Polyphenol cargo in circulating lipoproteins in diabetes: impact on endothelial health

Sara Rocha,

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Sara Rocha,
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Department of Chemistry and Biochemistry
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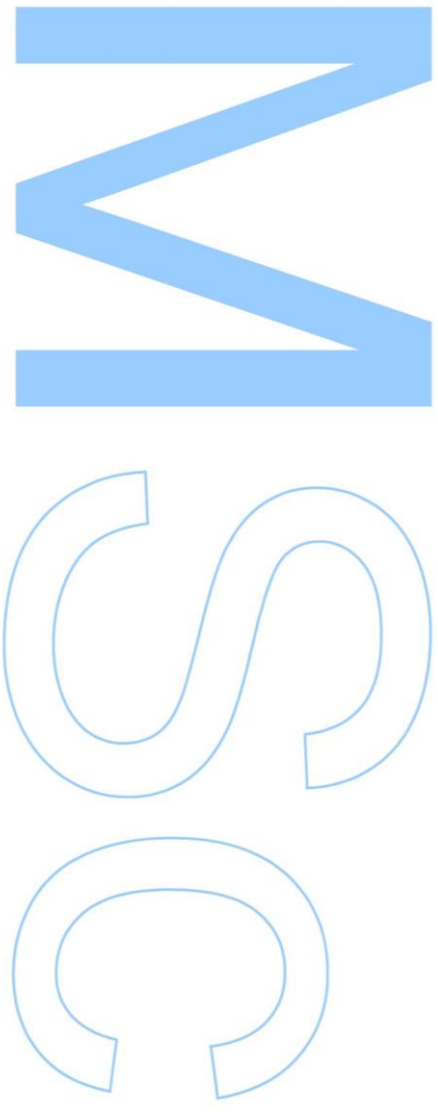
Supervisor
Doctor Ana Luísa Reis Pereira, Researcher, Faculty of Sciences of UP

Co-Supervisor
Prof. Doctor Victor Armando Pereira Freitas, Full Professor, Faculty of Sciences of UP



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Abstract

Diabetes is a disease that affects millions of people worldwide. The disease is characterized by a state of chronic inflammation that eventually leads to multiple vascular complications. Epidemiological studies have shown that the adoption of Mediterranean diets rich in polyphenols improves endothelial function and reduces the risk of vascular complications in diabetes, but to date, the cargo of polyphenol metabolites and their effect at physiological conditions in endothelial health remains poorly understood.

In this work, with the aim to evaluate the effect of polyphenol metabolites transported in lipoproteins on endothelial dysfunction generated by hyperglycemia, this study focused on the characterization of pooled lipoprotein sub-classes (VLDL and LDL) isolated from diabetic patients (n=15) before (Hyperglycemia Poor Control) and after drug and diet-treatment (Hyperglycemia Good Control) and from age-matched control group (n=15). Polyphenolic extracts prepared by solid-phase extraction (SPE) were characterized by UV/Vis methods in terms of total phenolic and flavonoid content; antioxidant capacity by the ferric reducing antioxidant power assay (FRAP) and anti-radicalar assay (DPPH). Polyphenol metabolites present in the extracts were tentatively identified by high-resolution mass spectrometry coupled with reverse-phase chromatography (UPLC-LC-MS) and quantified by UPLC-MS through SIM approach. The effect of two gut polyphenol metabolites identified in the extracts on the endothelial inflammatory response was evaluated *in* vitro by measuring the release of pro-inflammatory cytokines (IL-6 and IL-1β) in hyperglycemic medium by ELISA assays.

Spectrophotometric characterization of lipoprotein extracts revealed the content of polyphenols and flavonoids is higher in the VLDL population than LDL population. Hyperglycemia led to a marked decrease in VLDL polyphenol content (>50%) but not in LDL. This decline was reflected in the DPPH and FRAP assay in hyperglycemia conditions, though drug and diet treatment induced a slight improvement in the antioxidant capacity in VLDL population. The identification of polyphenol metabolites consisted mainly of gut polyphenol metabolites. The quantification by UPLC-MS showed a heterogeneous distribution of the polyphenol metabolites among lipoprotein populations. Hyperglycemia induced a decrease in the concentrations of the polyphenol metabolites. *In vitro* cellular studies revealed that gut metabolites were able to ameliorate the inflammatory response in hyperglycemia.

Key Words: Mediterranean Diet; Polyphenols; Metabolomic; Diabetes Mellitus Type 2; Endothelium; Inflammation.

Resumo

Diabetes é uma doença que afeta milhões de pessoas globalmente. A doença é caracterizada por apresentar um estado de inflamação crónica que eventualmente leva a complicações vasculares. Estudos epidemiológicos indicam que dietas mediterrâneas ricas em polifenóis melhoram a função endotelial e reduzem o risco de complicações vasculares em pacientes diabéticos, mas até à data, o painel de metabolitos de polifenóis e o seu efeito em condições fisiológicas na saúde endotelial é ainda desconhecida.

Neste trabalho, com o objetivo de avaliar o efeito de metabolitos de polifenóis presentes em lipoproteínas na disfunção endotelial causada por hiperglicemia, este estudo focou-se na caracterização de populações de lipoproteínas (VLDL e LDL) isoladas de pacientes diabéticos (n=15) antes (Hyperglycemia Poor Control) e após tratamentos com fármacos, mudança de dieta e exercício (Hyperglycemia Good Control) e de um grupo de controlo (n=15). Extratos ricos em polifenóis preparados por extração de fase sólida foram caracterizados quanto ao teor de polifenóis e flavonóides, atividade antioxidante (FRAP) e antirradicalar (DPPH) por métodos UV/Vis. Foi feita a identificação de potenciais metabolitos de polifenóis em extratos de lipoproteínas por espectrometria de massa de alta resolução acoplada a cromatografia (UPLC-LC-MS) e a quantificação de metabolitos de polifenóis por UPLC-MS através de SIM. Foi feita a avaliação da ação anti-inflamatória gerada por hiperglicemia de dois metabolitos de polifenóis *in vitro* ao medir a libertação de citocinas pró-inflamatórias (IL-6 and IL-1β) por ensaios de ELISA.

Caracterização espetrofotométrica dos extratos de lipoproteínas, mostra que o teor de polifenóis e flavonóides totais é maior em populações de VLDL que em LDL. Hiperglicemia levou a uma redução no conteúdo de polifenóis em extratos de VLDL (>50%), mas não de LDL. Esta redução foi igualmente observada na atividade antioxidante e antirradicalar em condições de hiperglicemia apesar de o tratamento (fármacos e mudança de dieta) levae a um ligeiro melhoramento na capacidade antioxidante da população de VLDL. A identificação dos metabolitos de polifenóis mostrou maioritariamente a presença de metabolitos sulfatados. A quantificação por UPLC-espectrometria de massa revelou uma distribuição heterogénea dos metabolitos de polifenóis entre as populações de lipoproteínas. Hiperglicemia induziu uma diminuição na concentração final dos metabolitos de polifenóis. Os ensaios *in* vitro mostrou que os metabolitos de polifenóis conseguiram melhorar o estado de inflamação.

Palavras-Chave: Dieta Mediterrânea; Polifenóis, Metabolómica; Diabetes Melitos tipo 2; Endotélio; Inflamação.

Index

List of Figures

List of Tables

List of Tables in Supplementary Information

List of Abbreviations

ACN- Acetonitrile

AGE- Advanced glycosylation endproducts

Ang II- Angiotensin II

Apo- Apolipoprotein

ATP- Adenosine triphosphate

BSA- Bovine serum albumin

CE- Catechin equivalent

CRP- C-Reactive protein

DHPPA- 3-(2, 4-Dihydroxyphenyl)propionic acid

DHPV- 3-(3',4'-dihydroxyphenyl)-γ-valerolactone

DPPH- 2,2'-Diphenyl-1-picrylhydrazyl

EGF- Epidermal growth factor

ELISA- Enzyme-linked immunosorbent assay

eNOS- endothelial NO synthase

ESI- Electrospray ionization

ET-1- Endothelin-1

FRAP- Ferric Reducing Antioxidant Power

GAE- Gallic acid equivalent

GI- Gastrointestinal

HMEC-1- Human microvascular endothelial cell

ICAM-1- Intercellular adhesion molecule-1

IL-1β- Interleukin 1β

IL-6- Interleukin 6

IS- Internal standard

JNK- c-Jun NH2-terminal kinase

LLE- Liquid-liquid extraction

LOD- Limit of detection

LOQ- Limit of quantification

LPDS- Lipoprotein depleted sample

MAPK- Mitogen-activated protein kinases

MCP-1- Monocyte chemoattractant protein-1

NADPH- Nicotinamide adenine dinucleotide phosphate

NF-κB- Nuclear factor kappa β

nM- Nanomolar

NO- Nitric oxide

PCA- Protocatechuic acid

PGI_2- Prostaglandin I

Pi- Inorganic phosphorous

PI3- Phosphoinositide-3 kinase

PL- Phospholipids

PPT- Protein precipitation

RAGE- Receptor for AGE

RNS- Reactive nitrogen species

ROS- Reactive oxygen species

SDS-PAGE- Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

SPE- Solid-phase extraction

TE- Trolox equivalent

TFC- Total flavonoid content

TNF-α-Tumor necroses factor α

TPC- Total phenolic content

TXA_2- Thromboxane

UPLC-MS- Ultra Performance Liquid Chromatography- Mass Spectrometer

VCAM-1- Vascular cell adhesion molecule-1

WHO- World Health Organization

XIC- Extracted ion chromatogram

µM- Micromolar

1. INTRODUCTION

1.1. Diabetes Mellitus Type 2

According to the World Health Organization (WHO) 1 in every 3 people have diabetes. There are over 422 million people worldwide diagnosed with diabetes and recent studies have evidence for the rising prevalence of diabetes in children and adolescents, making this disease a major health problem (1). Associated costs with hospitalizations and human resources is having a detrimental impact on national health budgets.

Type 2 diabetes is a chronic metabolic disorder that can be caused by genetic factors, though other risk factors like sedentarism, obesity, hypertension and elevated cholesterol contribute to the incidence of the disease (2, 3). This disease is characterized by hyperglycemia, a disproportionate amount of glucose in circulation, that is deleterious to the cells and ultimately leads to insulin resistance in insulin-dependent tissues and loss of β-cell mass in the pancreas (2-5). In insulin-dependent tissues like hepatic, muscle, and adipose, insulin resistance leads to a reduction of glucose uptake which in turn, gives rise to an increase in gluconeogenesis, increase formation of free fatty acids and decreased protein synthesis (6).

Before the onset of insulin resistance, hyperglycemia leads to an exacerbated consumption of glucose to form adenosine triphosphate (ATP), with an overuse of the mitochondrial electron chain that generates copious amounts of reactive oxygen species (ROS) like superoxide or hydrogen peroxide (4, 7). Other process that potentiates the formation of ROS are other pathways of glucose consumption (polyol and hexosamine), glucose autooxidation, formation of advanced glycosylation end products (AGE) by non-enzymatic glycation of proteins and lipids, lipid oxidation (4, 6, 7). The rise of ROS with hyperglycemia creates a state of oxidative stress characteristic of diabetes (4, 6).

Consequently, the rise of oxidative stress, hyperglycemia and high release of free fatty acids and leads to the activation of inflammatory pathways such as c-Jun NH2-terminal kinase (JNK) and nuclear factor kappa β (NF-κB), which triggers the release of inflammatory factors for instance cytokines namely tumor necroses factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) and ultimately to a chronic state of inflammation (4, 5, 8). Because of this, diabetes usually is accompanied by complications in the vascular system that affects high vascularized organs like heart, kidneys, brain and eyes (6).

1.1.1. Endothelial Dysfunction

The exacerbated oxidative stress and inflammatory status in diabetes mellitus type 2 is associated with endothelial dysfunction which constitutes an increased risk of cardiovascular diseases like atherosclerosis (9-11).

Endothelial cells form a single cell layer lining all blood vessels, the endothelium that controls vascular function (9, 12). To maintain vascular homeostasis, these cells control blood flow and pressure by the balance between the release of vasodilators and vasoconstrictors; control permeability of leukocytes; inhibit coagulation and promote fibrinolysis thus preventing the formation of thrombus (9, 12-14).

The principal vasodilator factor is nitric oxide (NO) generated by the endothelial NO synthase (eNOS) (15). It prevents the adhesion of leukocytes by the inhibition of the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin (9, 10, 14). By acting synergistically with another vasodilator (prostaglandin I, PGI_2), NO also inhibits platelet aggregation and factors involved in the formation of clots (9, 10, 14). The endothelium also releases vasoconstrictors like endothelin-1 (ET-1), angiotensin II (Ang II) and thromboxane A_2 (TXA_2) (9, 10, 14)

In a pathophysiological condition like diabetes, the hyperglycemia conditions result in a chronic state of oxidative stress and inflammation that results in endothelial dysfunction (10, 12). This condition is referred as the deficiency of the endothelium to maintain vascular homeostasis, characterized by the loss of NO, reduced ability of vasorelaxation with increase production of vasoconstrictors, prothrombotic properties and an increase in proinflammatory factors (9, 10, 12). Various mechanisms contribute to endothelial dysfunction. Insulin resistance promotes a reduction in NO production while the pathway to the expression of ET-1 remains unaffected, culminating in unopposed vasoconstriction (10, 12, 16). The elevated ROS in circulation can react with NO, forming more reactive nitrogen species (RNS), for example peroxynitrite, that might react with eNOS cofactors leading to the uncoupling of the protein complex (10, 12, 16). The state of hyperglycemia and high prevalence of free fatty acids also activates the complex nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) that produces more reactive species (10, 12, 16). In addition, due to the binding of AGE to its receptor (RAGE), expressed in the cell surface, stimulates ROS production (12, 17, 18).

Overall, the presence of ROS, the binding of AGE/RAGE and the pro-inflammatory cytokines in circulation leads to the activation of inflammatory signaling pathways such as NF-κB and Ras/MAPK in endothelial cells which culminates in the expression of

adhesion molecules (ICAM-1; VCAM-1 and E-Selectin) and the release of cytokines (TNF-α; IL-1β and IL-6) as represented in **Figure 1** (10, 12, 14, 16, 18).

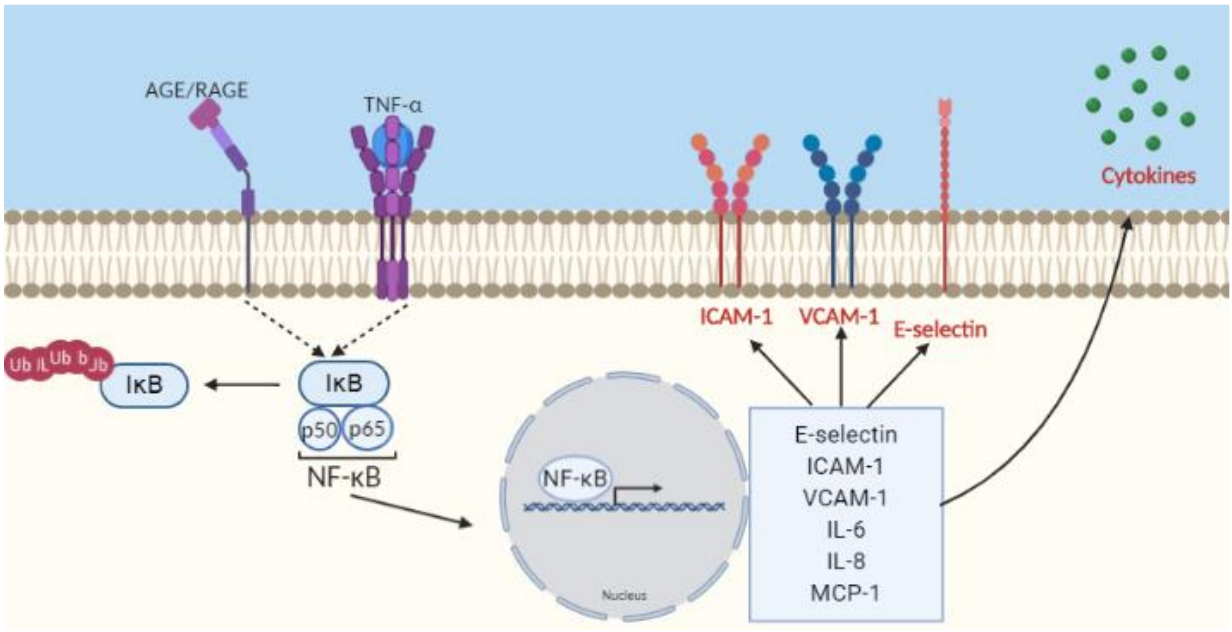


Figure 1: Inflammation pathway influenced in Diabetes Mellitus Type 2. Binding of TNF-α and AGE to their respective receptors activates adaptor proteins leading to the activation of the transcription factor NF-κB thus initiating the transcription of genes of adhesion molecules (ICAM-1; VCAM-1 and E-Selectin) and cytokines . Image created with BioRender.com.

The WHO recommends the adoption of Mediterranean and Nordic-type diets to prevent, reduce and help manage diet-related diseases such as diabetes and its complications (19, 20).

1.2. Polyphenol-rich diets as nutritional strategies in the management of diabetes

Mediterranean and Nordic countries have a diet characterized by polyphenol-rich foods such as fresh fruits, vegetables, nuts and vegetables (19, 20). Polyphenols are plant secondary metabolites that are widely distributed across fresh fruit, vegetables and beverages and have received great attention over the last decades due to their potential health benefits (21-23).

Polyphenols are a diverse group with over 8000 structures categorized in four different main classes namely, the phenolic acids (benzoic and cinnamic acids), flavonoids, stilbenes and lignans, depending on the number of phenolic rings and the elements that bind them as seen in **Figure 2**, that can be further modified at different position by sugar units or organic acids (23-25).

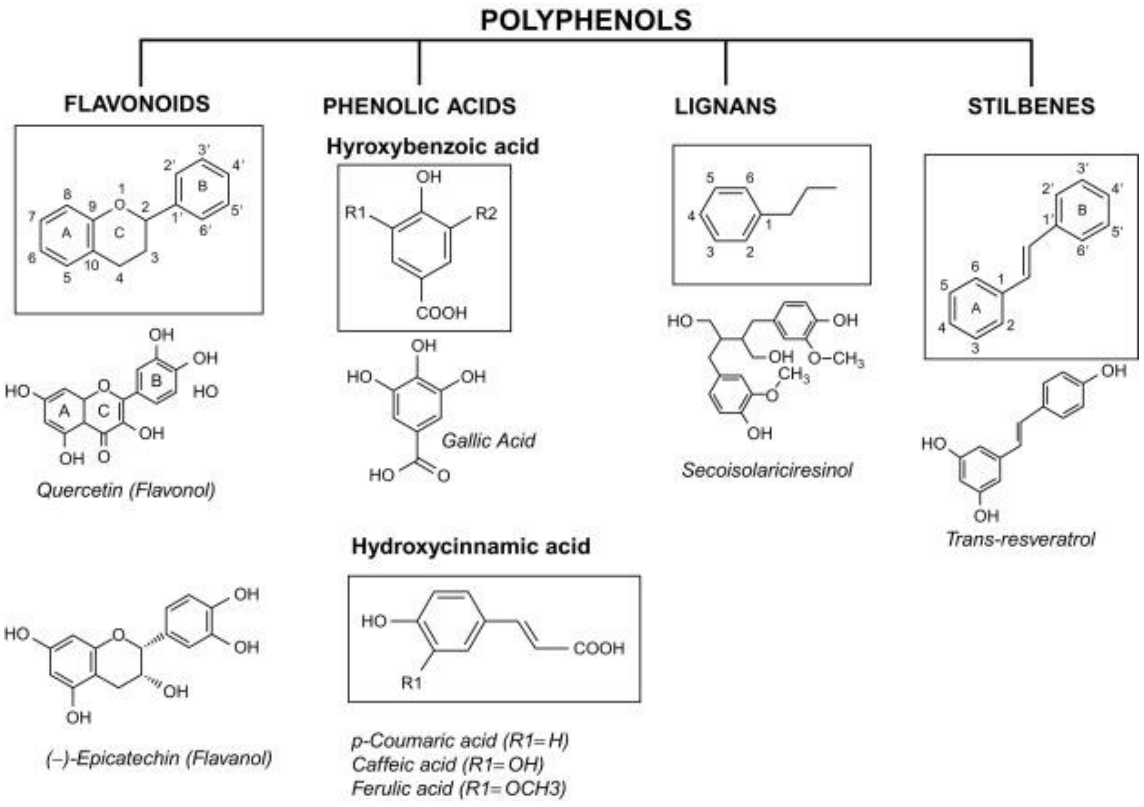


Figure 2: Structures of ingested polyphenols and selected examples. (reprinted with permission from Pathak et al., (2018), Polyphenols: Mechanisms of Action in Human Health and Disease (Second Edition))

Flavonoids is the most diverse class including six sub-groups with over 6000 structures found (23, 25). All of the sub-groups share the basic structure of flavonoids (**Figure 2**). Flavonoids are divided in six sub-groups: flavonols, flavones, isoflavones, flavanones, flavanols and anthocyanins. The hydroxylation pattern and variations in the C ring dictates which sub-group a certain polyphenol is included. (24, 25).

The amount of ingested polyphenols in Mediterranean-like diets can reach 1g/day, making an interesting option to prevent, treat and manage diabetes (26, 27). However, the amount of polyphenols ingested is dependent on our dietary choices and environmental factors like fruit ripeness, processing, storage conditions and cooking process (24, 28).

Epidemiological studies have shown an association between the consumption of polyphenol-rich diets, blood lipid-lowering effect, endothelial function and reduced risk of chronic diseases (29-33). The protective effect of polyphenols was mainly attributed to their antioxidant capacity, but more recently polyphenol have been associated with possible signaling properties and various beneficial effects, namely antiatherogenic, anti-inflammatory, neuro and vascular-protective, antithrombotic and anti-diabetic effects (23, 34-40). The anti-diabetic properties of polyphenols are mainly due to their ability to affect the metabolism of glucose by either inhibiting the enzymes α-amylase and α-glucosidase

or to ameliorate the uptake of glucose and increase insulin sensitivity. Ademiluyi and colleagues observed that extracts from different plants were able to inhibit both enzymes (41). Mulberry anthocyanin extracts were capable of increasing glucose uptake and glycogen content while diminishing glucose production (42). The anti-inflammatory effect of polyphenols including quercetin, hesperetin and genistein, was focused on the ability to suppress the expression of inflammatory mediators such as ICAM-1, VCAM-1, IL-6 and monocyte chemoattractant protein-1 (MCP-1) in endothelial cells (43-48).

