.01$ vs. control.

PsD, again, showed stronger inhibition effect on the metabolic activity of MEC-1 cell line. The lower concentrations of PsD did not affect the cells, but the highest concentration of PsD (50 µM) significantly decreased metabolic activity of cells (Figure 11).

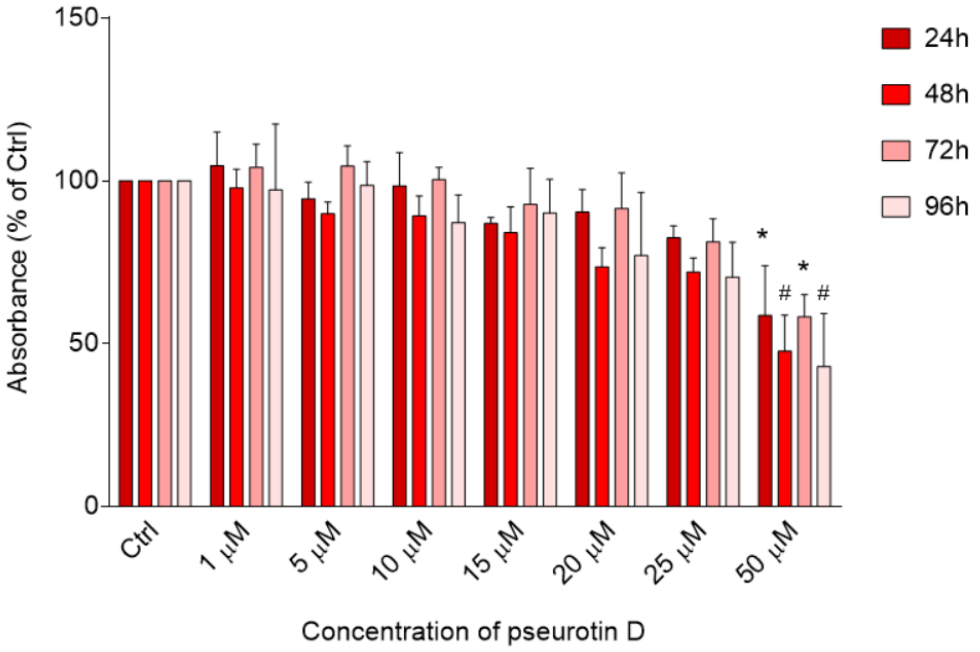

Figure 11. Effects of pseurotin D on metabolic activity of cells. Pseurotin D affected the metabolic activity of cells at the highest concentration at all time points. The results from four independent experiments were converted to a % of control and expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; * $p<0.05$; # $p<0.01$ vs. control.

3.1.3. Cytotoxicity

After exposure for 24, 48, 72 and 96 h to different concentrations (1 µM, 5 µM, 10 µM, 15 µM, 20 µM, 25 µM and 50 µM) of PsA and PsD, the effect of pseurotins on metabolic activity of cells was measured by using the Cytotoxicity Detection Kit ^{PLUS} LDH assay (Roche, Germany). LDH assay was used for measurement of LDH activity released from the cytosol of damaged cells. The percentage of released LDH in medium was compared to positive controls with the maximum release of LDH. Results showed that PsA did not affect the cytotoxicity of cells during 96 h (Figure 12). There were no statistically significant differences compared to the control group.

