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Department of Biology

BIOLOGICAL ACTIVITY OF ANTIOXIDANT COMPOUNDS IN MONOCYTES THP-1

DIPLOMA THESIS

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Rome and Hradec Králové 2010 Jan Hájek

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ABSTRACT

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
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Candidate: Jan Hájek
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Title of diploma thesis: Biological activity of antioxidant compounds in monocytes THP-1

Elaboration of this work is based on the experiments carried out in the Laboratory of Physiology at University Roma Tre in Rome, under the supervision of Prof. Sandra Incerpi. The team of this laboratory studies the antioxidants of different structures and evaluates their ability to prevent the production of reactive oxygen species (ROS). These reactive species are essential for many physiological processes, but they can also have deleterious effects and participate in the development of various diseases. In this work, I dealt with a study of antioxidant properties of different polyphenolic compounds (mosloflavone, negletein, 5,6-dihydroxyflavone, baicalein, gallic acid, and compound 1625) in the cell culture of human monocytes THP-1 and also in the *in vitro* test. Three different methods were used: intracellular ROS determination (dichlorofluorescein assay), cytotoxicity assay, and electron paramagnetic resonance spectroscopy (EPR). Mosloflavone showed scavenging ability neither in the cell culture nor during EPR. On the other hand, negletein possessed good scavenging ability in EPR but in the cell culture behaved as pro-oxidant at the concentration 10^{-5} M. Baicalein and 5,6-dihydroxyflavone exerted significant ROS scavenging ability in all three assays. Gallic acid was able to inhibit ROS production only at higher concentrations (10^{-5}-10^{-6} M), but due to its hydrophilic character is not capable of crossing the cell membrane. By contrast, its lipophilic derivative compound 1625, which proved to have comparable antioxidant ability as gallic acid, can cross the cell membrane and thus may serve as a transport form of gallic acid to the cells.

ABSTRAKT

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Název diplomové práce: Biologická aktivita antioxidačních látek v monocytech THP-1

Tato práce byla vytvořena na základě pokusů provedených v Laboratoři fyziologie na Univerzitě Roma Tre v Římě pod vedením prof. Sandry Incerpi. Tým laboratoře se zabývá studiem antioxidantů s různou strukturou a hodnocením jejich schopnosti zabránit tvorbě reaktivních forem kyslíku (ROS). Tyto reaktivní sloučeniny jsou nezbytné v mnoha fyziologických pochodech, ale mohou mít také škodlivé účinky a podílet se na rozvoji rozličných onemocnění. V této práci jsem se zabýval studiem antioxidačních vlastností různých polyfenolických látek (mosloflavon, negletein, 5,6-dihydroxyflavon, bajkalein, kyselina galová a sloučenina 1625) v buněčné kultuře lidských monocytů THP-1 a také v *in vitro* testu. Použil jsem tři různé metody: stanovení intracelulárních ROS (dichlorofluoresceinový test), test cytotoxicity a elektronovou paramagnetickou rezonanční spektroskopii (EPR). Mosloflavon neukázal žádnou vychytávací aktivitu ani v buněčné linii ani během EPR. Naproti tomu negletein měl dobrou vychytávací aktivitu při EPR, ale v buněčné linii se při koncentraci 10^{-5} M choval jako pro-oxidant. Bajkalein a 5,6-dihydroxyflavon projevily značnou schopnost vychytávat ROS ve všech třech metodách. Kyselina galová byla schopna inhibovat vznik ROS pouze ve vyšších koncentracích (10^{-5}-10^{-6} M), ale díky jejímu hydrofilnímu charakteru není schopna projít přes buněčnou membránu. Naproti tomu její lipofilní derivát sloučenina 1625, která měla srovnatelnou antioxidační aktivitu s kyselinou galovou, může projít přes buněčnou membránu a tak by mohla sloužit jako transportní forma kyseliny galové do buněk.

CONTENTS

1 INTRODUCTION

This work was carried out in the Laboratory of Physiology, Department of Biology, University Roma Tre under the supervision of Prof. Sandra Incerpi. The team of this laboratory deals with the evaluation of antioxidant activity of various polyphenols and their ability to prevent the production of reactive oxygen species (ROS).

Reactive oxygen species (ROS) commonly occur in human body. They are essentially for many physiological processes (e.g. as a first line of protection against pathogens) but under certain circumstances they can also cause serious damage to biomolecules. That is why the cells are provided by some protective mechanisms that decrease level of ROS and maintain redox homeostasis. If this balance is affected, ROS can irreversibly attack biomolecules and the cells may lose or change their functions or even die. This imbalance leads to the oxidative stress and oxidative stress-related diseases.

The team of prof. Incerpi tries to find an antioxidant, which would have a high activity to suppress the increase of ROS *in vitro* or in cell cultures. Production of ROS is during the experiments induced by cumene hydroperoxide or galvinoxyl (radical generators). This work was focused on the antioxidant properties of mosloflavone, negletein, 5,6-dihydroxyflavone, baicalein, gallic acid, and its derivative 1625 in a cell line of human monocytes THP-1 as well as in the *in vitro* experiments (EPR spectroscopy).

2 GENERAL PART

Free radicals, oxygen and nitrogen reactive species, oxidative stress, and also antioxidants are terms often connected with many diseases and everything has evolved from a simple molecule of oxygen or nitrogen, respectively.

2.1 OXYGEN

During evolution of aerobic life, our bioenergetics scheme has become completely dependent on the ability of oxygen to accept electrons, which is the only possibility how to make energy (McCord 2000). Nevertheless, oxygen is also responsible for generation of reactive oxygen species that may cause oxidative stress and development of various diseases.

2.1.1 HISTORY OF THE OXYGEN

The oxygen appeared in significant amounts in the atmosphere of Earth over 2.2 billion years ago due to the evolution of photosynthesis, when it was released into the atmosphere. This air pollution overgrew into a situation, when the concentration of O_2 in the atmosphere rose and enabled the formation of the ozone (O_3) layer in the stratosphere that protected living organisms from UV radiation, so the organisms could leave the sea and colonize land (Lane 2002, Halliwell 2006).

Nowadays, life on our planet has evolved into two main categories of life-forms. The first group constitutes of plants that capture solar energy and use it to make thermodynamically unfavorable reactions producing reduced carbonaceous compounds. The second group represents the rest of us, who eat these carbonaceous compounds and burn them in thermodynamically favorable reactions. The later reactions allow the hydrogen atoms from plants to join with O_2 from the air while releasing a large amount of utilizable energy (McCord 2000).

2.1.2 OXYGEN AS A MOLECULE

The molecule of O_2 that contains two unpaired electrons occupying two separated orbitals is called ground-state oxygen, which is the most stable state of oxygen molecule. In contrast, all the electrons in stable organic molecules are arranged in pairs with antiparallel spin states (*Fig. 1*). This unconventional distribution of electrons makes it impossible to accept a spin-matched pair of electrons and this spin restriction (*Fig. 2*) saves our lives from reacting explosively with an atmosphere and also makes

oxygen an ideal terminal electron acceptor for biological systems (Fridovich 1995, McCord 2000, Halliwell 2006).

The singlet oxygen, more reactive form of O_2, can be generated by an input of energy that rearranges the electrons and spin restriction is removed (Foote et al. 1985). One-electron reduction product is the superoxide radical ($O_2^{\bullet -}$) and when two electrons are transferred, then the product is called peroxide ion (*Fig. 1*) (McCord 2000).

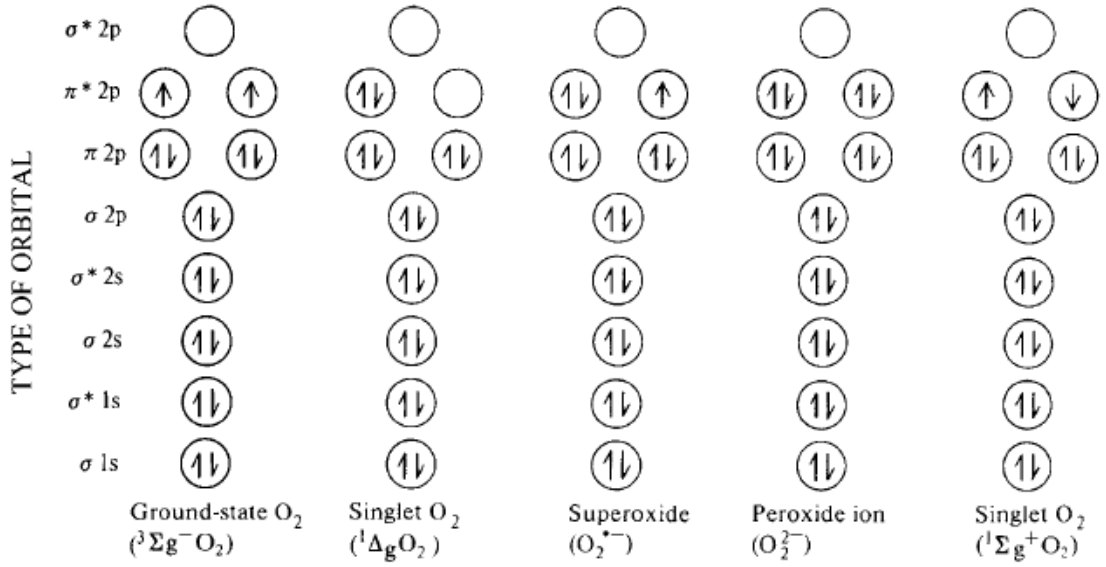


Fig. 1: A simplified version of bonding in the diatomic oxygen molecule and its derivatives (Halliwell 2006).

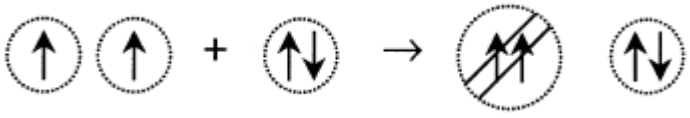


Fig. 2: A reaction between parallel electrons of oxygen and electrons of any stable compound cannot proceed due to a spin restriction (Fridovich 1999).

2.2 FREE RADICALS

Electrons are more stable, when are paired together. That is why the radicals are more reactive compounds. Free radicals can be generated in the initiation phase (eq. 1) as the products of homolytic, heterolytic as well as redox reaction. In the propagation phase (eq. 2), a radical might donate its unpaired electron or it might withdraw an electron from another molecule, which becomes a radical. The chain reaction is

completed by termination phase (eq. 3), where two radicals can combine their unpaired electrons to form a covalent bond and they share this pair of electrons (Halliwell 1989).

1) $A \rightarrow A^{\bullet} + A^{\bullet}$

2) $A + A^{\bullet} \rightarrow A - A^{\bullet}$

$A + A - A^{\bullet} \rightarrow A - A - A^{\bullet}$

3) $A - A - A^{\bullet} + A - A - A^{\bullet} \rightarrow A - A - A - A - A - A$

In the cells, the primary generated free radicals are superoxide and nitric oxide ($NO^{\bullet}$). Both can readily react to form a series of other reactive oxygen and nitrogen species, respectively (Halliwell 1989, Powers and Jackson 2008).

2.3 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are tiny molecules (also called reactive oxygen metabolites or active oxygen) and belong to a family of reactive intermediates resulting from the incomplete reduction of oxygen (McCord 2000, Bedard and Krause 2007).

2.3.1 SOURCES OF ROS

Source of ROS is any electron-transferring protein or enzymatic system that can result in the formation of ROS. The main producer of ROS (approximately 98% O_2 we metabolize) is mitochondrial cytochrome oxidase, a multiprotein complex that transfers four electrons to the molecule of oxygen and makes two molecules of water in several reduction steps. It consists of two hems and two copper ions, which are able to store a single electron each. Considering the isolation of this electron-transport is not perfect, at least two sites (Complex I and ubisemiquinone) where electrons may leak out and result in the formation of superoxide have been identified. It has been estimated that this leakage represents about 1% to 2% of total electron flux through the mitochondria (*Fig. 3*) (Babcock 1999, McCord 2000, Valko 2007).

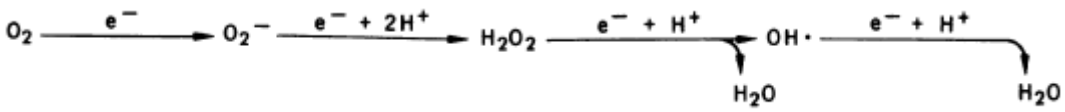


Fig. 3: The univalent pathway of oxygen reduction (Fridovich 1978).

The endoplasmic reticulum (ER) is another membrane-bound intracellular organelle that contains enzymes detoxifying lipid-soluble drugs and other metabolic products. They can also oxidize unsaturated acids and xenobiotics and reduce molecular O_2 to produce $O_2^{\bullet-}$ (Aust et al. 1972, Thannickal and Fanburg 2000).

Peroxisomes are other important source of ROS. They consist of many oxidative enzymes generating H_2O_2 (not $O_2^{\bullet-}$) to eliminate toxic molecules in liver and kidneys (Tolberts and Essner 1981) or to oxidize fatty acids (β-oxidation) (Alberts et al. 1994).

Intracellular membrane-associated oxidases such as aldehyde oxidase, tryptophan dioxygenase, flavoprotein dehydrogenase, dihydroorotate dehydrogenase and the most important xanthine oxidase also produce $O_2^{\bullet-}$ (Freeman and Crapo 1982).

Autoxidation of small molecules such as a dopamine, adrenaline, flavine, and hydroquinones may be a source of ROS as well (Freeman and Crapo 1982).

Plasma membrane-oxidases have been implicated as the sources of most growth factor and/or cytokine-stimulating oxidant production (Thannickal and Fanburg 1995), although the precise enzymatic sources have yet to be fully characterized. Among them, the best characterized enzyme is a phagocytic NADPH oxidase, a multicomponent enzyme that catalyzes one-electron reduction of O_2 to $O_2^{\bullet-}$. This enzyme plays a specialized function in host defense against invading microorganisms - respiratory burst. It has been suggested that NADPH oxidase is present even in non-phagocytic cells (Babior 1999, Thannickal and Fanburg 2000).

Ultraviolet light, atmospheric pollutants, such as ozone and nitrogen dioxide, cigarette smoke and also various xenobiotics are exogenous causes of ROS production (Young and Woodside 2001).

The major sources of ROS and the possible targets of their damage are mentioned in *Fig. 4*.