However, most works found in literature disregard the metabolization process polyphenols go through once ingested (22, 24, 49).

1.2.1. Metabolism of Polyphenols and Biological Actions of Polyphenol Metabolites

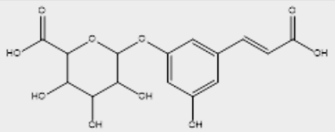
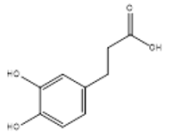
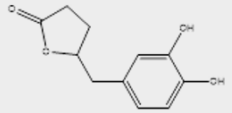
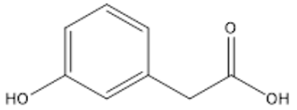
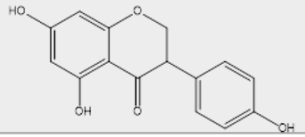
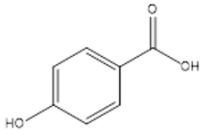
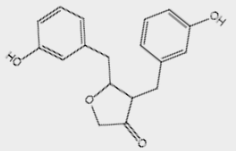
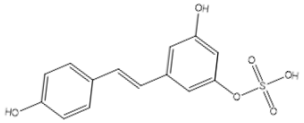
Ingested polyphenols are extensively metabolized in the gastrointestinal (GI) tract where they are exposed to different pH conditions that affects their chemical stability and are responsible for the biotransformation of polyphenols, a process that consequently changes their bioavailability and biological actions (22, 24, 49, 50).

The metabolization starts at the mouth by deglycosylation of polyphenols by salivary hydrolytic enzymes. In the stomach, the low pH affects the stability of some polyphenols like procyanidins or lignans opposite to glycoside polyphenols which are stable in these pH conditions (24, 49, 51). Most polyphenols reach the small intestine in their glycoside form and are hydrolyzed by intestinal enzymes. Polyphenols surviving the stomach and reaching the small intestine can also be conjugated, a process known as phase II metabolism involving enzymes like UDP-glucuronosyltransferase, catechol-O-methyltransferase or sulfotransferases leading to three main conjugation: glucuronidation, methylation and sulfation, respectively (22, 24, 49).

The large intestine will receive most ingested polyphenols where they are degraded by gut microbiota and conjugated polyphenols released from the enterohepatic circulation are further degraded (24, 49, 50). Degradation of flavonoids involves cleavage of the C-ring followed by the cleavage of the functional groups (dihydroxylation or demethylation) leading to aromatic compounds and phenolic acids that can be conjugated in the large intestine or liver (22, 49, 50). After metabolization, polyphenol metabolites in circulation only represent about 2% of the ingested polyphenol though the low bioavailability is not yet fully understood (24, 49).

In circulation polyphenol metabolites can be divided in two main categories: conjugated metabolites with a structure similar to the ingested polyphenols or metabolites resulted from the metabolization by gut microbiota (**Table 1**). In spite of the panel of polyphenols ingested being large it does not translate in a large panel of metabolites found in circulation (52). Recent studies focused on the polyphenol metabolome in urine and plasma samples revealed the occurrence of metabotypes, metabolic signatures of polyphenols despite inter-individual variability (53-56). The preliminary findings impact the research of polyphenols health benefits on diabetes mellitus or on endothelial cells (55, 56).

Table 1: Example of some of the most abundant gut metabolites found in circulation with representation of structures.

Class of Polyphenol	Metabolites Formed	Polyphenol Metabolites Structures	Reference
Phenolic Acids	3-(3-hydroxyphenyl) propionic acid Hippuric acid Caffeic acid 4-O-β-D-glucuronide*		(24-26, 30, 31)
Flavones **Flavanones**	Phloroglucinol 3-(3,4-dihydroxyphenyl) propionic acid*		
Flavanols	3-(3-hydroxyphenyl) propionic acid 5-(3'-hydroxyphenyl)-γ-valerolactone-3'-O-β-D-glucuronide 5-(3',4'-dihydroxyphenyl)-γ-valerolactone*		
Flavonols	3,4-dihydroxyphenylacetic acid 3-hydroxyphenylacetic acid* Homovanillic acid		
Isoflavones	Dihydrogenistein* 30,40,7-trihydroxyisoflavone 40,6,7-trihydroxyisoflavone		
Anthocyanins	Protocatechuic acid 4-hydroxybenzoic acid* Vanillic acid		
Lignans	Enterodiol Enterolactone*		
Stilbenes	Trans-resveratrol-3-O-glucoronide Trans-resveratrol-3-O-sulfate*		

*The structure of the polyphenol metabolite formed corresponds to the marked metabolite formed.

The different stages of absorption in the GI tract leads to the appearance of polyphenol metabolites at different time points as seen in **Figure 3** (57).

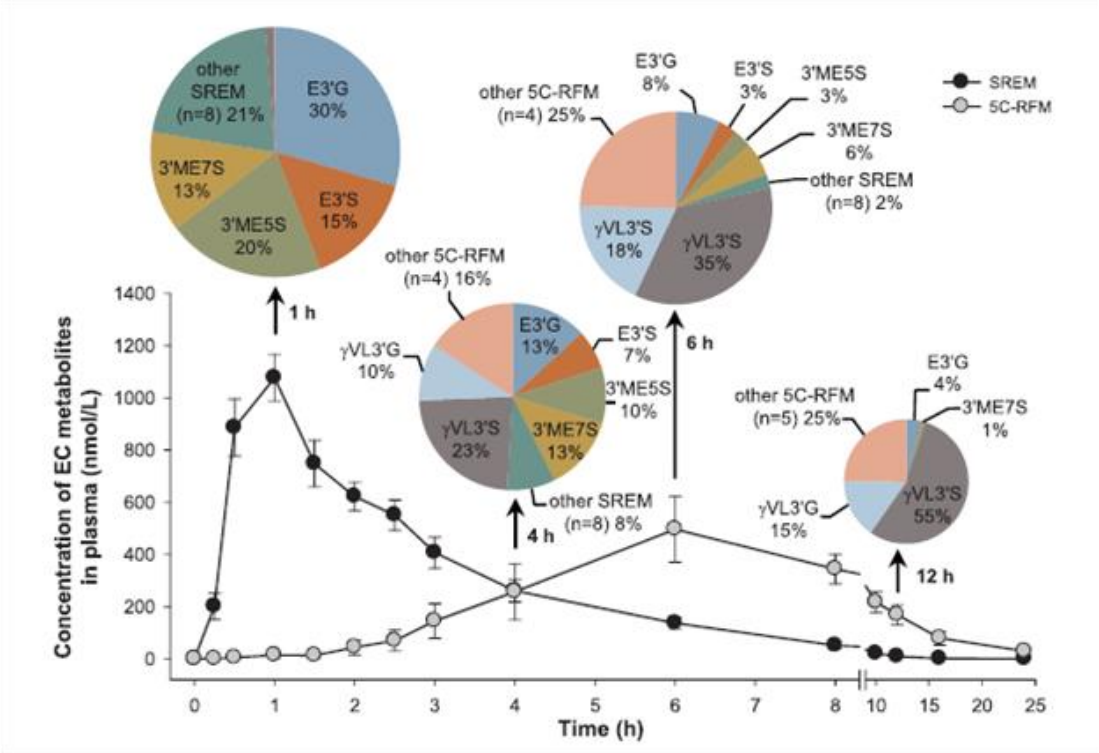


Figure 3: Profile of plasma [2-^{14}C](−)-epicatechin metabolites as a function of time. SREM: structurally-related (−)-epicatechin metabolites, include glucuronide conjugates (E3'G), sulfate derivatives (E3'S), and methyl-sulfate derivatives (3'ME7S, 3'ME5S, E3'S). 5C-RFM: 5-carbon ring fission metabolites include gamma-valerolactone sulfate and glucuronide conjugates (gamma-VL3'S, gamma-VL3'G). Data are expressed as mean values in nM ± SEM (n = 8). Insert: pie charts depict the relative amount (% of total) of individual SREM and 5C-RFM present in plasma at 1h, 4h, 6h and 12h after ^{14}C-EC ingestion (n = 8). (reprinted with permission from Ottaviani et al., (2016), Sci Rep, 6, 29034).

Pharmacokinetic studies conducted in humans supplemented with polyphenol-rich foods revealed that plasma concentrations of polyphenol metabolites reach micromolar (μM) values for the polyphenol conjugates while gut polyphenol metabolites reach nanomolar (nM) values (57-59), For instance, **Figure 3** shows the methyl-sulfate and glucuronide polyphenol metabolites predominate in circulation 1 hour after consumption whereas gut metabolites show their plasmatic peak much later at 6 hours after consumption (57, 58). Even though much is known about the panel of polyphenol metabolites in circulation after food supplementation in healthy individuals, there is still no work done that i) determines the basal levels for polyphenol metabolites in circulation; ii) demonstrates the distribution of polyphenols in the lipoprotein populations; iii) studies the impact hyperglycemia has on the panel and cargo of circulating polyphenol metabolites. To better understand the benefits of polyphenol-rich diets on endothelial health in diabetic patients, it is important to improve the knowledge on these aspects.

Research focused on the beneficial effects of polyphenol metabolites is still scarce, however it has been demonstrated that they possess anti-diabetic, anti-inflammatory,

anti-thrombotic, neuroprotective, anti-tumoral, and appetite modulatory actions (**Figure 4**) (45, 60-66).

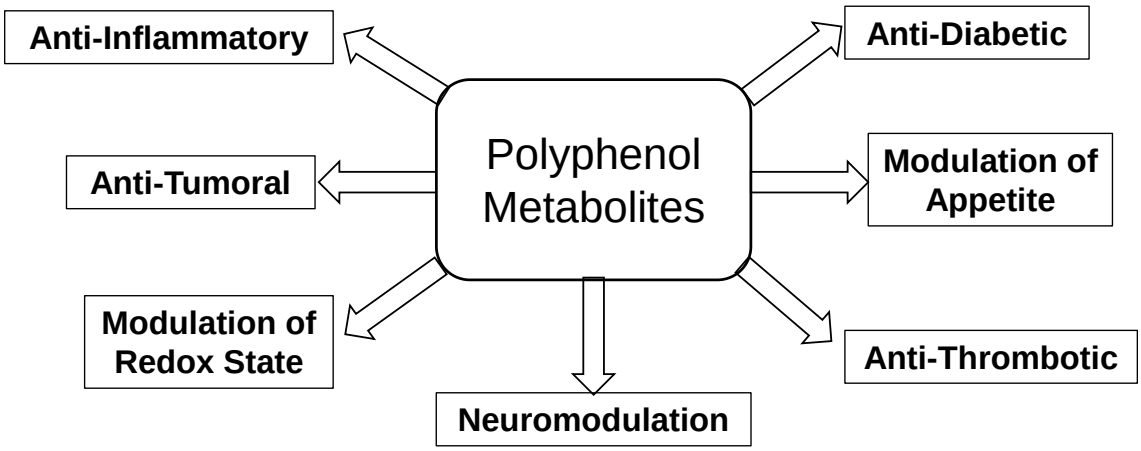


Figure 4: Beneficial health effects of polyphenol metabolites.

From the few studies reporting the biological action of polyphenol metabolites, Ho and colleagues found that metabolites expected from anthocyanins, for example, protocatechuic acid, vanillic acid, hippuric acid and 4-hydroxybenzoic acid were able to reduce the activity of α-amylase and α-glucosidase. The same authors also reported that all metabolites increased glucose uptake (67). It was also observed how polyphenol metabolites like protocatechuic acid, dihydroxyphenylacetic acids, urolithin A and B inhibit glycation and formation of AGE (65, 68, 69). Others reported that methyl, sulfate and glucuronide derivatives of catechin and quercetin metabolites decreased the expression of ICAM-1 and E-selectin in endothelial cells (60). Anthocyanin's gut metabolites reduced IL-6 production in an endothelial cell line (70).

Despite all the evidence that polyphenol metabolites have potential beneficial effects on diabetes the focus so far has been on polyphenols and their effect on endothelial cells under normoglycemia conditions using excessively long incubation times without taking in consideration the time of circulation of the metabolites (60, 65-70).

1.3. Objectives

The aim of this work was to improve the knowledge of ingested polyphenols present in polyphenol-rich diets typically found in Mediterranean and Nordic countries on the inflammatory response of endothelial cells exposed to hyperglycemia conditions. The role of lipoproteins in the transport of polyphenol metabolites and the effects of the polyphenol metabolites on the inflammatory response is also a topic scarcely studied.

With this in mind, the project had three main objectives:

- Characterization of lipoprotein extracts regarding their polyphenol and flavonoid content and their antioxidant ability by spectrophotometric methods;
- Identification and quantification of a circulating panel of polyphenol metabolites present in the lipoproteins extracts by UPLC-MS;
- *In vitro* evaluation of polyphenol metabolites in physiological relevant concentrations in an endothelial cell line exposed to hyperglycemia.

2.MATERIAL AND METHODS

2.1. Reagents and Materials

Reagents used in SDS-PAGE were acrylamide, trizma base, glycine, SDS, glycerol, dithiothreitol and Tris buffer from Sigma-Aldrich, Precision Plus Protein™ Unstained Protein Standard (Bio Rad) and Pierce coomassie staining (Thermo Scientific). For the extraction methods it was purchased ethyl acetate ($CH_3COOC_2H_5$) from Thermo Fisher, formic acid from Thermo Scientific, acetonitrile (ACN) from Chem-Lab, Oasis HLB cartridges from Waters and HybridSPE-Phospholipid cartridges from Supelco.

In the spectrophotometric methods it was used Coomassie plus reagent, gallic acid, sodium carbonate (Na_2CO_3), Folin-Ciocalteu reagent, sodium nitrate ($NaNO_2$), 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sodium acetate (CH_3COONa); ferric chloride ($FeCl_3$); 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), sodium molibdate ($NaMoO_4.H_2O$), ascorbic acid obtained from Sigma Aldrich, aluminum chlorite hexahydrate ($AlCl_3.6H_2O$) and catechin from Fluka, phosphate-buffered saline (PBS) buffer, sodium hydroxide (NaOH) and ferrous sulphate ($FeSO_4$) from PanReac AppliChem and perchloric acid ($HClO_4$) from VWR Chemicals. The manifold used was HyperSep™ Glass Block Manifold.

For LC-MS methods was used ultrapure water from a Milli-Q system (Rephile), acetonitrile (Chem-Lab) and formic acid (LC-MS grade, Thermo Scientific). Standards used were taxifolin from PureOri Biotech, 3-(2, 4-Dihydroxyphenyl)propionic acid (DHPPA) from Sigma-Aldrich and protocatechuic Acid (PCA) from HWI Group. The instruments used were Hypersil GOLD™ VANQUISH™ C18 UHPLC Colum purchased from Thermo Fisher, the speed-vacuum system CentriVap Benchtop Vacuum Concentrator from Labconco, HPLC (ACCELA 600, Thermo) coupled to a high resolution mass spectrometer LTQ Orbitrap XL from Thermo Scientific, Finnigan Surveyor series Liquid Chromatograph coupled to the mass detector Finnigan LCQ DECA XP MAX from Thermo Fisher.

The *in* vitro experiments consisted of Human microvascular endothelial cells (HMEC-1) from ATCC, RPMI 1640 medium and fetal bovine serum (FBS), penicillin/streptomycin from Invitrogen Life Technologies, Pierce BCA Protein Assay Kit from Thermo Scientific, sodium bicarbonate, HEPES, EGF, hydrocortisone (>98%), the polyphenol metabolite 3-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV), the ELISA kits from Sigma-Aldrich and the metabolite PCA from HWI group.

2.2 Lipoprotein Samples

Isolated lipoprotein samples were kindly supplied by a Prof José-Luis Sanchez-Quesada (Hospital de la Creu e Sant Pau, Barcelona, Spain) and their lipid content characterized.

The lipoprotein populations were obtained from pooled plasma collected from healthy (n=15) and type 2 diabetic (n=15) donors. Normoglycemic donors (Control group) consisted of 7 males and 8 females, mean age of 49±8 and body mass index (BMI) of 26.3±1.7. Diabetic donors (Poor control group, HbA1c > 8.5%) consisted of 7 males and 8 females, with mean age of 57±9, and BMI of 27.1±3.0. Patients with acute or chronic infections, clinically assessed cardiac disease, active inflammatory disease, treatment with anti-inflammatory drugs or CRP>20 mg/L were excluded from the study. The same diabetic patients were then subjected to a controlled diet, moderate exercise, and the drugs metformin and insulin to achieve good glycemia control (Hyperglycemia GC, HbA1c < 7%) and blood was again collected at this stage. Isolation of lipoprotein populations isolation was done by sucrose density gradient as previously described (71). **Table 2** shows the lipid content from the lipoprotein samples collected from three different study groups, namely normoglycemia, hyperglycemia under poor control (PC) and hyperglycemia under good control (GC).

Table 2: Lipid composition of lipoprotein fractions collected. Results expressed as % of lipoprotein mass.

Lipoprotein Population		Total Cholesterol	Triglycerides	Phospholipids	ApoB
VLDL	Normoglycemia	24.5	43.6	20.1	11.7
	Hyperglycemia PC	20.9	49.0	20.0	10.0
	Hyperglycemia GC	21.0	49.9	19.6	9.6
LDL	Normoglycemia	40.9	6.8	26.5	25.8
	Hyperglycemia PC	38.2	7.6	25.3	28.8
	Hyperglycemia GC	38.6	7.5	25.7	28.2

Informed consents were obtained in written form from patients. The study protocol was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Local Ethics Committee (IIBSP-REL-2017-27).

2.3 SDS-PAGE Protein Electrophoresis

The purity of the lipoprotein samples was verified using the method sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) based on the separation of proteins by their size.

SDS-PAGE electrophoresis of lipoproteins was carried out in a 4% acrylamide staking gel and 15% acrylamide resolving gel (1 mm). The lipoprotein samples (5 µL) contained 10 µL of Tris buffer (pH 6.8), 15 µL sample buffer (4% SDS and 350 mM dithiothreitol) and 15 µL marker while plasma sample (1 µL) contained 30 µL of Tris buffer, 30 µL samples buffer and 15 µL of marker. For denaturing conditions, the samples were heated for 5 min in a water bath at 100ºC then centrifuged at 10000g for 2 min. The running buffer was 250 mM Tris-buffer pH 8.3, 1.92 M glycine and 1% SDS. The separation was achieved by an electric current of 20 mA. The gels were then stained with Coomassie brilliant blue for 2 h in orbital shaker.

2.4 Extraction of Polyphenol Metabolites from Biological Samples

Polyphenol metabolites in plasma samples are often extracted using some of the most employed methods in literature such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE), although consensus has not yet been attained. To choose the best extraction method, it was used both in plasma samples.

2.4.1 Liquid-Liquid Extraction

For this, 50 µL of plasma and 150 µL of ethyl acetate ($CH_3COOC_2H_5$) were vortexed for 10 min with regular intervals where the samples were put in ice for 2 min, followed by centrifugation at 6500*g* for 2 min. The organic phase was then collected in separate eppendorf tubes. The process was repeated for the aqueous phase, and the organic phase was collected again. Both organic phases were pooled in one eppendorf and stores at -20ºC until further use. This procedure was done in triplicate.

2.4.2 Solid Phase Extraction

Plasma samples were fist diluted 1:2 (v/v) using water with 5% formic acid (v/v) solution. Then an Oasis HLB cartridge was activated with 2 mL of methanol with 5% formic acid (v/v) followed by 2 mL of water (5% formic acid). Next 200 µL of diluted plasma samples was loaded to the cartridges and washed under vacuum with 400 µL of acidified water and methanol (5% formic acid). Finally, the polyphenol metabolites fraction was eluted with the addition of 400 µL of methanol. The extracts were stored at

-20ºC until further use. To evaluate this extraction method plasma samples were used in triplicate.

Extraction of polyphenol metabolites using HybridSPE-Phospholipid cartridges was done according to manufacturer's instructions. Briefly, a solution of 1% of formic acid (v/v) in acetonitrile (ACN) is used in a ratio of 1:4 (v/v) to the samples. To 100 µL of plasma, 300 µL of ACN (1% formic acid) was added, vortexed for 10 seconds and centrifuged 3000*g* for 3 minutes. The pellet was discarded while the supernatant was loaded to the HybridSPE-Phospholipid cartridges and eluted using a vacuum manifold. This method was done in triplicate. The extracts were stored at -20ºC until further use.

2.5 Protein content by Bradford Assay

The protein content of the lipoprotein samples was estimated by spectrophotometry using the Bradford method (72).

To a 96 well plate was added 10 µL of each calibration solution, sample or blank then 10 µL of distilled water and 270 µL and Coomassie Plus Reagent was added making the final volume in each well was 300 µL. The lipoprotein samples were diluted 1:10 with PBS buffer for this assay and the blank was made from PBS buffer. The same buffer was used to prepare a stock solution of bovine serum albumin (BSA) 0.2 mg/mL and the consequently calibration solutions ranging from 7-200 mg/mL. The plate was shaken for 30 seconds and then incubated at 37ºC for 10 min. Absorbance values were measured in a microplate reader at λ=595 nm. The concentration of each well was plotted with its corresponding absorbance against the calibration curve and values were used to calculate the amount of protein in each sample. The results are expressed as mg protein per mL sample.

2.6 Characterization of Lipoprotein Extracts by Spectrophotometric Methods

The extracts of the lipoprotein samples were characterized using standard UV/Vis methods. In **Figure 5** depicts a scheme of the spectrophotometric methods used, quantification of polyphenols by the Folin-Ciocalteu method, quantification of flavonoids based on the aluminum chloride-flavonoid complexation, the antioxidant capacity using the FRAP assay and radical scavenging by de DPPH assay.