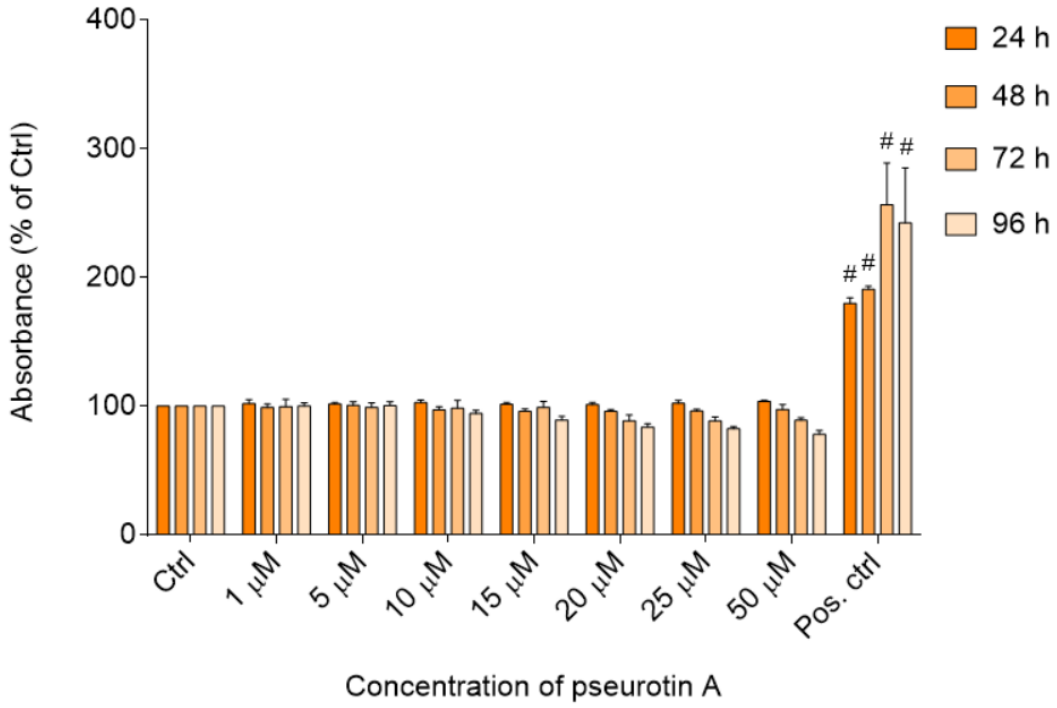

Figure 12. Effects of pseurotin A on cytotoxicity of cells. Pseurotin A did not affect the cytotoxicity of cells during 96 h. The results from four independent experiments were converted to a % of control and expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; * $p<0.05$; # $p<0.01$ vs. control.

Further, at the highest concentration of PsD (50 µM) an increase of LDH release into the medium was observed, but without statistically significant difference (Figure 13).

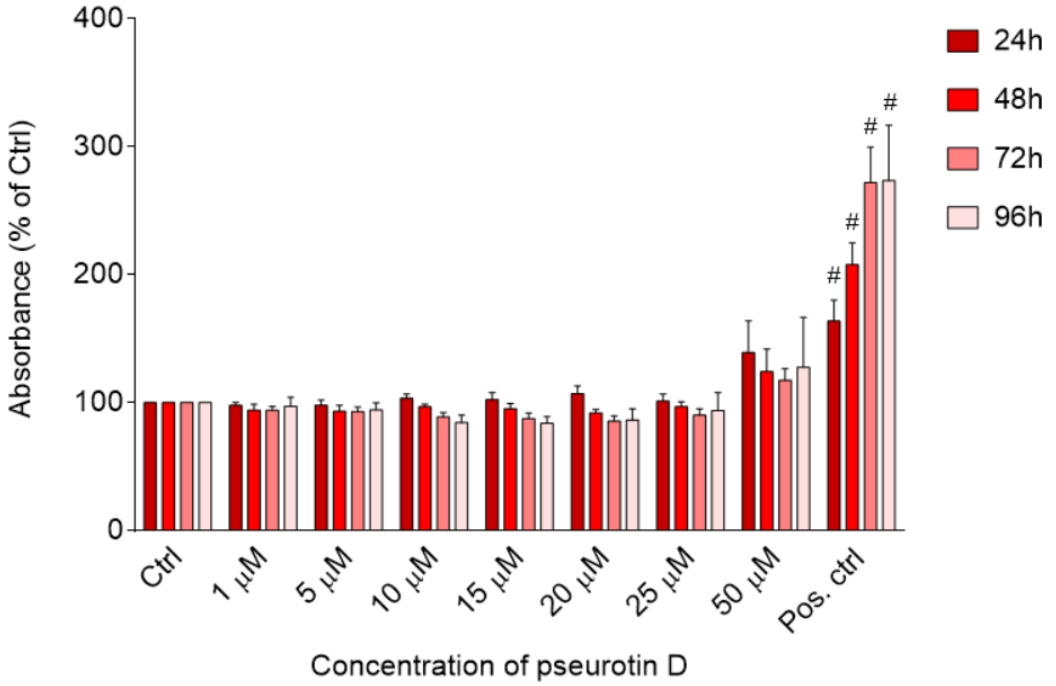

Figure 13. Effects of pseurotin D on cytotoxicity of cells. Pseurotin D increased LDH release into the medium. The results from four independent experiments were converted to a % of control and expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; * $p<0.05$; # $p<0.01$ vs. control.

3.2. The effect of pseurotins on cell cycle of lymphoma cell

3.2.1. Detection of cell cycle

For cell cycle experiments, cells were synchronized for 24 h by removing FBS from the RPMI 1640 medium. After synchronization, cells were exposed to different concentrations of PsD (1, 5, 10 and 25 μM) for 0, 12 and 24 h. For detection of cell cycle progression and synthesizing DNA Clic-iT-EdU Flow cytometry assay kit (Invitrogen, USA) was used. Results showed that PsD had an effect to cell cycle of MEC-1 cell line (Figure 14). Figure 15 represents the cell cycle distribution 24 h after synchronization. After 12 h, by increasing the concentration of PsD, the number of cells in S phase increased, but without statistically significant difference (Figure 16). However, after 24 h, PsD significantly inhibited the cell cycle. The cells slowed the transition through the S phase (Figure 17).