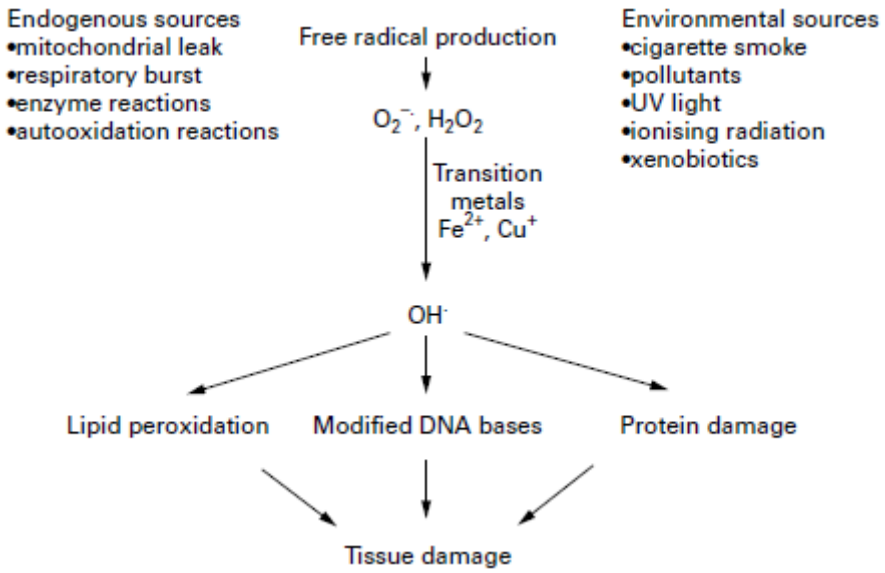


Fig. 4: Summary of major ROS sources and their potential damages (Young and Woodside 2001).

2.3.2 MAIN REPRESENTATIVES OF ROS

The most important representatives of ROS family are superoxide, hydrogen peroxide, hydroxyl radical and hypochlorous acid.

Superoxide – **$O_2^{\bullet-}$** is relatively membrane impermeable, because it is negatively charged and has a long half-life that enables its diffusion within the cell. This may be a cause of increasing number of potential targets. Although $O_2^{\bullet-}$ is generally considered quite unreactive compared to other ROS, it can react rapidly with $NO^{\bullet}$ and with iron-sulfur clusters in some proteins (Halliwell and Gutteridge 2007). Pathological production of $O_2^{\bullet-}$ may occur for example during the first part of oxygen reduction to water (eq. 4), which was already mentioned (Halliwell 1989, Powers and Jackson 2008).

4) $$O_2 + e^- \rightarrow O_2^{\bullet-}$$

Hydrogen peroxide – **H_2O_2** is a stable non-radical with relatively long half-life within the cell, which is able to permeate through the membranes. It is a weak oxidizing agent, which cannot oxidize DNA and lipids (Halliwell and Gutteridge 2007). The cytotoxicity of H_2O_2 occurs through its ability to generate hydroxyl radical in Fenton reaction (eq. 5) and Haber-Weiss reaction (eq. 6), which combines the Fenton reaction

and the reduction of Fe^{3+} by superoxide (eq. 6a and 6b). It occurs pathologically for example after damage to peroxisomes (Valko 2007, Powers and Jackson 2008).

5) $$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

6) $$O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe^{2+}/Cu^{+}} O_2 + {}^{\bullet}OH + OH^-$$

6a) $$Fe^{3+}/Cu^{2+} + O_2^{\bullet -} \rightarrow Fe^{2+}/Cu^{2+} + O_2$$

6b) $$H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow Fe^{3+}/Cu^{2+} + {}^{\bullet}OH + OH^-$$

Hydroxyl radical – **$^{\bullet}$OH** is a highly reactive compound with a strong oxidizing potential and that is why it is not membrane permeable. It is potentially the most damaging ROS present in biological materials. It can attack almost every molecule found in living cell with incredible speed. The best characterized biological damage caused by $^{\bullet}$OH is lipid peroxidation (chain reaction during which are formed alkoxyl and peroxyl radicals), when hydroxyl radical is generated close to a membrane and it damages the fatty acids of phospholipid bilayer. It can occur as a product of Fenton reaction (eq. 5) or when the tissues are exposed to gamma radiation. Radiation causes the splitting of oxygen-hydrogen bond in water molecule and creates two radicals (eq. 7). This is the major radiotherapy mechanism, by which malignant cells are killed (Halliwell 1989, Powers and Jackson 2008).

7) $$H - O - H \xrightarrow{\gamma} \underset{\text{stages}}{\text{intermediate}} \rightarrow H^{\bullet} + {}^{\bullet}OH$$

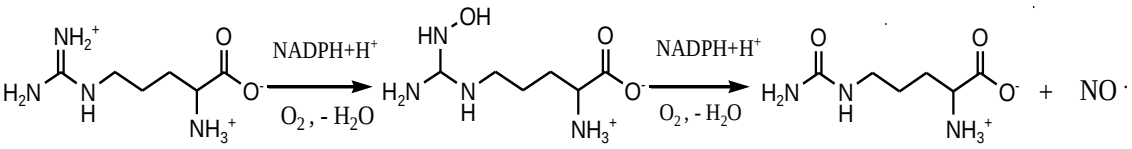
Hypochlorite – **HOCl** (**hypochlorous acid**) is predominantly formed in neutrophils by the action of myeloperoxidase utilizing H_2O_2 and chloride (eq. 8). It is powerful antibacterial agent, but it may also oxidize lipids, thiols and ascorbate and damage these molecules. This oxidant can cross the membranes as well and thus cause fragmentation and aggregation of proteins (Halliwell 1989, Halliwell and Gutteridge 2007, Powers and Jackson 2008).

8) $$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} HOCl + OH^-$$

2.4 REACTIVE NITROGEN SPECIES

Reactive nitrogen species (RNS) contain as central atom nitrogen instead of oxygen and include mainly nitric oxide and peroxynitrite.

Nitric oxide – **$NO^{\bullet}$** is a small molecule that contains one unpaired electron, and thus is the radical. $NO^{\bullet}$ is synthesized from the amino acid L-arginine by many cells, through several specific types of nitric oxide synthases (NOS) (*Fig. 5*). There are three types of NOS: NOS1 (neuronal), NOS3 (endothelial) and NOS2 (inducible). These enzymes are denominated according to the tissue, from which they were isolated for the first time, but they are present in more cell types. $NO^{\bullet}$ plays an important signaling role in a large variety of physiological processes. This radical is able to regulate blood pressure through binding of ferrous ion in guanylate cyclase, which is activated and produces cGMP. It also takes part in neurotransmission, smooth muscle relaxation, immune regulation and various defense mechanisms (Bergendi et al. 1999). Nitric oxide reacts with oxygen to form nitric dioxide and reacts very rapidly with superoxide to produce peroxynitrite (Valko 2007, Powers and Jackson 2008).



L-arginine L-citrulline

Fig. 5: Generation of $NO^{\bullet}$ by NOS utilizing NADPH and arginine via a five electron oxidative reaction (Ghafourifar and Cadenas 2005).

Peroxynitrite – **$ONOO^-$** is a product of cells of immune system during oxidative burst, when these cells generate both needed radicals – $NO^{\bullet}$ and $O_2^{\bullet-}$ (eq. 9). This strong oxidizing agent causes DNA fragmentation, lipid oxidation, and protein nitration and can lead to depletion of thiol groups. At physiological pH, $ONOO^-$ rapidly protonates to peroxynitrous acid (ONOOH), which may undergo homolytic fission (eq. 10). Peroxynitrite may also react with CO_2 (eq. 11a and 11b). Products of these reactions are powerful oxidizing agents too (Halliwell 2006, Valko 2007, Powers and Jackson 2008).

9) $$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$$

10) $$ONOOH \rightarrow NO_2^{\bullet} + {}^{\bullet}OH$$

11a) $ONOO^- + CO_2 \rightarrow ONOOCOO^-$

11b) $ONOOCOO^- \rightarrow NO_2^{\bullet} + CO_3^{\bullet -}$

The main reactive oxygen and nitrogen species (RONS) are summarized in *Tab. 1* and the physiological processes controlled by $NO^{\bullet}$ and $O_2^{\bullet -}$ are mentioned in *Tab. 2*.

Tab. 1. Reactive oxygen species and reactive nitrogen species (Halliwell 1996).

Radicals			Nonradicals	
ROS	Superoxide	$O_2^{\bullet -}$	Hydrogen peroxide	H_2O_2
	Hydroxyl	$OH^{\bullet}$	Hypochlorous acid	HOCl
	Peroxyl	$RO_2^{\bullet}$	Ozone	O_3
	Alkoxyl	$RO^{\bullet}$	Singlet oxygen	$^1\Delta g$
	Hydroperoxyl	$HO_2^{\bullet}$		
RNS	Nitric oxide	$NO^{\bullet}$	Nitrous acid	HNO_2
	Nitrogen dioxide	$NO_2^{\bullet}$	Dinitrogen tetroxide	N_2O_4
			Dinitrogen trioxide	N_2O_3
			Peroxynitrite	$ONOO^-$
			Peroxinitrous acid	ONOOH
			Nitronium cation	NO_2^+
			Alkyl peroxynitrites	ROONO

Tab. 2. Physiological processes summary of radicals $NO^{\bullet}$ and $O_2^{\bullet -}$ (Dröge 2002).

Type of radical	Source of radical	Physiology process
Nitro oxide	Nitric oxide synthase	Smooth muscle relaxation (control of vascular tone) and various other cGMP-dependent functions.
Superoxide and related ROS	NAD(P)H oxidase	Control of ventilation. Smooth muscle relaxation. Control of erythropoietin production. Signal transduction from various membrane receptors/enhancement of immunological functions.
Superoxide and related ROS	Any source	Oxidative stress responses and maintenance of redox homeostasis.

The preceding paragraphs give us information about the physiological roles of RONS as well as their harmful activity, which led to the development of some defense

mechanisms during evolution preventing us from accidental leakage of RONS, i. e. maintaining redox homeostasis.

2.5 ANTIOXIDANTS

One of the before mentioned defense mechanisms against RONS detrimental oxidative effect is antioxidant defense system. Antioxidants (AOX) are substances that either prevent the formation or react with and eliminate RONS before they could damage various cell components (McCord 2000, Young and Woodside 2001, Valko 2007) and so they contribute to maintaining redox homeostasis. Various authors report distinct classification of antioxidants. The classification used in this work is taken from Powers and Jackson (2008): dietary (exogenous) antioxidants, the antioxidant enzymes and endogenous non-enzymatic antioxidants.

2.5.1 DIETARY (EXOGENOUS) ANTIOXIDANTS

This work is focused mainly on polyphenols of different structure (mostly flavonoids) and that is why the classification of antioxidants begins with dietary antioxidants.

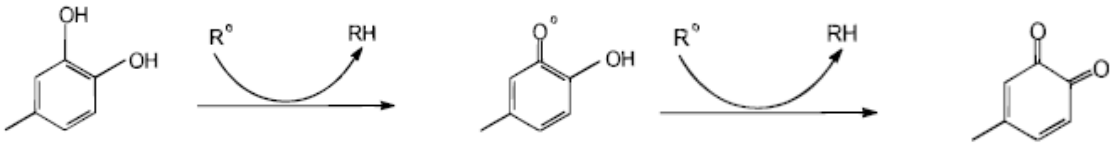
2.5.1.1 POLYPHENOLIC COMPOUNDS

Polyphenols are the most abundant exogenous antioxidants, which are widespread in all plants (D´Archivio et al. 2007) and often at high levels. According to Manach et al. (2004), polyphenols include flavonoids, phenolic acids, lignans and stilbenes, while Pietta (2000) classifies them into flavonoids, phenols and phenolic acids, tannins and lignans.

Flavonoids are naturally occurring phenolic compounds that are contained in various fruits and vegetables, but also in the seeds, nuts, grains, spices, medicinal plants and in beverages (red wine, tea and beer) (Kuhnau 1976). Flavonoid intake can range between 50 – 800 mg/day in the human diet. Their physiological functions in plants are: a visual signal for pollinating insect due to their colors, a defense system against insects due to their astringency, catalysts in the light phase of photosynthesis, regulation of iron channels, protection from UV radiation and of course scavenging of ROS. They have

been studied extensively in last decades for this ability to scavenge ROS (Pietta 2000, Firuzi 2005).

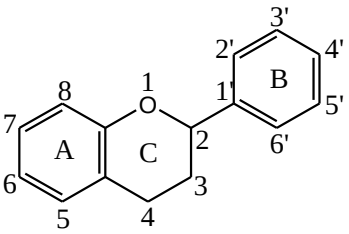
Flavonoids inhibit most of the enzymes responsible for ROS production and a number of flavonoids efficiently chelate trace metals that are potential enhancers of ROS formation. They are also able to reduce free radicals (superoxide anion, peroxyl, alkoxyl and hydroxyl radical), while flavonoids are changed into aroxyl radicals. Aroxyl radical may react with the other radical and becomes a stable quinone structure (*Fig. 6*). Nevertheless, there is also possible side reaction of the aroxyl radical, which could interact with oxygen generating quinone and superoxide anion. This reaction can occur in the presence of high levels of transition metal ions and is responsible for the undesired pro-oxidative effect of flavonoids (McCord 1995). Aroxyl may oxidize ascorbate, which in turn is generated by glutathione. This could be a reason, why plasma ascorbate and total glutathione decrease transiently (Pietta et al. 1998). Some flavonoids are able to regenerate α-tocopherol from its radical form. Flavonoids may stabilize free radicals involved in oxidative processes by complexing with them as well (Pietta 2000).



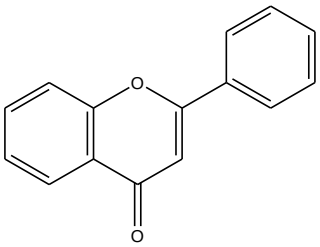
flavonoid aroxyl radical quinone

Fig. 6: Flavonoids with two radicals (R•) make a stable quinone (Pietta 2000).

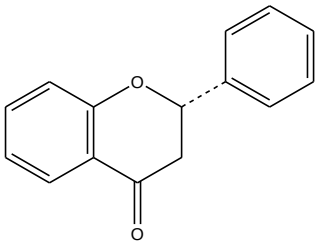
Flavonoids are formed from the aromatic amino acid phenylalanine or tyrosine together with malonate (Harborne 1986). The basic flavonoid structure is the flavan nucleus *(Fig. 7).* The various classes of flavonoids (*Fig. 7*) differ in the substitution of heterocyclic ring C, while individual compounds within a class differ in the substitution of aromatic rings A and B (Pietta 2000). The important structure-activity relationship of the antioxidant activity has been established.



