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Effects of plant-derived polyphenols
on glycation and oxidative stress related to vascular diseases

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ABSTRACT

There is a large amount of evidence indicating that oxidative stress and glycation play a crucial role in aging as well as in neurodegenerative and cardiovascular diseases. Oxidative stress is linked with reactive oxygen species (ROS), whose harmful effects can lead to several pathological conditions like vascular alterations, diabetes complications and inflammation. Moreover, ROS and oxidative stress can directly contribute to glycation reactions with the formation of reactive α-oxoaldehydes and advanced glycation end-products (AGES). The glycation process is characterized by non-enzymatic reactions between aldehyde or keto groups of sugars and free amino-residues of proteins, which lead to the formation of AGES. Glycation and AGES cause the irreversible modification of the proteins structure and the consequent loss of their functionality, causing detrimental effect in vasculature. Thus, it can be reasonable that compounds which counteract the effect of oxidative stress and glycation may assume an important role in the prevention of vascular damage. Natural phenolic compounds and flavonoids have received attention for their biological effects, such as antioxidant and antiglycative activities. Despite the health claims of polyphenols, it is known that their bioavailability can vary dramatically depending on many factors. The effects of the polyphenols, and of their in vivo circulating metabolites, will ultimately depend on the extent to which they associate with cells, either by interactions at the membrane or more importantly by their cellular uptake. Therefore, in order to assess the potential activity of natural polyphenols, it is primarily important to evaluate the intestinal permeability and bioavailability. In this research, human colorectal carcinoma cells (HT-29) were used as experimental model of intestinal cells, to evaluate the polyphenols ability to cross the cellular membrane and permeate into the cell. The polyphenols studied in this research are characteristic phytoconstituents of several medicinal plants widely used in traditional medicine such as: baicalein (from Scutellaria baicalensis G.), eupatorin (Eupatorium semiserratum DC.), galangin (Alpinia officinarum L.), magnolol (Magnolia officinalis L.), myricetin (Myrica rubra S. Z.), oleuropein (Olea europaea L.), and silybin (Silybum marianum L.).

The aims of the PhD project were firstly to evaluate the HT-29 intestinal cells permeability of natural compounds, and secondly, to investigate the in vitro antiglycation activity of polyphenols and their activity against endothelial cellular damage related to oxidation and glycation, using HUVEC cells as experimental model for endothelium function. The results showed that the ability of polyphenols to cross the HT-29 cell membrane depends on many factors, including molecular size and hydrophilic properties of each compound. The flavonoids baicalein, eupatorin and galangin were able to cross the HT-29 cell membranes and undergo biotransformation process; conversely, oleuropein seemed
to not been taken up in the HT-29 cells. The results of AGEs determination showed that most of the polyphenols, at the higher concentration, displayed antiglycative activity in all experimental conditions, and that baicalein, eupatorin and galangin showed the highest activity in counteract the AGEs formation.

In the experimental model involving HUVEC, the aim was to evaluate the potential role of several compounds related to hyperglycemia and glycation, such as glycated albumin, glyoxal and glucose at high concentration, in inducing cytotoxicity and oxidative stress. Moreover, the antioxidant activity of the selected natural compounds against the oxidative stress induced by those factors was assessed using the DCFH-DA assay. The results from ROS detection assay showed that the glycation products known as glycated albumin and glyoxal were not able to directly induce oxidative stress in HUVEC in the assay conditions, whereas glucose at high concentration displayed an effect of oxidative stress induction. Therefore, high glucose was used as oxidative stressor in the polyphenols evaluation assay. Baicalein, eupatorin and galangin were selected as natural compounds with a potential antioxidant activity, and their activity on ROS formation in HUVEC induced by high glucose was evaluated. The highest antioxidant activity was seen after the pre-treatment with the polyphenols at the concentration of 5 μM.

All together, the results obtained motivate further researches on the activities of polyphenols against oxidative and glycative damages in order to understand their potential role in the prevention of vascular diseases.
Abbreviations

AG: aminoguanidine

AGES: advanced glycation end-products

BSA: bovine serum albumin

CDDO: 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid

CML: N(ε)-carboxymethyllysine

CVD: cardiovascular disease

DAD: diode array detector

DCF: dichlorofluorescein

DCFH-DA: 2',7'-dichlorofluorescein diacetate

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EBM-2: endothelial basal medium

EGM-2: endothelial growth medium

eNOS: endothelial nitric oxide synthase

FBS: fetal bovine serum

gHSA: glycated human serum albumin

gly-LDL: glycated LDL

HSA: human serum albumin

HPLC: high performance liquid chromatography

HT-29: human colorectal cancer cells

HUVEC: human umbilical vein endothelial cells

LDL: low density lipoprotein

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH: nicotinamide adenine dinucleotide phosphate

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NO: nitric oxide

Nrf-2: nuclear factor erythroid 2–related factor 2

ORAC: oxygen radical absorbance capacity

ox-LDL: oxidized LDL

PBS: phosphate buffer saline

PKC: protein kinase C

RAGE: receptor for advanced glycation end-products

RCS: reactive carbonyl species

ROS: reactive oxygen species

SOD: superoxide dismutase

TNFα: tumor necrosis factor α

VCAM-1: vascular cell adhesion molecule 1

VEGF: vascular endothelial growth factor
1. INTRODUCTION

1.1. Vascular disease and diabetes

Vascular disease and diabetes are chronic diseases characterized by long duration and slow progression. Cardiovascular diseases (CVDs) are considered the first cause of death globally: an estimated 17.5 million people died from CVDs in 2012, representing 31% of all global deaths (World Health Organization - WHO, 2016). The deaths caused by CVDs include the conditions of concurrent diabetes, because of the higher risk of cardiovascular events in diabetic patients; in particular, they have a 2–3 fold increased risk of heart attack and strokes. Most CVDs can be prevented by addressing behavioral risk factors such as tobacco use, unhealthy diet and obesity, and people who are at high risk for cardiovascular complications need early detection and management of different parameters (hyperglycemia, hyperlipidemia, and hypertension) which could help prevention and an appropriate treatment of these diseases.

Diabetes mellitus is considered one of the most common chronic metabolic diseases in nearly all countries, and in particular type-2 diabetes represents a multifactorial proatherogenic disease involving various aspects of metabolic syndrome, a combination of hyperglycemia, hyperlipidemia, obesity and hypertension (Beckman et al., 2002). Type 2 diabetes may remain undetected for many years and its diagnosis is often made when the disease is in a later stage, and the complications have already occurred. Diabetics have a 2- to 4-fold higher risk for cardiovascular events, and nearly 80% of diabetes-associated deaths are caused by CVD (WHO, 2016). The vascular diabetic complications are related to prolonged exposure to hyperglycemia, as well as other risk factor such as hypertension and dyslipidemia. Diabetes-related macrovascular complications are mainly represented by atherosclerotic disease and its sequelae, whereas microvascular complications can cause retinopathy and nephropathy, up to blindness and renal insufficiency (fig. 1) (Creager et al., 2003).

1.1.1. Microvascular complications of diabetes

Diabetic retinopathy may be the most common microvascular complication of diabetes. The risk of developing diabetic retinopathy or other microvascular complications of diabetes depends on both the duration and the severity of hyperglycemia. Development of diabetic retinopathy in patients with type 2 diabetes was found to be related to both severity of hyperglycemia and presence of hypertension (Fowler, 2008). The exact mechanism by which diabetes causes retinopathy remains unclear, but several theories have been postulated to explain the typical course and history of the
disease (Vithian & Hurel, 2010). It occurs following hyperglycaemia-mediated damage within the retinal microvasculature. This earliest damage causes basement membrane thickening, increased capillary permeability and the formation of microaneurysms. These changes lead to intravascular coagulation, resulting in retinal ischaemia which drives the formation of new vessels within the retina; these new vessels are fragile and may rupture causing retinal bleeds and, if left untreated, up to retinal detachment. Furthermore, the lack of lymphatic drainage within the retina causes fluid accumulation resulting in macular edema. All these processes can cause rapid vision loss and the decrease of the quality of life of the diabetic patient (King et al., 1994).

**Figure 1.** The major diabetic complications: microvascular and macrovascular complications. From: Diabetes Atlas 3rd Ed, 2006.

Diabetic nephropathy occurs in 30–40% of patients within 25 years. Currently, diabetic nephropathy is the leading cause of chronic kidney disease in Western societies (Ritz & Orth, 1999). Diabetic nephropathy arises from the combination of hyperglycemia and hypertension driving glomerular
damage. This pathology is a clinical syndrome characterized by persistent albuminuria, progressive decline in the glomerular filtration rate and elevated arterial blood pressure. Microalbuminuria is the first step towards developing proteinuria, which is also a marker of vascular endothelial dysfunction. The pathological changes involve thickening of glomerular basement membrane, and the increase of the intraglomerular hypertension causes glomerular sclerosis. Furthermore, increase of matrix production and glycation of matrix proteins can occur. The resulting glomerular hyperfiltration leads to subsequent progressive loss of renal function (Gnudi et al., 2003). Good evidence suggests that early treatment delays or prevents the onset of diabetic kidney disease. This has consistently been shown in both type 1 and type 2 diabetes mellitus (Vithian & Hurel, 2010).

Diabetic neuropathy refers to a spectrum of various neurological disorders associated with diabetes (Boulton et al., 2005). Diabetic neuropathy affects all peripheral nerves including pain fibers, motor neurons and the autonomic nervous system. The most common form is a distal, symmetrical sensorimotor neuropathy which may be asymptomatic; other symptoms may include dysesthesia, erectile dysfunction and postural hypotension, and the main sequel of neuropathy is foot deformity, ulceration and Charcot arthropathy (Sohn et al., 2010). The combination of neuropathy, arteriopathy and infection are the driving factors behind most diabetic foot amputations.

1.1.2. Macrovascular complications of diabetes

The major pathological mechanism in diabetes-related macrovascular complications is atherosclerosis. Studies have shown that adults with diabetes have a 2-3-fold increased risk of heart attacks and strokes, and it is estimated that 75-80% of diabetic adults die from complications of atherosclerosis. This is probably due because the progression of atherosclerotic lesions is accelerated by diabetes (Suzuki et al., 2001). Atherosclerosis results from chronic inflammation and injury to the endothelium and arterial wall in the peripheral vascular system (Lusis, 2000). The initial step is the modification of low density lipoprotein (LDL), caused by oxidation and glycation process, which leads to the formation of oxidated LDL (ox-LDL) and glycated LDL (gly-LDL). The LDL modification triggers a chain of metabolic responses, the first of which is the adhesion of monocytes to endothelial cells through the link with adhesion molecules as VCAM-1 (Vascular Cell Adhesion Molecule 1), ICAM-1 (InterCellular Adhesion Molecule 1) and E-selectin, followed by their transmigration and infiltration into the subendothelial compartment. Under the influence of IL-1 (interleukin 1) from endothelium, infiltrated monocytes differentiate into macrophages, which start to avidly take up modified LDL via the scavenger receptor, ultimately leading to foam cells formation (fig. 2) (Di Marco et al., 2014). The uptake of ox-LDL induces macrophages to produced MSCF (Macrophages Colony Stimulating Factor),
which stimulates macrophages proliferation and increase of IL-2 and TNFα (Tumor Necrosis Factor α), which in turn stimulate VCAM-1 expression on the endothelial surface. VCAM-1 promotes the adhesion of circulating T-lymphocytes to endothelial cells and their migration in the subendothelial compartment. Vascular smooth muscle cells (VSMC) proliferation is the consequence of interferon-γ (IFN-γ) produced by T-lymphocytes in the arterial wall, and it is followed by elaboration of the extracellular matrix and accumulation of cross-linked collagen and proteoglycans. The net result of this process is the formation of a lipid-rich atherosclerotic lesion with a tick fibrous cap (Lusis, 2000).

The mechanism by which diabetes is linked with vascular disease is not completely defined, but evidences suggest a strong association between CVDs and diabetes. One mechanism by which high glucose condition may enhance the atherosclerotic process involves activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which leads to the expression of inflammatory genes and adhesion molecules that facilitate monocyte adhesion to endothelial cells (Yan et al.,

**Figure 2.** Hyperglycemia, hyperlipidemia and oxidative stress associated to diabetes lead to ox-LDL uptake, increase expression of adhesion molecules, macrophages migration into the intima and foam cells formation, to endothelial dysfunction and atherosclerotic plaque formation. From: Di Marco, 2014.
In addition, there is evidence of increase platelet adhesion and hypercoagulability in type 2 diabetes. Impaired NO (Nitric Oxide) generation and increased free radical formation in platelets, as well as altered calcium regulation, may promote platelet aggregation. In patients with diabetes, fibrinolysis may be impaired by elevated levels of PAI-1 (Plasminogen Activator Inhibitor type 1), and both increased coagulability and impaired fibrinolysis could increase the risk of vascular occlusion and other complications (Beckman et al., 2002).

In vitro experimental data from literature show that, when normal aortic rings are incubated in a hyperglycemic condition, endothelium-dependent relaxation is impaired, suggesting that hyperglycemia decreases endothelium-derived NO production (Tesfamariam et al., 1991). Other research studies suggest that hyperglycemia induces a series of cellular events that increase the production of reactive oxygen species (ROS, such as superoxide anion) that inactivate NO to form peroxynitrite (Beckman et al., 2002). Moreover, diabetes heightens migration of vascular smooth muscle cells into early atherosclerotic lesions, where they replicate and produce extracellular matrix, as important steps in mature lesion formation. Studies from literature indicate that hyperglycemia may play a role in the accelerated atherosclerosis and smooth muscle accumulation in the diabetic animals. Thus, the observed effects on VSMC may be mediated by combination of hyperglycemia with other factors, like hyperlipidemia and hypertension, which represent concurrent conditions in diabetic patients (Suzuki et al., 2001).

1.1.3. Molecular mechanism of diabetes-related complications

The pathogenesis of diabetes-related vascular complications is complex, and several molecular mechanisms related to hyperglycemia are involved. Hyperglycemia could contribute to the development of vascular injury through increased polyol pathway, PKC activation, hexosamine pathway, glycation and AGEs formation, oxidative stress and ROS generation (Brownlee, 2001).

1.1.3.1 Polyol pathway

The first molecular mechanism that was discovered was the polyol pathway, described in peripheral nerve in the 1966 *Science* paper (Brownlee, 2005; Gabbay et al., 1966). In physiological glucose condition, only a small fraction of glucose is metabolized through this pathway, whereas in diabetic states, augmented intracellular glucose levels can cause an increased flux through it. The polyol pathway is a cellular metabolic pathway which consists of two steps (fig. 3). The first involves the enzyme aldose reductase, which has the function of reducing aldehydes to inactive alcohols (it
reduces glucose to sorbitol). During this process, NADPH acts as cofactor. In the second step, the enzyme sorbitol dehydrogenase oxidizes sorbitol to fructose, using NAD\(^+\) as cofactor. Aldose reductase has low affinity for glucose, and, at the physiological glucose concentrations found in non-diabetics, metabolism of glucose through this pathway is a very small percentage of total glucose use.