12h

24h

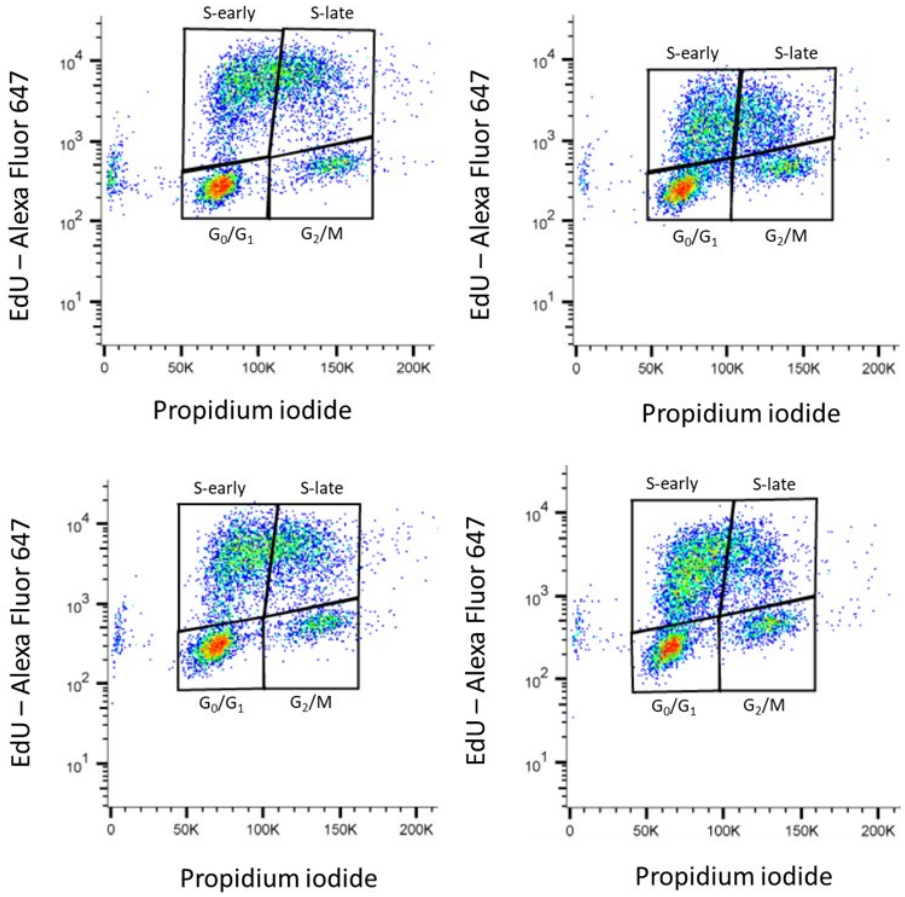

Figure 14. Effects of pseurotin D on the cell cycle. Representative results from Flow cytometry. After 12 h, by increasing the concentration of pseurotin D, increased the number of cells in S phase, but without statistically significant difference. After 24h, PsD significantly inhibited the cell cycle. The obtained data were analysed using Flowjo7.6.5 software

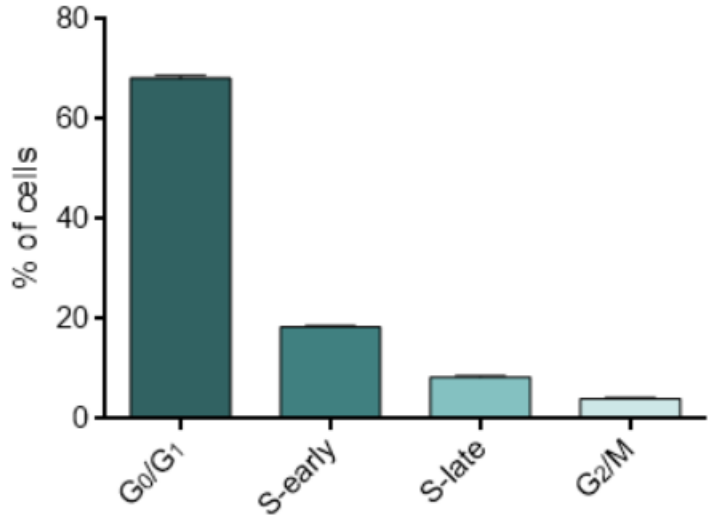

Figure 15. Cell cycle of synchronised cells. Most of the cells are in G_0/G_1 phase. The results from four independent experiments were expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; # $p<0.01$ vs. control.

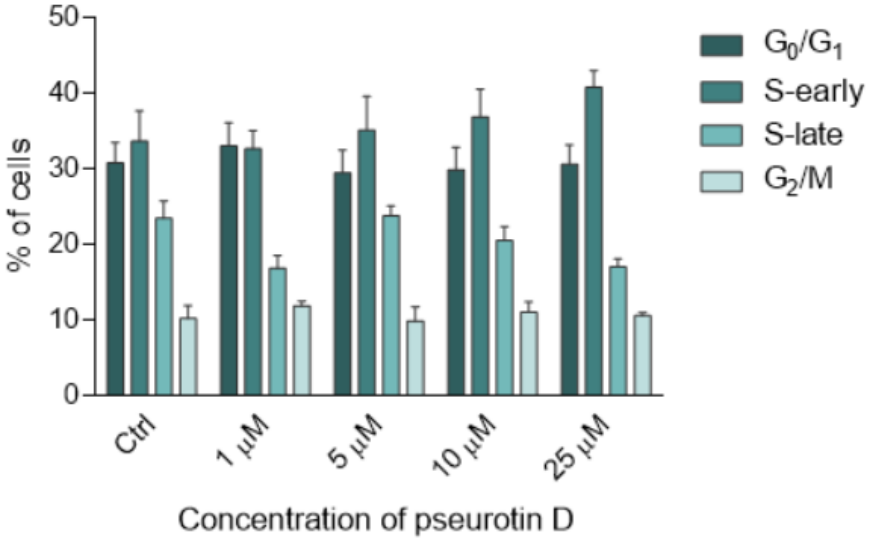

Figure 16. Effects of pseurotin D on the cell cycle after 12h. The highest concentration of pseurotin D increased the number of cells in S phase, but without statistically significant difference. The results from four independent experiments were expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; # $p<0.01$ vs. control.