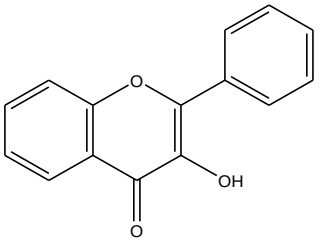
Flavan nucleus – basic flavonoid structure



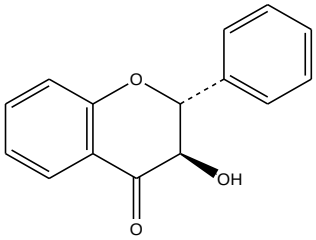
Flavones



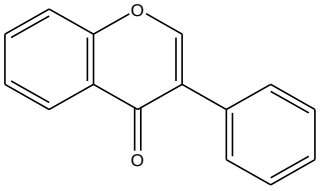
Flavanones



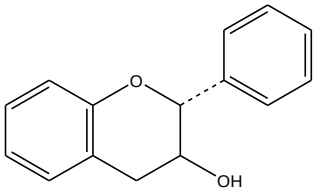
Flavonol



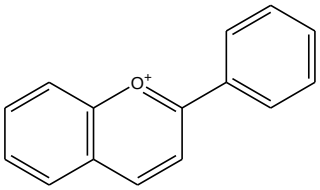
Flavanonol



Isoflavones



Flavan-3-ols



Flavylium salts

Fig. 7: Classes of flavonoids (Pietta 2000).

The presence of 2,3-double bond conjugated with 4-oxo group and catechol group in the ring B are the major determinants of increasing radical scavenging capability of the flavonoids, 3-hydroxyl group in the ring B or 5- and/or 7-hydroxyl/methoxyl group seem to be less important. An additional hydroxyl group in the ring B also enhances the antioxidant activity. In the case of isoflavones, the location of ring B at the position 3 of the heterocyclic ring greatly affects the radical scavenging capacity. On the other hand, the presence of only one hydroxyl group in the ring B or glycosylation of 3-hydroxyl group diminishes the activity of flavonoid (Pietta 2000).

Despite the increasing evidence for the *in vitro* antioxidant potential of flavonoids, little is known about their efficacy *in vivo*. The flavonoids (generally polyphenols) must overcome following barriers to become bioavailable: solubility, permeability, metabolism, excretion, target tissue uptake, and disposition (*Fig. 8*). Most of flavonoid glycosides, which are more hydrophilic, are initially hydrolyzed to their less hydrophilic aglycones. Nevertheless, these pure aglycones have poor solubility, which can result in slow dissolution rates and thus retard their absorption. Food composition plays an important factor that affects availability of flavonoids, e. g. alcohol and fats increase their bioavailability (Serafini et al. 1997), while protein diet decreases their availability probably due to the binding of polyphenols to the proteins (Ichikawa et al. 1992). The flavonoids that cross the intestinal membrane (only small portion) are partly transformed or metabolized by the liver enzymes into corresponding glucuronides and sulfate conjugates and further excreted into the urine or returned to the duodenum via the gallbladder. Transport of conjugates out of the liver and the intestines has been studied recently, but there is evidence suggesting that a variety of transporters may be involved in the transport of these conjugates in and out of cells. The major part of ingested flavonoids, which is not absorbed, is largely degraded by the intestinal microflora into phenolic acids. These phenolic acids can be reabsorbed; they may enter into the circulation and contribute to antioxidant protection of the body. Dietary flavonoids may display their first antioxidant defense in the digestive tract (Pietta 2000, Ming 2007).

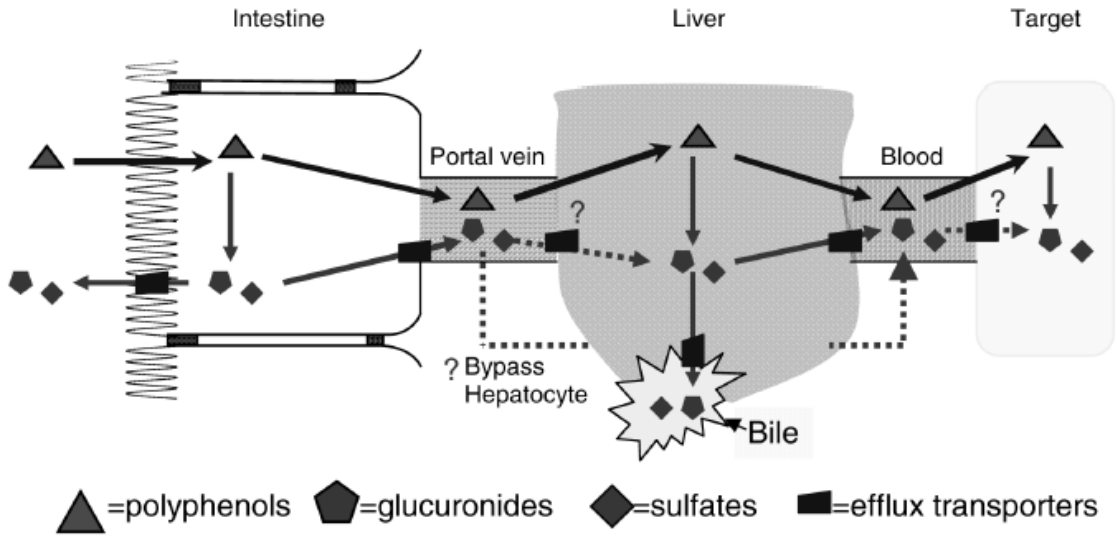
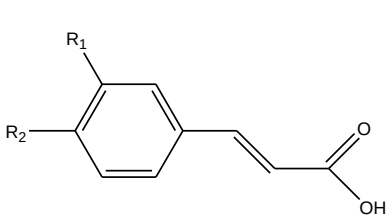
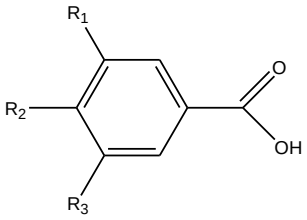


Fig. 8: Organ bioavailability barriers to polyphenols, ? = unknown mechanisms (Ming 2007).

Phenolic acids could be divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid *(Fig. 9).*



derivatives of cinnamic acid



derivatives of benzoic acid

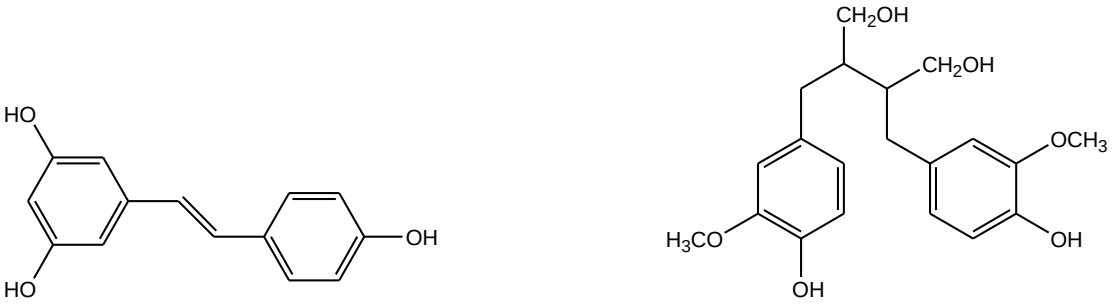
Fig. 9: Classes of phenolic acids (Manach et al. 2004)

Gallic acid (**GA, 3,4,5-trihydroxybenzoic acid**) and its derivative 1625 are the representatives of phenolic acids tested in this work. This type of polyphenols is ubiquitous in the plant kingdom and represents a large family of plant secondary polyphenolic metabolites. GA is found in three forms: methylated GA, galloyl conjugates of catechin derivatives, and polygalloyl esters (the last two groups are also called tannins), which are found in tea leaves, red berries (strawberries, raspberries and blackberries), black radish and onion together with protocatechuic acid, other representative of hydroxybenzoic acids (Manach et al. 2004, Lu et al. 2006).

The mechanism of GA radical scavenging effect (forming the stable quinone after exposure to the radicals) is the same as in the case of flavonoids (*Fig. 6*). GA has also other functions including antidiabetic activity or induction of apoptosis in cancer cells. The major GA metabolites are its methylated, decarboxylated or dehydroxylated derivatives (Lu et al. 2006).

The hydroxycinnamic acids (e.g. caffeic, ferulic and coumaric acid) are rarely found in the free form. They are usually present as glycosylated derivatives or esters. Caffeic acid is present in most of the fruits and ferulic acid can be found in cereal grains (D´Archivio et al. 2007).

Lignans (*Fig. 10*), formed by two phenylpropane units, are mostly found in linseed as well as in other cereals, fruits and certain vegetables. According to Manach et al. (2004), the last representatives of polyphenols are **stilbenes** (*Fig. 10*), which occur naturally in low quantities in wine. Their typical representative resveratrol has anticarcinogenic effects (Manach et al. 2004).



Stilbenes – resveratrol Lignans – secoisolariciresinol

Fig. 10: Structure of stilbenes and lignans (Manach et al. 2004)

2.5.1.2 OTHER EXOGENOUS ANTIOXIDANTS

Only the most important compounds that can contribute to scavenging of RONS, are mentioned in this chapter.

The best described role of **vitamin C** (**ascorbate**) is its participation in the synthesis of collagen, where it acts as a cofactor of prolyl and lysyl hydroxylases. It is a water soluble antioxidant as well and it is found mainly in fresh fruit and vegetables. Ascorbate has been shown to scavenge the superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, peroxyl radical, singlet oxygen and $NO^{\bullet}$. It can also reduce oxidized form of vitamin E (Gaté et al. 1999, Young and Woodside 2001).

There are eight naturally occurring forms of **vitamin E** – tocopherols α, β, γ, δ and tocotrienols α, β, γ, δ. The **α-tocopherol** is the most potent antioxidant among them and the most abundant form of vitamin E in humans. Vitamin E is the major lipophilic antioxidant, which is also present in butter, soybean, eggs and cereal grains. It may react with the oxygen or lipoperoxide radicals and so protects various types of cell membranes (Sies et al. 1992, Gaté et al. 1999, Young and Woodside 2001).

Carotenoids, the precursors of vitamin A (retinol), are found mainly in vegetables and milk. They are lipophilic compounds as vitamin E and thus can also protect biological membranes (Ciaccio et al. 1993) and LDL (Livrea et al. 1995) against oxidative stress (Gaté et al. 1999, Young and Woodside 2001).

On the other hand, all these compounds can contribute to oxidative stress, e.g. vitamin C together with Fe^{2+} increases the lipid peroxidation (Sadrzadeh and Eaton 1988), vitamin E is in high doses responsible for the propagation of lipid peroxidation (Witting et al. 1999), and carotenoids can also exhibit a pro-oxidant activity in particular conditions (Palozza 1998).

In fact, essential dietary constituents are not antioxidants, but those can be prepared from them, e. g. diet rich in proteins with sulfur containing amino acids is important for the synthesis of glutathione and trace elements such as copper, iron, zinc and magnesium are necessary for correct function of enzymes.

2.5.2 THE ANTIOXIDANT ENZYMES

Superoxide dismutases (**SODs**) catalyze the dismutation of superoxide to hydrogen peroxide and oxygen (eq. 12). They exist in three isoforms: with copper and zinc cofactor (CuZnSOD or SOD1), with manganese active-site (MnSOD or SOD2) and extracellular superoxide dismutase (EC-SOD or SOD3) that requires copper and zinc as cofactors. SOD1 is found in cytosol and in the mitochondrial intermembrane space. It consists of two protein subunits with catalytically active copper and zinc atoms. SOD2 is located in the mitochondrial matrix. This enzyme is tetramer with probably a single manganese atom. In contrast to the other SODs, SOD3 is synthesized only by a few cell types, such as fibroblasts and endothelial cells, and it is expressed on the cell surface. It may play a role in the regulation of vascular tone. SODs are the first line of cell defense against oxidative stress and they work in cooperation with enzymes removing H_2O_2 (Fridovich 1995, Gaté et al. 1999, Young and Woodside 2001, Halliwell 2006, Powers and Jackson 2008).

12) $$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SODs} H_2O_2 + O_2$$

Catalase (**CAT)** can convert hydrogen peroxide to water in two steps (eq. 13a and 13b). This enzyme is largely located in peroxisomes, especially in liver and erythrocytes. CAT consists of four identical protein subunits, where each subunit contains a hem prosthetic group and NADPH molecule (Gaté et al. 1999, Young and Woodside 2001, Powers and Jackson 2007).

13a) $$H_2O_2 + CAT - Fe^{3+} \rightarrow \text{compound I}$$

13b) $$\text{compound I} + H_2O_2 \rightarrow CAT - Fe^{3+} + 2H_2O + O_2$$

Glutathione peroxidase (**GPx**) is a selenium-dependent enzyme, which detoxifies H_2O_2 and organic hydroperoxides into water and alcohol with simultaneous

conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG), respectively (eq. 14a and 14b). GPx has five isoforms with different substrate specificity and cellular localization. Nevertheless, all isoforms play an important role in reparation of damage caused by lipid, protein and nucleic acid peroxidation. The highest concentration of glutathione peroxidase is in liver tissue (cytosol and mitochondria), but GPx is found also in other organs.

14a) $$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

14b) $$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

Regeneration of GSSG to GSH, which is enabled by a flavoprotein **glutathione reductase** (**GR**) in the presence of NADPH (eq. 15), is essential for maintaining GPx activity (Gaté et al. 1999, Young and Woodside 2001, Powers and Jackson 2007).

15) $$GSSG + NADPH + H^+ \xrightarrow{GSHRed} 2GSH + NADP^+$$

There are several other antioxidant enzymes that protect cells from RONS – the **thioredoxin** (TRX), **glutaredoxin** (GRX) and **peroxiredoxin** (PRX) (Powers and Jackson 2008).

The **thioredoxin system** comprises of TRX, thioredoxin reductase (TR) and NADPH. This system is primarily able to reduce the disulfide bonds of several proteins (*Fig. 11*), but it can also contribute to the reduction of lipid hydroperoxides, redox regulation of a number of transcription factors and control of apoptosis (Holmgren 1985, Arner and Holmgren 2000).