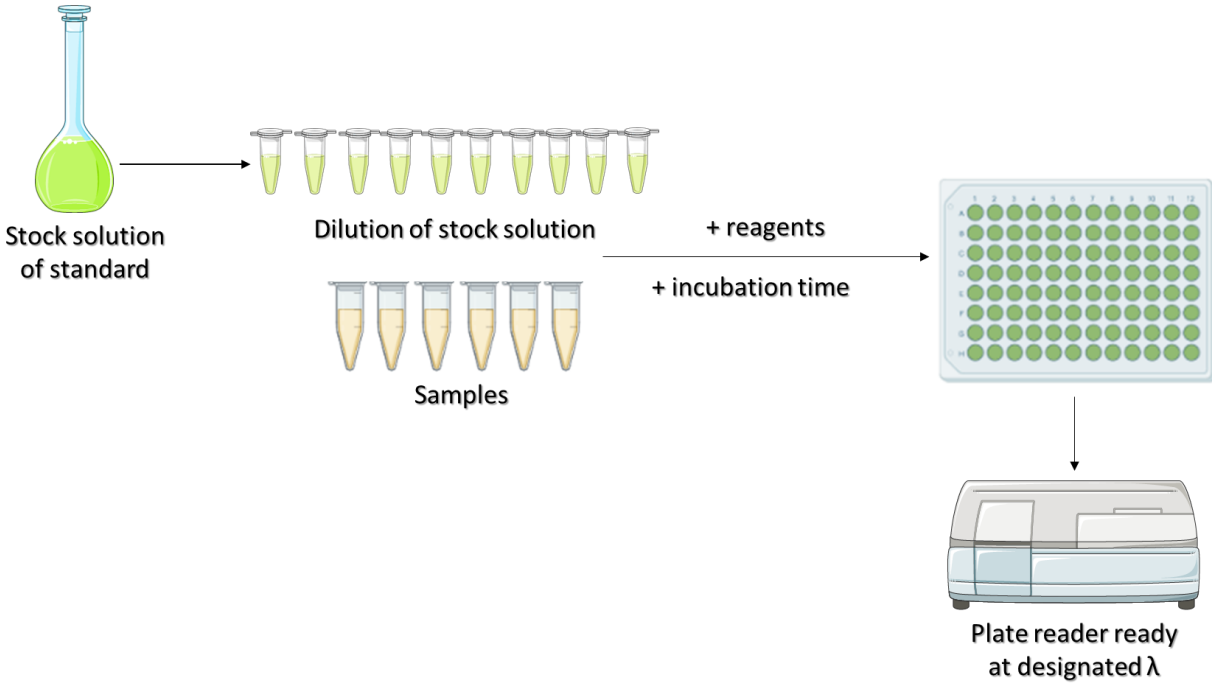


Figure 5: Schematic representation of the methods utilized to characterize the samples by UV/Vis methods.

2.6.1 Total Phenolic Content (TPC) Assay

The quantification of polyphenols in the sample extracts was done by the Folin-Ciocalteu method using gallic acid (GA) as the standard (73).

In a 96 well plate, 30 µL of each calibration solution, sample or blank was pipetted, alongside 150 µL of Folin-Ciocalteu solution (1:10 dilution of reagent with distilled water) and of 120 µL of a solution of 7.5% sodium carbonate (Na_2CO_3). The final volume in each well was 300 µL. The calibration curve was prepared after a stock solution of GA 1 mg/mL was diluted to concentrations ranging from 2-40 mg/L. The plate was put in a shaker for 30 seconds, incubated at 45ºC for 15 min and finally at 37ºC for 30 min. The absorbance was read at λ=765 nm. The concentration of each well was plotted with its corresponding absorbance for the calibration curve. The attained equation was then used to calculate the phenolic content of each sample. The results are expressed as gallic acid equivalent (GAE) per mL extract and mg of protein.

2.6.2 Total Flavonoid Content (TFC) Assay

The content of flavonoids in lipoprotein sample extracts was measured by the spectrophotometric method based on the complexation of flavonoids with the aluminum chloride solution as described before (74).

In a 96 well plate, 50 µL of each calibration solution, sample or blank was pipetted to a 96 well plate, 50 µL of distilled water and 6 µL of a solution of 1% sodium nitrate

($NaNO_2$) was then added. After 5 min, 6 µL of solution of 5% aluminum chlorite hexahydrate ($AlCl_3.6H_2O$) was added. Lastly, after resting another 1 min, 40 µL of a solution of 1 M sodium hydroxide (NaOH) and 48 µL of distilled water was put in each well. The final volume in each well was 200 µL. Catechin was used as the standard in this procedure and dissolved in drops of ethanol 96%, then added distilled water until a final concentration of 0.2 mg/mL of stock solution. The calibration curve was prepared by dissolving the catechin stock solution until obtained the concentrations of 0.8- 12 mg/L of catechin. The blank was made from distilled water. The plate was put in a shaker for 30 seconds. The absorbance was obtained at λ= 510 nm. The concentration of each well was against the calibration curve. The attained equation was then used to calculate the flavonoid content of each sample. The results in triplicate are expressed as catechin equivalent (CE) per mg of protein.

2.6.3 DPPH Radical Scavenging Capacity Assay

The evaluation of the radical scavenging ability of the lipoprotein extracts was done by a spectrophotometric method based on the electron transfer to the stable radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (75).

In a 96 well plate 30 µL of each calibration solution, sample or blank and 270 µL of a solution of 60 µM DPPH was pipetted (dissolved in methanol). The DPPH solution was allowed to rest 60 min protected from light before usage. The final volume in each well was 300 µL. A stock solution of trolox was prepared by dissolving the reagent in methanol to final concentration of 750 ppm, then dissolved to concentrations ranging from 1.5- 37.5 mg/L. The blank was made from methanol. The plate was put in a shaker for 30 seconds. The reaction progress of the reaction was monitored at λ=515 nm at every 5 min during 30 min. The concentration of each well was plotted with its corresponding absorbance for the calibration curve in the last point of time (30 min of reaction). The attained equation was then used to calculate the radical scavenging activity of each sample. The results are expressed as trolox equivalent (TE).

2.6.4 Ferric Reducing Antioxidant Power (FRAP) assay

The spectrophotometrically method to assess the reduction ability of the lipoprotein sample extracts was the FRAP assay developed by Benzie and Strain (76).

In a 96 well plate, 30 µL of each calibration solution, sample or blank was pipetted to a 96 well plate. The reagent FRAP was prepared by adding 12 mL of a 0.3 M pH 3.6 buffer solution of sodium acetate ($NaCH_3COO$), 1.2 mL of solutions of 10 mM TPTZ and 20 mM ferric chloride ($FeCl_3$) for a final volume of 14 mL. 270 µL of this reagent was

included in each well. The final volume was 300 µL. Stock solution of the standard 1 mM ferrous sulphate ($FeSO_4$) was prepared final concentration of 1 mM and consequently diluted to concentrations ranging from 10-300 µM of $FeSO_4$. The blank was made from distilled water. The plate was put in a shaker for 30 seconds and then incubated at 37ºC for 30 min. The absorbance was obtained at λ=595 nm. The concentration of each well was plotted with its corresponding absorbance for the calibration curve. The attained equation was then used to calculate the reducing ability of the samples. The results are expressed as µg of Fe^{2+}/mg of protein.

2.7 Quantification of Phospholipids

Quantification of phospholipids (PL) was done spectrophotometrically according to the method described by Bartlett and Lewis (77). Briefly, 5 µL of sample extracts were dried under N_2-stream in a pyrex test tube. To the dried extracts, 125 µL of 70% perchloric acid ($HClO_4$) was added and heated at 200ºC for 60 min. Phosphate standards (0-20 µL) were prepared from a phosphate stock solution of 100 µg/mL sodium phosphate (NaH_2PO_4). Thereafter, 125 µL 70% $HClO_4$, 825 µL of ultrapure water and 125 µL of 2.5% sodium molibdate ($NaMoO_4.H_2O$) were added to sample and standard test tubes and vortexed, followed by the addition of 125 µL of a solution of 10% ascorbic acid to standard and sample tubes and incubation in a water bath at 100 ºC for 10 min. After cool in water the tubes were centrifuged at 1000*g* for 5min. Absorbance values were measured in a microplate reader at λ=797 nm. This experiment was done in triplicate. The concentration of each standard was plotted with its corresponding absorbance for the calibration curve. The attained equation was then used to calculate the inorganic phosphorous. The results are expressed as ng of inorganic phosphorous (Pi)/ µL of extract.

2.8 Analysis and Identification of Polyphenol Metabolites in Lipoprotein Samples

Polyphenol metabolites transported by lipoproteins were identified in lipoprotein extracts prepared in section 2.4.2 using the HybridSPE-Phospholipid cartridge to extract polyphenol metabolites from 250 µL of lipoprotein samples. To the resulting extracts it was added 500 nM of taxifolin serving as internal standard (IS) and subsequently concentrated under vacuum at 30ºC. Once dry, the samples were stored at -20ºC until further use.

Separation and identification of polyphenol metabolites present in the lipoprotein extracts was achieved by reverse-phase chromatography coupled to mass spectrometry

(LC-MS). The separation of polyphenol metabolites was carried out in a Hypersil GOLD™ VANQUISH™ C18 UHPLC Colum (150 mm x 2.1 mm, 1.9 μm) in a HPLC equipped with autosampler and coupled to a high resolution mass spectrometer LTQ Orbitrap XL equipped with electrospray (ESI) source. The dried lipoprotein extracts were resuspended in 100 μL of 10% ACN in ultrapure water and the polyphenol metabolites separated by elution with ultrapure water with 1% formic acid (mobile phase A) and ACN containing 1% formic acid (mobile phase B). The elution program follows a gradient shown in **Table 3**. The volume injected consisted of 20 μL with flow rate of 150 μL/min with the autosampler at 10 ^{o}C.

Table 3: Elution gradient conditions used by liquid chromatography (LC)

Time (min)	Mobile Phase B (%)	Mobile Phase A (%)
0	2	98
3	2	98
12	20	80
17	95	5
25	95	5
29	2	98
35	2	98

The detection of polyphenol metabolites in extracts was conducted in full MS with an ionization voltage of -3.20 kV, the capillary voltage of -35 V, an ion source temperature of 275^{o}C and sheath flow gas 40 arbitrary units. The extracts of the samples were analyzed in triplicates with blank runs inserted between each three runs.

Analysis of LC-MS chromatograms and spectra was achieved in XCalibur software (version 2.2). Identification of potential ions attributed to polyphenol metabolites was done by analyzing monoisotopic mass, molecular structure, and retention time. All results achieved were compared to a homemade database and the online database, HMDB (https://hmdb.ca/spectra/ms/search), where the criteria applied for HMDB search was negative mode, molecular weight tolerance of ± 0.005 Da and adduct type of $[M-H]^-$ and $[M+FA]^-$.

2.9 Quantification of Polyphenol Metabolites by LC-MS in Lipoprotein Extracts

The quantification of the polyphenol metabolites was done using the Hypersil GOLD™ VANQUISH™ C18 UHPLC Colum (150 mm x 2.1 mm, 1.9 μm). The volume of sample injected was 25 μL with a flow rate of 150 μL/min with the autosampler at 20 ^{o}C in a Finnigan Surveyor series liquid chromatograph. The mobile phase A compromised

of ultrapure water with 1% formic acid while mobile phase B was ACN containing 1% formic acid. The elution program is presented in **Table 3**.

The mass detector used was a Finnigan LCQ DECA XP MAX quadrupole ion trap equipped with an atmospheric pressure ionization (API) source, using electrospray ionization (ESI) interface. Detection of standard and polyphenol metabolites was performed in selected ion mode (SIM) and negative mode with an ionization voltage of -5 kV, an ion source temperature of 325^{o}C and sheath gas flow 40 arbitrary units.

The standards employed were protocatechuic acid (PCA) and 3-(2,4-dihydroxyphenyl)propionic acid (DHPPA). For each standard, a TUNE acquisition file was obtained. The TUNE parameters from PCA and DHPPA are presented in **Table** of Supplementary Information. For the calibration curves, the injections of polyphenol standards were done in triplicate, in a random sequence with blank runs in between each three injection runs. The LC-MS chromatograms were then analyzed by XCalibur software (version 2.2) and the area under the curve (AUC) with smoothing of 7 for the analyte was taken for the various concentrations to build the calibration curve. The polyphenol metabolites were quantified in the extracts by plotting the peak areas against the calibration curve. The results are expressed as µmol/mg protein.

2.10 Evaluation of Extraction Performance by SPE

2.10.1 Validation of Quantification Method

To validate the method for quantification of the metabolites it was determined the limit of detection (LOD) and quantification (LOQ) and evaluation of reproducibility with intra-day and inter-day assessment. The calibration curve prepared for the taxifolin was also used to calculate the recovery percentage of the extraction method.

For this, a stock solution of taxifolin was prepared with a concentration of 0.5 mg/mL. Calibration curve was prepared by diluting the stock solution to final concentrations ranging from 20-160 nM and injected to the LC-MS as described in 2.9. Every concentration value was done in triplicate with blank runs in between each concentration.

The LC-MS chromatograms were then analyzed by XCalibur software (Version 2.2) and the peak area for the analyte was taken for the various concentrations to build the calibration curve. The peak area in the retention time for the taxifolin was also taken in the blank runs for the calculation of LOD and LOQ.

2.10.2 Extraction Recovery Percentage

For the percent recovery, a lipoprotein sample was spiked with 200 nM of taxifolin as internal standard (IS). The sample was then extracted following the procedure described for mass spectrometry using the method SPE with the HybridSPE-Phospholipid cartridges. The extracts were injected in triplicate in the LC-MS using the same elution program depicted in **Table 3**.

The area under the curve corresponding to IS was determined using XCalibur software (version 2.2). Using the equation attained previously for the validation of the quantification method, the final concentration was determined. Finally, the percentage of recovery was calculated. **Figure 6** shows a schematic representation of the method applied.

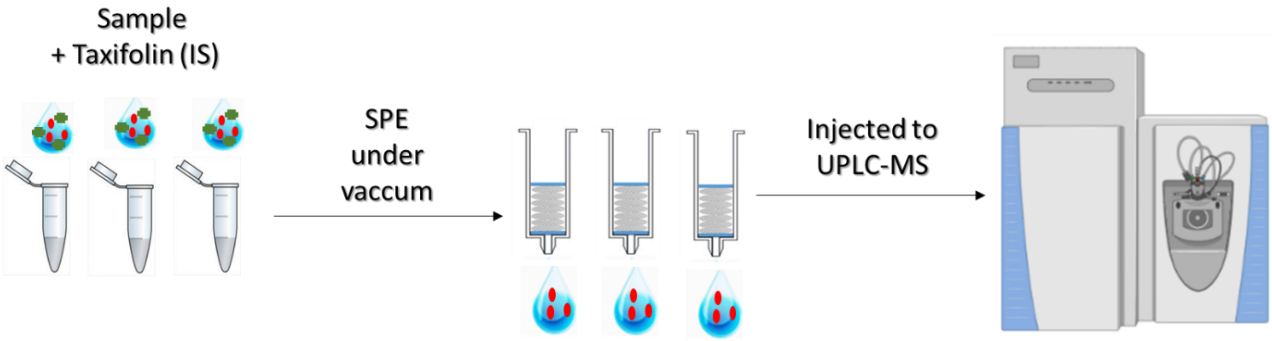


Figure 6: Schematic representation of determination of percent recovery of polyphenol metabolites by SPE.

2.10.3 Determination of LOD and LOQ

The LOD and LOQ were calculated using the peak areas of the blank runs in the retention time for the taxifolin (n=13). The standard deviation was calculated, then multiplied by 3 and 10, respectively.

2.10.4 Determination of Reproducibility and Repeatability

The intra-day reproducibility was obtained from 7 consecutively runs using the same LC-MS program as described in section 2.9. The inter-day repeatability was obtained by 13 analysis run on different days over one month.

2.11 *In Vitro* Evaluation of Anti-Inflammatory Response of Polyphenol Metabolites in Endothelial Cells

2.11.1 Preparation of Polyphenols for Cell Treatments

Standard stock solutions were prepared for 3-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) and protocatechuic acid (PCA) in ethanol (10, 100 and 500 μM). For treatments

of cells, it was added in the cell medium and assessed in concentrations of 0.1, 1 and 5 μM. The final concentration of ethanol present in cell culture was 0.1% (v/v).

2.11.2 Cell Culture of HMECs

Human microvascular endothelial cells (HMEC-1) were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1.176 g/L sodium bicarbonate, 4.76 g/L HEPES, 10 g/mL EGF and 1 mg/L hydrocortisone (purity >98%), and were maintained at 37ºC in a humidified 5% CO_2 atmosphere. All experiments were performed between cell passages 6 and 11. Treatment of HMEC-1 cells was done in serum-free cell medium with 5.5 mM, the normoglycemic control, or 30 mM glucose concentration, to mimic diabetic condition.

2.11.3 Cell Viability Assay

Cells at 70%-80% of confluence were sub-cultured at $2x10^5$ cells/mL of medium in 96-well plates. HMECs were treated for 24h in serum-free conditions with 5.5 mM or 30 mM glucose concentration. Next, the medium was replaced by fresh medium with glucose where it was added DHPV or PCA to final concentrations of 0.1, 1 and 5 μM and left to incubate for 1, 3 and 6 hours. The control used was 0.1% ethanol. Afterwards, the medium with the polyphenols was removed and substituted with medium with glucose and MTS. The absorbance at λ=492 nm was measured after 1h of incubation in the dark. The results are expressed as percentage change relative to control in 5.5 mM glucose medium.

2.11.4 Enzyme-Linked Immunosorbent Assay for IL-6 and IL-1β

To quantify the cytokines IL-6 and IL-1β (**Figure 7**), HMECs were seeded and allowed to grow to $24x10^4$ cells/well corresponding to 70%-80% confluence in 24-well plates. HMECs were treated in serum-free conditions with 5.5 mM or 30 mM glucose concentration for 24h. Next, to this medium it was added DHPV or PCA to final concentrations of 0.1, 1 and 5 μM and left to incubate for 1, 3 and 6 hours. The control used was 0.1% (v/v) ethanol. Cell supernatant were collected and stored at -20ºC until required.

The ELISA assays were done according to manufacturer's instructions. Briefly, 100 μL of cell supernatant was added in 96-well plates coated with anti-human IL-6 and IL-1β and incubated for 2.5h at room temperature. Each well was then incubated with the detection antibody for 1h followed by incubation with HRP-Streptavidin solution for 45

min at room temperature. Next it was added the substrate reagent, incubated for 30 min in the dark, and in the end, it was added the stop solution. Between each step it was carried out a washing step. The absorbance was measured at λ=450 nm. For each cytokine, a standard curve was done using the same procedure. The results are expressed in pg/mL.

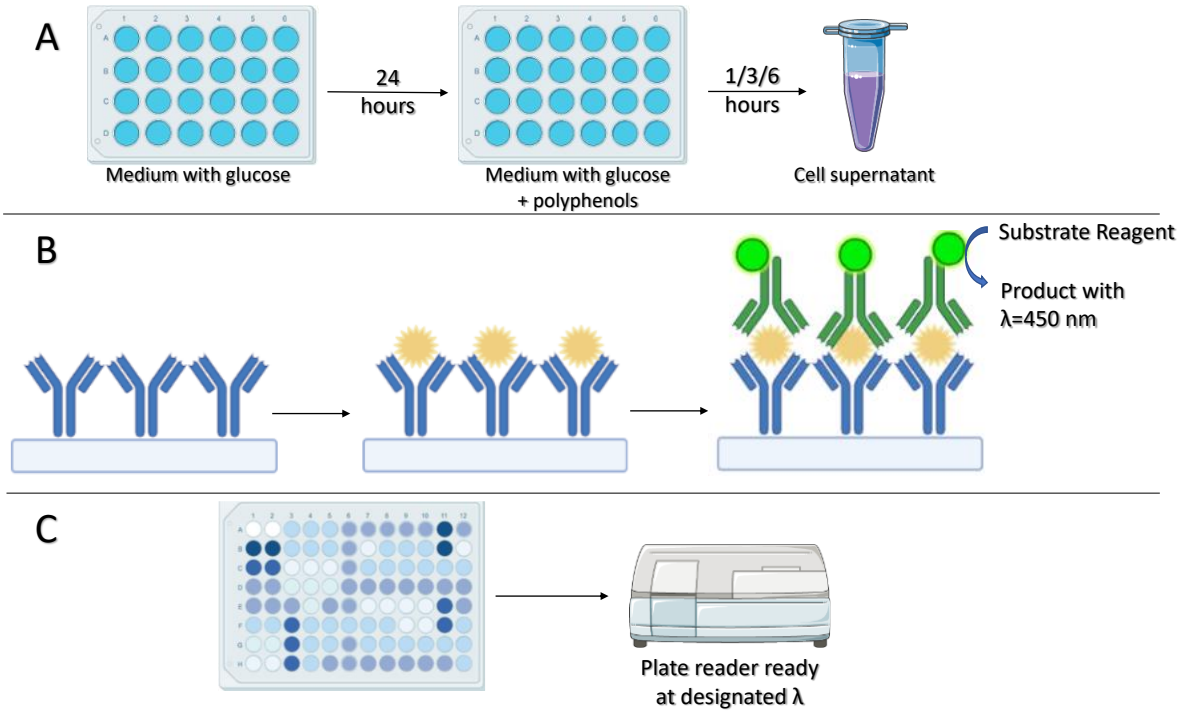


Figure 7: Illustrative example of procedure used for quantification of IL-6 and IL-1β. **A**- After incubation of cells with polyphenols (DHPV and PCA), the supernatant was collect to eppendorfs and stored until further use; **B**- Schematic representation of Sandwich Assay used to quantify the cytokines present in the cell supernatant; **C**- Immediately after using the stop solution in the final step, the absorbance was read at λ=450 nm.

2.11.5 Protein Quantification

The protein quantification of the cell supernatants collected was done using a commercially available BCA Protein Assay Kit, according to manufacturer's instructions, it was prepared a working reagent used to mix with cell supernatant and incubated for 30 min at room temperature. After it was read the absorbance at λ=550 nm.

2.12 Statistical Analysis

All the results are present as Mean ± SEM. For the statistical analysis of the UV/Vis characterization results it was used the One-way ANOVA followed by Kruskal-Wallis test, while for the statistical analysis of cell viability and ELISA assays it was used the One-way ANOVA and Bonferroni test in GraphPad Prism version 8.4.3 (GraphPad Software, USA). Significance was determined at $p<0.05$.

3. RESULTS

3.1. Sample Characterization

One of the aims of the work developed was to study lipoproteins and their role in the transport of polyphenol metabolites. In order to identify the panel of polyphenol metabolites transported in circulation and the effect diabetes mellitus type 2 might have on this transport, the samples received, comprised of triglyceride rich lipoproteins, VLDL and LDL, from three group studies, normoglycemia, hyperglycemia poor control and hyperglycemia good control were characterized.

