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Katedra farmakologie

ÚČINKY ROSTLINNÝCH EXTRAKTŮ Z BAZALKY, JALOVCE A VAVŘÍNU NA ŽIVOTASCHOPNOST SH-SY5Y BUNĚK NEUROBLASTOMU.

Diplomová práce

ve spolupráci s
UNIVERZITOU HELSINKI
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THE EFFECTS OF PLANT EXTRACTS FROM BASIL, JUNIPER AND LAUREL ON THE CELL VIABILITY IN SH-SY5Y NEUROBLASTOMA CELLS.

Diploma thesis

in cooperation with
UNIVERSITY OF HELSINKI
FACULTY OF PHARMACY
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ABSTRACT

Cancer is one of the major causes of death in the world, especially in developed countries. There are already many antiproliferative agents used to cure patients with cancer. However, many of the currently approachable medicines are synthetic chemicals, which are invasive and very often cause alterations in healthy cells. Because of these reasons, there is a tendency to turn back to natural agents used mainly traditionally in folk medicine, because some of them might be potentially effective against tumor development.

In this study, the cytotoxic effects of basil, laurel and juniper extracts on neuroblastoma cancer cells were tested by using MTT and LDH assays. The extracts at concentrations 2; 0,5; 0,1; 0,05 and 0,01 mg/ml were effecting the cells for 12 hours. Another method used for studying of extract's effects was western blotting analysis showing the level of tumor supressor p53 protein in the neuroblastoma cells after extract treatment. The results with treated cells were compared to the untreated cells serving as a control.

The results of MTT assay show decrease in cell viability of cells treated with all three plant extracts at concentration 2 mg/ml. Also four other concentrations of laurel and juniper extracts 0,5; 0,1; 0,05 and 0,05 mg/ml highly decreased the cell viability in comparison to control. Basil extract was effective only at concentration 2 mg/ml. The results of LDH assay show significant decrease in cell viability by cells treated with all three extracts at concentration 2 mg/ml. The other concentrations of all three extracts did not significantly decrease the cell viability compared to control. The reason for different results in MTT and LDH assay probably consists in different mechanisms of methods MTT and LDH assay. It is also possible that the mitochondria of cells does not work any more whilst the cell integrity is not disturbed yet. Western blotting analysis with 0,1 mg/ml concentration of laurel extract did not show any significant differences in p53 protein level compared to control even after longer incubation time (72 hours) with the extract. Cells treated with the basil extract at concentration 0,5 mg/ml showed little higher p53 level after 24 and 30 hours of incubation compared to control.

As the results of this study imply, laurel, basil and juniper extracts have cytotoxic effects on SH-SY5Y human neuroblastoma cells and might also influence the p53 level. These activitities depend on the concentration of extract and incubation time.

ABSTRAKT

Rakovina je jednou z hlavních příčin smrti ve světě, především ve vyspělých zemích. Existuje již celá řáda antiproliferativních látek, používaných k léčení pacientů. Ovšem mnoho při léčbě rakoviny dnes používaných látek jsou syntetické produkty, které jsou často invazivní a způsobují poškození rovněž zdravých buněk. To jsou důvody tendence vracet se zpět k přírodním látkám, které byly odedávna používany v lidové medicíně a některé z nich jsou potenciálně účinné proti rozvoji nádorů.

Ve své práci jsem studovala cytotoxické účinky extraktů z jalovce, vavřínu a bazalky na buňky neuroblastomu pomocí MTT a LDH testů. Extrakty v koncentracích 2; 0,5; 0,1; 0,05 a 0,01 mg/ml působily na buňky po dobu 12 hodin. Ke studiu účinků extraktů byla použita také western blotting analýza, pomocí níž byla zjišťována hladina tumor supresorového p53 proteinu v buňkách po působení extraktů. Ve všech testech byly výsledky experimentů s buňkami, na něž extrakt působil, srovnáván s výsledky experimentů s buňkami, na něž extrakt nepůsobil a které tedy sloužily jako kontrola.

Výsledky MTT testů ukazují pokles životaschopnosti buněk v experimentech, ve kterých byly použity všechny tři extrakty v koncentraci 2 mg/ml. Velmi účinná se ukázala být koncentrace 0,5 mg/ml extraktu z vavřínu. Také ostatní koncentrace vavřínu a jalovce značně snižovaly životaschopnost buněk ve srovnání s kontrolou. Bazalka v jiné koncentraci než 2 mg/ml životaschopnost buněk výrazně nesnižovala. Výsledky LDH testů ukazují výrazný pokles životaschopnosti buněk působením všech tří extraktů v koncentraci 2 mg/ml. Ostatní koncentrace všech tří extraktů nevykazují výrazné účinky. Rozdíly ve výsledcích MTT a LDH testů mohou být způsobeny tím, že mitochondrie již nefungují, ale zatím nedošlo k poruše integrity buňky, jinými slovy rozdílným mechanismem metod MTT a LDH assay. Výsledky western blotting analýzy s extraktem z vavřínu o koncentraci 0,1 mg/ml nevykazoval signifikantní rozdíly v hladině proteinu p53 oproti kontrolnímu vzorku, ani po delší inkubaci s extraktem po dobu 72 hodin. V případě extraktu z bazalky o koncentraci 0,5 mg/ml byly hladiny proteinu p53 oproti kontrole mírně zvýšeny po 24 a 30 hodinách inkubace s extraktem.

Výsledky této práce ukazují, že extrakty z jalovce, vavřínu a bazalky mají cytotoxický efekt na lidské SH-SY5Y buňky neuroblastomu a pravděpodobně mají vliv také na hladinu proteinu p53 v těchto buňkách. Tyto vlastnosti závisí především na koncentraci extaktů a na době, po kterou na buňky neuroblastomu působí.

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ABBREVIATIONS

AIF	apoptosis inducing factor
APS	ammonium persulfate
APAF-1	apoptosis protease activating factor 1
ATP	adenosintriphosphate
β-ACTIN	one of the non-muscle cytoskeletal actins
BCA	bicinchoninic acid
BCL-2, BCL-XL	anti-apoptotic proteins
BH	Bcl-2 homology
BID	pro-apoptotic protein
BAX, BAK	pro-apoptotic proteins
BSA	bovine serum albumin
CAPE	caffeic acid phenetyl ester
CCD	charge-coupled device
CD95	death receptor
DR5	death receptor
CO_2	carbonium oxide
DD	death domains
DED	death effector domain
DISC	death-inducing signalling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FADD	Fas-associating protein with death domain
FAS/APO-1	death receptor gene
FAS L	Fas ligand
FBS	foetal bovine serum
H_2O_2	hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HBSS	Hank's buffered salt solution

HCl	hydrochloric acid
IAP	apoptosis inhibitory protein
INT	iodonitrotetrazolium salt
IgG	immunoglobulin G
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MDM2	murine double minute oncogene 2
MDMX	murine double minute oncogene 4
$MgCl_2$	magnesium chloride
MOM(P)	mitochondrial outer membrane (pore)
MTT	{3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid}
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium hydrogen carbonat
Na_2HPO_4	sodium hydrogen phosphate
NEAA	non-essential aminoacids
NF- κB	nuclear factor kappa B
NOXA	protein modulator of p53-induced apoptosis
p53	tumor supressor protein
PAL	phenylalanine ammonialyase
PBS	phosphate buffered saline
PKC	protein kinase C
PUMA	protein modulator of p53-induced apoptosis
RT	room temperature
ROS	reactive oxygen species
SDS	sodiumlauryl sulphate
SDS-PAGE	sodiumlauryl sulphate polyacrylamide gel electrophoresis
SEM	standard error of a mean
SH-SY5Y	human neuroblastoma cells
SMAC/DIABLO	second mitochondrial activator of caspases/ direct IAP-binding protein of low isoelectric point

tBID	truncated Bid
TBS	tris-buffered saline
TP53	tumor supressor gene
TEMED	N, N, N‘, N‘-tetramethylethylenediamine
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor α
TNFR-1	tumor necrosis factor receptor 1
TRAIL	TNF-related apoptosis inducing ligand
TTBS	TBS with Tween
UV radiation	ultraviolet radiation
VEGF	vascular endothelial growth factor
XIAP	X-linked IAP

CONTENTS

1. INTRODUCTION

1.1. Cell death

1.1.1. Apoptosis and necrosis

Cell death plays an important role in the multicellular organisms. There are two distinct main ways of cell death – apoptosis and necrosis which vary morphologically and physiologically.

Apoptosis, in otherwords programmed cell death, is a physiologic, on genetical level highly regulated and energy-dependent cell suicide mechanism (controlled cell deletion), which appears to play a complementary but opposite role to mitosis. The cells participate in their own destruction (Kerr *et al*. 1972, Kinloch *et al*. 1999). Apoptosis is essential in multicellular (adult) organisms for removing of unwanted, damaged, extraneous, superfluous and old cells and is very important to maintain homeostasis under physiological conditions and in response to different internal and external stimuli. Apoptosis is important in tissue embryonic development during organogenesis and normal function maintence of the immune system (Mosmann 1983) and behaviour control during pathological states. Cells die during tissue regeneration in the adult organism (Ashkenazi and Dixit 1998). Apoptosis can be initiated by environmental stimuli, physiological and also pathological (Iacobini *et al*. 2001), such as cellular stress (genotoxic damage, oxidative stress or anti-neoplastic drugs) (Stennicke *et al*. 1998).

The main characteristics of apoptosis are cell shrinkage with cell integrity being not disturbed, plasma membrane blebbing, chromatin condensation, nuclear fragmentation and creation of nuclear bodies (Daugas *et al*. 2000, Adrain *et al*. 2001). The structure of organelles is preserved. The membrane integrity maintenances until very late stages in the death process (Kinloch *et al*. 1999). The mitochondrial membrane depolarization occurs in the initiation phase of apoptosis (Yang *et al*. 1997). There is no increased membrane permeability, depletion of ATP or changes in ion content at a time when chromatin and cytoplasm condensation occurs (Wyllie *et al*. 1980). The cells are separated from each other and form membrane bounds called apoptotic bodies. The apoptotic bodies contain closely packed fractions of cytoplasm and nucleus and are chemically and structurally intact (Kerr *et al*. 1972). The apoptotic bodies give signal by exposing phosphatidylserine on the outer surface of plasma membrane. Thus, they

are recognized, phagocyted and digested by phagocytes (Kerr *et al*. 1972, Iacobini *et al*. 2001) or taken up by the adjacent epithelial ingesting cells. Phagosomes containing apoptotic bodies fuse with the lysosomes of the ingesting cells and are digested by lysosomal enzymes. The nondigestable rests of cells stay in the secondary lysosomes, which disappear soon in cell defecation. Inflammation does not develop (Wyllie *et al*. 1980). The process of apoptosis is typified in Figure 1. (Kerr *et al*. 1972).

Poorly functioned cell death leads to diseases or disorders. Insufficient apoptosis can manifest as cancer or autoimmunity, while accelerated apoptosis is evident in acute and chronic degenerative diseases, such as Alzheimer's disease, developmental disorders, immunodeficiency and infertility (Richardson and Kumar 2002).

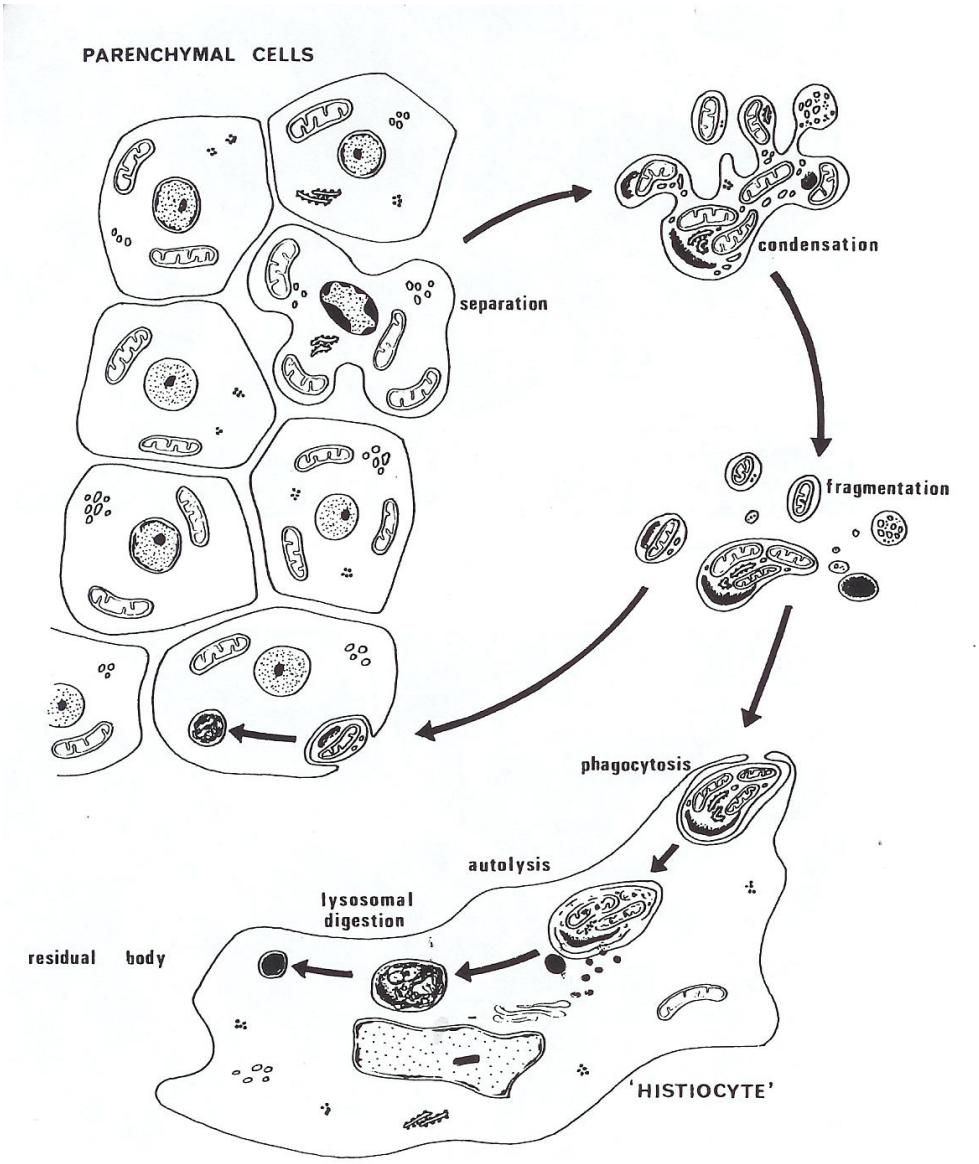


Figure 1. The process of apoptosis (Kerr *et al*. 1972).

Necrosis is a pathological type of cell death mostly caused by serious noxious (physical or chemical) stimuli (Kerr *et al*. 1972), such as toxins, ischemia, hyperthermia, hypoxia or metabolic poisons. It is probably the result of an irreversible disturbance of homeostatic mechanisms. This process does not control cell population and is not influenced by intrinsic factors as it is by apoptosis. Changes of tissues and organs, such as swelling of cytoplasm, rupture of nuclear and organelles, creation of flocculent matrix densities in mitochondria, desintegration of ribosomes, enhanced permeability of plasmalema and cellular membrane (causing changes in the cellular volume), are characteristic for necrosis. More calcium ions come into the cell with the increased permeability of cellular membrane. High concentrations of calcium ions within mitochondria damage respiratory apparatus. The cells lose energy in form of ATP. All these processes are not compatible with life of the cell. The result of necrosis is exudative inflammation, because groups of cells are damaged and several components release from the cytosol (Wyllie *et al*. 1980, Lossi and Merighi 2003). The main morphological, physiological and biochemical differences between apoptosis and necrosis are described in Table 1. for better orientation and clearness in the conception of apoptosis and necrosis (Pospěchová 2004).

NECROSIS	APOPTOSIS
Morfological features: - loss of membrane integrity - begins with swelling of cytoplasm and mitochondria - ends with total cell lysis - no vesicle formation, total lysis - desintegration of organelles	 - membrane blebbing, but no loss of integrity - begins with shrinking of cytoplasm and condensation of nucleus - ends with fragmentation of cell into apoptotic bodies - mitochondria becomes leaky due to pore formation involving proteins of the bcl-2 family
Physiological features: - affects group of contiguous cells - induced by non-physiological disturbancies (lytic viruses, ischemia, hypoxia, hypotermia, metabolic poisons) - phagocytosis by macrophages - significant inflammatory response	 - affects individual cells - induced by physiological stimuli (lack of growth factors, changes in hormonal environment) - phagocytosis by adjacent cells or macrophages - no inflammatory response
Biochemical features: - loss of regulation of ion homeostasis - no energy requirement (passive process, also occurs at 4 ºC) - random digestion of DNA - postlytic DNA fragmentation (= late event of death)	 - tighly regulated process involving activation and enzymatic steps - energy (ATP)-dependent (active process, does not occur at 4 ºC) - non-random mono- and oligonucleosomal length fragmentation of DNA - prelytic DNA fragmentation - release of various factors (cytochrome c, AIF) into cytoplasm by mitochondria - activation of caspase cascade - alterations in membrane asymmetry (i.e., translocation of phosphatidylserine from the cytoplasmatic to the extracellular side of the membrane)

Table 1. Morphological, physiological and biochemical differences between apoptosis and necrosis (Pospěchová 2004).

1.1.2. Molecular mechanism of apoptosis

The process of cell death is regulated by many different apoptosis–associated genes and proteins, which are located in cell membrane, cytosol, endoplasmic reticulum, mitochondrion and nucleus. The proteins can be divided into four groups: receptors, adaptors, regulators and executioners (Pospěchová 2004).

The executioners are presented by capases (cystein-aspartyl proteases). Caspases are constantly expressed in normal cells as inactive zymogens or pro-enzymes, called pro-caspases (Fadeel and Orrenius 2005). The pro-caspases undergo proteolytic process after receiving of death signal and create an active enzyme. Activated caspases cleave and activate other caspases and proteins during apoptosis. These processes are then responsible for many of the morphological changes, which the cells undergo during apoptosis (Slee *et al*. 1999).