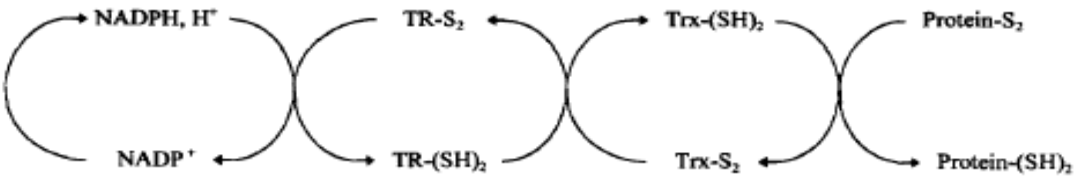


Fig. 11: Reduction of disulfide bonds by thioredoxin system (Gaté et al. 1999).

Glutaredoxin system is similar to thioredoxin system and is responsible for protection of protein and non-protein thiols. This system is composed of GRX, glutaredoxin reductase (GR), GSH and NADPH (*Fig. 12*) (Holmgren and Aslund 1995, Berndt et al. 2007).

Fig. 12: Reduction of disulfide bonds by glutaredoxin system (Gaté et al. 1999).

Peroxiredoxin is peroxidase capable of reducing both hydroperoxides and peroxynitrate with the use of electrons provided by a physiological thiol like TRX (Kim et al. 1985). PRX may defend cells against oxidative stress, although the importance of its antioxidant role in cells remains unclear (Flohe et al. 2003).

2.5.3 ENDOGENOUS NONENZYMATIC ANTIOXIDANTS

Tripeptide **glutathione** (**GSH**) belongs to the main endogenous non-enzymatic antioxidants in the body. GSH is a non-protein thiol, which is primarily synthesized in the liver, but it is found in every cell of our body. In contrast, very little amount of GSH is found in body fluids (DeLeve and Kaplowitz 1991). Glutathione can directly react with a variety of radicals by donating a hydrogen atom (Yu 1994), it acts as a coenzyme of GPx and xenobiotic-metabolizing enzyme glutathione S-transferase and it is also involved in regeneration of other antioxidants including vitamins C and E (Gaté et al. 1999, Scott and Powers 2008).

On the other hand, in the body fluids is predominant plasma protein **albumin** containing high number of thiol groups. It participates in the transport of fatty acid, binding of copper ions and thus inhibition of copper-dependent damage of lipids, and scavenging of phagocytic product HOCl. Because albumin itself is damaged, when it acts as an antioxidant, it has been viewed as a sacrificial molecule that prevents damage occurring to more vital species (Young and Woodside 2001).

Uric acid (**urate**) is a by-product of purine metabolism in humans, which acts as an antioxidant in biological fluids. Because uric acid is able to donate electron

(Halliwell and Gutteridge 2007), it has been considered as a scavenger of peroxyl radicals, hydroxyl radicals and singlet oxygen (Ames 1981). Urate is also able to chelate metal ions (Halliwell and Gutteridge 2007).

Bilirubin, the final product of hemoprotein catabolism, is a strong antioxidant against peroxyl radical and hydrogen peroxide. Mechanism of its antioxidant activity includes its oxidation to biliverdin and further reduction to bilirubin by biliverdin reductase (Baranano 2002).

Coenzym Q_{10} (**ubiquinone**) is essential carrier in mitochondrial electron transport chain, but it is also located in cell membranes (Halliwell and Gutteridge 2007). It scavenges peroxyl radical and inhibits lipid peroxidation *in vitro*, but *in vivo* its contribution remains unclear (Powers and Jackson 2008).

Taurine and its precursor **hypotaurine** are β-amino acids that are derived from cysteine metabolism (Malmezat 1998). Both compounds decrease lipid peroxidation by scavenging $^{\bullet}OH$ (Tadolini et al. 1995).

Special case in antioxidant defense system represents transition metal-binding proteins – **ferritin**, **transferrin**, **lactoferrin** (transport and storage of iron) and **ceruplasmin** (storage of copper). First curiosity of metal ion sequestration is the prevention from ROS formation (mainly of $^{\bullet}OH$ radical), while other antioxidants react with already formed RONS. The other important thing is that ceruplasmin might act also as antioxidant enzyme, because it catalyzes the oxidation of divalent iron (Halliwell 1996, Young and Woodside 2001).

2.6 OXIDATIVE AND NITROSATIVE STRESS

These terms mean a disturbance in the pro-oxidant-antioxidant balance in favor of the former leading to a potential damage (Sies 1991). Oxidative and nitrosative stress is a very complicated and complex process, which can be a result of only two events. The first one is overproduction of RONS and the second one is inadequate amount of diet-derived antioxidants (*Fig 13*). How do cells deals with the oxidative/nitrosative stress depends on their type and of course on the level of stress applied (Halliwell 1996, Valko 2007, Durackova 2007).

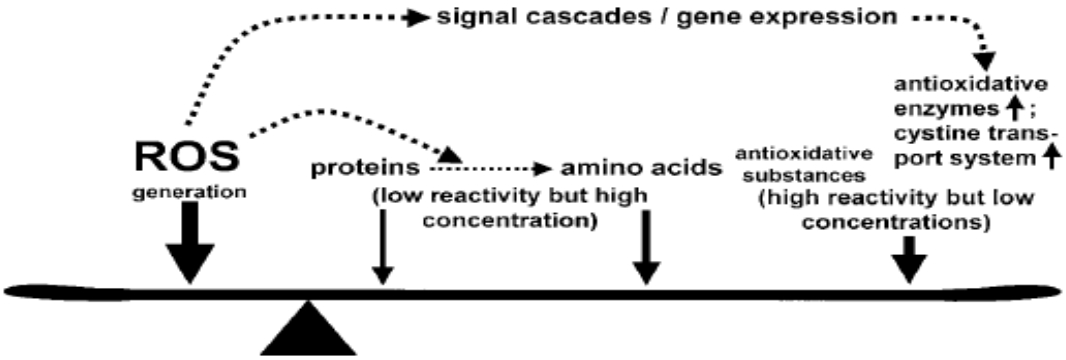


Fig. 13: Balance between RO(N)S production and AOX scavengers (Dröge 2002).

Cells often tolerate mild oxidative/nitrosative stress by up-regulation of antioxidant defense system synthesis (due to the signal cascades and gene expression). In the long run, this mechanism tends to maintain a stable state, but in case the RONS production is more intensive and persistent, the response of antioxidants may not be sufficient to reset original base level (*Fig. 14*) (Halliwell 1996, Dröge 2002).

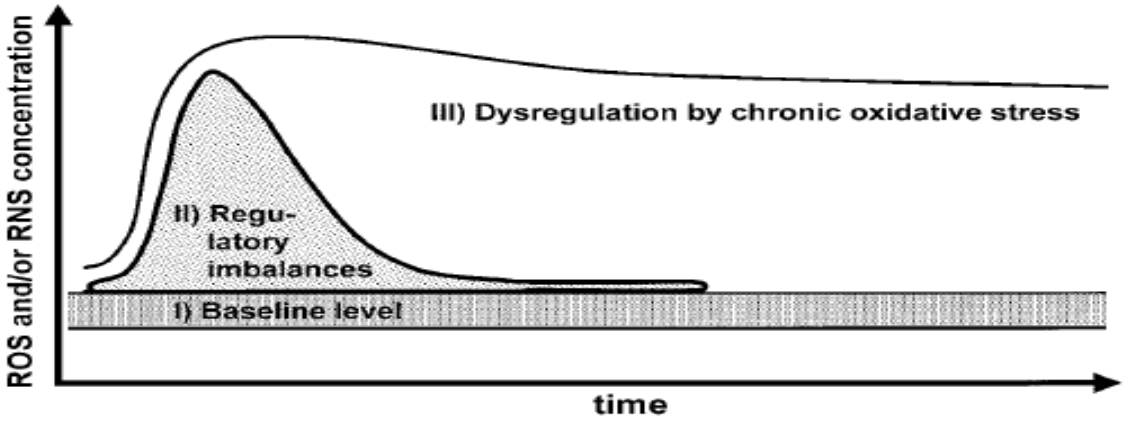


Fig. 14: Regulatory events and their dysregulation depend on the magnitude and duration of the change in RONS concentration (Dröge 2002).

If up-regulated synthesis of antioxidants is not sufficient, level of intracellular free Ca^{2+} rises and then the level of iron increases too. Iron may catalyze Fenton's reaction (eq. 5). Several cell types may respond to mild oxidative stress by proliferation. Moderate oxidative stress usually halts the cell cycle, it means that the cells are not able to divide anymore because $^{\bullet}OH$ from Fenton's reaction attacks guanine and may cause the DNA mutagenesis. $^{\bullet}OH$ oxidizes also polyunsaturated fatty acids or proteins, such as enzymes, transport systems or transduction systems. Harmful effect of rising level of

Ca^{2+} can damage the ion transporters and can lead to mitochondrial permeability transition or cytochrome c release. Severe oxidative damage may trigger cell death by apoptosis, necrosis or mechanisms with features of both (Gaté et al. 1999, Halliwell 2006).

2.7 AGING AND DISEASES

Aging is the progressive accumulation of changes with time associated with or responsible for ever-increasing susceptibility to disease and death, which accompanies advancing age. This process may be common to all living organisms. Nowadays, aging is the major risk factor for disease development and death in developed countries and is largely due to free radical reaction damage. The chance of death for humans drops steeply after birth to a minimum figure around puberty and then increases with age almost exponentially. Aging is under genetic control to some extent but manifestation of aging is decreased by improvements in general living condition (Harman 1981, Harman 1991).

Oxidative stress has been implicated in a variety of pathological conditions involving various diseases that fall into two major categories. To the first category belong diseases (cancer and diabetes mellitus) characterized by pro-oxidant shift in the systemic thiol/disulfide balance and impaired glucose tolerance. Mitochondria may be the major site of elevated ROS production. These conditions are referred to as „mitochondrial oxidative stress“. The second category is based on inflammation and referred to as „inflammatory oxidative conditions“, because these diseases are typically associated with an excessive stimulation of NAD(P)H oxidase activity by cytokines or other agents. This category involves chronic inflammation diseases, atherosclerosis or cardiovascular diseases, neurodegenerative diseases and so on (Dröge 2002, Valko et al. 2007).

3 AIM OF THE WORK

The aim of this work was:

- to establish the *in vitro* antioxidant activity of mosloflavone, negletein, 5,6-dihydroxyflavone, baicalein, gallic acid, and its derivative compound 1625 in the cell culture THP-1 using dichlorofluorescein and cytotoxicity assay

- to determine the *in vitro* antioxidant activity of the mosloflavone, negletein, 5,6-dihydroxyflavone and baicalein towards stable synthetic radical galvinoxyl using EPR spectroscopy

- to evaluate the antioxidant potential of tested compounds with respect to their structure

4 MATERIAL AND METHODS

4.1 REAGENTS

- Medium RPMI-1640 (Roswell Park Memorial Institute) + with L-glutamine was purchased from LONZA (Verviers, Belgium)
- Antibiotics (Penicillin 100 units/ml and streptomycin 100 μg/ml) and sterile plasticware for cell culture were from Flow Laboratory (Irvine, UK).
- Fetal bovine serum and sodium pyruvate were obtained from GIBCO (Grand Island, NY).
- Glucose, phosphate buffered saline (PBS), cumene hydroperoxide, galvinoxyl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxid (DMSO) were products of Sigma-Aldrich (St. Louis, USA).
- All flavonoids (mosloflavone, negletein, 5,6-dihydroxyflavone, baicalein) were synthesized and characterized by Prof. P. Bovicelli, Department of Chemistry, University of Rome "La Sapienza."
- Gallic acid and compound 1625 were synthesized in the laboratory of Prof. V. Parmar, Department of Chemistry, University of Delhi.
- 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR).

4.2 PREPARATION OF PHOSPHATE BUFFERED SALINE

The solution of phosphate buffered saline (PBS) was prepared by dissolving the phosphate buffered saline powder (pH 7.4) in 1000 ml of distilled water. One hundered ml of this PBS solution was supplemented with 90 mg of glucose (final concentration 5 mM) and heated before the experiment in the bath to 37 ºC.

4.3 CELL CULTURES THP-1

The human monocytic cell line THP-1 was first derived from the peripheral blood of a one-year-old male with acute monocytic leukemia. THP-1 cells easily divide and can be maintained in cell culture for a long period. THP-1 cells easily differentiate into macrophages (phagocytes) and adhere to cell culture flasks, when are stimulated with phorbol esters **(**PMA**)** or vitamin D_3. Thereby, THP-1 is widely used cell line with

properties similar to the properties of “human monocyte-derived macrophages” (Tsuchiya 1980).

The leukemic monocytes (THP-1 cells) were donated from the Laboratory of Virology of University Roma Tre. THP-1 cells were seeded in the plastic flasks (25 cm^2) in the presence of 8 ml of medium RPMI-1640 with sodium pyruvate, fetal bovine serum, penicillin 100 units/ml and streptomycin 100 μg/ml (to prevent bacterial contamination). The cells were maintained in an incubator at 37°C and 5% CO_2 in the atmosphere. The cells from seventh to twenty third passages approximately were used within experiments. The estimation of the amount of cells for seeding and for the experiment was about two millions cells/1 ml and about one million cells/1 ml, respectively.

4.4 INTRACELLULAR ROS DETERMINATION USING DICHLOROFLUORESCEIN ASSAY

The 2´,7´- dichlorofluorescin (DCFH) is a widely used fluorescent probe to measure oxidative stress in cells. Its diacetate form, 2´,7´- dichlorodihydrofluorescin diacetate (DCFH-DA), is able to diffuse across the cell membrane. DCFH-DA is inside the cells hydrolyzed by intracellular esterases into DCFH. In the presence of reactive oxygen species, DCFH is oxidized into highly fluorescent compound 2´,7´- dichlorofluorescein (DCF) (*Fig. 15*). The fluorescence intensity can be easily measured. This method constitutes the basis of cellular assays for oxidative stress (Lebel et al. 1992).

This probe is more suitable for detecting total ROS production (not individual radicals) in tissues or living cells, because in the cells are some substances that interfere with the formation of DCF. DCFH can be oxidized by several enzymes as peroxide oxidase or xanthine oxidase. DCFH oxidation can occur also by action of H_2O_2 in the presence of ions Fe^{2+}, when $^{\bullet}OH$, which causes the oxidation, is produced via Fenton reaction (eq. 5). DCFH is also sensitive to the presence of UVA radiation or visible light that causes generation of semiquinone radical ($DCF^{\bullet}$), which reacts with oxygen and gives rise to superoxide radical ($O_2^{\bullet-}$) that during its dismutation generates H_2O_2. This increased formation of H_2O_2 leads to an artificial rise of DCFH oxidation and consequently to an amplification of DCF fluorescence (Rota et al. 1999, Gomes et al. 2005).