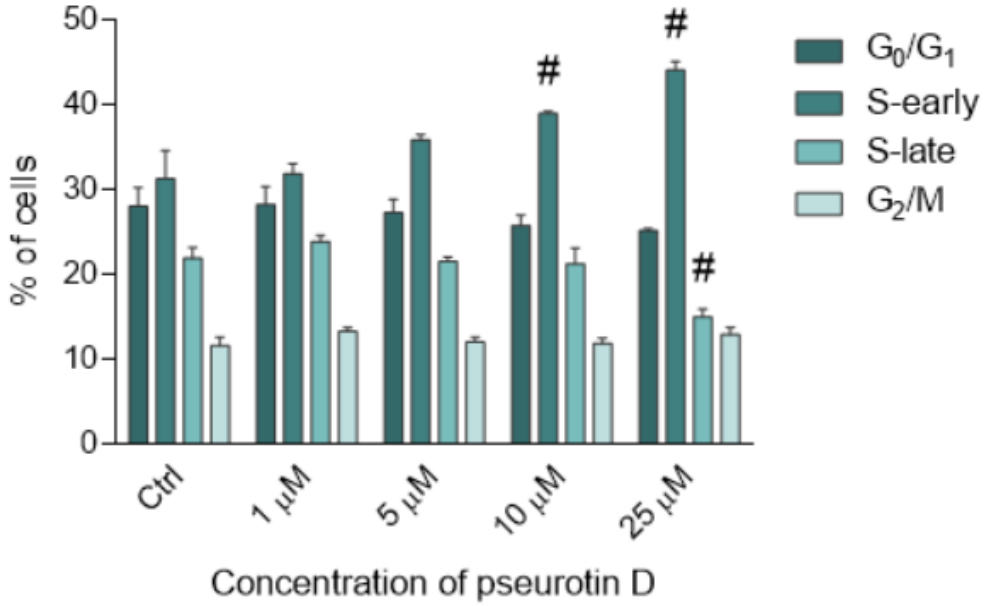

Figure 17. Effects of pseurotin D on the cell cycle after 24 h. The highest concentration of pseurotin D significantly increased the number of cells in S phase. The results from four independent experiments were expressed as the mean ± SEM. Data were statistically analysed using ANOVA, which was followed by Dunnett's multiple comparison test; # $p<0.01$ vs. control.

3.2.2. Detection of cyclins expression

For detection of cyclins expression, cells were synchronized for 24 h by removing FBS from the RPMI 1640 medium. After synchronization, cells were exposed to different concentrations of PsD (1, 5, 10 and 25 μM) for 0, 6, 8, 12 and 24 h. For detection of cyclins expression Western blot method was used. Only one repetition was made. Optical density of cyclins was measured and a decreasing trend is visible. Those, preliminary results showed that PsD decreased cyclins expression, but it is necessary to do several repetitions for statistical analysis and significant results (Figure 18, 19, 20, 21).

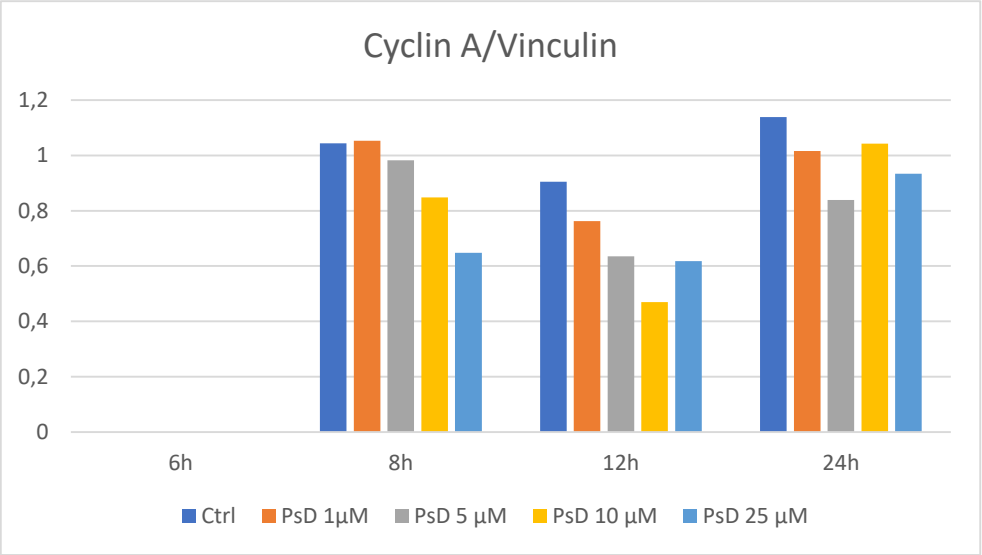

Figure 18. Optical density of Cyclin A. The results from one independent experiment.