The lipid composition of the lipoproteins studied, VLDL and LDL, was provided, showing the lipoproteins are mostly composed of triglycerides, esterified cholesterol, free cholesterol, and phospholipids (**Table 2**). The purity of the lipoproteins was verified by SDS-PAGE gel¸ as illustrated in **Figure 8**.

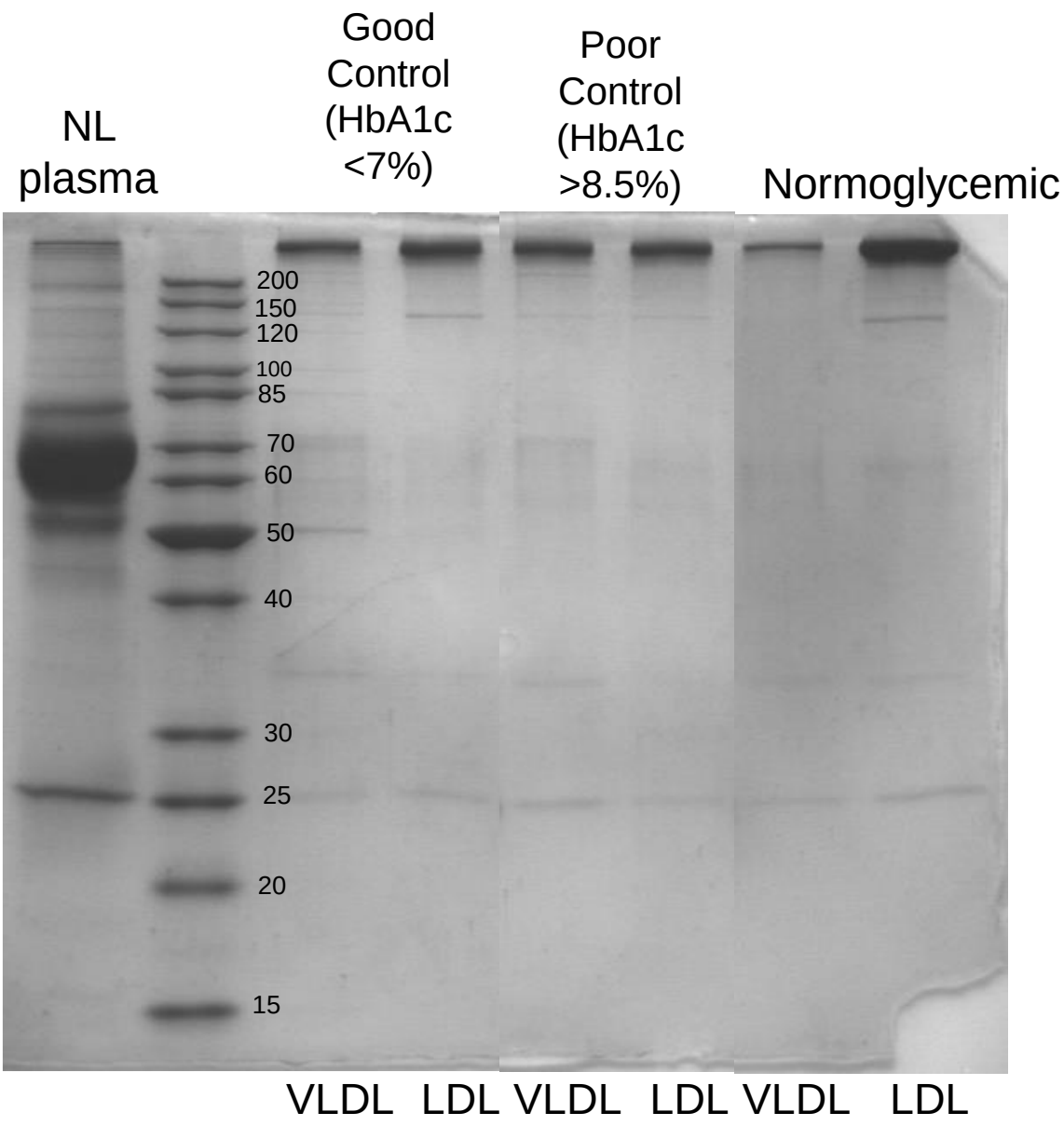


Figure 8: SDS-PAGE gel of lipoprotein fractions from the normoglycemia, hyperglycemia PC and GC study groups.

The SDS-PAGE gel of the VLDL and LDL populations shows a predominant band above the highest protein standard (200 KDa) corresponding to the apolipoprotein B-100 (ApoB-100). This corresponds to the main protein in triglyceride and cholesterol-rich lipoproteins, VLDL and LDL respectively (78, 79). The remaining proteins observed in the SDS gel correspond to other apolipoproteins such as apo-E and apo-C present in these populations which only represent about 5% of total protein (78, 79). The predominance of ApoB-100 protein in the gels confirms that the VLDL and LDL samples were of high purity.

Alongside the purified lipoprotein fractions received, it was also sent lipoprotein-depleted samples (LPDS) by our international collaborator (**Figure 9**).

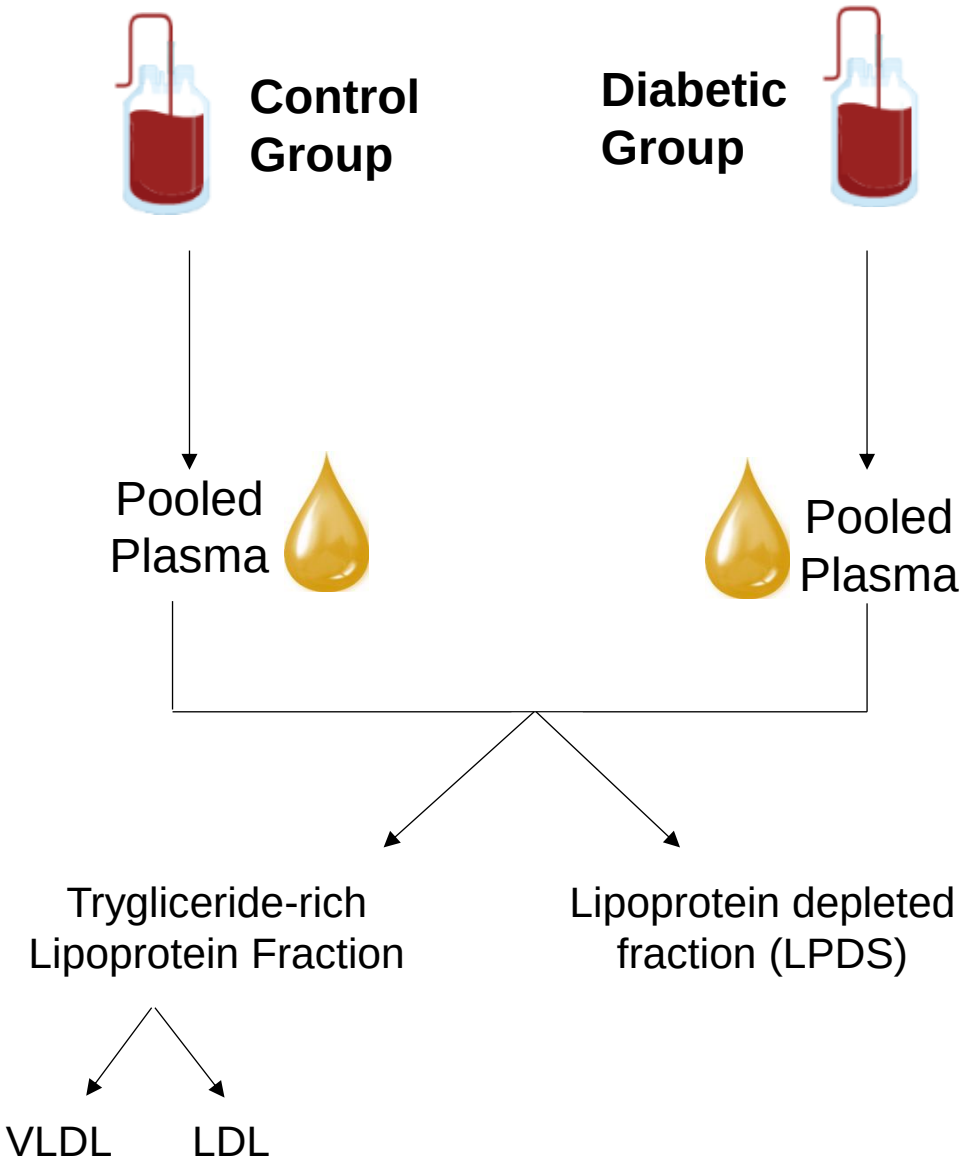


Figure 9: Schematic representation of the fractions received. From the pooled plasma samples, it was isolated and purified fraction of lipoproteins VLDL and LDL. Additionally, it was sent a lipoprotein depleted fraction (LPDS).

The lipoprotein and lipoprotein depleted fractions from the three study groups were characterized according to their protein and total phenolic content, thus enabling the comparative study of the distribution of polyphenol metabolites between both fractions. The concentration of protein in the lipoprotein samples is represented in **Table 4**. The protein content of the LPDS fractions is in **Table 2** of Supplementary Information.

Table 4: Protein concentration determined by Bradford assay in both lipoprotein populations from the normoglycemia, hyperglycemia PC and GC study groups. Results expressed as mg protein/ mL sample (n=3).

	VLDL			LDL		
	NL	**PC**	**GC**	**NL**	**PC**	**GC**

Mean (mg/mL)	0.266	0.552	0.696	1.264	1.751	1.737
SEM	0.034	0.040	0.039	0.061	0.129	0.006
CV (%)	22.1	12.7	9.69	8.35	12.7	0.574

The results show from both lipoprotein populations LDL presents the highest protein content. It also revealed the hyperglycemia condition, before and after drug and diet treatment, increased the protein content. Nonetheless, the LPDS fractions contained the highest protein content in comparison to both lipoprotein fractions and hyperglycemia induced a reduction of about 28% in the protein content.

Figure 10 shows the distribution of the polyphenol metabolites in both fractions in normoglycemia.

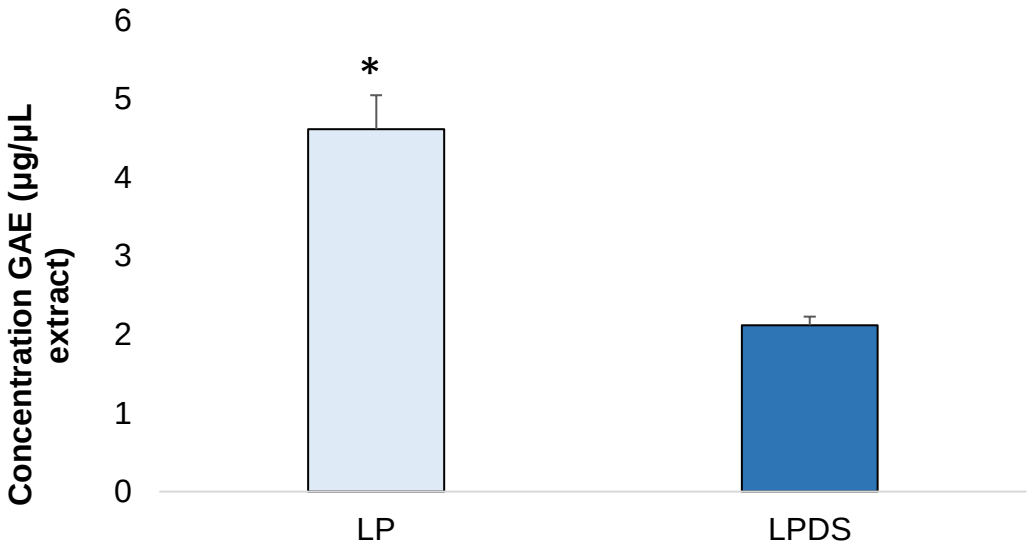


Figure 10: Distribution of total phenolic content in lipoprotein (LP) and lipoprotein-depleted sample (LPDS) in normoglycemia. *The lipoprotein fraction does not contain HDL, it is only the sum of VLDL and LDL. Results expressed as µg of GAE/µL of extract. Data shown as Mean + SEM (n=3).

Based on the results obtained, it is possible to notice how the lipoproteins are the main transporters of these compounds in plasma. Nonetheless, both fractions have different protein content (**Table 4**), so it was normalized to have this in consideration, represented in **Figure 11**.

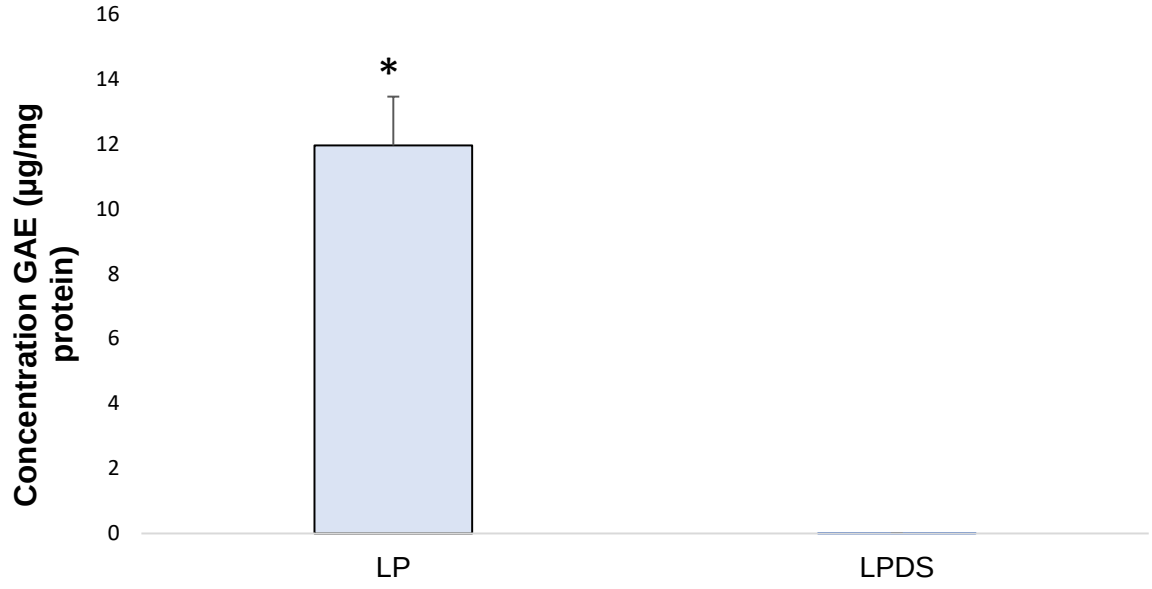


Figure 11: Distribution of total phenolic content in lipoprotein (LP) and lipoprotein-depleted sample (LPDS) in normoglycemia. *The lipoprotein fraction does not contain HDL, it is only the sum of VLDL and LDL. Results expressed as µg of GAE/mg of protein. Data shown as Mean + SEM (n=3).

The results show more clearly the main carriers of polyphenols are the triglyceride and cholesterol-rich lipoproteins in a normoglycemia situation.

Since the LPDS samples were also available from diabetic patients, it was compared the total phenolic content from both fractions in a hyperglycemia situation in order to see if the disease affected the transport of the polyphenol metabolites (**Figure 12**).

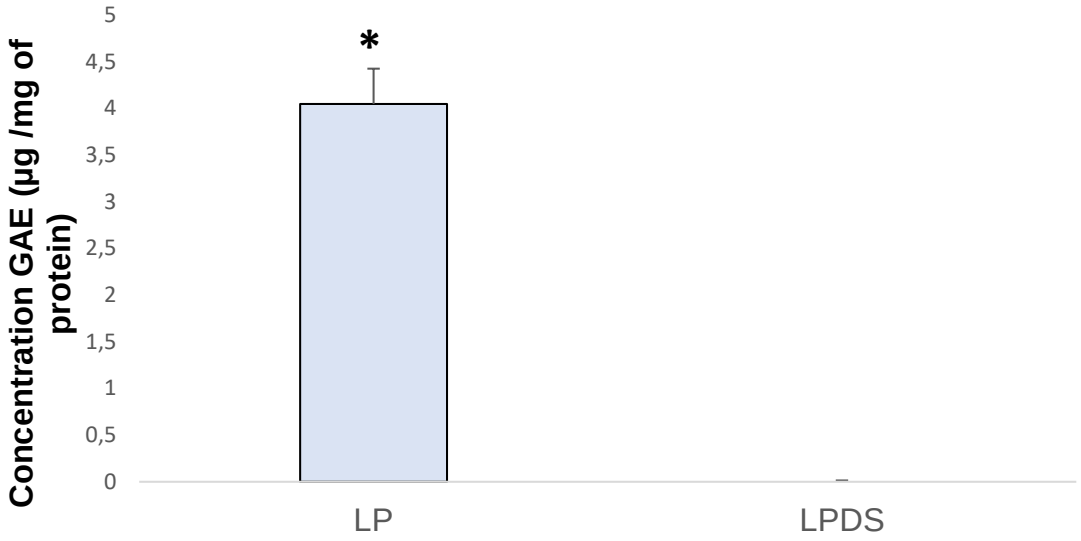


Figure 12: Distribution of total phenolic content in lipoprotein (LP) and lipoprotein-depleted sample (LPDS) in hyperglycemia. *The lipoprotein fraction does not contain HDL, it is only the sum of VLDL and LDL. Results expressed as µg of GAE/mg of protein. Data shown as Mean + SEM (n=3).

The profile between the LP and LPDS fractions for normoglycemia was mirrored in hyperglycemia conditions, as seen in the results obtained. This proves the VLDL and LDL lipoproteins are the main carriers of polyphenol metabolites and this distribution maintains in a situation of Diabetes Mellitus type 2.

These results overall reinforce the focus of this work in the VLDL and LDL lipoprotein populations.

3.2. Evaluation of Extraction Methods

Considering the numerous extraction methods used in the extraction of polyphenol metabolites reported in the literature, ranging from solid phase extraction (SPE) to liquid-liquid extraction (80-85), it was assessed the difference between both methods for the extraction of polyphenol from biological samples.

For this, three extraction methods were studied in plasma samples, namely LLE and SPE using two different cartridges (Oasis HLB from Waters and HybridSPE-Phospholipid from Supelco). The methods were evaluated based on two criteria namely on the ability to extract polyphenols without extracting other metabolites (for example, lipids). For this, the plasma extracts were characterized by spectrophotometric methods by measuring total phenolics content and phospholipids (PL), represented in **Figure 13**.

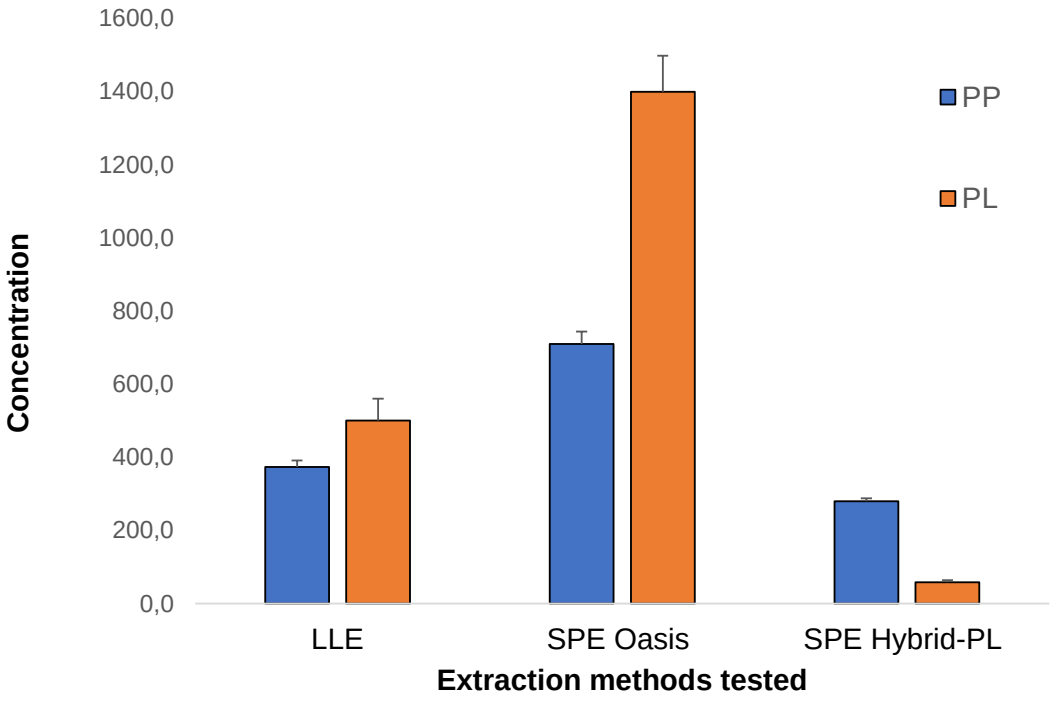


Figure 13: Comparison between three extraction methods employed in literature for polyphenol metabolites extraction (n=3).

The results of total phenolic content are expressed as µg of GAE/ µL of sample while the results for the quantification of PL is in ng of phospholipids/ µL of sample. Based on the results, it is possible to observe how the Oasis HLB cartridge can extract more polyphenols than the remaining methods. However, the Oasis HLB cartridge extracted more phospholipids in comparison to the remaining methods. LLE and the HybridSPE-Phospholipid cartridge extracted about 50% of the polyphenol methods when compared to the Oasis HLB cartridge. At the same time, it is interesting to notice how the HybridSPE-Phospholipid cartridge despite being the one that extracted less polyphenols overall it was the only method able to extract more polyphenols than phospholipids.

Based on these results, the HybridSPE-Phospholipid cartridge was selected as the extraction method for the preparation of the lipoprotein samples.

3.3. Evaluation of extraction performance of HybridSPE-Phospholipid cartridge

Based on the results of the evaluation of the extraction methods and the selection of the HybridSPE-Phospholipid cartridge for the preparation of the lipoprotein samples, it was tested the recovery percentage for this method.

For this purpose, an internal standard was chosen for the samples that is not a compound naturally present in biological samples. In this case it was chosen taxifolin, a dihydroquercetin with a structure similar to polyphenols and is also a commercially available polyphenol (**Figure 14**).

