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BIOLOGICAL ACTIVITY OF ANTIOXIDANT COMPOUNDS IN L-6 MYOBLASTS FROM RAT SKELETAL MUSCLES

Diploma Thesis

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ABSTRACT

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Title of diploma thesis: Biological activity of antioxidant compounds in L-6 myoblasts from rat skeletal muscles.

This work was 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 and oxidative stress. The reactive oxygen and nitrogen species play an important role as regulatory mediators of physiological responses. However, they can be also very harmful because they can damage DNA and proteins, cause peroxidation of lipids and injure other biomoleculs. In my work, I tested the antioxidant effect of different polyphenols (baicalein, 5,6-dihydroxyflavone, negletein, mosloflavone, gallic acid and its derivative) in the cell culture (L-6 myoblasts from rat skeletal muscles) and in the *in vitro* test. The state of oxidative stress was induced by cumene hydroperoxide (generator of cumene hydroperoxide radical) and galvinoxyl. The methods used to evaluate the antioxidant activity were the cytotoxicity assay (MTT), fluorescence assay (DCFH-DA method) and electron paramagnetic resonance spectroscopy (EPR). Baicalein, 5,6-dihydroxyflavone and the synthetic ester of gallic acid (methyl-3,4,5-triacetoxybenzoate) showed the significant scavenging ability of free radicals at concentrations 10^{-5}-10^{-7} M. Negletein showed prooxidative effect in the cells at concentration 10^{-5} M but in the *in vitro* test its scavenging ability was comparable with baicalein or 5,6-dihydroxyflavone. Mosloflavone did not show any scavenging ability at all. Gallic acid, because of its hydrophilic character, was not able to enter the cells and trap radicals. On the other hand, these compounds were able to inhibit the cell proliferation.

ABSTRAKT

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Název diplomové práce: Biologická aktivita antioxidačních látek v L-6 myoblastech ze svalů potkana.

Tato práce byla vytvořena v laboratoři fyziologie na univerzitě Roma Tre v Římě pod vedením Prof. Sandry Incerpi. Tato laboratoř se zabývá výzkumem antioxidantů různých struktur a vyhodnocováním jejich schopnosti chránit proti vzniku reaktivních forem kyslíku a oxidačnímu stresu. Reaktivní formy kyslíku a dusíku hrají důležitou roli jako regulační mediátory ve fyziologických odpovědích. Mohou však i škodit a způsobit poškození DNA a bílkovin, peroxidaci lipidů a poškození dalších biomolekul. Ve své práci jsem studovala různé polyfenoly (bajkalein, 5,6-dihydroxyflavon, negletein, kyselinu galovou a její derivát) v buněčné kultuře (L-6 myoblasty ze svalů potkana) a v *in vitro* testu. K vyvolání oxidačního stresu jsem použila kumenhydroperoxid (zdroj kumenhydroperoxid radikálu) a galvinoxyl. K vyhodnocení aktivity antioxidantů jsem použila test cytotoxicity (MTT), fluorescenční metodu (DCFH-DA metoda) a elektronovou paramagnetickou resonanční spektroskopii (EPR). Dobrou antioxidační aktivitu prokázal bajkalein, 5,6-dihydroxyflavon a ester kyseliny galové (methyl-3,4,5-triacetoxybenzoát) zejména v koncentracích 10^{-5}-10^{-7} M. Antioxidační aktivita negleteinu byla v *in vitro* testu srovnatelná s bajkaleinem a 5,6-dihydroxyflavonem, naproti tomu v buněčné kultuře vykazoval v koncentraci 10^{-5} M prooxidační účinek. Mosloflavone neprokázal žádnou antioxidační schopnost. Kyselina galová nebyla díky svému hydrofilnímu charakteru schopna prostupovat do buněk a tedy vychytávat volné radikály. Vedle antioxidačních vlastností bylo také zjištěno, že tyto látky tlumí buněčnou proliferaci.

Contents

1 INTRODUCTION

This work was carried out in the laboratory of physiology at University Roma Tre in Rome. The team of this laboratory under the supervision of Prof. Sandra Incerpi studies the antioxidants of different structures and evaluates their ability to prevent the production of free radicals.

In last several years the topic of free radicals, oxidative stress and production of reactive oxygen (ROS) and nitrogen species (RNS) is widely discussed. ROS and RNS play important role in the physiological functions, they act as regulatory mediators of many physiological responses (e.g. control of vascular tone, control of ventilation, redox homeostasis etc.) but they can be also harmful. These compounds can cause damage to DNA, lipids, proteins and other biomolecules and that is why they participate in the progress of many diseases as neurodegenerative diseases, atherosclerosis and other cardiovascular diseases, malignant diseases, diabetes mellitus etc. The free radicals and oxidative stress are also included in the theory of aging.

The antioxidants are substances that may decrease production of ROS and slow down the development of oxidative stress. Plants, fruits and vegetables are sources of exogenous (also called dietary) antioxidants that include vitamin C and E, carotenoids, and plant phenolic compounds (e.g. flavonoids and phenolic acids). Nowadays, some of these compounds are already used as dietary supplements. There are many studies suggesting that plant polyphenols have an ability to reduce free radical formation and to scavenge free radicals. They could be used in future in the prevention of oxidative stress related diseases. All these hypotheses became subject of present researches.

This work is focused on the evaluation of antioxidant activity of polyphenols different in structure.

2 GENERAL PART

2.1 THE OXYGEN AND ITS POTENTIAL TOXICITY

Oxygen itself is a biradical which contains two unpaired electrons with parallel spins. This is the most stable form of O_2 (also called ground state oxygen) which exists in the air around us (McCord 2000, Haliwell 2006). In contrast, all stable organic molecules have electrons arranged in pairs with antiparallel spins (Fridovich 1999). Thermodynamically, oxygen wants to accept additional electrons (two per atom, four per molecule) to produce water molecules, which have much lower free energy. It is not possible for oxygen to accept pair of electrons with antiparallel spin states due to unconventional distribution of electrons, until one of its unpaired electrons is able to spin inversion to make pairing possible. Fortunately, there is a kinetic barrier (large energy is needed to activate this oxidative reaction), which saves us from reacting with surrounding molecules. On the other hand, this kinetic restriction makes oxygen an ideal terminal electron acceptor for biological systems because enzymes can hold oxygen in contact and help it to overcome this kinetic barrier (McCord 2000). So the electrons are transferred to O_2 one at a time. Finally four electrons (and four protons) are needed to reduce O_2 into two molecules of water (Fig. 1) (Fridovich 1999).

$$O_2 \xrightarrow{e^-} O_2^{-\cdot} \xrightarrow{e^-,\,2H^+} H_2O_2 \xrightarrow{e^-} OH^- + OH^{\cdot} \xrightarrow{e^-,\,2H^+} 2H_2O$$

Fig. 1. The pathway of oxygen reduction (Fridovich 1999)

The ability of O_2 to accept electrons one at a time enables to break up electron pairs and results in free radical formation. If one electron is transferred to O_2, the product is the superoxide anion radical ($O_2^{\bullet-}$), if two electrons are transferred, it results in production of hydrogen peroxid (H_2O_2), which is a non-radical. The connection of the third electron to H_2O_2 causes lysis of the O-O bond and one fragment is reduced to water and other fragment is hydroxyl radical ($^{\bullet}$OH), very strong oxidant (McCord 2000).

2.2 FREE RADICALS

A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge 2007). Although most molecules *in vivo* are non-radicals, there are also many free radicals in the living organisms. Radicals can be formed by several mechanisms: adding of a single electron

to a non-radical (reduction), loss of a single electron from a non-radical (oxidation) or cleavage of covalent bond (homolytic fission) (Halliwell 2006). Free radicals can be products of homolytic, heterolytic, or redox reaction producing either charged or uncharged radicals species (Powers and Jackson 2008). Free radicals are very reactive due to an unpaired electron, because electrons are more stable when are paired together in orbital. When two radicals meet, they can combine their unpaired electrons and create a covalent bond (it means loss of activity). In case radical gives one electron to, or takes one from other molecule, this molecule becomes a radical. This type of reaction is called *chain reaction*: one radical begets other one, and so on (Halliwell 1989). The oxygen-centered radicals and also reactive non-radical derivates of oxygen (e.g. hydrogen peroxide) are called *reactive oxygen species* (ROS). Similarly, nitrogen radicals and other reactive molecules that contain nitrogen as reactive center are called *reactive nitrogen species* (RNS). The common term *reactive oxygen and nitrogen species* (RONS), that covers both types, is often used (Powers and Jackson 2008).

2.3 REACTIVE OXYGEN AND NITROGEN SPECIES

The reactive oxygen and nitrogen species are products of normal cellular metabolism and play a dual role. They have both positive biological effects, because they are involved in many physiological processes, and harmful effects, because they are able to cause biological damage (termed as oxidative and nitrosative stress) (Valko et al. 2007). Free radicals have important role in phagocytosis as microbicidal system and take part in other biochemical reactions (e.g. hydroxylation, carboxylation or peroxidation reactions, or in the reduction of ribonucleotides). They have also biomodulating and regulatory abilities in signal transduction processes during transduction of intercellular information (Ďuračková et al. 1999). Among other positive biological effects of free radicals belong their participation in the formation of the "second messenger" cGMP, control of smooth muscle relaxation, sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration, enhancement of signal transduction from various membrane receptors, ensuring of redox homeostasis, and inhibition of platelet adhesion (Tab. 1) (Dröge 2002).

Tab. 1. Important physiological functions that involve free radicals or their derivates (Dröge 2002).

Type of Radical	Source of Radical	Physiological Process
Nitric oxide ($NO^{\bullet}$)	Nitric oxide synthase	Smooth muscle relaxation and various other cGMP-dependent functions.
Superoxide ($O_2^{\bullet-}$)	NAD(P)H oxidase	Control of ventilation. Control of erythropoietin production, hypoxia-inducible functions. Smooth muscle relaxation. Signal transduction from membrane receptors of immunological functions.
Superoxide ($O_2^{\bullet-}$) and related ROS	Any source	Oxidative stress responses and the maintenance of redox homeostasis.

2.3.1 SOURCES OF REACTIVE SPECIES

The most important sources of oxidants in cells are:

a) The *aerobic respiration* when mitochondria consume O_2 and reduce it into H_2O. The undesirable products of this process are superoxide radicals, hydrogen peroxide and hydroxyl radicals.
b) *Phagocytic cells* that use the oxidative burst generating nitric oxide, superoxide, hydrogen peroxide and hypochlorite to destroy bacteria or viruses. The problem comes when chronic infection results in chronic phagocytic activity and chronic inflammation, which is a risk factor for various diseases and cancer.
c) *Peroxisomes*, organelles responsible for degradation of fatty acids and other molecules producing H_2O_2 as by-product, which is degraded by catalase and other enzymes. The part of H_2O_2 can escape and contribute to damage of DNA.
d) *Cytochrome P450*, one of the defense systems that prevents the formation of toxic molecules but also results in oxidant by-products.

There are various exogenous sources contributing to the production of reactive species (e.g. cigarette smoke, iron and cooper salts, excessive intake of phenolic compounds, air pollution and various sources of radiation) (Ames et al. 1993).

2.3.2 THE MAIN TYPES OF ROS

Superoxide ($O_2^{\bullet-}$) is formed by the reduction *(1)* of triplet-state molecular oxygen ($^3O_2$) (Fig. 2).

$$O_2 + e^- \rightarrow O_2^{-\cdot} \qquad (1)$$

This process includes enzymes such as NAD(P)H oxidase and xanthine oxidase, or it can proceed non-enzymatically by redox-reactive compounds of the mitochondrial electron transport chain such as semi-ubiquinone (Dröge 2002). Just the mitochondria are the main place of the $O_2^{\bullet-}$ production (Cadenas and Sies 1998). It has been suggested that 1-3 % of all electrons in the transport chain leak to oxygen to generate superoxide (Valko et al. 2007).

Superoxide is negatively charged and it is relatively membrane impermeable. Nonetheless, its relatively long half-life, compared with other free radicals, enables diffusion within the cells and consequently the increase in number of potential targets (Powers and Jackson 2008). Although superoxide is considered relatively unreactive, it can react rapidly with some radicals as $NO^{\bullet}$ and with some iron-sulphur clusters in proteins (Halliwell and Gutteridge 2007). This compound can reduce some biological materials (e.g. cytochrome *c*) and oxidize some other molecules, such as ascorbate. Superoxide undergoes dismutation into hydrogen peroxide and oxygen *(2)* by a reaction that is catalyzed by superoxide dismutase (SOD) (Fig 2.) and which provides a major source of hydrogen peroxide in cells (Powers and Jackson 2008).

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (2)$$

Some of the $O_2^{\bullet-}$ formation *in vivo* can be a chemical accident (e.g. in the mitochondria the electrons passing through the respiratory chain can leak from the electron carriers and pass directly onto oxygen) (Halliwell 1989). On the other hand, the production of superoxide can be required by organism, for example it is generated *in vivo* by activated phagocytes to kill some bacteria that can be harmful (Curnutte and Babior 1987). In case this essential defense mechanism goes wrong, the excessive activation of phagocytes can lead to tissues damage and this is a cause of several inflammatory diseases (Halliwell 1989).

Hydrogen peroxide (H_2O_2) is stable, permeable to membranes and it has a relatively long half-life within the cell. Hydrogen peroxide is cytotoxic, but it is considered a relatively weak oxidizing agent. Its cytotoxicity is a result of its ability to generate hydroxyl radical through metal-catalyzed reaction, such as *Fenton reaction (3*) (Fig. 2) (Powers and Jackson 2008). In biology, Fenton reaction is an important part of the *Haber-Weiss reaction (4),* where iron (or copper) is maintained in a reduced form by superoxide and this makes it able to catalyze the formation of hydroxyl radical from hydrogen peroxide (Halliwell 1995):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\cdot}OH + {}^{-}OH \qquad (3)$$

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe^{2+}/Cu^{1+}} O_2 + {}^{\cdot}OH + {}^{-}OH \qquad (4)$$

The Haber-Weiss reaction *(4)* involves two steps:

$$Fe^{3+} / Cu^{2+} + O_2^{\cdot -} \rightarrow Fe^{2+} / Cu^{+} + O_2$$

$$H_2O_2 + Fe^{2+} / Cu^{+} \rightarrow Fe^{3+} / Cu^{2+} + {}^{\cdot}OH + {}^{-}OH$$

Hydrogen peroxide is produced in peroxisomes, the major sites of oxygen consumption in the cells. Peroxisomes contain also enzymes that decompose hydrogen peroxide and prevent its accumulation. Hydrogen peroxide is converted into water mainly by glutathione peroxidase, or in the case of its excessive accumulation also by catalase (Dröge 2002, Valko et al. 2007).