There are two main types of caspases: initiators (upstream) and effectors (downstream) (Fadeel and Orrenius 2005). Initiators (also called signalling caspases), such as caspase -2, -8 and -10, are autocatalyticly activated and function as activators of the effector caspases (Fadeel and Orrenius 2005). Effectors (also called executioners), such as caspase -3, -6 and -7 are able to directly degradate multiple substrates including the structural and regulatory proteins in the cell nucleus, cytoplasm and cytoskeleton. They initiate apoptosis and their activation lead to typical apoptotical morphological features (Danial and Korsmeyer 2004, Thornberry and Lazebnik 1998). They respond to many apoptotic stimuli, such as ligation of death receptors of TNFR-1 receptor family, which results in cleavage and activation of other factors and manifesting of apoptosis. The executioners are not directly activated by receptor ligation, they are at first activated by upstream initiator caspases (Stennicke *et al*. 1998). Activation of caspase-3 is very important in the transmission of proteolytic signal (Stennicke *et al*. 1998).

Besides initiators and effectors, there are caspases, such as caspase -1, -4 and -5, that are involved in the cytokine maturation and thus in the process of inflammation. Caspase-12 is phylogenetically related to the cytokine maturation caspases. But in mice it has been proposed as a mediator of apoptosis induced by endoplasmic reticulum stress suggesting that it might contribute to the pathogenesis of Alzheimer's disease (Saleh 2004). Caspases contain two important domains - CARD, caspase recruitment domain, and DED, death effector domain. These domains are necessary for binding of caspases

to their adaptors, which allow their activation. For example, the CARD of caspase-9 is required for its recruitment by binding to Apaf-1 in DISC. CARD binds to the N-terminus of Apaf-1 (Richardson and Kumar 2002).

However, not only caspases, but also other proteases participate on caspase activation and apoptosis. For example, granzyme B is a serine protease that can activate several members of the caspase family, such as caspase -3 and -9 (Greenberg *et al*. 1996, Deiss *et al*. 1996). Perforin is required for the entrance of this enzyme into the cell. It forms pores in cell membrane allowing granzyme B to enter the cell and initiate apoptosis (Deiss *et al*. 1996).

1.1.3. Intrinsic (mitochondrial) pathway

In mammals, there are two main pathways of apoptosis, which are mediated by caspases – intrinsic and extrinsic (Fadeel and Orrenius 2005). Mitochondria plays an important role in the intrinsic apoptotic pathway. Several pro-apoptotic proteins are released from mitochondrial intermembrane space into the cytosol in response to apoptotic stimuli (chemical-, radiation- or oxidation-induced damage). These proteins promote apoptosis either by activating of caspases or by neutralizing of cytosolic inhibitors of this process (Danial and Korsmeyer 2004, Twiddy and Cain 2007).

For example, cytochrome c is released under apoptotic stimuli from the mitochondrial inner membrane into the cytosol, which occurs before mitochondria begins to swell (Danial and Korsmeyer 2004). Cytochrome c is released from the mitochondria either by rupture of the outer mitochondrial membrane or by so-called mitochondrial permeability transition. This transition is controlled by a voltage- and Ca^{2+}-sensitive pore (Blatt and Glick 2001). Released cytochrome c oligomerizes and creates a complex called apoptosome by binding to the cytosolic adaptor protein Apaf-1 (apoptotic protease-activating factor-1) (Yu *et al*. 2005, Twiddy and Cain 2007).

Apoptosome causes oligomerization and thus recruitment of procaspase-9. Autocatalyticly activated caspase-9 activates caspases, that lie downstream of caspase-9 (Nakabayashi *et al*. 2006, Twiddy and Cain 2007). Cytochrome c/ apoptosome-mediated caspase activation appears to result in swelling of the mitochondrial matrix and rupture of the inner and outer mitochondrial membrane (Adrain *et al*. 2001). After cytochrome c is released from mitochondria into cytosol, mitochondrial membrane

depolarization occurs together with morphological events, such as chromatin condensation and DNA fragmentation (Yang *et al*. 1997).

1.1.4. Extrinsic (death receptor) pathway

The extrinsic apoptotic pathway, also called death receptor-mediated pathway, involves death receptors. Death receptors are cell surface receptors, which are composed from proteins possessing protein motifs - cytoplasmic death domains (DD) and cystein rich extracellular death effector domains (DED) (Danial and Korsmeyer 2004). To the death receptors belong CD95 (also calles Apo-1 or Fas), tumor necrosis factor receptor, such as TNFR-1 (Hengartner 2000) and DR5, which all come from the tumor necrosis factor receptor superfamily (Walczak and Hass 2008, Thornberry and Lazebnik 1998).

Extrinsic apoptotic pathway is initiated by the oligomerization of death receptors by their ligands (FasL, TNF-alpha and TRAIL), that are members of the tumor necrosis factor (TNF) superfamily. Together they create a death-inducing signalling complex (DISC). Part of this complex is the adaptor protein FADD (Fas-associating protein with death domain). DISC activates procaspase-8 via binding to the DED of FADD (Walczak and Hass 2008, Blatt and Glick 2001). Procaspase-8 is autocatalyticly activated and subsequently functions as an activator of downstream effector caspases, such as caspase -3 and -7. Caspase-3 then mediates cleavage of proapoptotic Bid, which results in its translocation into the mitochondria and release of cytochrome c and Smac. The apoptosis is triggered through this process (Fadeel and Orrenius 2005). Caspase-8 activates also other downstream effector caspases such as caspase-9. This process also leades to apoptosis (Danial and Kosrmeyer 2004, Thornberry and Lazebnik 1998), because caspase-9 cleaves and activates other caspases, such as caspase -2, -3, -6, -7, -8 and -10, and thereby propagates the death signal (Slee *et al*. 1999).

Death receptors can activate death caspase within seconds after the ligand binds. Apoptotis demise of cells takes then a few hours (Thornberry and Lazebnik 1998). The apoptosis-inducing capacity of TNF receptors depends on the presence of death domains (DD) in the intracellular portion of receptor protein. The death domains are necessary for binding of death receptors to their ligands (Walczak and Hass 2008).

The integration between the death-receptor and mitochondrial pathway is provided by caspase-3 activation. The „cross-talk‘‘ is minimal under most conditions and these two apoptotic pathways work mostly undependently (Hengartner *et al*. 2000). In order to summarize the mechanisms of apoptosis, Figure 2. shows the scheme of apototic pathways (Maňáková 2003).

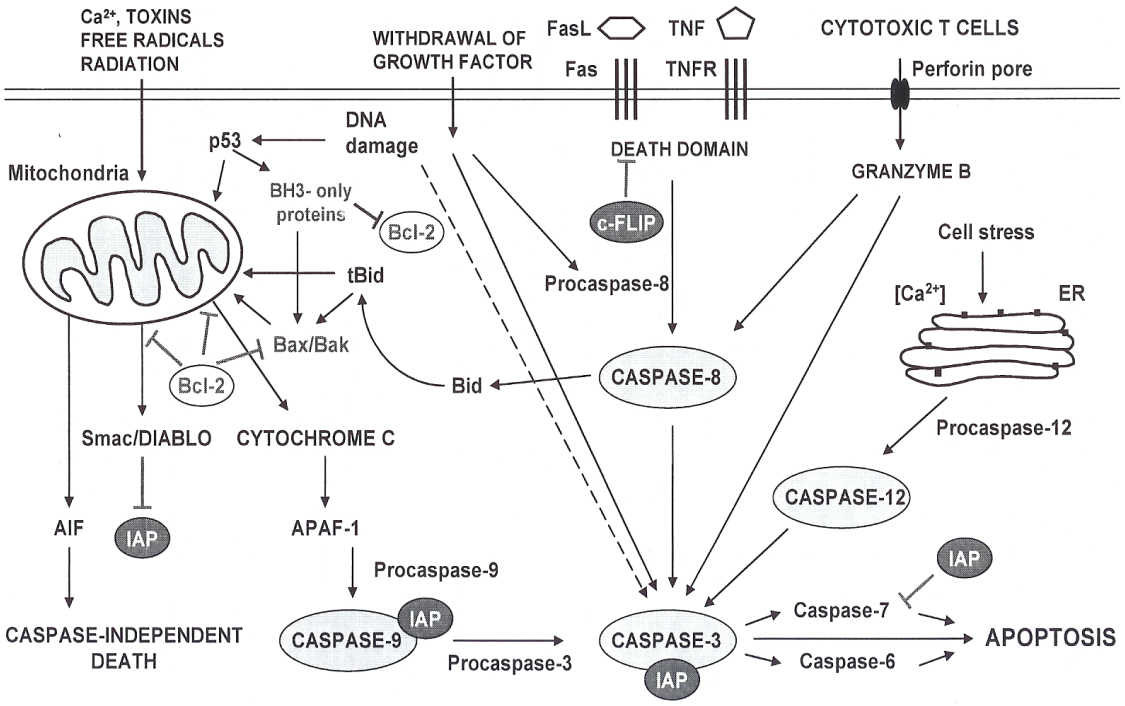


Figure 2. Scheme of the apoptotic pathways (Maňáková 2003).

1.1.5. p53

p53 is a protein, which functions biochemically as a transcription factor, that binds to a specific DNA sequence in order to activate transcription of target genes. p53 functions also biologically as a tumor supressor and as a stress sensor (Gomez-Lazaro *et al*. 2004, Benchimol *et al*. 2001).

p53 is activated upon stress and proliferative signals, such as genotoxic (gamma irradiation, UV radiation, cytotoxic drugs, chemical carcinogens) or non-genotoxic (hypoxia, hyperthermia), virus infection or DNA damage. Its activation leads to protection of cells and inhibition of tumor-cell growth. p53 lives shortly in most of the cell types, 5 to 20 minutes. The levels of the protein are influenced by several mechanisms. Increasing of the level and prolonging its half-life and therefore

stabilization, eventually activation of the protein can happen in stressful situations described (Gomez-Lazaro *et al*. 2004, Vousden and Lu 2002).

Two important proteins MDM2 and MDMX participate in the regulation of p53. MDM2 participates in the autoregulatory loop of p53. It is an ubiquitin ligase which targets p53 to degradation. Therefore, the activation and stabilization of p53 is principally associated with the inhibition of MDM2 function (Vousden and Lu 2002).

1.1.5.1. Functions of p53

The main function of p53 is to inhibit the cell growth, namely it makes the cells to undergo one of two processes: arrest in the G1 phase of the cell cycle or genetically programmed cell death (higher levels of p53) (Gomez-Lazaro *et al*. 2004). Low levels of p53 lead to the cell arrest in the G1 phase, which means that p53 stops the cell in the G1. Before the cell cycle continues, the cell has time to repair its DNA in order to conserve the genome. If the cell is not able to restore its DNA, p53 induces activation of apoptotic signalling pathway in the cell (Gomez-Lazaro *et al*. 2004).

The localization of p53 is very important for its function (Fridman and Lowe 2003). p53 in the latent form is situated in the cytoplasm, while p53 in the activated form translocates into other organelles (Gomez-Lazaro *et al*. 2004). p53-mediated apoptotic pathway can be devided into two groups: transcription-dependent, proceeding in the nucleus, and transcription independent, proceeding in the cytosol and mitochondria. These two ways work in concert to ensure that apoptotis proceeds efficiently (Fridman and Lowe 2003).

Transcription-dependent pathway in the nucleus involves p53-mediated induction of apoptosis by transcriptional regulation of its downstream target genes. This process leads to the activation of proapoptotic genes, transcriptional repression of anti-apoptotic genes and cellular survival genes, such as survivin in both mitochondrial and death receptor pathways. For example, p53-induced growth arrest is achieved mainly by transactivation of the direct target gene p21, which induces cell arrest in G1 phase and prevents the entry to S-phase (Sun 2006, Gomez-Lazaro *et al*. 2004). The nucleus localization is necessary also for supressor function of p53 (Gomez-Lazaro *et al*. 2004).

Transcription-independent p53-induced pathway plays an important role in the cytoplasmic pathway. Cytoplasmic p53 directly activates the proapoptotic protein Bax

and promotes the mitochondrial outer-membrane permeabilization (MOMP). When MOMP occurs, proapoptogenic factors cytochrome c and Smac are released from the mitochondria, caspases are activated and apoptosis is triggered (Chipuk *et al*. 2005). Transcription-independent pathway is involved also in the mitochondrial apoptotic pathway and occurs mainly in the presence of high intracellular levels of p53 (Moll *et al*. 2005). Upon apoptotic stimuli, p53 translocates into the mitochondria, where it participates in the induction of apoptosis by modulating activity of mitochondrial proteins (Sun 2006). For example, p53 interacts with the antiapoptotic Bcl-2 family members Bcl-2 and Bcl-xL and antagonizes their antiapoptotic stabilization of the outer mitochondrial membrane. Bcl-2 and Bcl-xL can also sequester the pro-apoptotic members of Bcl-2 family, such as Bax and Bak. p53 is able to liberate them from the complex with Bcl-2 and Bcl-xL and activate their oligomerization. p53 also supports mitochondrial membrane permeabilization and cytochrome c release (Moll *et al*. 2005).

p53 induces also other pro-apoptotic members of the Bcl-2 family of proteins, such as Puma and Noxa. Puma mediates p53-induced cell death through the cytochrome c/ Apaf-1-dependent pathway and caspase -3 and -9 pathway. Noxa translocates upon apoptotic signal from cytosol into mitochondria and interacts with the permeability pore and thus induces apoptosis (Gomez-Lazaro *et al*. 2004, Benchimol *et al*. 2001). All these fuctions mean, that p53 is working not only through the activation of target genes, but also through its interaction with other proteins (Gomez-Lazaro *et al*. 2004). p53 functions are regulated and controlled through several mechanisms, for example by p53 transcription and translation, protein stability, post-translational modifications, and subcellular localization (Gomez-Lazaro *et al*. 2004).

1.1.5.2. p53 and human diseases

About a hundred thousand cells are produced every second by mitosis and a similar number die by apoptosis in every human being. Therefore, the balance between cell death and proliferation are very important. A dysbalance or dysregulation of homeostasis and apoptosis (too little or too much of apoptosis, or apoptosis in the wrong time or at the wrong place) can cause several human diseases, either degenerative or proliferative (Fadeel and Orrenius 2005). For example, high levels of p53 were found in autoimmune diseases, ischemia/ reperfusion and heart failure,

cancer or neurodegenerative disorders (atherosclerotic lesions, multiple sclerosis, arthritis, Alzheimer's disease or Huntigton's disease). On the other hand, dysfunction of apoptosis leads to the cellular immortalization, genomic instability, inappropriate survival, allowing the continued proliferation and evolution of damaged cells (Fridman and Lowe 2003). It was also reported, that the inhibition of p53 in Parkinson's disease reduces the degeneration of dopaminergic neurons (Gomez-Lazaro *et al*. 2004, Fadeel and Orrenius 2005).

1.1.5.3. p53 and cancer

Mutations of p53 are very often observed in human cancer (Vousden and Lu 2002). Tumor cells, in which p53 is inactivated by mutations, are genetically unstable. These cells therefore accumulate mutations and chromosomal rearrangements, which lead to the progressive conversion of normal cells into cancer cells and to uncontrolled growth (Li *et al*. 1998, Fadeel and Orrenius 2005).

The mutations are caused by different mechanisms, such as mutation within the TP53 gene (encodes p53), mutation of downstream mediators of p53 function or disruption of cell-growth-inhibitor pathway (for example loss of components of caspase-cascade, such as caspase-9 or Apaf-1). Mutation of TP53 gene appears in about half of all cancers and leads to the expression of mutant p53 protein. Mutant p53 has a transformed activity, namely it contributes to tumor development (Vousden and Lu 2002), because of encoding of defective products (Benchimol *et al*. 2001). Mutations of p53 and apoptosis resistance lead to decreased apoptosis and typically to the failure in cancer therapy (Sun 2006)

Cancer cells, in comparisson to normal cells, are very sensitive to apoptotic stimuli and usually survive only because of the alterations in their p53 protein. Cell death is thus prevented by them. The alterations cause inactivation of the p53 tumor-supressor pathway and enable them to evade the death signals. Principally, loss of p53 is necessary for tumour cells to continue in growth and survival (Vousden and Lu 2002).

The mutation or alterations can affect not only p53, but any part or target of p53-dependent apoptotic pathway. For example, the disruption of Apaf-1 and caspase-9, which serve as downstream effectors of p53-dependent apoptosis, facilitates oncogenic

transformation and tumor development. Deregulation of members of the Bcl-2 family of apoptosis protein is also common in cancer (Fadeel and Orrenius 2005).

The goal of nowadays studies is to find „drugable‘‘ targets in p53 signalling pathway, that modulate p53-induced apoptosis. It can be for example apoptosis inducing genes (Sun 2006). Another goal is to find a therapy, which would reactivate p53 (Vousden and Lu 2002). Indeed, even these forms of cancer treatment would not have to be without problems. It was shown, that acute p53 activation can contribute to the side effects of cancer therapy and chronic p53 activation can contribute to aging (Fridman and Lowe 2003).

1.1.6. Bcl-2 protein family

The Bcl-2 family of proteins contains proapoptotic as well as antiapoptotic members in mammals. They can be derived into three main subclasses, which are defined through different degree of homology within four conserved parts called Bcl-2 homology BH1-4 domains. This domain homology influences the structure and functions of proteins and are necessary for binding of proteins with each other and thereby influencing each other in the functions (Danial and Korsmeyer 2004).

1.1.6.1. Bcl-2, Bcl-xL

The antiapoptotic members, Bcl-2 and Bcl-xL have conservation in all four BH1-4 domains (Danial and Korsmeyer 2004). They function as apoptosis inhibitors and regulate the activation of caspases (Hu *et al*. 1998). Bcl-2 and Bcl-xL are integral proteins, situated on the outer mitochondrial membrane. They present a critical intracellular checkpoint in the intrinsic apoptotic pathway (Danial and Korsmeyer 2004). Overexpression of these proteins prevents apoptosis by cells, because both Bcl-2 and Bcl-xL block the interaction between Apaf-1 from DISC and caspase-9. Thus, they obstruct release of cytochrome c from mitochondria into cytosol in the initiation phase of apoptosis. And they block also caspase-activation and DNA fragmentation, which would come after the release of cytochrome c (Yang *et al*. 1997, Hu *et al*. 1998).