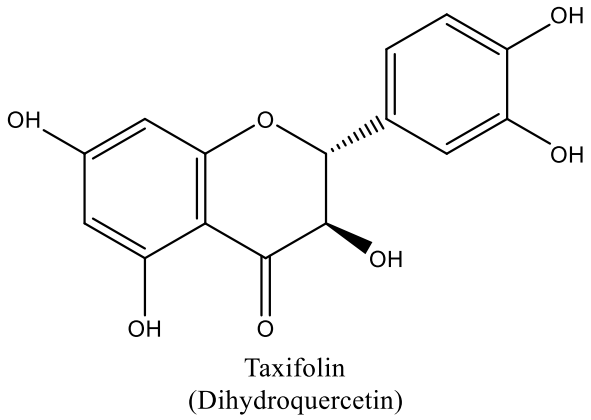


Figure 14: Structure of taxifolin (dihydroquercetin).

For the evaluation of the extraction method chosen, it was built a calibration curve for taxifolin (**Figure 15**), followed by determination of the LOD, LOQ, intra-day and inter-day variability to confirm the LC-MS method employed was detecting and quantifying the IS in the extracts.

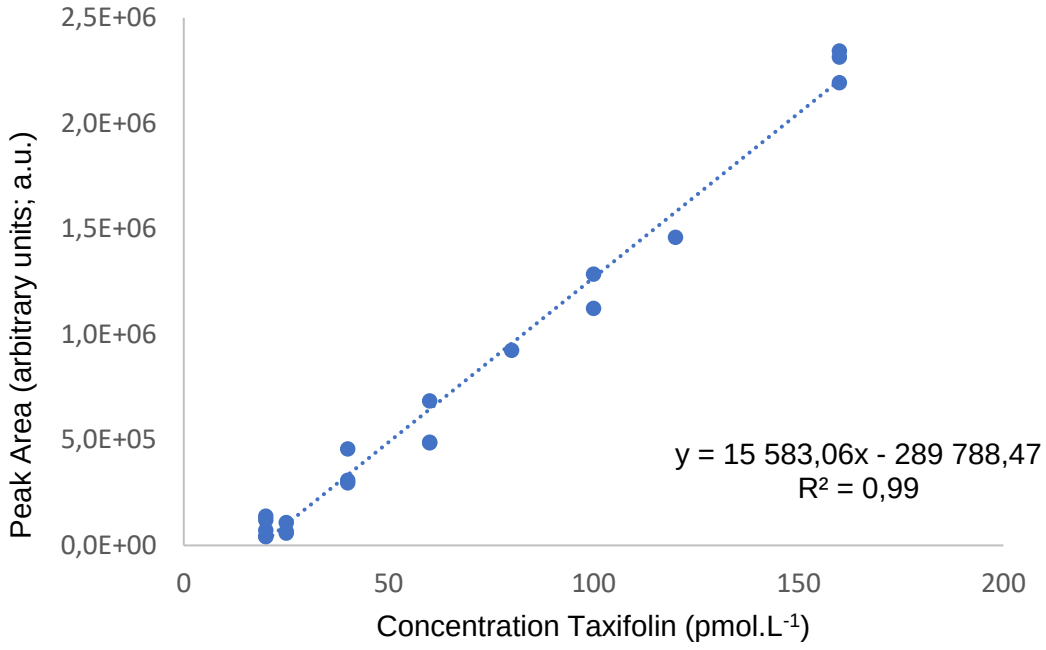


Figure 15: Calibration Curve obtained by LC-MS for taxifolin used for the quantification of the polyphenol metabolites in the lipoprotein extracts.

The LOD and LOQ for taxifolin was 20.1 nM and 24.6 nM, respectively. The intra-day variability was 10.4% and the inter-day variability was 58.9%. These results show the LC-MS method employed for the determination of the extraction recovery is reproducible and has good sensibility for low concentrations.

The recovery percentage of the extraction method performed was 110 ± 3%, demonstrating the HybridSPE-Phospholipid cartridge has a high recovery capacity for polyphenol metabolites.

3.4. Characterization of Lipoprotein Sample Extracts by Spectrophotometric Methods

The characterization of the lipoprotein extracts was done by spectrophotometric methods in the quantification of polyphenols and flavonoids, to evaluating the radical scavenging activity (DPPH assay) and antioxidant ability of the extracts (FRAP assay).

The total polyphenolic content was determined by the Folin-Ciocalteu method (73). The results obtained are represented in **Figure 16.**

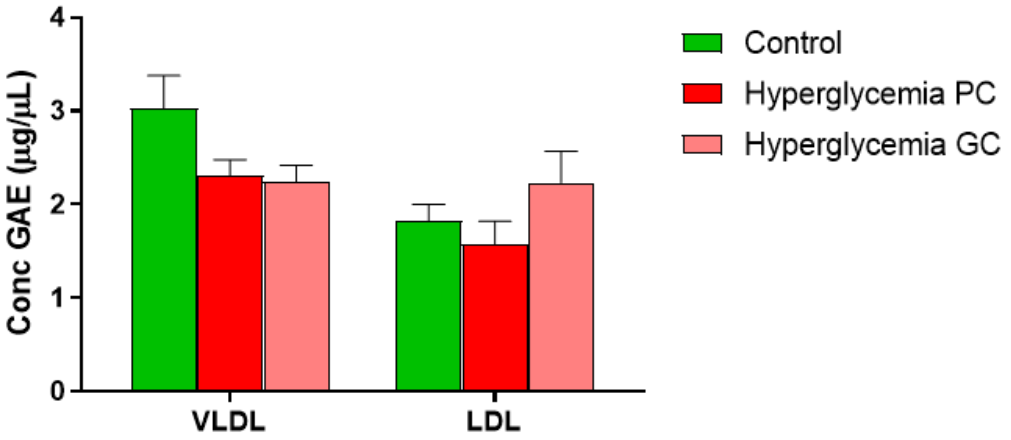


Figure 16: Total Phenolic Content determined by Folin-Ciocalteu method in both lipoprotein population extracts in normo and hyperglycemia study groups. Results expressed as µg of GAE/µL of sample. Data shown as Mean + SEM (n=3).

The results show both lipoprotein populations transport polyphenols, with VLDL presenting higher phenolic content than LDL. Diabetes lead to a reduction in the content of polyphenols, specially marked in the VLDL population. This reduction was ameliorated by drugs, exercise, and diet change in the LDL population.

However after taking into account the high differences in protein content (**Table 4**), the results were normalized to µg of GAE/mg of protein (**Figure 17**)**.**

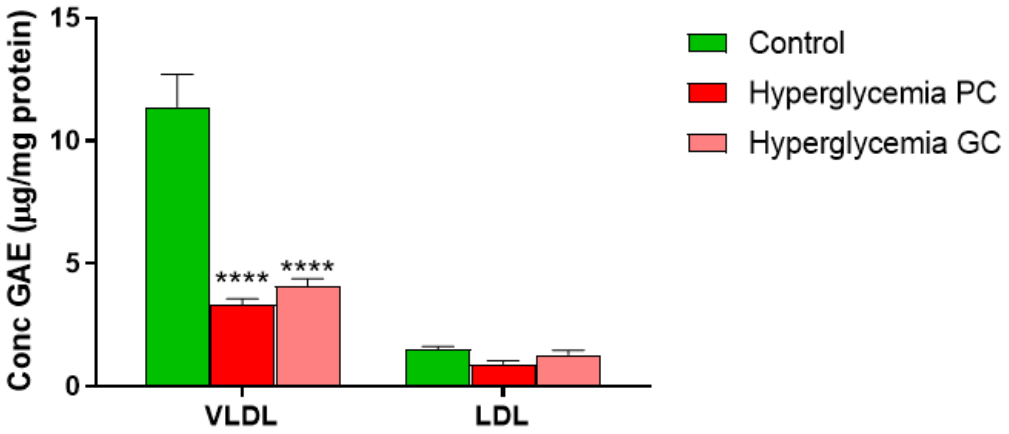


Figure 17: Total Phenolic Content determined by Folin-Ciocalteu method in both lipoprotein population extracts in normo and hyperglycemia study groups. Results expressed as µg of GAE/mg of protein in sample. Data shown as Mean + SEM (n=3). Significant difference relative to VLDL Control are expressed as ****p <0.0001.

VLDL population has a higher content in polyphenols when compared to the LDL population. There is a marked reduction of 70% on the total phenolic content in hyperglycemia PC. The data obtained also showed that treatment with drugs, exercise

and diet change did not lead to a statistically significant improvement in total phenolic content.

The total phenolic content is mirrored in the total flavonoid content in VLDL population visible in **Figure 18**.

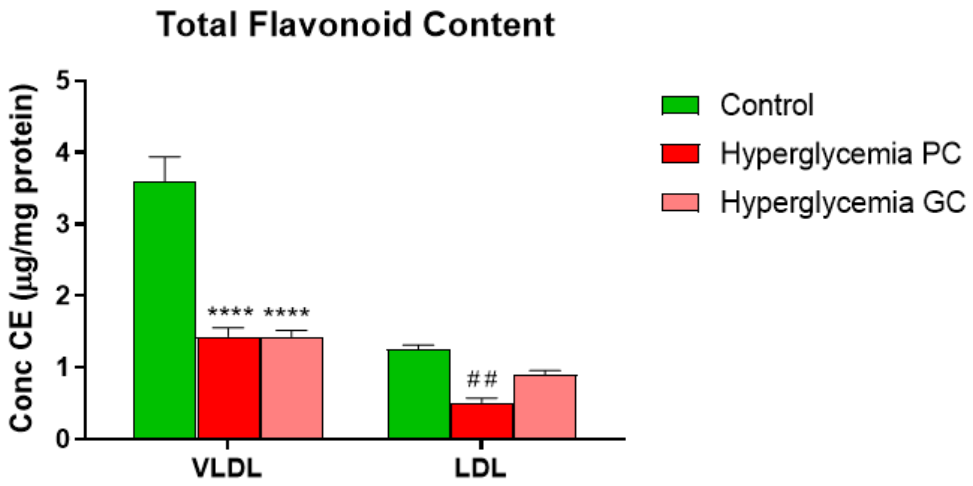


Figure 18: Total Flavonoid Content determined by Aluminum Chloride method in both lipoprotein population extracts. Results expressed as µg of CE/mg of protein in sample. Data shown as Mean + SEM (n=3). Significant difference relative to VLDL Control are expressed as ****p<0.001; Significant difference relative to LDL control are expressed as $^{\#\#}p$<0.01.

The VLDL population shows a higher flavonoid content than the LDL population and hyperglycemia generated a significant reduction (~60%). Hyperglycemia GC does not show improvement of the flavonoid content in the VLDL extract samples. Unlike the total phenolic content, the LDL population in this case shows the same marked reduction in total flavonoid content in case of hyperglycemia PC (~60%). Nonetheless, treatment with drugs, exercise and diet change resulted in an increase of flavonoid content to values similar to control group.

The radical scavenging activity was evaluated by the DPPH assay in the lipoprotein extracts. The results are visible in **Figure 19**.

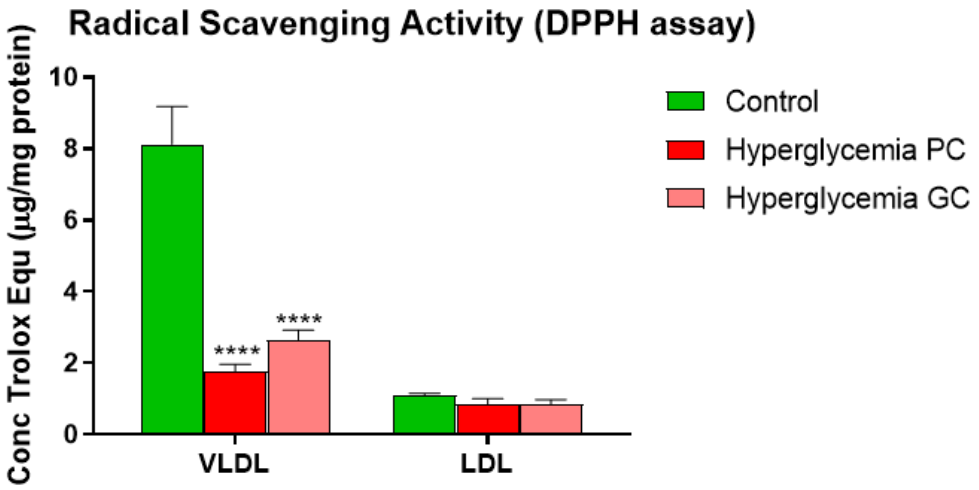


Figure 19: Radical Scavenging Activity determined by DPPH assay in both lipoprotein extracts in normo and hyperglycemia study groups. Results expressed as µg of TE/mg of protein in sample. Data shown as Mean + SEM (n=3). Significant difference relative to VLDL Control are expressed as ****p<0.0001.

Data obtained demonstrated VLDL population has higher radical scavenging activity than the LDL population, specially the control group. Hyperglycemia induced a reduction that only affected the VLDL population. This reduction was about 80% in hyperglycemia PC and 70% in hyperglycemia GC, demonstrating diet change, exercise and drugs did not improve the radical scavenging activity.

The results for the DPPH assay were mirrored in the measurement of antioxidant capacity of the extracts, in **Figure 20.**

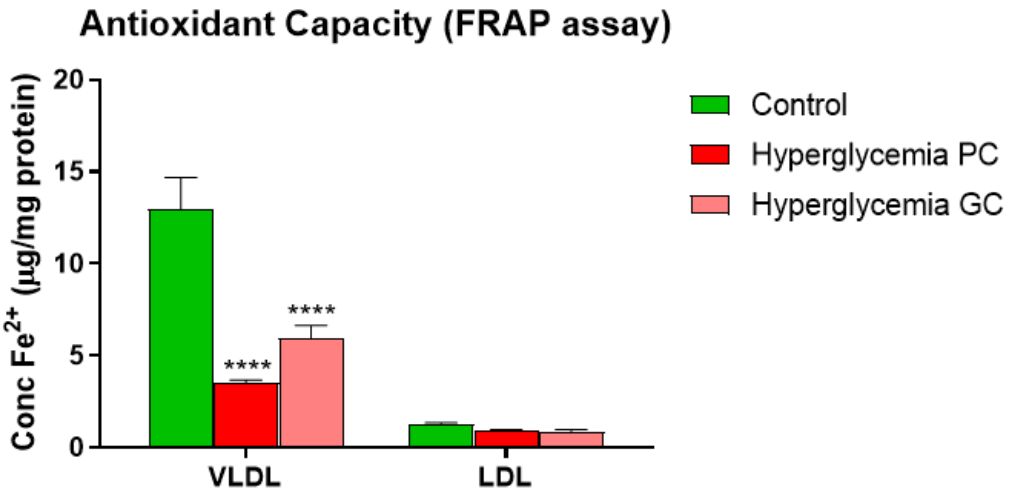


Figure 20: Antioxidant Capacity determined by FRAP assay in both lipoprotein extracts in normo and hyperglycemia study groups. Results expressed as µg of Fe^{2+}/mg of protein in sample. Data shown as Mean + SEM (n=3). Significant difference relative to VLDL Control are expressed as ****p<0.0001.

The results obtained in the FRAP assay show the VLDL extracts have higher antioxidant capacity than LDL extracts. Diabetes lead to a pronounced reduction (~70%)

in VLDL with no effect in the LDL extracts. Treatment with drugs, exercise and diet still was not able to recuperate the antioxidant capacity, with a reduction of about 55%.

3.5. Identification of Polyphenol Metabolites by Reverse-Phase LC-MS

Using spectrophotometric methods, it was determined the presence of polyphenol metabolites in the lipoprotein extracts. Based on these results, it was characterized the polyphenol metabolites content in the lipoprotein sample extracts by LC-MS, starting with the identification of possible metabolites in both VLDL and LDL extracts from all study groups.

The elution of the lipoprotein sample extracts by reverse-phase chromatography result in a typical LC-MS chromatogram, as exemplified in **Figure 21**.

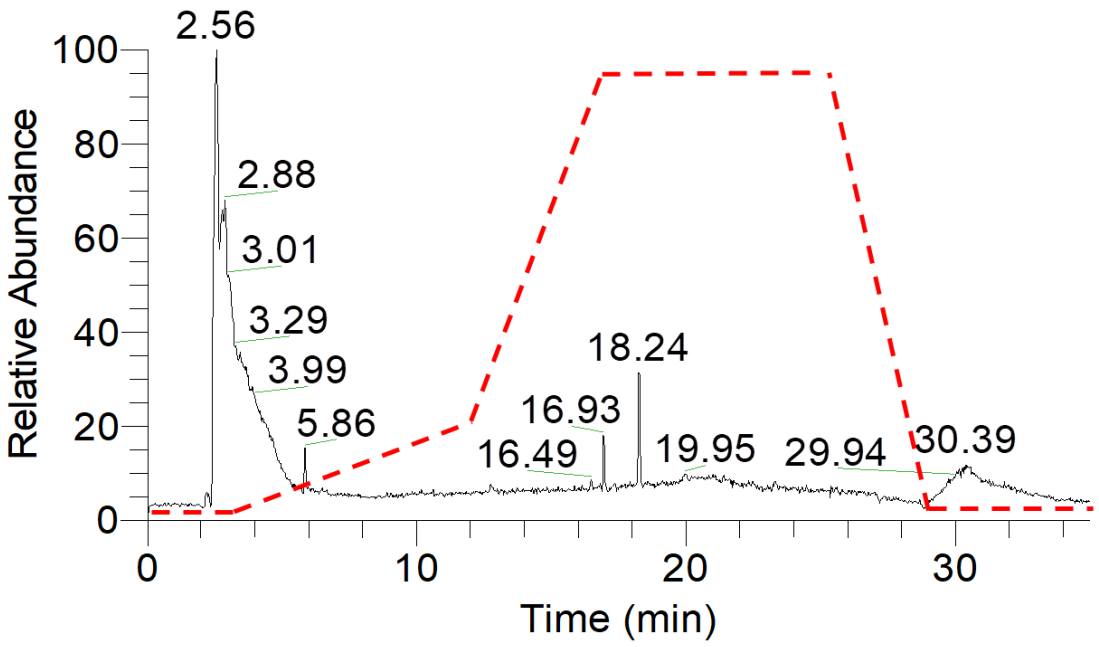


Figure 21: LC-MS chromatogram of lipoprotein extracts depicting the elution program (dotted line).

To determine the reproducibility of the detection in the various injections runs, taxifolin was spiked in all lipoprotein extracts and used as an internal standard (IS). The identification of polyphenol metabolites was achieved based on the monoisotopic mass observed for the polyphenol metabolite. The reproducibility of the elution times observed for taxifolin was high with a variability (%CV) of 0.091, while the error in detection of taxifolin ion (m/z 303.0510) was 4.48 ppm. Hence, all ions potentially attributed to polyphenol metabolites with an error below 5 ppm were accepted.

The panel of potential polyphenol metabolites identified in the lipoprotein extracts based on accurate mass measurements are present in **Table 5**.

Table 5: Polyphenol metabolites identified in the lipoprotein extracts. Line (-) indicates the metabolite was not found in the HMDB database.

Monoisotopic mass (m/z)	Retention time (min)	Metabolite identified	HMDB	Lipoprotein Population
179.0488	13.45	Caffeic Acid	HMDB0001964	VLDL
209.0950	9.04	5-(3', 4'-Dihydroxyphenyl)-valeric acid	HMDB0029233	VLDL
233.9842	6.43	Protocatechuic Acid Sulfate	HMDB0240382	LDL
261.0122	5.36	3-(2,4-Dihydroxyphenyl)propionic acid Sulfate	-	VLDL/LDL
369.0364	10.24	(-)-Epicatechin Sulfate	HMDB0012467	VLDL/LDL
477.0224	5.86	3-Hydroxy-2-[4-methoxy-3-(sulfooxy)phenyl]-7-sulfino-3,4-dihydro-2H-1-benzopyran-5-olate	HMDB0127897	LDL
479.0223	5.86	5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone-O-glucuronide-O-sulphate	-	LDL

Most of the proposed identified polyphenol metabolites can be attributed to sulfate conjugates of native polyphenols or of gut metabolites and exhibit a retention time lower than taxifolin (rt= 17.45 min) since polyphenol metabolites are more polar compounds, they will elute early in a reverse-phase column.

To improve the identification of the potential polyphenol metabolite, it was done a search of the same monoisotopic mass in the blank runs to confirm it was not derived from the system. For example, in **Figure 22**, it is represented the extracted ion chromatogram (XIC) of the ion with the m/z 369.0364.