Hydroxyl radical ($^{\cdot}OH$) is a highly reactive compound with a strong oxidizing potential. Due to its high reactivity, it is not able to cross the membranes and reacts immediately (in the range of nanoseconds) with all surrounding molecules (Powers and Jackson 2008). It can attack and damage almost every molecule found in living cells. The generation of hydroxyl radical is also the major mechanism by which malignant cells are killed during radiotherapy. Most of the energy of radiation (e.g. gamma-radiation) is absorbed by the cell water. This radiation causes splitting of oxygen-hydrogen covalent bond in water, giving a rise to two radicals, hydrogen radical H· and hydroxyl radical $^{\cdot}$OH *(5)*:

$$H-O-H \xrightarrow{radiation} H^{\cdot} + {}^{\cdot}OH \qquad (5)$$

When $^{\cdot}$OH is generated close to membranes, it can attack the fatty acid side-chains of the membrane phospholipids and cause biological damage called the *lipid peroxidation* (Fig. 2).

Singlet oxygen ($^1O_2$) is an electronically excited form of oxygen and it is not a radical. This compound has a very short half-life; it is capable of diffusion and permeation through the membranes. In the biological systems, it is formed for example during dismutation of superoxide anion in water [reaction *(2)* mentioned above] (Powers and Jackson 2008). The oxidizing ability of singlet oxygen is greatly increased compared to triplet oxygen. Singlet oxygen can directly oxidize proteins, DNA, and lipids (Foote et al. 1985). Some dietary plants contain photosensitizers of singlet oxygen (e.g. hypericin in *Hypericum perforatum*; psoralens in *Apium graveolens*). The same effect can be caused also by some drugs (e.g. fluoroquinolone antibiotics) (Morison 2004).

Hypochlorite (HOCl/OCl$^-$) is one of the strongest ROS, a highly reactive oxidant produced by activated phagocytes, neutrophils and monocytes via the reaction of H_2O_2 with Cl^- ions catalyzed by hem enzyme myeloperoxidase *(6)* (Kettle and Winterbourn 1997). The production of hypochlorite is one of the defense mechanisms against invading microorganisms, but it can be also a cause of tissue damage (Winterbourn and Kettle 2000). Hypochlorite can cause damage to various biomolecules (oxidizing thiols, lipids, ascorbate, NADPH) with the generation of various secondary products. Moreover this compound in the acid form (hypochlorous acid, HOCl) can cross cell membranes and cause fragmentation and aggregation of proteins by multiple reactions (Halliwell and Gutteridge 2007).

$$H_2O_2 + Cl^- \xrightarrow{myeloperoxidase} HOCl + {}^-OH \qquad (6)$$

2.3.3 THE MAIN TYPES OF RNS

Nitric oxide ($NO^{\bullet}$) is synthesized in higher organisms from the amino acid L-arginine by nitric oxide synthases (NOS). There are three main types of NOS: *neuronal NOS* (NOS1), which was first found in nervous tissue but it is present in most cell types; *inducible NOS* (NOS2) is widespread, but it is mainly found under inflammatory conditions; and *endothelial NOS* (NOS3), originally described in endothelial cells (Powers and Jackson 2008).

NO is an important biological signaling molecule, it plays role in various physiological processes, such as neurotransmission, regulation of blood pressure, defense mechanisms, smooth muscle relaxation, and immune regulation (Bergendi et al. 1999). NO is a weak reducing agent, which reacts with oxygen to form nitric dioxide and also with superoxide to produce peroxynitrite (Halliwell 1994). The latter mentioned reaction proceeds rapidly. The reaction between NO and superoxide *(7)* takes part in the regulation of vascular tone, because NO is identical to endothelium-derived relaxing factor (EDRF), which is produced by endothelium as an important mediator of vasodilator responses induced by pharmacological agents including acetylcholine and bradykinin. Therefore controlled production of NO and $O_2^{\bullet-}$ by endothelium provides one controlling mechanism for regulation of vascular tone (Halliwell 1989):

$$O_2^{-\cdot} + NO^{\cdot} \rightarrow ONOO^{-} \qquad (7)$$

The production of superoxide anion and nitric oxide occurs also during oxidative burst in the inflammatory processes and results in the formation of peroxynitrite anion (Fig. 2), which can damage DNA and cause lipid oxidation (Carr et al. 2000).

Peroxynitrite ($ONOO^{-}$) is a strong oxidizing agent. It can decrease the amount of thiol groups, damage molecule of DNA and can cause nitration of proteins. Other effect of the peroxynitrite formation is decrease in the bioavailability of $O_2^{\bullet-}$ and NO. The reaction between $O_2^{\bullet-}$ and NO to produce peroxynitrite *(7)* is three times faster than the dismutation of superoxide to hydrogen peroxide (Powers and Jackson 2008).

Both nitric oxide and peroxynitrite belong to RNS (reactive nitrogen species) and their overproduction causes imbalance called nitrosative stress, which may lead to

nitrosylation reactions that can change the structure of proteins and influence their normal functions (Valko et al. 2007).

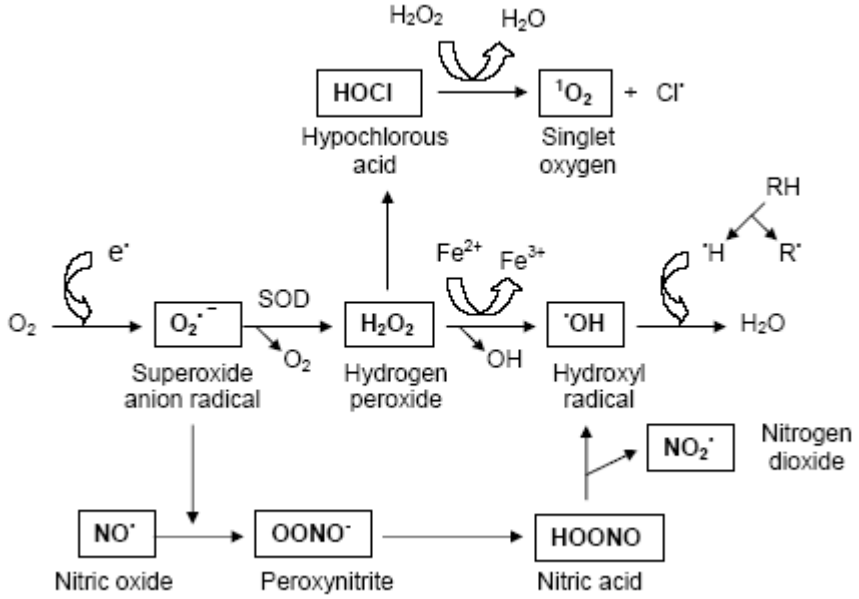

Fig. 2. Mutual association between free radicals and their reactive metabolites (Ďuračková 2009)

2.4 OXIDATIVE STRESS

The state of oxidative stress (OS) comes, when the balance between ROS and the available antioxidants is impaired. Sies (1997) defined oxidative stress as a disturbance in the prooxidant-antioxidant balance leading to a potential damage (Fig. 3). This damage to biomolecules, called oxidative damage, is a consequence of an attack of reactive oxygen species (ROS), which are components of living organisms (Halliwell and Whiteman 2004). The oxidative stress is very complicated and complex process and its impact on the organism depends on the type of oxidant, on the intensity of its production, on the composition and activities of various antioxidants, and on the ability of repair systems (Ďuračková 2007).

Oxidants are formed as products of normal metabolism, but they can be also produced in the inadequate amount under pathophysiological conditions (Sies 1997). One cause of OS can be decreased level of antioxidants and depletion of dietary antioxidants as well as of essential dietary constituents (copper, zinc, iron, magnesium) (Halliwell 2006). The OS can also result from toxins producing ROS or higher

production of ROS by natural system, e.g. inadequate activity of phagocytes (Halliwell and Gutteridge 2007). Other cause is exposure to a variety of agents present in the environment. Exogenous sources of ROS, which contribute to the increase in oxidative stress, are cigarette smoke, ionizing radiation, drugs, ethanol, chemical reagents, industrial solvents, and air pollution (Fig. 3) (Zadák et al. 2009).

What happens and how cells fight against the oxidative stress? It depends how serious the damage is. Cells can increase their proliferation, up-regulate their defense and repair systems, and use other protective mechanism. It might happen that the cells survive but cannot further proliferate. Severe oxidative damage, especially to DNA, can lead to apoptosis, necrosis, or mechanisms with features of both (Halliwell 2006).

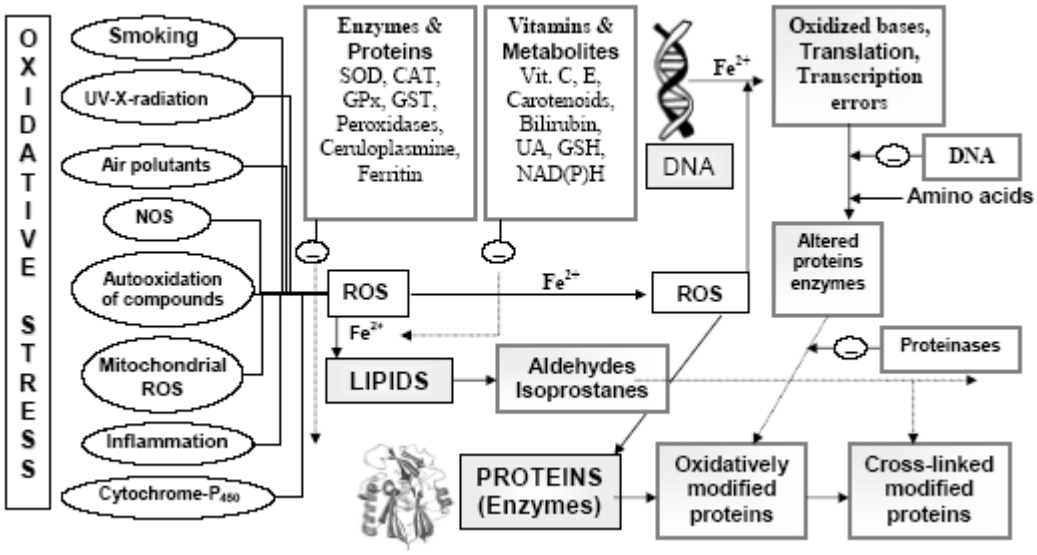

Fig. 3. Mutual association between oxidants and antioxidants (Ďuračková 2009)

NOS - NO-synthase, ROS - reactive oxygen species, CAT - catalase, SOD - superoxide dismutase, GPx - glutathione peroxidase, GST - glutathione S-transferase, UA - uric acid, GSH - reduced glutathione

2.5 ROS, HUMAN DISEASES AND AGEING

Oxidative stress plays a significant role in various pathological conditions. The important examples include cardiovascular diseases, cancer, diabetes, neurological disorders, ischemia, and HIV infection. These diseases can be divided into two groups. The first category, called „mitochondrial oxidative stress“ conditions (including diabetes mellitus and cancer), is characterized by imbalance in the thiol/disulfide redox state and impaired glucose tolerance. The second category may be called „inflammatory

oxidative conditions“. This group is characterized by the excessive stimulation of NAD(P)H oxidase activity by cytokines and some other agents, which leads to atherosclerosis and chronic inflammation, or xanthine oxidase that increases formation of ROS (e.g. ischemia and reperfusion injury) (Dröge 2002, Valko et al 2007).

The free radicals are involved also in the process of aging. The „free radical theory of aging“, first introduced in 1956 by Denham Harman (Valko et al. 2007), provides an explanation for the relation between disease and aging. The disease is a combination of changes that affect physiological functions and may lead to death. Nowadays the free radical diseases (cancer and atherosclerosis) are two major causes of death (Harman 1991). The free radical theory of aging is based on the fact that damaging effects of reactive species to DNA, lipids and proteins accumulate over time. The cells can repair much of the damage to nuclear DNA, but not to the mitochondrial DNA (mtDNA). This extensive mtDNA impairment accumulates, the cells die and the organism grows old. The repair activity declines with age, however antioxidant status does not change significantly with age (Valko et al 2007). The aging is also associated with accumulation of oxidized forms of proteins that results in the loss of catalyzing activities of enzymes (Stadtman 2004). The oxidative stress was also shown to cause shortening of telomeres, which are essential to the maintenance of genomic integrity. Telomere dysfunction plays also critical role in aging. There is evidence that the aging is a multifactorial process and not only DNA and protein damage is responsible for observed pathophysiological changes (Valko et al. 2007).

2.6 **ANTIOXIDANT DEFENSE SYSTEM**

The organisms were constraint on developing series of defense mechanisms due to long lasting exposure to free radicals (Cadenas 1997). Defense mechanisms against free radical-induced oxidative stress involve: preventative mechanisms, repair mechanisms, physical defenses, and antioxidant defenses (Valko et al. 2007). Antioxidant can be defined as: “any substance that delays or prevents the oxidation of a substrate” (i.e. all molecules found *in vivo*) (Halliwell and Gutteridge 2007). The antioxidant defenses can be divided into enzymatic and non-enzymatic ones (Tab. 2). *Enzymatic antioxidants* include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Compounds produced in the organism (endogenous antioxidants) such as uric acid,

lipoic acid, glutathione, ubiquinone (coenzyme Q_{10}), plasma protein thiols, and transport proteins, belong to the group of *non-enzymatic* antioxidants. Besides them exogenous antioxidants that are present in diet, e.g. vitamin C, vitamin E, carotenoids, flavonoids, and other polyphenolic compounds synthesized in plants, play important role in the protection of organisms against oxidative damage. Activities and intracellular levels of these antioxidants are under normal conditions in balance, which is essential for the survival of organisms and their health (Valko et al. 2007, Ďuračková 2008).

Tab. 2. The most important endogenous and exogenous antioxidants

Antioxidant defense system		
Enzymatic AOX	***Non-enzymatic AOX***	
endogenous		***exogenous***
Superoxide dismutase (SOD)	Glutathione	Vitamin C
Glutathione peroxidase (GPX)	Ubiquinone (Coenzyme Q_{10})	Vitamin E
Catalase (CAT)	Lipoic acid	Carotenoids
Thioredoxin and glutaredoxin	Uric acid	Plant polyphenols
	Transition metal binding proteins (transferrin, ferritin)	
	Plasma protein thiols	

2.6.1 ENZYMATIC ANTIOXIDANTS

Superoxide dismutase (SOD) is an enzyme that constitutes the first line of defense against superoxide radicals. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide and oxygen, i.e. dismutation of superoxide *(8)*.

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (8)$$

Although superoxide radicals are not highly toxic, it is essential for cells to take control of their level because they can attack and extract electrons from biological membranes and thus initiate a radical reaction. Superoxide radicals can also contribute to the formation of hydroxyl radical and react with NO to form peroxynitrite.

In mammals, there are three isoforms of SOD. *SOD1 (CuZnSOD),* primarily located in the cytosol and the mitochondrial intermembrane space, requires copper-zinc as a cofactor in its active site. *SOD2 (MnSOD)* is located in the mitochodrial matrix and contains manganese in the active site. *SOD3 (CuZnSOD)* is located in the extracellular space and also requires copper-zinc as cofactor (Powers and Jackson 2008). The active site metal is in all SODs reduced by one $O_2^{\bullet-}$ and then reoxidized by the next one. It means that it transfers an electron from one $O_2^{\bullet-}$ to the next while avoiding the electrostatic repulsion that would hinder from direct electron transfer between two $O_2^{\bullet-}$ (Fridovich 1999).