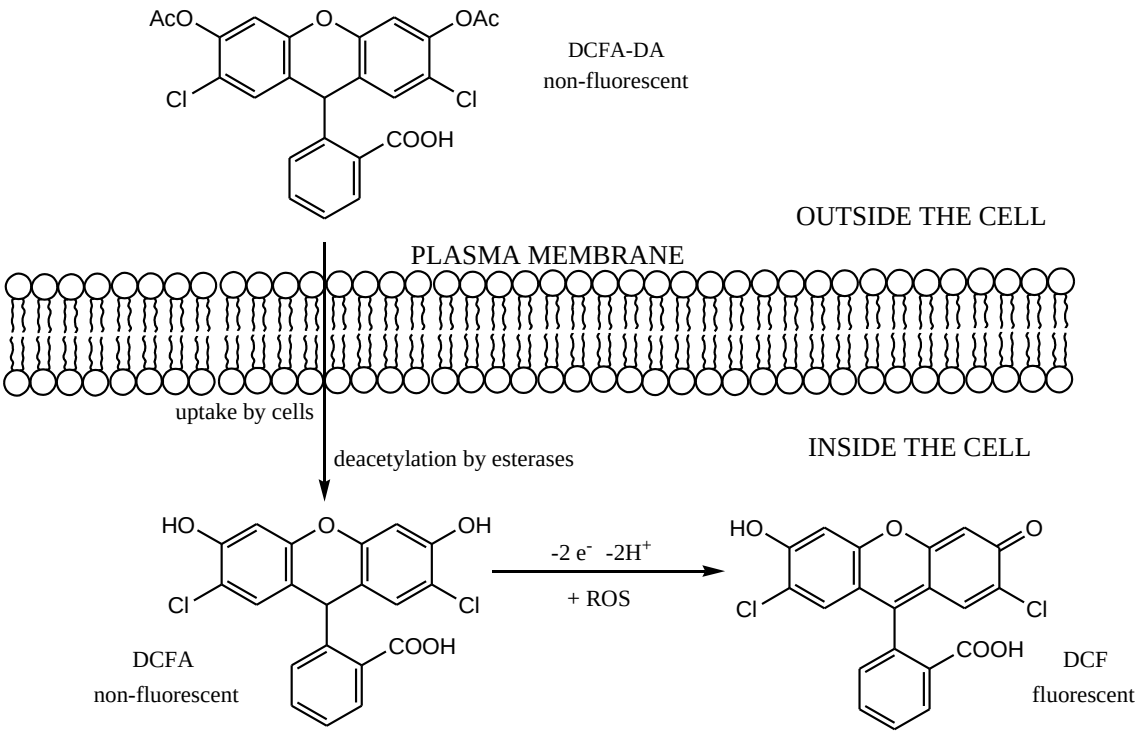


Fig. 15: DCFA-DA crosses the cell membrane, inside the cell is deacetylated by esterases to DCFA and gives rise to highly fluorescent compound DCF by a reaction with ROS (Lebel et al. 1992).

At the time of experiment, the cells were collected from 25 cm^2 flask, transferred to centrifuge tubes and centrifuged at 1200 rpm for 10 minutes. Then the cells were washed twice with 5 ml of PBS with glucose at 37°C. After each wash, the cells were centrifuged at 1200 rpm for 10 minutes. After the last centrifuge, the supernatant was discarded and the pellet re-suspended with a plastic Pasteur pipette in 8 ml of PBS with glucose.

Then 8 µl of the fluorescent probe (DCFH-DA) at a final concentration of 10 µM (from a stock solution of 10 mM in DMSO) were added. The incubation was carried out in the dark at 37°C for 30 minutes (Pallottini et al. 2005, Pedersen et al. 2007). The cells were gently re-suspended every 10 min; at the end of incubation cells were centrifuged at 1200 rpm for 10 min. The supernatant was discarded and the cell pellet was re-suspended in 8 ml of PBS with glucose and centrifuged again. The final supernatant was discarded and the cell pellet was re-suspended in 4 ml of PBS with glucose at a final concentration approximately 2 x 10^6 cells/ml. Before starting the experiment, a recovery of the cells was carried out at temperature 37°C in the water bath for one hour in the dark.

Intracellular fluorescence was measured under continuous gentle stirring in Perkin-Elmer LS 50B luminescence spectrometer (Norwalk, CT) at 37°C. Excitation and emission wavelengths were set at 498 nm and 530 nm using 5 and 10 nm slits for the two light paths, respectively. The assay was carried out in a final volume of 3 ml, containing 400 µl of cell suspension (i. e. approximately 800 x 10^3 cells/ml) and 2600 µl of PBS with glucose. Cumene hydroperoxide diluted 1:100 in DMSO was used as a radical generator (final concentration 222 µM); DMSO did not affect the fluorescence signal at the used concentrations. The antioxidant potency of individual antioxidants (3 µl) was determined by the decrease in the intracellular DCF fluorescence, reported as ΔF/10 min, and was calculated with respect to the fluorescence change induced by 222 µM cumene hydroperoxide alone (100%) (eq. 16). Cells were incubated with antioxidants for 10 min at 37°C before the addition of cumene hydroperoxide (Pedersen et al. 2007).

16) $$\text{ROS production (\%)} = \frac{\Delta F_{AOX+CUM}/10min}{\Delta F_{CUM}/10min} \times 100$$

AOX = antioxidant, CUM = cumene hydroperoxide

4.5 CYTOTOXICITY ASSAY

The cytotoxicity assay is a biological method using *in vitro* cell cultures for cytotoxicity testing. This assay is a good indicator of cell viability (vitality). The cells, which remain viable after exposure and incubation with a test chemical (in this case after exposure to ROS), are able to reduce yellow water-soluble salt MTT into insoluble intracellular metabolite blue formazan (*Fig. 16*) (Ozdemir et al. 2009). Cytotoxicity assay is also called MTT assay.

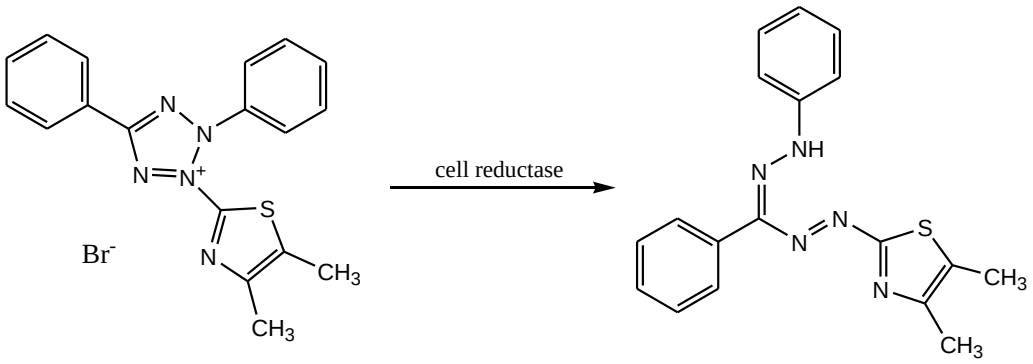


Fig. 16: Reduction of yellow MTT into blue metabolite formazan.

It has been hypothesized that this reaction is mainly result of activity of mitochondrial enzymes (reductases) and electron carriers (Marshall et al. 1995). However, there is evidence that the MTT reduction in mammalian cells is also catalyzed by a number of non-mitochondrial enzymes. This was confirmed also by the study of Bernas and Dobrucki (2002), who reported that only a fraction of MTT formazan is deposited in (or at the surface of) mitochondria, whereas most MTT is reduced in other cellular compartments in the cytoplasm and in the regions of plasma membranes. MTT may also interact with the outer side of the inner mitochondrial membrane (Bernas and Dobrucki 2002).

The product of this reaction, formazan, is solubilized by extraction with alcohol or dimethyl sulfoxide (DMSO) and can be quantified spectrophotometrically with results related to the portion of viable cells (Bean et al. 1995).

The method of Hansen et al. (1989) with some modifications was used to determine antioxidants toxicity during the experiments on intracellular ROS measurements. The experiment was carried out in six-well plates using 2 x 10^6 THP-1 cells at the confluent state per well (Pedersen et al. 2007).

The cells were collected with the Pasteur pipette from six-well plate into six centrifuge tubes, centrifuged at 1200 rpm for 10 minutes, and the supernatant was discarded. Then the cells were washed with 1 ml of PBS with glucose (37°C), the cells were centrifuged at 1200 rpm for 10 minutes and supernatant was discarded again. Then the cells were washed, centrifuged and the supernatant was discarded one more time.

At the time t_0, antioxidants at the final concentration 10 μM and 10 nM were added into four tubes and all tubes were incubated in water bath at 37°C for 30 min. After incubation, the cells in tubes were centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and 1 ml of PBS with glucose was added into each tube at 37°C. Then 30 μl of cumene hydroperoxide (diluted 1:100) was added into all tubes except for the control tube. Then the incubation in water bath at 37°C lasted for 30 min. After the incubation, tubes were centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the cells were re-suspended in 1 ml of MTT solution (concentration 1 mg/ml) in each tube and followed by incubation for 3 hours (atmosphere of 5% CO_2 at 37°C).

Thereafter, the cells were transferred with Pasteur pipette into eppendorf-tubes (1.5 ml) and centrifuged at 1200 rpm for 10 minutes; the supernatant was discarded, the

pellet was re-suspended in 300 µl of PBS with glucose and sonicated for 15 sec with an Ultrasonic W-225R (setting 4). Then the cells were centrifuged in the microcentrifuge at 13,000 rpm for 10 minutes. The supernatant was discarded and the final pellet re-suspended in 200 µl of dimethyl sulfoxide (DMSO). These 200 µl of re-suspended solution and 1800 µl of PBS with glucose were placed into cuvettes and the concentration of MTT formazan was measured using spectrophotometer at 560 nm (Pedersen et al. 2007).

4.6 ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY (EPR)

Electron paramagnetic resonance spectroscopy (also known as electron spin resonance spectroscopy or electronic magnetic resonance spectroscopy) is a magnetic resonance technique used for the study of paramagnetic materials, i. e. materials containing unpaired electrons such as free radicals and transition metal complexes (Atherton 1993). In a magnetic field, an unpaired electron can occupy one of two energy levels. These levels are characterized by a quantum number m_s (electron spin), which can have two values -1/2 or +1/2 (i. e. it has parallel state with, or antiparallel state against the field). These unpaired electrons interact with electromagnetic radiation (Cammack and Shergill 1995). The electromagnetic wave radiation of external magnetic field causes the excitation of unpaired electrons from the lower energy level to the higher level (Takeshita and Ozawa 2004). The energy accepted by electron is equal to the difference between the two energy levels (Cammack and Shergil 1995), i. e. state of different spin. This absorption of energy of the electromagnetic wave is detected by spectrometer. The EPR of the *in vitro* samples is usually measured with electromagnetic wave („X-band") 9-10 GHz (Takeshita and Ozawa 2004). The free radical signals are detectable down to levels of 0.1 µM, although higher concentration may give well-resolved EPR spectra (Cammack and Shergill 1995**)**.

Stock solutions of all tested flavonoids were prepared in ethanol 95% at the concentration of 10 mM. Galvinoxyl solution (10 mM in ethanol 95%) was freshly prepared immediately before the experiments. Galvinoxyl in solution is a stable radical that is converted to galvinol in the presence of an antioxidant (*Fig. 17*). Systematic screening of all compounds was made with final concentrations of 0.1, 1.0 and

10.0 μM in the presence of galvinoxyl at the constant concentration (10 μM). The solutions were drawn into glass capillaries and measured using ESP300 instrument (Bruker Spectrospin, Karlsruhe) equipped with a high sensitivity TM_{110} X-band cavity. Radical spectra were recorded at room temperature, using 0.6 G modulation, 1 mW microwave power and a scan time of 42 seconds for a 30 G spectrum. Usually, four spectra were accumulated to obtain a suitable signal-to-noise ratio. The kinetics of the reaction was followed for 15 minutes at room temperature or until the radical signal had disappeared (Pedersen et al. 2007).

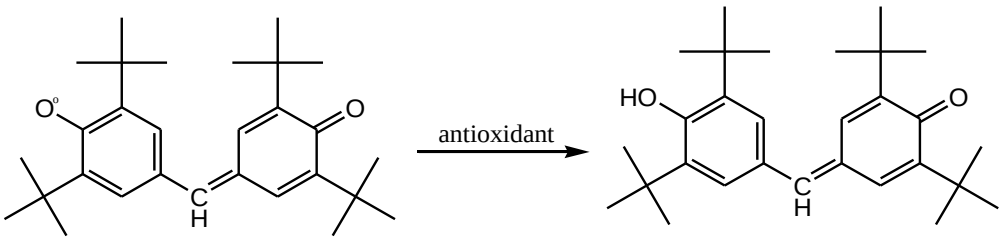


Fig. 17: Galvinoxyl transformation into galvinol in the presence of an antioxidant.

5 RESULTS

5.1 INTRACELLULAR ANTIOXIDANT ACTIVITY

The following *polyphenols* were tested as potential antioxidants – *flavones* **mosloflavone**, **negletein**, **5,6-dihydroxyflavone**, **baicalein** and *phenolic acids* **gallic acid** and its ester derivative **compound 1625** (Fig. 18). Two methods, **dichlorofluorescein assay** with the probe DCFH-DA and **cytotoxicity assay** with the probe MTT, were used in order to determine intracellular antioxidant activity of these compounds.