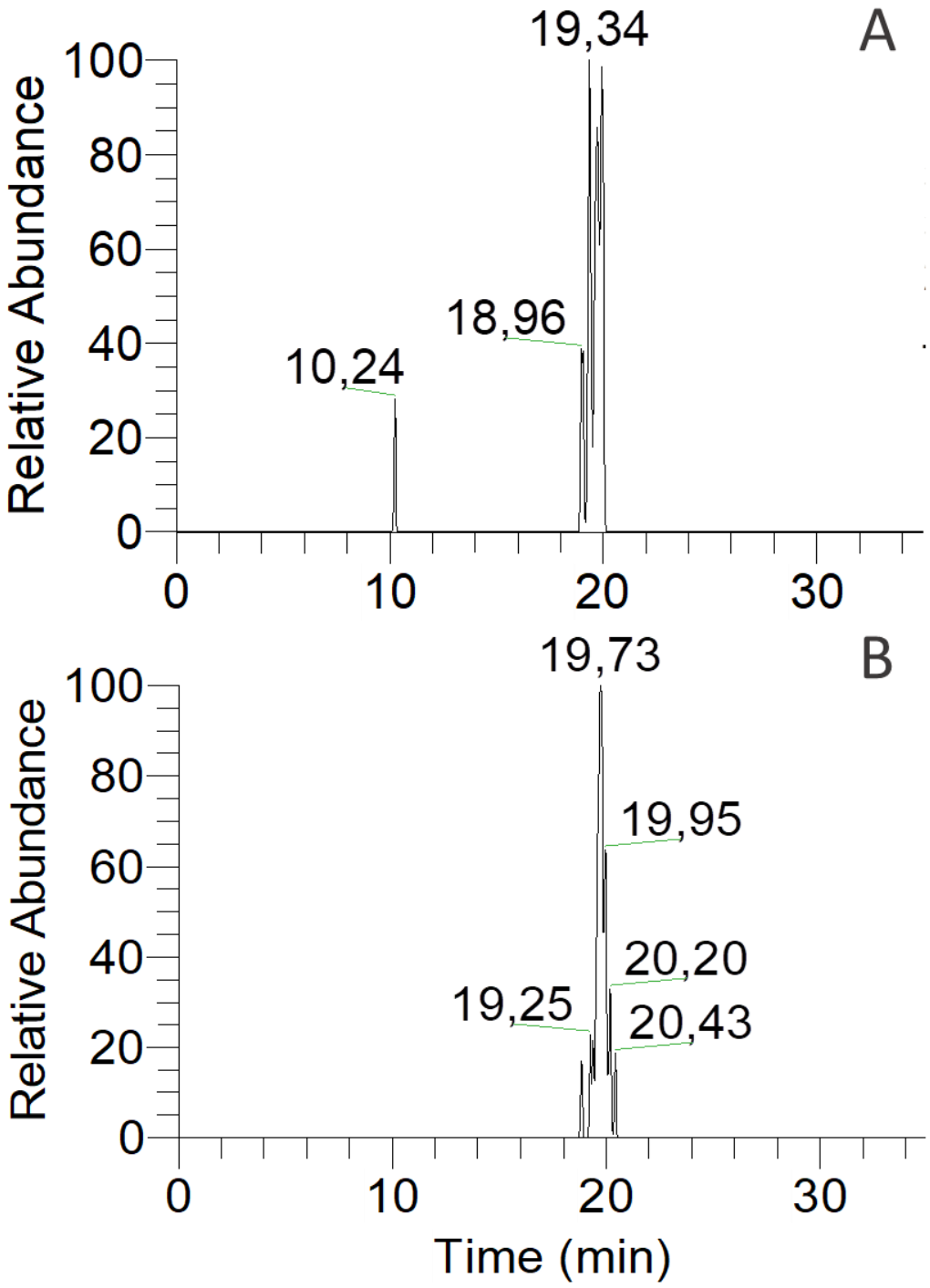


Figure 22: LC-MS chromatogram depicting the elution bands for the ion with m/z 369.0364, proposed as (-)-Epicatechin Sulfate. **A-** Chromatogram in a VLDL normoglycemia run; **B-** Chromatogram in a blank run.

The retention time for the ion with m/z 369, 0394 shows an elution time at 10.24 and 19.34 min in a normoglycemia VLDL sample extract (**Figure 22A**). In comparison, the XIC for the same m/z in the blank run (**Figure 22B**) shows only the elution at 20.01 min, confirming that the m/z 369.0394 at the retention time of 10.24 min was a potential polyphenol metabolite from the samples.

3.6. Quantification of Polyphenol Metabolites

Several polyphenol metabolites were identified in the lipoprotein extracts (**Table 5**) though many more are reported in literature (86-90). Despite this, polyphenol metabolites were tentatively quantified namely, protocatechuic acid (PCA) and 3-(2,4-dihydroxyphenyl)propionic acid (DHPPA) using a SIM approach.

The quantification of the polyphenol metabolites was done against a calibration curve for each metabolite standard. Due to the lack of commercially available standard of conjugated polyphenol metabolites, protocatechuic acid sulfate (PCA-Sulfate) and 3-(2,4-dihydroxyphenyl)propionic acid sulfate (DHPPA Sulfate) were also monitored at the m/z value expected in SIM mode and their concentrations estimated using the respective calibration curve.

The calibration curve of PCA is represented in **Figure 23A**. The calibration curve of DHPPA is depicted in **Figure 23B**.

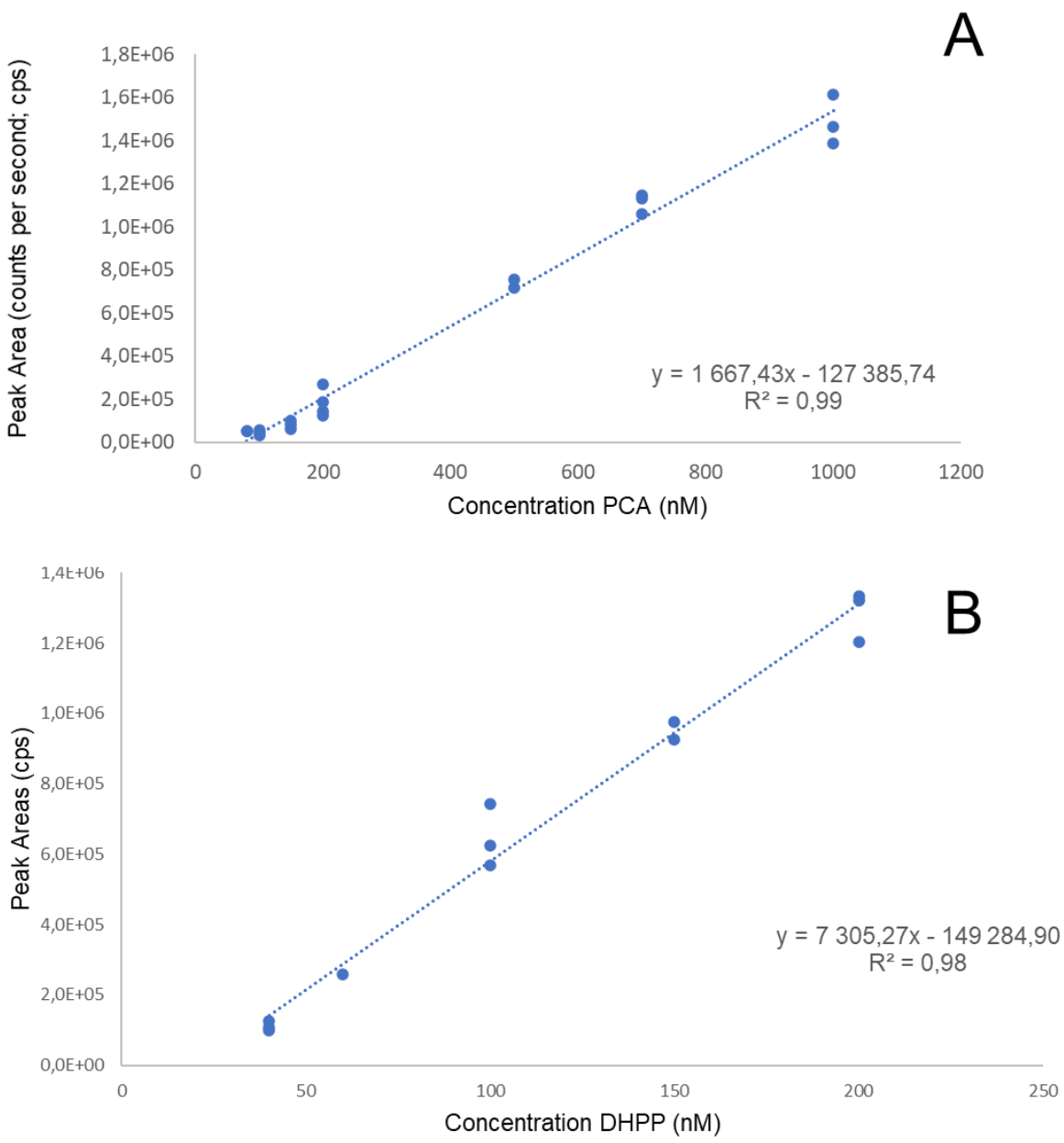


Figure 23: Calibration curves obtained by LC-MS for polyphenol metabolites. **A-** Calibration curve of PCA; **B-** Calibration curve of DHPPA

Using 14 blank runs (n=14) the LOD and LOQ of each standard was calculated to estimate the sensitivity of the method. For the standard PCA the LOD obtained was 82.47 nM and the LOQ was 96.64 nM. For DHPPA, the calculated LOD was 37.49 nM and the LOQ was 56.44 nM.

The concentration of the polyphenol metabolite PCA-Sulfate is portrayed in **Figure 24**.

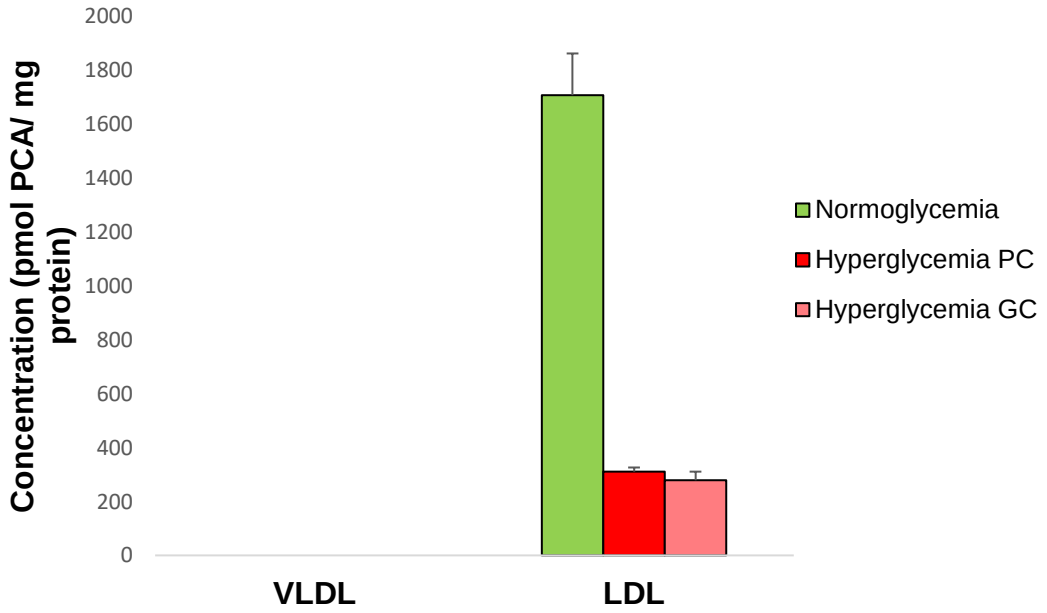


Figure 24: Concentration of PCA-Sulfate determined by LC-MS in lipoprotein sample extracts. Results expressed as pmol PCA/mg of protein in sample. Data shown as Mean + SEM (n=3).

The monitorization of PCA and PCA-sulfate showed only the sulfate conjugated metabolite was present in the lipoprotein extracts. In addition, the conjugated derivative only appeared in the LDL sample extracts, not in the VLDL extracts. The diabetic state induced a reduction (<63%) in the estimated amount of PCA-sulfate. Exercise, diet change and drugs did not recuperate the values closer to normoglycemia.

Data obtained for the polyphenol metabolite DHPPA-Sulfate is shown in **Figure 25**.

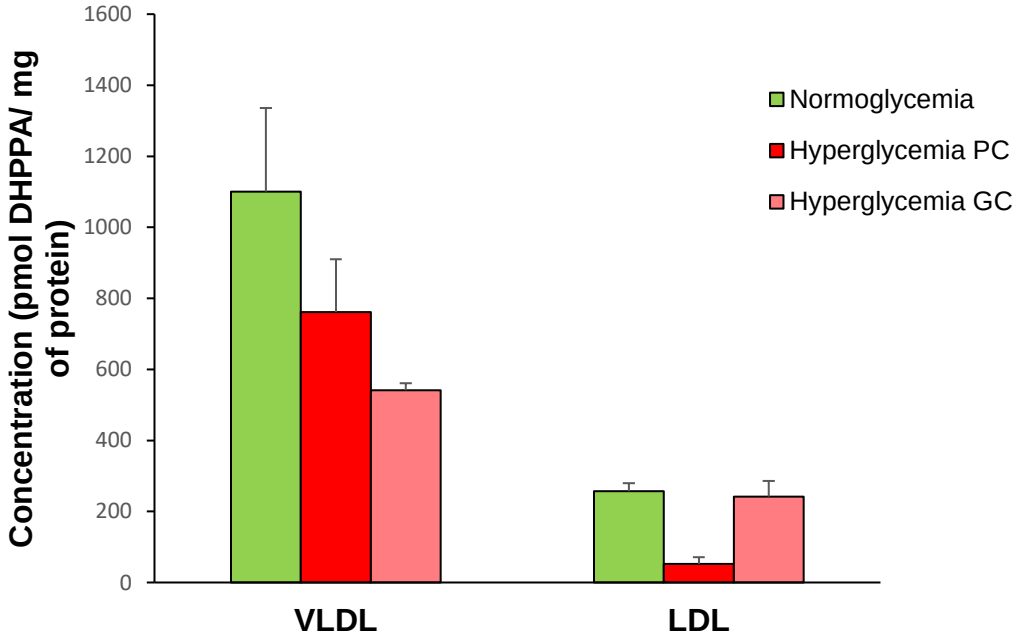


Figure 25: Concentration of DHPPA-Sulfate determined by LC-MS in lipoprotein sample extracts. Results expressed as pmol PCA/mg of protein in sample. Data shown as Mean + SEM (n=3).

The monitorization of the DHPPA and DHPPA sulfate indicated the presence of the sulfate conjugate only, in both lipoprotein sample extracts. VLDL sample extracts show the highest estimated amount of DHPPA-sulfate in comparison to the LDL sample extracts. The hyperglycemia condition induced a reduction in the amount of DHPPA-sulfate in both VLDL (<32%) and LDL samples (<77%). Management of diabetes (hyperglycemia GC) was able to improve the estimated concentration of DHPPA-sulfate in LDL samples, however this behavior was not observed for the VLDL samples.

3.7. Effect of Polyphenol Metabolites on Endothelial Inflammatory Response

3.7.1. Cell Viability

The chronic state of inflammation characteristic of diabetes mellitus type 2 with continuous release of inflammatory cytokines such as IL-6 and IL-1β was studied by ELISA assays (17, 91).

The anti-inflammatory response of the polyphenol metabolites 3-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) and protocatechuic acid (PCA) was evaluated since they are polyphenol metabolites reported in literature and release in circulation at

low concentrations (nM) in plasma (25, 49, 57, 58). The concentrations chosen, based on this literature, consisted of 0.1, 1 and 5 μM.

Human microvascular cells were incubated at two different glucose conditions, normoglycemia (5.5 mM glucose) and hyperglycemia (30 mM glucose), before exposure to polyphenols for 1, 3 and 6 hours at 0.1, 1 and 5 μM. **Figure 26** shows the results for cell viability after the 6h time point with DHPV and PCA.

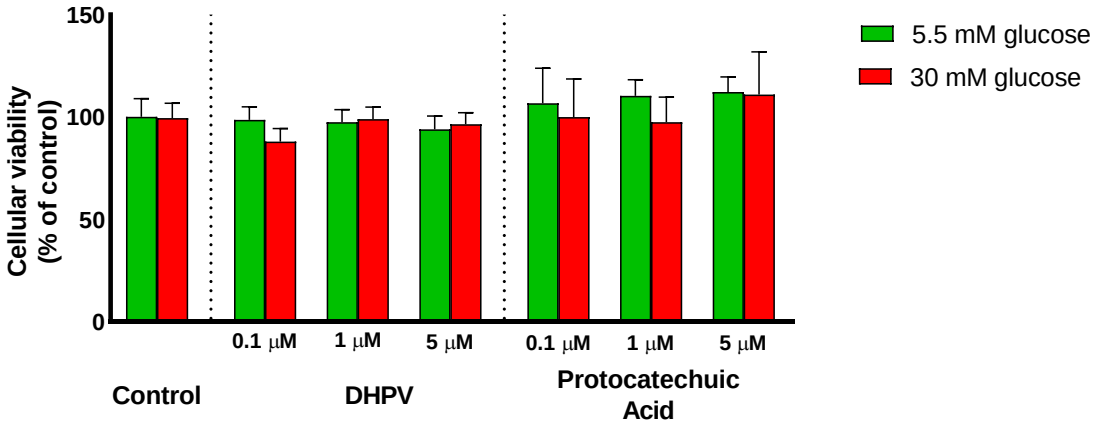


Figure 26: Comparison of cytotoxicity ability of DHPV and PCA. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), cells were exposed to polyphenol metabolites (0.1, 1 and 5 μM) for 6h. Cell viability was measured by MTS assay. Data expressed as Mean percentage ± SEM (n=3).

The results show the polyphenol metabolites did not compromise cell viability. As such it was maintained the same concentrations and incubation times in the evaluation of the anti-inflammatory effects of these polyphenol metabolites in endothelial cells.

3.7.2. Polyphenol Metabolites Effect on the Inflammatory Response

The anti-inflammatory ability of both metabolites was evaluated by measuring the IL-6 and IL-1β profile in cell media after exposure to normoglycemia or hyperglycemia for 24h, to mimic diabetes and as a stimulus to inflammation.

The data obtained (**Figure** , **Figure 4**, **Figure 5** and **Figure 6** in Supplementary Information) showed there was not a time-dependent effect until the 6 hour time point for both polyphenol metabolites. Based on these results it was selected to show only the 3h point for both metabolites. In addition to the data attained, it is also the amount of time reported in literature for DHPV and PCA to be in circulation (57).

The results regarding the release of IL-6 are presented in **Figure 27.**

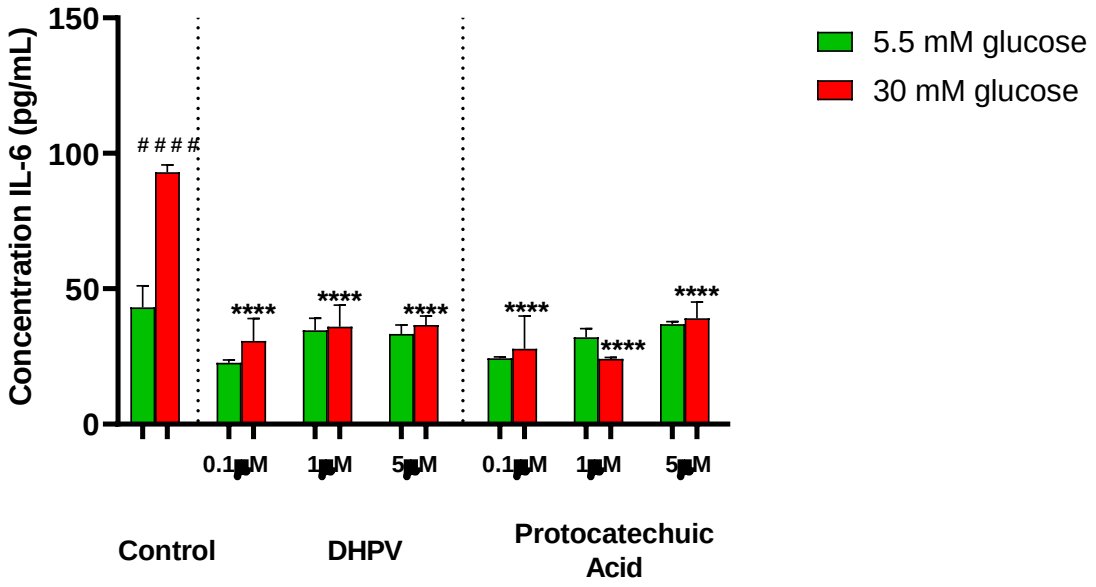


Figure 27: Effect of DHPV and PCA in the release of IL-6 in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 3h. Subsequently the cell supernatant was collected, and the production of IL-6 was assessed by ELISA. Data expressed as Mean ± SEM (n=2). Significant difference relative to 5.5 mM glucose control conditions are expressed as $^{\#\#\#\#}p<0.0001$; Significant difference relative to 30 mM glucose control conditions are expressed as ****p <0.0001.

Hyperglycemia induced a significant increase (~46%) in the release of IL-6 in comparison to the normoglycemia control. Both polyphenol metabolites reduced glucose-stimulated release of IL-6 by up to 40% relative to the cells treated with 30 mM glucose. This reduction was observed for all tested concentrations.

Figure 28 depicts the release of IL-1β and the influence of the polyphenol metabolites.

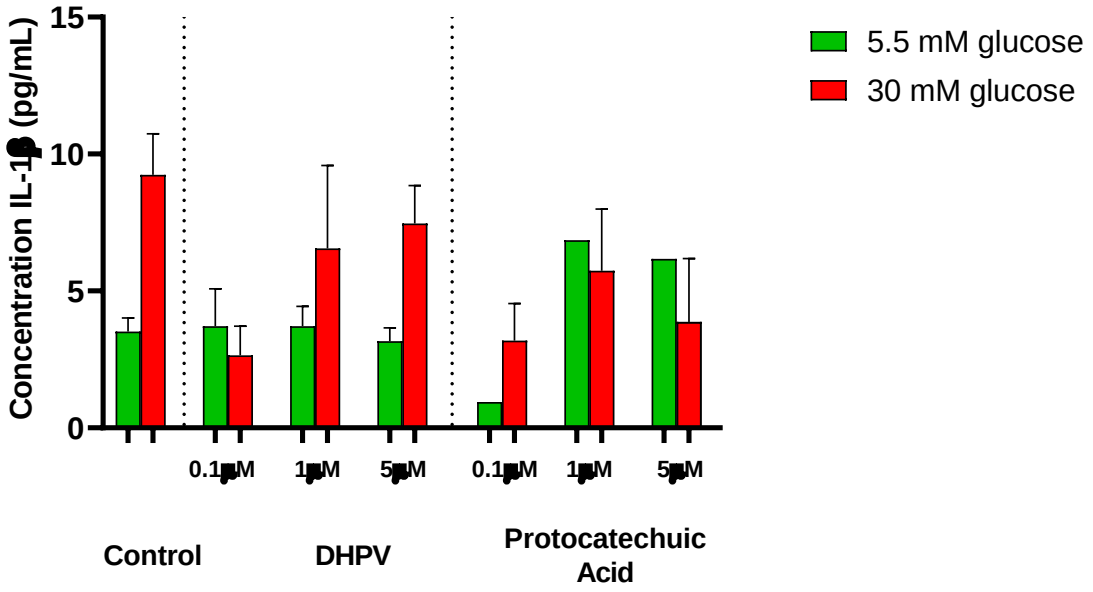


Figure 28: Effect of DHPV and PCA in the release of IL-1β in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 3h. Subsequently the cell supernatant was collected, and the production of IL-1β was assessed by ELISA. Data expressed as Mean ± SEM (n=2).

The results for the release of IL-1β did not show significance in the increase produced by hyperglycemia relative to the normoglycemia control. DHPV and protocatechuic acid also did not show significance in reducing the concentration of IL-1β.