![Figure 3. Polyol pathway: the enzyme aldose reductase reduces aldehydes and glucose to sorbitol, with NADPH as cofactor. From: Brownlee, 2001.](image)

In hyperglycemic environment, however, increased intracellular glucose results in its increased enzymatic conversion to the polyalcohol sorbitol, with concomitant decrease of NADPH. In experimental conditions, flux through this pathway during hyperglycemia varies from 33% of total glucose in the rabbit lens to 11% in human erythrocytes. Thus, the contribution of this pathway to diabetic complications may be species, site and tissue dependent (Brownlee, 2001). The increased flux of glucose in the polyol pathway determines the augmented consumption of NADPH. Since NADPH is also the essential cofactor for the regeneration of reduced glutathione (GSH), a critical intracellular antioxidant, the decrease of cellular NADPH changes the intracellular redox balance resulting in the reduced production of nitric oxide and increased oxidative stress. Decreased levels of GSH have in fact been found in the lenses of transgenic mice that overexpress aldose reductase, and this is the most likely mechanism by which increased flux through the polyol pathway has deleterious consequences. The effect of aldose reductase inhibition on diabetic neuropathy has, however, been confirmed in humans in a rigorous multi-dose, placebo-controlled trial with the potent aldose reductase inhibitor Zenarestat, though its development was terminated (Greene et al., 1999).
1.1.3.2. Protein kinase C (PKC) activation

Hyperglycemia increases diacylglycerol (DAG) content, which activates β and δ isoforms of PKC. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and plasminogen activator inhibitor-1 (PAI-1), and by activating NF-κB and NAD(P)H oxidases (Brownlee, 2001). Protein kinase C is a family of protein kinase enzymes with 15 isoforms that are involved in the regulation of protein function. Nine of these 15 PKC isoforms are activated by a lipid second-messenger, diacylglycerol, and the other PKC isoforms can also be activated by intracellular ROS in the absence of DAG. The regulatory domain of these PKC isoforms contains 2 pairs of zinc fingers with 6 cysteine residues and 2 zinc atoms, which can be oxidized by intracellular ROS. Oxidation alters zinc-finger conformation and activates PKC (Cosentino-Gomes et al., 2012). Hyperglycemia and elevated intracellular glucose levels increase DAG de novo synthesis in a variety of diabetic target tissue, and primarily activate the β and δ isoforms of PKC. Many cellular changes involved in diabetic vascular disease, as endothelial dysfunction, increased vascular permeability and impaired angiogenesis, have been linked to PKC activation. Data from literature show that hyperglycemia-induced activation of PKC-β promotes vascular inflammation and atherosclerosis in diabetic ApoE -/- mice by augmented expression of inflammatory mediators. Diabetic mice showed increased plaque formation, cholesteryl ester content, and macrophage infiltration (Kong et al., 2013). Abnormalities in renal blood flow and vascular smooth muscle cells functions are linked to hyperglycemia-induced PKC-β activation, perhaps by decrease of NO production by eNOS and increase expression of the vasoconstrictor endothelin-1. Moreover, PKC-α activation mediates increased permeability of endothelial cells induced by high glucose in cultured cells (Hempele et al., 1997). Hyperglycemia-induced activation of PKC has also been implicated in the overexpression of the fibrinolytic inhibitor PAI-1, the activation of NF-κB in cultured endothelial cells and vascular smooth muscle cells, and in the regulation and activation of various membrane-associated NAD(P)H-dependent oxidases (Yerneni et al., 1999).

1.1.3.3. Hexosamine pathway

The hexosamine pathway causes reversible post-translational modification of intracellular protein. The O-linked attachment of β-N-acetylglucosamine (O-GlcNAc) to serine/threonine residues of proteins is a dynamic and reversible process that is an essential signal transduction mediator in all cells (fig. 4). O-GlcNAc serves as a nutrient/stress sensor regulating cellular homeostasis by altering
signaling, transcription, metabolism, organelle biogenesis, cytoskeletal dynamics, and apoptosis (Hardivillé & Hart, 2014).

Figure 4. In hyperglycemic condition, the flux of glucose through the hexosamine pathway is increased. From: Brownlee, 2001.

In normal condition, the intracellular glucose is metabolized through glycolysis, given first glucose-6-phosphate and then fructose-6-phosphate, following the glycolytic pathway. However, in hyperglycemic condition, the increased fructose-6-phosphate is directed to a signaling pathway in which it is converted to glucosamine-6-phosphate and finally to UDP (uridine diphosphate) N-acetyl glucosamine. The enzyme O-GlcNAc transferase (OGT) uses UDP-GlcNAc to transfer N-acetylglucosamine to a variety of proteins, resulting in increased protein modification by N-acetylglucosamine. Thus, a protein over-modification induced by GlcNAc results in pathologic changes in gene expression. For example, increased modification of the transcription factor Sp1 results in increased expression of transforming growth factor-β1 and plasminogen activator inhibitor-1, both of which are detrimental for diabetic blood vessels (Marsh et al., 2014). It has been reported that hyperglycemia increased O-GlcNAcylation of endothelial NOS (eNOS) in bovine aortic endothelial cells, which was associated with a decrease in phosphorylation at Ser-1177, the site responsible for activation of the enzyme. The resultant increased protein modification by O-GlcNAc was responsible for decreased endothelium-dependent relaxation of the coronary arteries. In human
arterial endothelial cells, activation of eNOS by phosphorylation at Serine 1177 is inhibited directly by hyperglycemia-induced O-GlcNAcylation at this site (Du et al., 2001).

1.1.3.4. Glycation and AGEs formation

The non-enzymatic glycation of proteins is a process that links chronic hyperglycemia to physiopathological changes related to chronic diabetes complications. Glycation is characterized by non-enzymatic reactions between aldehyde or keto groups in sugars and dicarbonyl compounds, and free amino-residues in proteins, that lead to the formation of unstable adducts and Amadori compounds, up to the production of compounds known as Advanced Glycation End-products (AGEs) (fig. 5). Since its first description by Maillard in 1912, the link between glycation and various pathologies in the human body, including aging and diabetes, have been intensively studied.

![Glycation Diagram](image)

**Figure 5.** The glycation reaction schematically represented as the reaction between the carbonyl group of glucose and amino group of the lysine residue on a protein. All the main glycation steps are represented: Schiff base formation, Amadori products, and protein cross-links. From: Ahmed et al., 2003.

Glycation is a multistep reaction and three stages are recognized. The first step starts with the rapid formation of a non-stable Schiff base between a reducing sugar, as glucose, and the free amino group of lysine and arginine of proteins, nucleic acids or lipids. Then, following rearrangement leads to the formation of a more stable ketoamine (Amadori product), as fructosamine; all these steps are reversible. In the second stage, oxidation and dehydration reactions occur and Amadori products degrade into very reactive carbonyl compounds (glyoxal, methylglyoxal, 3-deoxyglcuronosone). These
reactive dicarboxyls can act as propagators and further react with free amino groups of proteins starting a new glycation process. The third stage involve oxidation, dehydration and cyclization reactions, up to the formation of the yellow-brown, often fluorescent, AGEs which are irreversible products that accumulate on long-living protein and, when formed at critical sites in enzymes or proteins, they may be associated with enzyme inactivation or protein altered function (Vistoli et al., 2013; Wautier & Schmidt, 2004).

Products that could alter protein structure and function are cross-linked AGEs, hydroimidazolone and lysine adducts; the best known of these compounds are GOLD (glyoxal-derived lysine dimer), MOLD (methylglyoxal-derived lysine dimer), DOLD (3-deoxyglucosone-derived lysine dimer), pentosidine and CML (carboxy-methyl-lysine), (Ahmed & Thornalley, 2003).

Glycation becomes relevant when it involves long-living proteins in the human body, impairing their function and contributing to diseases, such as diabetic retinopathy and atherosclerosis. AGEs are found in increased amounts in diabetic retinal vessels and renal glomeruli (Horie et al., 1997; Stitt et al., 1997).

Three main mechanisms by which AGEs cause tissue damage are recognized: intracellular glycation, cross-link formation, and interaction with specific cellular receptors (fig. 6), (Brownlee, 2005). AGEs accumulate rapidly inside macrophages, endothelial cells and vascular smooth muscle cells: in endothelial cells exposed to high glucose, intracellular AGE formation occurs within a week (McPherson et al., 1988). The AGES formation on intracellular proteins determines changes in their structure and alteration in function. Increased glycation of intracellular proteins in endothelial cells exposed to diabetic levels of hyperglycemia results in altered function of protein that regulate endothelial cells growth; basic fibroblast growth factor has been recognized one of the main AGE-modified proteins in endothelial cells (Giardino et al., 1994).

The AGE precursors formed inside the cells can diffuse outside the cells and modify extracellular matrix proteins (Charonis et al., 1990; McLellan et al., 1994). The mechanism of cross-link formation is non-receptor dependent and occurs especially on proteins with a slow turnover rate. The most important extracellular modification is abnormal cross-link on collagen and extracellular matrix proteins. The structural tissue modifications occurred in chronic complications of diabetes, such as vascular and arterial stiffening and basement membrane thickening, are related to structural and functional changes on collagen induced by glycation. Glycation of laminin and type I and type IV collagens, key molecules in the basement membrane, causes inhibited adhesion to endothelial cells for both matrix glycoproteins. In addition, AGE compounds in collagen may also trap macromolecules such as lipoproteins, immunoglobulin and albumin, contributing to vessel occlusion in atherosclerosis.
(Lapolla et al., 2005). In the extracellular compartment, it is reported that glycation can also occur on lipids and lipoproteins, leading to the formation of gly-LDL. Data from literature confirms that gly-LDL reduces nitric oxide production and suppresses uptake and clearance of LDL through its receptor on endothelial cells (Goldin et al., 2006).

Figure 6. The extracellular and intracellular effects of AGEs. The formation of AGEs on extracellular collagen and laminin alters the matrix structure and increase stiffness. AGEs that bind to RAGE on endothelial cells surface, start a signaling cascade with pro-oxidant and pro-inflammatory effects. From: Goldin, 2006.
In particular, AGEs play a physio-pathological role through the binding of specific receptors known as RAGE (Receptor for Advanced Glycation End-products). RAGE is a multiligand transmembrane receptor belonging to the immunoglobulin superfamily (Neeper et al., 1992; Schmidt et al., 2001). RAGE is widely localized in many cell types, and it is expressed on vascular smooth muscle cells, macrophages, fibroblasts, T-lymphocytes and endothelial cells. This receptor is expressed at low physiological levels in tissues and vasculature, but it can be abnormally up or down-regulated in pathological conditions, depending on ligand and signaling activation. RAGE is upregulated when AGE ligands accumulate, an example of positive-feedback activation, and this upregulation effect occurs on cells such as endothelial cells, smooth muscle cells, and mononuclear phagocytes in diabetic vasculature (Schmidt et al., 2001). RAGE is considered a pattern recognition receptor, because of its ability to bind several ligands in addition to AGEs: RAGE can bind proinflammatory cytokine-like mediators of the S100/calgranulin family, amphoterin (also known as high-mobility group B1, HMGB1), and amiloid-beta protein (Chavakis et al., 2003). The binding of RAGE with AGEs, as well as with S100 calgranulins and HMGB1, trigger inflammatory pathways. In the vasculature, the main pathological consequence of AGES interaction with RAGE is the induction of intracellular ROS, the generation of which seems to be linked to the activation of NADPH-oxidase. An important key target of RAGE signaling is NF-κB. Upon RAGE stimulation, the transcription factor NF-κB is translocated to the nucleus, where it increases transcription of several different proteins, including endothelin-1, VCAM-1, ICAM-1, E-selectin, tissue factor and RAGE itself (Goldin et al., 2006). In HUVEC, inhibitors of NF-κB greatly reduce high glucose-induced monocyte adhesion, suggesting that the activation of NF-κB is essential in AGE-induced monocyte adhesion and migration (Morigi et al., 1998).

In diabetes, the AGE-RAGE interaction may result in a long-lasting sustained activation of NF-κB, in the absence of decreased IκBα, via a protein synthesis-dependent pathway and sustained translocation of NF-κB from the cytoplasm into the nucleus. This mechanism may contribute to the persistent NF-κB activation observed in hyperglycemia (Bierhaus et al., 2001). Studies have shown that AGEs cause increased expression of macrophage scavenger receptor (MSR) and CD36 receptors, leading to increased ox-LDL uptake and foam cell formation. AGEs may contribute to the expression of oxidized LDL (ox-LDL) receptors in human monocyte-derived macrophages. Activation of monocytes by AGE-modified human serum albumin also leads to expression of IL-1β and TNFα mRNA (Webster et al., 1997).

Interestingly, RAGE is not the only receptor for AGEs, other receptors known to recognize and bind AGEs include class A macrophage scavenger receptors (MSRs), oligosaccharyl transferase-48 (OST48, also known as AGE-R1), 80 K-H phosphoprotein (or AGE-R2), galectin-3 (AGE-R3), lectin-like oxidized low density lipoprotein receptor-1 (Lox-1), fasciclin EGF-like, laminin-type EGF-like, and link domain-
containing scavenger receptor-1/2 (FEEL1/2) and CD36. Nevertheless, they have not been shown to transduce cellular signals after engagement by AGEs. Instead, they may cause the clearance and possible detoxification of AGEs (Bucciarelli et al., 2002).

### 1.1.3.5. Oxidative stress and ROS formation

The hyperglycemia-related process besides all the cited mechanisms of vascular damage (polyol pathway, PKC activation, enhanced flux through hexosamine pathway, glycation and AGEs formation) can be recognized as the overproduction of superoxide by the mitochondrial electron transport chain (Shah & Brownlee, 2016). Enhanced intracellular glucose transport and oxidation leads to mitochondrial overproduction of superoxide (fig. 7). Many studies have shown that diabetes and hyperglycemia increase oxidative stress, but the specific pathway is not clear. It has now been established that all of the different pathogenic mechanisms described above derive from a single hyperglycemia-induced process: the overproduction of superoxide by the mitochondrial electron-transport chain. Superoxide is the initial oxygen free radical formed by the mitochondria, which is then converted to other more reactive species that can damage cells in numerous ways (Wallace, 1992).

![Figure 7.](image)

**Figure 7.** The overproduction of ROS by the mitochondrial electron-transport chain is the initial step of oxidative stress, which could lead to lipid peroxidation, protein oxidation and DNA damage, up to cellular dysfunction (Baughman & Mootha, 2006).
The mitochondrial isoform of the enzyme SOD degrades this oxygen free radical to hydrogen peroxide, which is then converted to H₂O and O₂ by other enzymes. In primary arterial endothelial cells in culture, intracellular high glucose concentration increases the voltage across the mitochondrial membrane above the critical threshold necessary to increase superoxide formation and, subsequently, increases production of ROS (Giacco & Brownlee, 2010). Evidences indicate that the mitochondria are required for the initiation of hyperglycemia-induced superoxide production, and that this higher radical production may then amplify the original damaging effect of hyperglycemia, including redox changes, NADPH oxidase, and uncoupled eNOS. The initiating role of mitochondrial ROS is suggested by the observation that cells lacking mitochondrial electron transport chain function (ρ₀ cells) fail to increase ROS production in response to high glucose (Brownlee, 2005). The excess of ROS production leads to cellular dysfunction, inflammation, apoptosis, and fibrosis in cells exposed to high glucose flux. The importance of ROS generation in these processes is confirmed by the fact that cellular damage can be prevented when the transgenic expression of the enzyme superoxide dismutase determined the reduction of hyperglycemia-mediated ROS generation (Shen et al., 2006).

In cardiovascular physiology, ROS production occur also in normal conditions, and ROS species act as signaling molecules essential for cellular homeostasis. Physiological level of ROS is essential for cell differentiation, autophagy, growth factor stimulation, and generation of physiological inflammatory response. In specific, low concentrations of superoxide anion (O₂•⁻) and hydrogen peroxide (H₂O₂) are produced in cells by mitochondria and NADPH oxidases. They are controlled by endogenous antioxidants, manganese and copper/zinc superoxide dismutase (MnSOD, Cu/Zn SOD), catalase, and glutathione peroxidases. Together with nitric oxide (•NO), ROS act as cell signaling initiators, for example regulating VSMC contraction and relaxation (Cohen & Tong, 2010). Moreover, it is well known that the signal propagation upon receptor binding of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor, and endothelin-1, as well as cytokines such as TNFα and IL-1β, is mediated through the generation of O₂•⁻ and H₂O₂. To maintain cellular redox balance and prevent potential damage induced by radical species, enzymatic antioxidant defense systems (catalase, superoxide dismutase, glutathione peroxidase) remove and scavenge ROS. In pathological conditions, the increase in ROS production and the not adequate efficiency of the antioxidant systems lead to oxidative stress. The excessive ROS production and the oxidative stress in vascular compartment are believed to be crucial contributors to the pathogenesis and progression of diabetes-related vascular disease. Increased ROS formation leads to endothelial dysfunction, impaired vascular relaxation, increased VSMC growth and migration, and increased of extracellular matrix deposition (Brown & Griendling, 2015).
1.2. Natural products and polyphenols

Nature has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine (Cragg & Newman, 2001). The use of plants in the traditional medicine systems of many cultures has been extensively documented, and these plant-based systems continue to play an essential role in health care: it has been estimated by World Health Organization that approximately 80% of people worldwide rely on traditional medicines for their primary health care, and plant products play an important role in the health system for 20% of the population. Especially in Africa and some developing countries, traditional medicine is often the primary source of health care (Farnsworth et al., 1985; World Health Organization, 2005).