Bcl-2 prevents mitochondrial membrane depolarization, which appears in the initiation phase of apoptosis. In otherwords, Bcl-2 principally protects cells from losing

of their mitochondrial membrane potential and thus release of cytochrome c (Yang *et al*. 1997). Bcl-2 and Bcl-xL bind to BH3-only molecules, so that proapoptotic Bax and Bak can not be activated (Danial and Korsmeyer 2004). (more about BH-3 only molecules, Bax and Bak in the chapters 1.1.6.2. and 1.1.6.3). Bcl-2 prevents apoptosis, which is triggered by genotoxic damage, glucocorticoids, chemotherapeutic drugs, but it does not prevent apoptosis induced by death receptors (Stennicke *et al*. 1998).

1.1.6.2. Bax and Bak

The propapoptotic members, Bax and Bak possess BH1, BH2 and BH3 domain. They play an important role in the intrinsic apoptotic pathway. Bax and Bak exist in their inactive form of monomers in the viable cells. Inactive Bax can be found in cytosol and inactive Bak resides in the mitochondria. When the death signal comes or under apoptotic stimuli, both Bax and Bak interpose into mitochondrial outer membrane (MOM). This process is accompanied with the releasing of intermembrane space proteins, such as cytochrome c (Danial and Korsmeyer 2004).

Bax and Bak activity can be blocked by binding to the anti-apoptotic members of Bcl-2 family of proteins, such as Bcl-2 or Bcl-xL. Bax and Bak are active in homodimeric form, but inactive in the heterodimeric form with the anti-apoptotic members, so they are regulated indirectly by downregulation of its anti-apoptotic antagonists (Cregan *et al*. 1999).

1.1.6.3. BH3-only proteins

This group of pro-apoptotic members of Bcl-2 family has homology only within one death domain, the BH-3. Therefore, they are called BH3-only proteins (Danial and Korsmeyer 2004). The BH3-only proteins members (Bid, Bad, Bim) selectively respond to specific death and survival signals. They can participate on the extrinsic and also on the intrinsic apoptotic pathway. For example, Bid is normally localized in the cytosol, but it is cleaved upon apoptotic stimuli (upon activation of surface death receptors) by caspase-8 and in the truncated form tBid is translocated into the

mitochondria. Through this process, the death receptor pathway activates the mitochondrial pathway (Li *et al*. 1998, Danial and Korsmeyer 2004).

Bid cooperates with other mitochondrial proteins in the mitochondria, which leads to the release of apoptogenic proteins, such as Bax and Bak. Upon interaction with BH3-only proteins through BH3 domain (Danial and Korsmeyer 2004), Bax and Bak undergo conformational change and oligomerization. They create pores, cause mitochondrial damage and release of cytochrome c and other apoptogenic proteins (Adrain *et al*. 2001, Wang *et al*. 2006, Cui *et al*. 2002). Bid seems to not be cleaved only by caspase-8, but also by other caspases, such as caspase-3. Bid may therefore serve as a general integrator and amplifier for many apoptotic signals (Wang *et al*. 2006).

The activities of BH3-only proteins and proteins Bax and Bak can be neutralized by the antiapoptotic members of Bcl-2 family of proteins, Bcl-2 and Bcl-xL. These proteins do not affect the translocation of the BH3-only proteins to the mitochondria, but they block the oligomerization of Bax and Bak (Wang *et al*. 2006).

1.1.7. Other proteins involved in apoptosis

1.1.7.1. Apoptosis-inducing factor

Apoptosis-inducing factor (AIF) is a flavoprotein, which functions as an apoptotic effector. AIF is normally situated in the mitochondrial intermembrane space. The outer mitochondrial membrane becomes unspecifically permeabilized after induction of apoptosis (Daugas *et al*. 2000). AIF translocates from the mitochondria into the nucleus and causes chromatin condensation and DNA fragmentation (Wang *et al*. 2006). But AIF does not create apoptotic bodies in the cell. This intrinsic apoptotic signalling pathway is caspase-independent. The mitochondrion-nuclear distribution of AIF is blocked by Bcl-2 proteins (Daugas *et al*. 2000).

1.1.7.2. Inhibitor of apoptosis protein

The inhibitor of apoptosis protein (IAP), containing BIR domain, regulates function of caspases. The N–terminal part of BIR domain binds to the substrate place of caspases, so that the protein substrate can not collocate to the active caspase (Fadeel and Orrenius 2005). IAP protein antagonizes caspase-3 activation (Hengratner *et al*. 2000).

Several mechanisms, such as binding of Smac/Diablo control mammalian IAPs (Danial and Korsmeyer 2004). XIAP–associated factor 1 is a cytosolic protein, which antagonizes IAPs inhibition of caspases, and ensures the redistribution of XIAP (X-linked IAP) (Fadeel and Orrenius 2005).

1.1.7.3. Smac / Diablo

Smac/ Diablo is a mitochondrial protein released during apoptosis. Smac/ Diablo contains IAP binding motif and antagonizes IAP inhibition of caspases. For example, XIAP binds to the procaspase-9 matured form, which leads to the inhibition of caspase-9 activity (Wang *et al*. 2001, Danial and Korsmeyer 2004). Smac displaces XIAP from the caspase-9, so XIAP does not repress active caspase-9 within the apoptosome anymore (Adrain *et al*. 2001). Smac releases from mitochondria into cytosol upon UV- and γ-irradiation, cytotoxic drugs, DNA damage and ligation of the CD95 death receptor. Translocation of Smac from mitochondria is blocked, when Bcl-2 is overexpressed. Unlike cytochrome c release is caspase-independent, Smac release depends on caspase activation (Adrain *et al*. 2001).

1.1.8. Cytosine arabinoside

1-β-D-arabinofuranosylcytosine (AraC) is a pyrimidine antimetabolite, nucleoside analog (Fadeel and Orrenius 2005). AraC is the most important agent used for induction of remission in acute leukemia in children and adults, because it effects antiproliferativelly (Iacobini *et al*. 2001, Mc Donald *et al*. 1991).

AraC appears to be selectively incorporated into the nuclear, rather than mitochondrial DNA, so most probably does not alter mitochondrial functions. AraC probably incorporates into newly synthetized DNA of the cells, causes oxidative DNA damage and genotoxic stress, induces the formation of reactive oxygen species (ROS) and also DNA single-strand breaks. These processes lead to the increased p53 protein level, caspase-3 activation and to p53-dependent apoptosis. It is not clear, how AraC causes ROS generation. AraC increases ATP production and activity of cytochrome c, which might lead to ROS production (Fadeel and Orrenius 2005, Maňáková 2003). Besides this effect, AraC increases the activity of stress-activated protein kinases, such as Jun kinase, which promote apoptosis (Geller *et al*. 2001).

AraC induces apoptosis dose- and time-dependently also in healthy cells, it is therefore very toxic for dividing tissues. Myelosupression, gastrointestinal toxicity and neurotoxicity (cerebellar syndromes, myelopathy, peripheral neuropathy and encephalopathies) are the main side effects of AraC and the common and potential dose-limiting complications. Especially high doses can damage any part of the nervous system. These toxic effects of AraC on healthy cells happen probably through oxidation (Iacobini *et al*. 2001, McDonald *et al*. 1991, Geller *et al*. 2001).

Cell death caused by AraC can be abrogated by several anti-apoptotic agents or reduced by treatment with free-radical scavengers, such as dipyridamol, uric acid or vitamin E. Also antioxidants might have neuroprotective effects and could therefore be used to reduce neurotoxicity and DNA damage in AraC chemotherapy. Because cell death induced by AraC is p53 dependent, the toxicity of AraC is reduced in the depletion of p53 (Geller *et al*. 2001).

1.2. Plant - derived polyphenols

Phenolic compounds in plants are their secondary metabolites, which are identified in many edible and nonedible plant materials, such as berries, fruits, vegetables, herbs, cereals, plant sprouts, seeds or tree materials. Their role in plants is to protect them from photosynthetic stress, reactive oxygen species, wounds and herbivores. They are important in normal growth development and defense against infection and injury. They have antinutritional effect, because they bind proteins and minerals and thus decrease absorption and digestability of food. They cause

adstringency of many fruits due to precipitating of salivary proteins (Yang *et al*. 2001, Kähkönen *et al*. 1999). Some of them, such as flavonoids give color to many flowers, fruits and leaves (Kähkönen *et al*. 1999).

The plant-derived polyphenols possess many biological effects, such as anti-inflammatory, antibacterial, antiallergic, hepatoprotective, antithrombic, antiviral, vasodilatatory and anticarcinogenic activity (Soobrattee *et al*. 2005, Yang *et al*. 2001). Many phenolics are potential therapeutic agents against a wide range of ailments, such as neurodegenerative diseases, cancer, diabetes, heart disease, cardiovascular dysfunction, inflammatory diseases and also aging. Their medicinal actions, such as antioxidant capacity, free radical scavenging, chelatation of redox active metal ions, modulation of gene expression and interaction with the cell signalling pathways might participate in their therapeutic activity (Soobrattee *et al*. 2005, Kähkönen *et al.* 1999).

The activities of plant-derived polyphenols very much depend on the structure. Conjugation system of bonds and the number and configuration of H-donating hydroxyl groups in the structure are of fundamental importance for the antioxidant function (Soobrattee *et al*. 2005). In the form of extracts, the activities can be supported from other extract components, such as sugars or acsorbic acid (Kähkönen *et al*. 1999). They can be used as natural antioxidants for the preservation and as prophylactic agents mainly in oxidative and nitrisative stress, which cause many acute and chronic clinical disorders (Soobrattee *et al*. 2005). Next to the chemical structure the availability and prevalence in food plants are important (Soobrattee *et al*. 2005).

1.2.1. Biosynthesis

All plant phenolic compounds arise from the aminoacid phenylalanin or its close precursor shikimic acid. Phenylalanine ammonia-lyase (PAL), the main enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism, is important for the formation (Herrmann *et al*. 1995).

The presence of polyphenols in different parts of plants is largely influenced by genetic factors and environmental conditions. For example, formation of flavones and flavonols strongly depends on light. The highest concentrations are therefore in leaves and outer parts of plants. Also other factors, such as germination, degree of ripeness, variety, processing and storage influence the content of polyphenols.

Many of the phenolic compounds are very often glycosylated with sugars, such as glucose, rhamnose, galactose and arabinose (Yang *et al*. 2001, Goldberg *et al*. 2003, Bravo *et al*. 1998).

1.2.2. Bioavailability

The bioavailability of plant-derived polyphenols *in vivo* differs between different polyphenols, but it is quite low by many dietary polyphenols. They are extensively metabolised *in vivo*, mainly during transfer across the small intestine, by colonic microflora and in the liver. Their blood and tissue concentrations usually do not reach the levels used in *in vitro* experiments, thus they are usually not able to display the effects observed *in vitro*. Their activities, especially redox potentials are in this step very often alterated (Yang *et al*. 2001).

The main factors influencing bioavailability of polyphenols are chemical properties of polyphenols, deconjugation/ reconjugation in the intestine, intestinal absorption, mechanism of biotransformation and enzymes available for metabolism (Yang *et al*. 2001, Soobratte *et al*. 2005).

1.2.3. Plant - derived polyphenols and cancer

It was proved, that cancer is a preventable and highly susceptible to dietary factors. Phenolic compounds are potential chemopreventive agents. Many of the molecular alterations associated with carcinogenesis occur in cell signalling pathways, that regulate cell proliferation and differentation. To the components of these pathways belong kinases, such as mitogen-activated protein kinases (MAPK) and protein kinase C (PKC), which contribute to homeostasis maintenance. Another component of signalling pathway is nuclear transcription factor NF- κB, that regulates the expression of various genes involved in inflammation and carcinogenesis. Abnormal activation or silencing of these kinases and factors can result in uncontrolled cell growth and therefore lead to malignant transformation (Fresco *et al*. 2006).

Polyphenols can activate cell death signals, induce apoptosis in precancerous and malignant cells and inhibite cancer development and progression by modulating cell signalling pathways and its components (Fresco *et al*. 2006). Other possible

mechanisms of anticarcinogenic effects can be inhibition of carcinogen uptake, inhibition of formation or activation of carcinogen, deactivation or detoxification of the carcinogen or preventing the carcinogen binding to DNA. Also antioxidant properties include scavenging oxygen radicals and inhibiting of arachidonic acid metabolism play an important role (Nichenametla and Taruscio 2006).

1.2.4. Groups of plant - derived polyphenols and their biological effects

Polyphenols are very heterogenous group of compounds (Soobrattee *et al*. 2005). They are complex substances with two or more phenolic rings joined together. Phenolic acids, flavonoids and stilbenes are the most abundantly occuring polyphenols in plants. Flavonoids and phenolic acids account for 60% and 30% respectively of total dietary polyphenols (Nichenametla and Taruscio 2006). The most abundant flavonoids are flavanols (catechins and proanthocyanidinins), anthocyanins and their oxidation products. The total daily intake is approximately 1g/day (Scalbert and Williamson 2000). Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins (Bravo *et al*. 1998).

1.2.4.1. Phenolic acids and derivates

This group of plant phenols involves derivates of hydroxybenzoic and hydroxycinnamic acids, such as curcumin, caffeic, gallic and ferulic acid. They occur in food very often, mostly as esters. The general structures of hydroxybenzoic and hydroxycinnamic acids are in Figure 3. (Fresco *et al*. 2006).

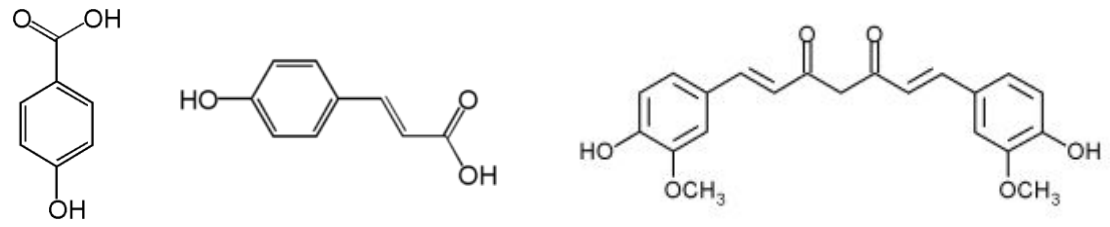
The phenolic acids inhibit mutagenesis, have antimetastatic activity, modulate immune function and induce apoptosis. Besides their chemopreventive activities they can also promote carcinogenity via reactive oxygen species or tumor promoting activity (Nichenametla and Taruscio 2006).

Caffeic acid is found in many fruits, such as apples, plums, tomatos and grapes (Soobrattee *et al*. 2005). Caffeic acid phenetyl ester (CAPE) exhibits dose-dependent, selective toxicity and growth supressive properties against cancer cells, but not against normal cells. CAPE supresses the motility of cancerogenic cells, which might be the

mechanism of metastasis inhibition (Nichenametla and Taruscio 2006). Other caffeic acid derivates inhibit several enzymes, such as 5-lipooxygenase or protein kinase C, that are involved in pathological processess, such as allergic diseases, inlammation, cataracts or neuropathies (Soobrattee *et al*. 2005).

Ferulic acid is found in food sources, such as wheat bran (Bravo *et al*. 1998) and is known as a chemopreventive and antioxidant agent. It decreases the number of lung and skin tumors, but on the other hand its metabolite seems to be carcinogenic to liver. The carcinogenic and anticarcinogenic activity of caffeic acid may be dose specific. It is carcinogen at 2 %, tumor-promoter at 1% and anticarcinogenic at 0.05-0.5% (Nichenametla and Taruscio 2006).

Curcumin, found in Curcuma longa L. is the major yellow pigment in turmeric and mustard and is used as food preservative and yellow coloring agent for food, drugs and cosmetics (Yang *et al*. 2001). The chemical structure of curcumin is possible to see in Figure 3. (Yang *et al*. 2001).



a. b. c.

Figure 3. Chemical structures of phenolic acids and derivates: a. hydroxybenzoic acid; b. hydroxycinnamic acid (Fresco *et al*. 2006); c. curcumin (Yang *et al*. 2001).

1.2.4.2. Flavonoids

Flavonoids belong to the group of natural benzo-γ-pyran derivates. They appear as aglycones, glycosides and methylated derivates. In according to degree of central pyran ring oxidation, they can be divided into several groups: flavones, flavonols, flavonones, isoflavones, flavans, flavanols and anthocyanins. Polyphenolic biflavonoids are proanthocyanidins (Škerget *et al*. 2005).

To flavanols (flavan-3-ols) belong catechins (epicatechin-3-gallat, epigallocatechin, epicatechin, catechin and epigallocatechin-3-gallat) abundant in tea, red wine, berries and chocolate (Nichenametla and Taruscio 2006). The chemical structure of epicatechin is in Figure 4. (Fresco *et al*. 2006). Catechins are able to induce apoptosis and have antiproliferative and growth controlling properties in cancerous cell lines. Catechins are involved in prevention of metastasis. They inhibit dose-dependently vascular endothelial growth factor-induced angiogenesis in human microvascular endothelial cells (Nichenametla and Taruscio 2006).

Catechins are potential agents against neurodegenerative diseases, because they decrease the expression of proapoptotic genes, such as Bax and others. Thus, they maintain the mitochondrial membrane integrity. They are also potentially effective against cancer, diabetic retinopathy and chronic inflammation. They supress angiogenesis by downregulating of VEGF production in tumor cells and inhibit capillary cell proliferation and blood vessel formation. They are also potent antioxidants in lipid systems (Soobrattee et al. 2005). Epigallocatechin has antioxidant, immunomodulatory and antimicrobial activity (Nichenametla and Taruscio 2006).

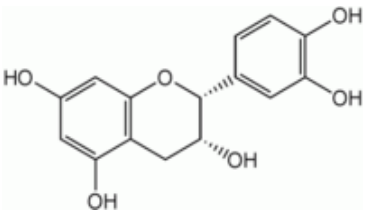


Figure 4. Chemical structure of epicatechin (Fresco et al. 2006).

Next to flavanols belong to this group of polyphenols flavonols, such as quercetin, myricetin and kaempferol (occurs as glycoside in fruits). Glycosidic form of quercetin is rutin (Nichenametla and Taruscio 2006). The chemical structure of quercetin is in Figure 5. (Fresco *et al*. 2006, Škerget *et al*. 2005).

Quercetin inhibits cell proliferation, cancer cell growth and cessation of cell cycle. Kaempferol reduces estrogen receptor-positive breast cancer cells and functions as growth inhibitor. But these functions of kaempferol depend on concentration, because it enhances cell growth at low concentration (1-10 μM) by acting as an estrogen receptor agonist and enhancing DNA synthesis (Nichenametla and Taruscio 2006).