4. DISCUSSION

The World Health Organization recommends the adoption of diets rich in fresh fruits, vegetables and nuts found in Mediterranean and Nordic countries to improve and ameliorate endothelial dysfunction and help manage diabetes mellitus (19, 20). Fresh fruit, vegetables and nuts are rich in polyphenol with potential beneficial health effects in chronic diet-related diseases like diabetes and cardiovascular diseases (9, 21, 23, 25, 92). However, once ingested polyphenols are extensively metabolized and have low bioavailability thus their effects in human health are still not fully understood (49, 57, 93). To date, most cellular-based works focus on the effects of dietary polyphenols in endothelial cells rather than the polyphenol metabolites in circulation (9, 94, 95). In addition, the work done for polyphenols or polyphenol metabolites employ supraphysiological concentrations, the time polyphenol metabolites are in circulation is not taken in consideration in the time of incubation used in cellular works, and most works do not recreate the pathological situation they are studying, for example, hyperglycemia to study the anti-diabetic effects. Finally, cellular work using polyphenols do not consider chemical degradation in cell medium and how the effects described for polyphenols might be derived from the degradation products. Concomitantly, the transport of polyphenol metabolites in circulation is still not completely explained raising the question if it is done by low or high molecular weight proteins. Ultimately, the question whether the full extent of how the Mediterranean diets can improve endothelial function in diabetes needs to be studied.

In order to improve the knowledge on the impact of diabetes on the transport profile and cargo of circulating polyphenol metabolites and the effect of these polyphenol metabolites in endothelial function, this work characterized triglyceride and cholesterol-rich lipoprotein populations from healthy and diabetic groups using spectrophotometric methods and high-resolution mass spectrometry coupled with reverse-phase chromatography (UPLC-MS) and studied the impact of polyphenol metabolites on the inflammatory response in cultured endothelial cells.

The estimated total phenolic content of VLDL and LDL lipoprotein fractions (LP) and lipoprotein depleted samples (LPDS) in normoglycemia (**Figure 10**) revealed a heterogeneous distribution of polyphenols in circulation, between high- and low-molecular weight proteins. However, when the absolute values of each fraction were normalized to protein content it showed VLDL and LDL populations were the main transporters of polyphenols in circulation (**Figure 11**). These findings are in agreement with previous results obtained after supplementation of resveratrol to plasma where Belguendouz and colleagues demonstrated that polyphenols could be transported in lipoproteins in normoglycemia conditions (96), however, the authors did not shown this

for hyperglycemia conditions. It was also observed that the distribution of polyphenols in circulation in normoglycemia conditions were mirrored in hyperglycemia conditions (**Figure 12**). This is the first work to address the distribution of circulating polyphenols in diabetes mellitus type 2.

Based on the literature available, extraction methods implemented for polyphenol metabolites in biological samples are not standardized, with several approaches described involving liquid-liquid extraction (LLE) and solid-phase extraction (SPE) procedures (80-85). In this work, comparison between LLE and SPE in the extraction of polyphenol metabolites from plasma samples (**Figure 13**) revealed the Oasis HLB cartridge extracted more polyphenol metabolites than HybridSPE-Phospholipid cartridge and LLE which extracted about 50% less. The Oasis HLB cartridge is described as more prone to retain polar analytes while having some lipophilic abilities thus able to retain some phospholipids, that will then eluate together. Quantification of phospholipids in the extracts obtained (**Figure 13**) showed the content in PL was higher for the Oasis HLB cartridge, opposite to the HybridSPE-Phospholipid cartridge which showed the lowest content in PL. The HybridSPE-Phospholipid cartridge is specific for metabolomic analysis in the removal of phospholipids, although it has never been used for the extraction and characterization of polyphenol metabolites. The removal of phospholipids from the plasma sample extracts is desirable as the phospholipids can cause ion suppression thus compromising the detection and characterization of polyphenol metabolites in the extracts by LC-MS. In view of these results, the HybridSPE-Phospholipid cartridge was selected for the extraction of polyphenol metabolites as the method that can remove more phospholipids from the samples while also extracting polyphenol metabolites from the lipoprotein samples.

Interestingly, the results are in concordance with others as Mena and colleagues also tested three methods of extraction, protein precipitation (PPT), hybrid PPT and SPE-mediated phospholipid removal and, lastly, SPE Oasis HLB (97). From all methods tested they concluded how protein precipitation prior to reverse-phase SPE methodology exhibit a higher efficient recovery for most polyphenol classes specially metabolites of phase II (97). The authors reported how the use of Oasis HLB 96-well plates, despite the higher performance, lead to the loss of polar metabolites and for some medium-polarity metabolites and possible losses of metabolites that are expected to be in very low concentrations (57, 58, 97). Additionally, previous studies also used an extra step of enzymatic hydrolysis for the glucuronide and sulfate conjugates of phase II metabolization (98-100). Despite the quantification and analyzing process being facilitated, it was proven recently that it leads to lower recovery and high degradation of

the polyphenols in their aglycone form and consequently lead to an underestimation of the concentration of the polyphenol metabolites in circulation (97, 101).

The extraction of polyphenol metabolites with HybridSPE-Phospholipid cartridge showed high recovery percentage (110±3%), supporting that the extraction method employed is very efficient and would not impair the quantification of polyphenol metabolites which are expected to be in nanomolar (nM) concentrations (22, 49, 57, 58).

One of the aims of this work was to evaluate the polyphenol cargo in lipoproteins and how the diabetes mellitus type 2 could affect the amount and type of metabolites present in the VLDL and LDL lipoproteins. Since it was already shown these lipoproteins were the main carriers of polyphenol metabolites in circulation when compared to a lipoprotein depleted fraction, extracts of triglyceride and cholesterol-rich lipoproteins (VLDL and LDL) prepared by the HybridSPE-Phospholipid cartridge were characterized by spectrophotometric methods.

Determination of total phenolic content (**Figure 16**) revealed the VLDL population had a higher phenolic content than LDL. Hyperglycemia led to a reduction in total phenolic content in both lipoprotein populations, however treatment of diabetic patients with drugs, diet and exercise was able to improve the phenolic content in LDL extracts samples. Since the LDL population is generated by the loss of triglycerides in the VLDL population, leading to a smaller protein-to-lipid ratio, the protein content is different for both populations (**Table 4**) (78, 79). Based on this notion, it was normalized the absolute values to consider the protein content of lipoprotein population where it revealed the distribution of polyphenol content is heterogeneous among lipoprotein studied where the VLDL populations appear to be the main carriers of polyphenol metabolites in circulation. Unmanaged hyperglycemia affected the content of total phenolics in VLDL population leading to a decrease, though this is not observed for the LDL samples (**Figure 17**). Despite the same diabetic patients being subjected to a drug, diet and exercise regime, this lead only to a small amelioration.

The assessment of the flavonoid content depicted in **Figure 18** showed the same profile as the polyphenol content, with the VLDL population being the main transporter of this polyphenol class. Hyperglycemia induced a slight reduction (~60%) where drug and diet treatment lead only to a small improvement. There is no literature to compare the flavonoid content since so far it has only been measured in different food extracts and not biological fluids (102-104).

The decrease of total phenolic content observed in lipoprotein populations with diabetes lead to a concomitant decrease in antioxidant ability for both assays performed

(**Figure 19** and **Figure 20**). The treatment with drugs, diet and exercise slightly improved this reduction. In spite of the lack of literature in lipoproteins for these assays, it is interesting to note that literature describes polyphenol metabolites to not have high antioxidant ability (105, 106). This might be explained as the spectrophotometric FRAP and DPPH assays are still mostly utilized in food and extracts and are not specific for biological samples thus lipophilic antioxidants like ubiquinol-10 might contribute to the measurement of the antioxidant and scavenging ability of our extracts leading to overestimations (107).

Most of the works published describe the polyphenol metabolome in plasma samples after food supplementation, because of this, this study had the challenge with the lack of values to compare with for the lipoproteins (108-111). Nonetheless, the normoglycemia values were compared to the literature found for basal levels of polyphenols in plasma samples where the lipoprotein sample extracts showed a higher content in polyphenols (110-112) and higher antioxidant ability (109-111, 113-115). This is the first study characterizing and describing the polyphenol, flavonoid and antioxidant capacity in purified lipoproteins collected from healthy and diabetic patients. As such, there is no literature to compare the results concerning the effect of hyperglycemia PC and hyperglycemia GC against normoglycemia.

In overview, the spectrophotometric characterization of lipoprotein extracts showed that among the triglyceride and cholesterol-rich lipoproteins the polyphenol metabolites are unevenly distributed. It also showed that diabetes leads to a reduction in the amount of polyphenols in circulation and consequently the antioxidant capacity of the lipoproteins. The reason for the reduction of total phenolic and flavonoids content alongside the antioxidant capacity with hyperglycemia is still not known, though it can be postulated that perhaps the changes in lipid content (**Table 2**) of lipoproteins that occur with diabetes (dyslipidemia) or the glycation of lipoproteins might impair the incorporation of the polyphenol metabolites in those lipoproteins and account for the reduction observed (116, 117).

Based on the results from the characterization of the lipoprotein extracts by spectrophotometric methods where it showed the presence of polyphenol metabolites in the lipoprotein samples, it was performed a more thorough characterization by reverse-phase chromatography coupled with high-resolution mass spectrometry (UPLC-MS) to identify and quantify potential polyphenol metabolites.

The panel of potential polyphenol metabolites obtained (**Table 5**) showed the predominance for sulfate derivatives of gut polyphenol metabolites which was as

expected since these polyphenol metabolites were described in plasma samples of healthy individuals (58, 59, 118-123), however this is the first study to identify the polyphenol metabolome signature in purified VLDL and LDL populations and the influence of hyperglycemia in this profile, as such so there is no literature to compare the results obtained.

Based on the identification of polyphenol metabolites (**Table 5**) and on the panel of polyphenol metabolites described in the literature for plasma samples in normoglycemia, two polyphenol metabolites were quantified in the lipoprotein extracts by UPLC-MS, namely protocatechuic acid (PCA) and its sulfate conjugate (PCA sulfate) as well as 3-(2,4-dihydroxyphenyl)propionic acid (DHPPA) and its sulfate conjugate (DHPPA sulfate) (55, 56, 111, 116-120). The results obtained and present in **Figure 24** and **Figure 25** show an uneven distribution between the lipoprotein populations, confirming earlier results, where PCA sulfate was only found in the LDL population whereas DHPPA-sulfate was found in both VLDL and LDL extracts. The VLDL population showed higher concentrations overall. In both cases, hyperglycemia induced a decrease in the concentrations of this polyphenol metabolite, where the treatment with drugs, diet and exercise did recuperate the concentrations to normal values. The concentrations of DHPPA sulfate are more heterogeneous between lipoprotein population and study group. In both populations, unmanaged diabetes reduced the final concentrations. However, diet, drug and exercise treatment induced a reduction of estimated concentration values in VLDL. Interesting, in the LDL population the effect was the opposite, with the treatment favoring an increase of the final concentration for the polyphenol metabolite DHPPA-sulfate.

The heterogeneous distribution observed for the polyphenol metabolites between VLDL and LDL could be explained by the different lipid content or protein to lipid ratio which could influence the interaction of the polyphenol metabolites within the different lipoprotein populations. Moreover, there is still no knowledge of where the polyphenol metabolites interact in lipoproteins to determine what influences their location. The different behavior for the hyperglycemia GC group may be partly derived by the drugs or type of diet change the diabetic patients were subjected to. Nevertheless, the quantification of these polyphenol metabolites in lipoproteins in healthy or diabetic patients has never been done, so there is no literature to corroborate the results obtained.

The characterization of the extracts from triglyceride-rich lipoproteins demonstrated the presence of several polyphenol metabolites among the VLDL and LDL lipoproteins

in normo- and hyperglycemia conditions, considering this, one additional objective of this work was to assess the role of polyphenol metabolites in endothelial function in hyperglycemia despite their low concentrations. For this, it was measured the release of cytokines IL-6 and IL-1β to the cell medium as both IL-6 and IL-1β are pro-inflammatory cytokines elevated in a situation of inflammation, as seen in diabetes mellitus type 2 and have been suggested as possible therapeutic targets (124).

The *in vitro* evaluation of the anti-inflammatory response of polyphenol metabolites tested, namely 3-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) and protocatechuic acid (PCA) showed in **Figure 27** and **Figure 28** both PCA and DHPV induced a reduction of IL-6 released in a situation of hyperglycemia. The anti-inflammatory effect observed was derived from the action of the polyphenol metabolites and not by compromising cell viability as both polyphenol metabolites did not affect the cell viability assay (**Figure 26**). The same anti-inflammatory effect was not seen for IL-1β (**Figure 28**), showing no marked effect.

Both PCA and DHPV are major gut derived metabolites, that can be found in circulation in very low concentration and during a few hours (57-59, 118, 125, 126). For the anti-inflammatory evaluation, the incubation times tested were up to 6h, however the time point chosen to present was 3h as based on pharmacokinetic studies, it mimics the amount of time gut metabolites remain in circulation above a basal level before excretion in urine (57, 58).

Nevertheless, the anti-inflammatory response observed in this study for PCA and DHPV at physiological concentrations in normoglycemia conditions is in agreement with others reported in literature (43-48). Amin and colleagues reported a reduction in IL-6 reduction by protocatechuic acid in human umbilical vein endothelial cells (HUVEC) in the same range of concentrations (0.1, 1 and 10 μM) (70). *In vitro* evaluation with PCA in other cell lines showed this reduction for IL-6 and IL-1β release (127, 128), however in one of the articles the release of IL-1β was not affected by this metabolite (128). The research done with DHPV regarding their effect in the release of cytokines is very scarce and it was not found literature for IL-6 and IL-1β. Nonetheless, DHPV induced a reduction in the expression and secretion of cell adhesion molecules and chemokines (VCAM-1 and MCP-1), an increase in phosphorylation of IKK and reduction in NF-κB transcriptional activity in human cell lines (129, 130) confirming that DHPV affects the inflammatory signaling pathway and consequently could affect the release of cytokines.

Results obtained in this work also show that for both metabolites the response is not in a dose-dependent manner for IL-6 and IL-1β where higher concentrations of

polyphenol metabolites do not mean higher anti-inflammatory response. The effects of polyphenol metabolites on the IL-1β release did not show time-dependent effects up to 6 hours (**Figure 5** and **Figure 6** in Supplementary Information), while for IL-6 it was possible to observe some time-dependent effects between 1h and 3h time points (**Figure 7** in Supplementary Information and **Figure 27**) for both polyphenol metabolites.

The inconsistency in behavior observed for IL-6 and IL-1β may be related to the fact that IL-6 is expressed in the mature form, yet IL-1β is translated to a precursor form, pro-IL-1β, that requires the cleavage to the mature form (124). These cytokines mediate inflammation by interacting with their respective receptor and activate signaling pathways (NF-κB; JAK-STAT; MAPK) which in turn leads to the release of more cytokines and more IL-6 and IL-1β (14, 124, 131, 132). Our results show that both polyphenol metabolites might have a selective action since only IL-6 release was affected, however it should be noted that additional studies are required since the number of replicas is low and might influence the significance in the release of IL-1β. If this trend of only IL-6 release being affected maintains, it should be further studied to understand how the metabolites might affect the signaling pathways of each cytokine. Furthermore, the results concerning IL-1β release might not be statistically significant and yet it could be biologically significant that does not translate in *in vitro* experiments since epidemiological studies have shown a reduction of this cytokine after consumption of polyphenols (133, 134).

Although the literature describing the anti-inflammatory effects of these polyphenol metabolites in endothelial cell lines is scarce, this is the first study that describes the behavior of polyphenol metabolites in an inflammatory situation while taking into account cell culture conditions to mimic physiological conditions such as polyphenol metabolite concentration and incubation time and inducing hyperglycemia to mimic diabetic condition.

In overview, the results presented in this work show for the first time the triglyceride and cholesterol-rich lipoproteins (VLDL and LDL) as the main carriers of polyphenol metabolites in circulation. It is also the first work describing the cargo and distribution of polyphenol metabolites in VLDL and LDL while also assessing how diabetes mellitus type 2 affects these parameters. In addition, it is also a pioneer in describing the anti-inflammatory effect of gut metabolites using physiological concentrations and taking in consideration the time they are in circulation, in hyperglycemia conditions.

In the future, additional research should be done particularly on:

- A more thorough quantification of the polyphenol metabolites present in all lipoproteins, to include additional phase II and gut polyphenol metabolites.
- Understand if the selective distribution of the polyphenol metabolites is because of the lipid and/or protein content.
- Inclusion of phase II conjugated or gut polyphenol metabolites in *in vitro* studies in endothelial cells
- Evaluation of ROS and NO production and the antioxidant response in endothelial cells exposed to hyperglycemia.
- Assessment of polyphenol metabolites on the modulation of proteins that regulate inflammatory signaling pathway.
- Additional studies to understand if polyphenol metabolites are able to directly enter the cell or interact with cell membrane receptors.

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6. SUPPLEMENTARY INFORMATION

Table 1: Optimized electronic parameters in tune files used for the quantification of polyphenol metabolites in SPE extracts of isolated lipoproteins form normo- and hyperglycemic patients.

Parameters	Protocatechuic acid	3-(2,4-dihydroxyphenyl)propionic acid
Capillary voltage (V)	44	18
Tube lens (V)	60	60
Multipole RF	400	400
Multipole 1 offset (V)	9	9
Multipole 2 offset (V)	12.50	14.50
Interpole	16	26
Entrance lens	88	62
Trap DC offset (V)	10	10

Table 2: Protein concentration determined by Bradford assay in the lipoprotein depleted fractions (LPDS) from the normoglycemia, hyperglycemia PC and GC study groups. Results expressed as mg protein/ mL sample.

	LPDS		
	NL	**PC**	**GC**
Mean (mg/mL)	239.29	210.13	172.52
SEM	19.19	5.37	17.68
CV (%)	8.02	2.56	10.25

Table 3: Total phenolic content determined by the Folin-Ciocalteu method in the Lipoprotein-depleted samples (LPDS) from the normoglycemia, hyperglycemia PC and hyperglycemia GC study groups. Results expressed as mg GAE/ mL extract or mg GAE/mg of protein (n=3).

	LPDS		
	NL	**PC**	**GC**
Mean (mg/mL)	2.12	1.77	1.94
SEM	0.11	0.51	0.36
CV (%)	8.96	49.75	32.15
Mean (mg GAE /mg protein)	0.01	0.01	0.01
SEM	0.001	0.003	0.002
CV (%)	11.11	51.42	35.20

Table 4: Concentration of polyphenol metabolites determined by LC-MS in the lipoprotein sample extracts. Results expressed as nM (n=3).

Polyphenol Metabolite		VLDL			LDL		
		NL	PC	GC	NL	PC	GC
PCA-Sulfate	Mean (nM)	-	-	-	2159,9	544,9	486,3
	SEM	-	-	-	195,5	28,0	55,4
	CV (%)	-	-	-	15,7	8,9	19,7
DHPPA Sulfate	Mean (nM)	292,84	420,21	376,64	325,47	92,37	419,77
	SEM	62,55	82,36	14,15	28,27	32,38	77,87
	CV (%)	37,00	33,95	6,51	15,05	60,72	32,13

Line (-) indicates the metabolite was not found in the extract.

Table 5: Concentration of polyphenol metabolites determined by LC-MS in lipoprotein sample extracts. Results expressed as pmol/ mg of protein (n=3).

Polyphenol Metabolite		VLDL			LDL		
		NL	PC	GC	NL	PC	GC
PCA-Sulfate	Mean (pmol PCA/mg protein)	-	-	-	1708,7	311,2	280,0
	SEM	-	-	-	154,7	16,0	31,9
	CV (%)	-	-	-	15,7	8,9	19,7
DHPPA Sulfate	Mean (pmol DHPPA/mg protein)	1100,90	761,24	541,15	257,49	52,75	241,66
	SEM	235,15	149,20	20,33	22,37	18,49	44,83
	CV (%)	37,00	33,95	6,51	15,05	60,72	32,13

Line (-) indicates the metabolite was not found in the extract.

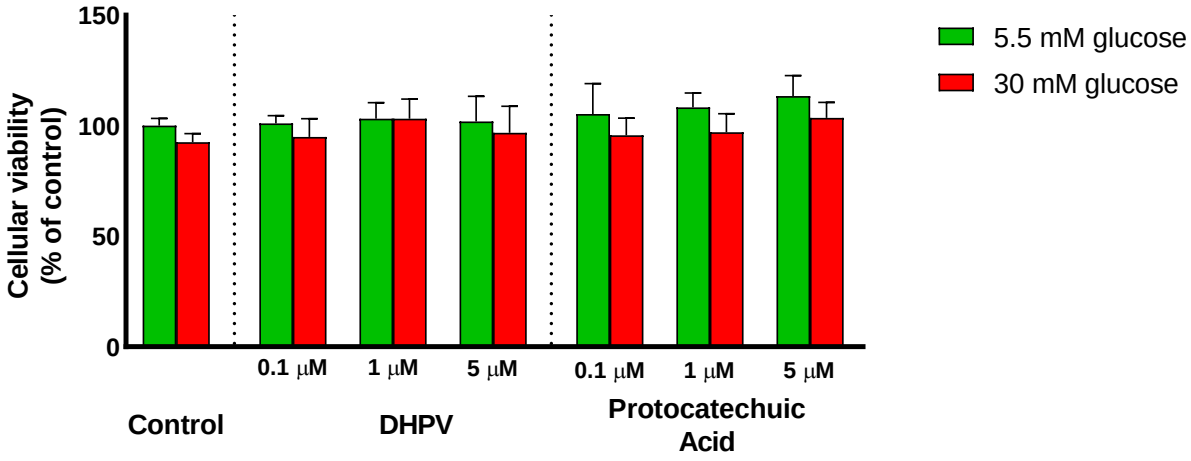

Figure 1: Comparison of cytotoxicity ability of DHPV and PCA. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), cells were exposed to polyphenol metabolites (0.1, 1 and 5 μM) for 1h. Cell viability was measured by MTS assay. Data expressed as Mean percentage ± SEM (n=3).