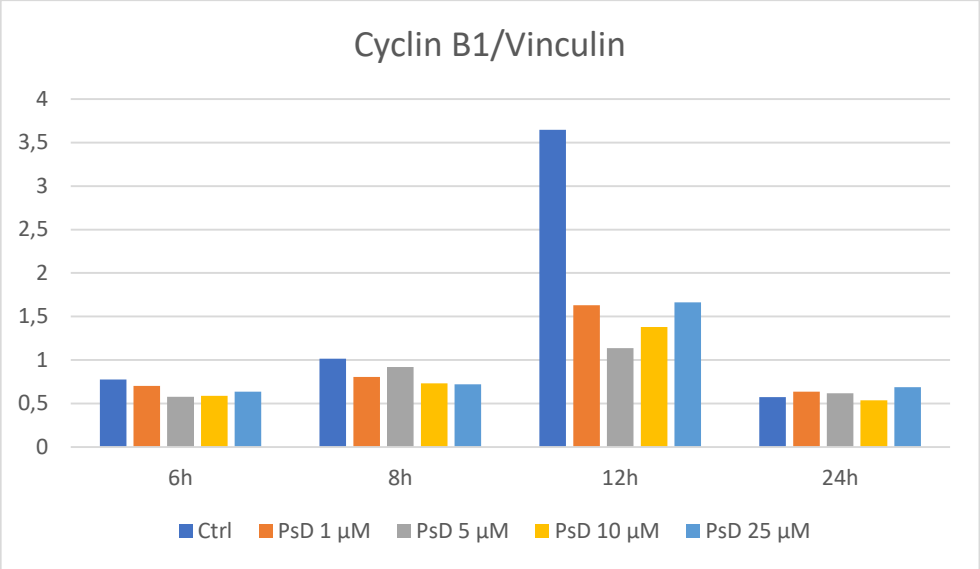

Figure 19. Optical density of Cyclin B1. The results from one independent experiment.

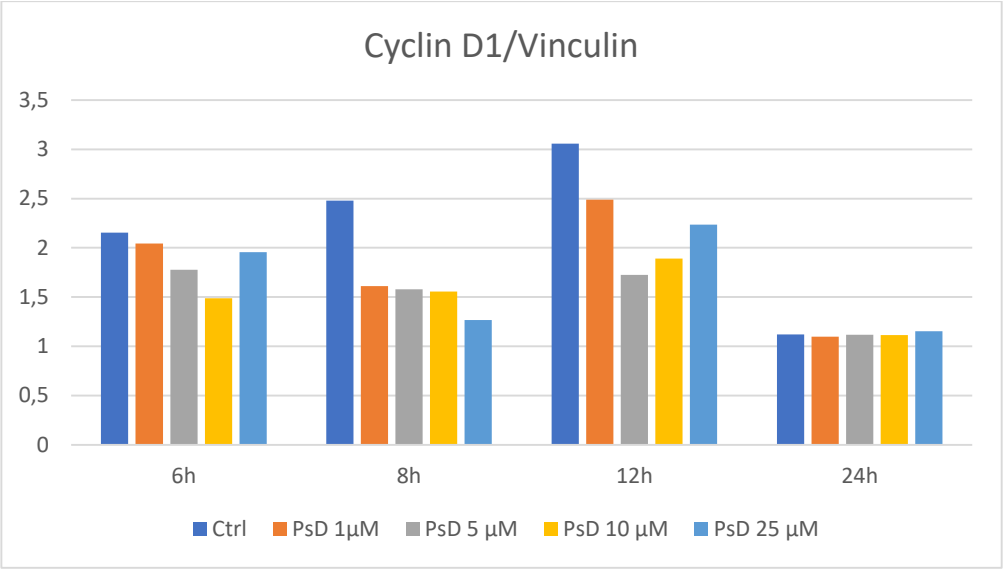

Figure 20. Optical density of Cyclin D1. The results from one independent experiment.