Plant-derived natural products have importance not only in traditional medicine, but they also represent an interesting source for drug discovery (Newman & Cragg, 2016). Since natural products are made from living organisms, they possess properties that are evolutionary optimized for having different biological functions (for example binding to specific target proteins or other biomolecules), (Appendino et al., 2010). The most interesting thing is that drug discovery from nature could lead to the isolation of active compounds with therapeutic activity, which can be improved thanks to chemical and structural modifications of the starting compound. In a recent review, Newman and Cragg have shown that 34% of current drugs are natural-product-inspired or derived (semi-synthetic natural products, or active compounds inspired from nature) (Newman & Cragg, 2012).

Plant-derived natural products approved for therapeutic use in the last thirty years (1984-2014) are used for the treatment of various disease conditions and modulate a large range of molecular targets. Drug discovery from medicinal plants in the 19th century led to the purification of several early drugs including aspirin, codeine, digitoxin, morphine, and quinine, which are all still widely used. Moreover, still today natural products are an important source for the discovery and development of new drugs. The most recent plant-derived products approved as drugs by FDA are Veregen® (sinocatechins; green tea (*Camellia sinensis* L. leaf extract), Fulyzaq® (crofelemer; extract from the red latex of *Croton lechleri* Müll.Arg.), and Grastek® (Timothy grass (*Phleum pretense* L.) pollen allergen extract), (Waltenberger et al., 2016).

Thus, medicinal plants and natural products still represent an important pool for identification of new pharmacological leads today. Scientific interest in plant-derived natural product-based drug discovery is renewing, as seen from the analysis of PubMed publications trends (Atanasov et al., 2015).
Considering the huge morbidity and mortality burden related to vascular and metabolic disorders, there is a high interest in the discovery of novel compounds as well as novel pharmacological targets that might be effective in the treatment or prevention of cardiovascular and/or metabolic disorders.

Several natural compounds have been shown to affect vascular and metabolic disorders through different mechanisms, such as anti-inflammatory activity, changes of blood lipid profiles, improvement of insulin sensitivity, or normalization of blood glucose levels (Lacroix & Li-Chan, 2014; Ríos et al., 2015). Often the underlying molecular targets mediating these beneficial effects are not well understood. However, there are several molecular targets or pathways that are already well established to mediate the beneficial effects of natural compounds in the context of cardiovascular and metabolic disorders. Of those, the AMP-activated protein kinase (AMPK), cyclooxygenase (COX)-1 and -2, the dipeptidyl peptidase-4 (DPP-4), the endothelial nitric oxide synthase (eNOS), NF-kB, nuclear factor-erythroid 2-related factor 2 (Nrf2), (Waltenberger et al., 2016).

The most widespread compounds present in medicinal plants which claim a potential therapeutic activity belong to the class of polyphenols, flavonoids, terpenes, and alkaloids. Among these, flavonoids are secondary metabolites widely distributed throughout the plant kingdom with well known and proven antioxidant and anti-inflammatory activities (Vasanthi et al., 2012).

In the present research, seven plant-derived polyphenols were studied for their potential activity in the treatment or prevention of the vascular damage induced by oxidative stress and glycation. The polyphenols studied in this research are from medicinal plants widely used in traditional medicine: baicalein (from Scutellaria baicalensis G.), eupatorin (Eupatorium semiserratum DC.), galangin (Alpinia officinarum L.), magnolol (Magnolia officinalis L.), myricetin (Myrica rubra S. Z.), oleuropein (Olea europaea L.) and silybin (Silybum marianum L.), (fig. 8).
Figure 8. Chemical structure of baicalein, eupatorin, galangin, magnolol, myricetin, oleuropein and silybin: polyphenols from medicinal plants widely used in traditional medicine.
1.2.1. Baicalein

Baicalein is a flavone isolated primarily from the roots of *Scutellaria baicalensis* G., but its presence is also reported in *Oroxylum indicum* B. *Scutellaria baicalensis* and *Oroxylum indicum* are medicinal plants used in Chinese and eastern traditional medicine. *Scutellaria baicalensis* is one of the 50 fundamental herbs used in traditional Chinese medicine, which has been widely used as anti-inflammatory, anti-viral, anti-bacterial and anti-cancer compound (Huang et al., 2006; Kumagai et al., 2007). *Oroxylum indicum* has been used for centuries as important herbal medicine in many Asian countries to cure various diseases. The traditional uses of *Oroxylum indicum* comprise the treatment of fever, bronchitis, intestinal worms, asthma, inflammation and dysentery; in Traditional Chinese medicine, the seeds of the plant have been widely used for the treatment of cough, bronchitis, pharyngitis, pertussis and other respiratory disorders (Dinda et al., 2015).

Baicalein is one of the major flavonoids present in the dried roots of *Scutellaria baicalensis*, and it is reported that possesses a multitude of pharmacological activities. Baicalein is a potent free radical scavenger and xanthine oxidase inhibitor, thus improving endothelial function and having cardiovascular protective actions against oxidative stress-induced cell injury (Huang et al., 2005). Many researches were conducted to study its potential role in vascular diseases and in particular its activity on blood pressure. The results show that baicalein lowers blood pressure in renin-dependent hypertension in rats, and the in vivo hypotensive effect may be partly attributed to its inhibition of lipoxygenase, resulting in reduced biosynthesis and release of arachidonic acid-derived vasoconstrictor products (El-Bassossy et al., 2014). Baicalein was studied, together with its glucuronide form baicalin, for the antioxidant and anti-inflammatory activities. These two flavonoids have shown anti-inflammatory effects and improvement of mitochondrial dysfunction through redox-dependent mechanisms (de Oliveira et al., 2015). In addition, it has shown that baicalin and baicalein protect mitochondria from oxidative stress both in vitro and in vivo. Data from literature show that baicalein protects different types of cells against oxidative stress, involving the inhibition of 12-lipoxygenase and 15-lipoxygenase. By inhibiting 12-lipoxygenase and up-regulating Nrf2, baicalein blocks the increase in ROS level in NG108–15 neuroblastoma x glioma hybrid cells, which have been widely used to study many aspects of neuronal function and differentiation, from H$_2$O$_2$-induced activation of the mitochondrial apoptotic pathway (Yeh et al., 2015).

Regarding the research for natural products that modulate vascular inflammatory response, it has been found that baicalein and baicalin had anti-inflammatory effects on LPS-mediated inflammation in vitro and in vivo. Recent investigations showed that baicalein can exert anti-inflammatory effects on LPS-stimulated macrophages by inhibiting TNF-α, COX-2, and iNOS gene expression, as well as
inhibiting the production of NO, IL-1β, TNF-α, COX-2, iNOS, and PGE₂ via estrogen receptor-dependent pathway (Fan et al., 2013).

Baicalein was also studied for its potential role as anti-tumor agent. Research studies reveal that baicalein exerts this effect by promoting the apoptosis and inhibiting the proliferation of cancer cells. In specific, treatment with baicalein in vitro resulted in hepatocellular carcinoma cells growth inhibition, with an accumulation of cancer cells in G0/G1 phase. The in vivo studies on mice carrying xenograft tumors showed that the treatment with baicalein determined the reduction of tumor weight in a dose-dependent manner (Zheng et al., 2014).

1.2.2. Eupatorin

Flavone eupatorin is isolated from Orthosiphon stamineus B., a medicinal herb used in folk medicine in South East Asia for the treatment of various disorders. This plant is used in traditional medicine and leaves are consumed as herb tea for body detoxification, to treat fever and influenza, rheumatism, urinary and biliary lithiasis. Extensive pharmacological investigations on various extracts from Orthosiphon stamineus were carried out to characterize its therapeutic potential. The results revealed that Orthosiphon stamineus exhibits a large spectrum of activities, including diuretic, antioxidant, uricosuric, anti-hypertensive, hepatoprotective, and anti-inflammatory (Olah et al., 2003).

The polymethoxylated flavonoids sinensetin, eupatorin and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone are found as dominant compounds in the chloroform fraction of the extracts, and are among the most therapeutically important constituents (Yam et al., 2016). Studies from literature show that eupatorin has anti-proliferative effect against several cancer lines, including human cervical adenocarcinoma (HeLa), human gastric adenocarcinoma (MK-1), murine melanoma (B16F10), murine colon carcinoma (26-L5), or human breast cancer cell line (MDA-MB-468), (Androutsopoulos et al., 2008; Lee et al., 2016). Anti-angiogenic activity of eupatorin was also tested, using HUVEC as experimental model of angiogenesis in vitro. Eupatorin determined the decrease of cell migration and also the decrease of the cell ability of tube formation (Dolečková et al., 2012). In addition to this, due to its structural similarity to known protein kinase inhibitors, the effect of eupatorin to inhibit representative human protein kinase was examined, and the results show that eupatorin is a non-specific inhibitor of protein kinases (Singh & Agarwal, 2006).

Other researches from literature set the aim to study the anti-inflammatory activity of Orthosiphon stamineus extracts and isolated flavonoids, including eupatorin. The studies on inflammatory gene expression in vitro and carrageenan-induced paw inflammation in vivo showed that flavonoids
eupatorin and sinensetin inhibited the expression of inflammatory mediators as PGE2, and TNFα. Furthermore, eupatorin and sinensetin inhibited carrageenan-induced paw inflammation (Laavola et al., 2012). Eupatorin seems to display also vasorelaxation effect, as seen by Yam and colleagues in rats aortic ring (Yam et al., 2016).

1.2.3. Galangin

Galangin is a polyphenolic compound isolated from the rhizome of *Alpinia officinarum* H., which is used as traditional herb remedy in the Middle East, for rheumatic pains and arthritis (Su et al., 2013). It is also used traditionally for hiccups, nausea, chronic gastritis and ulcers in the digestive tract. It has been reported that *Alpinia officinarum* has analgesic, anti-pyretic, and antimicrobial activities. The flavonol galangin has been studied with the aim to detect its potential antioxidant, anti-inflammatory and anti-cancer activity. In the context of cancer, the activity of galangin on leukemia cancer cells was studied. It is reported that galangin could induce a G0-G1 cell cycle arrest and decrease Bcl-2 levels, which leads to apoptosis of imatinib-resistant Bcr-Abl expressing leukemia cell lines (Tolomeo et al., 2008). Furthermore, the activity of galangin on hepatocellular carcinoma was extensively studied. The results from literature reveal that galangin significantly reduce the viability of hepatocellular carcinoma cells via mitochondrial pathway, triggering Bax translocation to the mitochondria and inducing overexpression of Bcl-2 (Zhang et al., 2010). The anti-cancer activity has been further studied, and the results reveal that galangin induced ER (endoplasmic reticulum) stress in liver cancer cells leading to proliferation inhibition (Su et al., 2013).

A potential vascular activity of galangin has been hypothesized, thus, its effect on isolated rat thoracic aorta was examined (Morello et al., 2006). In the study, the flavonoid causes both concentration- and endothelium-dependent relaxation of rat thoracic aortic rings pre-contracted with phenylephrine. Data from literature confirm also the antioxidant activity of galangin on isolated rat liver mitochondria, because of its protective effect against membrane lipid preroxidation (Dorta et al., 2008).

1.2.4. Magnolol

Magnolol is a hydroxylated biphenyl compound isolated from the stem bark of *Magnolia officinalis* L.; this is a Chinese herb used in traditional medicine in South Korea, China and Japan for gastrointestinal disorders, cough, anxiety, depression, and allergic diseases (Chen et al., 2011; Maruyama et al., 1998). *Magnolia officinalis* bark is rich in two biphenyl compounds, magnolol and...
honokiol, that have been extensively investigated. A large number of pharmacological activities have been reported for magnolol, including anti-inflammatory, antimicrobial, antioxidant, neuroprotective, and anti-tumor activities. In the context of the anti-inflammatory activity, magnolol was found to inhibit NO production in LPS-activated macrophages, and to suppress inflammatory cytokines production in THP-1 monocyte cells (Park et al., 2004). Research reports that magnolol inhibits LPS-induced pro-inflammatory cytokines by interfering with toll-like receptor 4, and that this is related to the inhibition of NF-κB and MAPK signaling pathway (Fu et al., 2013).

The beneficial effects of magnolol in the context of vascular disease have been studied, and the results show in vitro and in vivo evidences of a potential activity. Treatment of human aortic endothelial cells (HAECs) with magnolol 5 μM significantly suppresses the TNFα-induced expression of VCAM-1. Furthermore, in vivo, magnolol attenuates the intimal thickening and TNFα and VCAM-1 protein expression seen in the thoracic aortas of cholesterol-fed rabbits. In addition, it inhibited the binding of the human monocytic cell line, U937, to TNFα-stimulated HAECs and decreased H₂O₂ levels in control and TNFα-treated HAECs (Chen et al., 2002). These results suggest that magnolol may play an important role in the prevention of atherosclerosis.

1.2.5. Myricetin

Myricetin is a polyhydroxylated flavonol isolated from Myrica rubra S. Z., which is a traditional medicinal plant from Asia. Myricetin is a common plant-derived flavonoid and is well recognized for its beneficial health value; it is largely used as preserving agent to extend the shelf life of foods containing fats, and this use is attributed to its ability to protect lipids against oxidation (Semwal et al., 2016).

This polyphenol compound is considered a powerful anti-oxidant. Extensive researches from literature reveal that myricetin exhibits the scavenging activity towards a large number of radicals and ions. The inhibition of ABTS⁺ and DPPH radicals by myricetin was found to be polyphenol oxidase-dependent (Jiménez & García-Carmona, 1999). However, other researchers reported that although the compound exerts a strong scavenging activity against DPPH radicals, it does not have activity against ROS in menadione-stressed HL-60 cells (Rusak et al., 2005). On the other hand, it was also found to protect cells against H₂O₂-induced cell damage via inhibition of ROS generation and activation of antioxidant enzymes at the concentration of 30 μM (Bertin et al., 2016; Kang et al., 2010). Several studies suggested that myricetin protects tert-butylhydroperoxide (t-BHP)-induced oxidative stress in erythrocytes from type 2 diabetic patients (Li & Ding, 2012). Myricetin along with other flavonoids decreases LDL glycation and electrophoretic mobility, which reduce the
atherosclerotic risk of patients with diabetes mellitus (Ghaffari & Mojab, 2007). The high reactivity and antioxidant activity of myricetin is supposed to be due to the number of hydroxyl substituents present in the chemical structure. Myricetin, with its six hydroxyl groups, could be expected to have a strong peroxyl radical absorbing capability.

Extensive research on the anticancer activities of myricetin has indicated that the compound is cytotoxic towards a spectrum of human cancer cell lines, including hepatic, skin, pancreatic and colon cancer cells. It is reported that myricetin possesses anti-proliferative activity against human acute leukemia HL-60 cells and the activity was enhanced with increasing concentration (Chang et al., 2007). Research conducted on human lung carcinoma A549 cells reveal that myricetin inhibits metastasis of this cancer cells in vitro by limiting the adhesion, invasion and migration of cancer cells without showing cytotoxicity against normal cells (Shih et al., 2009). Other research studied the potential activity of myricetin against inflammation. The results show that myricetin displays anti-inflammatory activity by inhibiting the production of LPS-induced prostaglandins (Takano-Ishikawa et al., 2006). At a concentration of 10 μM, myricetin inhibited NO production in endotoxin-stimulated RAW264.7 murine macrophages, without evident cytotoxicity (Wang et al., 2006).