Catalase (CAT) is one of the hydrogen peroxide-removing enzymes, which collaborates with SOD. CAT converts H_2O_2 to oxygen and two molecules of water *(9).* It is located in the peroxisomes of the most mammalian cells.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \qquad (9)$$

Most CATs are hem-containing enzymes; in their active site is present iron as cofactor (Fridovich 1999).

Glutathione peroxidase (GPX) is the most important hydrogen peroxide-removing enzyme in animals (Brigelius-Flohe 1999). There were identified five glutathione peroxidases in mammals (GPX1-GPX5), which differ in the substrate specificity and the cellular localization (Brigelius-Flohe 1999).

GPX is selenium-containing metalloenzyme that catalyzes the reduction of H_2O_2 *(10)* or organic hydroperoxide (ROOH) *(11)* to water (H_2O) and alcohol (ROH) using reduced glutathione (GSH), which is oxidized to glutathione disulfide (GSSG) (Powers and Jackson 2008). The regeneration of GSH is provided by glutathione reductase, where NADPH has the reducing power (Meister and Anderson 1983).

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad (10)$$

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O \qquad (11)$$

The cells contain also several other enzymes, such as thioredoxin and glutaredoxin antioxidant systems. Both of them act as disulfide reductases and help to maintain the reduced forms of protein as well as non-protein thiols. In this way, they contribute to the maintenance of the redox balance (Powers and Jackson 2008).

2.6.2 ENDOGENOUS NON-ENZYMATIC ANTIOXIDANTS

Glutathione (GSH), a tripeptide γ-L-glutamyl-L-cysteinyl-L-glycine, is the major thiol antioxidant in the body. It is found mainly in organs which are exposed to toxins such as the kidneys, liver, lungs and intestines (DeLeve and Kaplowitz 1991). Glutathione is highly present in the cytosol, nuclei and mitochondria and is the major soluble antioxidant in these cell compartments. The oxidized form of glutathione is GSSG, glutathione disulphide (Valko et al. 2007). Oxidized glutathione is accumulated inside the cells and its high concentration may cause oxidative damage to many enzymes. That is why the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Nogueira et al. 2004). GSH plays role in the protein synthesis, amino acid transport, DNA synthesis, and generally in the cellular detoxification. It is involved in the reaction catalyzed by glutathione peroxidase, it also takes part in the reduction of lipid hydroperoxides and in the elimination of xenobiotics (Gaté et al. 1999). GSH is able to regenerate the most important antioxidants, such as vitamin C and E, back to their active forms (Valko et al. 2007).

Another endogenous antioxidant is for example **ubiquinone-10 (coenzyme Q_{10})**, a lipid-soluble compound, whose main biological function is to act as an electron carrier of the respiratory chain in mitochondria. Its reduced form ubiquinol-10 is an efficient scavenger of free radicals occurring in the membranes (Frei et al.1990). It can scavenge lipid peroxide radicals, prevent the lipid peroxidation, and also regenerate α-tocopheryl radical (Lass and Sohal 1998).

Lipoic acid is a naturally occurring compound that is synthesized in small amounts by plants and animals, including humans (Smith et al. 2004). Its antioxidant properties include the ability to scavenge reactive oxygen species (ROS) and to regenerate endogenous antioxidants (as glutathione and vitamins E and C). This compound has also metal chelating activity resulting in reduced ROS production. Lipoic

acid can trap radicals in both aqueous and lipid surrounding due to its amphiphilic character. Lipoic acid contains two thiol groups, which may be oxidized or reduced. The reduced form, dihydrolipoic acid, is a potent reducing agent (Singh and Jialal 2008).

Uric acid is an end-product of purine metabolism in humans and other primates. Uric acid is a powerful scavenger of peroxyl radicals, hydroxyl radicals and singlet oxygen (Powers and Jackson 2008). Urate can act as an electron donor and it is also able to chelate metal ions such as iron and copper and prevent the formation of hydroxyl radicals (Halliwell and Gutteridge 2007).

The **transition metal binding proteins** (ferritin, transferrin, lactoferrin, caeruloplasmin) are able to scavenge free metal ions, iron and cooper, so these metals are not able to catalyze formation of hydroxyl radical via the Fenton reaction (Young and Woodside 2001).

2.6.3 EXOGENOUS NON-ENZYMATIC ANTIOXIDANTS

However, despite of these developed body´s endogenous defense systems, some ROS still escape and can cause damage and so there are needed other mechanisms to prevent the cumulative oxidative effects. Part of our diet is constituted of plants containing various compounds that have also antioxidant properties due to their structure. These compounds derived from food are called **exogenous (dietary) antioxidants** (Pietta 2000). This group includes well-known antioxidants as *vitamin C*, the most important antioxidant in aqueous phase; *vitamin E* (tocopherols α, β, γ, δ and tocotrienols α, β, γ, δ), the major lipophilic antioxidant that traps peroxyl radicals and prevents the lipid peroxidation in cell membranes and lipoproteins; or *carotenoids* (Sies et al. 1992).

In recent years, the interest is focused on plant polyphenols, very important class of defense antioxidants.

2.6.3.1 POLYPHENOLS

Polyphenols (i.e. molecules with several phenolic groups on aromatic ring) are the most abundant antioxidants in our diet and are widespread constituents of higher plants

(they are found in fruits, vegetables, cereals, olives, dry legumes, chocolate and beverages, such as tea, coffee and wine). Current research is focused on their possible antioxidant abilities to help in the prevention of various diseases associated with oxidative stress. The health effects of polyphenols depend on the consumed amount and on their bioavailability, which can be decreased during their metabolism. The polyphenols are secondary metabolites of plants, where they play role in defense against ultraviolet radiation or aggression by pathogens. Polyphenols are classified into different groups according to their structure: *phenolic acids, flavonoids, stilbenes,* and *lignans* (Manach et al. 2004).

Flavonoids create the largest group of polyphenols, which represents the important source of dietary antioxidants. Antioxidant activities of flavonoids, gallic acid and its derivative became a subject of study in this work.

Gallic acid

Gallic acid (GA, 3,4,5-trihydroxybenzoic acid, Fig. 4.) and its derivatives are widely present in the plant kingdom. GA belongs to the hydroxybenzoic acids, the group of plant polyphenols (Lu et al. 2006).

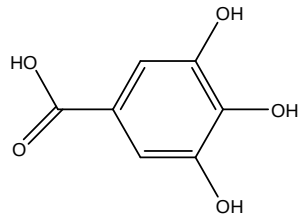

Fig. 4. Structure of gallic acid

GA can be found in various natural products, like gallnuts, sumac, tea leaves, oak bark, green tea, apple-peels, grapes, strawberries, pineapples, bananas, lemons, and in red and white wine (Sun et al. 2002). GA can be present as free molecule or as a part of tannin molecule. GA and its derivatives have shown many beneficial effects, as antiallergic, antimutagenic, anti-inflammatory and anticarcinogenic effects (Madlener et al. 2007). The three hydroxyl groups present in the structure of GA, especially the OH group in the *para*-position to the carboxylic group, are responsible for the free radical scavenging activity. GA is quickly metabolized in the body. The main GA metabolites are products of methylation (4-O-methylgallic acid), decarboxylation (unconjugated and conjugated pyrogallol), and dehydroxylation (resorcinol) (Lu et al. 2006).

Flavonoids

Flavonoids belong to the group of plant polyphenols, which constitutes an important class of defense antioxidants. Flavonoids are formed in plants from the malonate and aromatic amino acids phenylalanine and tyrosine by shikimate pathway (Harborne 1986). The basic flavonoid structure (Fig. 5) is the flavan nucleus, which consists of 15 carbon atoms arranged into three rings (A, B, C).

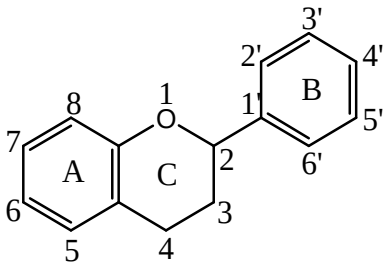

Fig. 5. Basic flavonoid structure (flavan nucleus) (Pietta 2000)

The flavonoids of various classes differ in the level of oxidation and substitution of the ring C (Fig. 6). Nevertheless, the individual compounds within each class differ in the substitution of the rings A and B (Pietta 2000).

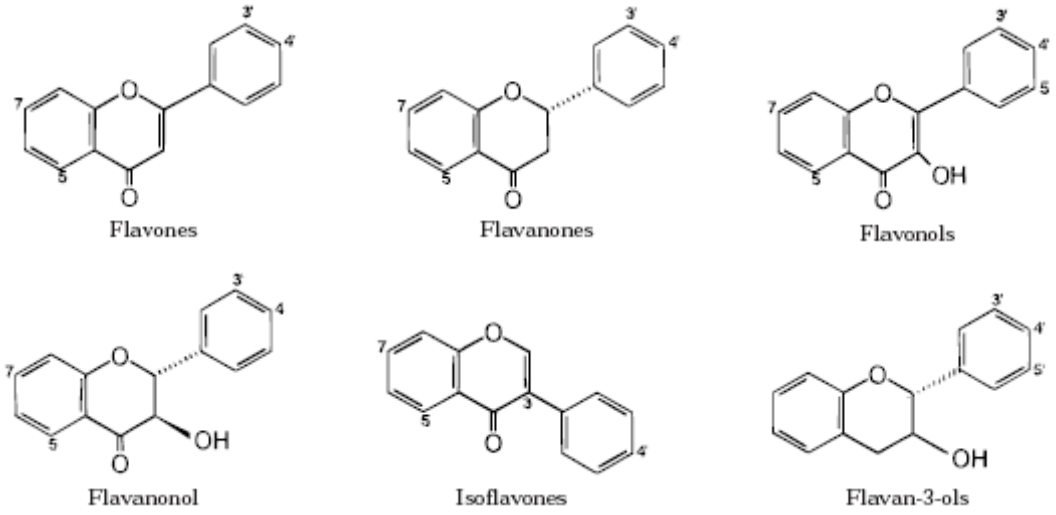

Fig. 6. Structures of flavonoids (Pietta 2000)

Flavonoids occur in various vegetables and fruits, spices, and in different medical plants and beverages, such as wine (particularly red wine), tea, and in lower levels also in the beer (Kuhnau 1976). Apart from their physiological roles in plants where they act as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants and

protectors against UV radiation, flavonoids are important components of the human diet (Pietta 2000). Flavonoid intake can range between 50-800 mg/day (Larson 1988). The present epidemiological studies prove the possible protective role of dietary flavonoids. Diet rich in polyphenols is associated with lower risk of developing of some age-related diseases in humans. Antioxidant action of flavonoids includes:

a) inhibition of enzymes responsible for superoxide anion production, such as xanthine oxidase, protein kinase C, and another enzymes involved in the generation of ROS (cyclooxygenase, lipoxygenase, monooxygenase, glutathione S-transferase, NADH oxidase)
b) ability to chelate trace metals (iron and cooper), which are potential enhancers of ROS production (e.g. reduction of H_2O_2 to $^{\bullet}OH$)
c) due to their lower redox potentials ($0.23 < E^0 < 0.75$ V), flavonoids are able to reduce highly oxidizing free radicals, which have redox potentials in the range of 2.13-1.0 V by hydrogen atom donation (Buettner 1993). The rising aroxyl radical (Fl-$O^{\bullet}$) may react with a second radical creating a stable quinone structure (Fig. 7) (Pietta 2000).

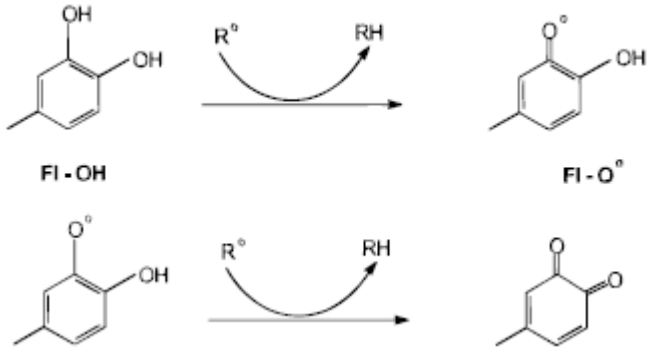

Fig. 7. Scavenging of ROS ($R^{\bullet}$) by flavonoids (Pietta 2000)

The possible side reaction of the aroxyl radical, when Fl-$O^{\bullet}$ interacts with oxygen generating quinones and superoxide anion, exists. This reaction can occur in the presence of high levels of transient metal ions and is responsible for the undesired prooxidant effect of flavonoids (McCord 1995). Some of these aroxyl radicals (those with $E^0 > 0.282$ V) may also oxidize ascorbate, which is in turn regenerated by glutathione. That causes a decrease in the plasma concentrations of ascorbate and glutathione. On the other hand, flavonoids as quercetin and tea catechins ($E^0 = 0.22$ and

≈ 0.4 V, respectively) may be able to regenerate α-tocopherol from the α-tocopheryl radical (Pietta 2000).

The relationship between flavonoid structure and its scavenging activity was established on the basis of many studies. The major determinants for radical-scavenging activity are:

a) the presence of a catechol group in the ring B (two hydroxyl groups in *ortho* position), which has better electron donating properties and is a radical target
b) 2,3-double bond conjugated with the 4-oxo group, which is responsible for the electron delocalization
c) the activity can be enhanced by the presence of 3-hydroxyl group in the heterocyclic C ring, or an additional hydroxyl group in the ring B (Pietta 2000).

Many people are hoping that one day some pill, that will provide the same benefit effects of dietary polyphenols as fruits and vegetables, will be invented. However, there is a problem with bioavailability of polyphenols. The barriers that must be overcome include: solubility, permeability, metabolism, excretion, target tissue uptake and disposition (Hu 2007).

The dietary flavonoids may be used as first antioxidant defense in the digestive tract by scavenging and restricting formation of ROS (Papas 1996). Flavonoids are completely absorbed from gastrointestinal tract only exceptionally. They are present in the glycosidic forms that are usually too polar or too large to penetrate through the intestinal membrane (Liu and Hu 2002). Intestinal enzymes break them to aglycone forms that are highly permeable, but the bioavailability is still limited because of the poor solubility of aglycones in water (Hu 2007). So there is a question if the amount of polyphenols absorbed is sufficient for the antioxidant function? Plasma levels of unconjugated flavonoids are not higher than 1 μM and the metabolites have a lower antioxidant activity, because the radical-scavenging hydroxyl groups are blocked by methylation, sulphation or glucuronidation (Manach and Donovan 2004, Williamson et al. 2005). It has been proposed that protective effect of flavonoids occurs before absorption, i.e. within the stomach, intestines and colon. The gastrointestinal tract is constantly exposed to reactive species (some are produced by GIT itself, some come from food). It explains the suggested ability of flavonoids to protect against gastric and possibly colonic cancer (Halliwell 2007).