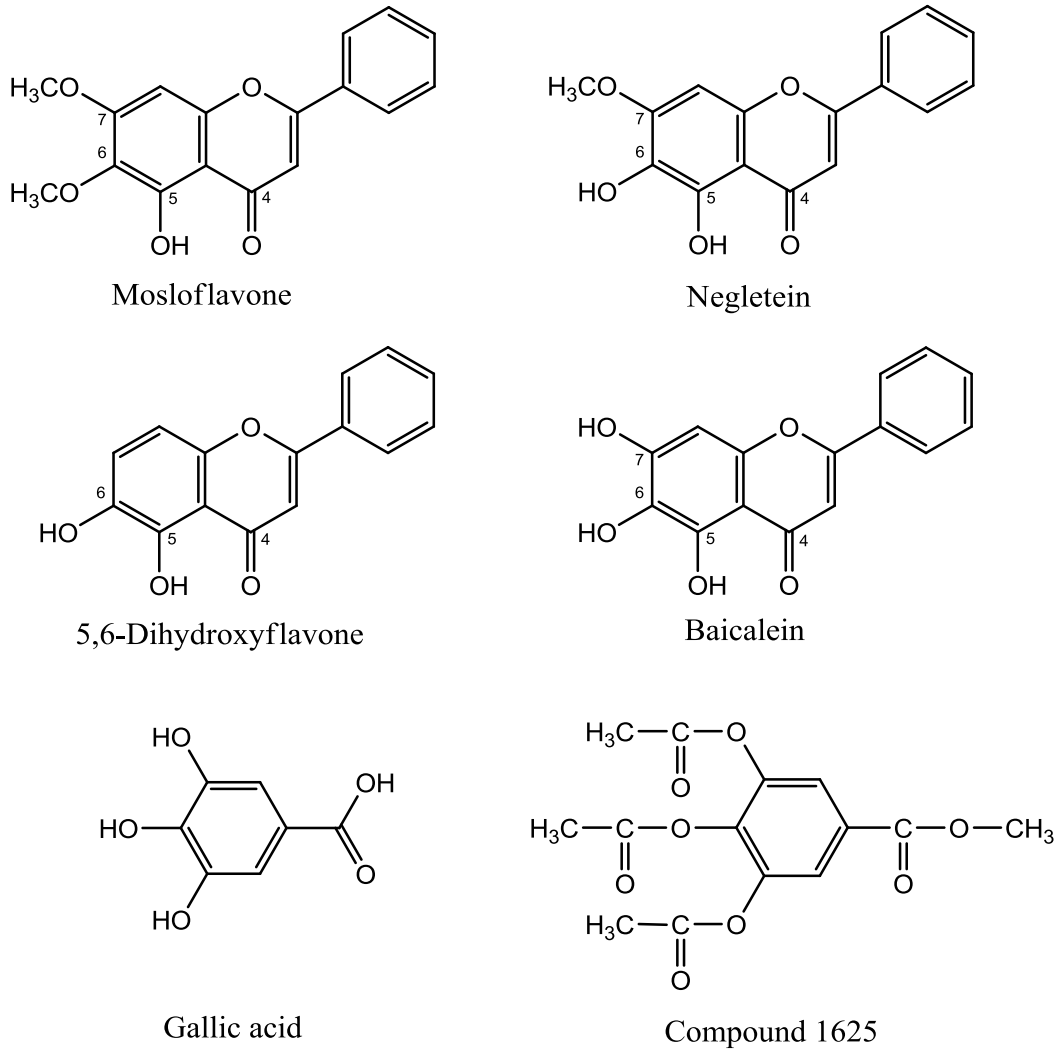


Fig. 18: Chemical structures of studied compounds.

5.1.1 MOSLOFLAVONE

Mosloflavone has the 5-hydroxyl group and 6,7-methoxyl groups in the ring A (*Fig. 18*). This compound was tested in the range of concentrations 10^{-5}-10^{-9} M. The results showed that mosloflavone had very low ability to decrease intracellular ROS production and it even exerted mild, but statistically insignificant pro-oxidative effect at the highest concentration (*Fig. 19*).

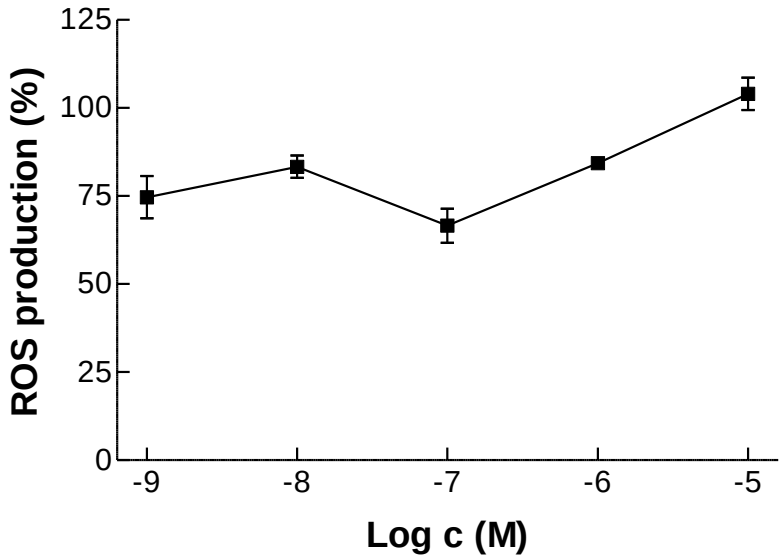


Fig. 19: The effect of mosloflavone (10^{-5}-10^{-9} M) on the level of intracellular ROS formed in THP-1 cells after the exposure to cumene hydroperoxide. The ROS production (%) was calculated according the eq. 16. The data are expressed as the mean ± SD of n = 3 independent experiments.

The cytotoxicity assay was performed using two concentrations of mosloflavone (10^{-5} and 10^{-8} M). The results of cytotoxicity assay did not confirm results of dichlorofluorescein assay. Mosloflavone was able to partially reverse effect of cumene hydroperoxide and thus protected cells. Higher protective effect was observed at the concentration 10^{-5} M (cell viability 83.5%) than at the concentration 10^{-8} M (62.5%) compared to cumene hydroperoxide (44.7%) (*Fig. 20*).

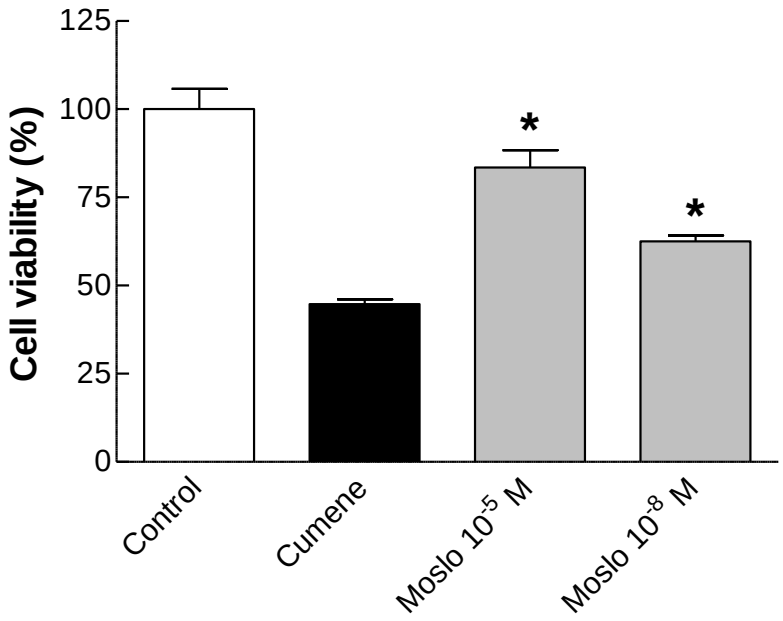


Fig. 20: The effect of mosloflavone (moslo, 10^{-5} and 10^{-8} M) on the cell viability (%) of THP-1 cells after exposure to cumene hydroperoxide. The data are expressed as the mean ± SD of n = 3 independent experiments (* data with $p < 0.05$; Student's *t*-test).

5.1.2 NEGLETEIN

Negletein has two hydroxyl groups in positions 5 a 6 and one methoxyl group in the position 7 of the ring A (*Fig. 18*). Negletein was tested at the concentrations 10^{-5}-10^{-9} M. The result showed that negletein was able to decrease ROS production in the concentrations 10^{-6}-10^{-9} M, but this effect was not statistically significant. Slight pro-oxidative effect similar to that of mosloflavone was noted at the concentration 10^{-5} M (*Fig. 21*).

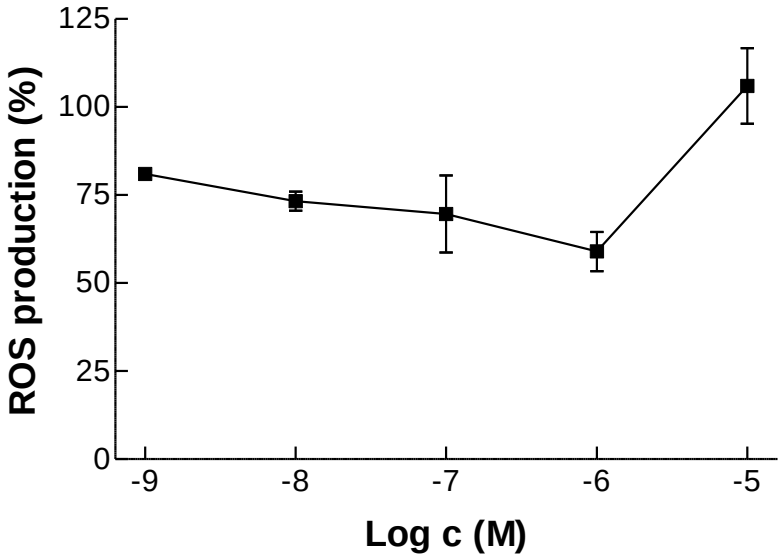


Fig. 21: The effect of negletein (10^{-5}-10^{-9} M) on the level of intracellular ROS formed in THP-1 cells after the exposure to cumene hydroperoxide. The ROS production (%) was calculated according the eq. 16. The data are expressed as the mean ± SD of n = 1-5 independent experiments.

The cytotoxicity assay was performed using two concentrations of negletein (10^{-5} and 10^{-8} M). The results of cytotoxicity assay confirmed outcomes of dichlorofluorescein assay, because negletein was not able to protect cells against the effect of cumene hydroperoxide (*Fig. 22*). Cell viability was similar in the samples containing cumene hydroperoxide (53.6%) and in the samples containing cumene hydroperoxide with negletein (45.8 and 59.4%).

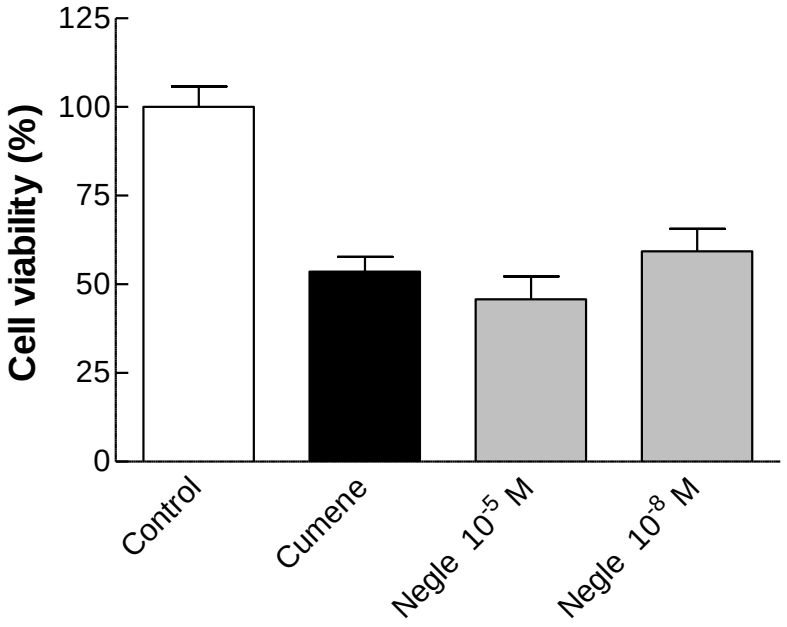


Fig. 22: The effect of negletein (negle, 10^{-5} and 10^{-8} M) on the cell viability (%) of THP-1 cells after exposure to cumene hydroperoxide. The data are expressed as the mean ± SD of n = 3-4 independent experiments.

5.1.3 5,6-DIHYDROXYFLAVONE

5,6-dihydroxyflavone (**5,6-DHF**) has two hydroxyl groups in position 5 a 6 of the ring A (*Fig. 18*). 5,6-DHF was tested in the range of concentrations 10^{-5}-10^{-11} M. Its ability to decrease ROS production in the dichlorofluorescein assay was significant in the concentrations 10^{-5}-10^{-9} M, e.g. 5,6-DHF 10^{-5} M inhibited ROS production by 81% compared to the control sample with cumene hydroperoxide (*Fig 23*).

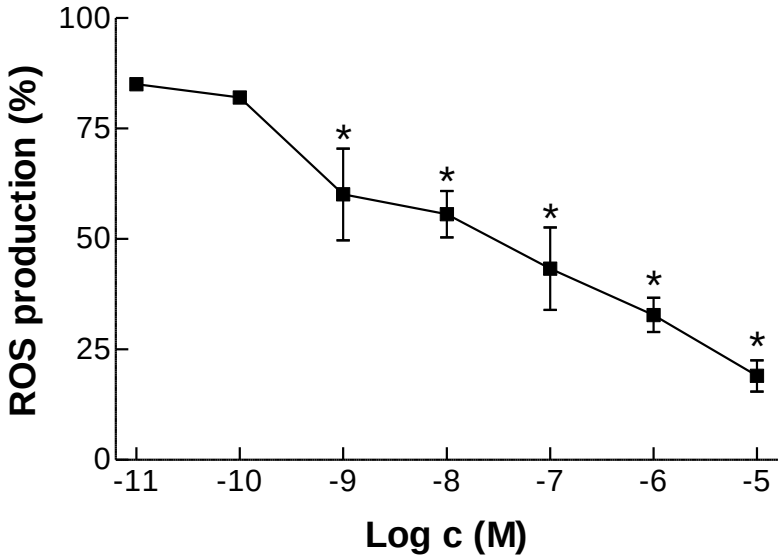


Fig. 23: The effect of 5,6-dihydroxyflavone (10^{-5}-10^{-11} M) on the level of intracellular ROS formed in THP-1 cells after the exposure to cumene hydroperoxide. The ROS production (%) was calculated according the eq. 16. The data are expressed as the mean ± SD of n = 1-5 independent experiments (* data with $p < 0.05$; Student's *t*-test).

The results of cytotoxicity assay confirmed good scavenging ability of 5,6-DHF observed in the dichlorofluorescein assay (*Fig. 24*). The cell viability determined at the concentration 10^{-5} M of 5,6-DHF was 82.3%, while the viability in the samples containing cumene hydroperoxide only was 44.3%.