1.2.6. Oleuropein

Oleuropein is a polyphenol isolated from the leaf of *Olea europea* L., and it is generally the most prominent phenolic compound in olive cultivars. A large number of researches have been conducted on oleuropein, and several pharmacological properties of this polyphenolic compound have been detected. It is reported that oleuropein possess many activities, including antioxidant, anti-inflammatory, anti-atherogenic, anti-cancer, antimicrobial and antiviral (Tripoli et al., 2005; Visioli et al., 1998; Visioli et al., 2002). The antioxidant activity of oleuropein has been extensively studied, and the results show that its antioxidant potential is mainly related to its ability to improve radical stability, as assessed in vitro by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) test, demonstrating antioxidant potential similar to those exerted by ascorbic acid (vitamin C) and α-tocopherol (vitamin E) (Visioli et al., 1998). With its catecholic structure, oleuropein is able to scavenge the peroxyl radicals and break peroxidative chain reactions, producing very stable resonance structures (Barbaro et al., 2014).

Moreover, it is reported the protective activity of oleuropein against LDL oxidation: in vitro, oleuropein strongly and dose-dependently inhibits copper sulphate-induced oxidation of LDL (Visioli & Galli, 1994). Other authors conducted in vivo studies with rabbit fed with special diet rich in olive oil and oleuropein; the results indicate that the compound increases the ability of LDL to resist
oxidation and, at the same time, reduces the plasma levels of total, free, and esterified cholesterol. Furthermore, oleuropein has additional beneficial effects on several aspects of cardiovascular disease because of its potential anti-platelet aggregation, vasodilation, anti-ischemic and hypotensive properties (Coni et al., 2000). Other effects of this compound have been highlighted, in particular oleuropein administration has a hepatoprotective and therapeutic effect on carbon tetrachloride-induced liver damage in mice, and a diet supplemented with oleuropein reduces hepatic steatosis and progression to non-alcoholic steatohepatitis in mice fed with a high fat diet (Domitrović et al., 2012; Park et al., 2011). Several in vitro evidences have been collected demonstrating the antiproliferative and proapoptotic effects of oleuropein in different cancer cell lines (Casaburi et al., 2013).

1.2.7. Silybin

Silybin is the major active constituent of silymarin, a standardized extract of the Silybum marianum L. fruits. Silybin itself is a mixture of two diastereoisomers, silybin A and B, present in approximately equimolar ratio. Silybum marianum has been largely used in traditional medicine against intoxication and to promote bile flow, as well as to treating liver diseases. Silybin exhibits a large number of pharmacological effects, especially in the liver. Several clinical trials have been conducted, and there are some clinical evidences that highlighted the beneficial effect of silybin for alcoholic liver cirrhosis, chronic hepatitis C infection, non alcoholic fatty liver disease, and strong inhibitory effects on the proliferation of hepatocellular carcinoma and cirrhosis-associated insulin resistance (Federico et al., 2006; Lirussi et al., 2002; Singh et al., 2008). Most of the biological effects of silybin have been attributed to its antioxidant capacity, which has been largely studied. It is reported that silybin inhibits and blocks the high glucose-dependent increase of intracellular superoxide production and NADPH-oxidase activity, protecting the cells from apoptosis (Khazim et al., 2013; Varga et al., 2004). In vitro studies show that silymarin has both antioxidant and anti-inflammatory effects, including inhibition of superoxide production in Küpffer cells, and in addition, silybin prevents hydrogen peroxide-induced apoptosis of endothelial cells (Dehmlow et al., 1996). In another experimental study of H$_2$O$_2$ apoptosis model, the treatment with silybin exerts in vitro radical scavenging activity and restored cell viability with a reduction in H$_2$O$_2$-induced apoptotic DNA damage, and decreased the expression of caspase-3 (Wang et al., 2005). In addition, there is considerable data showing strong inhibitory effects of silybin on the proliferation and survival of different cancer cells in vitro and in vivo (Singh et al., 2008).
2. AIM

Considering the huge morbidity and mortality burden related to vascular and metabolic disorders, there is high interest in the discovery of plant-derived compounds that might be effective in the treatment or prevention of cardiovascular and/or metabolic disorders.

The aims of this study were to investigate and evaluate:

- the cellular uptake of several polyphenols on HT-29 cells, an experimental model of intestinal cells, in order to examine their ability to cross intestinal cell membrane;
- the antiglycative activity of polyphenols against albumin glycation induced by glucose, ribose and glyoxal;
- the anti-inflammatory activity of baicalein and eupatorin against the TNFα-induced VCAM-1 expression on HUVECtert cells;
- the effects of selected compounds involved in pathological conditions linked with hyperglycemia and vascular disease, like glycated albumin, glyoxal and glucose at high concentration, on viability and ROS formation in HUVEC;
- the antioxidant activity of selected polyphenols against the ROS formation induced by glucose at high concentration in HUVEC.

The polyphenols studied in this research are characteristic phytoconstituents of several medicinal plants widely used in traditional medicine such as: baicalein (from *Scutellaria baicalensis* G.), eupatorin (*Eupatorium semiserratum* DC.), galangin (*Alpinia officinarum* L.), magnolol (*Magnolia officinalis* L.), myricetin (*Myrica rubra* S. Z.), oleuropein (*Olea europaea* L.) and silybin (*Silybum marianum* L.).
3. METHODS

3.1. HT-29 cells

HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology (fig. 9). In addition to being a xenograft tumor model for colorectal cancer, the HT-29 cell line is also used as an *in vitro* model to study absorption, transport, and secretion by intestinal cells. Under standard culture conditions, these cells grow as a non-polarized, undifferentiated multilayer (Collett et al., 1996). This cell line was established in 1964 from the primary tumor of a 44-year-old Caucasian woman with colorectal adenocarcinoma. HT-29 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2% glutamine and 1% penicillin/streptomycin. Cells were grown in 10 mm dish and subcultured when reached 80-90% confluence, using enzymatic digestion with trypsin-EDTA. The HT-29 cells were used as reference model of intestinal cells to study the cellular uptake of the tested polyphenols.

![Figure 9. Human colorectal adenocarcinoma cell line HT-29.](image)

3.1.1. HT-29 cytotoxicity assay

The cytotoxicity assay was performed to see whether the polyphenols could be toxic for the cells at the concentrations chosen for the cellular uptake assay. The cellular viability after the treatment with the selected compound, at the concentration range of 1-10 μM, was determined using the MTT assay (van Meerloo, Kaspers, & Cloos, 2011). This experimental method is a colorimetric assay for evaluating the cellular metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide (yellow) to its insoluble formazan, which has a purple color. This assay could be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of the tested compounds.

HT-29 cells were seeded in a 96-well plate at the density of 5,000 cells/well and let grown overnight; the day after cells were treated with each polyphenols for 24 hours, and then an aliquot of MTT solution was added to each well, to reach the final concentration of 500 μg/mL. After the reduction of the MTT by cellular enzymes, the medium was removed and the insoluble formazan salts were solubilized with 2-propanol/0.33% HCl (36%). The absorbance of each purple formazan solution was measured using a PerkinElmer 2030 Victor™X3, setting the wavelength at 520 nm.

3.1.2. Cellular uptake in HT-29 cells

A critical point in the debate on health effects of polyphenols is indeed their bioavailability, therefore the human colorectal carcinoma cells (HT-29) were used to evaluate the polyphenols ability to cross the cellular membrane and permeate into the cells. To assess the polyphenols cellular uptake, HT-29 cells were seeded in 6-well plate in complete medium and grown until confluence for 48 hours. Then, culture medium was removed, and cells were washed with PBS and treated for three hours with 5 μM polyphenols solution in HBSS.

After the incubation, an aliquot of the extracellular solution was taken from each well and stored at -20 °C for further analysis. Then the medium was removed, cells were washed two times with PBS and prepared for the following steps:

a) protein quantification - the cells were lysed with 200 μL of lysis buffer (RIPA buffer) and the wells were washed with 200 μL of PBS;

b) cellular uptake evaluation - the cells were collected gently scraping with PBS, centrifuged at 1200 rpm for 5 minutes, and 500 μL of 80% methanol solution in water/0.1% acetic acid was used to extract the intracellular content. The cells were mixed with the methanol solution, put on ice for 15 minutes, sonicated and centrifuged at 10,000 rpm for 10 minutes. The precipitated proteins were excluded and the supernatant solution kept for chromatographic analysis.

The lysates and the intracellular extracted solution were stored at -20 °C for further analysis.
3.1.3. HPLC analysis

The chromatographic analysis of extracellular and intracellular solutions was performed using a reverse phase HPLC system consisting of a 1525 binary HPLC pump (Waters) equipped with Waters® 2998 Photodiode array detector, and a reversed phase C18 column Waters® Symmetry® C18 3.5 μm 4,6 x 75 mm. Methanol/0,1% acetic acid and water/0,1% acetic acid were used as mobile phase, in different proportions depending on the compound tested, and an appropriate gradient elution was chosen for the analysis (tab. 1). All analysis were performed at room temperature (24-25°C) using a linear gradient and a flow rate of 1 mL/min. The amount of each polyphenol compound in the extra- and intracellular solutions was calculated comparing the results with the standard calibration curve. The last step included the analysis of the lysate protein content using the Lowry protein assay, to obtain the final results expressed as nmol of polyphenol per mg of cellular proteins.

Table 1. Gradient elution set for the extra- and intracellular solution of the polyphenols uptake in HT-29 cells; eluent A is H2O/0,1% acetic acid, eluent B is MeOH/0,1% acetic acid, flow rate 1 mL/min.

<table>
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<th>Baicalein</th>
<th>Eupatorin</th>
<th>Galangin</th>
<th>Magnolol</th>
<th>Myricetin</th>
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3.1.4. Lowry protein assay

The Lowry protein assay is a colorimetric assay useful for the determination of the total level of proteins. Under alkaline conditions, the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes
reduced to molybdenum/tungsten blue, whose intensity can be measured with a spectrophotometer setting the wavelength of 595 nm (Lowry et al., 1951). This assay was used to determine the protein content of the lysates obtained in the cellular uptake assay.

Briefly, 100 μL of each sample were added to 1 mL of Lowry reagent (tab. 2). They were incubated for 30 minutes, then, 100 μL of Folin reagent were added. After 30 minutes of incubation, in the dark, at room temperature, the absorbance of the experimental mixtures was measured using a spectrophotometer. The same procedure was performed using the solutions of the standard protein (BSA) in PBS at the concentration of 0 - 1.0 mg/mL, used as calibration standard.

The results were obtained comparing the absorbance values of the samples with those of the BSA standard curve, and expressed as mg protein/mL.

<table>
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<th>Table 2. Composition of Lowry reagent.</th>
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<td>Reagent</td>
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<td>Lowry reagent</td>
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3.2. In vitro BSA glycation assay

The in vitro BSA glycation assay was performed to evaluate the polyphenols inhibitory activity of protein glycation. AGEs were determined using bovine serum albumin (BSA) as protein substrate and glucose, ribose or glyoxal as glicative agents. Glucose and ribose are sugars found in vivo in physiological conditions, while glyoxal is a highly reactive dialdehyde, a metabolite produced during the autoxidation of glucose and the glycation reaction (Sliman et al., 2010). At first, we performed preliminary experiments to detect the ability of glucose, glyoxal and ribose to induce the AGEs formation on BSA. In the beginning, BSA solution was incubated with glucose, glyoxal and ribose solution in a black 96-well plate and kept in the dark at 37°C. The first reading with the fluorimeter
was performed after 24 hours, and then it was repeated every day, for seven days. The analysis of these first experimental results suggested the most suitable incubation time for each glyating agent. After the preliminary experiments, we performed the glycation assay with the selected polyphenols, and the phytocompounds concentrations used were 5-10-50 μM for each compound. Briefly, 100 μL of BSA solution was incubated with 100 μL of glucose, glyoxal or ribose solution, and with 10 μL of each tested compound using black 96-well plates. The final concentrations of the reagents are listed in the following table (table 3). The protein and sugar solutions were all prepared in PBS pH 7.4 with 0.02% sodium azide as aseptic agent. The protein, the sugar and the prospective inhibitor were included in the mixture simultaneously. Aminoguanidine, a well-known inhibitor of AGEs formation, was used as positive control at the final concentration of 2.5 mM (Edelstein & Brownlee, 1992). The plate was kept at 37°C in the dark and incubated for 5 days (glycation by ribose) or 7 days (glycation by glyoxal or glucose). After the incubation, fluorescence intensity of the test solutions was measured using a microplate reader, setting excitation wavelength at 355 nm and emission wavelength at 460 nm. The final results were obtained comparing the fluorescence intensity between the reaction mixture with the tested compound and the reaction mixture with only BSA and glyating agent.

Table 3. Concentrations of the reagents used for the BSA glycation assay.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>[C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>50 mg/mL</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.8 M</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>35 mM</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.1 M</td>
</tr>
</tbody>
</table>
3.3. HUVEC

HUVEC are endothelial cells isolated from the umbilical vein of the cord from voluntary donors. Since the umbilical cord has no function in the postnatal period, it is an attractive source for human endothelial cells in research. These cells, at the initial passages, maintain nearly all of the features of native vascular endothelial cells including the expression of specific markers such as von Willebrand factor and endothelial specific adhesion molecule (CD31), expression of receptors for growth factors, cytokines, and specific signaling pathways for vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-β, tumor necrosis factor (TNF)-α and angiotensin II. Thus, HUVEC are primary cells that mimic many processes that occur also in vivo and their use has played a major role in the development of the field of vascular biology and has therefore become a classical model to study many aspects of function and disease of the human endothelium.

Figure 10. Umbilical vein endothelial cells (HUVEC).

HUVEC were isolated from human umbilical cord of healthy donors and used at passage 3-6 (fig. 10). Briefly, the serum-free PBS containing type II collagenase (10 mg/mL) was used to perfuse the umbilical vein in order to detach the endothelial cell layer. After dissociation, the cells were collected in 10 mm dishes and cultured in endothelial cell growth medium (EGM-2 BulletKit, LONZA, Verviers, Belgium) and maintained in a humidified atmosphere containing 5% CO₂ at 37°C. The culture medium was replaced every 2-3 days until the first subcultivation. For sub-cultivation, cells were washed with PBS and detached using trypsin-EDTA 0.05% solution in PBS for 2-5 minutes at 37°C. The enzymatic activity of trypsin was then stopped by addition of a solution 10% FBS in PBS to the cell culture dish. The cell suspension was collected in a falcon and centrifuged at 1,000 rpm for 5 minutes. The
supernatant was discarded and the cell pellet resuspended in EGM-2 medium. Cell density of the suspension was measured and obtained data were used to calculate the required volume of cell suspension which was transferred to a new sterile culture dish. HUVEC were cultured in EBM-2 supplemented with EGM-2 Single Quots. Cells were grown in 10 mm dish and subcultured when reached 80-90% confluence, using enzymatic digestion with trypsin-EDTA. Cells were cultured and used for the experiments at passage number 3-6. Cells treatment with compounds of interest was done in culture medium starvation, using EBM-2 supplemented with 1% FBS as medium.

### 3.3.1. HUVEC cytotoxicity assay

The cytotoxicity assay was performed to see whether the selected polyphenols could be toxic for the HUVEC cells at the concentrations chosen for the *in vitro* assays. The cytotoxicity assay was also performed to see a potential cytotoxic activity given by the selected compounds involved in the pathological condition linked with hyperglycemia and vascular disease, like glycated albumin, glyoxal and glucose at high concentration. The cellular viability after the treatment with the selected compound was determined using the MTT assay, as seen before in this chapter.