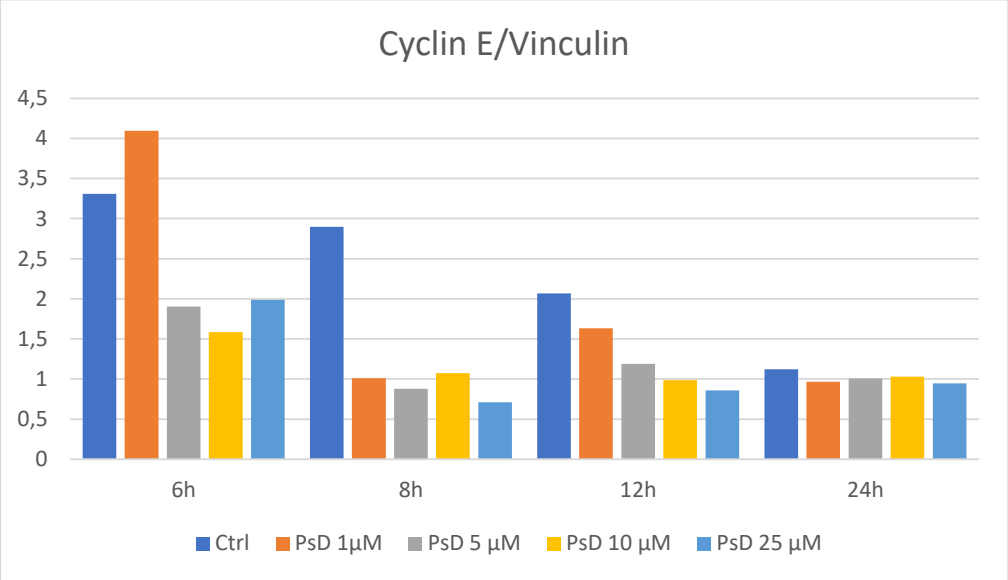

Figure 21. Optical density of Cyclin E. The results from one independent experiment.

3.3. The effect of pseurotins on apoptosis of lymphoma cell

For cell cycle experiments, cells were synchronized for 24 h by removing FBS from the RPMI 1640 medium. After synchronization, cells were exposed to different concentrations of PsD (1, 5, 10 and 25 µM) for 24, 48 and 72h. For detection of apoptosis of lymphoma cells, Annexin V Apoptosis Kit-FITC (Apronex biotechnologies, Czech Republic) was used. Results showed that PsD did not affect apoptosis of MEC-1 cell line in these concentrations after 24, 48 and 72 h (Figure 22, 23 and 24).

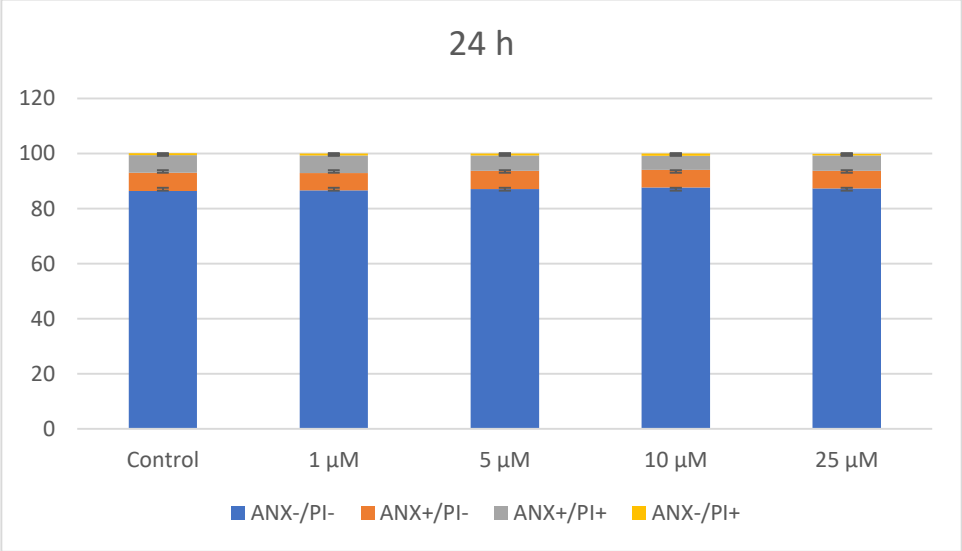

Figure 22. Effects of pseurotin D on the apoptosis after 24h. The results from two independent experiments were expressed as the mean ± SEM.

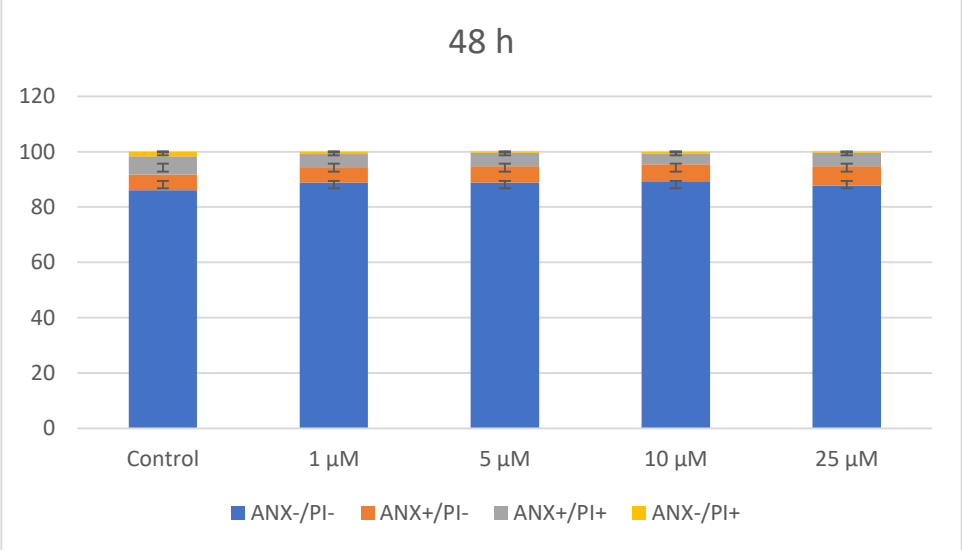

Figure 23. Effects of pseurotin D on the apoptosis after 48h. The results from two independent experiments were expressed as the mean ± SEM.