2.7 ROS AND MUSCLES

In this work, the myoblasts from rat skeletal muscles were used as a cell culture model for experiments, in which the ability of selected antioxidants to decrease ROS formation was evaluated. Due to this fact I introduce here the main sources of ROS and RNS in skeletal muscles and possible conditions that may lead to the increase in their concentration.

The evidence that skeletal muscles are potential sources of ROS and RNS and the exercise is associated with an increase of their formation is known for more than 20 years. The primary free radicals generated by skeletal muscle, both at rest and during activity, are NO and superoxide, that may lead to the formation of hydrogen peroxide, peroxynitrite, or in the presence of catalytic transition metals into hydroxyl radicals (Fig. 8). The skeletal muscles have a well-developed system to regulate ROS and to prevent its possible injury (Jackson et al. 2007.) The skeletal muscles appear to function quite well in relatively high oxidizing environments. They are exposed to such quantity of mechanic and metabolic insults during exercise that could kill most of other cell types (Clanton et al. 1999). The protective systems include mitochondrial and cytosolic isoforms of superoxide dismutase (MnSOD and CuZnSOD), catalase and glutathione peroxidase enzymes, and direct scavengers of ROS (e.g. glutathione, vitamin E, and ascorbate) (Jackson et al. 2007).

The endogenous sites for ROS generation in skeletal muscles include mitochondria, sarcoplasmic reticulum, plasma membrane, phospholipase $A_{2,}$ and probably also xanthine oxidase (Jackson et al. 2007).

Mitochondria are the major site of superoxide generation in tissues (mainly the complexes I and III of the electron transport chain). There is also CuZnSOD in the mitochondrial intermembrane space to minimize the possibility of the superoxide release to the cytosol (Muller et al. 2004). The production of superoxide in mitochondria is increased during aerobic contraction and it is directly related to the elevated oxygen consumption that occurs with increased mitochondrial activity. There is also evidence that the ROS formation in muscle mitochondria grows up with age (Jackson et al. 2007).

There were identified NADH oxidase enzymes in the *sarcoplasmic reticulum* (SR). The superoxide generated by these enzymes appears to influence calcium release by the SR (Xia et al. 2003).

Numerous studies have indicated that skeletal muscle cells release superoxide into the extracellular space. This is probably due to *plasma membrane* redox systems that enable the electron transport across the plasma membrane. One of these systems can be the NAD(P)H oxidase complex localized in the region of the plasma membrane. The relevance of these processes to skeletal muscle contractions has not been established, but it is feasible that such systems are activated during contractile activity (Jackson et al. 2007).

Phosholiphase A_2 (PLA_2) is an enzyme that cleaves arachidonic acid from membrane phosholipids. The activation of PLA_2 may stimulate NAD(P)H oxidases and ROS generation in muscle mitochondria and can release ROS into the extracellular space (Zuo et al. 2004).

The levels of *xanthine oxidase* were found to be increased in rat skeletal muscles in contrast to human skeletal muscles. However, xanthine oxidase probably plays a role in exercise-induced ROS production (Jackson et al. 2007).

Skeletal muscles normally contain the *NOS1* and *NOS3* isoforms of NO synthase that is responsible for NO formation. NOS1 is localized in the muscle sarcolemma, while NOS3 in the muscle mitochondria. Higher release of NO was demonstrated during contractile activity and also during passive stretching of muscle (Kobzik et al. 1995).

The presence of some amount of oxidized glutathione as well as lipid and protein oxidation products during exercise is normal state and perhaps may be important in cell signaling. It has been hypothesized that ROS play important roles in the normal contracting myocytes to regulate Ca^{2+} metabolism, contractile behavior, and perhaps utilization and control of energy substrates. The excessive ROS formation during fatigue and hypoxia probably through signal pathway can function to protect the muscles from the overstimulation and injury. Despite of this ability severe ischemia, hypoxia, heat stress, septic shock and stretch induced injury may lead to oxidant-mediated injury to myocytes and result in the mechanic dysfunction (Clanton et al. 1999).

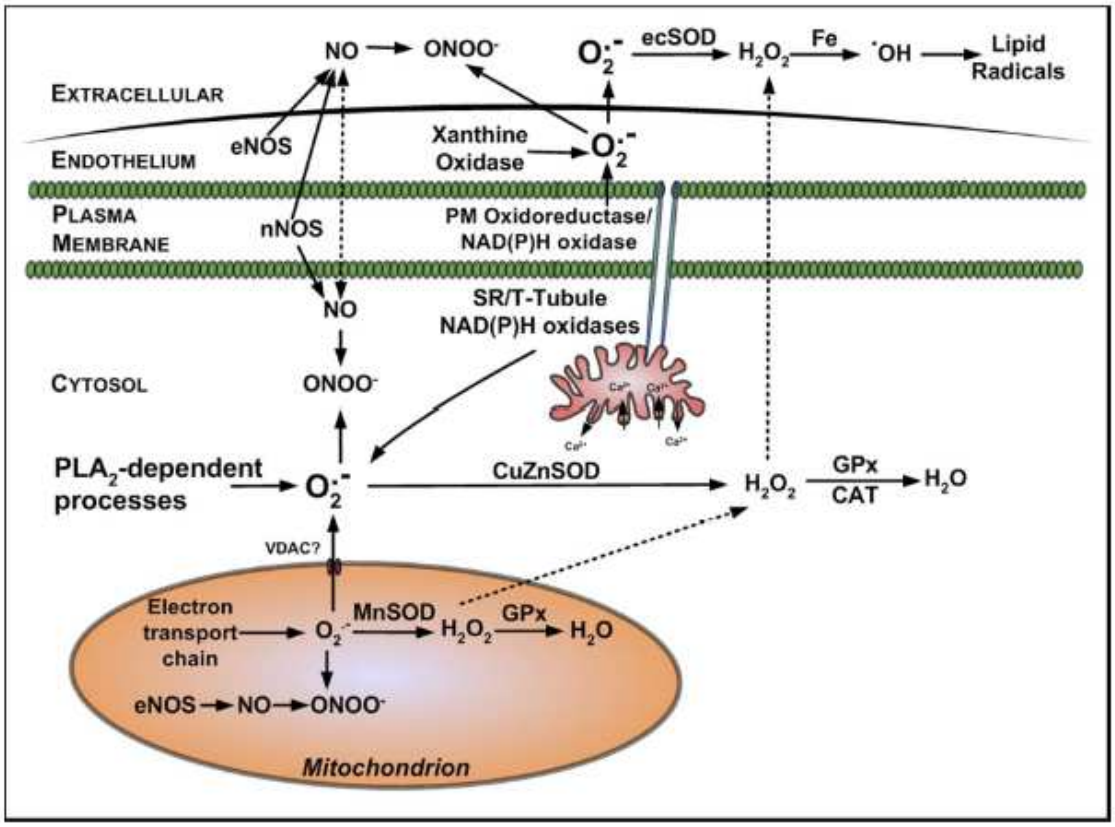

Fig. 8. Potential sites for the production of superoxide and nitric oxide in skeletal muscles (Powers and Jackson 2008)

Fe – iron, NOS - nitric oxide synthase, SOD – superoxide dismutase, PM – plasma membrane, SR – sarcoplasmic reticulum, PLA_2 – phospholipase A_2, GPx – glutathione peroxidase, CAT - catalase

3 AIM OF THE WORK

The main goals of this work were:

a) to investigate potential antioxidant activity of selected polyphenols (baicalein, 5,6-dihydroxyflavone, negletein, mosloflavone, gallic acid and its derivative 1625) in cell culture (L-6 myoblasts from rat skeletal muscles) using fluorescence assay with DCFH-DA probe and cytotoxicity assay

b) to study the antioxidant activity of the above mentioned compounds *in vitro* using electron paramagnetic resonance spectroscopy (EPR)

c) to evaluate their ability of preventing the formation of ROS (reactive oxygen species) that may cause the oxidative stress

4 MATERIALS AND METHODS

4.1 REAGENTS

- All flavones (baicalein, mosloflavone, negletein, 5,6-dihydroxyflavone) were synthesized and characterized by Prof. P. Bovicelli, Department of Chemistry, “La Sapienza” University of Rome.
- Gallic acid and the compound 1625 were synthesized and characterized in the laboratory of Prof. V. Parmar, Department of Chemistry, University of Delhi.
- The cumene hydroperoxide, galvinoxyl, glucose, phosphate buffer saline (PBS) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (St. Louis, MO, USA).
- 2'7'-dichlorodihydrofluorescin diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR, USA).
- Dulbecco´s Modified Eagle´s medium (DMEM), antibiotics, and sterile plastic ware for cell culture were products of Flow Laboratory (Irvine, UK).
- Fetal bovine serum was purchased from GIBCO (Grand Island, NY, USA).

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. Then, 100 mL of this PBS solution was supplemented with 90 mg of glucose (final concentration 5 mM) and warmed up before the experiment in the bath to 37 ºC.

4.3 PREPARATION OF FETAL BOVINE SERUM AND ITS ENRICHMENT WITH ANTIBIOTICS

The fetal bovine serum (FBS, 100 mL) was thawed and warmed for 30 min in the bath of 57 ºC and immediately it was dispensed in aliquots of 5 ml into 50 mL tubes and supplemented with 1 mL of antibiotics (solution containing 100 µg/mL of streptomycin and 100 U/mL of penicillin). After it all tubes containing FBS + ATB were stored in the freezer at -20 ºC. Five hundred mL of Dulbecco´s Modified Eagle´s medium (DMEM, containing 4.5 g/L of glucose) was supplemented with 5 mL of 100 mM pyruvate and

10 mL of 200 mM glutamine. The tube containing FBS + ATB was thawed in time of need and together with the bottle of DMEM was warmed up in the bath to 37 ºC. Then 45 mL of DMEM was added into the tube containing FBS + ATB, and this solution was used for the cell seeding and for the trypsinization.

4.4 CELL CULTURE

The L6 cells from rat skeletal muscles obtained from American Type Culture Collection (Rockville, MD) were used in all experiments. Cells were seeded in flasks for tissue culture and grown in DMEM supplemented with 10% FBS, glutamine, pyruvate, and antibiotics (100 µg/mL of streptomycin and 100 U/mL of penicillin) in the atmosphere of 5% CO_2 at 37°C. The cells reached confluence after five days and were kept in culture as myoblasts by continuous passages (D'Arezzo et al., 2004).

4.4.1 TRYPSINIZATION

For the propagation, cells were seeded in 25 cm^2 flasks for tissue culture and grown in DMEM supplemented with 10% FBS, glutamine, pyruvate, and antibiotics (100 µg/ml of streptomycin and 100 U/ml of penicillin) in the atmosphere of 5% CO_2 at 37°C. They reached confluence (about 2 x 10^6 cells) after five days. It was necessary to detach cells from flask's surface and bring them to suspension in order to accomplish following passage. This detaching was achieved by addition of trypsin solution diluted 1:10.

DMEM (supplemented with FBS + ATB) was discarded from flask (25cm^2) and cells were washed twice with 5 ml of DMEM (without FBS + ATB). Then 2 ml of trypsin solution diluted 1:10 were added to cells for 45 seconds. After this time, a part of trypsin solution was discarded and the flask was kept for 8-10 min. in the incubator (5% CO_2 at 37°C). The detached cells were collected from the flask´s wall with 5 ml of DMEM (supplemented with FBS + ATB) and the cell suspension was transferred to a centrifuge tube and centrifuged at 1000 rpm for 5 min. After centrifugation, the supernatant was discarded and cells were resuspended in 10 ml of DMEM (supplemented with FBS + ATB).

The appropriate amount of the cell suspension was seeded for the experiments.

4.5 INTRACELLULAR ROS DETERMINATION

2´,7´-Dichlorofluorescin (DCFH) is widely used fluorescent probe for measuring the extent of oxidative stress in cells. Its diacetate form 2´,7´-dichlorodihydrofluorescin diacetate (DCFH-DA) is able to diffuse through the cell membrane, inside the cell is hydrolyzed by intracellular esterases into DCFH, which is in the presence of ROS oxidized into highly fluorescent compound 2´,7´-dichlorofluorescein (DCF) (Fig. 9). The fluorescence intensity can be easily measured and it represents the principle of the cellular assays for determination of oxidative stress extent.

This probe is more suitable for detecting total ROS production (not individual radicals) in living cells or tissues, because several substances present in the cells may interfere with the formation of DCF. DCFH can be oxidized by several enzymes as peroxide oxidase or xanthine oxidase. Its oxidation can also occur by action of H_2O_2 in the presence of Fe^{2+} ions, when $^{\bullet}OH$ causing the oxidation is produced via Fenton reaction. DCFH is also sensitive to the presence of visible light or UVA radiation that causes generation of semiquinone radical ($DCF^{\bullet}$), which reacts with oxygen and forms superoxide radical generating H_2O_2 during its dismutation. This increasing 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).