#### Table 4. Concentrations of the selected compounds used for the MTT assay in HUVEC.

<table>
<thead>
<tr>
<th>Selected compound</th>
<th>[C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols</td>
<td>1-10 μM</td>
</tr>
<tr>
<td>gHSA</td>
<td>200-500 μg/mL</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>500-5 μM</td>
</tr>
<tr>
<td>High glucose</td>
<td>25 mM</td>
</tr>
</tbody>
</table>

HUVEC cells were seeded in a 96-well plate at the density of 10,000 cells/well in EGM-2 and let grown for 48 hours; then cells were treated with the selected compounds in EBM-2 medium for 2-24 hours, following the scheme reported in the table 4. After the treatment, an aliquot of MTT solution was
added to each well, to reach the final concentration of 500 μg/mL. After the reduction of the MTT by cellular enzymes, the medium was removed and the insoluble formazan salts were solubilized with 2-propanol/0.33% HCl (36%). The absorbance of each purple formazan solution was measured using a PerkinElmer 2030 Victor™X3 setting the wavelength 520 nm.

### 3.3.2. ROS detection in HUVEC

The ROS formation caused by glycated albumin, glyoxal and high glucose concentration, as well as the effect of natural polyphenols on ROS-induced oxidative stress in HUVEC, was determined by a fluorimetric assay using DCFH-DA. DCFH-DA assay uses the reagent 2’, 7’-dichlorofluorescein diacetate (DCFH-DA), a fluorogenic dye that allow to measure hydroxyl, peroxyl and other reactive oxygen species (ROS) within the cell. After diffusion into the cell, DCFH-DA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2’, 7’-dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 485 nm and 538 nm. The fluorescence intensity of DCF is proportional to the ROS formed within the cells (H. Wang & Joseph, 1999).

<table>
<thead>
<tr>
<th>Selected compound</th>
<th>[C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols</td>
<td>1-5 μM</td>
</tr>
<tr>
<td>gHSA</td>
<td>200-500 μg/mL</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>500-5 μM</td>
</tr>
<tr>
<td>High glucose</td>
<td>25 mM</td>
</tr>
</tbody>
</table>

Table 5. Concentrations of the selected compounds chosen for the ROS detection assay on HUVEC cells.

Following the experimental protocol, HUVEC cells were seeded in a 96-well plate at the density of 10’000 cells/well in EGM-2 and let grown for 48 hours; then cells were treated with the selected
compounds in starvation medium for 2-24 hours, following the scheme reported in the table 5. In order to evaluate the polyphenols activity, cells were pre-incubated for 2 hours with each phytoconstituent (1-5 μM), and then treated with high glucose for 24 h. HUVEC were then incubated with DCFH-DA 25 μM for 30 minutes. The fluorescence intensity was measured at 485 nm excitation and 538 nm emission wavelengths using a PerkinElmer Victor™ X3 microplate reader (Waltham, MA, USA).

3.4. HUVEC tert

In vitro, normal somatic cells undergo a non-dividing state termed cellular replicative senescence. The erosion of telomeres, due to the inactivity of telomerase, a reverse transcriptase which synthesizes telomeric DNA, has been suggested to contribute to cellular replicative senescence (Levy, Allsopp, Futcher, Greider, & Harley, 1992). Like most human somatic cells, HUVEC undergo replicative senescence in vitro after a finite number of divisions, which can vary between 40 and 60 population doublings. In order to save time and resources in the research, it is necessary to increase the lifespan of endothelial cells in vitro. A way to bypass senescence has been achieved by ectopic expression of the human telomerase reverse transcriptase (hTERT) gene. HUVEC tert are HUVEC immortalized by infection with lentiviral particles encoding the hTERT (fig. 11), (Schiller, Szekeres, Binder, Stockinger, & Leksa, 2009).

Figure 11. HUVEC tert cells.
The experiments performed using HUVECtert cells were done during the research exchange project in Vienna, at the “Molecular Target group” of the Department of Pharmacognosy, under the supervision of Ass. Prof. Dr. E. Heiss. HUVECtert were cultured in Endothelial Basal Medium (EBM™) supplemented with EGM™ SingleQuots™. Cells were grown in 10 mm dish and subcultured when reached 80-90% confluence, using enzymatic digestion with trypsin-EDTA. The treatment with compounds of interest was done in starvation, using EBM/1% FBS as medium.

3.4.1. VCAM-1 expression in HUVECtert

VCAM-1 (vascular cell adhesion molecule 1) is an immunoglobulin-like adhesion molecule expressed on activated endothelial cells. Endothelial cells up-regulate the expression of the adhesion molecules in response to NF-κB signaling activation, for the recruitment of leukocytes as one of the major events in the development of inflammation (Cook-Mills, Marchese, & Abdala-Valencia, 2011). VCAM-1 binds to α4β1 integrin, which is constitutively expressed on lymphocytes, monocytes, and eosinophils. VCAM-1 mediates leukocyte-endothelial cell adhesion and signal transduction and may play a role in the development of atherosclerosis and other inflammatory diseases. Thus, VCAM-1 signaling is a target for intervention by pharmacological agents and by antioxidants during inflammatory diseases.

In this work, flowcytometry was used to detect the pro-inflammatory cell adhesion proteins VCAM-1 in HUVECtert. Briefly, cells were seeded into pre-coated (0.1 % gelatin in PBS incubated 10-15 minutes at 37°C) 12-well-plates in 2 ml of HUVEC culture medium and grown overnight; then, cells were pre-incubated with compounds of interest (5-10 μM) or solvent vehicle as control for 30 minutes, and then treated with TNFα 20 ng/mL. After the incubation at 37°C, 5% CO₂ for 18-20 hours, cells were washed with PBS and detached using trypsin. Cells suspension was transferred to FACS-vials and centrifuged for 4 minutes at 150 rpm; then supernatant was discarded and after two washing with PBS/2%BSA, 35 μL of anti-FITC-VCAM-1 antibody were added into each tube, which was incubated on ice in the dark for 60 minutes. After the incubation, 1 mL of PBS/BSA was added, and after washing two times with PBS/BSA, the pelleted cells were resuspended in 250 μL of PBS/BSA and analyzed immediately using a flowcytometer.
4. RESULTS

4.1. Cellular uptake in HT-29 cells

Information about natural compounds ability to cross biological membranes is of great interest and importance to deepen the study about their potential in vivo biological activities. The cellular uptake assay was performed to see in what extent the tested phytocompounds were able to cross the intestinal HT-29 cell membrane and be found in the cytosol. HT-29 cells were treated with the 5 μM polyphenols solution and the chromatographic analysis was performed using HPLC-DAD apparatus.

At first, the chromatographic analysis related to the intracellular content and the extracellular solution were performed, and the chromatograms were recorded at the wavelength range of 200-400 nm, using a PDA detector. Successively, the appropriate wavelength applicable for the analysis of each tested polyphenols was selected, relying on the UV spectrum and λ max of the standard solution of the tested compound (tab. 6). We proceeded to the peak identification, comparing the chromatogram relative to the intracellular and the extracellular contents with the chromatogram of the analytical standard, to detect whether the compounds were able to cross the cellular membrane.

![Intracellular content (A)](image1)

![Extracellular medium (B)](image2)

**Figure 12.** Intracellular content of polyphenols measured in HT-29 cells after 3 h treatment; the concentration is expressed as nmol of compound per mg of cellular protein (A). The panel B show the results relative to the concentration of each polyphenols measured in the extracellular medium after 3 h treatment of HT-29 cells; results are expressed as μM concentration. M = metabolite; ND = not detected. Each value represents mean ± SD of at least three experiments.
Table 6. UV spectra of polyphenols and selected $\lambda$ max used to detect each compound.

<table>
<thead>
<tr>
<th>Compound</th>
<th>UV spectrum</th>
<th>$\lambda$ max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalein</td>
<td><img src="image" alt="Baicalein" /></td>
<td>276 nm</td>
</tr>
<tr>
<td>Eupatorin</td>
<td><img src="image" alt="Eupatorin" /></td>
<td>345 nm</td>
</tr>
<tr>
<td>Galangin</td>
<td><img src="image" alt="Galangin" /></td>
<td>358 nm</td>
</tr>
<tr>
<td>Magnolol</td>
<td><img src="image" alt="Magnolol" /></td>
<td>290 nm</td>
</tr>
<tr>
<td>Myricetin</td>
<td><img src="image" alt="Myricetin" /></td>
<td>376 nm</td>
</tr>
<tr>
<td>Oleuropein</td>
<td><img src="image" alt="Oleuropein" /></td>
<td>250 nm</td>
</tr>
<tr>
<td>Silybin</td>
<td><img src="image" alt="Silybin" /></td>
<td>288 nm</td>
</tr>
</tbody>
</table>
The study of the extracellular and intracellular solutions was some time a difficult task because of the presence of several and different peaks in the chromatograms, with different retention times and UV spectra from the reference compound. The experimental conditions applied in the assay led to the presence of compounds with different retention time and UV absorption than the tested polyphenol, suggesting the presence of metabolites of the original compound. Data from literature helped the peak identification, where possible, with the comparison of the λ max and UV spectra.

In the table 7 are summarized the data from the polyphenols cellular uptake assay. The intracellular content of the polyphenols is express as nmol compound/mg cellular protein, whereas the extracellular concentration is express as μM.

In general, the results from the cellular uptake showed that the flavonoid eupatorin and the neolignan magnolol are able to cross the HT-29 cell membranes, considering that they are found in the cytosol after 3 hours of incubation. Moreover, we observed that baikalein and galangin not only were able to pass through the cell membrane, but also underwent biotransformation process. Indeed, peaks which did not correspond to the original compound were found in the intra- and extracellular solution. Conversely, oleuropein and silybin seemed not able to be taken up by the HT-29 cells, and they were found in the native form only in the extracellular solution (fig. 12).

In particular, as seen in figure 18, baikalein was not detected in the extracellular medium after 3 hours of incubation. Baicalin was taken up in the cells during the assay, and moreover it was transformed in different compounds not identifiable with baikalein itself. The differences in the retention times and the UV spectra of the different peaks suggested the presence of metabolites of the flavonoid; data from literature suggest the intestinal metabolism of baikalein into glucuronidated forms, especially in C-7 (fig. 13).

**Figure 13.** Molecular structure of baikalein (on the left), and its conjugated form baikalein-7-glucuronide (on the right). From: Zhang et al., 2007.
The chromatographic analysis with baicalein-7-glucuronide standard solution was performed, and the comparison between the chromatogram from the uptake assay and the chromatogram of the standard of baicalein-7-glucuronide validated the presence of this metabolite in the intracellular compartment (fig. 14). The retention time and the UV spectrum of baicalein-7-glucuronide standard and the ones from the unknown compound were overlapping; so the major metabolite has been identified as baicalein-7-glucuronide (Rt 6.53 min), a conjugated form of baicalein, which was quantified equal to 0.60 ± 0.20 nmol/mgprot.

The flavonoid eupatorin was able to permeate the intestinal HT-29 cells; as seen in figure 15 the peak corresponding to the standard eupatorin is present in both the chromatograms (intra- and extracellular compartments), and the UV spectrum and the retention time of the peaks were comparable with the analytical standard of eupatorin. The amount of eupatorin detected in the cells was 0.3 ± 0.02 nmol/mgprot. The experimental conditions chosen for the assay allowed the partial internalization of the flavonoid, thus, after 3 hours of incubation, eupatorin was quantified in the extracellular solution at the concentration of 1.87 ± 0.3 μM. No other peaks were found in either the intra- or extracellular solutions, so in these conditions no metabolism reactions occurred.

Table 7. Cellular uptake in HT-29 cells: intracellular and extracellular content detected in HT-29 cells after 3 h treatment with each polyphenols at the concentration of 5 μM.

<table>
<thead>
<tr>
<th>Treatment [5 μM]</th>
<th>Intracellular content</th>
<th>[C] nmol/mg protein</th>
<th>Extracellular content</th>
<th>[C] μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalein</td>
<td>Bai-7-glucuronide</td>
<td>0.60 ± 0.20</td>
<td>Bai-7-glucuronide</td>
<td>0.91 ± 0.24</td>
</tr>
<tr>
<td>Eupatorin</td>
<td>Eupatorin</td>
<td>0.30 ± 0.02</td>
<td>Eupatorin</td>
<td>1.87 ± 0.30</td>
</tr>
<tr>
<td>Galangin</td>
<td>Galangin</td>
<td>0.12 ± 0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Magnolol</td>
<td>Magnolol</td>
<td>0.25 ± 0.02</td>
<td>Magnolol</td>
<td>2.71 ± 0.15</td>
</tr>
<tr>
<td>Myricetin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oleuropein</td>
<td>-</td>
<td>-</td>
<td>Oleuropein</td>
<td>4.81 ± 0.54</td>
</tr>
<tr>
<td>Silybin</td>
<td>-</td>
<td>-</td>
<td>Silybin</td>
<td>2.81 ± 0.10</td>
</tr>
</tbody>
</table>

*: not detected.
Figure 14. The HPLC chromatographic analysis of baicalein content in HT-29 cells. In the figure, the chromatograms relative to the intracellular content, extracellular content and analytical standard are reported. The panels on the right show the UV spectra of the peaks revealed during the chromatographic analysis. The peak 1 has been identified as baicalein-7-glucuronide. Intracellular and extracellular flavonoids levels are compared with that of baicalein standard (peak 4, Rt 6.53 min) and baicalein-7-glucuronide standard (peak 1, Rt 3.83 min).
The extracellular medium from galangin treated cells yielded several peaks, as shown in figure 16; the peak at 6.46 min relative to galangin was not detected, but new peaks were detected at Rt 4.98 and 5.83. The UV spectra of the two peaks are reported in figure 16, these were similar to galangin UV spectrum, suggesting the presence of its metabolites. Furthermore, the intracellular content of galangin treated cells showed the presence of several peaks (Rt 5.83, 6.46, 7.40 min), and the one at Rt 6.46 min, with absorbance maxima at 265 and 358 nm, corresponded with the galangin peak in the analytical standard chromatogram. The additional peaks had UV spectra similar to galangin, suggesting the presence of possible galangin metabolites.

The results about cellular uptake of magnolol are reported in figure 17, which shows the chromatographic analysis of intra- and extracellular solutions. In both the chromatograms was detected a peak at 9.98 and 10.08 minutes, nearly the same retention time of magnolol standard (Rt 10.05 min). The UV spectra of the two peaks confirmed the presence of magnolol inside and outside the cells. The intracellular content of magnolol was quantified equal to 0.25 ± 0.02 nmol/mgprot and the extracellular concentration was 2.71 ± 0.15 μM.

The chromatograms relative to the analysis of the cellular uptake of myricetin are reported in figure 18. In both chromatograms of intra- and extracellular contents, recorded at 376 nm, the peak relative to myricetin (Rt 4.25 min) was not detected. The chromatograms were analyzed at all the wavelengths recorded from 200 to 400 nm, but no peaks were revealed. This could be due to changes in myricetin structure induced by oxidation reaction, leading to the formation of not detectable compounds.

The data from the uptake assay of oleuropein showed that the compound was not able, in the experimental conditions, to cross cellular membranes, and the peak relative to oleuropein was found only in the chromatogram of extracellular solution (fig. 19). The peak was identified as oleuropein by comparison with the retention time and UV spectrum of the analytical standard. In the intracellular content chromatogram no peaks were found at all the wavelengths analysed. The determination of oleuropein concentration in the extracellular solution was performed using the standard curve, and the result showed that oleuropein was present in the extracellular compartment at the concentration of 4.81 ± 0.54 μM.