Anticancerogenic activity of flavonols may be due to their antioxidant acivity. Quercetin and kaempferol increase levels of glutathione, a natural antioxidant. Quercetin, myricetin and kaempferol also induce apoptosis by induction of caspase-3, procaspase-9, elevation of reactive oxygen species and release of mitochondrial cytochrome c. Quercetin, myricetin and kaempferol have effectively supressed the activity of several cell proliferative enzymes, such as cyclooxygenase-2, topoisomerases I and II and ornithin decarboxylase (Nichenametla and Taruscio 2006).

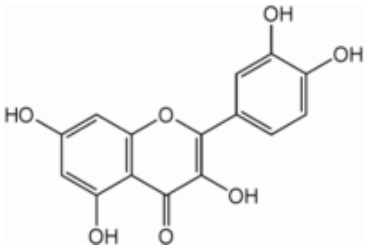


Figure 5. Chemical structure of quercetin (Škerget *et al*. 2005).

Another group of flavonoids flavones are represented by luteolin and apigenin. To the group of isoflavones belong genistein and daidzein, which mostly occur in soybeans and are considered as phytoestrogens (Yang *et al*. 2001). They have been suggested to protect against hormone-related types of cancer, such as breast or prostate, and menopausal symptoms (Soobrattee *et al*. 2005). The chemical structure of apigenin is in Figure 6. (Fresco *et al*. 2006).

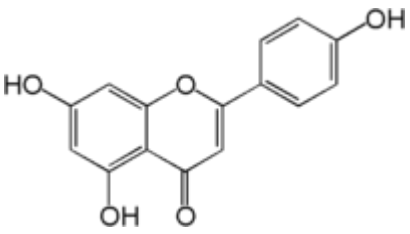


Figure 6. Chemical structure of apigenin (Fresco *et al*. 2006).

Anthocyanins, such as cyanidin are water-soluble pigments, which have antioxidant activity and cancer preventive activity. Berry extracts containing a lot of anthocyanins have differential effects on normal and cancerous cells. For example, grape seeds induce cytotoxicity in several types of cells, but increase the viability

in normal human cells (Nichenametla and Taruscio 2006, Fresco *et al*. 2006). The structure of cyanidin is in Figure 7. (Fresco *et al*. 2006).

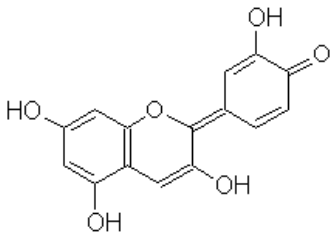


Figure 7. Chemical structure of cyanidin (Fresco *et al*. 2006).

1.2.4.3. Stilbenes

Most stilbenes act as antifungal antialexins, compounds, which are usually synthesized only in response to infection or injury. To this group belongs for example resveratrol, which is present in grapes, wine and peanuts (Yang *et al*. 2001). The chemical structure of resveratrol is in Figure 8. (Fresco *et al*. 2005).

Stilbenes inhibit cellular events associated with carcinogenesis, including tumor initiation, promotion and progression, so they are chemopreventive and cytotoxic. Resveratrol also inhibits the expression of vascular endothelial growth factor and thus tumor neovascularization. But it does not distinguish between physiological and pathological angiogenesis, so it delayes wound repair (Nichenametla and Taruscio 2006).

Resveratrol inhibits free radical formation and effects production and activation of several enzymes. For example it decreases activity of ornithine decarboxylase, which is enhanced in cell cancer proliferation. Resveratrol induces apoptosis and increases the expression of p53 by enhancing the Bax activity (Nichenametla and Taruscio 2006).

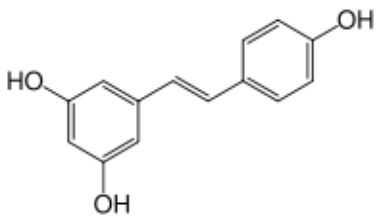


Figure 8. Chemical structure of resveratrol (Fresco *et al*. 2006).

1.2.4.4. Tannins, lignans and coumarins

The best known hydrolyzable tannin is tannic acid. Tannins can form insoluble complexes with carbohydrates and proteins. This function of tannins is responsible for the adstringency of tannin-rich food, because of the salivary proteins precipitation (Bravo *et al*. 1998). The structure of tannic acid is in Figure 9. (Fresco *et al*. 2006).

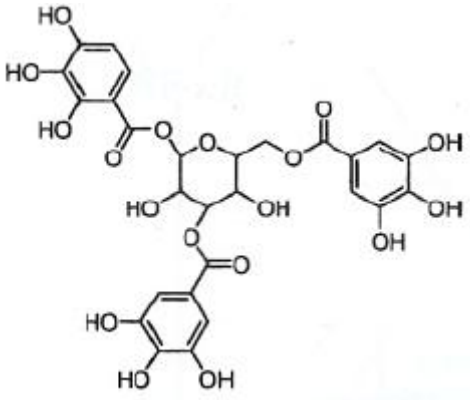


Figure 9. Chemical structure of tannic acid (Fresco *et al*. 2006).

Lignans, such as sesamin, enterodiol and enterolacton, have antioxidant properties. They function as phytoestrogens, which potentially decrease the mammary tumorigenesis (Yang *et al*. 2001). The structure of sesamin is in Figure 10. (Fresco *et al*. 2006).

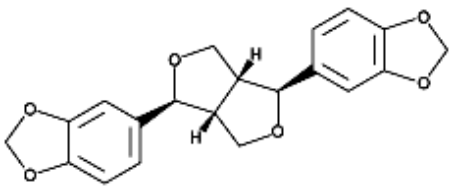


Figure 10. Chemical structure of sesamin (Fresco *et al*. 2006).

Coumarins, such as esculetin have antioxidant properties (Fresco *et al*. 2006). The chemical structure of coumarin is in Figure 11. (Fresco *et al*. 2006).

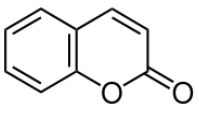


Figure 11. Chemical structure of coumarin (Fresco *et al*. 2006).

1.2.5. Polyphenols in different plants

1.2.5.1. Basil (*Ocimum basilicum*)

Basil is an annual plant, belonging to the Lamiaceae family. The aromatic character of this plant depends on the major chemical compounds of essential oils primarily consisting of monoterpenes and phenylpropanoids (Miele *et al*. 2001).

Generally, the essential oil of *Ocimum basilicum* leaves, stems and flowers ranges from 0.07 to 1.92 % in dry herbage. The main components of essential oil are monoterpnene alcohol (Lewinsohn *et al*. 2000) linalool (30-70%), methylchavicol (0.1-70%), eugenol (8-30%) and methyleugenol (1-91%). To other components belong delta-cadinene, alpha-pinene, alpha-humulene, camphene, eucalyptol, isobornyl acetate, camphor and trans-caryophyllene (Zheljazkov *et al*. 2008).

There are big differences in the content of essential oils between the wild and cultivated type of basil. They consist in different environmental conditions, such as temperature, length of summer, soil characteristics, number of cut and production system. Stress (chemical elicitators, heavy metals, cutting) causes enhancement of polyphenol production and accumulation (Zheljazkov *et al*. 2008).

The essential oil has a pleasant aroma and antimicrobial and insecticidal activity, and is therefore used as aromatic agent to food, culinary (leaves), pharmaceuticals, cosmetic, aromatherapy industries and natural therapies (Zheljazkov *et al*. 2008). Linalool is used in domestic products, such as soaps and shampoos. Camphor is used as a moth repellent (Miele *et al*. 2001). Eugenol is used in dentistry as an antiseptic and analgesic agent, but it is in a dose-dependent manner genotoxic (Maralhas *et al*. 2006).

The essential oil has antioxidant, antimicrobial, free radical scavenging, inhibitory lipid peroxidation, spasmolytic, carminative, hepatoprotective and antiviral properties. Minor basil oil constituents delta-cadinene, 3-carene, alpha-humulene, citral and trans-cyrophyllene have antileishmanial activity and in the same time do not have cytotoxic effect on mammalian cells, except citral (Zheljazkov *et al*. 2008).

The major antioxidant compound of *Ocimum basilicum* is rosmarinic acid. (Jayasinghe *et al*. 2003), which has suppressive effect on mesangioproliferative glomerulonephritis (proliferation of mesangial cells and glomerular matrix expansion)

(Makino *et al*. 2002). The antioxidant activity of basil is concentration-dependent (Gülçin *et al*. 2007).

The essential oil of basil has antiproliferative effect against epidermal carcinoma and murine leukemia (Manosroi *et al*. 2006). Basil extract supresses carcinogenesis and can act as a protective agent, so it might be used in cancer treatment (Aruna *et al*. 1990).

1.2.5.2. Laurel (*Laurus nobilis*)

The laurel plant is an evergreen shrub or tree with aromatic smell from the family Lauraceae. Its leaves and essential oil have been traditionally used as condiment and against cancer, bronchitis, dermatitis, digestive problems and anxiety (Škerget *et al*. 2005, Conforti *et al*. 2006, Ferreira *et al*. 2006, Longo *et al*. 2005). Bay leaves are used as food preservatives, due to their antimicrobial and insecticide activities, because of aroma also in cosmetic industry (Longo *et al*. 2005). The essential oil exhibits also significant analgesic, anti-inflammatory and sedative effect. The sedative effect may be related to the presence of eugenol and methyleugenol, which possess anesthetic and muscle relaxant activities. β-pinene, α-pinene and sabinene possess anti-inflammatory activity (Sayyah *et al*. 2003).

Laurus nobilis essential oil has cytotoxic effect on mellanoma cells, renal cell carcinoma, breast cancer cell line, prostate carcinoma. This oil inhibits human tumor cell growth (Loizzo *et al*. 2007). The main component of essential oil prepared from bayleaves *Laurus nobilis* is 1, 8-cineole. This substance induces apoptosis and thereby supresses growth specifically in human leukemia cells in dose- and time-dependent manner (Moteki *et al*. 2002). Sesquiterpene costunolide from the essential oil has strong growth inhibitory, pro-apoptotic effect against human leukemia cells. This activity increases with concentration (Komiya *et al*. 2004).

The leaves oil contains cinnamtannin B-1, a proanthocyanidin, which is a radical scavenger with a great antioxidant activity (Ben Amor *et al*. 2007). Cinnamtannin B-1 enhances caspase-3 activity and causes apoptosis in tumoral cells, but not in normal cells. The reason for different effect of cinnamtannin B-1 in apoptosis in normal and tumoral cells has not been fully investigated. The concentration does not influence this fact (Bouaziz *et al*. 2007). Polyphenolic flavonol aglycones isoquercetin and kaempferol are responsible for the exhibited antioxidant activity (Soobrattee *et al*.

2005). Isoquercetin from the leaves possesses very high alkyl peroxy radical scavenging activity (Kang *et al*. 2002). Secondary metabolites, contained in laurel leaves, eventually in fruits are sesquiterpenes, such as santamarine, reynosine and costunolide, which have antimicrobial, immunomodulating and cytotoxic activities in human leukemia cells (Matsuda *et al*. 2002).

1.2.5.3. Juniper (*Juniperus communis*)

Juniperus communis from the family Cupressaceae is an evergreen, aromatic shrub or tree. Seeds, dried leaves and fruits, in form of extract, are traditionally used to treat bronchitis, common cold, nose bleed, hypertension, inflammation, as expectorans, diuretics and for rheumatic symptoms. Nowadays juniper is used as an aroma in foods, drugs and cosmetics (Emami *et al*. 2007). All organs of this plant contain essential oil, but it is obtained mainly from the berries, needles and branches (juniper foliage). The oil content is around 0,2-3,4%, depending on geographic location, altitude, degree of ripeness, and other factors (Barjaktarović *et al.* 2005).

Juniperus communis grows widely in both hemispheres, primarily in lower elevations. The main content of leaves essential oil are monoterpenes, such as alpha-pinene (40-60%), sabinene (1-10%), camphene, beta-pinene, myrcene, alpha-phellandrene, 3-carene and terpinen-4-ol (Filipowicz *et al*. 2006). The juniper essential oil (Juniper aetheroleum) from juniper berry contains mainly monoterpenes (58-85%), such as alpha-pinene (10-76%, mostly around 30%), beta-pinene (around 18%), sabinene (1-28%, mostly around 15%), beta-myrcene, terpinen-4-ol and sesquiterpenes (Pepeljnjak *et al*. 2005, Barjaktarović *et al*. 2005). To the flavonoids contained in juniper belong catechins and epitcatechins (Iida *et al*. 2007).

The methanol extract from fruits and leaves of *Juniperus communis* possess antioxidant acitivity, even higher than alpha-tocopherol (Emami *et al*. 2007). Juniper berry extract is able to kill a large amount of liver and colon carcinomas (Bayazit *et al*. 2004) and decreases the growth of breast cancer cells (Slambrouck *et al*. 2007). Decoction from juniper berries decreases glycemic levels in normoglycemic due to increasing of peripheral glucose consumption (De Medina *et al*. 1994). Juniper also possess antimicrobial activity against dermatophytes, Gram-positive and Gram-negative bacterial species (Pepeljnjak *et al*. 2005).

2. AIM OF STUDY

Plants and herbs are used in folk medicine against many human diseases, including cancer, for a long time. Previous studies showed that plant-derived polyphenols have many properties, including antioxidant and anticancerogenic activities, which can effect apoptosis. They can directly interact with specific steps and/or proteins regulating apoptotic process in different ways depending on their concentration, the cell system, the cell type or the stage of pathological process (Giovannini *et al.* 2007).

The aim of the experimental part of this work was to examine the activities of several plant extracts containing polyphenols, concretely their effect on cell viability. Several studies comparing the effects of plant extracts and isolated phenolic compounds from these extracts have proven, that the whole extract has much higher activities than the individual compounds. It is possible, that the compounds within the extract have synergistic influence on each other (Giovannini *et al.* 2007).

The experiments were performed on the SH-SY5Y neuroblastoma cells, which are human neuroblast highly metastatic cancer cell line. Previously, these cells were used for example in experiments involved in the Parkinson's disease research (Maňáková *et al*. 2003), in the examination of nicotinic receptors in these cells (Ridley *et al*. 2001) or in the experiments examining effect of cytarabin, resveratrol and kurkumin on apoptosis (Lantto 2006). In the experiment were used extracts from laurel, juniper and basil, which were prepared at the University of Helsinki, Department of Pharmaceutical biology (Hinneburg *et al*. 2006)

To the used methods belong Western blotting analysis, MTT and LDH assay. The level of p53 protein in cells was measured in Western blotting analysis. The cell viability was examined in MTT and LDH assay. MTT assay measures the amount of formazan created from MTT by reducing enzymes in the living cells. LDH assay measures the enzyme lactate dehydrogenase, which is produced in the damaged cells. The aim of study in MTT and LDH assays was to compare the effects, production of enzymes, of different extract concentrations after the same time of incubation. The main goal in Western blotting analysis was to show how the level of p53 protein (commonly used marker of apoptosis) is changing upon effect of one extract concentration depending on incubation time.

3. MATERIALS AND METHODS

3.1. Principles of methods

3.1.1. Cell viability assays

3.1.1.1. MTT assay

MTT is a laboratory test and a standard colorimetric assay. This method is used to measure cell viability and proliferation, cell population's response to external factors and to screen the effect, eventually cytotoxicity of anticancer drugs. It is based on the reduction of tetrazolium salt (MTT).

Pale yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a water soluble dye, which is reduced by metabolically active cells by their dehydrogenase enzymes. This reaction leads to the creation of reducing equivalents, such as NADH and NADPH. In principle, it is a NADH dependent reduction. MTT is reduced to water insoluble purple to dark blue crystalic formazan. The amount of formazan generated per cell depends on the level of energy metabolism in the cell, in otherwords on cell viability (Mosmann 1983). Formazan product is only partially soluble in medium (Mosmann 1983), so a solubilization solution, either dimethyl sulfoxide (DMSO) or detergent sodium dodecyl sulfate solution in dilute hydrochloric acid is added to dissolve formazan product into a colored solution. The absorbance of colored formazan solution can be measured and quantified at a certain wavelength (usually between 500 and 600 nm) at a spectrophotometer (Mossmann 1983).

The low absorbance means absence of cells or decreased cell viability. Higher absorbance means an increase in cell proliferation. Measuring of the absorbance allows to quantify change in the rate of cell proliferation. The linear relationship between cell number and signal produced is established for each cell type.

Reduction of MTT, in other words cleavage of the MTT ring, takes places only in active living cells, in which reductase enzymes and dehydrogenases are active. The amount of created formazan is directly related to the number of viable cells. Increased viable cell number results in increased amount of formed MTT formazan and an increase in absorbance. When the amount of formazan produced by treated cells is compared with the amount of formazan produced by untreated cells, the effectivness

of the agent in causing cell death can be deduced from a dose-response curve. This method does not detect death cells. The cell population should be homogenous (Mosmann 1983).

3.1.1.2. LDH assay

LDH test is a fluorometric assay and a cell viability test, based on the fact, that damaged cells have decreased integrity of cell membrane, which allows the entrance of enzym lactate dehydrogenase (LDH) outside the cell. LDH is an oxidoreductase which catalyzes the interconversion of lactate and pyruvate. The LDH assay is based on the reduction of iodonitrotetrazolium salt (INT) in a NADH-coupled enzymatic reaction to red colored formazan. Formazan exhibits an absorption maximum at 492 nm and can be measured colorimetrically. The intensity of the formed red or purple color is increased in the presence of increased LDH activity (Larsen 2005).