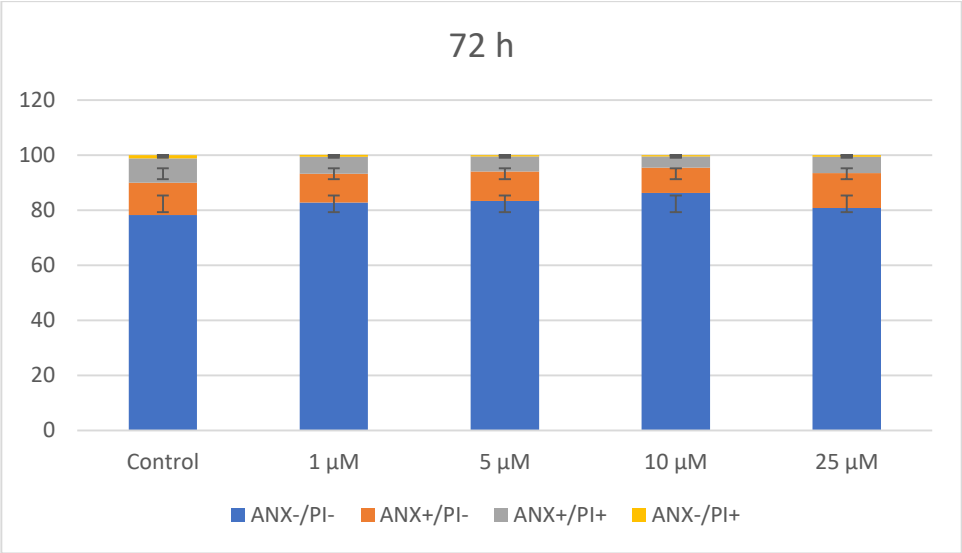

Figure 24. Effects of pseurotin D on the apoptosis after 72h. The results from two independent experiments were expressed as the mean ± SEM.

4. DISCUSSION

In this research, the main goal was to investigate the effect of natural pseurotins, precisely pseurotin A and Pseurotin D, on human lymphoma cell and bring new knowledge about their potential therapeutic use. For this purpose, MEC-1 cell line, derived from human chronic B cell leukemia, was treated with different concentrations of PsA and PsD.

Pseurotins are secondary metabolites of filamentous fungi, or specifically the moulds (Whitlow et.al., 2010). Pseurotins family have interesting and diverse bioactivity on immunological system in humans because of their unique structure, biosynthetic origins and their production of a different fungal species. Because of these characteristics a significant interest in these compounds has increased (Jin et al., 2009). In two independent studies, Vašíček et al. (under review 1 and 2) showed positive results in which pseurotins affected certain type of cells. In first study, pseurotins were shown to inhibit the activation of mouse B-cells via a STAT signalling pathway, resulting in decreased proliferation and differentiation of B-cells (Vašíček et al., under review 1). Although the detailed mechanism of action has not been clarified yet, there is some thoughts that pseurotins could directly interfere with STATs, act upstream of STATs interaction with cytokine receptor intracellular domain, or operate through another JAK-independent pathway (Wang et al., 2007). Second study showed that both pseurotins inhibited RAW264.7 macrophage proliferation and suppressed their responses to LPS-stimulation without any toxic effects up to 50 μM. These effects were connected with inhibition activation of several key signalling pathways since pseurotins decreased phosphorylation of several members of the STAT signalling family and EKR1/2 from the MAPK family in LPS activated macrophages (Vašíček et al., under review 2). Therefore, the next step was to investigate effect on lymphoma cells as a potential drug for treatment.

The lymphomas include a large number of heterogeneous malignancies that originate in lymphocytes. They are classified as Hodgkin's disease and Non-Hodgkin's lymphoma (NHL) (Harris et al. 1994, 1999). Lymphoma tumor cells are a malignant form of these precursor lymphocytes. The transformation of these cells represents a multi-step process which leads to the clonal expansion of malignant B- or T-cells, and the formation of leukemic or solid or solid malignant (Fisher and Fisher, 2004).

B-chronic lymphocytic leukemia (CLL) is characterized by the clonal proliferation and accumulation of neoplastic B lymphocytes in the bone marrow, lymph nodes, blood and spleen (Rozman and Montserrat, 1995). In Western countries, it is the most common

leukemia in adults. Because of a lot of mutations and differences in gene expression, CLL is such a heterogenic disorder with a very variable clinical course. (Chiorazzi et al., 2005). To treat patients with CLL, glucocorticoid hormones are commonly used. They reduce the number of peripheral lymphocyte (McConkey et al., 1991). Another way of treatment is combination of chemo-radiotherapy, stem cell transplantation and chemotherapy. Nairismägi et al. (2018) considered that JAK / STAT signal pathway, which is important in cell proliferation, could have an important role in treatment. In recent years, mycotoxins, secondary metabolites of filamentous fungi gained attention of researchers for the development of organic drugs which will provide the effective treatment.