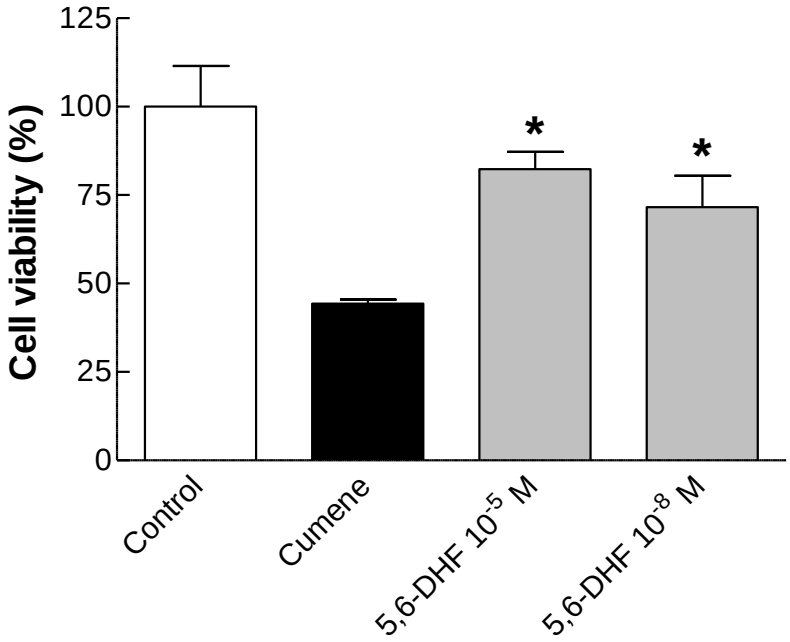


Fig. 24: The effect of 5,6-dihydroxyflavone (5,6-DHF, 10^{-5} and 10^{-8} M) on the cell viability (%) of THP-1 cells after exposure to cumene hydroperoxide. The data are expressed as the mean ± SD of n = 3 independent experiments (* data with $p < 0.02$; Student's *t*-test).

5.1.4 BAICALEIN

Baicalein has three hydroxyl groups in position 5, 6 and 7 of the ring A (*Fig. 18*). Baicalein was tested in the range of concentrations 10^{-5}-10^{-13} M. The results of dichlorofluorescein assay showed that this compound possessed the highest scavenging ability from all tested compounds. Baicalein was able to significantly reduce ROS production up to the concentration 10^{-11} M (*Fig 25*).

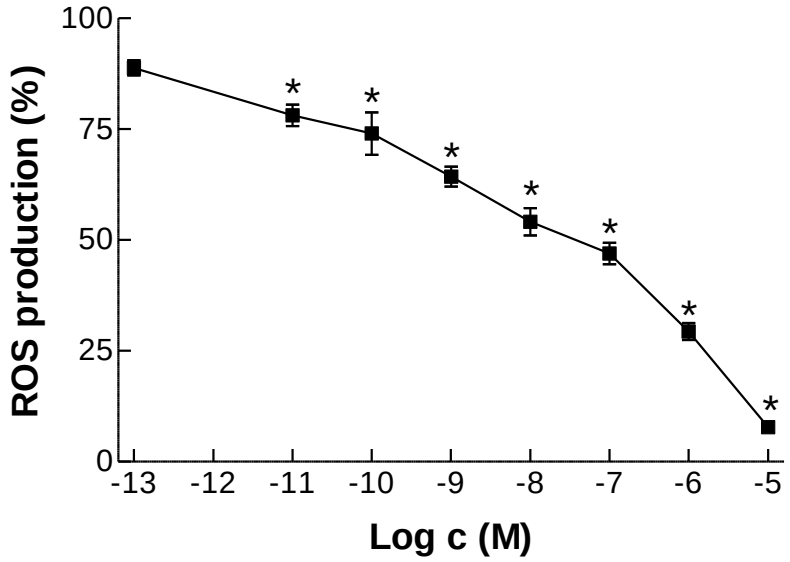


Fig. 25: The effect of baicalein (10^{-5}-10^{-13} M) on the level of intracellular ROS formed in THP-1 cells after the exposure to cumene hydroperoxide. The ROS production (%) was calculated according the eq. 16. The data are expressed as the mean ± SD of n = 3-7 independent experiments (* data with $p < 0.005$; Student´s *t*-test).

The cytotoxicity assay was performed using two concentrations of baicalein (10^{-5} and 10^{-8} M). The results of this assay confirmed excellent scavenging ability of this compound observed in the dichlorofluorescein assay. Baicalein 10^{-5} M completely protected cells against the effect of cumene hydroperoxide (*Fig. 26*).

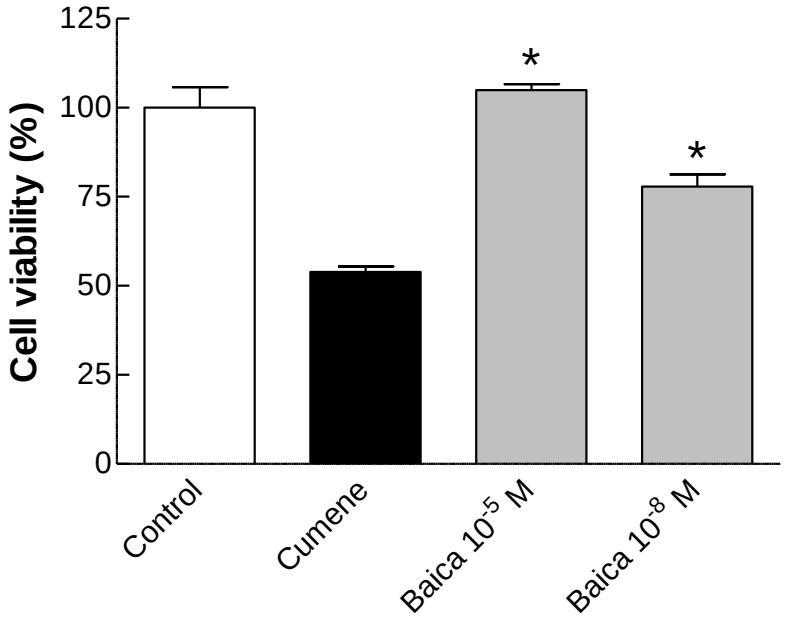


Fig. 26: The effect of baicalein (baica, 10^{-5} and 10^{-8} M) on the cell viability (%) of THP-1 cells after exposure to cumene hydroperoxide. The data are expressed as the mean ± SD of n = 3 independent experiments (* data with $p < 0.05$; Student´s *t*-test).

5.1.5 GALLIC ACID

Gallic acid has one carboxyl group and three hydroxyl groups in the position 3, 4 and 5 of the benzene ring (*Fig. 18*). There is a presumption that negatively charged gallic acid should not be a good scavenger, which was confirmed by dichlorofluorescein as well as cytotoxicity assay. Gallic acid was tested in the range of concentrations 10^{-5}-10^{-8} M. Statistically significant reduction in the ROS production was observed at 10^{-6} and 10^{-5} M concentration, where the ROS production decreased by 29% and 64%, respectively (*Fig 27*).

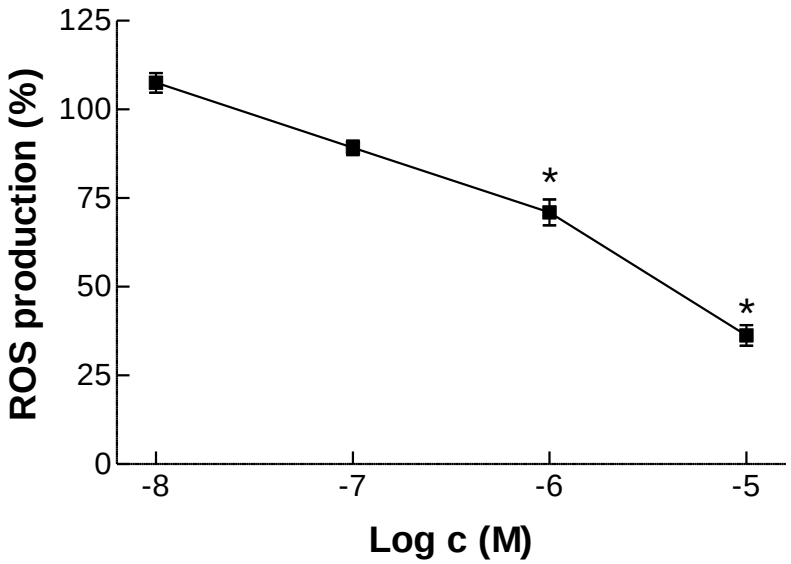


Fig. 27: The effect of gallic acid (10^{-5}-10^{-8} M) on the level of intracellular ROS formed in THP-1 cells after the exposure to cumene hydroperoxide. The ROS production (%) was calculated according the eq. 16. The data are expressed as the mean ± SD of n = 3-4 independent experiments (* data with $p < 0.005$; Student´s *t*-test).

The cytotoxicity assay was performed using two concentrations of gallic acid (10^{-5} and 10^{-8} M). This compound was not able to protect cells against the effect of cumene hydroperoxide and even slightly intensified its effect (*Fig. 28*).

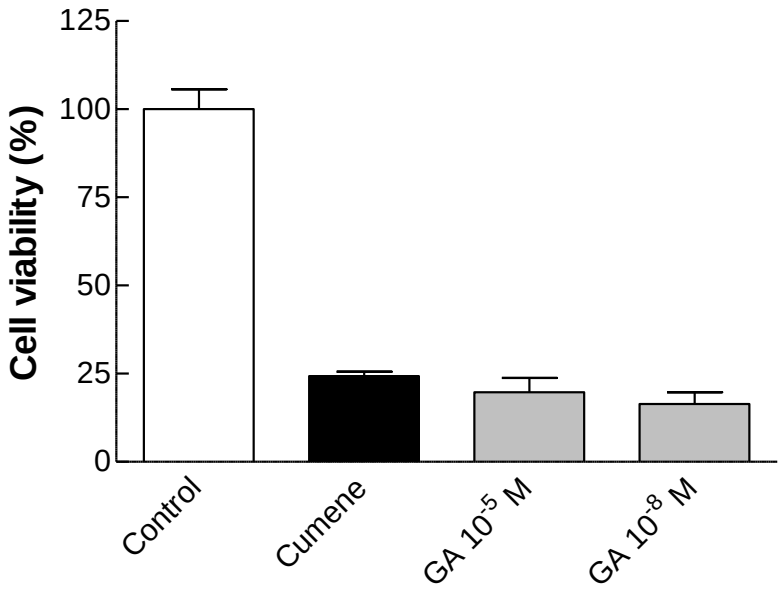


Fig. 28: The effect of gallic acid (GA, 10^{-5} and 10^{-8} M) on the cell viability (%) of THP-1 cells after exposure to cumene hydroperoxide. The data are expressed as the mean ± SD of n = 2 independent experiments.

5.1.6 COMPOUND 1625

Compound 1625 [methyl 3,4,5-tris(acetyloxy)benzoate] is ester derivative of gallic acid (*Fig. 18*). This compound was tested in the same range of concentrations as gallic acid, i.e. 10^{-5}-10^{-8} M. The result of dichlorofluorescein assay showed remarkable ROS scavenging ability of this compound (10^{-5} M), which was comparable to that of baicalein. Unfortunately, this activity dramatically decreased in other tested concentrations (*Fig 29*).

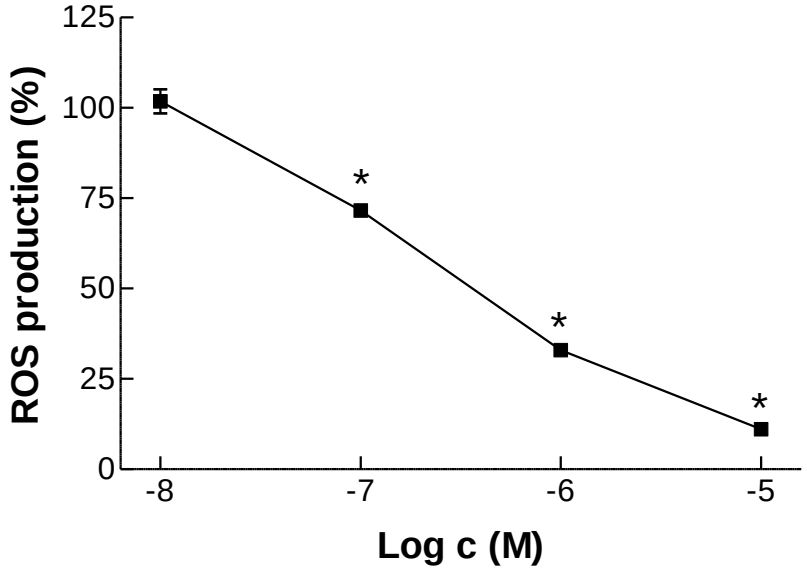


Fig. 29: The effect of compound 1625 (10^{-5}-10^{-8} M) on the level of intracellular ROS formed in THP-1 cells after the exposure to cumene hydroperoxide. The ROS production (%) was calculated according the eq. 16. The data are expressed as the mean ± SD of n = 4 independent experiments (* data with $p < 0.001$; Student´s *t*-test).

Surprisingly, the cytotoxicity assay did not confirm the results obtained by dichlorofluorescein assay and compound 1625 was not able to protect cells from the effect of cumene hydroperoxide at any of the tested concentrations, i.e. 10^{-5} and 10^{-8} M (*Fig. 30*).

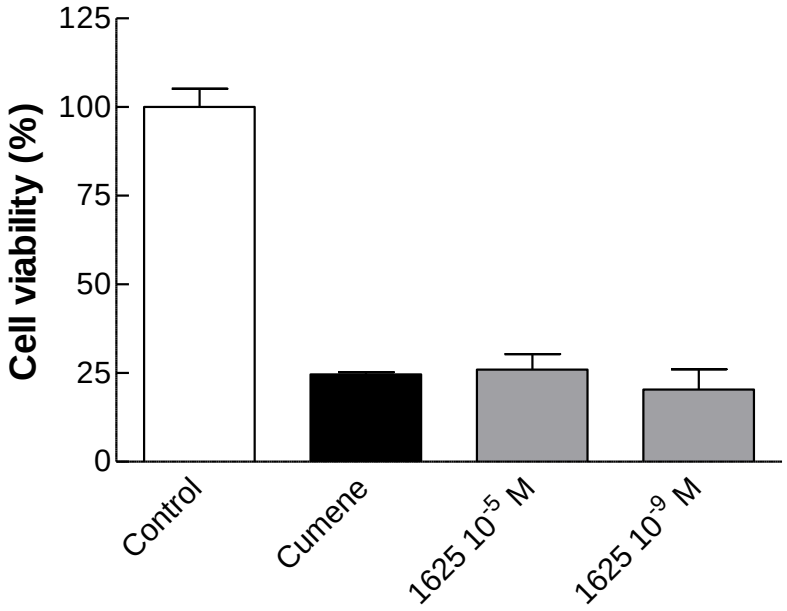


Fig. 30: The effect of compound 1625 (1625; 10^{-5} and 10^{-8} M) on the cell viability (%) of THP-1 cells after exposure to cumene hydroperoxide. The data are expressed as the mean ± SD of n = 3 independent experiments.