Scheme of the reaction catalysed by LDH:

$NAD^+ + \text{lactat} \rightarrow \text{pyruvat} + NADH$

$NADH + \text{iodonitrotetrasolium-diaphorase} \rightarrow NAD^+ + \text{formazan}$

3.1.2. Western blotting

Western blotting is a modern technique, which is used for analysis and characterization of proteins, for purifying and identifying of biochemicals on the basis of charge, size and conformation, and to determine their molecular sizes (weights). Purity is indicated by the number of stained bands in the electropherogram. One band usually means, that only one detectable component is present. In this case the sample is homogenous or „electrophoretically pure“. Two or more bands indicate, that the sample contains more components, contaminants or inpurities and is therefore heterogenous. Sometimes there is just one component, but there are more bands found. This can

happen, when the component degrades during electrophoresis. The principle of this method is the transfer and detection of proteins that react with antibodies (Boyes 2000). Western blotting has several steps. The first step is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE serves mainly to the fractionation of protein mixture. The samples have to be denaturated in order to fractionate proteins properly (Boyes 2000). The protein samples are transferred into the wells of polyacrylamide gel. The polyacrylamide gels are prepared by the free radical polymerization of acrylamide and the cross-linking agent N,N‘-methylene-bis-acrylamide. Chemical polymerization is controlled by an initiator-catalyst system, ammonium persulfate-N,N,N‘,N‘-tetramethylenediamine (TEMED). Standard gel for protein separation contains 7,5 % polyacrylamide and is used to identify proteins with molecular size 10,000 to 1,000,000 daltons with the fact, that best results are reached in the range of 30,000 to 300,000 daltons. The resolving power and molecular size range of a gel depend on the concentration of acrylamide and bis-acrylamide. Using low concentrations a gel with larger pores is created, which is used to analyse higher-molecular-weight biomolecules. Higher concentration of acrylamide gives the gel with smaller pores, therefore used to analysis of lower-molecular-weight biomolecules (Boyes 2000).

It is possible to use prepared, so called pre-cast gels or to prepare our own gel. In this experiment the gel was prepared. It is so called slab gel. The polyacrylamide slab is prepared between two glass plates, that are separated by spacers. The spacers allow a thickness of 0.5 to 2.0 mm, which is appropriate for analytical procedures. Slab gels are usually 8x10 cm or 10x10 cm big. The plastic combs inserted into the top of the slab gel create wells. There are usually 10 or 12 wells created. After the gel is polymerized, the comb is removed and the wells should be rinsed with electrophoresis buffer to remove salt and unpolymerized acrylamide. The gel plate is clamped into place between two buffer reservoirs and a sample is loaded into each well (Boyes 2000).

During SDS-PAGE the proteins are in the electric field. Voltage stays constant during electrophoresis. The movements of charged particles through the gel depend directly on the electric field and charge and inverselly on the viscozity (Boyes 2000).

To the main components of the SDS buffer belong SDS and mercaptoethanol. The detergent SDS disrupts secondary, tertiary and quarternary structure to produce linear polypeptide chains coated with negatively charged SDS molecules. Mercaptoethanol assists in protein denaturation by reducing all disulfide bonds.

The detergent binds to hydrophobic regions of the denaturated protein chain. The bound detergent molecule carrying negative charges mask the native charge of the protein. Thus, a polypeptide chain of a constant charge ratio and uniform shape is prepared. The electrophoretic mobility of the SDS-protein complexes depends very much on the molecular size. The larger molecules are going slower through the gel because of gel sieving, whilst small molecules have greater mobility (Boyes 2000).

After the first step, proteins are deeply embedded in the gel, so they are not accessible for analytical reagents. Thus, in the second step proteins separated by SDS-PAGE are transferred (blotted) from the gel to support matrix, which can be nylon, polyvinyl-difluoride or nitrocellulose membrane. The nitrocellulose membrane is the most used. The membrane strongly binds and immobilizes proteins. Binding of proteins to nitrocellulose membranes is noncovalent, mostly hydrophobic (Boyes 2000).

Effective transfer is reached by using of an electrophoretic blotter. A sandwich of filter paper, gel, membrane and special sponges is prepared in a cassette, which is placed between platinum electrodes. An electric current is passed throught the gel and causes the electrophoresing of proteins out of the gel onto the membrane. The migration of molecules is influenced by electric field (Boyes 2000). The protein blots are on the membrane better accessible for chemical or biochemical reagents, so in this form ensure further analysis of proteins, which have specific structure and function. Highly specific and sensitive immunological detection technique for analysis is called Western blotting (Boyes 2000).

After blotting it is necessary to probe the protein bands and detect a specific protein or group of proteins among the blots, in other words to visualize the specific protein blots. Specific identification is based on immunology, which means antigen-antibody interactions. Protein binding sites on the membrane, which are not occupied by blotted proteins, have to be blocked before detection. The reason is that antibodies used for detection are also proteins, so they could interfere with these proteins. Protein binding sites still remaining on blotted membranes can be blocked with solution of casein, which is the major protein in milk. Milk solution is the most used blocking buffer.

When all protein binding sites of the blotted membrane are occupied, it can be treated with analytical reagents for detection of specific proteins. The blotted membrane is incubated with so called primary antibody, antibody, which is specific for protein of interest. The primary antibody creates with the protein of interest antigen-antibody

complex. However, the interaction between protein and antibody usually does not create any visible signal. The blot is therefore incubated with a secondary antibody, which is directed against general class of primary antibody. For example, if the primary antibody was prepared in rabbit serum, the secondary antibody will be anti-rabbit IgG, usually from a goat or a horse. The secondary antibody is labeled (conjugated), so the interacion between primary and secondary antibody produces visible signal. Secondary antibody is usually tagged with an enzyme, usually horseradish peroxidase (HRP). When the treated blot is incubated in a substrate solution, the conjugated enzyme catalyzes conversion of the substrate into a visible product, that precipitates at the blot site. The protein is situated on the place, where the colored band is present (Boyes 2000).

The reaction catalyzed by conjugated enzymes is:

4-chloro-1-naphtolreduced + H_2O_2 → 4-chloro-1-naphtoloxidized + H_2O_2 + 1/2 O_2

Western blotting, also called immunoblotting assay of proteins, have many advantages, such as small reagent volumes, short processing time, relatively inexpensive equipment and ease of performance (Boyes 2000).

3.1.3. β-actin

Beta-actin is one of six different actin isoforms which have been identified in humans. Beta-actin belongs to the non-muscle cytoskeletal actins. Actins are highly conserved proteins, that are involved in cell motility, structure and integrity. Beta-actin is a constituent of filaments attached to the nuclear pore complexes and extending into the nucleus (Pederson *et al*. 2005).

Beta-actin is implicated in the regulation of cell spreading and membrane organisation. The intracellular location of beta-actin mRNA has been shown to be regulated by growth factor stimulation of signal transduction pathways (Gunning *et al*. 1997).

3.2. Methods

3.2.1. Cell culture

The SH-SY5Y chemosensitive neuroblastoma cells were used in the experiments. Stock cultures of SH-SY5Y human neuroblastoma cells were maintained in a DMEM: F12 (1:1) (Gibco) modified medium supplemented with 1% of non-essential amino-acids (NEAAs, 100x) (Gibco), 15% foetal bovine serum (FBS) (heat-inactivated +56 °C, 30 minutes) (Gibco), Penicillin-streptomycin (170 U/ ml penicillin G sodium, 170 μg/ ml streptomycin sulfate) (Gibco). Cultures were seeded into 75 cm^2 standard tissue culture plastic flasks containing 20 ml of supplemented medium and maintained at 37°C in 5% CO_2 humidified air. Stock cultures were passaged 1:2 to 1:4 weekly and fed every other day.

3.2.1.1. Preparing of a new cell culture

The cell suspension was freezed in liquid nitrogen. The new cell culture was prepared by melting of cell suspension in vial in water bath for 1-2 minutes. Then the cell suspension was added into 40-50 ml of medium in a 75 cm^2 flask. After 6-8 hours the medium was changed.

3.2.1.2. Preparing of a cell subculture

The old medium was sucked out for binding assays. The SH-SY5Y cells were detached by adding 2,5 ml of Trypsin-EDTA (pH 7,4) and incubated for 3 minutes at 37 °C to loosen cells. 10 ml of medium was added and the cells were thus transferred into a falcon tube. The suspension was then cetrifuged at 500 x g, 3 minutes, 20 °C to separate the cells from medium. After that the supernatant was removed.

The cells with confluency 90-95 % were used for preparing the experiment. The cells were suspendend in 1 ml of medium. 10 μl of suspended cell suspension was added into 990 μl of medium in the tube and the cells were counted in Bürker chamber

in 5 patches, which did not neighbour with each other with their sides, only with their corners.

3.2.2. Plant extracts

In the experiments the extracts from laurel, juniper and basil were used. The aqueous extracts were prepared by water extraction. The essential oils were removed by hydrodistillation. The content of total phenols in the extracts is in the Table 2. (Hinneburg *et al.* 2006).

Species	Total phenols (mg/g)
Laurel (*Laurus nobilis* L.)	92,0 ± 2,45
Basil (*Ocimum basilicum* L.)	147 ± 1,60
Juniper (*Juniperus communis* L.)	18,5 ± 0,62

Table 2. Content of total phenols in the extracts from juniper, laurel and basil (Hinneburg *et al.* 2006).

3.2.3. Cell viability assays

3.2.3.1. Cell culture for cell viability assays

For the cell viability assays the cells were seeded in 96-well plates in 200 µl of medium with 6 x 10^4 cells per each well. For measuring background six wells were filled only with medium. Residual wells were filled with sterile H_2O. The cells needed some time to become confluent, to recover from handling and to attach to the wells, thus they stayed in the incubator at 37°C and 5% CO_2 over night. The old medium was removed and 150 µl of fresh medium containing 5% of FBS was added into the wells.

The cells were treated with extracts from juniper, laurel and basil. The stock solutions with concentration 30 mg/ml were prepared by dissolving 30 mg of each extract in 1 ml of medium. New solutions were prepared from the stock solutions by dissolving in medium, in a way, that by adding of 10 µl of these new solutions to the 150 µl of medium, the concentrations were in sequence 2; 0,5; 0,1; 0,05 and 0,01 mg/ml. In according to reach homogenity within the wells, the plate was shaked after

adding of extracts. AraC in the concentration 100 μM has been in the sixth column. Only cells in 150 μl of medium without extract (serve as a control) have been in the seventh column. Only 150 μl of medium has been in the eigth column. The cells were exposed to the extracts for 12 hours in the incubator at 37°C and 5% CO_2. After 12 hours 50 μl of medium without cells was transferred from the transparent plate into a new 96-well white-walled plate.

3.2.3.2. MTT assay

MTT solution in concentration 5mg/ml was prepared by dissolving in HBSS solution (Hank's balanced salt solution, 5,36 mM KCl; 441 μM KH_2PO_4; 137 mM NaCl; 4,17 mM $NaHCO_3$ and 337 μM Na_2HPO_4, pH 7,4) and 10 μl of this solution was added to the rest of the fluid in the old plate. The final concentration of MTT in each well was thus 0,5 mg/ml. Gentle shaking is necessary to mix the MTT into media.

Incubation with MTT solution lasted 2,5 to 3 hours at 37°C until the purple precipitate was visible. Afterwards the medium was carefully removed in the corners of the plate to avoid removing of blue crystals on the well's bottom. Then 200 μl of DMSO (dimethyl sulfoxide) was added into every well and resuspended until the blue crystals fully dissolved. At the end, the absorbance, otherwords optical density was measured in a microtiter plate reader (Bio-Rad, model 550, Japan) at the wavelength 550 nm with the reference filter set to 655 nm.

3.2.3.3. LDH assay

The LDH assay kit was used for the LDH test and the assay was performed following manufacturer's instructions. The 96-well white-walled plate with 50 μl of medium in each well was equilibrated at RT for 20-30 minutes. Then 50 μl of CytoTox-One reagent was added into each well. The plate was wrapped into aluminium folia and shaked for 10 minutes at 300 rpm, followed with further 20 minutes of incubation at RT.

After 30 minutes 25 μl of Stop solution was added (in the same order as addition of reagent). The Stop solution stops the reactions and stabilizes the fluorescent signal.

The adding of CytoTox reagent had to be quick, because the reagent is sensitive to the light. Also adding of Stop solution was quick in according to react in all wells for the same time.

After adding the Stop solution, the plate was shaking for a few seconds at 300 rpm and right after that the fluorescence (the amount of created LDH) was measured spectrophotometrically on Varioskan machine with excitation 560 nm and emission 590 nm. It was necessary to avoid air bubbles while pipetting (using needles to explode them) and keep the plate in dark (aluminium folia) the whole time after adding the reagents until measuring. The measuring should be provided after adding the Stop solution as soon as possible.

3.2.4. Western blotting

3.2.4.1. Cell culture for Western blotting

The treatment of cells for Western blotting was similar to the one for cell viability assays. After counting of cells, 2,5 x 10^6 cells were cultured in the sterile, plastic plates in 5 ml of medium. After 18-24 hours of incubation at 37°C and 5% of CO_2, the cells were treated with plant extracts from basil and laurel, concretelly basil was used in the concentration 0,5 mg/ml and laurel 0,1 mg/ml. These extracts were the same as for cell viability tests.

This experiment was examining time response. All cells were treated with the same concentration of plant extract, but they differed in the time of exposure to the plant extract. After certain time the plates with cells were taken out of the incubator, put onto the ice. The medium was removed away and 2,5 ml of ice cold physiological solution (PBS, phosphate buffered saline, 137 mM NaCl; 2,7 mM KCl; 7,9 mM Na_2HPO_4 and 1,5 mM KH_2PO_4) was added. After the washing of cells with PBS two times, 1 minute each washing, the cells were collected by scraping into 1,25 ml of ice-cold PBS. The tubes with cells were centrifuged (Eppendorf 5415D, Germany) at 4°C and 5000 x g for 5 minutes. The supernatant was removed and the cell pelets were stored at –80°C.

3.2.4.2. Protein extraction and BCA protein assay

The proteins were extracted from cells by a lysis buffer (20 mM N-2-hydroxyethylpiperazin-N1-2-ethanesulfoc acid (HEPES); 20% glycerol; 500 mM NaCl; 1,5 mM $MgCl_2$; 0,2 mM EDTA and 0,1% Triton X-100) with Complete Mini Stock solution protease inhibitor (Roche). The cells were then incubated on ice for 30 minutes. After that the tubes with samples were briefly shaked on Vortex and then sonicated 3 x 10 seconds (Rinco Ultrasonics, Switzerland). In order to remove DNA and pieces of cell membrane from the protein sample, the samples were centrifuged (Eppendorf 5415D, Germany) at 4°C and 16 100 x g for 15 minutes. The supernatant was then trasnferred into new tubes.

The protein samples were diluted in the rate of 1:10 with the EMSA buffer and 10 µl of each protein sample was transferred twice behind the BSA standards. 200 µl of a solution, created by mixing of two liquides-reagents A and B (in the rate 50:1), acids from BCATMProtein Assay Kit, Pierce, was added into each well. After 30 minutes of incubation in the folio at 37°C, the plate was cooled down. Then the absorbance, and thus protein concentration, was measured on spectrophotometer (Bio-Rad, malli 550, Japan) at 550 nm of wavelength.

Due to the BSA standards the protein concentration was measured, and it was counted, how much of the sample is necessary to prepare a loading sample for Western blotting. In this experiment 20 µg of protein per well was used. The pipetting volume was 20 to 30 µl in according to the ascertained protein content (when the protein content was to low, the loading volume was smaller). In according to get comparable results, all the samples contained the same amount of protein, which was reached by dilution of the samples with higher concentration with lysis buffer. Before loading and starting the western bloting, 4x electrophoresis sample buffer (loading buffer) was added to the protein samples. The loading buffer (125 mM Tris (pH 6,8); 50% glycerol; 4% natriumlaurylsulphat (SDS, sodium dodecyl sulphate); 10% 2-merkaptoethanol and 0,02% bromophenol) denaturalizes the proteins and dilutes the protein sample in the rate 1:3. The whole time the samples had to be in ice.

3.2.4.3. SDS-PAGE

The protein samples were loaded into the prepared sodium laurylsulphat-polyacrylamid gel (SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis). The gel contained two parts. The stacking (upper) gel containing 5% of polyacrylamide was used to concentrate the sample and to make thin starting layer for proteins before they enter the separating gel. The separating (lower) gel containing 12% of polyacrylamide was used to separate the proteins in according to their molecular weight. At first the resolving gel was prepared. It takes about 45 minutes until the gel stiffts. During stiffting, above the gel there was destilled water, which was removed when the stacking gel was prepared. The gel could be used immediatelly after the preparation or it could be stored in the refrigerator over night, covered with a moist paper and plastic folia.

Also Prestained protein MW marker, Fermentas (5µl/ well) with the molecular weight 10,000-70,000 was loaded into the gel. This marker is necessary to identify the searched proteins in according to their molecular weight on the membrane in the latter phase of Western blotting and for detection (especially when there are two or more proteins searched in one experiment). Electrophoresis proceeded in the electrophoresis buffer (25 mM Tris; 192 mM glycine and 0,1% SDS) at Bio-Rad (PowerPac HC, USA) machine at constant voltage 120 V and 25-30 mA per slab gel approximatelly for 1,5 to 2 hours, until the bromophenol blue marker dye reaches the bottom of the gel. The speed depends on voltage. When the voltage is higher, the marker and samples wander through the gel quicker.

3.2.4.4. Transfer of proteins onto membrane

The proteins were transferred from the gel into the nitrocellulose membrane (Protran BA 85, Schleicher amd Schuell). A sponge, 2 or 3 3MM Whatmann filter papers, membrane, gel, again filter papers and sponge were put into a special cassette. The transferring of proteins into the membrane proceeded in the 4°C cold transfer (blotting) buffer containing 20% methanol and 1 x TBS (Tris-buffered saline, 25 mM Tris; 137 mM NaCl and 2,7 mM KCl), in the presence of ice (in order to avoid destruction of proteins by heat), at Bio-Rad machine (PowerPac HC, USA) at the

voltage 100 V for about 1 to 1,5 hour. The nitrocellulose membrane, filter papers and sponges should be incubated in transfer buffer in the fridge for about 10-15 minutes before transferring of the gel.

After transferring of proteins into the membrane the non-specific bindings and sites on the membrane were blocked in the blocking buffer at RT with mild shaking for 1,5 hours. Blocking buffer contains 1 x TBS solution with 0,05% Tween 20 (also called TTBS) and 0,5% non-fat dried milk powder. Mainly milk has the blocking properties. Tween 20 serves as a surfactant. The blocking buffer helps to increase sensitivity and prevent non specific signal caused by cross-reactivity of the antibody and the blocked reagent. After this phase the membrane was incubated in primary antibody solution.

3.2.4.5. Primary antibody

Primary monoclonal antibodies p53 and β-actin were diluted to the concentration 1:1000 (p53, NCL-p53-DO7, Novocastra) and 1:3000 (β-actin, A1978, Sigma-Aldrich) in TTBS with 0.5% non-fat dried milk powder. The membrane was cut in a way, that p53 was identified in one part and β-actin in the other part. The membranes were put into the primary antibody solutions. This phase lasted at 4°C without shaking overnight.