The extracellular medium from silybin treated cells yielded only two peaks (fig. 20). The comparison with retention time and UV spectrum of the analytical standard confirm the identification of the peaks as silybin A and B. The concentration of silybin found in the extracellular compartment was equal to 2.81 ± 0.10 μM. No peaks related to silybin were found in the intracellular compartment.
Figure 15. HPLC chromatographic analysis of eupatorin content in HT-29 cells. In the figure, the chromatograms relative to the intracellular content, extracellular content and analytical standard are reported. The panels on the right show the UV spectra of the peaks revealed during the chromatographic analysis. The peak 1 has been identified as eupatorin. Intracellular and extracellular flavonoid levels are compared with that of eupatorin standard (peak 1, Rt 6.32 min).
Figure 16. HPLC chromatographic analysis of galangin content in HT-29 cells. In the figure, the chromatograms relative to the intracellular content, extracellular content and analytical standard are reported. The panels on the right show the UV spectra of the peaks revealed during the chromatographic analysis. The peak 2 has been identified as galangin. Intracellular and extracellular flavonoid levels are compared with that of galangin standard (peak 2, Rt 6.46 min).
Figure 17. HPLC chromatographic analysis of magnolol content in HT-29 cells. In the figure, the chromatograms relative to the intracellular content, extracellular content and analytical standard are reported. The panels on the right show the UV spectra of the peaks revealed during the chromatographic analysis. The peak 1 has been identified as magnolol. Intracellular and extracellular polyphenol levels are compared with that of magnolol standard (peak 1, Rt 10.05 min).
Figure 18. HPLC chromatographic analysis of myricetin content in HT-29 cells. In the figure, the chromatograms relative to the intracellular content, extracellular content and analytical standard are reported. The panels on the right show the UV spectrum of the peak revealed during the chromatographic analysis. The peak 1 represent the standard myricetin (peak 1, Rt 4.25 min).
Figure 19. HPLC chromatographic analysis of oleuropein content in HT-29 cells. In the figure, the chromatograms relative to the intracellular content, extracellular content and analytical standard are reported. The panels on the right show the UV spectra of the peaks revealed during the chromatographic analysis. The peak 1 has been identified as oleuropein. Extracellular polyphenol level is compared with that of oleuropein standard (peak 1, Rt 5.08 min).
Figure 20. HPLC chromatographic analysis of silybin content in HT-29 cells. In the figure, the chromatograms relative to the intracellular content, extracellular content and analytical standard are reported. The panels on the right show the UV spectra of the peaks revealed during the chromatographic analysis. The peaks 1 and 2 have been identified as silybin (the two isomer of silybin A and B). Extracellular polyphenol level are compared with that of silybin standard (peak 1, Rt 5.48 min; peak 2, Rt 5.60 min).
4.2. *In vitro* BSA glycation assay

AGEs are the final products of the non-enzymatic reaction between reducing sugar and amino groups in protein, lipoproteins and nucleic acids. AGEs can play a physio-pathological role through the binding of specific receptors, initiating a signal cascade with pro-oxidant and pro-inflammatory effects. This is the reason why they are well known agents related to hyperglycemia, which worsen the vascular damage induced by atherosclerosis. Considering all the dangerous effects of the glycation process, a strategy for the prevention of the AGEs formation could be promising and helpful in reducing the negative consequences.

![Figure 21](image)

*Figure 21.* The effects of glucose (G), glyoxal (GO) and ribose (R) on the AGEs formation on BSA. BSA was incubated with glucose 0,8 M, glyoxal 35 mM and ribose 0,1 M, then the fluorescence intensity of the reaction mixture was measured setting λ excitation 355 nm and λ emission 460 nm. (A) Time-dependent evaluation of AGEs formation. (B) Evaluation of AGEs formation measuring the fluorescence intensity after 5 days of incubation of BSA with ribose, and after 7 days of incubation with glucose or glyoxal.

The preliminary experiments allowed us to investigate the ability of glucose, glyoxal and ribose as inducer of AGEs formation in BSA. A time-course assay was performed, and the results showed that glucose, glyoxal and ribose have a different ability of inducing the AGEs formation. Ribose was the fastest *in vitro* glycation inducer, as seen in figure 21, with a maximum of fluorescent AGEs formation after five days of incubation with BSA. Glyoxal showed a good glycating activity after seven days of incubation, whereas glucose needed more time to complete the glycation reaction, reaching a lower
level of fluorescent AGEs after seven days. These results were compared with the ones from the literature, thus, the end-point chosen for the fluorimetric measures were five days for ribose and seven days for glyoxal and glucose. The graph in figure 21 shows the fluorescence intensity of AGEs formed during the assay and measured after five or seven days. It seemed clear that ribose is not only the fastest glycation inducer but also the strongest, with the highest value of fluorescence intensity. Glucose showed a little but significant increase in fluorescence intensity, compared to the not treated BSA.

The following experiments were conducted to evaluate the inhibitory activity of the polyphenols on the AGEs formation.

**Figure 22.** Antiglycative activity of the selected polyphenols expressed as inhibition of AGEs formation induced by glucose (G). Aminoguanidine (AG) was used as positive control. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs BSA treated with glucose alone (ctrl G).

The figure 22 shows the results of the inhibitory activity of the polyphenols on the glycation induced by glucose. All the tested polyphenols showed antiglycative activity at the concentration of 50 μM, with different percentage of inhibition. Interestingly, baicalein, eupatorin, myricetin and silybin also
inhibited the AGEs formation at the concentration of 10 μM, whereas baicalein was the only compound that exerted the antiglycative activity at 5 μM.

In the BSA-glyoxal assay (fig. 23), galangin and magnolol at the 50 μM concentration showed the highest activity. Together with baicalein and eupatorin, they showed inhibitory activity also at the concentration of 10 μM. Moreover, magnolol and eupatorin 5 μM were able to inhibit the AGEs formation induced by glyoxal.

**Figure 23.** Antiglycative activity of selected polyphenols expressed as inhibition of AGEs formation induced by glyoxal (GO). Aminoguanidine (AG) was used as positive control. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs BSA treated with glyoxal alone (ctrl GO).

Baicalein, galangin and magnolol showed good antiglycative activity in the BSA-ribose assay, too, with a particular and significant inhibition of AGEs formation at 10-50 μM (fig. 24). Although silybin exerted a significant activity against the glycation induced by glucose, in the BSA-glyoxal and BSA-ribose assay silybin showed the inhibitory activity only at 50 μM. A very weak antiglycative activity was shown by oleuropein in all the tested conditions, except for oleuropein 50 μM in the BSA-glucose assay. Analysing the results obtained so far, the polyphenols with the highest activity in preventing
the AGES formation were baicalein, eupatorin and magnolol, with elevated activities in all three glycation conditions and significant activities also at the lower concentration of 5 μM.

**Figure 24.** Antiglycative activity of selected polyphenols expressed as inhibition of AGES formation induced by ribose. Aminoguanidine (AG) was used as positive control. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs BSA treated with ribose alone (ctrl R).
4.3. VCAM-1 expression in HUVECtert

Endothelial cells upregulate the expression of the adhesion molecules in response to inflammation signaling, for the recruitment of leukocytes as one of the major events in the development of inflammatory diseases. To see whether the compounds were able to induce or reduce the expression of the adhesion molecules, the flowcytometric analysis on VCAM-1 was performed (fig. 25).

![Flowcytometric analysis of VCAM-1 expression on HUVECtert. The cells were incubated for 30 minutes with the tested compounds (or vehicle control), and then treated with TNFα. CDDO (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) was used as positive control (100 nM). The fluorescence of the anti-FITC-VCAM-1 antibody on the selected cells was measured and all the results were compared to the TNFα-treated cells. The panels shown in the figures are relative to the flowctometric analysis of HUVECtert treated with TNFα, and of cells pre-incubated with CDDO, eupatorin 5 μM and eupatorin 10 μM.](image-url)
Figure 26. Relative VCAM-1 expression in HUVEC tert evaluated using flowcytometry. Glycated albumin (gHSA) was not able to induce VCAM-1 expression compared to the not-treated cells (ctrl). TNFα was used as stimulus, and CDDO as positive control. The evaluation of flavonoids ability to inhibit TNFα-induced VCAM-1 expression showed that baicalein (5-10 μM) had no effect in inhibiting the TNFα activity, whereas eupatorin (5-10 μM) displayed a significant activity. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs TNFα treated cells (TNFα).

The first experiments evaluated if glycated albumin was able to induce an inflammatory event in the endothelial HUVEC tert cells, as the VCAM-1 expression; moreover, the aim was to examine if the flavonoid compounds baicalein and eupatorin were able to reduce the VCAM-1 expression induced by TNFα. The concentrations used in this assay were 200 μg/mL for gHSA, and 5 and 10 μM for the flavonoids. CDDO (2-cyano-3,12-dioxoolene-1,9-dien-28-oic acid) was used as positive control at the concentration of 100 nM. The obtained results were compared to the TNFα-treated cells, and they were expressed as relative percentage of VCAM-1 expression. In figure 26 it can be seen that HUVEC tert did not increase the expression of adhesion molecules after the treatment with glycated albumin. Thus, gHSA was not able to induce the inflammatory event like VCAM-1 expression. Moreover, the flavonoid baicalein at both concentrations tested in the assay did not counteract the effect of TNFα in inducing VCAM-1 expression, whereas eupatorin at 5 and 10 μM exerted a significant anti-inflammatory activity, showing a VCAM-1 expression inhibition of 23.7 ± 0.10% and 51.6 ± 0.07%.
4.4. HUVEC cytotoxicity assay

The MTT viability assay was performed to see a potential cytotoxic activity given by the selected compounds involved in the pathological condition linked with hyperglycemia and vascular disease, like glycated albumin, glyoxal and glucose at high concentration.

At first, the effect of gHSA on the viability of HUVEC was evaluated. The results, reported in figure 27, showed that glycated albumin was not cytotoxic for HUVEC after 24 hours of treatment, but it seemed to have a proliferative effect, which was significant for the concentration 200 and 500 μg/mL. The highest concentration tested of gHSA determined an increase in cells viability of about 158 %.

![Figure 27. Effect of glycated albumin (gHSA) on the viability of HUVEC, assessed using the MTT assay. The concentrations chosen for gHSA were 200-500 μg/mL. Bovine serum albumin (BSA), not glycated, was used as protein control. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs not treated cells (ctrl).](image)

The cytotoxic effect of glyoxal on HUVEC was performed choosing a concentration range of 0.2-5.0 mM. The MTT assay was performed after glyoxal treatment of 2 and 24 hours. The results, reported in figure 28, showed low cytotoxic activity of glyoxal after 2 hours treatment; at the highest concentration tested glyoxal reduced the cells viability to 64.3%. After 24 hours treatment, glyoxal displayed a stronger effect on HUVEC, reducing the cells viability starting from the 0.5 mM concentration. After 24 hours treatment with glyoxal 5 mM the HUVEC viability was reduced to 5.9%.
**Figure 28.** Cytotoxic effect of glyoxal (GO) on HUVEC expressed as percentage of viable cells. Cells were treated with different concentrations of glyoxal for 2 h (A) or 24 h (B). Each value represents mean ± SD of at least three experiments, * p < 0.05 vs not treated cells (ctrl).

It was further detected the activity on HUVEC of glucose at high concentration (hGLU; 25 mM), to see whether it could have a toxic effect on endothelial cells after 24 hours treatment. The results, reported in figure 29, showed that high glucose did not influence the HUVEC viability in the conditions chosen for the assay.

In summary, glycated albumin had not cytotoxic activity on HUVEC, conversely it showed a proliferative effect on endothelial cells. It was also observed that glyoxal determined a reduction of the cells viability especially after 24 hours, whereas high glucose did not impaired cellular viability after 24 h treatment.

**Figure 29.** The effect of glucose at high concentration (hGLU; 25 mM) on the viability of HUVEC. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs not treated cells (ctrl).
4.5. ROS detection in HUVEC

Oxidative stress is described as the imbalance between the ROS production and the antioxidant defenses. The excess of ROS production could have negative consequences in the organism and is associated with hyperglycemic condition, diabetes and atherosclerosis. Several factors related to hyperglycemia and diabetes could promote oxidative stress and ROS formation, therefore in this research work the activity of glycated albumin, glyoxal and high glucose were evaluated using the DCFH-DA assay.

![ROS 2 h](image)

**Figure 30.** The effects of glycated albumin (gHSA), glyoxal (GO) and high glucose (hGLU) on ROS formation in HUVEC, measured using DCFH-DA. Cells were treated with the selected compounds and ROS formation was measured within 2 h. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs not treated cells (ctrl).

At first, we evaluated potential effects of the selected compounds in inducing ROS formation in HUVEC after 2 hours treatment (fig. 30). Glycated albumin, as well as glyoxal, was not able to induce ROS formation in the cells, at the concentrations chosen for the assay. Instead, the treatment of 2 hours with glucose at high concentration determined an increase of ROS in HUVEC, with a rise of about 134%.
The following step has been the evaluation of the activity of glyoxal and high glucose on the ROS formation after a longer treatment. After 24 hours of incubation, high glucose was able to increase ROS production in HUVEC cells of 134.9 ± 9.9 %, whereas glyoxal did not increase ROS formation (fig. 31).

![ROS 24 h](image)

**Figure 31.** Oxidative stress in HUVEC: ROS formed after treatment with glyoxal 0.5 mM (GO) and high glucose (hGLU) for 24 h. Each value represents mean ± SD of at least three experiments, * p < 0.05 vs not treated cells (ctrl).

After the evaluation of the activity of glycated albumin, glyoxal and high glucose on ROS formation in HUVEC, it was clear that high glucose was the only compound able to induce oxidative stress in the experimental conditions.

To prevent and counteract ROS formation in endothelial cells assumes an important role in the treatment of oxidative stress-related pathologies. Natural products from medicinal plants could be good candidate as agents that protect ROS-induced damage, therefore the antioxidant activity of the polyphenols baicalein, eupatorin and galangin was evaluated using the DCFH-DA assay.
At first, we evaluated the activity of the selected polyphenols in reducing ROS production in HUVEC in basal condition, without any oxidative stressor. The cells were treated for 2 hours with each natural compound, and then DCFH-DA was added. Fluorescence of reduced DCF was measured and the results are reported in figure 32. In basal condition, baicalein and galangin at 1 and 5 μM were able to significantly reduce the ROS formation in HUVEC, whereas eupatorin did not displayed any antioxidant activity at both concentrations tested. The ROS formation in HUVEC after the treatment with baicalein 1 and 5 μM was inhibited of 18.3 ± 6.1 and 32.0 ± 5.7 %. Also galangin 1 and 5 μM displayed antioxidant activity, reducing ROS formation of 19.5 ± 4.6 % and 39.0 ± 5.8 % (fig. 32).
Afterwards, we evaluated the antioxidant activity of the polyphenols after stimulation of HUVEC with high glucose, chosen as oxidative stressor (fig. 33). Cells were pre-treated with each compound at the concentrations of 1-5 μM, and then cells were treated with high glucose (25 mM) for 24 hours. The pro-oxidant effect of high glucose on the ROS formation was referred as 100% of ROS production. All the polyphenols seemed to counteract the ROS formation induced by high glucose treatment, except for eupatorin at the concentration of 1 μM. The ROS formation in HUVEC after pre-treatment with baicalein 1 and 5 μM was 84.2 ± 7.2% and 63.7 ± 6.6%, and after pre-treatment with galangin 1 and 5 μM was 77.2 ± 11% and 65.4 ± 3.9%. Eupatorin showed an antioxidant activity at the concentration of 5 μM, reducing the ROS formation to 70.3 ± 4.8%.

Figure 33. Antioxidant activity of baicalein, eupatorin and galangin expressed as percentage of ROS formation in HUVEC after 24 hours treatment with high glucose (hGLU). Each value represents mean ± SD of at least three experiments, * p < 0.05 vs HUVEC treated with high glucose (hGLU).
5. DISCUSSION

5.1. Cellular uptake in HT-29 cells

Medicinal plants and natural products still represent important sources for the identification of new pharmacological leads today. Several natural compounds have been shown to affect vascular and metabolic disorders, via different mechanisms, such as anti-inflammatory activity, changes of blood lipid profiles, improvement of insulin sensitivity, or normalization of blood glucose levels (Lee et al., 2006; Pan et al., 2010; Yugarani et al., 1992). The polyphenols have aroused great scientific interest, since their beneficial effects are attributed to several activities including antioxidant capacity. It is well known that the phenolic compounds can act as metal chelators and radical scavengers, and might also trigger changes in cells and in the signaling pathways, as by preventing the LDL oxidation, platelet aggregation, and ROS generation. It has become clear that the health effects of polyphenols greatly depend on their permeability and bioavailability, namely the bioactive forms in vivo. The polyphenols bioactive form is not necessarily the natural chemical structure, for example their aglycones or their glycosides, but instead their conjugates and metabolites, arising from their metabolism during the absorption phase. There are now strong evidences for the extensive phase-I deglycosylation and phase-II metabolism of the aglycones such as quercetin, hesperidin and epicatechin to glucuronides, sulphates and O-methylated forms during transfer across the small intestine and again in the liver (Spencer et al., 2004). The cellular effects of polyphenols (or their metabolites) will ultimately depend on the extent to which they associate with cells, either by interaction at the membrane and uptake into the cytosol. Circulating glucuronides, sulphates and methylated forms of polyphenols are believed to be the most present forms in vivo and the most likely to exert bioactivity. Thus, information regarding uptake of polyphenols and their modification into metabolites has become increasingly important to understand their potential role as modulators of intracellular signaling crucial for cellular function. The informations about permeability, metabolism and bioavailability of natural compounds are important even because they represent essential and usefull parameters that have to be considered in the process of drug formulation.