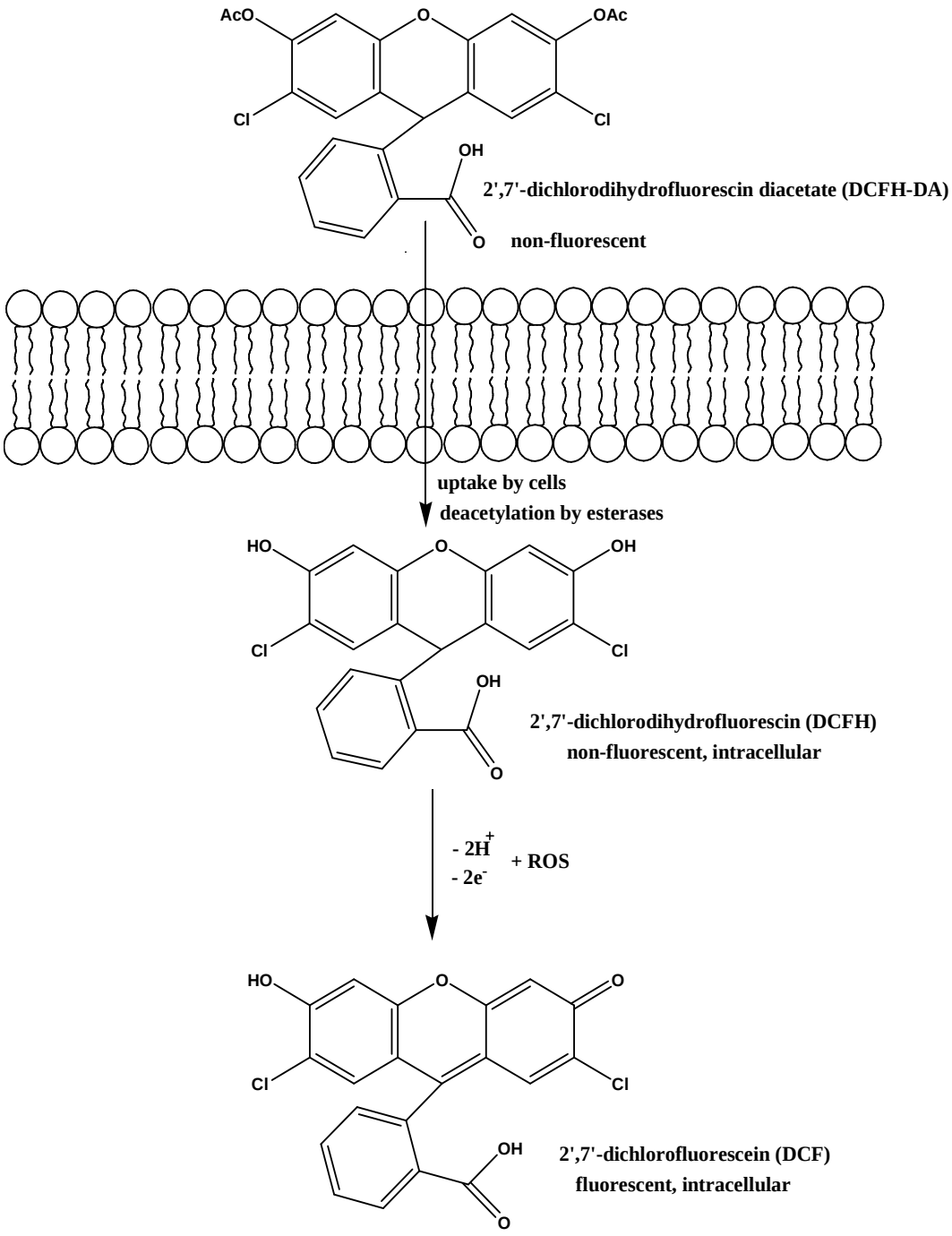

Fig. 9. Mechanism of entry of DCFH-DA into the cell

L-6 cells were seeded in 75 cm^2 flasks for tissue culture and grown in DMEM supplemented with 10% FBS, glutamine, pyruvate, and antibiotics (100 µg/ml of streptomycin and 100 U/ml of penicillin) in 5% CO_2 at 37°C. At the time of experiment, the cells reached confluence about 6 x 10^6 cells. DMEM was discarded and cells were washed twice with 5 mL of PBS containing glucose 5 mM at 37°C. Cells were gently scraped off in three following steps - twice with 3 mL and once with 2 mL

of PBS with glucose 5 mM (i.e. totally 8 mL) at 37°C. The cell suspension was transferred to a centrifuge tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the pellet re-suspended with a plastic Pasteur pipette in 5 mL of PBS with glucose 5 mM. Then 5 µL of the stock solution (10 mM in dimethyl sulfoxide, DMSO) of fluorescent probe DCFH-DA (final concentration 10 µM) were added. The incubation was carried out in the dark for 30 minutes at 37°C. The cells were gently re-suspended every 10 min and centrifuged at 1200 rpm for 5 min at the end of incubation. The supernatant was discarded and the cell pellet was re-suspended in 5 mL of PBS with glucose 5 mM and centrifuged again. The final supernatant was discarded and the pellet was re-suspended in 2 mL of PBS obtaining the final concentration of 3 x 10^6 cells/mL. Prior to the experiment, a recovery was carried out in the dark for 45 min at 37°C (Pedersen et al. 2007).

Intracellular fluorescence was measured using luminescence spectrometer Perkin-Elmer LS 50B (Norwalk, CT) under continuous gentle magnetic stirring at 37°C. Excitation and emission wavelengths were set at 498 nm and 530 nm using 5 and 10 nm slits, respectively. The assay was carried out in the final volume of 3 mL of PBS with glucose 5 mM containing appropriate amount of cell suspension. When the base line of cell fluorescence was stable, 3 µL of studied antioxidant were pipetted into the cuvette and the resulting solution was incubated for 10 min at 37°C. Then 10 µL of cumene hydroperoxide diluted 1:100 in DMSO (final concentration 300 µM; radical generator) was pipetted into the cuvette and the fluorescence was registered for 10 min. DMSO at the concentrations used during these experiments did not affect the fluorescence signal. The antioxidant ability of studied compounds to decrease the production of ROS was determined by the decrease in the intracellular DCF fluorescence, reported as ΔF/10 min relative to the fluorescence change induced by 300 µM cumene hydroperoxide alone (100%), was calculated according to the equation *(12)* (Pedersen et al. 2007).

$$ROS\ production\ (\%) = \frac{\Delta F_{AOX+CUM}/10min}{\Delta F_{CUM}/10min} \times 100 \qquad (12)$$

AOX = antioxidant, CUM = cumene hydroperoxide

4.6 CYTOTOXICITY ASSAY (MTT)

The MTT (cytotoxicity) assay is a biological method using *in vitro* cell culture for cytotoxicity testing. This assay is a good indicator of cell viability. The cells, which remain viable after exposure and incubation with a studied chemical (in this case after exposure to ROS), are able to reduce yellow water-soluble salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] into insoluble, intracellular metabolite blue formazan crystals (Fig. 10) (Ozdemir et al. 2009).

It has been proposed 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 observation was confirmed also by the study of Bernas and Dobrucki (2002), who reported that only small fraction of MTT-formazan is deposited in (or at the surface of) mitochondria whereas most of MTT is reduced in other cellular compartments, e.g. 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 DMSO and can be quantified spectrophotometrically, where the optical density is directly related to the number of viable cells (Bean et al. 1995).

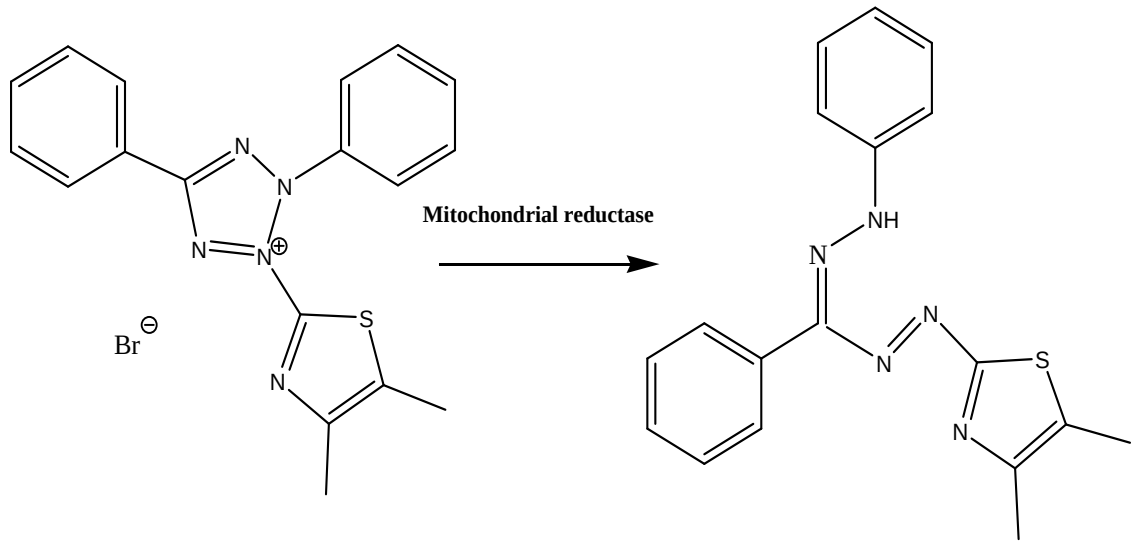

Fig. 10. Reduction of yellow MTT into blue metabolite formazan

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

DMEM was discarded with Pasteur pipette from each well and cells were washed twice with 1 mL of PBS with glucose 5 mM per well at 37°C (i.e. totally 2 mL/well). Then 1 mL of PBS with glucose 5 mM was pipetted into each well at 37°C. Thirty µL of cumene hydroperoxide diluted 1:100 (final concentration 300 µM) and antioxidants in desired concentration were added into one and four wells at the time t_0, respectively. The six-well plates were placed into incubator (5% CO_2 at 37°C) for 10 min. At the time t_{10min}, 30 µL of cumene hydroperoxide diluted 1:100 (final concentration 300 µM) were added into the wells, where cells were treated with antioxidant. The six-well plates were put back into incubator for 20 min. After incubation, the solution was discarded from all wells with Pasteur pipette and cells were washed with 1 mL of PBS with glucose 5 mM per well at 37°C. Then 1 mL of MTT solution (concentration 1 mg/mL) was pipetted into each well and the plate was incubated for 3 h (5% CO_2 at 37°C).

Thereafter, cells were transferred into 1,5 mL eppendorf-tubes with Pasteur pipette and centrifuged at 1300 rpm for 10 min. The supernatant was discarded, the pellet was re-suspended in 300 µL of PBS with glucose 5 mM, sonicated on ice for 15 sec using Ultrasonic W-225R (setting 4), and then centrifuged in microfuge at 13000 rpm for 10 min. The supernatant was discarded and the final pellet re-suspended in 200 µL of DMSO. This resulting solution (200 µL) was placed into cuvettes together with 1800 µL of PBS with glucose 5 mM and MTT formazan was measured using the spectrophotometer at 560 nm (Pedersen et al 2007).

4.7 ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY (EPR)

Electron paramagnetic resonance spectroscopy (or also known as electron spin resonance 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 take 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 to the higher energy level (Takeshita and Ozawa 2004). The energy accepted by electron is equal to the difference between these two energy levels (Cammack and Shergill), i.e. state of different spin. This absorption of energy of the electromagnetic wave is detected by spectrometer. The EPR of *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 flavones were prepared in 95% ethanol at the concentration of 10 mM. A galvinoxyl solution (10 mM in ethanol 95%) was freshly prepared immediately before each experiment. Galvinoxyl in solution is a stable radical that is converted to galvinol in the presence of an antioxidant (Fig. 11). Systematic screening of studied flavones was made with the final concentrations of 0.1, 1.0 and 10 μM in the presence of 10 μM galvinoxyl. The solutions were drawn into glass capillaries and measured using the ESP300 instrument (Bruker Spectrospin, Karlsruhe, Germany) equipped with highly sensitive TM_{110} X-band cavity. Radical spectra were recorded at room temperature using 0.6 G modulation, 1 mW microwave power and scan time of 42 s for a 30 G spectrum. Normally, four spectra were accumulated to obtain a suitable signal-to-noise ratio. The kinetics of the reaction was followed for 15 min at room temperature or until the radical signal had disappeared (Pedersen et al. 2007).

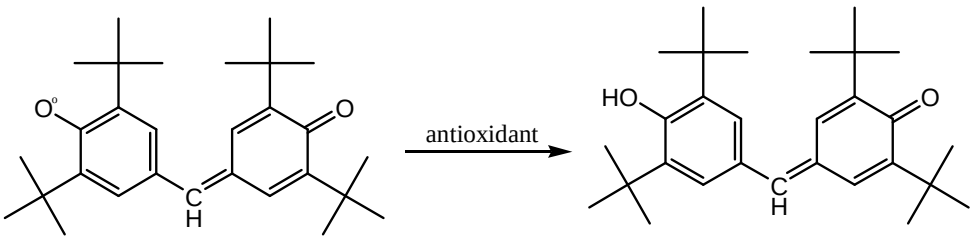

Fig. 11. Reduction of galvinoxyl to galvinol in the presence of an antioxidant

4.8 THE CELL PROLIFERATION ASSAY

L-6 cells were seeded in Petri dish (30 mm x 15 mm) and grown in DMEM supplemented with 10% FBS, glutamine, pyruvate, and ATB (100 µg/mL of streptomycin and 100 U/mL of penicillin) in 5% CO_2 at 37°C. The achieved confluence of cells was 1 x 10^6 cells/dish (in 2 mL of medium) at the day of the cell counting. The medium was changed after 24 hours. The solution of studied compound was added 48 h after the cell seeding (t = 0).

At the time of the cell counting, DMEM was discarded from Petri dish and cells were washed twice with 1 mL of DMEM without serum (i.e. 2 mL totally). Then 1 ml of trypsin solution diluted 1:10 was added to the dish with cells for 45 seconds. After this time, a part of trypsin solution was discarded and the dish was kept for 5 min. in the incubator (5% CO_2 at 37°C). The detached cells were re-suspended, collected in 1 mL of DMEM supplemented with FBS and ATB, and transferred from Petri dish into the tube.

The cells were re-suspended with Pasteur pipette to ensure the homogenity of sample before the cell counting. Then 30 µL of the cell suspension was transferred into each chamber, where the cell counting followed. The cells were counted in five quadrants of the chamber, i.e. in four corners and in the middle. The cell counting was repeated every 24 hours (up to 96 hours). The number of cells in 1 mL has been calculated according to the equation *(13):*

$$N = \overline{N}\, x\, 10^4 \qquad (13)$$

N = number of cells in 1 mL; $\overline{N}$ = the mean of the cells in five quadrants; 10^4 is the dilution factor, which is constant for Neubauer chamber

5 RESULTS

5.1 INTRACELLULAR ANTIOXIDANT ACTIVITY

The intracellular antioxidant activity was determinate using the standard assay based on the fluorescence of DCFH-DA probe and cytotoxicity assay was performed using the MTT probe. Four flavonoids from the group of flavones and two representatives of phenolic acids were studied.

Fluorescence assay

Baicalein showed the highest antioxidant activity of all studied flavonoids. 5,6-Dihydroxyflavone and the synthetic ester of gallic acid (methyl-3,4,5-triacetoxybenzoate) proved the significant antioxidant activity especially at concentrations 10^{-5}-10^{-7} M. Negletein had a prooxidative effect in the cells at concentration 10^{-5} M but at the lower concentrations (10^{-6}-10^{-8} M) it decreased ROS production approximately by 60%. Mosloflavone did not prove any scavenging ability and it even acted as a prooxidant at the highest concentrations (10^{-5}-10^{-6} M). Gallic acid showed only slight ability to inhibit ROS production.

Cytotoxicity assay (MTT)

5,6-Dihydroxyflavone proved a good antioxidant activity at the concentration of 10^{-5} M, when it protected 100% of cells. However, no protective effect was observed at concentration 10^{-8} M.

Unfortunately, MTT assay with baicalein, negletein and mosloflavone was not successful and all cells died during the experiment. This result was surprising, because baicalein should have the protective effect on ROS production according to the results of DCFH-DA method. Based on these findings, the effect of studied flavones on the cell proliferation was tested.

Gallic acid and its ester (methyl-3,4,5-triacetoxybenzoate) did not show any protective effect in any tested concentration.

Proliferation assay:

I found out that all tested flavones inhibit the cell proliferation at concentration 10^{-5} M. 5,6-Dihydroxyflavone and baicalein exerted a slight inhibitory effect on cell proliferation, while negletein and mosloflavone proved to be strong inhibitors.