3.2.4.6. Secondary antibody

On the next day the membranes were washed by suspending in the washing buffer TTBS 4 x 8 minutes (increasing of washing buffer volume and the number of washes may reduce background, briefly rinsing membrane in washing buffer after incubation can increase wash efficiency), followed by incubation with secondary antibody solution at RT with shaking for 60 min. As secondary antibody was used horse radish peroxidase (HRP) conjugated immunoglobuline G (Ig G) in concentration 1:2000, diluted in TTBS with 0.5% non-fat milk powder. The secondary antibody specifically binds to the primary antibody and enables chemiluminiscence visualization. (The concentration depends on the HRP conjugate and the amount of antigen on the membrane). After this phase the membrane has been washed in TTBS 4 x 8 minutes

to remove non-bound HRP-conjugates. (Large blocking and washing buffer volumes may result in reduced non-specific signals).

3.2.4.7. Visualization and analysis of proteins

The commercial chemiluminescent reagent (Super Signal West Pico, Pierce) was used to visualize the protein bands. The secondary antibody conjugated with HRP enzymatically effects these substrates, which causes a chemical reaction connected with the production of light, in other words chemiluminiscence.

The nitrocellulose membrane was put into the mixture (10 to 15 ml) of two reagents (Luminol/Enhancer and Stable Peroxide) in the rate 1:1 on the shaker for 5 minutes, followed by putting the membrane into a plastic wrap, removing of the excess liquid and bubbles, and taking a chemiluminiscence picture 5 x 50 s CCD (charge-coupled device) by a camera (GeneSnap with GeneGnome, Syngene). The pictures were analysed with the GeneTools programme (Syngene).

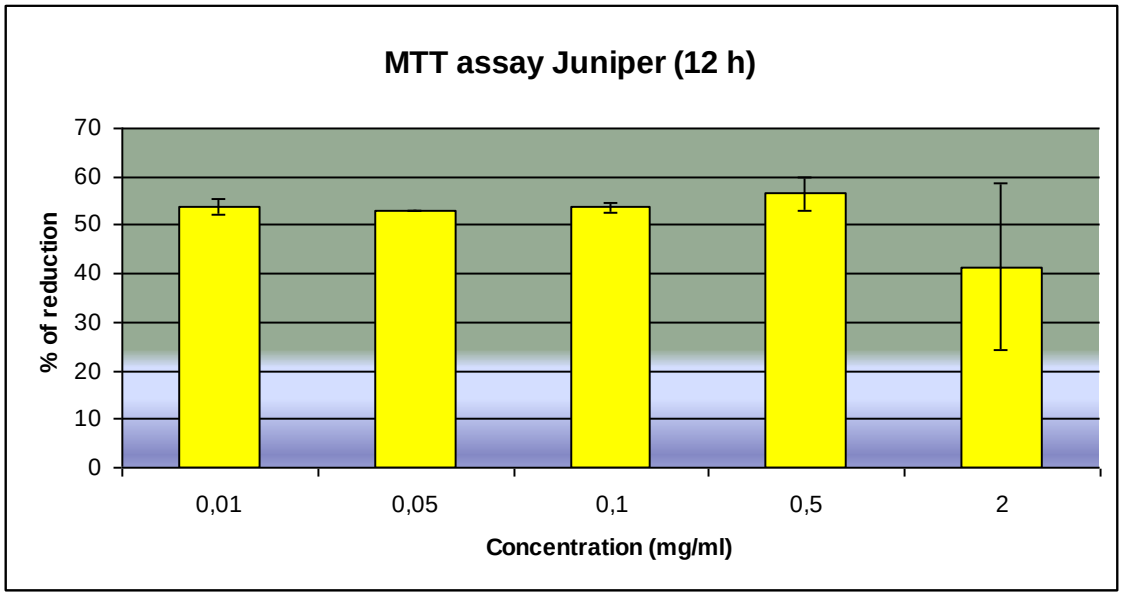
4. RESULTS

The aim of the MTT assay was to estimate the mitochondrial enzymatic activity, in otherwords if and how much the cells, treated with different juniper, laurel and basil extract concentrations, enzymatically reduce MTT and create formazan in comparisson to the untreated cells (creating of formazan is connected with the viable cells). In the LDH assay the amount of created enzyme lactat dehydrogenase produced in the damaged cells was measured. The SH-SY5Y neuroblastoma cells have been incubated with the extracts for 12 hours. The untreated cells were used as an experiment control. The MTT and LDH assay graphs show, how many percent of the control results were reached by the treated cells results. The experiments were done three times with three different, but similar cell samples. The average data of these three experiments were used for the creation of the graphs. The aim in Western blotting analysis was to determine the p53 protein level in the SH-SY5Y cells treated with the laurel and basil extracts. The beta-actin level has been used as a control. The untreated cells have been used as an experiment control.

4.1. Juniper extract

4.1.1. MTT assay

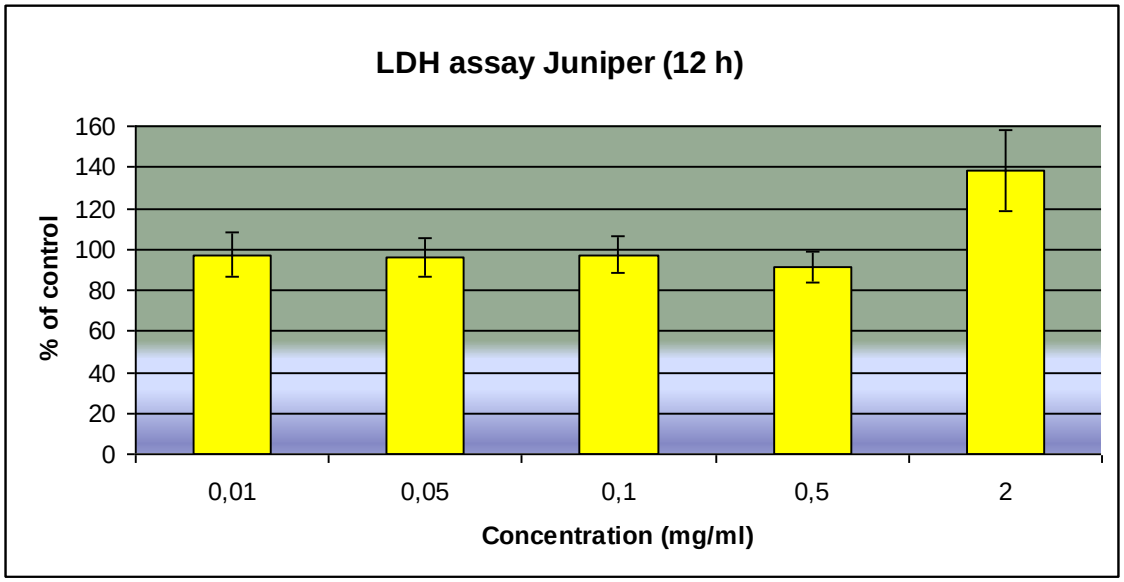
The Graph 1. shows, that 12 hours of incubation with the extract invoked significant decrease in MTT reduction in cells treated with the extract concentration 2 mg/ml. The results did not reach 45% of the control. The results in four other concentrations 0,1; 0,5; 0,01 and 0,05 mg/ml reached less than 60% of the control results.



Graph 1. Reduction of MTT by SH-SY5Y-neuroblastoma cells after 12 hours of treatment with the juniper extract (n=3).

4.1.2. LDH assay

The Graph 2. shows, that only in the case of cells treated with the extract concentration 2 mg/ml the amount of created LDH was much higher compared to control, almost 140% of the control. There were almost no differences between the treatments with the other extract concentrations 0,5; 0,1; 0,05 and 0,01 mg/ml. The production of LDH by SH-SY5Y neuroblastoma cells treated with these four extract concentrations was similar to the control.

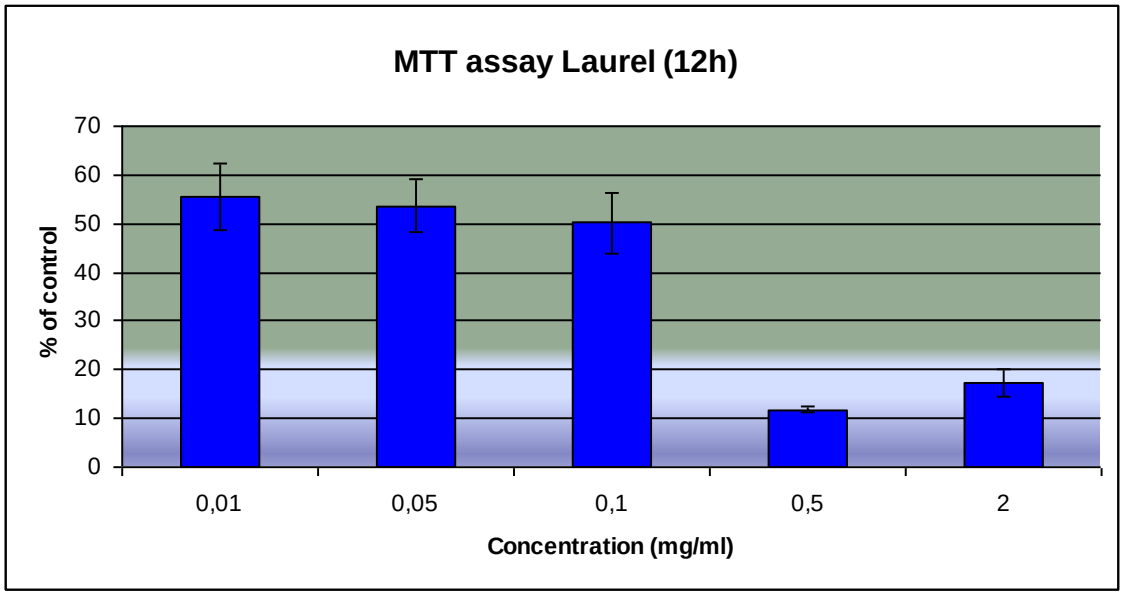


Graph 2. Production of LDH by SH-SY5Y-neuroblastoma cells after 12 hours of treatment with the juniper extract (n=3).

4.2. Laurel extract

4.2.1. MTT assay

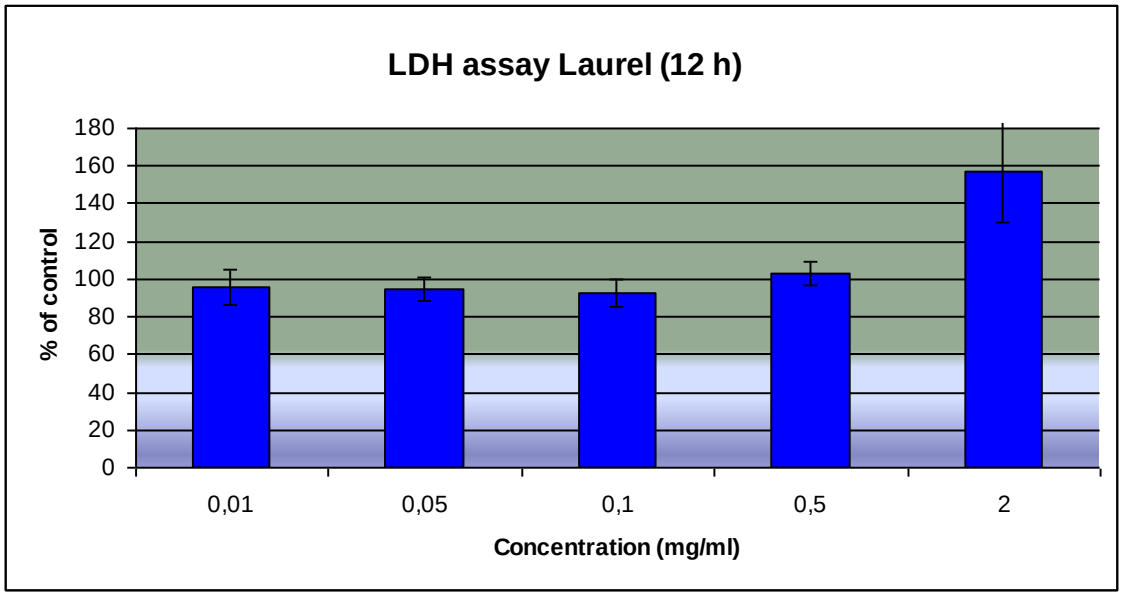
After 12 hours of incubation with the extract, as the Graph 3. shows, significant decrease in MTT reduction was observed in cells treated with the extract concentrations 0,5 and 2 mg/ml. The results did not reach 20% of the control. The results in three other concentrations 0,1; 0,05 and 0,01 mg/ml reached less than 60% of control results.



Graph 3. Reduction of MTT by SH-SY5Y-neuroblastoma cells after 12 hours of treatment with the laurel extract (n=3).

4.2.2. LDH assay

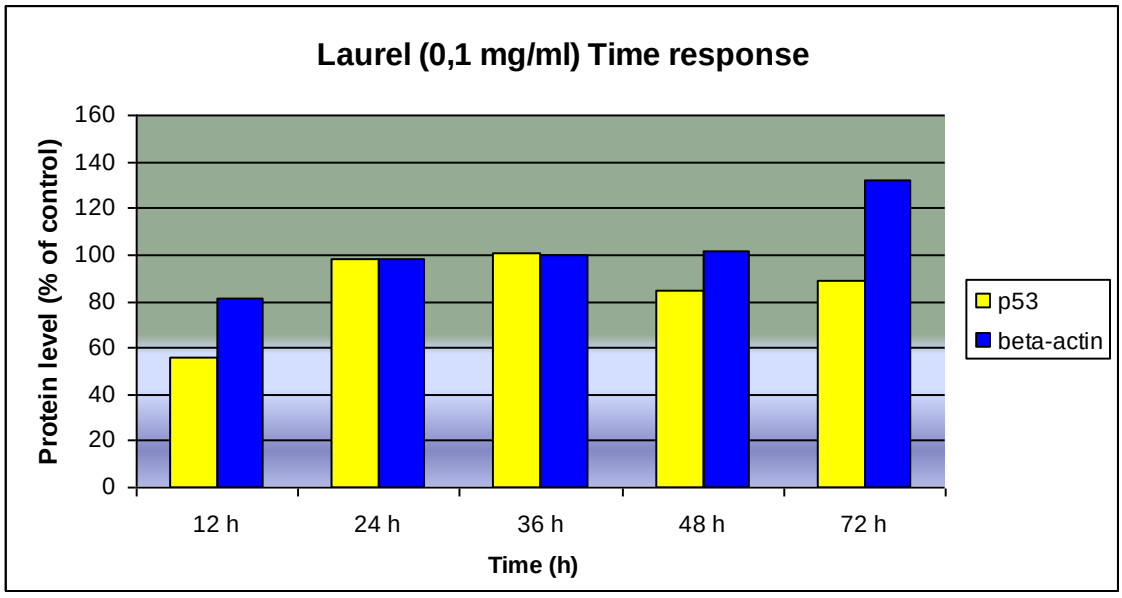
The Graph 4. shows, that only in the case of cells treated with the extract concentration 2 mg/ml the amount of created LDH was much higher compared to control, almost 160% of the control. The cells treated with the other extract concentrations 0,5; 0,1; 0,05 and 0,01 mg/ml did not show significant differences between each other and the amount of created LDH was similar to the control. Only in the case of cells treated with the extract concentration 0,5 mg/ml was the amount of created LDH just a few percent higher than in control.



Graph 4. Production of LDH by SH-SY5Y neuroblastoma cells after 12 hours of the laurel treatment (n=3).

4.2.3. Western blotting analysis

The cells were treated with the extract concentration 0,1 mg/ml for different time periods (time-response experiment), which are shown in the Graph 5. From the graph is possible to see, that the cells treated with the extract even for long time period did not show increased p53 level compared to the control cells. These results come from only one experiment, so more experiments are needed to get reliable results.



Graph 5. Results of Western blotting analysis showing the p53 level in the SH-SY5Y-neuroblastoma cells treated with the laurel extract for different time periods (n=1).

In the Figures 11. to 13. there are pictures of SH-SY5Y neuroblastoma cells. These cells were used in the MTT test and their medium supernatant in the LDH test. These pictures were taken under microscope with the photocamera. In the Figure 11. there are the untreated control cells after 12 hours of incubation. In the Figure 12. there are cells after 12 h of incubation with the laurel extract concentration 0,5 mg/ml. In the Figure 13. there are cells after 12 h of incubation with the laurel extract concentration 2 mg/ml. In the Figures 12. and 13. it is possible to see the morphological changes of treated cells in comparison to the untreated cells in Figure 11. The morphological changes mark the destruction of cells.

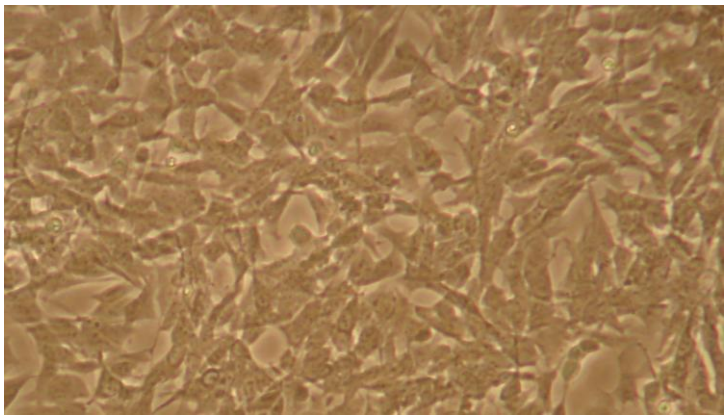


Figure 11. SH-SY5Y neuroblastoma control cells after 12 hours of incubation without extract.

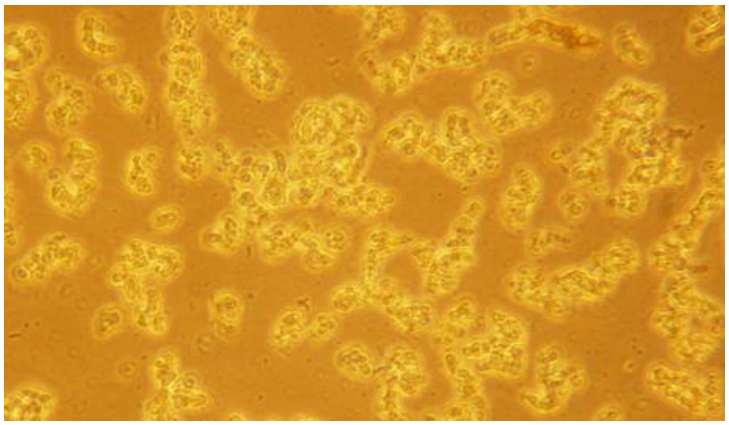


Figure 12. SH-SY5Y neuroblastoma cells after 12h of incubation with the laurel extract concentration 0,5 mg/ml.