5.2 ANTIOXIDANT ACTIVITY *IN VITRO* – EPR

The height of EPR signal is directly proportional to the concentration of a free radical galvinoxyl that gives a typical EPR spectrum, it means, if potential antioxidant is added then the signal would disappear *(Fig. 31).*

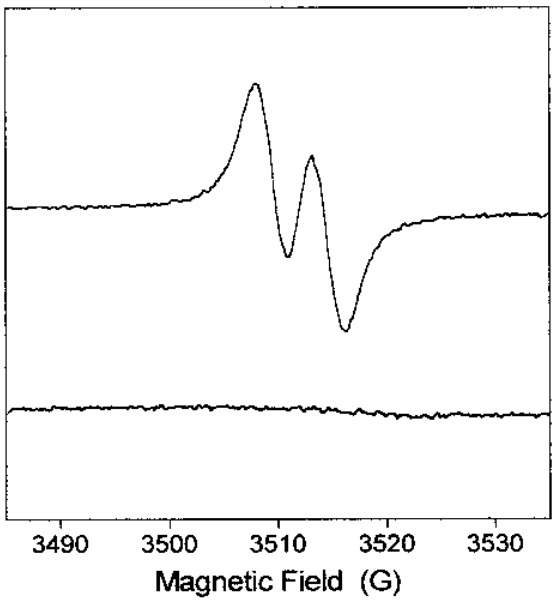


Fig. 31: The typical EPR spectrum of a free radical galvinoxyl 10^{-5} M (top); the EPR spectrum of galvinoxyl 10^{-5} M with an antioxidant, which has a good scavenging ability, free radical is eliminated completely and the signal disappears (bottom).

The amount of remaining galvinoxyl in the sample depends on the scavenging ability of antioxidant, its quantity, and time of incubation.

The EPR spectrum was measured with a free radical galvinoxyl 10^{-5} M alone and compared to galvinoxyl 10^{-5} M with the antioxidant in concentrations 10^{-7}, 10^{-6} and 10^{-5} M. The EPR spectrum was measured in the time 5, 10 and 15 min. The following flavones were tested: **mosloflavone**, **negletein**, **5,6-dihydroxyflavone** and **baicalein** (*Tab. 3*).

Tab. 3. The data show the concentrations of galvinoxyl alone and galvinoxyl that remained in a sample after 5, 10 and 15 min of incubation with different antioxidants. The initial concentration of galvinoxyl was 10^{-5} M. The data are expressed as the mean ± SD of n = 3 independent experiments.

Sample	Time of incubation		
	5 min	10 min	15 min
Galvinoxyl standard 10^{-5} M	10.0 ± 0.1	10.0 ± 0.0	9.9 ± 0.1
+ Baicalein 10^{-5} M	0.3 ± 0.2	n.d.	n.d.
+ Baicalein 10^{-6} M	5.8 ± 1.1	1.3 ± 0.4	0.1 ± 0.1
+ Baicalein 10^{-7} M	9.4 ± 0.8	6.8 ± 0.4	6.2 ± 0.7
+ 5,6-DHF 10^{-5} M	n.d.	n.d.	n.d.
+ 5,6-DHF 10^{-6} M	4.7 ± 0.7	0.9 ± 0.6	0.2 ± 0.2
+ Mosloflavone 10^{-5} M	9.7 ± 0.2	9.9 ± 0.1	9.6 ± 0.2
+ Negletein 10^{-5} M	n.d.	n.d.	n.d.
+ Negletein 10^{-6} M	6.4 ± 0.8	2.0 ± 0.4	0.8 ± 0.4

6 DISCUSSION

This work is focused on establishing antioxidant activity of some polyphenols that would have potential to decrease the level of reactive oxygen species and resulting oxidative stress, which is closely related to the aging and various diseases.

Four flavones and two representatives of phenolic acids were tested using two types of radical generators (cumene hydroperoxide and galvinoxyl) by three types of methods: dichlorofluorescein assay and cytotoxicity assay (methods for determination of intracellular antioxidant activity) and electron paramagnetic resonance spectroscopy (method for determination of antioxidant activity *in vitro*). The first part of the discussion is dedicated to the effects of flavones and results obtained with gallic acid and its derivative 1625 are discussed in the second part.

Mosloflavone (5-hydroxy-6,7-dimethoxyflavone) is an active component of plant *Desmos chinensis* (Van Kiem et al. 2005), while **negletein** (5,6-dihydroxy-7-methoxyflavone) is naturally occurring flavonoid found in the plants *Colebrookea oppositifolia* (Righi et al. 2010) and *Centauria clementei* (Yang et al. 1996). **Baicalein** (5,6,7-trihydroxyflavone) is present in *Scutellaria baicalensis* (Hamada et al. 1993) and **5,6-dihydroxyflavone** is found in *Primula modesta*. Although all flavones used in this work are naturally occurring compounds as is indicated by foregoing sentences, the chemicals employed in our experiments were chemically synthesized by Prof. Bovicelli at the Department of Chemistry, University of Rome "La Sapienza".

Flavonoids with maximal antioxidant effect have the following structural features in their molecules: 3',4'-*o*-dihydroxy structure in the B ring (presence of three hydroxyl groups in the B ring further increases antioxidant activity); 2,3-double bond in conjugation with 4-oxo function in the C ring; 3 and 5-hydroxyl groups with 4-oxo function in A and C rings (Pietta 2000). None of the tested flavones fulfill the first condition (presence of catechol group in the B ring), but they are substituted by hydroxyl and/or methoxyl groups in the position 5, 6 or 7 in the ring A that perform a similar function as the catechol group in the ring B.

Evaluation of intracellular ROS production by dichlorofluorescein assay proved the statement of Pietta (2000). The molecule of 5,6-DHF up to the concentration 10^{-11} M and baicalein even up to the concentration 10^{-13} M were still able to decrease ROS production. This effect could be explained by the presence of two hydroxyl groups in *ortho*-position in the molecule of 5,6-DHF, which may create stable quinone during the reaction with two molecules of free radicals (Pietta 2000). In case of baicalein, there

are even three hydroxyl groups next to each other that have stronger scavenging effect than just two hydroxyl groups. Baicalein may protect the cells also by some other mechanism that has not been fully explained yet (Shao et al. 2002). Mosloflavone is a compound which contains two methoxyl groups and only one hydroxyl group and negletein has one catechol group and one methoxyl group. Both these flavones seem to possess low antioxidant activity. Mosloflavone does not have any catechol group and so it cannot trap free radical and in the molecule of negletein is methoxyl group that probably sterically shields the reaction between antioxidant and free radical. These structural features could explain their mild scavenging activity at low concentrations (10^{-6}-10^{-9} M). The reason of their rather pro-oxidant activity in the highest tested concentration (10^{-5} M) may be caused due to their methoxyl group(s) or generated metabolites that could somehow impair cells or interfere with their regular metabolism. However, no explanation of the mechanism of this pro-oxidative effect or additional information on this topic has been found in the scientific literature.

The results of cytotoxicity assay obtained with 5,6-DHF and baicalein confirmed their high antioxidant activity. 5,6-DHF protected over 82% of cells and baicalein even 104.9% at the concentration 10^{-5} M. On the other hand, negletein exerted slightly pro-oxidative effect at the concentration 10^{-5} M, which means that in the sample containing cumene hydroperoxide alone survived 56.32% of cells, while in the sample containing also negletein 10^{-5} and 10^{-8} survived only 45.8% and 59.35% of cells, respectively. The hypothesis based on the possibility that methoxyl group present in the molecule of negletein may be responsible for the decrease of its antioxidant ability in the cells is supported by obtained results. However, that theory needs to be further explored and verified. Mosloflavone is not supposed to be a good scavenger, because of its structure, but surprisingly it protected cells against the activity of cumene hydroperoxide to the similar extent as 5,6-DHF. Unfortunately, no scientific article that would elucidate this phenomenon was found.

The *in vitro* antioxidant activity of all four flavones towards synthetic radical galvinoxyl was tested using EPR spectroscopy. Baicalein and 5,6-DHF (10^{-5} M) exerted very high scavenging ability at all measured times, when both compounds trapped all galvinoxyl radicals in the sample. This scavenging ability weakened with their decreasing concentration, but both compounds (10^{-6} M) were still able to trap merely all radicals after 15 min of incubation. Both compounds proved to have comparable scavenging ability towards galvinoxyl. EPR spectrum of baicalein 10^{-7} M indicated that

it was still able to reduce galvinoxyl. These outcomes confirmed that 5,6-DHF and baicalein are good antioxidants and their antioxidant activity is probably regarded to their catechol group in the ring A, which traps free radicals. In the case of baicalein, its third hydroxyl group supports the catechol group in scavenging of free radicals. Mosloflavone has no catechol group suggesting low or no scavenging ability of this compound. This assumption was confirmed by results of EPR, because mosloflavone (10^{-5} M) did not decrease the galvinoxyl level at any measured time. Surprisingly, the effect of negletein in EPR showed antioxidant activity comparable with that of 5,6-DHF or baicalein. This is interesting result, because negletein did not show similar effect in other two assays in cell culture and it had even pro-oxidative effect at the concentration 10^{-5} M.

Gallic acid (**GA**, 3,4,5-trihydroxybenzoic acid) is naturally occurring compound that is found in various natural products like tea leaves, oak bark, grapes, red and white wine, strawberries, pineapples, bananas, and lemons (Sun et al. 2002). GA and its derivative **compound 1625** [methyl 3,4,5-tris(acetyloxy)benzoate] were synthesized by Prof. Parmar at the Department of Chemistry, University of Delhi.

Dichlorofluorescein assay with GA showed that GA is less active than tested flavones. It was able to decrease the ROS production to 36% at the highest concentration 10^{-5} M, but its activity steeply decreased at lower concentrations of GA. In contrast, the results of compound 1625 showed high scavenging activity. This discrepancy has been already solved out in the work of Lu et al. (2006), where the authors reported that hydrophobicity of an antioxidant is a particularly important factor. GA (pKa ~ 4.0) is negatively charged compound with hydrophilic character therefore it is not able to cross the cell membrane. The molecule of compound 1625 is more hydrophobic and may easily enter to the cells and attenuate ROS formation. The hypothesized mechanism and explanation for greater scavenging ability of compound 1625 is that this compound crosses the cell membrane, its ester bonds are cleaved by cell esterases and resulting GA is able to react with free radicals. Therefore, this compound serves as a "pro-drug" of GA, which has the ability to cross cell membranes and thus deliver active antioxidant to the cell.

Outcomes of both molecules in the cytotoxicity assay did not confirm results obtained in dichlorofluorescein assay, not even at the concentration 10^{-5} M. According to the results of the Candelotti (2009), compound 1625 has better inhibitory effect on

ROS production in THP-1 cells after 30 minutes of pre-incubation with this antioxidant and EPR spectroscopy (data not included in this thesis) confirmed that this compound can scavenge a free radical galvinoxyl after longer time period. Nevertheless, compound 1625 suppresses the cell proliferation, which could be an explanation of its incapability to protect THP-1 cells in the cytotoxicity assay. Longer pre-incubation with this antioxidant may be needed. Other possible reason of its low protective effect in the cytotoxicity assay could be too high concentration of cumene hydroperoxide (222 μM) used throughout the experiments.

7 CONCLUSION

- Good ROS scavenging ability of baicalein and 5,6-dihydroxyflavone in all tested concentrations was proved using dichlorofluorescein assay, while negletein and mosloflavone exerted mild pro-oxidative effect at the highest concentration (10^{-5} M). Gallic acid and compound 1625 had lower antioxidant ability compared to the flavones baicalein and 5,6-DHF. In the cytotoxicity assay, baicalein and 5,6-DHF effectively protected THP-1 cells against the effect of cumene hydroperoxide, while the other tested compounds had no effect.

- EPR spectroscopy confirmed excellent scavenging ability of baicalein and 5,6-DHF. Surprisingly, also negletein (10^{-5} M) was able to trap all galvinoxyl radicals in the sample. On the other hand, mosloflavone possessed no antioxidant activity.

- The structure-activity relationship in the group of polyphenols was found. The flavones containing catechol group in the ring A showed much higher antioxidant activity (baicalein and 5,6-dihydroxyflavone) than compound with lacking catechol group (mosloflavone). Negletein containing the catechol and methoxyl group in the ring A had very low antioxidant activity compared to 5,6-DHF and baicalein, which could be caused by its methoxyl group that may cause decrease of its antioxidant ability in the cells. This hypothesis is supported by the results of EPR assay, where negletein showed to be a good scavenger with effect comparable to that of 5,6-DHF or baicalein. The GA has a potential to be a good scavenger *in vitro*, but it hardly crosses the cell membrane due to its negative charge. Its ester compound 1625 showed a stronger antioxidant activity in dichlorofluorescein assay, but it had also inhibitory effect on the cell proliferation, which could be a reason of its lower protective activity observed in the cytotoxicity assay. This compound has a potential to be a good scavenger in living organisms and deserves further research.

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8 LIST OF ABBREVIATIONS

AOX	Antioxidant(s)
CAT	Catalase
DCF	2',7'-dichlorodihydrofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescin diacetate
DMSO	Dimethyl sulfoxide
EPR	Electron paramagnetic resonance spectroscopy
GRX	Glutaredoxin
GSH	Reduced glutathione
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorite, hypochlorous acid
LDL	Low density lipoprotein(s)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamid adenine dinucleotide phosphate
$NO^{\bullet}$	Nitric oxide
$O_2^{\bullet-}$	Superoxide radical
O_2^{2-}	Peroxide ion
$^{\bullet}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
ONOOH	Peroxynitrous acid
PBS	Phosphate buffered saline
RNS	Reactive nitrogen species
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SOD	Superoxide dismutase
TR	Thioredoxin reductase
TRX	Thioredoxin

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