5,6-DIHYDROXYFLAVONE

5,6-Dihydroxyflavone belongs to the group of flavones. This compound contains two hydroxyl groups in the *ortho*-position in the ring A (Fig. 12). It was dissolved in DMSO and its ability to decrease production of ROS in L-6 cells was tested in the final concentrations 10^{-5}-10^{-11} M. 5,6-Dihydroxyflavone showed a good scavenging ability at the concentrations of 10^{-5}-10^{-7} M, when it was able to restrict the ROS production to 10-22% (Fig. 13).

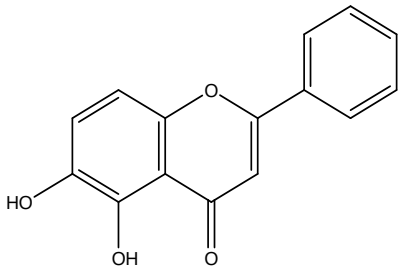

Fig. 12. Structure of 5,6-dihydroxyflavone

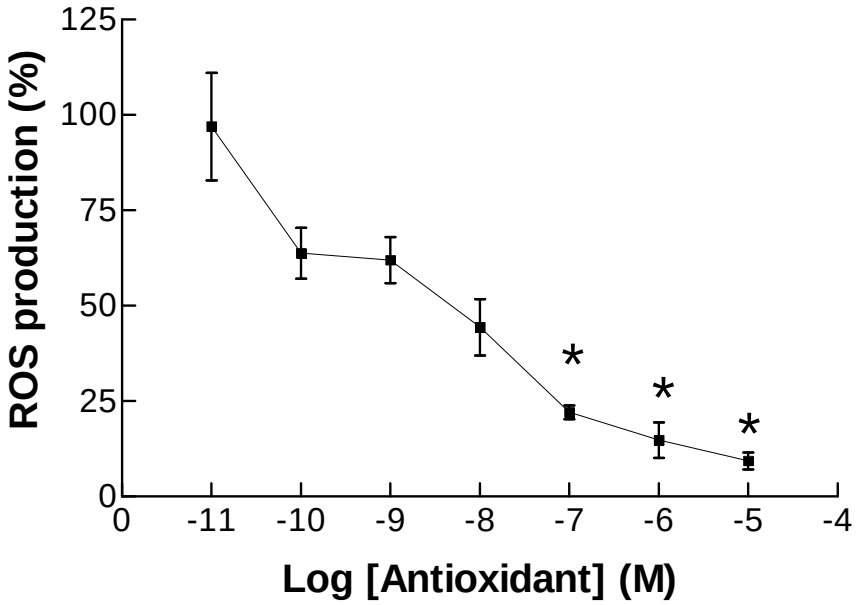

Fig. 13. The effect of 5,6-dihydroxyflavone on the level of intracellular ROS formed in L-6 myoblasts after the exposure to cumene hydroperoxide. The production of ROS was measured through DCF fluorescence assay. The ROS production (%) was calculated according the eq. 13. as a decrease in the intracellular DCF fluorescence, ΔF/10 min, considering the fluorescence of 300 μM cumene hydroperoxide to be 100%. The data are expressed as the mean ± SD of n = 2-6 independent experiments. * p < 0.001, Student's t-test.

Only two concentrations of 5,6-dihydroxyflavone (10^{-5} and 10^{-8} M) were tested using MTT assay. The results of this method confirmed the data obtained from DCFH-DA method. 5,6-Dihydroxyflavone at the concentration of 10^{-5} M showed a good antioxidant activity and protected 100% of cells against damage caused by ROS. However, nearly no protective effect was observed at concentration 10^{-8} M (Fig. 14).

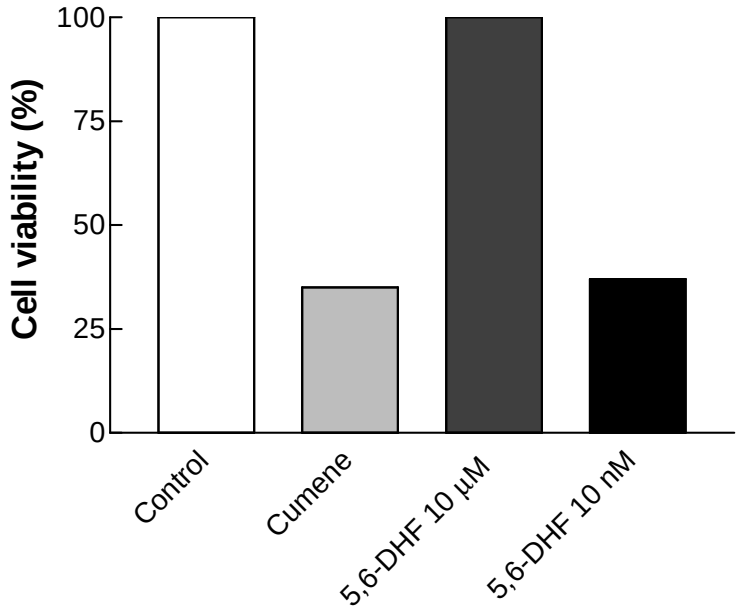

Fig. 14. The effect of 5,6-dihydroxyflavone (10 µM and 10 nM) on the cell viability (%) of L-6 myoblasts after exposure to cumene hydroperoxide (300 µM). The blue crystals of MTT formazan, which correspond to the amount of viable cells, were measured at 560 nm using spectrophotometer. The graph shows results of representative experiment. 5,6-DHF stands for 5,6-dihydroxyflavone.

BAICALEIN

Baicalein is flavone with three hydroxyl groups next to each other (5,6,7-trihydroxy) in the ring A (Fig. 15). This compound was dissolved in DMSO and its ability to decrease production of ROS in L-6 cells was tested in the range of final concentrations 10^{-5}-10^{-10} M. Baicalein showed a good scavenging ability in all concentrations used, even at the concentration 10^{-10} M was still able to decrease ROS production to ~ 46% (Fig. 16).

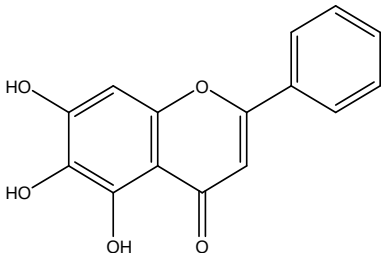

Fig. 15. Structure of baicalein

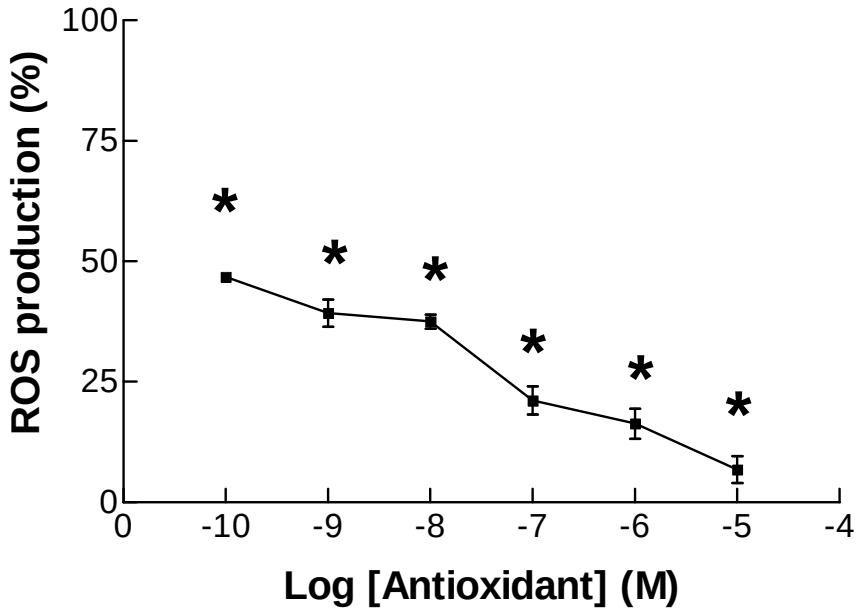

Fig. 16. The effect of baicalein on the level of intracellular ROS formed in L-6 myoblasts after the exposure to cumene hydroperoxide. The production of ROS was measured through DCF fluorescence assay. The ROS production (%) was calculated according the eq. 13. as a decrease in the intracellular DCF fluorescence, ΔF/10 min, considering the fluorescence of 300 μM cumene hydroperoxide to be 100%. The data are expressed as the mean ± SD of n = 3 independent experiments. * p < 0.005, Student's t-test.

NEGLETEIN

Structure of negletein is similar to the structure of baicalein. The difference is in the methylation of hydroxyl group in the position 7 of ring A (Fig. 17). This compound was dissolved in DMSO and its ability to decrease production of ROS in L-6 cells was tested in the range of final concentrations 10^{-5}-10^{-10} M. This compound was able to reduce ROS production to ~ 45% at the concentrations 10^{-6}-10^{-8} M, surprisingly this scavenging ability dramatically decreased at the concentration 10^{-5} M (Fig. 18).

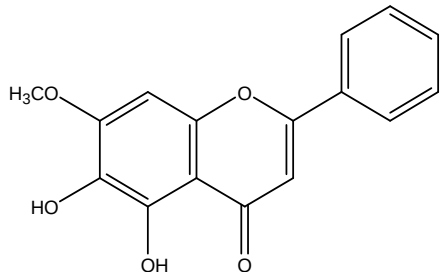

Fig. 17. Structure of negletein

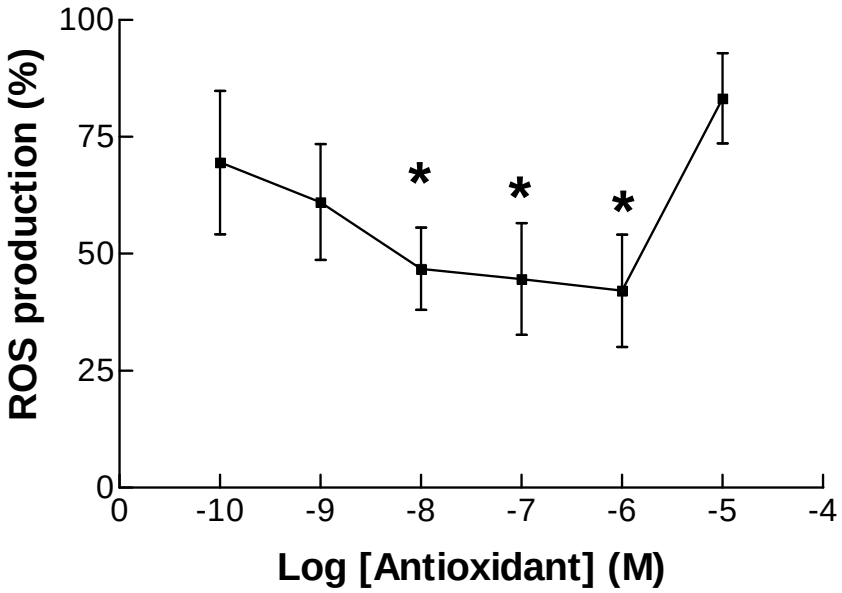

Fig. 18. The effect of negletein on the level of intracellular ROS formed in L-6 myoblasts after the exposure to cumene hydroperoxide. The production of ROS was measured through DCF fluorescence assay. The ROS production (%) was calculated according the eq. 13. as a decrease in the intracellular DCF fluorescence, ΔF/10 min, considering the fluorescence of 300 μM cumene hydroperoxide to be 100%. The data are expressed as the mean ± SD of n = 2-8 independent experiments. * p < 0.001, Student's t-test.

MOSLOFLAVONE

The structure of mosloflavone is similar to that of baicalein, just two hydroxyl groups in the position 6 and 7 of the ring A are methylated (Fig. 19). This compound was dissolved in DMSO and its ability to decrease production of ROS in L-6 cells was tested in the range of final concentrations 10^{-5}-10^{-10} M. Mosloflavone did not show any scavenging activity in all tested concentrations. Moreover, it showed prooxidative effect in two highest concentrations (10^{-5} and 10^{-6} M) (Fig. 20).

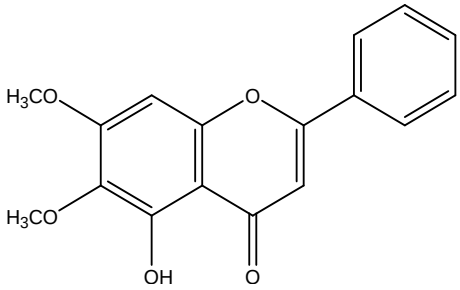

Fig. 19. Structure of mosloflavone

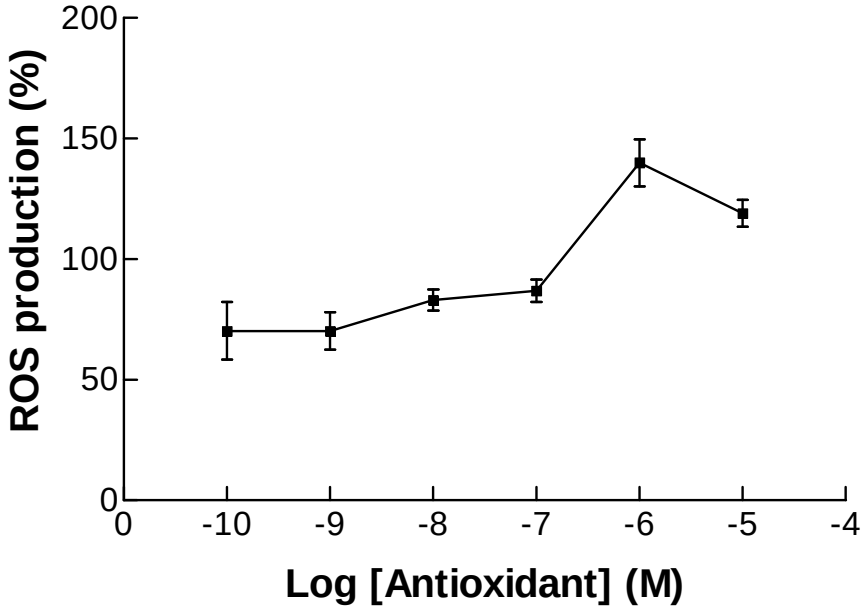

Fig. 20. The effect of mosloflavone on the level of intracellular ROS formed in L-6 myoblasts after the exposure to cumene hydroperoxide. The production of ROS was measured through DCF fluorescence assay. The ROS production (%) was calculated according the eq. 13. as a decrease in the intracellular DCF fluorescence, ΔF/10 min, considering the fluorescence of 300 μM cumene hydroperoxide to be 100%. The data are expressed as the mean ± SD of n = 2-5 independent experiments.

L-6 cells were counted every 24 hours (up to 96 hours) to discover, if the cell propagation can be affected by the presence of antioxidant. All antioxidants were used at the final concentration 10^{-5} M. The results showed that all tested flavones inhibited cell proliferation. Negletein and mosloflavone proved to be strong inhibitors of cell proliferation (Fig. 21), while 5,6-dihydroxyflavone and baicalein exerted slight inhibitory effect (Fig. 22).