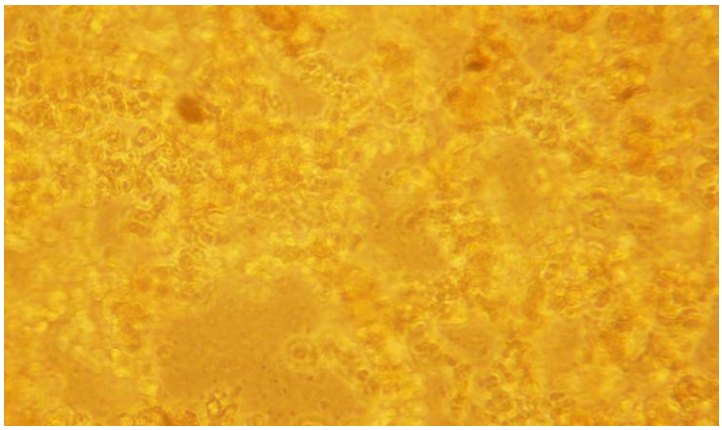
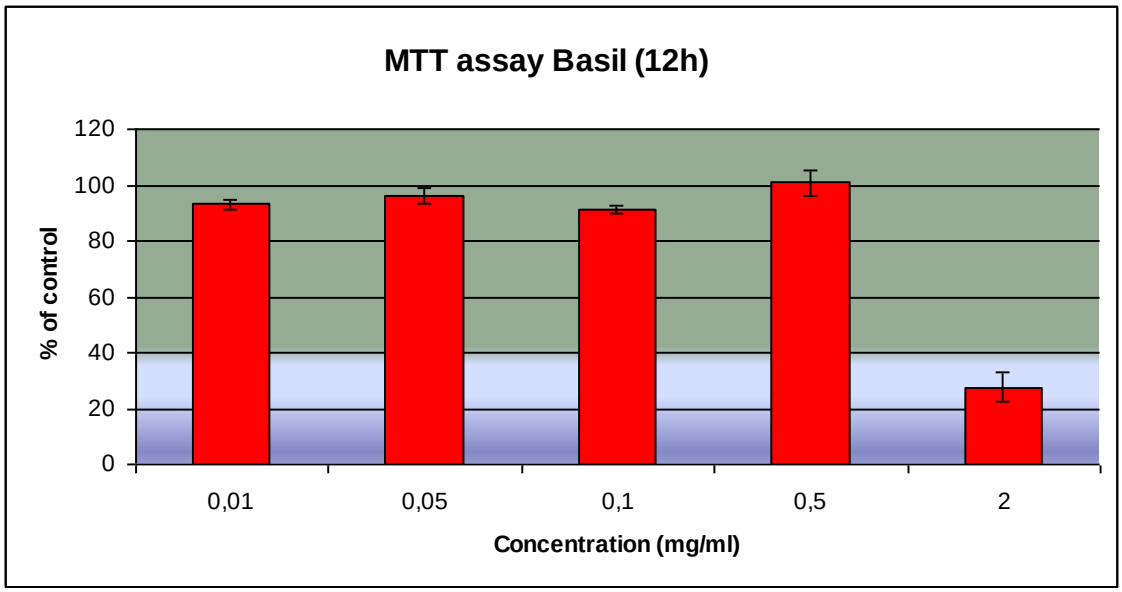


Figure 13. SH-SY5Y neuroblastoma cells after 12 h of incubation with the laurel extract concentration 2 mg/ml.

4.3. Basil extract

4.3.1. MTT assay

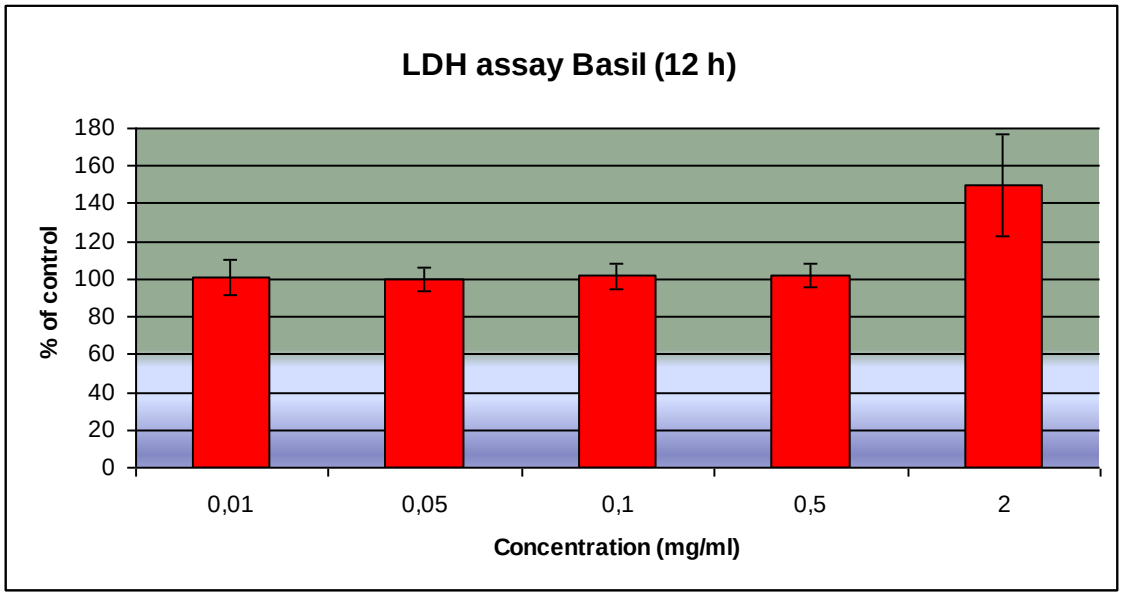
The Graph 6. shows, that 12 hours of incubation with the extract invoked the significant decrease in MTT reduction in cells treated with the extract concentration 2 mg/ml. The results reached about 30% of the control. The cells treated with the other extract concentrations 0,5; 0,1, 0,01 and 0,05 mg/ml did not show big differences between each other and the reduction of MTT was in all four cases similar to the control.



Graph 6. Reduction of MTT by SH-SY5Y neuroblastoma cells after 12 hours of treatment with the basil extract (n=3).

4.3.2. LDH assay

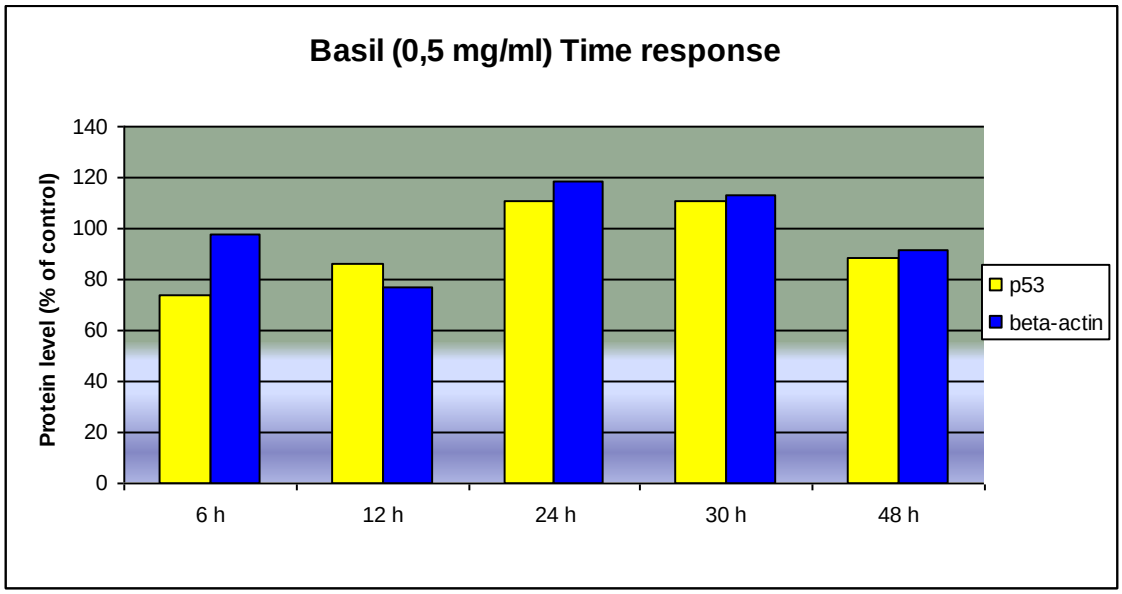
The Graph 7. shows, that only in the case of cells treated with the extract concentration 2 mg/ml the amount of created LDH was much higher compared to control, almost 150% of the control. The cells treated with the other extract concentrations 0,5; 0,1; 0,05 and 0,01 mg/ml did not show significant differences between each other and the amount of created LDH was similar to the control.



Graph 7. Production of LDH by SH-SY5Y neuroblastoma cells after 12 hours of basil extract treatment (n=3).

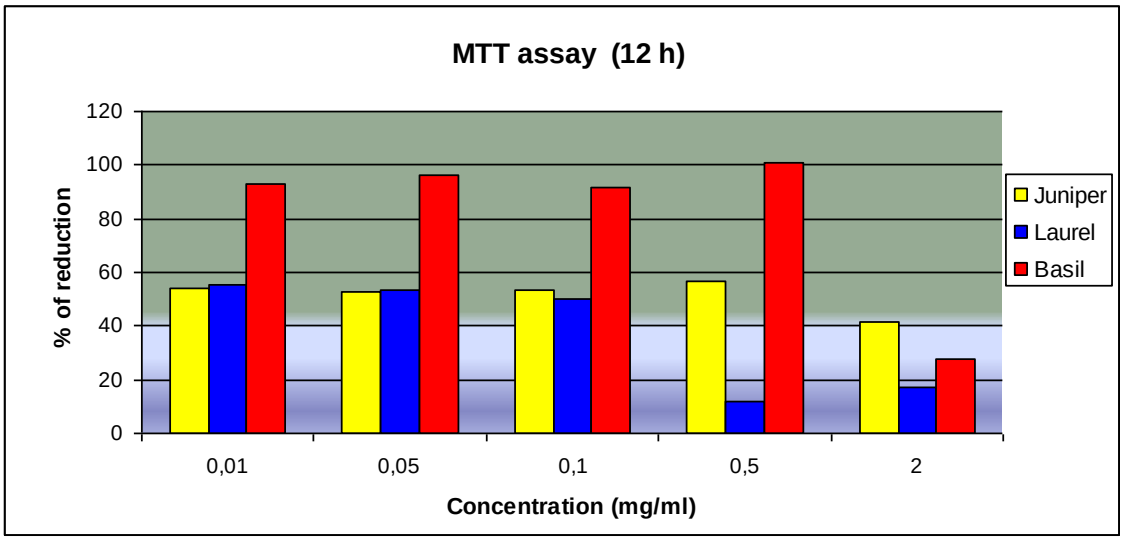
4.3.3. Western blotting analysis

The cells were treated with the extract concentration 0,5 mg/ml for different time periods (time-response experiment), which are marked in the Graph 8. The graph shows, that the cells treated with the extract for 24 and 30 hours embody a little bit higher p53 level compared to control, but the difference is only about 10%. After shorter incubation time with the extract the cell‘s p53 level was lower compared to the control. These results come from just two experiments, so more experiments are necessary to get reliable results.

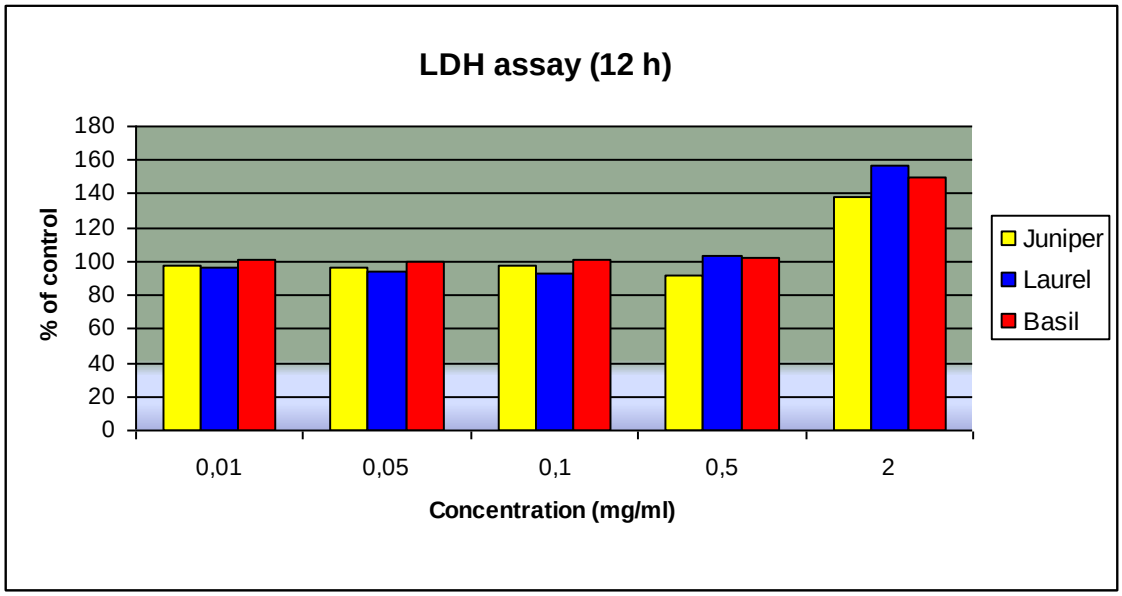


Graph 8. Results of Western blotting analysis showing the p53 level in the SH-SY5Y neuroblastoma cells treated with the basil extract for different time periods (n=2).

In order to compare the effects of juniper, laurel and basil extracts, the results of MTT and LDH assays with all three extracts are shown in the Graph 9. and 10.



Graph 9. Reduction of MTT by SH-SY5Y-neuroblastoma cells after 12 hours of treatment with the juniper, laurel and basil extracts (n=3).



Graph 10. Production of LDH by SH-SY5Y-neuroblastoma cells after 12 hours of treatment with the juniper, laurel and basil extracts (n=3).

5. DISCUSSION

Plant-derived polyphenols and other drug substances are easily approachable, because they occur in high amounts in plants, herbs, fruits and vegetables, which are part of the human diet and which were and still are used against many human diseases in the traditional medicine all over the world (Fresco 2006).

Nowadays, polyphenols are of a big interest in research, because they have many therapeutical properties. Some of these properties are not discovered yet and there are not many information about the discoverd activities. Especially the mechanisms of action are very often missing and are questions for research.

However, the anticancerogenic properties of phenolic compounds are well known. It has been shown cancer to be a largely preventable disease, highly susceptible to modulation by dietary factors (Fresco *et al*. 2006, Yang *et al*. 2001). For example, a diet high in fruits reduces oxidative damage to DNA, which may be one critical step in the onset of some types of cancer. Protective effect of fruits and vegetables intake was proved against lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder, pancreas and ovary cancer (Nichenametla *et al*. 2006). Increased tea consumption was associated with lower risk for breast cancer metastasis and recurrence. Frequent consumption of green tea showed lower incidence of esophageal cancer (Yang *et al*. 2001). The phenolic compounds might affect the molecular events in the initiation, promotion and progression stages of carcinogenesis (Yang *et al*. 2001).

In this work, a cell model was used. The cells were treated with plant extracts from laurel leaves, juniper berries and basil leaves. The effects of plant extracts on cell viability were examined. The effects of laurel and basil on p53 protein level in the SH-SY5Y neuroblastoma cells were examined already earlier (Lantto 2006). It was also shown, that basil essential oil has antioxidant activity (Zheljazkov *et al*. 2008), the major antioxidant compound in basil is rosmarinic acid (Jayasinghe *et al*. 2003) and the antioxidant activity is dose-dependent (Gülçin *et al*. 2007). Basil has antiproliferative effect on mesangial cells (Makino *et al*. 2002) and its extract supresses carcinogenesis (Aruna *et al*. 1990). The antioxidant activity might participate in the effect against cancer, but more experiments are needed to prove this potential property of basil.

The main component of laurel essential oil 1,8-cineole induces apoptosis and supresses the growth of human leukemia cells (Moteki *et al*. 2002). Other important component cinnamtannin B-1 has radical scavenging activity with antioxidant properties. Cinnamtannin B-1 enhances caspase-3 activity and induces apoptosis in the cancer cells, not in the healthy cells (Ben Amor *et al*. 2007, Bouaziz *et al*. 2007). Also polyphenolic flavonol aglycones isoquercetin and kaempferol have antioxidant properties (Soobrattee *et al*. 2005).

Juniper extract from berries and leaves possesses antioxidant activities (Emami *et al*. 2007) and decreases the growth of breast cancer cells, liver and colon carcinoma cells (Bayazit *et al*. 2004, Slambrouck *et al*. 2007).

In this work in the MTT and LDH assay the extracts in the concentrations 0,01; 0,05; 0,1; 0,5 and 2 mg/ml were effecting the cells for 12 hours. In the Western blotting analysis the cells were treated with the laurel extract concentration 0,1 mg/ ml for 12, 24, 36, 48 and 72 hours, and with the basil extract concentration 0,5 mg/ml for 6, 12, 24, 30 and 48 hours.

In the MTT test, a significant effect was observed by all extracts in the concentration 2 mg/ml. In the case of juniper the concentration of created formazan did reach around 40 % of the control, under influence of basil not even 40 % and by laurel less than 20 % of the control. Laurel was very effective also in the concentration 0,5 mg/ml - the activity of reducing enzymes did not reach 15 % of the control. All other concentrations of laurel and juniper extract showed also significant activity with the results reaching less than 60% of the control. It is possible, that longer exposure of the extracts, especially of laurel, could show bigger differences in the results in comparison to the controls.