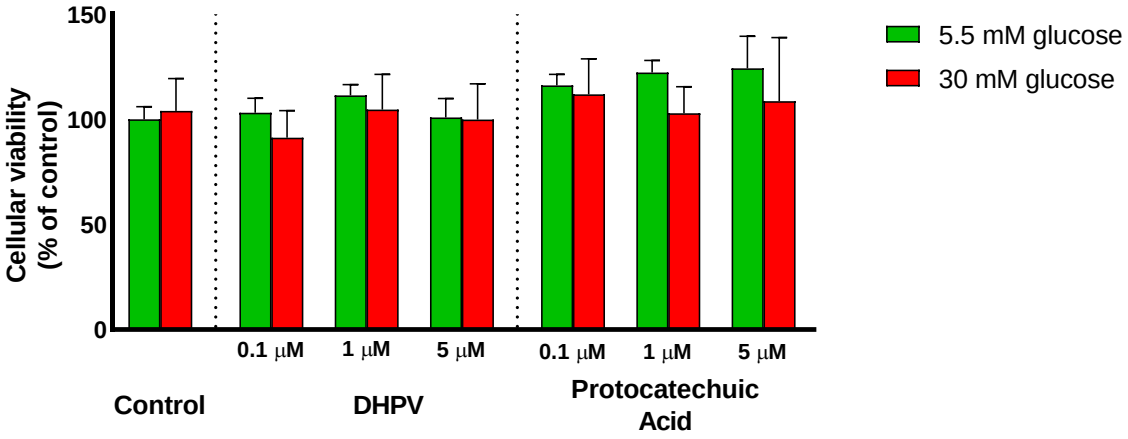

Figure 2: Comparison of cytotoxicity ability of DHPV and PCA. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), cells were exposed to polyphenol metabolites (0.1, 1 and 5 μM) for 3h. Cell viability was measured by MTS assay. Data expressed as Mean percentage ± SEM (n=3).

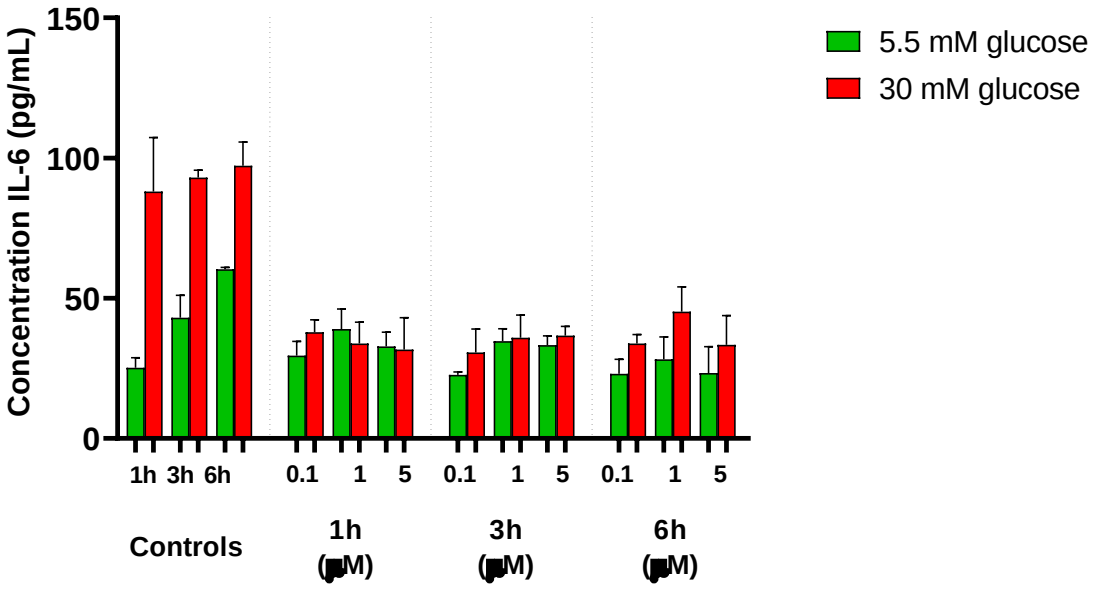

Figure 3: Effect of DHPV through time in the release of IL-6 in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 1, 3 and 6h. Subsequently the cell supernatant was collected, and the production of IL-6 was assessed by ELISA. Data expressed as Mean ± SEM (n=2).

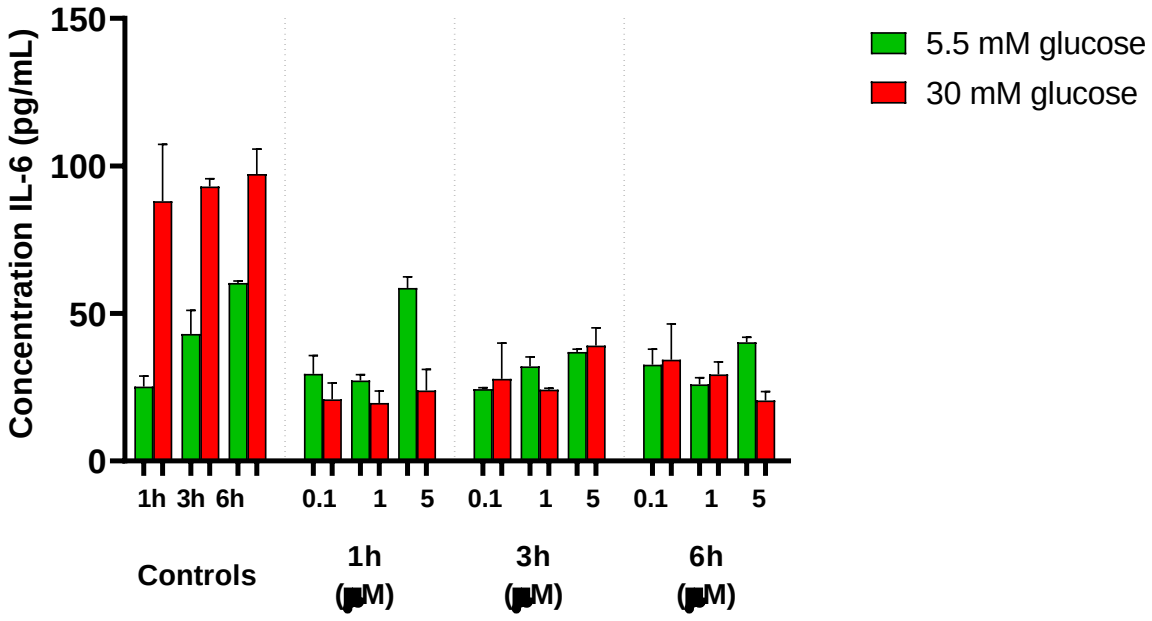

Figure 4: Effect of PCA through time in the release of IL-6 in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 1, 3 and 6h. Subsequently the cell supernatant was collected, and the production of IL-6 was assessed by ELISA. Data expressed as Mean ± SEM (n=2).

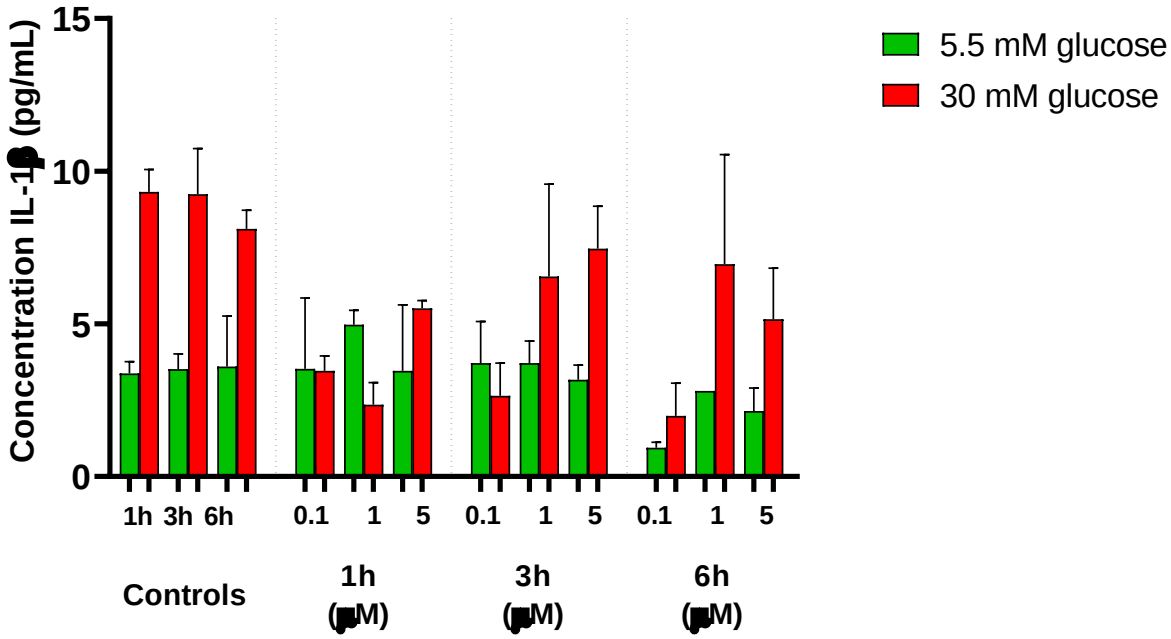

Figure 5: Effect of DHPV through time in the release of IL-1β in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 μM) and incubated for 1, 3 and 6h. Subsequently the cell supernatant was collected, and the production of IL-1β was assessed by ELISA. Data expressed as Mean ± SEM (n=2).

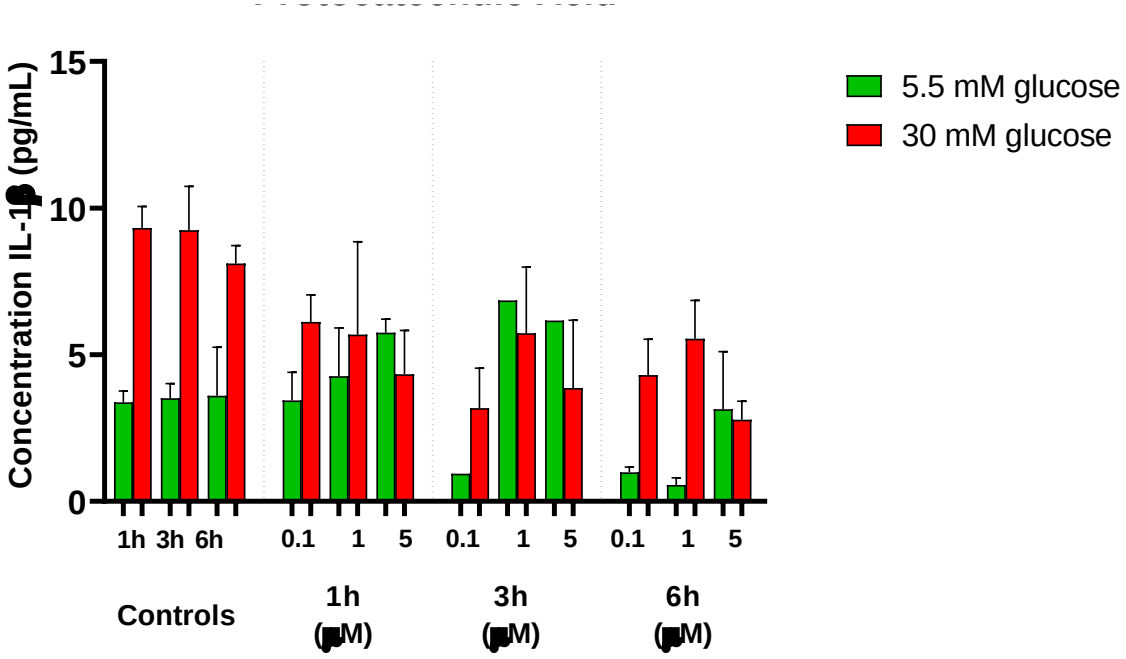

Figure 6: Effect of PCA through time in the release of IL-1β in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 μM) and incubated for 1, 3 and 6h. Subsequently the cell supernatant was collected, and the production of IL-1β was assessed by ELISA. Data expressed as Mean ± SEM (n=2).

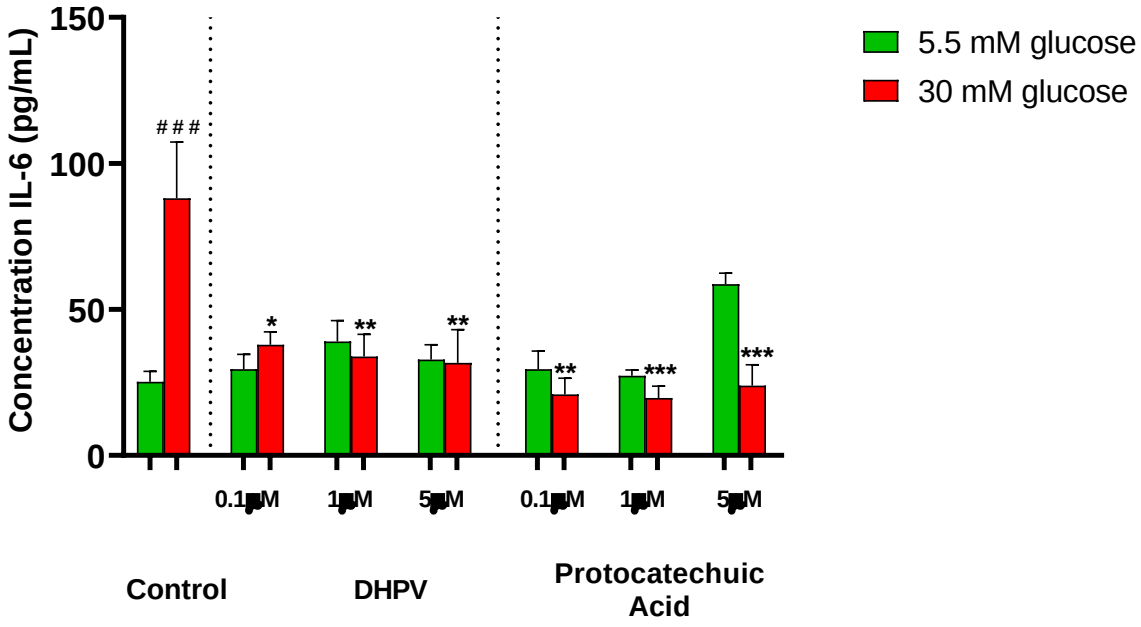

Figure 7: Effect of DHPV and PCA in the release of IL-6 in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 1h. Subsequently the cell supernatant was collected, and the production of IL-6 was assessed by ELISA. Data expressed as Mean ± SEM (n=2).Significant difference relative to 5.5 mM glucose control conditions are expressed as $^{\#\#\#\#}p<0.001$; Significant difference relative to 30 mM glucose control conditions are expressed as $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$.

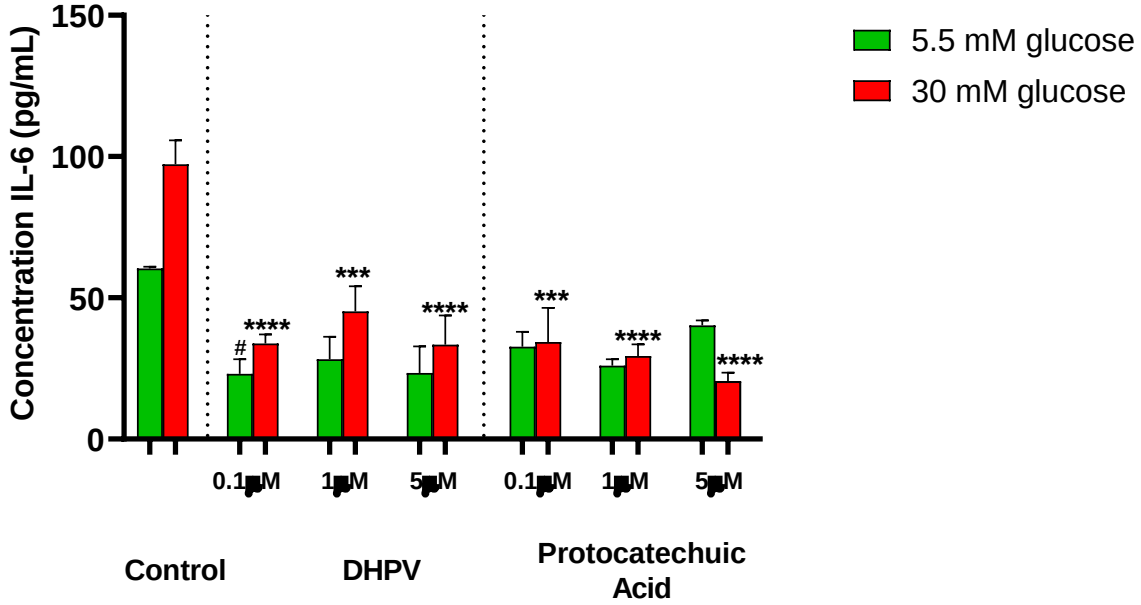

Figure 8: Effect of DHPV and PCA in the release of IL-6 in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 6h. Subsequently the cell supernatant was collected, and the production of IL-6 was assessed by ELISA. Data expressed as Mean ± SEM (n=2).Significant difference relative to 5.5 mM glucose control conditions are expressed as $^{\#}p<0.05$; Significant difference relative to 30 mM glucose control conditions are expressed as $^{***}p<0.001$ and $^{****}p<0.0001$.

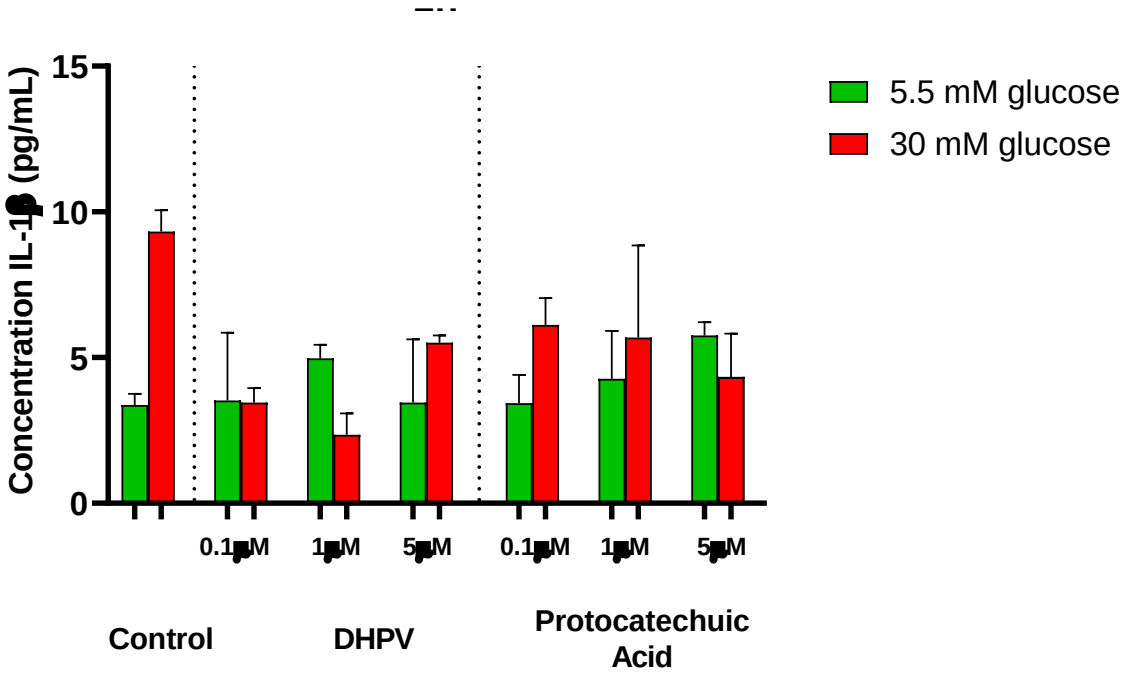

Figure 9: Effect of DHPV and PCA in the release of IL-1β in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 1h. Subsequently the cell supernatant was collected, and the production of IL-1β was assessed by ELISA. Data expressed as Mean ± SEM (n=2).

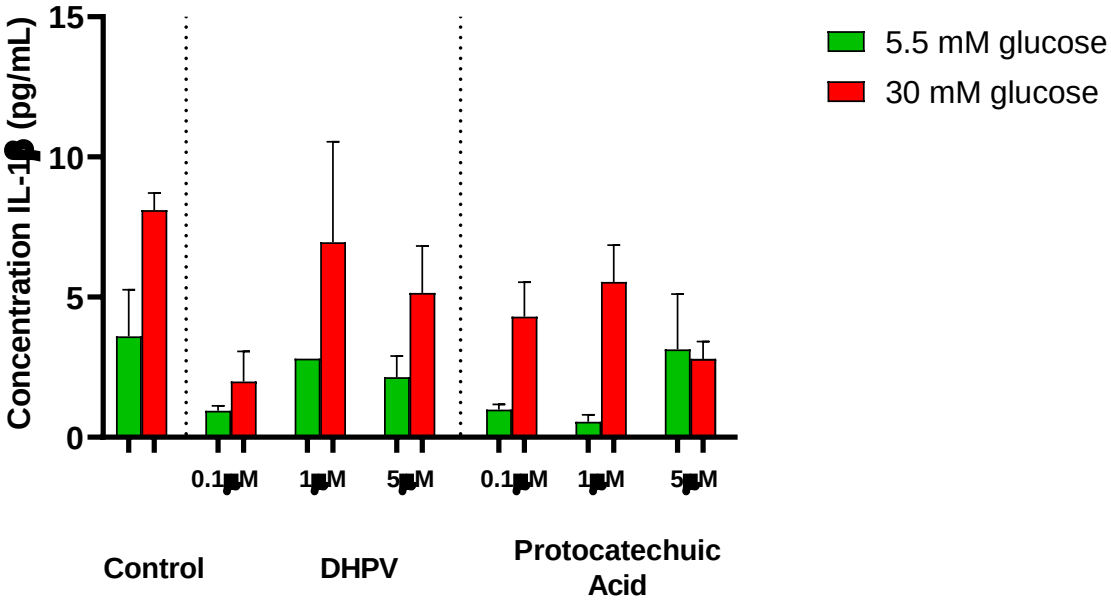

Figure 10: Effect of DHPV and PCA in the release of IL-1β in HMECs exposed to hyperglycemia. After 24h incubation in medium with glucose (5.5 and 30 mM glucose), it was added the polyphenol metabolites (0.1, 1 and 5 µM) and incubated for 6h. Subsequently the cell supernatant was collected, and the production of IL-1β was assessed by ELISA. Data expressed as Mean ± SEM (n=2).