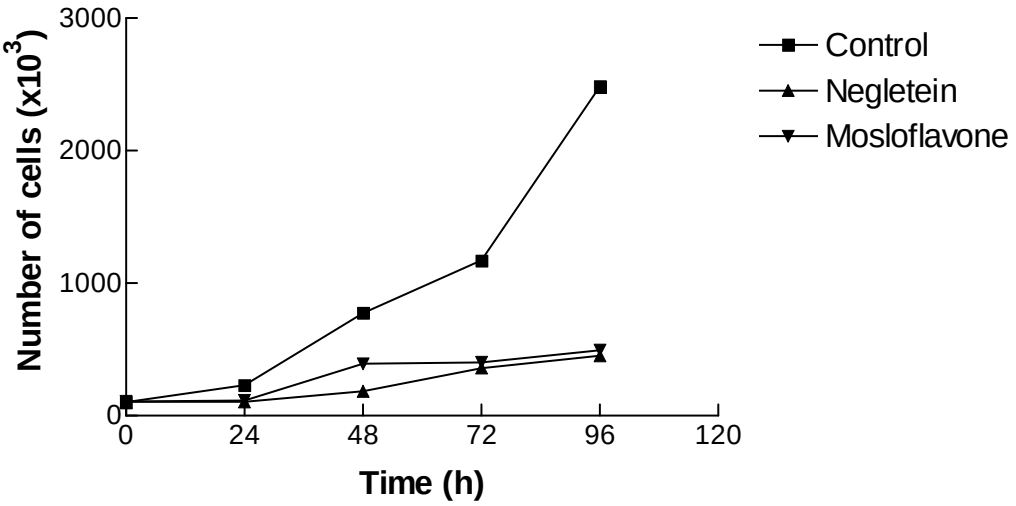

Fig. 21. The effect of negletein and mosloflavone (10^{-5} M) on the cell proliferation. The counting of cells was repeated every 24 hours for four following days. The graph shows representative experiment.

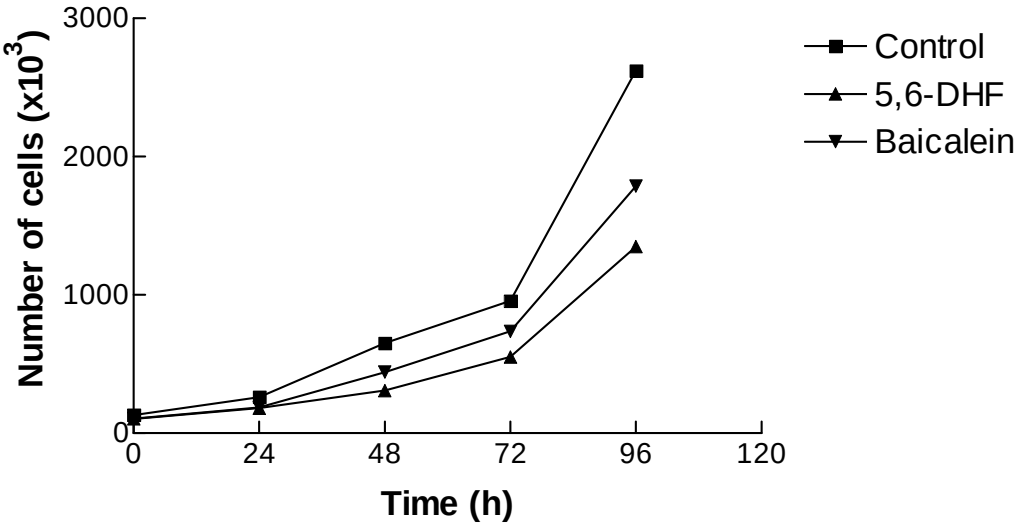

Fig. 22. The effect of 5,6-dihydroxyflavone and baicalein (10^{-5} M) on the cell propagation. The counting of cells was repeated every 24 hours for four following days. The graph shows representative experiment. 5,6-DHF stands for 5,6-dihydroxyflavone.

GALLIC ACID

Gallic acid, 3,4,5-trihydroxybenzoic acid (Fig. 23), was used at the final concentrations 10^{-5}–10^{-8} M. The results of DCFH-DA method in L-6 cell exerted significant scavenging ability of this compound only at the concentration of 10^{-5} M (Fig. 24).

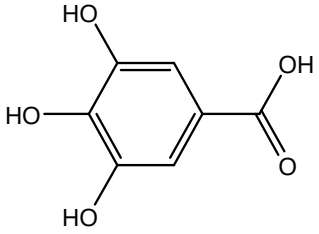

Fig. 23. Structure of gallic acid

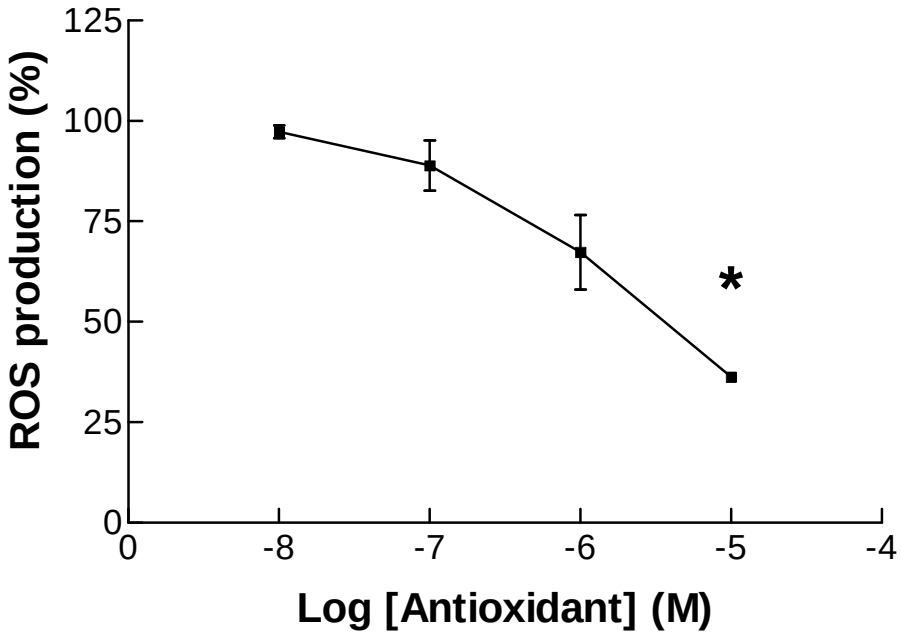

Fig. 24. The effect of gallic acid on the level of intracellular ROS formed in L-6 myoblasts after the exposure to cumene hydroperoxide. The production of ROS was measured through DCF fluorescence assay. The ROS production (%) was calculated according the eq. 13. as a decrease in the intracellular DCF fluorescence, ΔF/10 min, considering the fluorescence of 300 μM cumene hydroperoxide to be 100%. The data are expressed as the mean ± SD of n = 3-4 independent experiments. * p < 0.005, Student's t-test.

The MTT assay was performed with gallic acid at the concentrations 10^{-5} and 10^{-8} M, but no protective effect against cumene hydroperoxide-induced damage in L-6 cells was observed (Fig. 25).

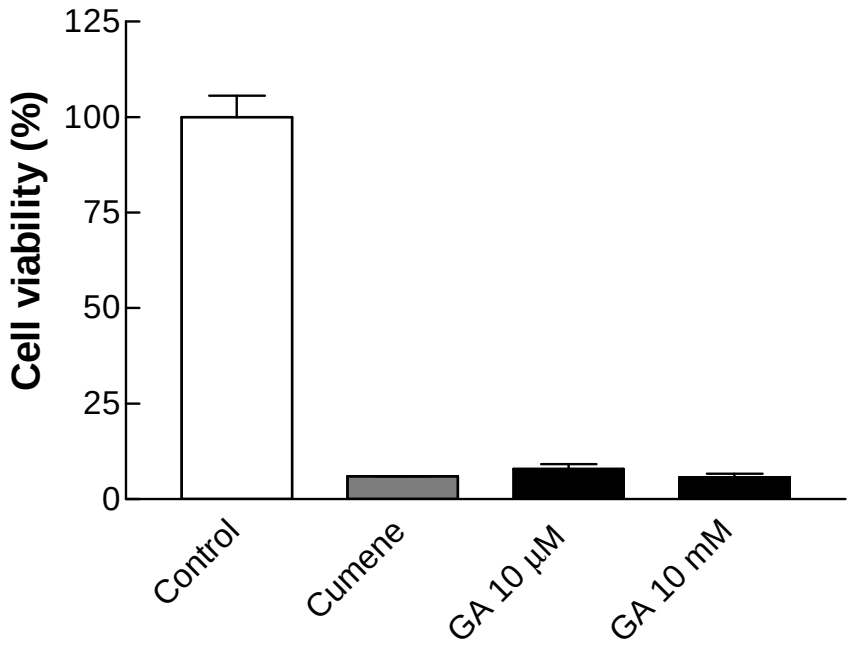

Fig. 25. The effect of gallic acid (10 μM and 10 mM) on the cell viability (%) of L-6 myoblasts after exposure to cumene hydroperoxide (300 μM). The blue crystals of MTT formazan, which correspond to the amount of viable cells, were measured at 560 nm using spectrophotometer. The data are expressed as the mean ± SD of n = 2 different experiments. GA stands for gallic acid.

COMPOUND 1625

The compound 1625, methyl-3,4,5-triacetoxy benzoate (Fig. 26), was synthesized as the ester derivative of gallic acid. It was dissolved in DMSO and its ability to decrease production of ROS in L-6 cells was tested in the range of concentrations 10^{-5}-10^{-9} M. In contrast to gallic acid, it showed good scavenging ability at the concentrations 10^{-5} M and 10^{-6} M. Compound 1625 was able to inhibit ROS production by 83-88% (Fig. 27).

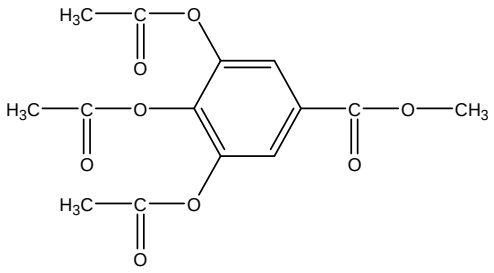

Fig. 26. Structure of compound 1625

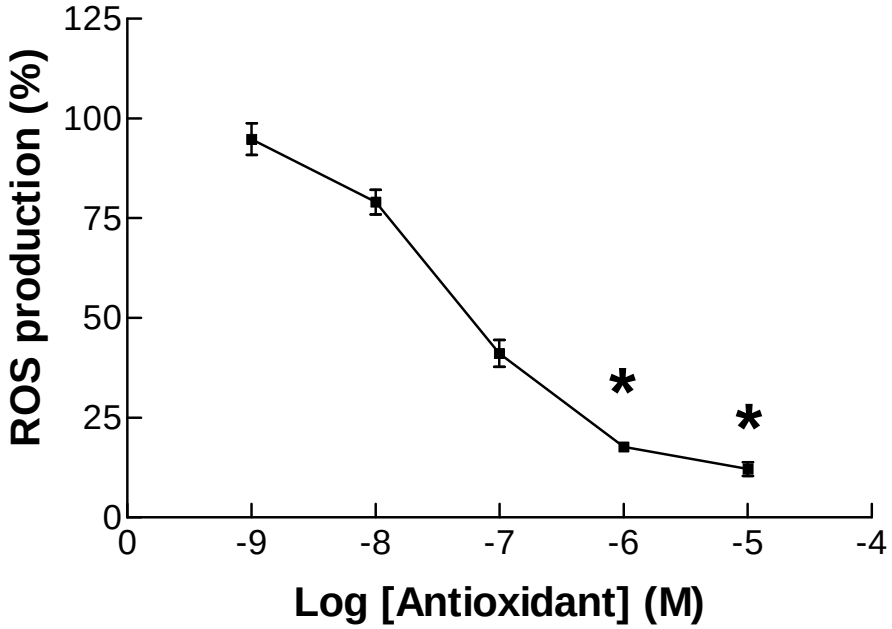

Fig. 27. The effect of compound 1625 on the level of intracellular ROS formed in L-6 myoblasts after the exposure to cumene hydroperoxide. The production of ROS was measured through DCF fluorescence assay. The ROS production (%) was calculated according the eq. 13. as a decrease in the intracellular DCF fluorescence, ΔF/10 min, considering the fluorescence of 300 μM cumene hydroperoxide to be 100%. The data are expressed as the mean ± SD of n = 3 independent experiments. * p < 0.001, Student's t-test.

The MTT assay with compound 1625 did not confirm the results obtained by DCFH-DA method. Surprisingly, this compound (10^{-5} and 10^{-8} M) did not protect cells against free radical-induced damage at all (Fig. 28).

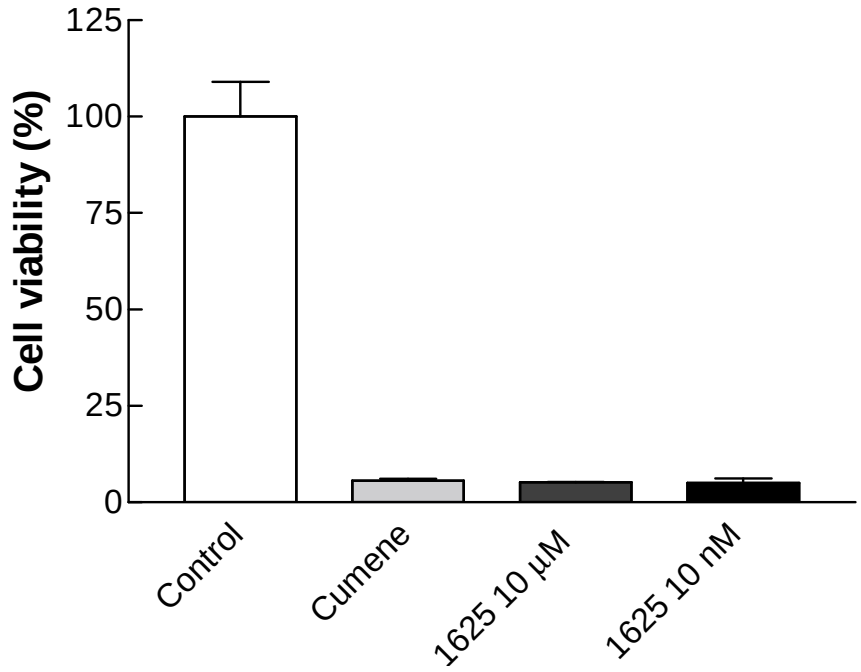

Fig. 28. The effect of compound 1625 (10 µM and 10 nM) on the cell viability (%) of L-6 myoblasts after exposure to cumene hydroperoxide (300 µM). The blue crystals of MTT formazan, which correspond to the amount of viable cells, were measured at 560 nm using spectrophotometer. The data are expressed as the mean ± SD of n = 2 different experiments.