It is well established that the gastrointestinal tract plays a significant role in the metabolism of polyphenols before entering the hepatic and systemic circulation. Therefore, in order to evaluate the polyphenols ability to cross the intestinal cells membrane and permeate into the cells, in the present research work human colorectal carcinoma HT-29 cells were used as reference model of intestinal barrier (Collett et al., 1996). It should be noted that the assessment of polyphenols cellular uptake using in vitro cell culture models may not fully reflect uptake in vivo. Despite this, the polyphenols
cellular uptake studies still provide important information on which natural compounds or their metabolites are most able to cross cellular membrane and permeate into cells.

Baicalein is a bioactive flavone isolated from *Scutellaria radix*, a traditional Chinese medicinal herb derived from *Scutellaria baicalensis* G. Despite the variety of beneficial effect of baicalein, investigations on its metabolism are not complete. Studies from literature have demonstrated that there is an extensive first-pass metabolism of baicalein and that conjugated metabolites were predominant in systemic circulation after oral administration in rats (Zhang et al., 2011). Pharmacokinetic studies in rats discovered that baicalein-glucuronide is the major metabolite found in circulation after oral administration. To fully understand the mechanism behind the metabolism of baicalein, several studies were conducted on cultured cells and intestinal and liver microsome from humans and rats (Zhang et al., 2007). Caco-2 (human epithelial colorectal adenocarcinoma) cells have been used as model for intestinal absorption of drugs, and results indicated the same first-pass metabolism of baicalein observed in rats, showing that over 90% of baicalein was rapidly converted to baicalein-glucuronide (Zhang et al., 2005). The results from the present research showed that baicalein in cultured HT-29 cells undergoes intestinal-like metabolism, since the HPLC analysis of the intracellular content revealed three different peaks, apart from baicalein. The major peak has been identified as baicalein-glucuronide, confirming that phase-II intestinal metabolism of baicalein occurs, also in HT-29 cultured cells. The other two peaks could be related to other baicalein metabolites, and, comparing the data from literature, they may be associated with baicalein-glucuronide isomer and baicalein-sulphate (Zhang et al., 2007). Apart from cellular uptake, several peaks were revealed also in the extracellular compartment, suggesting a potential cellular transport of baicalein-glucuronide. Carrier-mediated transport is a cellular system that mediates the flux of drugs and phase-II conjugated metabolites through cellular membranes, and this system could play a role during the intestinal absorption of baicalein. Further studies are needed to fully elucidate the fate of baicalein in the human organism; however, part of the pharmacological effects observed for baicalein may be due to the biological activity of baicalein-glucuronide.

Eupatorin is a polymethoxylated flavones isolated from *Orthosiphon stamineus* B., a medicinal herb used in folk medicine in South East Asia for treatment of various disorders. It is known that the main reason for the low oral bioavailability of the flavonoids is the extensive conjugation of the free hydroxyl groups (Wen & Walle, 2006). The exclusive feature in the chemical structure of eupatorin is the polymethoxylation of the hydroxyl groups. The characteristics of polymethoxylated flavonoids are their multiple methoxyl groups, instead of free hydroxyl groups, and the higher lipophilic properties. These features result in increased metabolic stability and membrane transport in the intestine and in the liver, improving the oral bioavailability (Walle et al., 2007). The bioavailability of
different formulations containing polymethoxylated flavonoids was evaluated by Evans and colleagues, and the results show that the serum samples from healthy subjects after oral administration demonstrate detectable amounts of unmodified compounds (Evans et al., 2012). The results from the present research confirmed that the presence of multiple methoxyl substituents in the chemical structure protected eupatorin against metabolism conjugation. Unlike other flavonoids studied in this research, as baicalein or galangin, the only peaks revealed in the HPLC analysis of the intra- and extracellular contents of HT-29 cells after the treatment with eupatorin, were those relative to eupatorin. The compound was able to penetrate into the cells and no metabolism reaction occurred during the time frame of the uptake experiment, since eupatorin was present in the native form both inside and outside the cells. Further studies are needed to deepen the research on the bioavailability of eupatorin and to find the actual amount of the natural compound achievable in vivo.

Galangin is a flavonol compound isolated from the rhizome of Alpinia officinarum H., a traditional herb medicine used for rheumatic pains and arthritis. The metabolism of flavonols, such as quercetin, has been largely studied in cultured cells, rat intestine and liver cells (Walle, 2004). It is reported an extensive pre-systemic metabolism by glucuronidation, sulfation and methylation in the intestine and in the liver, therefore accurate and complete pharmacokinetic information on flavonols is useful to understand their bioactivity and the in vivo circulating form, as well as the actual compound which reach the target sites in the organism. Overall, in vivo pharmacological studies have confirmed that galangin could have lots of beneficial effects (Chen et al., 2015). However, these effects could be attributed to galangin metabolites, thus it is important to study the effect of intestinal cells to this natural compound. In vitro studies have revealed that galangin can be oxidized to kaempferol, but galangin mainly undergoes glucuronidation and sulfation in human hepatocytes (Otake et al., 2002). In vivo studies in rat plasma suggest that galangin is preferentially metabolized into glucuronide and sulphate, rather than oxidation (Chen et al., 2015). The results from the present research showed that galangin, in cultured HT-29 cells, undergoes metabolism. The HPLC analysis of the intracellular content revealed a small peak relative to galangin, and two other minor peaks which could be related to metabolites of galangin. The HPLC analysis of extracellular content revealed two peaks, none of which was relative to galangin. Comparing these results and the UV spectra of the peaks with the data from literature, it seems likely that the unknown peaks revealed in the intra- and extracellular contents are related to metabolites of galangin, especially glucuronidated and sulphate forms (Wu et al., 2011). Further studies are needed to clearly identify the in vivo metabolites of galangin, and to clarify their role and their potential bioactivity in the human organism.
Magnolol is a polyphenolic lignan compound isolated from the bark of *Magnolia officinalis* L., a popular Chinese plant used in traditional medicine for the treatment of anxiety, fever and headache (Chen et al., 2011). Most of the bioactivities evaluated *in vitro* for magnolol have been attributed to the native form of magnolol, although the analysis regarding the metabolism of this compound has shown that it undergoes extensive glucuronidation (Zhu et al., 2012). The pharmacokinetics and tissue distribution of magnolol and its metabolites have been studied in various experimental conditions, in order to obtain more detailed information about the actual molecules working *in vivo*. Studies from literature were conducted in rats, and the results reveal the presence of metabolites after administration of magnolol *in vivo* (Lin et al., 2011). The results indicated that magnolol-glucuronide is the major conjugated form present in circulation. The tissue distribution analysis show that, despite the high metabolism of the compound, magnolol is the major form in various organs, and this is probably due to the metabolic hydrolysis of the glucuronide and sulphate conjugates into the cells of the various organs (Lin et al., 2011). The HPLC analysis performed in the present research revealed that, in the intra- and extracellular compartments, only one main peak was present. Comparing these chromatograms with that of magnolol standard, the only peak found after treatment of HT-29 cells with magnolol was magnolol itself. No peaks relative to conjugates or metabolites were revealed, suggesting that the metabolism of this compound may be different in human cells, than in rats, either for different pharmacokinetic time or for different expression of specific enzymes. Additional studies are needed to deepen the research on the potential *in vivo* forms of magnolol and their biological action.

Myricetin is a polyhydroxylated flavonoid isolated from *Myrica rubra* S. Z., which is a traditional plant from Asia. Several beneficial properties have been attributed to myricetin, especially scavenging and antioxidant activities, and the contribution of these activities *in vivo* have been explored in many research. In previous studies, authors evaluated the potential metabolic transformation of polyhydroxylated flavonoids, including myricetin, after oral administration in rats (Griffiths & Smith, 1972). The results showed that the hydroxyl groups in the B-ring of the selected flavonoids were susceptible of metabolism, leading to phenolic and phenyl-acetic acids as main products. Lin and colleagues also studied the *in vivo* biotransformation of myricetin in rats, and they identified myricetin and its metabolites in the urine, whereas in plasma samples only metabolites were revealed (Lin et al., 2012). Few other researches from literature investigated the *in vitro* cellular uptake of flavonoids, including myricetin, using Caco-2 cells as experimental model, with the aim to evaluate the ability of the compounds to cross intestinal cell membrane. Although other flavonoid aglycones studied were incorporated into Caco-2 cells, myricetin was not detected (Yokomizo & Moriwaki, 2006). In the present research, the *in vitro* cellular uptake of myricetin was evaluated.
using HT-29 cells as experimental model. The HPLC analysis revealed that no peaks relative to myricetin have been found in both the intra- and extracellular compartments. All the wavelengths analyzed were scanned but the presence of other peaks relative to possible metabolites of myricetin was not detected. These results were similar to that from literature on Caco-2 cells. The authors supposed that the reason behind the absence of peaks relative to myricetin or its metabolites might have been its instability under the experimental conditions (Miura et al., 1998; Yokomizo & Moriwaki, 2006).

Oleuropein is a polyphenolic compound isolated from the leaf of Olea europea L., and it is one of the main phenolic constituents of olive oil. Studies from literature indicate that the consumption of olive oil, as seen with the Mediterranean diet, gives low incidence of cardiovascular diseases and certain types of cancer (Carluccio et al., 2003; Visioli et al., 2002). Olive oil is a source of at least 30 different phenolic compounds, as oleuropein, which possess beneficial properties, like antioxidant activity (Carluccio et al., 2003). Many in vitro studies have been performed to identify the bioactivity of the polyphenols from olive oil, and the results showed that these compounds possess strong radical scavenging activity. Moreover, they also act as inhibitors of LDL oxidation in vitro, and they are able to break the peroxidative chain reaction (Bertin et al., 2016; Tripoli et al., 2005). Despite the evaluation of the in vitro biological activity, there are not enough data in the literature about the biological fate and the absorption of oleuropein. Some authors investigated the absorption of oleuropein using an isolated perfuse rat intestine (Edgecombe et al., 2000). The results indicate that oleuropein is capable of permeating the intestinal barrier, but the amount of oleuropein that reaches the systemic circulation seems very low. The HPLC analysis, performed in the intra- and extracellular contents after the treatment of HT-29 cells with oleuropein, indicated that the peak relative to oleuropein was present only in the extracellular compartment, and no other peaks were revealed, both in intra- and extracellular compartments. During the time frame of the experiments, oleuropein was not taken up by HT-29 cells, and no metabolism reactions occurred. This is probably due to the high hydrophilic properties of this compound, which has free hydroxyl groups, and this does not allow the permeability and passive diffusion of oleuropein across the HT-29 cell membranes. It is possible that other reactions occur in vivo on oleuropein before it enters the intestinal lumen, and that potential different ways of absorption exist. Therefore, further studies are needed to understand how oleuropein interacts with human organism.

Silybin is a polyphenolic compound and the major active constituent of silymarin, a well known flavonolignan complex from Silybum marianum L. The possible therapeutic applications of this flavonoid are hampered by its poor enteral absorption, which accounts for its very low bioavailability. Silymarin exerts a wide range of biological and pharmacological effects, and in vivo clinical studies
evidence and demonstrate clinical efficacy, although the limited oral bioavailability (Wellington & Jarvis, 2001). Only 20-50% of oral silymarin is absorbed from the gastrointestinal tract, where it undergoes extensive enterohepatic circulation (Wu et al., 2009). It is reported that the limited bioavailability of silybin is due to four major causes: extensive phase-II metabolism, low permeability across intestinal epithelial cells, low water solubility, and rapid excretion in bile and urine (Javed et al., 2011). The results from the present research confirmed the poor uptake of silybin across the HT-29 cell membranes. The HPLC analysis revealed that silybin was not present inside the cells, since the peaks were absent in the intracellular compartment. In the extracellular content, two peaks relative to the two isomers of silybin were revealed, thus, silybin was not able to permeate into the HT-29 cells. Because of the poor bioavailability, recently, authors formulated different kind of complex, in order to improve silybin and silymarin in vivo bioavailability (Javed et al., 2011).

5.2. Polyphenols activity against glycation and oxidative stress

Protein glycation is a non-enzymatic reaction between the carbonyl group of a reducing sugar and the free amino group of a protein, leading to the structural and functional changes in the native protein. Chronic hyperglycemia can cause non-enzymatic protein glycation because the carbonyl group of glucose reacts with the free amino groups of proteins to initiate a complex cascade of repeated condensations, rearrangements, oxidative modifications, resulting in the reversible formation of a Schiff’s base (Vistoli et al., 2013). This is further rearranged into more stable structures called Amadori products, which undergo further oxidation, generating dicarbonyl compounds to form cross-linked structures (AGEs). Although an elevated level of glucose had been thought to play a primary role in the Maillard reaction, the formation of AGEs is now known to result also from the action of various metabolites other than glucose, such as glyoxal and dicarbonyl compounds (Sadowska-Bartosz et al., 2014). It has been clearly demonstrated that the accumulation of AGEs in body tissue is the leading cause of several age-related degeneration, atherosclerosis and diabetic complications such as retinopathy, nephropathy and neuropathy (Vlassara & Palace, 2002). For this reason, the inhibition of AGEs formation could represent a therapeutic strategy against diabetic complications.

Aminoguanidine (AG), used as positive control in the present research, is a small synthetic hydrazine-like compound, well-known for its anti-glycation activity. AG has shown promising results in terms of in vitro inhibition of AGE formation, and clinical trials were also conducted. However, AG has shown some toxicity reaction in diabetic nephropathy, such as gastrointestinal problems and anemia (Bolton et al., 2004). Therefore, the effort has been directed in finding phytochemical compounds from
medicinal plants that effectively inhibit protein glycation and AGEs formation. It is well known that phenolic compounds have attracted scientific interest because of their antioxidant properties. The polyphenols exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents and metal ion chelating properties (Brewer, 2011; Perron & Brumaghim, 2009). The mechanism behind the glycation reaction is linked with oxidation and ROS formation, thus the antioxidant properties of polyphenols could represent a strategy against protein glycation. A recent study has shown that compounds with combined anti-glycation and antioxidant properties, such as aminosalicylic acid, are more effective than aminoguanidine in protecting endothelial cells against the harmful effects of high glucose and AGEs in vitro (Duraisamy et al., 2003). The present study reports the inhibitory effects of selected polyphenols from medicinal plant, namely baicalein, eupatorin, galangin, magnolol, myricetin, oleuropein and silybin, on glucose-, ribose-, and glyoxal-mediated BSA glycation. These three glycating agents were selected between other compounds because of their potential role in hyperglycemic condition and diabetes complications. Glucose and ribose are sugars found in vivo in physiological conditions, while glyoxal is a high reactive dicarbonyl compound derived from the autoxidation of glucose and from the glycation reactions (Wells-Knecht et al., 1995). The data from the present research indicated that the glycation induced by glucose determined lower AGEs formation than glyoxal and ribose, suggesting that glucose has a slower rate of glycation. This is probably because the rate of Schiff base formation is directly proportional to the percentage of sugar in the open chain form. Indeed, the rate of glycation for intracellular sugars as glucose-6-phosphate and glyceraldehyde-3-phosphate is considerably faster than the rate for glucose (Giardino et al., 1994). The elevated levels of these compounds occurring during hyperglycemia would therefore modify circulating and intracellular protein. It has been hypothesized that increased glycation of intracellular proteins in endothelial cells exposed to diabetic levels of hyperglycemia would result in altered function of proteins that regulate endothelial growth (Giardino et al., 1994). Ribose is another naturally occurring sugar present in living cells. It is reported that ribosylation is a rapid process that produces AGEs formation and causes protein aggregation in vitro and in vivo (Wei et al., 2012). The present research confirms that ribose has the faster and stronger ability to induce in vitro glycation and AGEs formation on albumin. During diabetes, there is increased intracellular accumulation of dicarbonyl compounds such as glyoxal and methylglyoxal (MGO), potent cross-linking agents believed to be of significance in diabetic complications (Akhand et al., 2001; Yamawaki & Hara, 2008). In the present research, the effect of glyoxal on in vitro BSA glycation was evaluated, as well as its effect on HUVEC. Glyoxal is a highly reactive α-oxoaldehyde, endogenously produced via autoxidation of carbohydrates and ascorbate, degradation of glycated proteins and lipid peroxidation. AGEs and fructosamine can be oxidized, contributing to glyoxal production (Thornalley et al., 1999). Lipid peroxidation is also a potentially
important source of glyoxal. The initiation step occurs when polyunsaturated fatty acids such as linoleic and linolenic acid undergo nonenzymatic lipid peroxidation. The resulting product is a hydroperoxide which then degrades into a variety of products, including glyoxal (Yin & Porter, 2005).