In the LDH test, the cells treated with all three extracts at concentration 2 mg/ml did show significantly higher amount of the enzyme lactate dehydrogenase and its activity compared to the control. In the case of laurel and basil the reasults reached around 150 % of the control, by juniper a little bit less than 140 % of the control. The cells treated with all three extracts at four other concentrations did not produce significantly more lactate dehydrogenase enzyme than the control cells.

It is possible to see the differences between the MTT and LDH assay results. These dissimilarities might occur, because the principle of MTT and LDH assay vary and various substrates are used to measure the viability of cells. It is also possible, that

mitochondria does not work anymore, which shows MTT test, while there are no changes in the integrity of cell membrane yet, which is shown in the LDH test.

From Western blotting analysis by cells treated with basil extract is possible to see a little bit higher p53 level after 24 and 30 hours of treatment in comparison to control. After 6, 12 and 48 hours the p53 level in cells was lower compared to control. Western blotting analysis with laurel extract does not show any significantly higher p53 levels in cells compared to control, not even after 72 hours of treatment. However, only one experiment was done with the laurel extracts and two with the basil extract. The results are therefore not valid and more experiments are necessary to get reliable results.

Important point of view in all three used methods is the fact, that the enzyme activity and p53 level is measured within small 96-well plate or in small plastic plates, where the cells grow principally isolated and they are not part of the whole organism. Thus, it is very important to implement the in vivo experiments, which would show, how the extracts behave inside the organism. It could be possible to see, if the extracts reach the effective concentrations in the blood after peroral or intravenous application, and how the cells react within the body.

It was also proved, that some substances exude apoptosis in low concentrations, whilst in higher concentrations cause necrosis. It is therefore very important to examine, if apoptosis or necrosis proceeds in the cells, and what can influence the ,,decision‘‘ of the cells for apoptosis.

6. CONCLUSION

From the experiment results on cell models in this study imply, that the plant extracts are effecting the human SH-SY5Y-neuroblastoma cells and their viability. These properties very much depend on the concentration and exposure time. My results show, that the effect is higher with higher concentration and exposure time. All the three extracts or their components embody antioxidant activity, which might in according to previous studies participate on their anticancerogenic properties. It is necessary to examine the extracts on the animal models to further understand the biological effects of these three extracts, especially the mechanism of their activity, their eventuall toxicity in high doses in the organism and eventually to discover their unknown active components.

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8. SUPPLEMENTS

8.1. Time-response experiments with laurel and basil extracts using Western blotting analysis

The bands in Figure 1. (chemiluminiscence picture) show the time-dependent p53 and beta-actin level in the cells treated with the laurel extract concentration 0,1 mg/ml for 12, 24, 36, 48 and 72 hours. The mark c means control, no treatment with the extract. In the Figure 1 there are two pictures, because the samples were transferred into two gels in one experiment (for the case, something would not work in one of the gels).

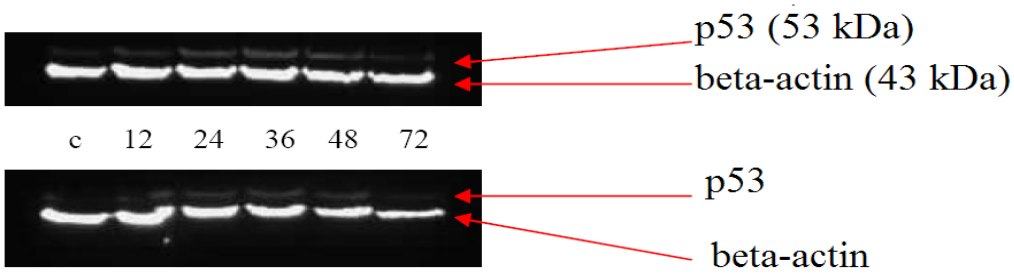

Figure 1. Chemiluminiscence picture of the nitrocellulose membrane, showing the time-dependent p53 and beta-actin level in the SH-SY5Y neuroblastoma cells after treatment with the laurel extract concentration 0,1 mg/ml in the time. The time points were 12, 24, 36, 48 and 72 hours, c means control (0h).

The bands in the Figure 2. (chemiluminiscence picture) show the time-dependent p53 and beta-actin level in the cells treated with the basil extract concentration 0,5 mg/ml for 6, 12, 24, 30 and 48 hours. The mark c means control, no treatment with the extract. In the Figure 2. there are two pictures, because the samples were transferred into two gels in one experiment.

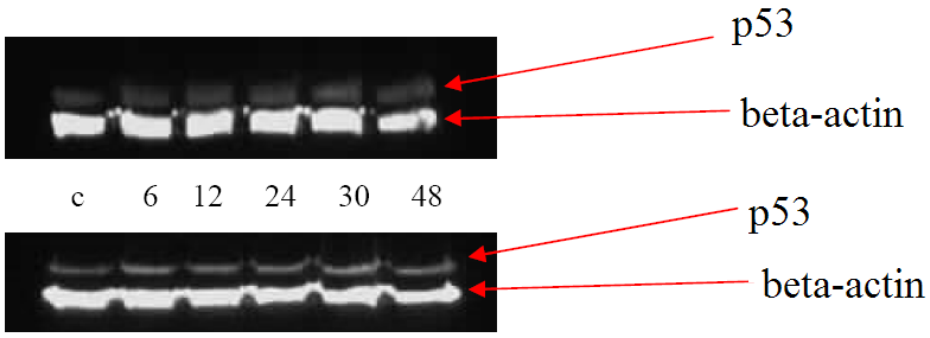

Figure 2. Chemiluminiscence picture of the nitrocellulose membrane, showing the time-dependent p53 and beta-actin level in the SH-SY5Y neuroblastoma cells after treatment with the basil extract concentration 0,5 mg/ml in the time. The time points were 6, 12, 24, 30 and 48 hours, c means control (0h).

9. APPENDIX - SUMMARY IN CZECH

Apoptóza hraje důležitou roli v procesu udržování homeostázy v dospělém organismu. Podílí se na odstraňování nepotřebných, nadbytečných a poškozených buněk a je v rovnováze s mitózou. Nadměrná nebo naopak nedostatečná apoptóza se může projevit jako onemocnění člověka, např. Alzheimerova choroba nebo nádory.

V dnešní době se k léčbě rakoviny užívají především chemická léčiva, která mají hodně nežádoucích účinků. Proto se současná věda zabývá zkoumáním i přírodních látek, jejichž některé účinky jsou známé z užívání v tradiční medicíně a některé potenciálně působí protinádorově. Ve své práci jsem se zabývala působením rostlinných extraktů z vavřínu, jalovce a basalky na životaschopnost lidských SH-SY5Y buněk neuroblastomu.

Existují dva druhy buněčné smrti - apoptóza a nekróza. Nekróza je proces patologický, vyvolaný většinou škodlivými chemickými a fyzikálními faktory, který zpravidla vede k zánětlivé reakci. Apoptóza je fyziologický a geneticky naprogramovaný proces buněčné smrti, který je charakteristický scvrkáváním buněk, chromatinovou kondenzací a tvorbou apoptotických tělísek. Není doprovázena zánětlivou reakcí. Apoptóza je regulována mnoha různými proteiny. Mezi důležité regulátory patří kaspázy, cystein-aspartyl proteázy, které jsou v buňce produkovány jako neaktivni proenzymy, takzvané prokaspázy. Prokaspázy musí být aktivovány proteolytickým štěpením, aby mohly plnit svou funkci. Kaspázy jsou rozděleny do dvou skupin, na efektory a iniciátory. Efektory (nazývané též exekutory), jako jsou kaspáza-3, -6 a -7, spouštějí apoptózu a jejich aktivace vede k typickým morfologickým změnám buněk. K jejich aktivaci jsou nutné iniciátory, jako jsou kaspáza -2, -8, -9 a-10, které jsou schopny aktivovat se samy autolytickou reakcí. Aktivace kaspáz může být dosažena dvěma různými mechanismy, a sice mitochondriální neboli vnitřní cestou a death receptor neboli vnější cestou.

Vnitřní cesta se odehrává v mitochondrii. Po příchodu apoptotického signálu do mitochondrie, je z její vnitřní membrány uvolněn cytochrome c. V cytosolu cytochrome c oligomerizuje a s cytosolickým adaptor proteinem Apaf-1 vytváří komplex zvaný apoptosome. Apoptosome působí na prokaspázu-9, která se autolyticky štěpí na kaspázu-9. Kaspáza-9 pak aktivuje kaspázy-3,-6 a -7.

Death receptory jsou situovány na povrchu buněk a patří do skupiny TNF proteinů. Death receptor cesta je spuštěna při navázání death receptorů (Fas, TNFR-1, DR5) k jejich ligandům (FasL, TNF-alpha, TRAIL), které také patří do skupiny TNF proteinů. Tato vazba vede k oligomerizaci death receptorů, které navázáním adaptorového proteinu FADD vytváří tzv. death-inducing signalling komplex (DISC). DISC aktivuje prokaspázu-8. Prokaspáza-8 se autolyticky štěpí na kaspázu-8, která aktivuje kaspázu-3 a -7. Kaspáza-3 poté štěpí proapototický protein Bid, který se přemístí do mitochondrie. Bid v mitochondrii vyvolává uvolnění cytochromu c, což dále rozvíjí proces apoptózy.

p53 je protein, který inhibuje růst buněk. V nižší koncentraci zastavuje buněčný cyklus ve fázi G1, ve vyšší koncentraci způsobuje apoptózu. p53 působí biologicky jako tumor supressor a biochemicky jako transkripční faktor, který reguluje transkripci cílových proteinů. Aktivuje pro-apoptotické geny (Bax, Puma, Noxa), potlačuje anti-apoptotické geny (Bcl-2) a geny zajišťující přežití buňky (survivin). p53 hraje důležitou roli v mitochondriální i death-receptor apoptotické cestě. Poruchy produkce proteinu p53 mohou vést k různým lidským chorobám. Nadměrná funkce p53 vede např. k autoimunitním chorobám a k neurodegenerativním chorobám. Snížená funkce proteinu p53 může být příčinou vzniku rakoviny. Právě mutace p53 jsou velmi často pozorovány u rakovinných onemocnění.

Mitochondriální apoptotická cesta je regulována pro- a antiapoptotickými členy Bcl-2 skupiny proteinů. Mezi proapoptotické členy, které apoptózu vyvolávají, patří Bid, Bad, Bim, Bax a Bak. Mezi antiapoptotické členy, které apoptózu inhibují, patří Bcl-2 a Bcl-xL. Aktivace nebo inhibice apoptózy závisí na tom, které z faktorů převažují. K dalším důležitým proteinům, které ovlivňují apoptózu patří apoptosis-inducing factor, který po příchodu apoptotického signálu způsobuje chromatinou kondenzaci, a inhibitor of apoptosis protein, který antagonizuje aktivaci kaspázy-3. Důležitý je rovněž Smac/ Diablo protein, který neutralizuje apoptosis-inducing factor. Obě apoptotické cesty se sbíhají na úrovni kaspázy-3, která štěpí další buněčné substráty.

Cytosine arabinoside (AraC) je pyrimidinový antimetabolit, který je v dnešní době hlavní látkou používanou v léčbě akutní leukemie. Jeho přesný mechanismus účinku není známý. Pravděpodobně se inkorporuje do nově syntetizované DNA buňky, způsobuje poškození DNA. Podporuje rovněž tvorbu reaktivního oxygen species, což vede k produkci p53 proteinu a indukci apoptózy. Ovšem AraC indukuje apoptózu

také zdravých buněk v závislosti na čase a dávce. Proto je limitujícím faktorem v užívání AraC při léčbě leukemie jeho hlavní nežádoucí účinek neurotoxicita.

Rostlinné polyfenoly jsou jejich sekundární metabolity, které jsou syntetizovány z phenyalaninu takzvanou šikimátovou syntézou. Vyskytují se ve velkém množství v ovoci, zelenině a bylinách. Mohou být tedy bohatě zastoupeny v potravě člověka. Pro rostliny mají především význam ochranný (před škůdci, fotosyntetickým stresem) a jsou důležité pro jejich růst a rozvoj.

Polyfenoly mají také řadu biologických funkcí, jako jsou antibakteriální, antivirové, protizánětlivé, vazodilatační nebo protirakovinné. Důležité jsou také vlastnosti antioxidační a schopnost shášet volné radikály, které se mohou podílet nejen na protirakovinném účinku, ale také mohou být potenciálně použity v léčbě diabetu nebo kardiovaskulárních onemocnění. Limitujícím faktorem pro klinické užívání rostlinných polyfenolů je jejich velmi nízká biologická dostupnost v krvi, protože procházejí mohutnou metabolizací v lidském trávicím traktu. Polyfenoly jsou velmi rozsáhlou skupinou rostlinných sloučenin. Mezi nejznámější patří flavonoidy, fenolické kyseliny, stilbeny, taniny, lignany a kumaríny.

Bazalka je rostlina patřící do čeledi Lamiaceae. Hlavními obsahovými látkami esenciálního oleje z natě bazalky jsou linalool, methylchavikol, eugenol a methyleugenol. Zastoupení jednotlivých složek závisí zejména na prostředí, ve kterém rostlina roste. U esenciálního oleje byly prokázány účinky antioxidační (hlavní antioxidační látkou je kyselina rozmarinová), antimikrobiální, hepatoprotektivní a karminativní. Důležitý ja také účinek protirakovinný. Byl prokázán např. antiproliferativní účinek na leukemické a epidermální rakovinné buňky.

Vavřín patří do čeledi Lauraceae a hlavními obsahovými látkami jeho listů jsou 1,8-cineol, cinnamtannin B-1 a seskviterpeny. 1,8-cineol indukuje apoptózu a potlačuje růst leukemických buněk v závislosti na čase a dávce. Cinnamtannin B-1 působí silně antioxidačně, sháší volné radikály a zvyšuje aktivitu caspázy-3, čímž indukuje apoptózu, jak bylo zjištěno, pouze v tumorálních buňkách, nikoli v buňkách zdravých. Toto působení zatím nebylo jasně vysvětleno. Také seskviterpeny projevily cytotoxickou aktivitu v lidských leukemických buňkách.

Jalovec patří do čeledi Cupressaceae. Mezi hlavní obsahové látky esenciálního oleje z listů i plodů patří alfa-pinen, beta-pinen a sabinen. Kromě účinků antimikrobiálních a antioxidačních, je důležitý také protirakovinný efekt např. proti jaterním nebo střevním karcinomatickým buňkám.

Vliv extraktů z bazalky, vavřínu a jalovce na apoptózu jsem ve svých experimentech zjišťovala pomocí metod MTT a LDH assay, které zjišťují životaschopnost buněk, a metodou western blotting analýzy.

Výsledky MTT assay udávají množství vzniklého formazanu, který je vytvářen z MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) činností dehydrogenáz a redukujících enzymů v živých buňkách. Množství vytvořeného formazanu se měří spektofotometricky a je principielně úměrné množství živých buněk. Laktát dehydrogenáza (LDH) je enzym, který je produkován poškozenými buňkami. V LDH testu laktát dehydrogenáza přeměňuje laktát na pyruvát, přičemž při této reakci vzniklý NADH katalyzuje přeměnu iodonitrotetrazolia na formazan. Množství vzniklého formazanu je měřeno spektrofotometricky. Cílem western blotting analýzy bylo zjistit hladiny proteinu p53 v buňkách po různých dobách působení extraktů. Ve všech testech byly výsledky experimentů s buňkami, na něž extrakt působil, srovnáván s výsledky experimentů s buňkami, které byly po příslušnou dobu inkubovány, ale na něž extrakt nepůsobil, a které tedy sloužily jako kontrola.

V MTT a LDH testu působily na buňky všechny tři extrakty v koncentraci 2; 0,5; 0,1; 0,05 a 0,01 mg/ml po dobu 12 hodin. U všech tří extraktů v koncentraci 2 mg/ml byl v MTT testu zaznamenán signifikantní pokles redukce MTT. V případě jalovce tvořila redukce MTT 40% kontroly, v případě vavřínu méně než 20 % a u bazalky kolem 30%. Velmi účinná se ukázala být v případě vavřínu také koncentrace 0,5 mg/ml, jejímž působením klesla redukce MTT pod 15% kontroly. Působení ostatních tří koncentrací vavřínu a čtyř koncentrací jalovce způsobily pokles redukce MTT pod 60% kontroly. V případě basalky byl vidět nepatrný rozdíl mezi kontrolou a buňkami, na něž působil extrakt v ostatních čtyřech koncentracích. Ve výsledcích LDH testu je možné pozorovat výsledky částečně podobné. Projevil se opět velký účinek všech tří extraktů v koncentraci 2 mg/ml, kdy množství vytvořeného LDH dosáhlo v případě jalovce skoro 140%, u basalky kolem 150% a u vavřínu skoro 160% kontroly. Jiné koncentrace všech tří extraktů nezpůsobily signifikantní rozdíl v množství vytvořeného LDH ve srovnání s buňkami kontrolními. Rozdíly ve výsledcích MTT a LDH testu jsou dány pravděpodobně tím, že mitochondrie už nefungují, ale zatím nebyla porušena integrita celulární membrány. Rozdíly mohou být způsobeny také rozdílnými principy metod MTT and LDH assay.

Ve Western blotting analýze byly buňky vystaveny působení roztoku vavřínu v koncentraci 0,1 mg/ml po dobu 12, 24, 36, 48 a 72 hodin, a roztoku bazalky

v koncentraci 0,5 mg/ml po dobu 6, 12, 24, 30 a 48 hodin. V případě vavřínu nebyla pozorována zvýšená hladina proteinu p53 oproti kontrole, ani po inkubaci 72 hodin. V případě bazalky byla hladina proteinu p53 mírně zvýšena oproti kontrole pouze po inkubaci 24 a 30 hodin.

Z výsledků mých experimentů vyplývá, že všechny tři extrakty jsou schopny ovlivňovat životaschopnost SH-SY5Y buněk neuroblastomu. Tato schopnost silně závisí na koncentraci extraktu. Se zvýšenou koncentrací extraktu jeho účinek roste. Ve svých experimentech jsem používala v případě MTT a LDH testu inkubační čas pouze 12 hodin. Je možné, že po delší inkubaci s extraktem by mohly být i nižší koncentrace účinnější. Důležitou roli v těchto experimentech hraje poznatek, že buňky jsou živé organismy, které se mohou chovat velmi různorodě podle podmínek, ve kterých se nacházejí a žijí. Tento fakt může mít velký vliv na výsledky experimentů.