5.2 **ANTIOXIDANT ACTIVITY *IN VITRO* (EPR)**

The samples of antioxidants were prepared in the concentration 0.1, 1 and 10 μM by dilution of their stock solutions (10 mM) with ethanol 95% (see Materials and methods section 4.7). The EPR spectra of prepared samples were measured. Galvinoxyl 10 μM was used as a free radical standard because this compound gives typical EPR spectrum consisting of two parts. The height of EPR signal is directly proportional to the concentration of the radical, when it is reduced by an antioxidant, its spectrum disappears (Fig. 29). The amount of galvinoxyl, which remains in a sample, depends on the scavenging ability of an antioxidant, its quantity, and on the time of incubation.

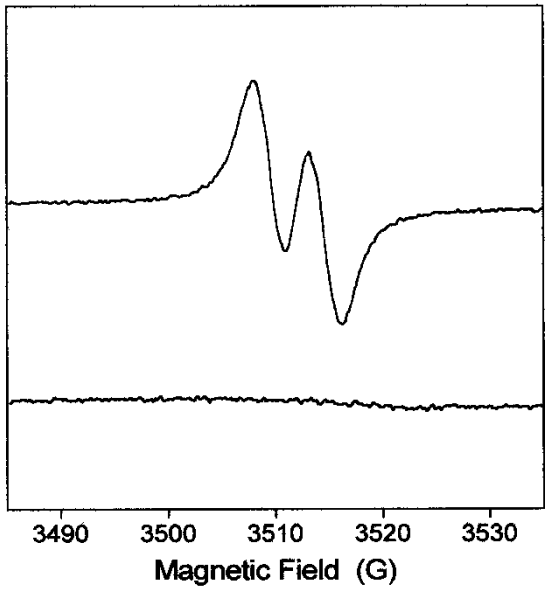

Fig. 29. EPR spectrum of 10 μM galvinoxyl in ethanol (top), and after addition of an antioxidant with good scavenging ability (bottom). In this case spectrum of the radical disappeared, i.e. galvinoxyl radical was trapped completely.

This method was used to evaluate *in vitro* antioxidant activity of flavonoids 5,6-dihydroxyflavone, negletein, mosloflavone, and baicalein. The good scavenging ability of negletein, 5,6-dihydroxyflavone and baicalein was proved also by EPR method. They were able to trap all galvinoxyl radicals at 10 μM concentration also in short time interval. Mosloflavone did not show any scavenging ability (Tab. 3).

Tab. 3. Reaction between flavonoids and galvinoxyl. The data show the concentration of galvinoxyl that remained in a sample after 5, 10 and 15 min of incubation. The initial concentration of galvinoxyl was 10 μM. The data are reported as mean ± SD of n = 3 different experiments.

Sample	5 min	10 min	15 min
Galvinoxyl standard 10 μM	10.0 ± 0.1	10.0 ± 0.0	9.9 ± 0.1
+ Baicalein 10 μM	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
+ Baicalein 1 μM	5.8 ± 1.1	1.3 ± 0.4	0.1 ± 0.1
+ Baicalein 0.1 μM	9.4 ± 0.8	6.8 ± 0.4	6.2 ± 0.7
+ 5,6-dihydroxyflavone 10 μM	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
+ 5,6-dihydroxyflavone 1 μM	4.7 ± 0.7	0.9 ± 0.6	0.2 ± 0.2
+ Negletein 10 μM	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
+ Negletein 1 μM	6.4 ± 0.8	2.0 ± 0.4	0.8 ± 0.4
+ Mosloflavone 10 μM	9.7 ± 0.2	9.9 ± 0.1	9.6 ± 0.2

6 DISCUSSION

This work was focused on the evaluation of antioxidant activity of selected flavones (baicalein, 5,6-dihydroxyflavone, mosloflavone, and negletein) and representatives of phenolic acids (gallic acid and its derivative 1625). Both flavonoids and phenolic acids belong to the group of plant polyphenols, widespread constituents in higher plants, which exert antioxidant properties. Due to their antioxidant ability, the research in last years has focused on the possible preventive activity of these compounds in the development of various diseases associated with oxidative stress (Manach et al. 2004).

Baicalein is one of the major flavonoids of *Scutellaria baicalensis*, herb used in Chinese and Japanese folk medicine. It has been reported that baicalein possesses antioxidant and other pharmacological effects. It is able to scavenge reactive oxygen species and inhibit lipid peroxidation (Hamada et al. 1993). Baicalein also inhibits proliferation of myeloma cells. The cell proliferation is suppressed by reduction of 12-lipoxygenase activity that is overexpressed in tumor tissues such as prostate cancer, breast cancer and so on. Baicalein is also able to induce apoptosis through the activation of caspase-3 in human leukemia HL-60 cells (Li et al. 2006). **Mosloflavone** is present in plants *Desmos chinensis* (Van Kiem et al. 2005) or *Mosla soochowensis Matsuda* (Wu et al. 1981). **Negletein** is found in *Centaurea clementei* (Righi et al. 2010) or *Colebrookea oppositifolia*, herb used in Chinese folk medicine for the treatment of fractures, traumatic injuries and rheumatoid arthritis (Yang F. et al. 1996). **5,6-Dihydroxyflavone** is a chemical constituent of *Primula modesta*. However, the potential antioxidant properties of these compounds (except baicalein) have not been widely studied yet. All flavones used in this work were synthesized by Prof. Paolo Bovicelli at the Department of Chemistry, "La Sapienza" University of Rome.

The **fluorescence assay with DCFH-DA** probe and cytotoxicity test were used to evaluate the ability of tested compounds to inhibit ROS production induced by cumene hydroperoxide in L-6 cells. Both baicalein and 5,6-dihydroxyflavone in higher concentrations (10^{-5} M and 10^{-6} M) showed good protective effect against oxidative damage. This scavenging ability is probably related to the structure of tested compounds. The **structure-activity relationship** of various flavonoids was described. Flavonoids with good scavenging ability possess a catechol group in the ring B (two hydroxyl groups in *ortho*-position) and a 2,3-double bond conjugated with the 4-oxo group in the ring C (Pietta 2000). All tested compounds lack the catechol group in the

ring B but 2,3-double bond conjugated with the 4-oxo group in the ring C is present in their structure. In addition, 5,6-dihydroxyflavone has two hydroxyl groups and baicalein even three hydroxyl groups in the ring A. This pyrogallol structure in the ring A is probably responsible for potent free radical scavenging and antioxidant effects of baicalein (Gao et al. 1999), just like two hydroxyl groups in the position 5 and 6 for the scavenging activity of 5,6-dihydroxyflavone. The possible explanation is that hydroxyl groups in adjacent positions may share an electron trapped from a free radical and create a stable quinone structure. Also **cytotoxicity assay** confirmed good scavenging ability of 5,6-dihydroxyflavone at the concentration of 10^{-5} M. Unfortunately, MTT assay with baicalein was not successful because all cells died during the experiment. That is why the **cell proliferation assay** with studied flavones was performed. The obtained results showed that both 5,6-dihydroxyflavone and baicalein inhibited cell proliferation. Such interaction between cells and antioxidant could be one of the reasons, why the cells died during MTT assay. Other possible explanation of cell death during experiment could be either too long incubation with cumene hydroperoxide or its too high concentration. Unfortunately, the real cause of this problem remains unknown and its solution would become a part of further study targeted to optimization of MTT assay.

In contrast, negletein showed lower ability to inhibit ROS production and mosloflavone was not able to inhibit the ROS production at all. Latter mentioned compound even exerted the prooxidative effect at higher concentrations (10^{-5} and 10^{-6} M). Both compounds lack the catechol group in the ring B, but they have 2,3-double bond conjugated with the 4-oxo group in the ring C as baicalein and 5,6-dihydroxyflavone. Negletein has similar structure to baicalein but its third hydroxyl group in the position 7 is methylated into methoxy group. This methoxylation is probably the reason for the decrease in the scavenging activity of negletein. We have supposed that this methoxy group could cause a steric hindrance and it may disable the *o*-hydroxyl group from trapping of free radicals. Mosloflavone has two methoxy groups in the position 6 and 7 and only one hydroxyl group in the position 5. The presence of these two methoxy groups instead of two hydroxyl groups in *ortho*-positions is probably the reason why it has nearly no protective effect on ROS production. These structural features may explain why negletein and mosloflavone did not protect cells during MTT assay. Other possible reason of unsuccessful MTT assay could be inhibition of the cell proliferation by negletein and mosloflavone, which was even more pronounced than in the case of 5,6-dihydroxyflavone and baicalein.

EPR assay is the *in vitro* method, which was employed in order to confirm the scavenging ability of tested antioxidants using stable radical galvinoxyl. The scavenging ability of tested compounds was evaluated as decrease in concentration of galvinoxyl in the sample. The obtained results confirmed the good scavenging ability of 5,6-dihydroxyflavone (10 μM) and baicalein (10 μM) that reduced the galvinoxyl level to zero, i.e. all radicals in the sample were trapped. The scavenging activity of negletein (10 μM) was surprising, because it was also able to trap galvinoxyl completely in contrast to the results obtained in cell culture. Mosloflavone showed again nearly no scavenging ability. Several questions regarding antioxidant ability of tested flavones remain unsolved, e.g. why negletein 10 μM decreased the ROS production only by 20% in cells but *in vitro* was able to trap all the radicals? The hypothesis that methoxy group next to the *ortho*-hydroxyl groups has some steric influence on the antioxidant scavenging ability should be investigated, because results of EPR showed that this is probably not the main reason of negletein's lower scavenging ability in cells. It is also possible that negletein at 10 μM concentration is cytotoxic (because its lower concentrations exerted better protective effect). Other explanation of its lower scavenging ability in cells may be that the final effect of antioxidant in cell culture depends also on the amount of antioxidant that enters to the cell and also on the possible metabolic changes of antioxidant within the cell. Finally, the fact that antioxidants react with artificially prepared stable radical galvinoxyl during EPR method while during DCFH-DA method react with ROS should be also considered.

The obtained results confirmed that the relationship between the structure of flavonoids and their scavenging activity exists, especially relationship between good scavenging ability and the presence of *ortho*-hydroxyl group in the structure of flavonoids.

The second part of this work was focused on the evaluation of antioxidant activity of **gallic acid** and its derivative **compound 1625** (methyl-3,4,5-triacetoxy benzoate). Gallic acid 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). Gallic acid and its derivatives have shown to possess many beneficial effects, e.g. antiallergic, antimutagenic, anti-inflammatory, and anticarcinogenic effects (Madlener et al. 2007). The gallic acid and its derivative 1625 used in this work were synthesized and

characterized in the laboratory of Prof. V. Parmar at the Department of Chemistry, University of Delhi.

The intracellular antioxidant activity of gallic acid and compound 1625 were evaluated with the fluorescence and cytotoxicity assay in L-6 cells. Gallic acid is reported to be a strong natural antioxidant (Gali et al. 1992), which is known to suppress reactive oxygen species and enhance the levels of GSH (Slaga 1995). These good antioxidant properties of gallic acid are connected with its structure, where three hydroxyl groups, especially the OH group in the *para* position to the carboxyl group, are responsible for its free radical scavenging activity (Lu et al. 2006). Our results of **fluorescence assay** showed some scavenging effect of gallic acid only at 10^{-5} M concentration, but there was no significant effect to inhibit ROS production at its lower concentrations. However, not even the concentration 10^{-5} M protected the cells during **MTT assay**. In contrast to gallic acid, its ester compound 1625 showed a great scavenging ability. It decreased the ROS production to 12-17% at concentration 10^{-5} and 10^{-6} M measured by **fluorescence assay with DCFH-DA**. Lu et al. (2006) reported that gallic acid showed weaker protective effects than its esters because of its hydrophilic properties. The hydrophobicity is an important factor for antioxidant activity within cell systems. A hydrophobic antioxidant may easily enter the cytoplasm and suppress ROS formation. The hypothesized mechanism of compound 1625 and also the explanation of its better scavenging ability is that this compound is able to easily cross the plasma membrane, its ester bond is cleaved by esterases within the cell and the released gallic acid can trap free radicals. But similarly to baicalein, the **MTT assay** did not confirm the results of compound 1625 obtained by DCFH-DA method and all cells died during MTT assay. Why was compound 1625 disable to scavenge free radicals as in the case of DCFH-DA method? According to the results mentioned in the diploma thesis of Elena Candelotti (2009), the compound 1625 had better inhibiting effect on ROS production in THP-1 cells after 30 min of pre-incubation with antioxidant, the proliferation assay showed that compound 1625 suppresses the cell propagations in L-6 cells and also in THP-1 cells, and the EPR assay confirmed that compound 1625 is able to trap free radicals (galvinoxyl) after longer time. Based on these results, the reason why the cells died during MTT assay could be because of the inhibiting effect of compound 1625 on the cell proliferation (as in the case of baicalein, negletein and mosloflavone). One of the suggestions for further experiments is to prolong the pre-incubation of cells with antioxidant before the cumene hydroperoxide is added.

7 CONCLUSIONS

Baicalein proved the highest antioxidant activity of all studied polyphenols in the cell culture. 5,6-Dihydroxyflavone demonstrated a comparable antioxidant activity with baicalein particularly at highest concentrations (10^{-5}-10^{-7} M). This good ROS scavenging ability is probably associated with the presence of *ortho*-hydroxyl groups in their structure. Mosloflavone did not show any antioxidant activity, even in concentration 10^{-5} and 10^{-6} M it had a prooxidant effect. Negletein acted as prooxidant at the highest measured concentration 10^{-5} M, but at the lower concentrations it proved a decrease of ROS production but not so significant as baicalein or 5,6-DHF. On the other hand, negletein proved to reduce the concentration of free radical galvinoxyl to the same level as baicalein or 5,6-DHF in the *in vitro* EPR assay. The potential steric effect of methoxy group on the antioxidant activity should become a subject of further study. The disability of tested flavones to protect cells during MTT assay (except 5,6-DHF) is probably associated with observed side effects on the cell proliferation.

The data of gallic acid and its ester derivative 1625 showed the latter mentioned compound had a better antioxidant activity and the ability to prevent ROS production probably due to its hydrophobic properties that allow this compound to enter to the cells.

There still remain many questions about these compounds that should be investigated in the future.

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8 LIST OF ABBREVIATIONS

ATB	Antibiotics
CAT	Catalase
cGMP	Cyclic guanosin monophosphate
DCF	2',7'-dichlorodihydrofluorescein
DCFH	2',7'-dichlorodihydrofluorescin
DCFH-DA	2',7'-dichlorodihydrofluorescin diacetate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDRF	Endothelium derived relaxing factor
EPR	Electron paramagnetic resonance spectroscopy
FBS	Fetal bovine serum
$Fl\text{-}O^{\bullet}$	Aroxyl radical
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulphide
H_2O_2	Hydrogen peroxide
$HOCl/OCl^{-}$	Hypochlorite, hypochlorous acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
$NO^{\bullet}$	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\bullet -}$	Superoxide radical
$^{\bullet}OH$	Hydroxyl radical
$ONOO^{-}$	Peroxynitrite
OS	Oxidative stress
PBS	Phosphate buffered saline
PLA_2	Phospholipase A_2
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum

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