According to the obtained results, PsA did not affect the cells in significant way in concentrations used (1 – 50 μM) in this research. During 96 h, PsA did not affect cell proliferation, mitochondrial activity and cytotoxicity of human lymphoma cell line which indicate that PsA cannot be used as a potential cytostatic. Those results are opposite to previous two studies about PsA effect on cancer cells. Namely, Komagata et al. (1996) found that PsA induce the cell differentiation and exhibited cytotoxicity with an IC_{50} value of 12 μg/mL in A2780 human ovarian carcinoma cell. In second study it was shown that PsA exhibited cytotoxicity against the human leukemia, HL-60 cell line (IC_{50} = 67 μM) (Wang et al., 2010). The difference in response to PsA in MEC-1 cell line and those two could be in different type of cells and different source of compounds. Also, the effective dose is more than 50 μM. Earlier studies discovered that PsA has several biological activities. PsA is an inhibitor of monoamine oxidase (Maebayashi et al., 1985). Wink et al. (1993) discovered that PsA has apomorphine antagonistic activity, and Wenke et al. (1993) discovered that PsA is an inhibitor of chitin synthase. It was reported that PsA is inhibitor of immunoglobulin E (IgE) production in vitro (IC_{50} = 3.6 μM) (Ishikawa et al, 2009).

Contrary to PsA, PsD affected the tested MEC-1 cells. Results indicate effect of PsD on viability and cytotoxicity of lymphoma cells. PsD decreased the number of cells and metabolic activity of cells and increased LDH release into the medium. These results could be explained by the slight difference in a very similar structure of those two compounds. PsD carries a different C2 side chain. Because of that PsD was an even more potent inhibitor of IgE production Vašiček et al. (under review, 1).

The effect of PsD on cell cycle of lymphoma cell includes detection of cell cycle and detection of cyclins expression which PsD affected both. After cell synchronisation, most of

the cells were in G0/G1 phase. During 24h exposure to PsD (10 μM and 25 μM), the cell cycle was inhibited. The cells slowed the transition through the S phase. No details are known about this effect and mechanism of PsD on cell cycle. The possible explanation could be connected with inhibitory potential of pseurotins on several signalling pathways but there is more place for further investigation. Results obtained in this research indicate that PsD decreased cyclins expression but since it was tested only on one occasion it is necessary to do more repetitions to confirm observed effect.

The last research aim of this study was to investigate effect of PsD on apoptosis of lymphoma cell. Results showed that PsD did not affect apoptosis of MEC-1 cell line in concentrations of 1, 5, 10 and 25 μM after 24, 48 and 72 h. For better understanding, additional research should be done with higher concentrations and longer exposure.

Previous studies showed that Pseurotins family have interesting and diverse bioactivity on immunological system in humans. Still, little is known about PsD effect. This is a relative new area of research so more study is necessary. However, there is a couple of studies who did a research about PsD and his effect on immunological system in humans. They all have similar results. Martinez-Luis et al. (2012) obtained similar results and showed that PsD has anticancer activity on MCF-7, human breast cancer cell line (IC_{50} = 15.6 μM). In the same study it was showed that all mycotoxins isolated from *Aspergillus sp*. strain F1544 (pseurotin A, 14-norpseurotin A, FD-838, and pseurotin D, and fumoquinone) have good anticancer and antiparasitic and activity, and especially presented strong activity against the parasite that causes leishmaniasis (*L. donovani*) concluding that they can be used as a promising drug against it. Cytotoxic activities of PsD is also reported by Saiaraiva et al. (2014). They showed that PsD affected tumour cell line HCT-116 (human colon carcinoma). Comparation of results from present research and results from previous studies has indicate cytotoxic activities of PsD. According to these results, PsD could be used as a cytostatic. Also, PsD was shown to have apomorphine antagonistic activity (Wink et al., 1993).

5. CONCLUSIONS

To investigate the effects of natural pseurotins on human lymphoma cell line and bring new knowledge about their potential therapeutic use, MEC-1 cell line, derived from human chronic B cell leukemia, was treated with different concentrations of PsA and PsD. Main conclusions of the present research are:

1. PsA did not affect MEC-1 cell line, in used concentrations (1, 5, 10, 15, 20, 25 and 50 μM), during 96 h.
2. PsD did affect cell proliferation, cellular metabolism and cytotoxicity of lymphoma cell in concentration of 20, 25 and 50 μM.
3. After 24 h, PsD significantly inhibited the cell cycle. The cells slowed the transition through the S phase.
4. Preliminary results showed that PsD decreased cyclins expression, but it is necessary to do several repetitions to confirm observed effect.
5. Results showed that PsD did not affect apoptosis of MEC-1 cell line in used concentrations (1, 5, 10 and 25 μM) after 24, 48 and 72 h.

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