Studies from literature have shown that most of the glycation inhibitory phytochemicals constituents from medicinal plants possess polyphenolic structure. For example, the active glycation inhibitory constituents of green tea have been regarded as polyphenols (Peng & Zhang, 2014). Other authors suggested the inhibitory capacity of flavonoids against protein glycation, which was remarkably related to their scavenging effect on free radicals derived from glycoxidation process (Wu & Yen, 2005). Moreover, a number of plant derived flavonoids (quercetin, rutin and kaempferol) with antioxidant activity have been reported to inhibit glycation, at least in vitro (Vinayagam & Xu, 2015).

The results from the present research showed that baicalein and eupatorin displayed the best antiglycative activity in the BSA-glucose assay and, together with galangin, they were able to effectively inhibit AGEs formation in the BSA-ribose assay. The results from the BSA-glyoxal assay showed that baicalein and eupatorin inhibited the glycation in a lesser extent, whereas galangin and magnolol exerted a strong effect on the glycation reactions and on AGEs formation. Comparing the antiglycative activities evaluated in the present research, we can conclude that baicalein and eupatorin displayed the strongest inhibitory activity against AGEs formation. Since a correlation between tissue AGEs concentrations, protein oxidative modification and the severity of some chronic diseases has been demonstrated, the antiglycative activity displayed by baicalein, eupatorin and galangin suggest that these natural compounds could be considered promising candidates for further studies about their potential effects against glycation.

Studies have indicated that several factors related to hyperglycemia, dyslipidemia and hyperinsulinemia may play roles in the development of diabetes-associated vascular disease and atherosclerosis (Creager et al., 2003). A further aim of this research was to examine the effect of the factors related to hyperglycemia and diabetes, such as glycated albumin, glyoxal and glucose at high concentration on endothelial cells, using HUVEC as experimental model. Among various glycated proteins, serum glycated albumin has been reported to be a useful and rapid indicator of glycemic control for diabetic patients, since the turnover of serum albumin is much shorter than that of glycated hemoglobin (HbA1c). It has been reported a better correlation between gHSA in patients with type 2 diabetes with various parameters of glycemic profiles than HbA1c, therefore gHSA could be a more accurate marker for evaluating glycemic control (Inaba et al., 2007). Thus, it is important to study the effect of glycated albumin, because it could play an important role in diabetes-associate vascular disease.
Endothelial cells up-regulate the expression of the adhesion molecules in response to NF-kB signaling activation, for the recruitment of leukocytes as one of the major events in the development of inflammation. VCAM-1 mediates leukocyte-endothelial cell adhesion and signal transduction, and may play a role in the development of atherosclerosis and other inflammatory diseases (Cook-Mills et al., 2011). Thus, VCAM-1 signaling is a target for intervention by pharmacological agents and by antioxidants during inflammatory diseases. In this work, flowcytometry was used to detect the pro-inflammatory cell adhesion proteins VCAM-1 in HUVECtert. The results showed that glycated albumin had not the effect of VCAM-1 expression inducer, suggesting that gHSA do not exert this pro-inflammatory activity. Many types of study, ranging from in vitro experiments to epidemiological studies, have shown that AGEs could play a central role in the pathogenesis of accelerated atherosclerosis in diabetes (Basta et al., 2004). Indeed, AGEs and their receptor (RAGE) interaction evokes oxidative stress generation and vascular inflammation (Goldin et al., 2006). The experimental results obtained in this work showed that the early product of glycation gHSA had not the same effect reported in literature about AGEs, and it is not directly involved in the pro-inflammatory activation of HUVEC. Concerning the oxidative stress, the results obtained showed that gHSA did not display a pro-oxidant effect in HUVEC. Many studies from literature proposed that the damaging effect of AGEs is predominantly induced by RAGE signaling thus increasing ROS formation and inflammatory processes (Ott et al., 2014). Glycated-HSA, which is an early glycation product mainly composed of fructosamine, seems to have a very different effect than the advanced glycation end products AGEs (Thornalley et al., 1999). This could be mainly due to the differences of structure and reactivity between the different glycation products (Paradela-Dobarro et al., 2016). In the literature, there are several studies reporting the effect of glycated albumin in vasculature (Basta et al., 2002; Higai et al., 2006; Rubenstein et al., 2010). However, some critical aspects about these works must be considered. Many studies used AGEs prepared in vitro by incubating a protein, often albumin, with a reducing sugar or dicarbonyl compound, using however different parameters as concentrations of albumin and sugar, incubation time, analysis of the final products. Therefore the final products can be vary and different, and the effect experimentally detected can be produced by various agents as AGEs, Amadori products or even other modification products. Without knowing the exact nature of the products involved, it is difficult to determine the distinct role of each glycation product in diabetes-associated vascular complications. The glycated albumin used in this research work is an early glycation product which has a degree of substitution expressed as 1-5 mol of hexose (as fructosamine) per mol of albumin. The present study showed that the early glycated product, such as gHSA, is not responsible of increased oxidative stress in HUVEC during the two hours of incubation. Moreover, a potential effect of gHSA on the viability of endothelial cells was evaluated. The results showed that gHSA has not cytotoxic activity, but conversely a slight proliferative effect in cultured
HUVEC. In order to better understand the vascular complications associated with diabetes, the link between glycation and angiogenesis has been explored in several researches. Recently, it has been demonstrated that AGEs induce a significant increase in the number of viable skin microvascular endothelial cells and that this effect is mainly mediated by the paracrine secretion of VEGF (Roca et al., 2014). These effects seem to be due to RAGE activation. However, the effects of AGEs on angiogenesis are not that straightforward. While endothelial cells stimulation by AGE-BSA or -HSA seems to increase VEGF expression, capillary tube formation and promote angiogenesis, some types of AGEs appear to exert the opposite effect. Glycation seems to be involved in this paradox because it is both proangiogenic (with the ability to activate inflammation and ROS production pathways) and antiangiogenic (glycated extracellular matrix proteins result in an unfavorable environment for angiogenesis). Further, pro- and anti-angiogenic factors are proteins which can also be glycated (Roca et al., 2014). In a prolonged hyperglycemic condition, the glycation of growth factors could modify their respective activities, impairing the angiogenesis. The results from the present study revealed that gHSA could have a pro-angiogenic effect on HUVEC; these data have to be still study in deep to learn more about the molecular mechanisms involved in these events.

During hyperglycemia and diabetes, there are high blood glucose levels as well as fluctuation in glucose concentration. This condition has been linked to vascular disease and endothelial dysfunction. High glucose levels in circulation promote glycation process and the formation of the reactive α-oxoaldehydes glyoxal and methylglyoxal (Ott et al., 2014). Glyoxal is the most prominent reactive α-oxoaldehyde, together with methylglyoxal, derived from glucose oxidation, lipid peroxidation, and glycation reaction. Glyoxal can furthermore react with different amino acid residues of protein targets becoming a further cause of AGEs formation (Sliman et al., 2010; Thornalley et al., 1999). Glycation during hyperglycemic conditions and oxidative stress-mediated lipid peroxidation contribute to the formation of reactive carbonyl species (RCS) and induction of carbonyl stress. Oxidative stress plays a major role in the development of micro- and macrovascular complications of diabetes. Accumulation of free radicals in the vasculature of diabetic patients is responsible for the activation of redox signaling and epigenetic changes contributing to vascular inflammation and ROS generation (Creager et al., 2003). Reduction in cellular viability and increased oxidative stress can lead to endothelial dysfunction; therefore, in this study we evaluated the effect of glyoxal and high glucose on HUVEC viability and ROS formation. Studies from literature revealed that glyoxal cause detrimental effect in vasculature seen as cytotoxicity, barrier dysfunction and inhibition of angiogenesis in bovine pulmonary artery endothelial cells (Sliman et al., 2010). In the present study, glyoxal displayed cytotoxic activity seen as reduction in HUVEC viability after 24 hours of incubation. Glucose at high concentration did not display a direct cytotoxic activity on HUVEC, but
the mechanism by which it contributes to endothelial dysfunction is reported to be mainly related to oxidative stress and ROS formation (Quagliaro et al., 2005). Therefore, the activity on ROS formation in HUVEC has been the subsequent effect evaluated in the present research. The experimental study using DCFH-DA assay did not show a direct effect of glyoxal on ROS formation, during the 24 hours of incubation. Thus the cytotoxic effect of glyoxal on HUVEC is not directly related to oxidative stress, and further studies are needed to find the molecular mechanisms involved in the negative effect of glycation-derived dicarbonyl compounds. As reported in the literature, GO seems to determine changes in HUVEC morphology after 24 h treatment, and to examine whether the morphological changes are associated with vascular inflammatory states, the expression of inflammation-related proteins was measured. Chronic treatment of HUVEC with GO (500 μM, 24 h) induced COX-2 protein expression, but not VCAM-1 expression (Yamawaki & Hara, 2008). Overproduction of ROS by mitochondria is considered as a causal link between elevated glucose and the major biochemical pathways involved in the development of vascular complications of diabetes. The high levels of glucose are proposed to mediate pathological effects in vascular cells through the production of ROS. Cells cultured in high glucose and cells from diabetic subjects have been shown to release higher levels of ROS (Pitocco et al., 2013). Several mechanisms have been proposed for this increased ROS production, such as the mitochondrial electron transport chain, the advanced glycation end products and the PKC-dependent activation of NAD(P)H oxidase. The results obtained in this research work confirmed the data from literature, showing an increase of ROS formation in HUVEC cells after the treatment with high glucose.

Considering the harmful effect of glucose at high concentration on ROS production and oxidative stress in endothelial cells, antioxidant therapy may be an interesting choice to reduce diabetes-mediated vascular abnormalities. Previous studies showed that there is an improved endothelium-dependent relaxant response with various antioxidant agents, including superoxide dismutase (SOD) (Voinea et al., 2004). Thus, antioxidant approach is an option that could be used in combination with other therapies to treat vascular diseases. Natural compounds from medicinal plants have received particular attention in the context of the antioxidant activity. In recent decades, herbal remedies and natural products have undisturbedly attracted much research attention in the context of prevention and treatment of cardiovascular and metabolic diseases. Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. In the last decade, there has been much interest in the potential health benefits of plant polyphenols as antioxidants. The antioxidant activity of polyphenols is characterized by the phenolic structure; however, different classes of polyphenols may have different significant effects on vascular disease as evidenced by the study done by Loke and colleagues (Loke et al., 2010). In the present work, the
polyphenols studied for their antioxidant activity in in vitro endothelial cells were baicalein, eupatorin and galangin. These natural compounds have been chosen from a larger group of polyphenols from medicinal plants widely used in traditional medicine. It has been found that baicalein, eupatorin and galangin display a good in vitro antioxidant activity in DPPH and ORAC assay (Bertin et al., 2016) and the results from the glycation assay, described in the “Results” chapter, showed that these three phytocompounds determined the best inhibitory activity of protein glycation induced by glucose and glyoxal.

Regarding the activity of baicalein and eupatorin on the TNFα-induced VCAM-1 expression, the results showed that only eupatorin displayed the inhibitory activity, at both the concentration tested of 5 and 10 μM. In the literature is reported that the effect on adhesion molecule expression was strongly dependent on the chemical structure of the flavonoids (Lotito & Frei, 2006). These were previously reported to prevent adhesion molecule expression in endothelial cells, and that flavonols, as apigenin, are the most effective flavonoids in inhibiting cytokine-induced expression of E-selectin, ICAM-1, and VCAM-1 in HUVEC (Gerritsen et al., 1995). Specific research from literature about the structure-relation activity of flavonoids found that flavanones and flavanol were not able to inhibit adhesion molecule expression, whereas hydroxyl flavones and flavanols displayed the strongest inhibitory activity (Lotito & Frei, 2006). The results from the present research showed that eupatorin, a polymethoxylated flavone, was more effective in inhibiting the VCAM-1 expression on HUVEC than baicalein, a hydroxyl flavone. Differently it happened for the antioxidant activity. It is reported that baicalein is a potent antioxidant agent, and displays anti-inflammatory properties in vitro and in vivo (Shen et al., 2003; Tsai et al., 2014). Evidences indicate that baicalein has multiple biological activities, including cardioprotective and neuroprotective (Chang et al., 2011; Zhang et al., 2012). Studies from literature suggested that the mechanisms of action underlying these protective effects are mainly associated with antioxidant activity, which has been evaluated as inhibition of lipid peroxidation, up-regulation of HO-1, and activation of Nrf-2 (Im et al., 2005; Zhang et al., 2012). In the present research, ROS determination assay was performed on HUVEC, and the results showed that baicalein was able to inhibit the ROS production in HUVEC in basal condition. Baicalein displayed also antioxidant activity against the oxidative stress induced in HUVEC by high glucose, reducing the ROS formation at both the concentration tested of 1 and 5 μM. Data from literature show that galangin protects in vitro cultured cells from cytotoxicity and oxidative stress induced by glutamate, low levels of glutathione, and glucose starvation in culture medium, and that these activities could be attributed to the three hydroxyl groups of this compound. The effects seen for galangin have been attributed to its antioxidant activity (Ishige et al., 2001). In the present research, galangin displayed a good antioxidant activity against the ROS formation in HUVEC, both in basal condition and after high
glucose-induced oxidative stress. Conversely, eupatorin was not able to inhibit the ROS production in HUVEC in basal condition. However, after the induction of oxidative stress by high glucose, eupatorin 5 μM displayed antioxidant effect seen as inhibition of ROS formation. The differences in the structure of selected polyphenols are represented by the hydroxyl groups of baicalein and galangin, and the methoxyl groups of eupatorin, suggesting that the free hydroxyl groups lead to antioxidant activity.

In conclusion, the results showed that high glucose could have an important role in vascular complication caused by hyperglycemia and diabetes. The mechanisms behind glycation-related pathologies are very complex, and the activity of glycated albumin and glyoxal have to be further evaluated.

Regarding the activity of polyphenols, the results suggest that baicalein, eupatorin and galangin have high antioxidant and antiglycative activities against in vitro glycation of albumin (BSA), and the antioxidant activity has been displayed also in cultured HUVEC. Baicalein and galangin effectively decrease the ROS formation induced by high glucose on HUVEC, whereas the most important activity of eupatorin was the anti-inflammatory activity against the TNFα-induced VCAM-1 expression. Thus, these natural compounds may represent promising candidates for more detailed studies about their potential effects against glycation and oxidative stress related to vascular diseases.

All together, the results obtained motivate further researches on the activities of natural polyphenols against oxidative and glycative damages in order to understand their potential and actual role in the prevention of diabetes-related vascular diseases.
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